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SPECIAL REPORT

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Adenosine A_3 receptors[1] mediate hypotension in the angiotensin II-supported circulation of the pithed rat

[2]J.R. Fozard & A.M. Carruthers

Preclinical Research, Sandoz Pharma Ltd., CH-4002 Basel, Switzerland

The cardiovascular effects of N^6-2-(4-aminophenyl)ethyladenosine (APNEA), which when radiolabelled with ^{125}I shows high affinity for the newly described adenosine A_3 receptor, have been investigated in the angiotension II-supported circulation of the pithed rat. APNEA induces hypotensive responses which are unaffected by high doses (20–40 mg kg^{-1}) of the broad spectrum, adenosine receptor antagonist, 8-(*p*-sulphophenyl)theophylline (8-SPT). 8-SPT-resistant falls in blood pressure are also seen, in the absence of bradycardia, with 5'-N-ethylcarboxamidoadenosine (NECA) and the **R**- and **S**-enantiomers of N^6-phenylisopropyladenosine (PIA). Xanthine insensitivity, high potencies of APNEA, NECA and **R**-PIA, and an enantiomeric selectivity favouring **R**- over **S**-PIA are distinguishing features of the adenosine A_3 receptor. We suggest that hypotension in the pithed rat may be a functional correlate of this site.

Keywords: 5'-N-ethylcarboxamidoadenosine (NECA); **R**-N^6-phenylisopropyladenosine (**R**-PIA); **S**-N^6-phenylisopropyladenosine (**S**-PIA); N^6-2-(4-aminophenyl)ethyl adenosine (APNEA); hypotension; A_3 adenosine receptors

Introduction Recently, Zhou *et al.* (1992) described the cloning, expression and functional characterization of a novel adenosine receptor which they designated A_3. The A_3 receptor can be labelled with high affinity by the A_1 receptor agonist radioligand, [^{125}I]-APNEA (N^6-2-(4-amino-3-iodophenyl)ethyladenosine. We here report that low doses of APNEA induce hypotension in pithed rats with their blood pressures raised to normal by angiotension II. Significantly, the response shows the major features of the A_3 receptor in being resistant to blockade by 8-(*p*-sulphophenyl)theophylline (8-SPT), a xanthine-based, broad spectrum, adenosine receptor antagonist, mimicked by low doses of 5'-N-ethylcarboxamidoadenosine (NECA) and **R**-N^6-phenylisopropyladenosine (**R**-PIA) and manifesting enantiomeric selectivity favouring **R**-PIA over **S**-PIA.

Methods Male Sprague-Dawley rats weighing 284–389 g, supplied by Biological Research Laboratories (Füllinsdorf, Switzerland) were anaesthetized with sodium pentobarbitone, 60 mg kg^{-1}, i.p., and pithed as described previously (Mir *et al.*, 1989). Rats were artificially respired with pure oxygen (Palmer Pump, 1 ml 100 g^{-1}; 60 strokes min^{-1}) and catheters inserted into the right carotid artery for recording blood pressure and heart rate and into the jugular veins for drug administration. Body temperature was maintained at 37°C by means of a heated table and lamp. All parameters were displayed on a Beckman R-SIIA polygraph.

Following pithing, blood pressure was routinely raised to 100–120 mmHg by infusion of angiotensin II, 0.2–0.5 μg kg^{-1} min^{-1} and a 15 min stabilization period was allowed before any further intervention. Dose-response curves to the adenosine receptor agonists were established by cumulative bolus injection, the intervals between doses being sufficient to allow a plateau response to develop. In the experiments with the non-selective A_1/A_2 receptor antagonist, 8-SPT, either 8-SPT, 20 or 40 mg kg^{-1} was injected intravenously 5 min before establishing the dose-response curves to the agonists. Only one agonist dose-response curve was generated per animal.

The following compounds were used: N^6-2-(4-aminophenyl)ethyladenosine (APNEA), gift of Professor R. Olsson, University of South Florida, Tampa, Fla, U.S.A.; 5'-N-ethylcarboxamidoadenosine (NECA), **R**- and **S**-N^6-phenylisopropyladenosine (**R**- and **S**-PIA), Sigma, Buchs, Switzerland; angiotensin II in the form of the amide analogue, Ciba-Geigy, Basel, Switzerland; 8-(*p*-sulphophenyl)theophylline (8-SPT), synthesized by Dr F. Gadient at Sandoz, Basel, Switzerland; sodium pentobarbitone (Sagatal), May and Baker Ltd., Dagenham, U.K. Final dilutions of all drugs were prepared in 0.9% NaCl.

Results Cumulative intravenous administration of APNEA, 1–30 μg kg^{-1} induced dose-related falls in blood pressure in the angiotensin II supported circulation of the pithed rat accompanied at the higher doses by short-lived (<1 min) falls in heart rate (Figure 1a). NECA, 0.3–10 μg kg^{-1}, **R**-PIA, 1–30 μg kg^{-1} and **S**-PIA, 10–300 μg kg^{-1} also induced dose-related falls in blood pressure (Figure 1b–d); in each case these were accompanied by bradycardia (Figures 1b–d) which was sustained relative to that seen with APNEA.

8-SPT, injected intravenously at a dose of 20 mg kg^{-1}, abolished the fall in heart rate induced by APNEA but did not influence the fall in blood pressure; a similar result was obtained following 40 mg kg^{-1} (Figure 1a). Following 40 mg kg^{-1} 8-SPT, the bradycardia following NECA, **R**-PIA and **S**-PIA was essentially abolished. In contrast, falls in blood pressure persisted in the presence of 8-SPT although the dose-response curves were displaced to the right at the ED_{50} level by 4.9, 2.7 and 1.5 fold for NECA, **R**-PIA and **S**-PIA, respectively (Figure 1b–d, Table 1).

Discussion The major finding of the present study is that APNEA, which when radiolabelled with ^{125}I shows high affinity for the newly described A_3 adenosine receptor (Zhou *et al.*, 1992), induced hypotensive responses in the pithed rat which are resistant to blockade by the broad spectrum adenosine receptor antagonist, 8-SPT. The response is not dependent on the use of angiotensin II to raise blood pressure since APNEA manifests similar hypotension in pithed rats infused

[1] Footnote: There is confusion in the literature with respect to the term 'adenosine A_3 receptor'. In this paper 'adenosine A_3 receptor' refers to the novel, structurally defined, adenosine receptor cloned from rat brain by Xhou *et al.* (1992) and not to the quite different 'A_3 receptor' suggested on pharmacological grounds to be present in certain peripheral neurones (Ribeiro & Sebastião, 1986).

[2] Author for correspondence.

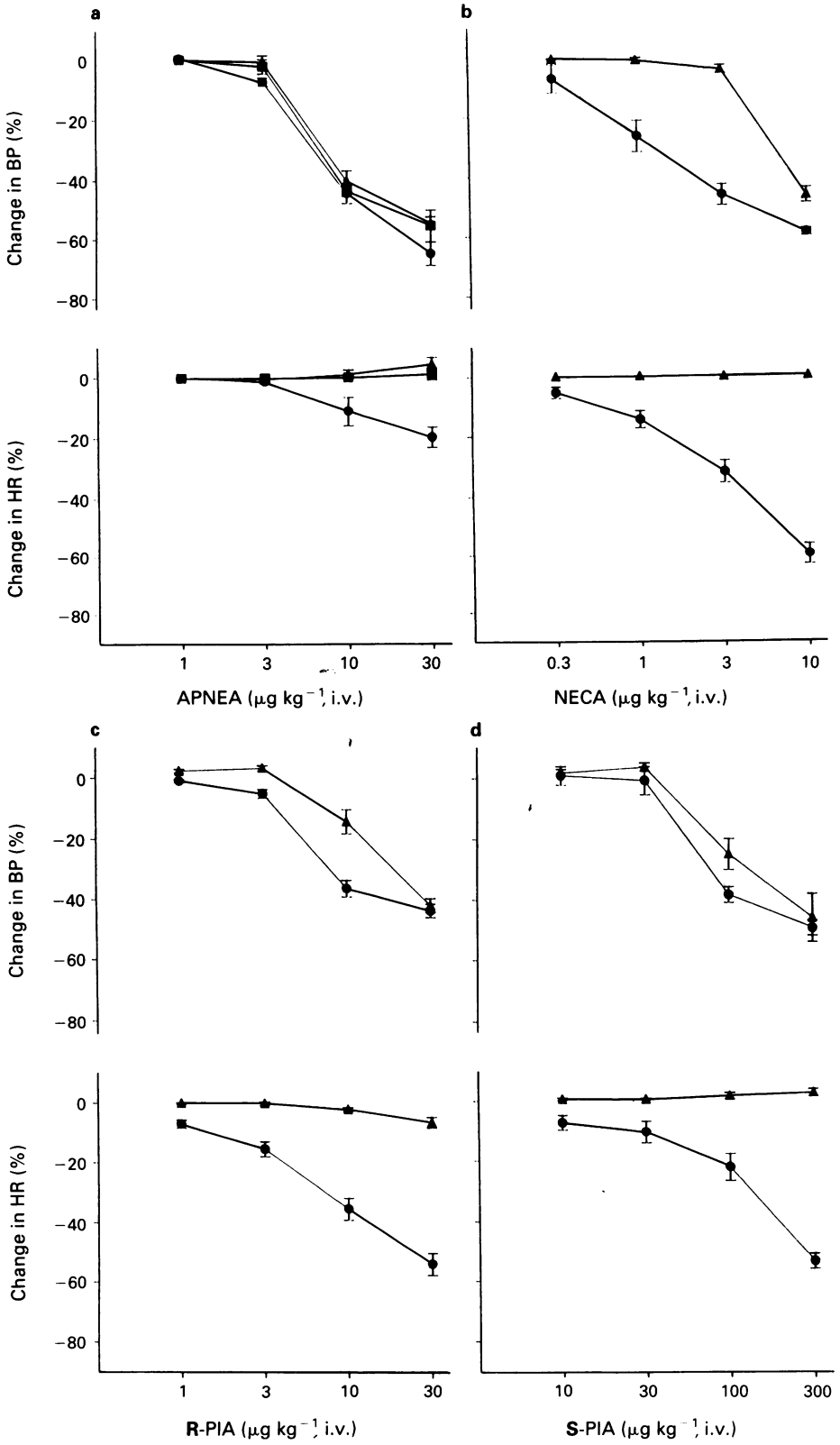


Figure 1 Effects of 8-(*p*-sulphophenyl)theophyline (8-SPT) on the cardiovascular responses to N^6-2-(4-aminophenyl)ethyl adenosine (APNEA) (a), 5'-N-ethylcarboxamidoadenosine (NECA) (b), **R**-N^6-phenylisopropyladenosine (**R**-PIA) (c) and S-PIA (d) in pithed rats with blood pressure supported by angiotensin II: (●) vehicle-treated controls; (■) animals treated with 20 mg kg^{-1} 8-SPT; (▲) animals treated with 40 mg kg^{-1} 8-SPT. The mean baseline blood pressure (BP) and heart rate (HR) values just prior to starting the injection sequences were:

APNEA	(vehicle)	110 ± 4	280 ± 6	(4)
APNEA	(8-SPT; 20)	110 ± 3	293 ± 7	(4)
APNEA	(8-SPT; 40)	108 ± 2	285 ± 7	(4)
NECA	(vehicle)	102 ± 3	299 ± 10	(4)
NECA	(8-SPT; 40)	102 ± 1	293 ± 9	(4)
R-PIA	(vehicle)	103 ± 5	267 ± 10	(4)
R-PIA	(8-SPT; 40)	113 ± 3	288 ± 6	(9)
S-PIA	(vehicle)	106 ± 4	283 ± 11	(4)
S-PIA	(8-SPT; 40)	108 ± 3	287 ± 12	(4)

Table 1 Cardiovascular effects of adenosine receptor agonists and their blockade by 8-(*p*-sulphophenyl)theophyline) (8-SPT)

	Control			*8-SPT-treated*[b]			
	BP, ED_{30}[a] ($nmol\ kg^{-1}$)	*HR, ED_{30}*[a] ($nmol\ kg^{-1}$)	n	*BP, ED_{30}*[a] ($nmol\ kg^{-1}$)	*HR, ED_{30}*[a] ($nmol\ kg^{-1}$)	*BP* Shift[c]	n
APNEA	16 ± 1	>78	4	20 ± 2	>259	1.2	4
NECA	3.7 ± 0.9	7.8 ± 1.5	4	18.2 ± 0.7	>88	4.9	4
R-PIA	21 ± 2	20 ± 3	4	56 ± 6	>259	2.7	9
S-PIA	198 ± 19	337 ± 58	4	292 ± 35	>778	1.5	4

[a]Dose to lower BP and HR by 30%; [b]40 mg kg^{-1} given i.v. 5 min prior to first dose of agonist; [c]Ratio: BP, ED_{30} (control)/BP, ED_{30} (8-SPT); For abbreviations, see text.

with phenylephrine (unpublished data). The high doses of 8-SPT used are sufficient to block the bradycardia induced by NECA and **R**-PIA which is known to result from activation of the A_1 receptor of the sino-atrial node (Linden, 1991). Moreover, a dose of 40 mg kg^{-1} 8-SPT blocked hypotension in the pithed rat induced by 2-[*p*-(2-carboxyethyl)phenylethylamino]-5'N-ethylcarboxamidoadenosine (CGS 21680), a selective A_{2A} receptor agonist (Hutchinson *et al.*, 1989), by 19 fold (Fozard & Carruthers, 1993). Thus, the fall in blood pressure induced by APNEA is not a consequence of activation of either A_1 or A_{2A} receptors. Further, an effect on A_{2B} receptors can be excluded since 8-SPT is at least as active at this particular subtype as at either the A_1 or the A_2 receptors (Bruns *et al.*, 1986; Hargreaves *et al.*, 1990).

Apart from a high affinity for APNEA, the principal pharmacological characteristics of the A_3 receptor are xanthine insensitivity, high affinities of the A_1/A_2 receptor agonist, NECA, and the nominally selective A_1 receptor agonist, **R**-PIA and an enantiomeric selectivity favouring **R**-PIA over **S**-PIA (Zhou *et al.*, 1992). It bears emphasis that low doses of NECA and **R**-PIA induce dose-related falls in blood pressure in the presence of a dose of 8-SPT adequate to block A_1, A_{2A} and A_{2B} receptors and that **S**-PIA is some 6 fold less active than **R**-PIA in this respect.

In conclusion, our data indicate that hypotension in the angiotensin II-supported circulation of the pithed rat is a functional correlate of the A_3 adenosine receptor and confirm the activity of the classical adenosine receptor agonists, NECA and **R**-PIA, at this site. It is significant that the selectivity of the nominally A_1 receptor selective agonist, **R**-PIA, is substantially less when comparisons are made with the A_3 receptor rather than either of the A_2 receptor subtypes. Moreover, since a number of claimed A_1 selective agonists induce 8-SPT-resistant hypotension in our model (Carruthers *et al.*, 1992), significant activity at this site may be a widespread property among adenosine receptor agonist ligands traditionally used to discriminate between adenosine A_1 and A_2 receptor subtypes. Clearly, a redefinition of the value of such tools and a reappraisal of conclusions made on the basis of their use would seem appropriate.

It is a pleasure to thank Dr Fulvio Gadient for synthesizing 8-SPT and Professor Ray Olsson for supplying APNEA.

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Propofol stimulates nitric oxide release from cultured porcine aortic endothelial cells

[1]Andy J. Petros, *Richard G. Bogle & *Jeremy D. Pearson

Intensive Therapy Unit, St. George's Hospital, Blackshaw Road, London SW17 0QT and *Vascular Biology Research Centre, Biomedical Sciences Division, King's College London, Campden Hill Road, London W8 7AH

Propofol, an intravenous anaesthetic agent, causes marked vasodilatation *in vivo*. In the present study the effects of propofol on the release of nitric oxide (NO) from vascular endothelial cells was determined *in vitro*. Application of propofol to co-cultures of porcine aortic endothelial and smooth muscle cells resulted in a rapid increase in cyclic GMP formation. This increase was significantly inhibited following pretreatment of the cells with either N^G-nitro-L-arginine (L-NOARG) or in the presence of haemoglobin. When applied to smooth muscle cells alone, propofol did not result in an increase in cyclic GMP levels. These results demonstrate that propofol stimulates the production and release of NO from cultured endothelial cells and suggest that the vasodilatation and hypotension observed when propofol is given *in vivo* may be due to NO release.

Keywords: Endothelial cells; nitric oxide; N^G-nitro-L-arginine (L-NOARG); propofol

Introduction The synthesis of nitric oxide (NO) from L-arginine is a widespread pathway modulating both the cardiovascular and the immune system. Vascular endothelial cells generate NO from L-arginine via a Ca^{2+}/calmodulin-dependent constitutive NO synthase enzyme. This results in elevation of guanosine 3':5'-cyclic monophosphate (cyclic GMP) levels in smooth muscle cells and subsequent vascular relaxation (Moncada *et al.*, 1991).

Propofol (2,6 diisopropyl-phenol) is a rapidly acting intravenous anaesthetic agent, which upon administration results in a marked reduction in systemic vascular resistance (Grounds *et al.*, 1985). In the present study we have examined whether propofol stimulates the release of NO from cultured vascular endothelial cells.

Methods Porcine aortic endothelial cells (PAEC) were isolated and cultured in Dulbecco's modified Eagle's medium supplemented with penicillin (100 units ml^{-1}), streptomycin (100 $\mu g\ ml^{-1}$), glutamine (4 mM), foetal calf serum (10% v/v) and newborn calf-serum (10% v/v). Endothelial cells were used at either passage one or two (Bogle *et al.*, 1991). Porcine aortic smooth muscle cells (PASMC) were isolated by a modified explant technique (Crossman *et al.*, 1990). When confluent, endothelial cells were mixed with smooth muscle cells (1:1) and plated into 24 well plates for use 24 h later.

Co-cultures were rinsed twice with HEPES-buffered Krebs solution of the following composition (mM): NaCl 131, KCl 5.5, $CaCl_2$ 2.5, $MgCl_2$ 1, NaH_2PO_4 1, $NaHCO_3$ 25, glucose 5.5, HEPES 20, pH 7.4, 37°C. Following this, cells were exposed to either propofol alone or propofol in the presence of inhibitors of NO formation for 5 min. Cells were pretreated with inhibitors for 10 min. Subsequently, 0.5 ml ice-cold hydrochloric acid (0.1 N) was added and plates placed on ice for 20–30 min to allow extraction of cyclic nucleotides. Cyclic GMP levels were determined by specific radioimmunoassay following acetylation (Crossman *et al.*, 1990). Cell number was determined after trypsinization by counting the number of cells in representative wells using a Coulter counter.

Propofol (2,6 diisopropyl-phenol) was obtained from ICI. Intralipid, is an oil in water emulsion containing fractionated soybean oil, egg phospholipids and glycerol, pH 7.0 obtained from KabiVitrum Ltd., Bucks.

[1] Author for correspondence.

Concentration-dependence data were log transformed and a pooled estimate of the standard deviation calculated. A Dunnett's t test was performed on this pooled estimate. Other data were analysed by an unpaired t test. $P<0.05$ was considered as significant.

Results In co-cultures of endothelial and smooth muscle cells, application of bradykinin (100 nM, 1 min) resulted in a significant increase in cyclic GMP production (control: 8.0 ± 2.8 fmol/10^6 cells, bradykinin 31.2 ± 11.4 fmol/10^6 cells, $P<0.01$, $n=3$). Similarly, application of sodium nitroprusside (SNP, 0.1 mM, 1 min) resulted in a large increase in cyclic GMP production (control: 8.0 ± 2.8, SNP: 91 ± 31, $P<0.01$, $n=3$).

Figure 1 shows the concentration-dependence of propofol-induced cyclic GMP formation in co-cultures of porcine aortic endothelial and smooth muscle cells. Application of propofol (0.03–1 mM) resulted in a rapid increase in cyclic GMP formation. Propofol vehicle (dimethyl sulphoxide, DMSO 0.1%) or clinical vehicle (intralipid 10%) had no significant

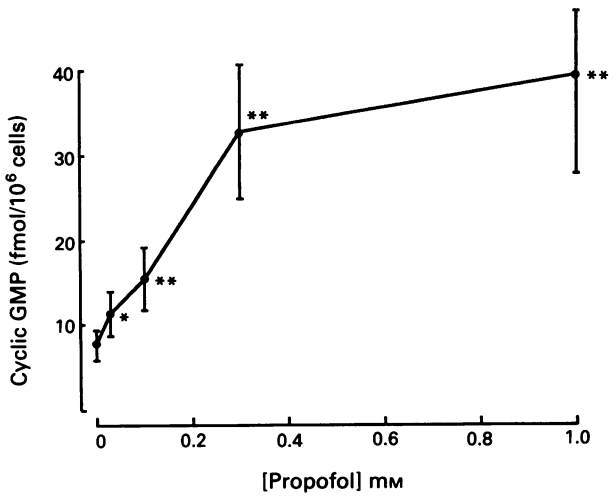


Figure 1 Concentration-dependence of propofol-induced cyclic GMP production. Porcine aortic endothelial and smooth muscle cell co-cultures were incubated with propofol (0.03–1.0 mM; 5 min). Results are mean ± s.d. obtained from 4 experiments on different cell batches. *$P<0.05$; **$P<0.01$ vs control.

effect on co-culture cyclic GMP levels (control cyclic GMP: 8.0 ± 2.8 fmol/10^6 cells, DMSO 6.9 ± 4.4 fmol/10^6 cells, intralipid 6.6 ± 3.9 fmol/10^6 cells, $n = 3$). When propofol (0.3 mM, 5 min) was added to cultures of smooth muscle cells no significant increase in cyclic GMP production was observed (8.5 ± 3.9 fmol/10^6 cells).

Figure 2 shows the effects of propofol on cyclic GMP production following pretreatment of co-cultures with inhibitors of NO production or action. Pretreatment of cells with either N^G-nitro-L-arginine (L-NOARG, 0.1 mM, 10 min) or haemoglobin (10 μM, 10 min) resulted in a significant inhibition of the effects of propofol on cyclic GMP production.

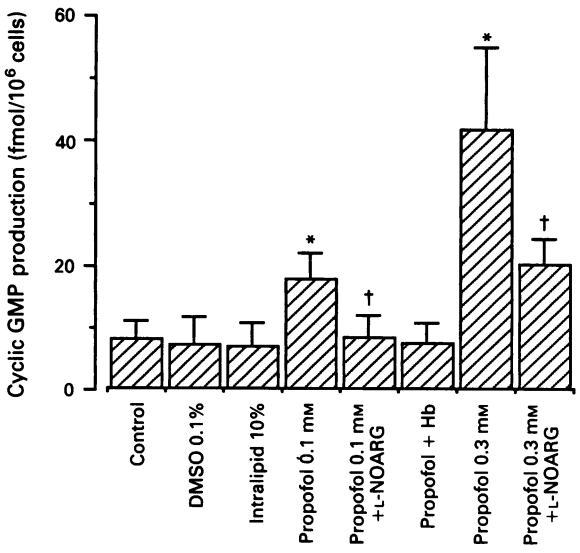


Figure 2 Role of nitric oxide in propofol-induced cyclic GMP production. Co-cultures of porcine aortic endothelial cells and porcine aortic smooth muscle cells were incubated with propofol (0.1 mM or 0.3 mM; 5 min) in the presence of either N^G-nitro-L-arginine (L-NOARG) 0.1 mM or haemoglobin (Hb) 0.01 mM. Results are mean ± s.d. obtained from 3 experiments on different cell batches. *$P < 0.05$ vs control; †$P < 0.05$ vs propofol alone.

Discussion The results of this study indicate that propofol stimulates NO production in a concentration-dependent manner from cultured endothelial cells. This increase in NO synthesis and release results in a subsequent cyclic GMP formation in vascular smooth muscle cells *in vitro*. Intralipid, the vehicle in which propofol is suspended for clinical use, did not increase NO production. This observation suggests that the *in vivo* effects of propofol on systemic vascular resistance are unlikely to be mediated by its vehicle.

To confirm that the increase in cyclic GMP observed in these experiments were due to NO, we demonstrated that an inhibitor of NO synthesis, L-NOARG, blocked the effects of propofol. Furthermore, haemoglobin an agent which binds and inactivates NO also inhibited propofol-induced cyclic GMP formation.

A recent study examined the effects of propofol on rat aortic and pulmonary artery rings and demonstrated a marked relaxation, which was endothelium-independent (Park *et al.*, 1992). This report suggested that in the rat, vascular smooth muscle may be more sensitive to the actions of propofol than the endothelium. In contrast our results would suggest that the predominant action of propofol on NO production is endothelium-dependent since there was no significant increase in NO production when propofol was applied to smooth muscle cells alone. These differences could be accounted for either by the different experimental techniques employed (isolated vascular rings versus cultured cells) or species differences.

Intracellular cyclic GMP levels may increase as a result of either increased production or inhibition of breakdown by phosphodiesterase enzymes (Waldman & Murad, 1987). This latter effect is unlikely to contribute to the changes observed in our experiments since the effects of propofol were abolished following inhibition of NO generation.

Although it is evident from this study that propofol stimulates NO production from endothelial cells it is possible that it may in addition reduce peripheral vascular resistance and blood pressure by depressing sympathetic drive, as do other general anaesthetic agents. Whether NO release is the predominant mechanism responsible for the vasodilatation requires further study.

In conclusion, we suggest that stimulated release of NO is a possible mechanism responsible for the reduction in systemic vascular resistance and hypotension observed when propofol is used *in vivo*. Further animal studies are needed to investigate the usefulness of NO synthase inhibitors in preventing hypotension associated with intravenous injection of propofol.

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Acetylcholine-induced relaxation in bovine isolated mesenteric arteries is suppressed by polymorphonuclear leukocytes

[1]Sjef J. De Kimpe, Dicky Van Heuven-Nolsen & Frans P. Nijkamp

Department of Pharmacology, Faculty of Pharmacy, Utrecht University, P.O. Box 80.082, 3508 TB Utrecht, The Netherlands

1 The endothelium plays a critical role in maintaining vascular tone via generation of potent vasoconstrictor and dilator substances. We examined the effect of bovine purified polymorphonuclear leukocytes (PMN) on the endothelium-dependent relaxation to acetylcholine in isolated mesenteric arteries.

2 In the presence of PMN (2.5×10^6 cells ml^{-1}) the maximal relaxation to acetylcholine was decreased from $76.1 \pm 2.4\%$ to $44.9 \pm 7.4\%$ of the precontraction ($P < 0.001$). This effect was inhibited by superoxide dismutase and N^G-mono-methyl-L-arginine, but not by catalase or indomethacin.

3 PMN were not able to influence significantly the endothelium-independent relaxation to nitroprusside.

4 Removal of PMN after preincubation and prior to precontraction and relaxation did not influence the acetylcholine-induced relaxation, indicating that no irreversible vascular damage had occurred.

5 Superoxide anion production by unstimulated PMN was less than 10% compared to phorbol myristate acetate-activated PMN, measured by chemiluminescence and reduction of ferricytochrome c.

6 We conclude that small amounts of superoxide anions produced by unstimulated PMN contribute to a decrease in relaxation to acetylcholine by interfering with endothelium-derived nitric oxide.

Keywords: Acetylcholine; endothelium-derived relaxing factor (EDRF); superoxide anion; polymorphonuclear leukocytes; mesenteric arteries

Introduction

The vascular endothelium is widely recognised as a tissue with a variety of functions, including regulation of vascular tone. The response of isolated blood vessels to several vasoactive agonists is modulated by the presence of endothelial cells. Indeed, these cells can release both dilator and constrictor substances (Vanhoutte, 1989; Lüscher, 1990). Imbalances between the release of these factors is observed in aging and diseases such as hypertension, atherosclerosis and ischaemia (Lüscher, 1988; Koga *et al.*, 1989; Shimokawa & Vanhoutte, 1989). The major vasodilator substances produced by endothelium are endothelium-derived relaxing factor (EDRF), identified as nitric oxide derived from L-arginine, and prostacyclin, a metabolite of the arachidonic acid pathway. Endothelin, produced by the endothelium, is one of the most potent vasoconstrictors.

Several pathological states, such as ischaemia, hypertension and inflammatory disorders, are associated with neutrophil accumulation and activation (Chobanian *et al.*, 1984; Schmid-Schönbein & Engler, 1986; Werns & Lucchesi, 1990). Neutrophils are capable of releasing a variety of compounds, like lipid mediators, oxygen-derived free radicals and proteolytic enzymes. These neutrophil-derived factors are able to influence vascular tone directly via an action on smooth muscle or indirectly via the endothelium. Lipid mediators such as platelet-activating factor relax vascular tissue in an endothelium-dependent manner (Siren & Feuerstein, 1989); however, others like leukotrienes constrict the vascular smooth muscle directly (Feigen, 1983). Superoxide anion breaks down endothelium-derived nitric oxide and has also been reported to contract vascular smooth muscle (Gryglewski *et al.*, 1986; Auck-Schwellk *et al.*, 1989). Hydrogen peroxide relaxes vascular smooth muscle via the generation of guanosine 3':5'-cyclic monophosphate (cyclic GMP) (Burke & Wolin, 1987). Endothelial prostacyclin release has been reported to be stimulated (Harlan & Callahan, 1984) or depressed (Whorton *et al.*, 1985) by oxygen-derived free radicals depending on the concentration of radicals present (Taylor *et al.*, 1983). Furthermore, oxygen-derived radicals and proteolytic enzymes are cytotoxic and both can injure the endothelium (Ward & Varani, 1990).

Several studies have demonstrated the ability of neutrophils to contract (Gillespie *et al.*, 1986; Sessa & Mullane, 1990) or relax (Mehta *et al.*, 1989; Lee *et al.*, 1990) vascular smooth muscle. The role of the endothelium herein is diverse and not yet well established. A study by Carden *et al.* (1990) showed that adhesion of the neutrophils to endothelium was required to increase vascular resistance *in vivo*. In another study the vasoconstriction induced by neutrophils *in vitro* was caused by a metabolic interaction, in which the endothelium converted leukotriene A_4 from the neutrophils to the vasoconstrictor peptidergic leukotrienes (Nishida *et al.*, 1990). Others described damage to the endothelium as a cause of altered vascular function (Carpenter *et al.*, 1987; Wolin, 1991).

In a previous study we demonstrated that bovine neutrophils caused an endothelium-dependent increase in sensitivity for noradrenaline in isolated mesenteric arteries (De Kimpe *et al.*, 1992). In the present study we determine the effect of bovine neutrophils on the endothelium-dependent relaxation of bovine isolated mesenteric arteries to acetylcholine.

Methods

Preparation of bovine polymorphonuclear leukocytes (PMN) (Henricks *et al.*, 1987)

Whole blood was obtained from cows in heparinised tubes. Plasma was removed after centrifugation at 400 *g* for 10 min. Erythrocytes were lysed by hypotonic treatment using a phosphate buffer. The white blood cells were suspended in Krebs buffer. Krebs buffer consisted of (mM): NaCl 118, KCl 4.7, $MgSO_4$ 1.1, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 7.6 and was gassed with 95% O_2:5% CO_2. The

[1] Author for correspondence.

cell suspension was layered on Percoll (Pharmacia, Uppsala, Sweden; density 1.077 g ml^{-1}) and centrifuged for 20 min at 400 *g* to remove mononuclear cells. The neutrophil-rich pellet was suspended in Krebs' buffer and was washed three times with Krebs buffer, first at 400 *g* and then twice at 100 *g* to reduce platelet contamination. The PMN suspension was kept on ice at a concentration of $1-2 \times 10^7$ cells ml^{-1} and washed just before use in the organ bath experiments. The PMN suspension contained $77.5 \pm 10.1\%$ neutrophils, $14.2 \pm 8.5\%$ eosinophils and $8.4 \pm 0.9\%$ mononuclear cells (mean ± s.e.). Viability was always greater than 95% as assessed by trypan blue exclusion. Platelet contamination of the PMN suspension was less than one platelet per PMN.

Bovine mesenteric arteries

Bovine mesenteric arteries were obtained from a local slaughterhouse and transported to the laboratory in ice-cold Krebs solution. Adherent tissue was removed and in some experiments the arteries were kept overnight at 4°C under a continuous overflow of Krebs buffer gassed with 95% O_2:5% CO_2. Preliminary experiments revealed that this storage procedure did not affect the responsiveness to acetylcholine. Arteries from the third or fourth branch of the mesenteric artery with a diameter of 2 to 2.5 mm were cut into rings of 2 mm length. In some experiments the endothelium was removed by careful rubbing of the intima with cotton wool.

Organ bath experiments

The isolated rings were mounted between two stainless steel wire hooks and placed in 12 ml siliconised water-jacketed organ baths which were filled with Krebs buffer and gassed with 95% O_2:5% CO_2 continuously. Temperature was maintained at 37°C. The tension was measured by a Harvard isometric transducer and recorded by a semi-automatic set up. Preload of the mesenteric rings was adjusted during 30 min to 50 mN, which was optimal to generate a maximal contraction as determined with 50 mM KCl.

The isolated mesenteric arteries were allowed to stabilize for 45 min during which time the tissues were washed three times. The rings were contracted by cumulative addition of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$ 1–100 nM) in the organ bath. At maximal contraction the arteries were exposed to 3 μM acetylcholine to 'challenge' the tissue and to test the functional integrity of endothelium. The tissues were rinsed several times and allowed to restabilize. Next the rings were incubated with 2.5×10^6 PMN ml^{-1} in the organ bath. After a 20 min incubation period the mesenteric arteries were precontracted with $PGF_{2\alpha}$ (30–100 nM) to attain a submaximal contraction. After stabilization a cumulative concentration-effect curve with acetylcholine ($10^{-9}-10^{-5}$ M) or nitroprusside ($10^{-9}-10^{-4}$ M) was obtained. Only preparations of which the control tissue from the same artery showed a relaxation in response to acetylcholine of at least 35% of the precontraction were included in the study.

Some experiments were carried out in the presence of the cyclo-oxygenase inhibitor indomethacin (1 μM) or the oxygen-radical scavengers superoxide dismutase (SOD, 200 u ml^{-1}) or catalase (2000 u ml^{-1}), which respectively scavenge superoxide anion or hydrogen peroxide. These compounds were added to the organ bath 15 min before the incubation with PMN.

To characterize the acetylcholine-induced relaxation in bovine isolated mesenteric arteries inhibition of nitric oxide synthesis from L-arginine was studied. N^G-monomethyl-L-arginine (L-NMMA; 30, 100 and 300 μM) was added to the organ baths after the precontraction and the arterial rings were incubated for 15 min. A cumulative concentration-effect curve to acetylcholine was obtained. The highest concentration of L-NMMA was also used in combination with PMN. In this case PMN were added 10 min before the precontraction.

In all experiments the arteries were exposed for 25 min to the PMN before the concentration-effect curve was started. In one study we investigated the possibility of irreversible damage by the PMN by washing them out of the organ bath after a 25 min incubation period just prior to the precontraction and subsequent relaxation.

Superoxide anion production

Two methods selective for superoxide anion were used to study the production of oxygen radicals by PMN. Firstly lucigenin-enhanced chemiluminescence by PMN was measured (Gyllenhammar, 1987), with a LKB-Wallac 1251 luminometer (Finland). PMN suspension (final cell number 2.5×10^6 ml^{-1} Krebs buffer) and lucigenin (end concentration 0.5 mM) were added to Krebs buffer in a cuvette to a total volume of 0.5 ml. The cuvettes were placed in the luminometer and chemiluminescence was measured every 90 s during 45 min. The temperature was maintained at 37°C.

Secondly the ability of superoxide anion to reduce ferricytochrome c was used (Babior *et al.*, 1973). PMN suspension (2.5×10^6 cells ml^{-1}) was incubated with ferricytochrome c (1.1 mg ml^{-1}) in Krebs buffer for 45 min at 37°C. The suspension was stirred throughout the experiment. Incubation fluid was collected and centrifuged to remove the PMN at 400 *g* for 5 min at 4°C. The amount of reduced ferricytochrome c was determined by measuring the absorbance at a wavelength of 550 nm with a double-beam spectrophotometer and using an extinction coefficient of 2.1×10^4 M^{-1} cm^{-1}. A solution of ferricytochrome c was used as reference. Paired reactions with and without SOD (200 u ml^{-1}) were performed to enable the calculation of superoxide anion-dependent reduction only. Catalase (2000 u ml^{-1}) was present during the whole experiment to prevent reoxidation of reduced ferricytochrome c by hydrogen peroxide.

The generation of superoxide anions by PMN stimulated with phorbol myristate acetate (PMA; 100 ng ml^{-1}) was also determined by the two above described methods.

Drugs

Acetylcholine, sodium nitroprusside, superoxide dismutase (SOD), N^G-monomethyl-L-arginine (L-NMMA) were purchased from Sigma (St. Louis, U.S.A.) and catalase from Boehringer (Mannheim, Germany). These compounds were dissolved in saline (0.9% NaCl). Indomethacin (10 mM; Merck Sharp & Dome, Haarlem, The Netherlands) was dissolved in 1 M Tris (2-amino-2-hydroxymethyl-propane-1,3-diol) buffer (pH = 7.8). Ferricytochrome c (Type III, Sigma) was dissolved and diluted in Krebs buffer. All these solutions were prepared freshly before each experiment. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; Upjohn Company, Kalamazoo, U.S.A.) at a stock concentration of 1 mM in methanol and lucigenin (Sigma; 5 mM in Krebs buffer) were stored at − 20°C. Phorbol myristate acetate (PMA; Sigma) was dissolved in dimethylsulphoxide (1 mg ml^{-1}) and kept at − 80°C.

Data analysis

All data are expressed as mean ± standard error of the mean (s.e.mean). pD_2-values ($-\log EC_{50}$) are used to indicate sensitivity to acetylcholine-induced relaxation. The extent of relaxation was calculated relative to the force of precontraction after $PGF_{2\alpha}$. Statistical analysis was performed by a two-tailed Student's *t* test for unpaired observations.

Results

Effect of PMN on arterial relaxation

Addition of PMN (2.5×10^6 cells ml^{-1}) to mesenteric arterial rings had no significant effect on the basal tension. The

relaxation of mesenteric arteries to acetylcholine, however, was found to be impaired (Figure 1). The maximal relaxation was decreased from 76.1 ± 2.4% to 44.9 ± 7.4% of the precontraction ($P < 0.001$). The sensitivity to acetylcholine was not altered significantly. The pD_2-values were 6.80 ± 0.05 for control and 6.67 ± 0.16 for PMN-incubated rings. The precontraction was not influenced significantly by the PMN, being 216 ± 10 mN for control vs 236 ± 14 mN for PMN-treated arterial rings. If the PMN were washed out of the organ bath just prior to precontraction, the relaxation to acetylcholine was not attenuated (maximal relaxation: 82.9 ± 7.6 % for PMN preincubation). If nitroprusside was used to relax the mesenteric arteries independently from the endothelium, the responsiveness was not altered significantly by addition of PMN to the organ bath (Figure 2).

Characterization of the response to acetylcholine

Experiments were performed to determine the mediators involved in the relaxation to acetylcholine in bovine isolated mesenteric arteries (Figure 3). The relaxation to acetylcholine

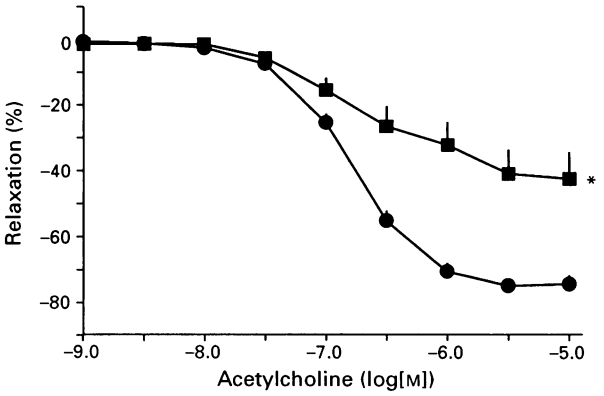


Figure 1 The effect of polymorphonuclear leukocytes (PMN) on the relaxation by acetylcholine in bovine isolated mesenteric arteries. Concentration-effect curves to acetylcholine in arterial rings after precontraction with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 30–100 nM) were obtained in the absence of PMN (●, $n = 49$) and in the presence of 2.5×10^6 PMN ml^{-1} (■, $n = 14$). The results are expressed as a percentage of the precontraction. Mean are given, with s.e.mean shown as vertical lines. The maximal relaxations were significantly different as calculated by the unpaired two-tailed Student's t test (*$P < 0.001$).

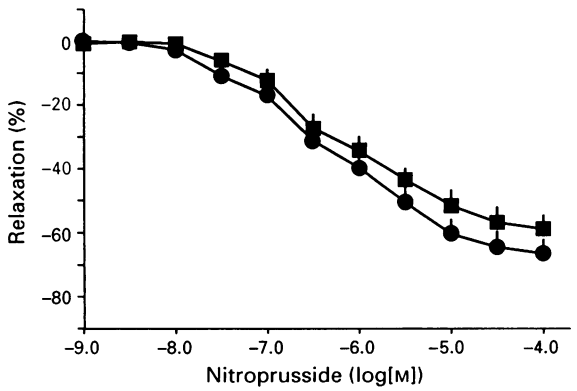


Figure 2 Concentration-effect curves of nitroprusside in bovine isolated mesenteric arteries in the absence (●, $n = 8$) and presence of 2.5×10^6 polymorphonuclear leukocytes (PMN) ml^{-1} (■, $n = 8$). The relaxation is expressed as a percentage of the precontraction with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 30–100 nM). The force of precontraction was 244 ± 35 mN for control and 242 ± 28 mN for PMN incubation. Mean are given with s.e.mean shown as vertical lines.

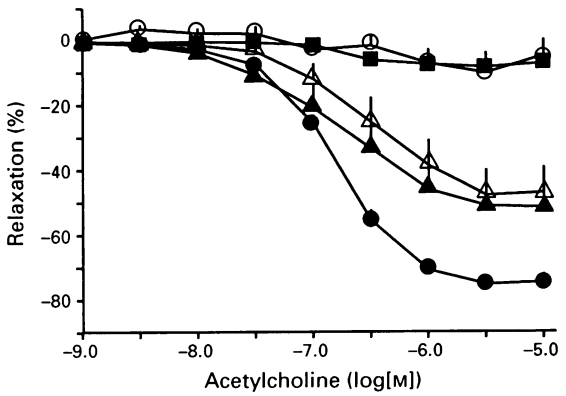


Figure 3 Characterization of the acetylcholine-induced relaxation in bovine isolated mesenteric arteries. Acetylcholine concentration-effect curves were obtained in intact vascular rings (control, ●, $n = 42$), in the presence of 1 μM indomethacin (▲, $n = 10$), in the presence of 300 μM N^G-monomethyl-L-arginine (L-NMMA; △, $n = 12$), in the presence of both L-NMMA (300 μM) and indomethacin (1 μM) (■, $n = 5$), and in endothelium-denuded rings (○, $n = 5$). The relaxation is expressed as the percentage of the precontraction with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 30–100 nM). Mean are given with s.e.mean shown as vertical lines. All treatments significantly decreased the maximal relaxation to acetylcholine, as determined by means of an unpaired two-tailed Student's t test ($P < 0.001$).

was abolished in endothelium-denuded rings. Inhibition of nitric oxide synthesis from L-arginine by L-NMMA (30–300 μM) resulted in a concentration-dependent decrease of the maximal relaxation to acetylcholine. However the maximal vasodilatation was not inhibited completely, 76.1 ± 2.4% for control vs 48.0 ± 8.0% for L-NMMA (300 μM) ($P < 0.001$). The cyclo-oxygenase inhibitor indomethacin (1 μM) also decreased significantly the maximal relaxation to acetylcholine (maximal relaxation: 52.0 ± 7.9%, $P < 0.001$). If the mesenteric arterial rings were incubated simultaneously with L-NMMA (300 μM) and indomethacin (1 μM) the responsiveness to acetylcholine was blocked completely. The precontraction to $PGF_{2\alpha}$ was not influenced by the different treatments.

Effect of indomethacin, L-NMMA and oxygen-radical scavengers

To elucidate the mechanism of action of PMN on the acetylcholine-induced relaxation, the experiments were repeated in the presence of indomethacin, L-NMMA and the oxygen free-radical scavengers SOD and catalase (Figure 4). As indicated above, indomethacin or L-NMMA (300 μM) reduced the maximal relaxation to acetylcholine. Treatment of the arterial rings with both indomethacin and PMN resulted in a further significant reduction of the maximal relaxation. In contrast, simultaneous incubation of L-NMMA (300 μM) and PMN did not result in a further statistically significant reduction. Both oxygen radical scavengers SOD (200 u ml^{-1}) or catalase (2000 u ml^{-1}) had no effect on the maximal relaxation to acetylcholine. SOD, simultaneously incubated with the PMN prevented the decrease in relaxation. However, in the presence of catalase, PMN still diminished the responsiveness of the arterial rings (Figure 4). Table 1 summarizes the force of precontraction, pD_2-value, extent of maximal relaxation and number of independent observations (n) for all acetylcholine experiments.

Superoxide anion production

Lucigenin-enhanced chemiluminescence measurement is indicative for the superoxide anion concentration in the PMN suspension, while total production of superoxide anions is

correlated with the amount of reduced ferricytochrome c. The results on oxygen radical production by PMN are presented in Figure 5. The PMN used in the organ bath studies were not stimulated. Strikingly, basal superoxide anion production is less than 10% compared to the production by PMA-stimulated PMN for both the chemiluminescence test (maximal chemiluminescence: 9.2 ± 3.1%, $n = 6$) and the cytochrome C test (4.8 ± 1.5%, $n = 6$).

Discussion

The present study demonstrates that bovine PMN added to organ baths containing segments of bovine mesenteric arteries decrease the relaxation to acetylcholine. The diminished response is restored by treatment with SOD, which is indicative of involvement of oxygen-derived free radicals. The importance of the endothelium is stressed by the observation that the relaxation to nitroprusside is not affected by addition of PMN.

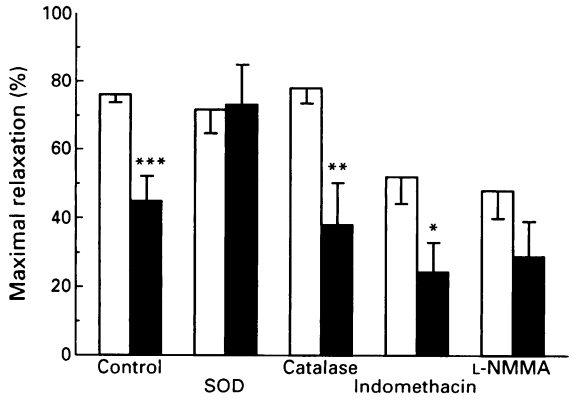


Figure 4 Influence of treatment with indomethacin, N^G-monomethyl-L-arginine (L-NMMA) or oxygen radical scavengers on the polymorphonuclear leukocyte (PMN)-induced decrease in maximal relaxation to acetylcholine in bovine isolated mesenteric arteries. Maximal relaxation to acetylcholine in the absence (open columns) and presence of 2.5×10^6 PMN ml^{-1} after a 25 min incubation period (closed column) is presented as the percentage of the precontraction with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 30–100 nM). Superoxide dismutase (SOD; 200 u ml^{-1}; $n = 7$ resp. 6), catalase (2000 u ml^{-1}; $n = 7$), indomethacin (1 μM; $n = 10$ resp. 6) were added to the organ bath 15 min before the PMN incubation period. L-NMMA (300 μM; $n = 12$ resp. 7) was added after the precontraction 10 min prior to the acetylcholine addition. Means are given with s.e.mean shown as vertical lines. $***P < 0.001$, $**P < 0.01$ and $*P < 0.05$ vs treatment without PMN incubation (two-tailed Student's t test).

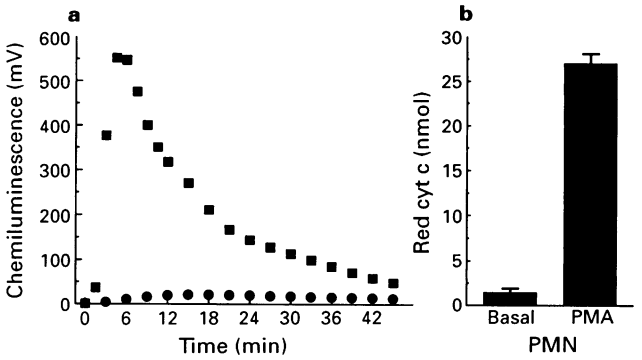


Figure 5 Superoxide anion production by polymorphonuclear leukocytes (PMN) is measured by lucigenin-enhanced chemiluminescence (a) and reduction of ferricytochrome c (Red cyt c; b). Chemiluminescence was measured every 90 s during 45 min in a PMN suspension (2.5×10^6 ml^{-1} in Krebs buffer) containing lucigenin (0.5 mM) at 37°C. The trace is representative for 6 experiments. Reduction of ferricytochrome c was measured after incubation of PMN (2.5×10^6 cells ml^{-1} Krebs buffer) with ferricytochrome c (1.1 mg ml^{-1}) for 45 min at 37°C using a spectrophotometer. The amount of superoxide anion-dependent reduced ferricytochrome c was calculated from paired incubations with and without superoxide dismutase. The actual amount was calculated from the absorbance at 550 nm using the molar extinction coefficient (2.1×10^4 M^{-1} cm^{-1}). Mean of 6 experiments are given with s.e.mean shown as vertical bars.

Table 1 Summary of parameters of vascular relaxation to acetylcholine[a]

		Acetylcholine-induced relaxation		
Treatment	n	*$PGF_{2\alpha}$-precontraction* (force in mN)	*pD_2-values* (− log[M])	*Maximal effect* (% precontraction)
Control	49	216 ± 10	6.80 ± 0.05	76.1 ± 2.4
+ PMN	14	236 ± 14	6.67 ± 0.16	44.9 ± 7.4†††
+ PMN and wash	5	197 ± 24	6.93 ± 0.09	82.9 ± 7.6
SOD (200 u ml^{-1})	7	217 ± 23	6.50 ± 0.10†	71.7 ± 6.9
+ PMN	6	234 ± 23	6.45 ± 0.23	73.2 ± 11.5
Catalase (2000 u ml^{-1})	7	230 ± 16	6.35 ± 0.17††	78.0 ± 4.4
+ PMN	7	227 ± 13	6.02 ± 0.15†	38.1 ± 12.1§§
Indomethacin (1 μM)	10	201 ± 15	6.66 ± 0.13	52.0 ± 7.9†††
+ PMN	6	199 ± 13	6.59 ± 0.13	24.4 ± 8.6§
L-NMMA				
30 μM	5	237 ± 28	6.69 ± 0.13	67.0 ± 9.2
100 μM	9	175 ± 12	6.63 ± 0.10	56.2 ± 8.0††
300 μM	12	212 ± 18	6.50 ± 0.15	48.0 ± 8.0†††
+ PMN	7	251 ± 27	6.70 ± 0.21	28.9 ± 10.4
+ indomethacin	5	218 ± 25	–	8.3 ± 2.2†††
Endothelium denuded	5	175 ± 12	–	14.0 ± 5.5†††

[a]Relaxation to acetylcholine in bovine isolated mesenteric arteries, which were precontracted submaximally with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 30–100 nM). Force of precontraction, pD_2-values and maximal relaxation are presented as mean ± s.e.mean. n represents the number of observations. Polymorphonuclear leukocytes (PMN: 2.5×10^6 ml^{-1}) were incubated in the organ bath for 25 min before the acetylcholine relaxation curve was obtained. Superoxide dismutase (SOD), catalase and indomethacin were added 15 before the PMN. N^G-monomethyl-L-arginine (L-NMMA) was added after the $PGF_{2\alpha}$-precontraction and incubated for 15 min.
Statistical analysis with a two-tailed Student's t test for unpaired observations: $\S P < 0.05$, $\S\S P < 0.01$ vs treatment without PMN incubation; $\dagger P < 0.05$, $\dagger\dagger P < 0.01$, $\dagger\dagger\dagger P < 0.001$ vs control.

Many neurohumoral compounds, e.g. acetylcholine, are able to evoke the release of vasodilator substances from the endothelium (Vanhoutte, 1989; Vane *et al.*, 1990). Two potent dilators released from the endothelium are nitric oxide and prostacyclin. The relaxation to acetylcholine is completely dependent on an intact endothelium. The relaxation to acetylcholine is only partially inhibited by the nitric oxide synthesis inhibitor L-NMMA or the cyclo-oxygenase inhibitor indomethacin. Simultaneous use of these inhibitors blocks the relaxation totally. Therefore it is likely that the endothelium of bovine isolated mesenteric arteries releases two substances, nitric oxide and prostacyclin, which mediate relaxation.

In contrast to acetylcholine, nitroprusside causes relaxation by direct action on the vascular smooth muscle via stimulation of soluble guanylate cyclase. In this way nitroprusside mimics the vasodilator action of endothelium-derived nitric oxide. From the observation that PMN only decrease relaxation to acetylcholine and not to nitroprusside, it is concluded that the effect of the PMN is not related directly to the action of nitric oxide on vascular smooth muscle.

PMN can produce cytotoxic oxygen free radicals. These reactive oxygen metabolites are associated with cell injury in various pathological states, such as acute hypertension, ischaemia and shock (Wei *et al.*, 1985; Wang *et al.*, 1990; Werns & Lucchesi, 1990). Damage to endothelial cells may lead to an altered responsiveness of the vasculature. However, in this study the acetylcholine response is only influenced in the presence of PMN and not when the PMN are washed out of the organ bath after a preincubation period prior to the precontraction. This finding indicates that no irreversible functional damage has occurred. From the literature it is known that oxygen radicals may influence the endothelium-derived vasodilators, nitric oxide (Gryglewski *et al.*, 1986) and prostacyclin (Taylor *et al.*, 1983; Whorton *et al.*, 1985). The PMN-dependent decrease in responsiveness to acetylcholine is similar in the absence or presence of the cyclo-oxygenase inhibitor, indomethacin. This result indicates that PMN affect the nitric oxide pathway, because the prostacyclin release is inhibited by indomethacin. Furthermore, in the presence of the nitric oxide synthesis inhibitor L-NMMA (300 μM) PMN induce only a minor decrease which is not found to be significant. Therefore, the PMN influence the nitric oxide pathway, but probably do not mediate alterations in the release or action of prostacyclin.

One of the pharmacological criteria to identify endothelium-derived relaxing factor (EDRF) as nitric oxide is that its action is augmented by SOD in a superfusion cascade. Nitric oxide is a labile substance and its stability is decreased by superoxide anion, but not by other oxygen-derived reactive metabolites, such as hydrogen peroxide (Gryglewski *et al.*, 1986). Indeed, we find that the decrease in relaxation to acetylcholine by PMN is completely restored by SOD, but not by the hydrogen peroxide scavenger catalase.

Unstimulated PMN used in the present investigation are able to produce superoxide anions as shown by reduction of ferricytochrome c and chemiluminescence in the presence of lucigenin. The amount of radicals produced is limited compared to stimulated PMN. However, the behaviour of PMN near the arterial rings in the organ bath may be different. Adhesion and activation may occur. In the literature, activated PMN and oxygen radical generating systems like xanthine/xanthine-oxidase, have been shown to cause irreversible change in arterial reactivity (Carpenter *et al.*, 1987; Pieper & Gross, 1989; Lawson *et al.*, 1990; Todoki *et al.*, 1992; Tsao *et al.*, 1992). However, the present finding indicates that the endothelium-dependent relaxation was completely preserved, if the PMN were washed out of the organ bath after the incubation period prior to the precontraction and acetylcholine relaxation. Therefore, adhesion of PMN and endothelial injury did not seem to play a role.

In summary, bovine PMN present in the organ bath cause a SOD-inhibitable suppression of the acetylcholine-induced relaxation of bovine mesenteric arteries. It is concluded that small amounts of superoxide anions produced by PMN impair endothelium-dependent relaxation by direct interference with endothelium-derived nitric oxide.

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[Leu^8]des-Arg^9-bradykinin inhibits the angiogenic effect of bradykinin and interleukin-1 in rats

De-En Hu & [1]Tai-Ping D. Fan

Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ

1 Subcutaneous implantation of sterile polyether sponges in rats elicited a reproducible neovascular response over 14 days, as determined by measurements of relative sponge blood flow by a ^{133}Xe clearance technique. The angiogenic response was verified by quantitation of haemoglobin contents and histological evaluation of vascularized sponges.

2 Daily administration of 1 nmol of bradykinin (BK) into the implants significantly enhanced the basal sponge-induced neovascularization, leading to higher ^{133}Xe clearance values, increased haemoglobin contents, cellularity and vascularity.

3 When given alone, lower doses of BK (10 pmol) or recombinant human interleukin-1 α (IL-1α, 0.3 pmol) produced no apparent effects on the basal sponge-induced angiogenesis. However, co-administration of these two peptides produced an angiogenic response similar to that elicited by 1 nmol of BK.

4 The BK/IL-1α-induced neovascularization was abolished by the bradykinin B_1 receptor antagonist, [Leu^8]des-Arg^9-BK (1 nmol day^{-1}), but not by the B_2 receptor antagonist Ac-D-Arg-[Hyp^3,D-Phe^7,Leu^8]-BK (1 nmol day^{-1}).

5 Thus, if such interaction between BK and IL-1α contributes to the excessive neovascularization in chronic inflammatory diseases, the blockade of B_1 receptors may provide an effective treatment.

Keywords: Angiogenesis; bradykinin; interleukin-1; angiosuppression; B_1 and B_2 receptor antagonists

Introduction

Angiogenesis is an important physiological process closely involved with normal embryonic development, endometrial regeneration and wound healing. Angiogenic diseases such as rheumatoid arthritis, diabetic retinopathy and cancer are thought to be due to the imbalance between stimulators and inhibitors of angiogenesis (Folkman & Klagsbrun, 1987). One potentially attractive approach to achieve angiosuppression is to block the activity of angiogenic factors at the receptor level. However, little work in this area has been undertaken.

Bradykinin (BK) is an endogenous peptide which causes vasodilatation, oedema and pain. It is detected in large quantities in rheumatoid synovial fluid (reviewed in Regoli & Barabe, 1980; Proud & Kaplan, 1988). More recent *in vitro* studies showed that BK also interacts with the inflammatory cytokine interleukin-1 (IL-1) to stimulate proliferation of BALB/3T3 fibroblasts (Kimball & Fisher, 1988) and prostaglandin E_2 (PGE_2) release by human synovial cells (O'Neill & Lewis, 1989) and gingival fibroblasts (Lerner & Modeer, 1991). These observations have implicated BK in the pathogenesis of chronic inflammatory diseases.

The aim of the present study was to investigate whether BK, alone or in combination with IL-1, would stimulate angiogenesis *in vivo*. Furthermore, we attempted to characterize the BK receptors associated with such a response using B_1 and B_2 receptor antagonists.

Methods

The sponge implant model

Sterile circular polyether sponge discs (5 mm thick, 1.2 cm diameter) with central cannulae (1.3 cm long, 1.4 mm internal diameter) were implanted subcutaneously in male Wistar rats (180–200 g) after induction of neuroleptanalgesia by Hypnorm (0.315 mg ml^{-1} fentanyl citrate and 10 mg ml^{-1} fluanisone; 0.1 ml kg^{-1}, i.m.). Test substances (50 μl) were injected daily into the sponges through the cannulae and the neovascular response was assessed as a function of blood flow through the implants by use of a ^{133}Xe clearance technique described by Andrade *et al.* (1987).

Two additional approaches were taken to verify the angiogenic response. First, the sponges were homogenized in phosphate buffered saline (PBS) and their haemoglobin contents measured colorimetrically with a commercial kit. Second, sponge sections (10 μm) were stained with haematoxylin and eosin (H&E) or an endothelial cell marker *Bandeirea simplicifolia* lectin I, isolectin B_4 (BSL-B_4) for the assessment of cellularity and vascularity, respectively. The specimens were analysed 'blind' and recorded on Ektachrome ASA 64T film.

Materials

Recombinant human IL-1α was kindly supplied to Dr Jerry Saklatvala of Strangeways Research Laboratory, Cambridge. Other materials were purchased from the following companies: BK, [Leu^8]des-Arg^9-BK (Peninsula Laboratories, UK); Ac-D-Arg-[Hyp^3,D-Phe^7,Leu^8]-BK (Novobiochem Co., UK); ^{133}Xenon injection (10 mCi in 3 ml saline, Amersham International, UK); polyether foam sheet (R.E. Carpenter & Co., Suffolk, UK); polythene tubings (Portex Ltd., UK); Hypnorm (Janssen Pharmaceuticals, UK); *Bandeirea simplicifolia* lectin I, isolectin B_4 (BSL-B_4, Vector Laboratories Ltd., Peterborough, UK); phosphate buffered saline (PBS) and haemoglobin kit (Sigma Chemical Co. Ltd., UK). All solutions were prepared daily in PBS and sterilized by membrane filtration (0.22 μm) before use.

Statistical analysis

Statistical analysis was performed by two-tailed Student's *t* test for unpaired observations.

[1] Author for correspondence.

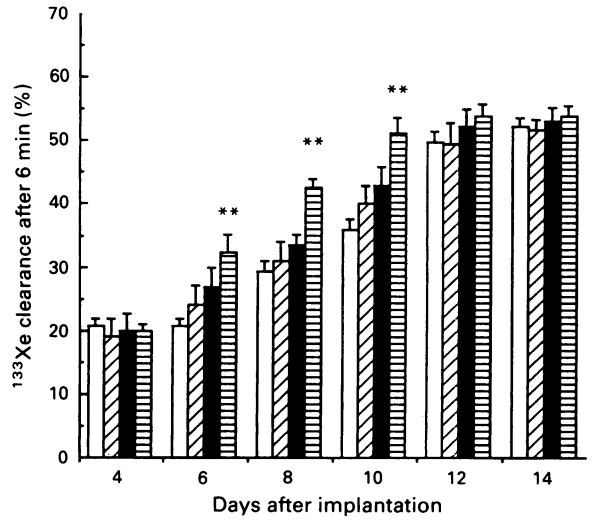


Figure 1 Effect of bradykinin (BK) on sponge-induced angiogenesis. Symbols represent sponges treated daily with phosphate buffered saline (□), 10 pmol BK (▨), 100 pmol BK (■) and 1000 pmol BK (▤). Data represent the mean ± s.e. $n = 4-10$. $^{**}P < 0.01$ (1000 pmol BK vs PBS).

Results

Basal sponge-induced angiogenesis

Subcutaneous implantation of sterile polyether sponges in rats elicited a reproducible neovascular response over 14 days, as demonstrated by (i) a gradual increase in the ability of the vascularized sponges to clear ^{133}Xe-saline (Figure 1) and (ii) a corresponding elevation of haemoglobin contents in sponges recovered at various intervals (Figure 2).

Enhanced angiogenic response induced by bradykinin and interleukin-1

Figure 1 also shows daily administration of BK into the implants caused a dose-dependent enhancement of ^{133}Xe clearance, suggesting that 1 nmol doses BK augmented the basal sponge-induced neovascular response. This enhanced angiogenic response was verified histologically. Figure 3a shows the increased cellularity in the test sponges as compared to controls (Figure 3b). Furthermore, the BK-treated sponges showed a distinct increase in vascularity as identified by the endothelial cell marker *Bandeirea simplicifolia* lection I, isolectin B_4 (Figure 3d and 3e).

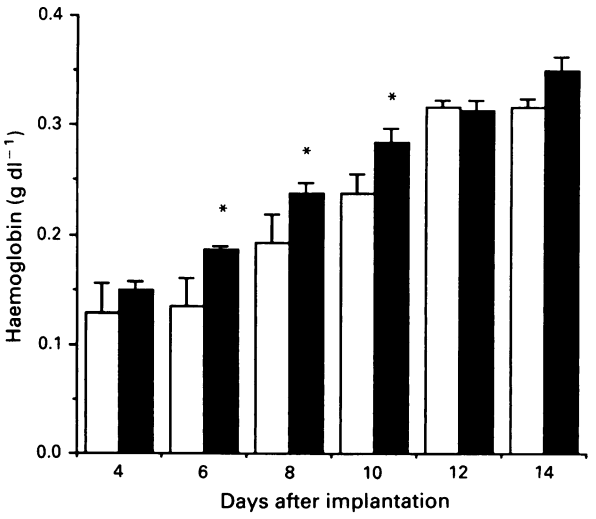


Figure 2 Effect of bradykinin (BK) and interleukin-1α (IL-1α) on the haemoglobin contents in sponge implants. Symbols represent sponges treated daily with phosphate buffered saline (□) or a combination of BK and IL-1α (■). Data represent the mean ± s.e. $n = 4-10$. $^{*}P < 0.05$.

Lower doses of BK (10 pmol or 100 pmol) did not influence the basal sponge-induced neovascular response (Figure 1). However, subthreshold doses of BK (10 pmol) interacted with subthreshold doses of IL-1α (0.3 pmol) to produce an enhancement of angiogenesis (Figure 4) which was similar in magnitude to that elicited by 1 nmol BK (Figure 1). Consistently higher levels of haemoglobin were detected in sponges treated with 10 pmol BK plus 0.3 pmol of IL-1α (Figure 2). The angiogenic effect of BK/IL-1α was confirmed histologically (Figure 3c and 3f).

Blockade of bradykinin-induced angiogenesis by B_1 receptor antagonist

Since kinins act through at least two different receptors (B_1 and B_2), we attempted to characterize the receptor subtype associated with the angiogenic activity of BK. Figure 5a shows that the effect of BK was abolished by the B_1 receptor antagonist [Leu^8]des-Arg^9-BK (1 nmol), but not by the same doses of B_2 receptor antagonist Ac-D-Arg-[Hyp^3,D-Phe^7,Leu^8]-BK (Figure 5b).

Discussion

The two most significant findings of this paper are that subthreshold doses of BK and IL-1α can interact to produce an intense neovascular response in rats, and that this angiogenic response can be blocked by the B_1 receptor antagonist [Leu^8]des-Arg^9-BK. These results not only provide further evidence for an important role of BK in the pathogenesis of chronic inflammatory diseases, but also suggest the possible involvement of B_1 receptors in angiogenesis.

Angiogenesis is a multi-step process that involves degradation of basement membrane by metalloproteases, migration and proliferation of endothelial cells and capillary tube formation (Folkman & Klagsbrun, 1987). BK and IL-1 can elicit new blood vessel formation by multiple pathways. IL-1 may have a direct action since it is potent inducer of collagenase (Dinarello, 1991) and a mitogen for microvascular endothelial cells (Detmar *et al.*, 1992). In contrast, it is not known if BK has a direct action in angiogenesis. The effect of BK may result in part from the generation of active metabolites. For example, cleavage of the C-terminal arginine residue leads to the formation of the biologically active metabolite des-Arg^9-BK, which is a B_1 agonist. Although the physiological role of B_1 receptors is not well established, they appear to be related to the regulation of tissue repair and cell growth. Indeed, B_1 receptors are synthesized *de novo* following tissue injury (Regoli & Barabé, 1980) and the responsiveness of these receptors is up-regulated by IL-1 (Deblois *et al.*, 1988). Furthermore, des-Arg^9-BK has been shown to stimulate DNA synthesis by cultured lung fibroblasts. This action of des-Arg^9-BK is inhibited by the B_1 antagonist [Leu^8]des-Arg^9-BK (Goldstein & Wall, 1984). It is also possible that BK causes the release of substance P from capsaicin-sensitive sensory nerves (Geppetti *et al.*, 1991). Substance P has already been shown to stimulate endothelial cell proliferation *in vitro* and angiogenesis *in vivo* (Ziche *et al.*, 1990).

BK has been shown to stimulate phophatidylinositol turnover, calcium mobilization, arachidonate release, and production of prostaglandins, platelet-activating factor and endothelium-derived relaxing factor in endothelial cells, via the activation of B_2 receptors (see Proud & Kaplan, 1988). Lerner & Modeer (1991) demonstrated that both B_1 (des Arg^9-BK) and B_2 (BK, Lys-BK) receptor agonists synergize with IL-1β to stimulate PGE_2 production by human gingival fibroblasts. Our data suggest that B_1 receptors play a prominent role in angiogenesis.

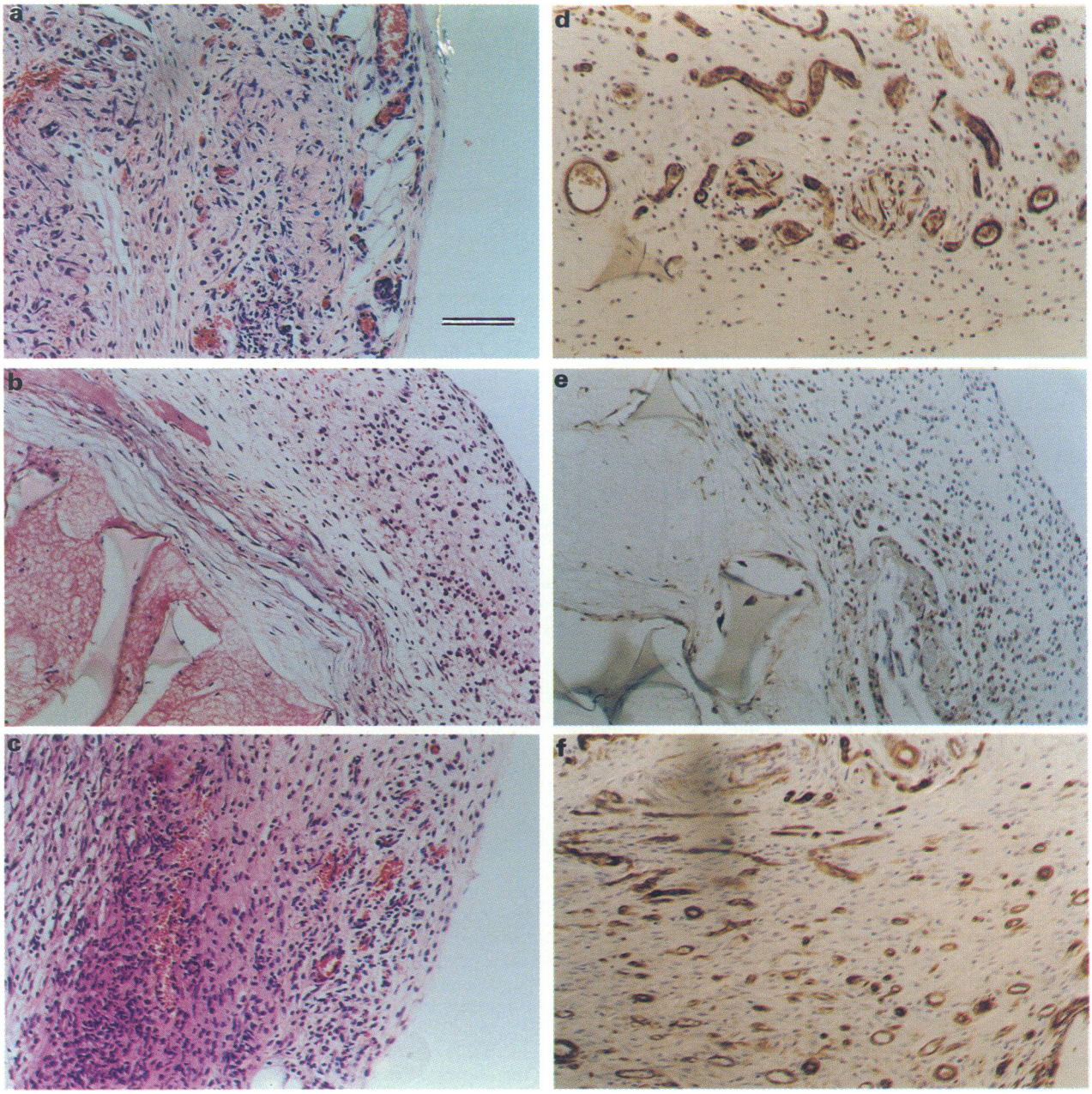
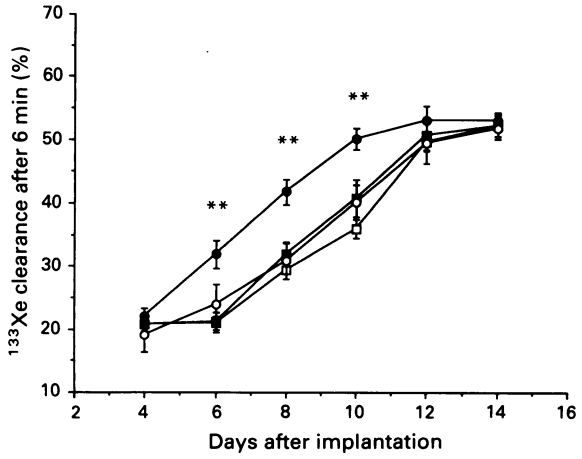


Figure 3 Histological sections of 10-day old sponges illustrating the intense neovascular response induced by bradykinin (BK, 1 nmol day^{-1}) or bradykinin/interleukin-1α (BK/IL-1α, 10 pmol/0.3 pmol day^{-1}). Bar = 100 μm. (a) Sponge treated with BK (1 nmol day^{-1}; haematoxylin and eosin (H&E) stained). (b) Sponge treated with phosphate buffered saline (PBS, 50 μl; H&E stained). (c) Sponge treated with BK/IL-1α (10 pmol/0.3 pmol day^{-1}; H&E stained). (d) Sponge treated with BK (1 nmol day^{-1}; BSL-B_4-stained, see Methods). (e) Sponge treated with PBS (50 μl; BSL-B_4-stained). (f) Sponge treated with BK/IL-1α (10 pmol/0.3 pmol day^{-1}; BSL-B_4-stained).



It might be argued that vascular reactivity to BK might interfere with the assessment of angiogenesis by ^{133}Xe measurement. However, the new vessels will have to be developed before being influenced by BK. To exclude their possible acute effects on the microvasculature (dilatation or constriction), the test substances were always injected via the cannulae into the sponges 24 h before ^{133}Xe measurements. As we have previously reported, laser Doppler flowmetry studies showed that BK (1 nmol) produced only a transient

Figure 4 Effect of bradykinin (BK) and interleukin-1α (IL-1α) on sponge-induced angiogenesis. Symbols represent sponges treated daily with phosphate buffered saline (PBS) (□), 10 pmol BK (○), 0.3 pmol IL-1α (■) and 10 pmol BK plus 0.3 pmol IL-1α (●). Data represent the mean ± s.e. $n = 4-10$. $^{**}P < 0.01$ (BK plus IL-1α vs PBS).

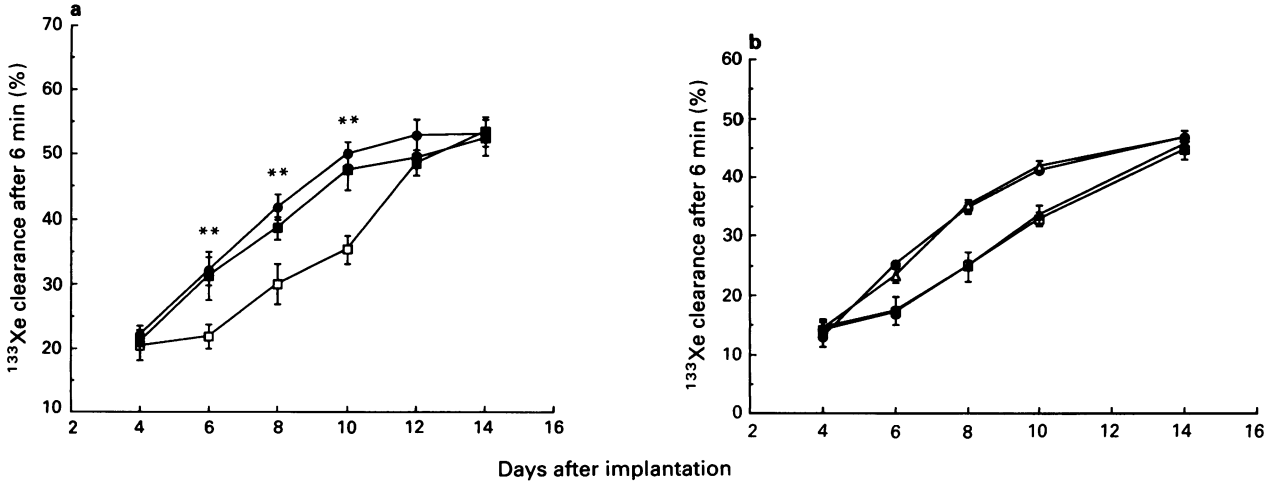


Figure 5 (a) Effect of [Leu^8]des-Arg^9-BK on angiogenesis induced by bradykinin (BK) and interleukin-1α (IL-1α) (10 pmol and 0.3 pmol). Symbols represent sponges treated daily with BK and IL-1α plus phosphate buffered saline (PBS) (●) or BK and IL-1α plus 100 pmol (■) or 1000 pmol (□) of the antagonist. Data represent the mean ± s.e. $n = 4-10$. $^{**}P < 0.01$ (BK and IL-1α plus 1000 pmol vs BK and IL-1α plus PBS). (b) Effect of Ac-D-Arg-[Hyp^3,D-Phe^7,Leu^8]-BK on angiogenesis induced by BK and IL-1α. Symbols represent sponges treated daily with PBS (□), BK and IL-1α plus PBS (●), BK-IL-1α plus 1000 pmol (△) or 100 pmol (▲) of the antagonist. Data represent the mean ± s.e. $n = 4-10$. The effect of the B_2 antagonist + BK + IL-1α was not statistically different from PBS + BK + IL-1α. This was not due to any agonist effect of the B_2 antagonist since the results with the antagonist were not significantly different from the PBS control.

(< 10 min) increase in rat cutaneous blood flow (Fan *et al.*, 1992). On the other hand, intradermal injection of 10 pmol BK, 0.3 pmol IL-1α or co-injection of these two peptides failed to show any local blood changes. Parallel studies with the ^{133}Xe clearance technique to follow the effects of BK and other peptides at 6 h, 12 h and 24 h after injection also failed to show any protracted vascular responses (unpublished data). Furthermore, these peptides did not show any vasoactivity in 14-day old sponges.

In conclusion, we have shown that [Leu^8]des-Arg^9-BK can inhibit the angiogenic response elicited by a combination of subthreshold doses of BK and IL-1. These data illustrate the potentially attractive approach to achieve angiosuppression by blocking the activity of angiogenic factors at the receptor level.

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Role of cyclic AMP in promoting the thromboresistance of human endothelial cells by enhancing thrombomodulin and decreasing tissue factor activities

Gisèle Archipoff, [1]Alain Beretz, Katalin Bartha, Christine Brisson, Corinne de la Salle, Catherine Froget-Léon, Claudine Klein-Soyer & [2]Jean-Pierre Cazenave

Centre Régional de Transfusion Sanguine, INSERM U.311, 10 rue Spielmann, 67085 Strasbourg Cédex, France

1 The effects of forskolin, prostaglandin E_1 (PGE_1), dibutyryl cyclic AMP (db cyclic AMP), dibutyryl cyclic GMP (db cyclic GMP) and 3-isobutyl-l-methyl-xanthine (IBMX) were investigated on the expression of tissue factor and thrombomodulin activities on the surface of human saphenous vein endothelial cells (HSVEC) in culture.

2 Forskolin (10^{-6} to 10^{-4} M), PGE_1 (10^{-7} to 10^{-5} M) and db cyclic AMP (10^{-4} to 10^{-3} M) caused a concentration-dependent decrease of cytokine-induced tissue factor activity.

3 Similar concentrations of forskolin, PGE_1 and db cyclic AMP enhanced significantly constitutive thrombomodulin activity and reversed the decrease of this activity caused by interleukin-1 (IL-1).

4 IBMX (10^{-4} M) decreased tissue factor activity and enhanced the effect of forskolin on tissue factor and thrombomodulin activities.

5 Forskolin (10^{-4} M) decreased the IL-1-induced tissue factor mRNA and increased the thrombomodulin mRNA level. IL-1 did not change the thrombomodulin mRNA level after 2 h of incubation with HSVEC in culture.

6 Dibutyryl cyclic GMP (10^{-4} M to 10^{-3} M) did not influence tissue factor or thrombomodulin activity.

7 Our data suggest that elevation of intracellular cyclic AMP levels may participate in the regulation of tissue factor and thrombomodulin expression, thus contributing to promote or restore antithrombotic properties of the endothelium.

Keywords: Endothelial cells; thrombomodulin; tissue factor; cyclic AMP; forskolin; prostaglandin E_1; interleukin-1; thrombin; cyclic GMP

Introduction

One of the remarkable features of the coagulation system is its ability to respond rapidly to seal a wound site without the haemostatic plug extending to occlude the vessel lumen. This property is due to both surface properties and secreted products of endothelial cells. The endothelial cell surface participates in the activation or inactivation of coagulation factors and their cofactors, by binding them to receptors or to negatively charged membrane phospholipids. Endothelium synthesizes antithrombotic agents such as prostacyclin, endothelium-derived relaxing factor (EDRF), plasminogen activators, 13-hydroxyoctadecadienoic acid (13-HODE) and heparan sulphates (Fajardo, 1989). One of the anticoagulant mechanisms involves thrombomodulin, a membrane glycoprotein which is constitutively expressed by endothelium and is a specific receptor for thrombin. Thrombomodulin exhibits two complementary anticoagulant functions: one is to decrease the ability of thrombin to catalyze clot formation and the other is to convert thrombin into a potent protein C activator (Freyssinet & Cazenave, 1988; Esmon, 1989; Dittman & Majerus, 1990). Thrombin forms a 1:1 complex with thrombomodulin and this complex activates protein C into activated protein C. Activated protein C functions as an anticoagulant by inactivating activated factors V and VIII (FVa and VIIIa), two essential cofactors of two serine-proteases, respectively factors Xa and IXa. Activated protein C thus exerts a potent negative feedback control on the generation of thrombin.

Under normal conditions, endothelial cells present a non-thrombogenic surface, which is non-reactive to platelets or coagulation factors. But when injured, they rapidly become procoagulant and capable of initiating blood coagulation. In this activated state, they synthesize tissue factor (TF), a transmembrane glycoprotein which is essential in the initiation of the extrinsic coagulation pathway. Tissue factor is the receptor for factors VII and VIIa; the complex TF-FVII/VIIa catalyzes the conversion of factors X to Xa and IX to IXa (Nemerson, 1988). These activated factors contribute to the generation of thrombin at the surface of the cells Endothelium synthesizes tissue factor in some pathological situations or when exposed to stimuli such as cytokines, thrombin or endotoxin (Boeri *et al.*, 1989; Zuckerman & Surprenant 1989; Conway *et al.*, 1989; Almus *et al.*, 1989; Noguchi *et al.*, 1989; Crossman *et al.*, 1990; Archipoff *et al.*, 1991; Pettersen *et al.*, 1992). Some of these stimuli have also been described as reducing thrombomodulin activity *in vivo* or *in vitro*: endotoxin (Moore *et al.*, 1987), interleukin 1 (IL-1) (Nawroth *et al.*, 1986; Hirokawa & Aoki 1991), tumour necrosis factor (Gerlach *et al.*, 1989; Moore *et al.*, 1989; Scarpati & Sadler, 1989; Hirokawa & Aoki, 1991). The effect of thrombin is debated. Thrombin was found to cause the internalization of the thrombin:thrombomodulin complexes by human umbilical cord vein endothelial cells and A549 lung cancer cells (Maruyama & Majerus, 1985), but such endocytosis was not observed in human saphenous vein endothelial cells or in the EA.hy 926 cell line obtained by fusing human umbilical vein endothelial cells with the A549 lung cancer cells (Beretz *et al.*, 1989).

[1] Present address: Laboratoire de Pharmacologie Cellulaire et Moléculaire, URA CNRS 600, Université Louis Pasteur, Faculté de Pharmacie, BP 24, 67401 Illkirch-Graffenstaden, France.
[2] Author for correspondence.

Endothelial cells are thus able to regulate the expression of tissue factor and thrombomodulin, two glycoproteins with opposed functions. Under normal conditions, the constitutive expression of thrombomodulin and the absence of induced tissue factor on the intact endothelial cell surface contributes to the maintenance of an anticoagulant state of the endothelium. Abnormalities in the balance between pro and anticoagulant properties of the endothelium could contribute to the pathogenesis of human thrombotic diseases (Gimbrone, 1986). Pharmacological manipulation of these endothelial properties would therefore be useful in the prevention or treatment of such thrombotic disorders. Cyclic AMP is known to be an important intracellular second messenger involved in a wide variety of endothelial cell functions. Our aim was to study the role of cyclic AMP in the modulation of tissue factor and thrombomodulin activities. We show here that agents which elevate cyclic AMP enhance thrombomodulin activity in HSVEC and reverse the effects of cytokines on tissue factor and thrombomodulin activities.

Methods

Cell culture

Human endothelial cells (HSVEC) were collected from fragments of saphenous veins obtained during coronary-bypass surgery (Beretz *et al.*, 1989) and cultured as previously described (Klein-Soyer *et al.* 1986). The culture medium was M199/RPMI 1640 (1:1) containing 10 mM HEPES, 2 mM L-glutamine, antibiotics (100 units penicillin and 100 μg streptomycin ml^{-1}), fungizone (0.25 $\mu g\ ml^{-1}$) and 30% human serum. It was verified by a Limulus chromogenic assay (Coatest: Kabi Vitrum, Mölndal, Sweden) that all media and buffers had an endotoxin content which never exceeded 0.04 $ng\ ml^{-1}$. The cells were frozen at the second passage and used in experiments from the 3rd to the 10th passage. Preliminary experiments were carried out in order to verify that the number of passages did not alter cell responses. The cells were subcultured in 96-multiwell plates at an initial cell density of 10^4 cells cm^{-2} for concentration-effect and kinetics experiments and in 6-well plates for extraction of mRNA. All plates were precoated with purified human fibronectin (0.5 $mg\ ml^{-1}$).

Assays for thrombomodulin and tissue factor activities

For each experimental condition simultaneous measurements of tissue factor and thrombomodulin activities were performed on the same cell population.

Concentration-effect studies The culture medium was changed 24 h before each assay. For the 24 h incubation period, this medium contained either forskolin, PGE_1, dbcyclic AMP or dbcyclic GMP. Before the last 4 h of incubation, the cells were rinsed 3 times with RPMI/M199 (v/v) and then incubated with RPMI/M199 containing 10 $mg\ ml^{-1}$ HSA and either forskolin PGE_1, IBMX, dbcyclic AMP or dbcyclic GMP and Il-1, TNF or thrombin. In forskolin, PGE_1 and IBMX concentration-effect studies, IL-1 was used at 0.2 $u\ ml^{-1}$ to induce tissue factor activity and at 1 $u\ ml^{-1}$ to decrease thrombomodulin activity. In dbcyclic AMP and dbcyclic GMP concentration-effect studies, 1 $u\ ml^{-1}$ IL-1 (57 nM), 1 $ng\ ml^{-1}$ TNF (22 pM) and 1 $u\ ml^{-1}$ thrombin (8.8 nM) were used in both tissue factor and thrombomodulin assays. After 4 h of incubation at 37°C, the cells were rinsed 3 times with PBS. Tissue factor and thrombomodulin activities were then measured. For this purpose, the cell monolayers were covered with 200 μl of RPMI/M199 without phenol red, containing 3 $mg\ ml^{-1}$ HSA. For tissue factor assays, purified human factors VII (5 nM final concentration) and X (400 nM final concentration) were added to each well. After 20 min at 37°C, the supernatants were transferred into 96 multiwell plates for reading. The amidolytic activity of generated factor Xa was measured at 405 nm, using the specific chromogenic substrate S2765 (0.2 mM final concentration) in a kinetic microplate reader coupled to a microcomputer (Molecular Devices, Palo Alto, CA., U.S.A.). Thrombomodulin activity was evaluated in the same way by addition to the wells of thrombin (0.25 NIH $u\ ml^{-1}$, 2.2 nM final concentration) and protein C (65 nM final concentration). After 2 h of incubation at 37°C, the reaction was blocked by addition of hirudin (1.25 a.t.u. ml^{-1}). The amidolytic activity of generated activated protein C was measured as described above, using the substrate S2366 (0.1 mM final concentration). Each experimental condition was assayed in sextuplate wells. The concentration of APC or factor Xa formed was determined by reference to standard curves established with known amounts of factor Xa or APC. Cells from 3 wells per experimental condition were detached with trypsin-EDTA and then counted (Coulter Counter ZM, Coultertronics, Margency, France); the cell number was used to express thrombomodulin and tissue factor activities normalized per 10^4 cells. Cells from the remaining wells were fixed with 2% paraformaldehyde, 20 min at room temperature, and then stained with May-Grünwald Giemsa for subsequent control of the morphology of the cells in the monolayer.

Kinetic studies The cells were first incubated with RPMI/M199 containing 30% human serum and either forskolin (FK) (10^{-5} M), dbcyclic AMP (5×10^{-4} M), IBMX (10^{-4} M) or control buffer. Cells were incubated at 37°C for various times, all incubations being stopped at the same time. Before the last 4 h of incubation, the cell monolayers were washed 3 times with RPMI/M199 (v/v) and then incubated with RPMI/M199 containing 10 $mg\ ml^{-1}$ HSA and the same agents (FK, dbcyclic AMP, IBMX or buffer) plus IL-1 for 4 h at 37°C. IL-1 was used at 0.2 $u\ ml^{-1}$ to induce tissue factor activity and at 1 $u\ ml^{-1}$ to decrease thrombomodulin activity. Thrombomodulin and tissue factor activities were then measured as described above.

Cyclic AMP assay

HSVEC were cultured in 35 mm diameter culture dishes. At confluence the cells were washed 3 times in M199/RPMI medium (v/v), and then incubated at 37°C for 15 min with 1 ml of M199/RPMI containing 1% human albumin and 100 μM IBMX (final concentration), with or without forskolin or PGE_1. All following manipulations were done on ice. The cells were rinsed twice with ice cold PBS and 500 μl ice cold PBS plus 50 μl 6.6 N perchloric acid was added. After 10 min, the supernatant was removed and particulate material was pelleted by centrifugation. The supernatant was transferred to a 5 ml polystyrene tube and cyclic AMP was extracted as described by Khym (1975) with a mixture of trioctylamine and freon (33 ml/42 ml); 2 ml of this mixture were added to the supernatant and mixed thoroughly for 2 min. The upper aqueous phases was transferred to an Eppendorf tube, lyophilized and the dry extract dissolved in the buffer provided in the commercial radioimmunoassay kit (Amersham) used for cyclic AMP measurement. The cells were then fixed with 2% paraformaldehyde for 20 min at room temperature, stained with May-Grünewald Giemsa and counted in order to normalize the level of cyclic AMP per 10^6 cells.

Immunoelectron microscopy

HSVEC grown in 35 mm dishes were fixed in paraformaldehyde-glutaraldehyde mixture (2%–0.5%), followed by permeabilization with digitonin (10^{-6} M). The cells were incubated first with specific Fab-fragments of rabbit anti-TM antibodies (30 $\mu g\ ml^{-1}$). Fab-fragments of goat anti rabbit-IgG antibodies coupled to peroxidase (Biosys, Compiègne,

France) were used as a second antibody. Peroxidase activity was visualized according to Graham & Karnovsky (1966). Post fixation in osmium tetroxide and embedding in Epon were performed according to Tougard & Picart (1986). Ultrathin sections were counterstained with uranyl acetate and examined with a Siemens Elmiskop 10.

Extraction of RNA from endothelial cells

As for concentration-effect studies, after washing, cells were incubated with M199/RPMI containing 10 mg ml^{-1} HSA and either 10^{-4} M forskolin, 1 u ml^{-1} IL-1 or both or control buffer for 2 h at 37°C. RNA was extracted from about 4×10^5 cells from each well by a classical technique with guanidium isothiocyanate (Chirgwin *et al.*, 1979). Briefly, 1 ml of guanidium isothiocyanate (4 M) cellular lysate was overlaid onto a 0.5 ml caesium chloride (5.7 M) cushion and centrifuged 3 h at 55,000 r.p.m. in the TLS rotor of a Beckman TL 100 centrifuge. The pellet was resuspended in 200 µl of 0.3 M sodium acetate and precipitated with ethanol. After centrifugation, the pellet was dried and resuspended in water and the RNA concentration was determined by O.D. at 260 nm.

Reverse transcription and polymerase chain reaction method

For each assay condition, reverse transcription (RT) was performed on 100 ng of total cellular RNA. The mRNA was reverse transcribed directly in the polymerase chain reaction (PCR) buffer (50 mM KCl, 10 mM Tris HCl, 1.5 mM $MgCl_2$, 0.001% gelatine, pH 8.3) with 3 pmol of the 3' PCR specific primer (respectively for thrombomodulin, tissue factor or glyceraldehyde phosphodeshydrogenase (GAPDH)), 1.25 mM of each dNTP and 20 units of MMLV reverse transcriptase in a final volume of 30 µl, at 37°C for 30 min. Then 10 pmol of each 3' and 5' primer was added and the reaction mixture was adjusted to a final volume of 50 µl with PCR buffer, pH 10. After an initial denaturation step of 1.5 min at 94°C, the tubes were put on ice and 1 unit of Taq polymerase was added. The mixture was then covered with about 40 µl of mineral oil to avoid evaporation and the tubes were transferred to a DNA thermal cycler for amplification by PCR (Perkin Elmer Cetus).

The primers used for thrombomodulin amplification were 5' primer: 5'CATGTGCGACCGGCTACCGGCTGGCGG 3', corresponding to nucleotides 1071 to 1100, and 3' primer: 5'AGGGGCTGGCACTGGTACTCGCAGTTGGC 3', corresponding to nucleotides 1262 to 1291, according to Wen *et al.* (1987). PCR conditions were 30 cycles, each consisting of 2 min denaturation at 94°C, 2 min hybridization at 51°C and 3 min elongation at 72°C.

The primers used for tissue factor amplification were 5' primer: 5' ACTACTGTTTCAGTGTTCAAGCAGTGATTC 3', corresponding to nucleotides 722 to 751, and 3' primer: 5' ATTCAGTGGGGAGTTCTCCTTCCAGCTCTG 3', corresponding to nucleotides 925 to 954, according to Scarpati *et al.* (1987). PCR conditions were 30 cycles of 1.5 min denaturation at 94°C, 2 min hybridization at 62°C and 3 min elongation at 72°C.

GAPDH mRNA was chosen as housekeeping gene (Maier *et al.*, 1990). The GAPDH mRNA level was thus used as a control to correct for errors in RNA concentration in the starting material an amplification of GAPDH being performed on the same aliquot of cellular RNA for each condition tested in thrombomodulin and tissue factor assay. The primers used were 5' primer: 5' CCACCCATGGCAAATTCCATGGCA 3' and 3' primer: 5' TCTAGACGGCAGGTCAGGTCCACC 3', according to Maier *et al.* (1990). PCR conditions were 30 cycles of 1.5 min denaturation at 94°C, 2 min hybridization at 62°C and 3 min elongation at 72°C.

An aliquot of each sample was loaded onto a 2% agarose gel. After migration the gel was stained with ethidium bromide and photographed with Polaroid 665 negative film. Quantification was performed by scanning the bands with a Biocom 2000 Image Analyzer (Les Ulis, France) and by comparison with the bands obtained for each condition with the GAPDH housekeeping mRNA on the same gel.

Reagents

Human protein C, α-thrombin (3000 NIH u mg^{-1}) and factors X and VII were purified from human plasma as previously described (Freyssinet *et al.*, 1988). Purified human fibronectin was prepared according to a published method (Ruoslahti *et al.*, 1982). Materials were obtained from the following sources: cell culture media (M199, RPMI 1640, HEPES, L-glutamine, penicillin, streptomycin, fungizone and trypsin-EDTA solution [trypsin (0.5 g l^{-1})/EDTA (0.2 g l^{-1})/NaCl (0.85 g l^{-1})]) were from Gibco, Paisley, UK; phosphate buffered saline (PBS) and endotoxin-free human serum albumin (HSA) were from the Centre Régional de Transfusion Sanguine, Strasbourg, France; hirudin was from Stago, Asnières, France; chromogenic substrates S2366 (L-pyroglutamyl-L-prolyl-L-arginine-*p*-nitroanilide hydrochloride) and S2765 (N-α-benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-*p*-nitroanilide hydrochloride) were from Kabi Vitrum, Stockholm, Sweden; human recombinant tumour necrosis factor-α (TNF) (2.10^7 u mg^{-1}, Mr 45,000) and human recombinant interleukin-1-β (IL-1) (10^7 u mg^{-1}, Mr 17,500) were from Boehringer, Mannheim, Germany; forskolin, 3-isobutyl-1-methyl-xanthine (IBMX), prostaglandin E_1. (PGE_1), N^6, 2'-O-dibutyryladenosine 3':5'-cyclic monophosphate (dbcyclic AMP), N^2, 2'-O-dibutyrylguanosine 3':5'-cyclic monophosphate (dbcyclicGMP), agarose type II_A and mineral oil were from Sigma, St. Louis, MO, U.S.A. Human serum was prepared from a pool of 100–150 healthy blood donor negative for hepatitis B virus or HIV. The serum was complement inactivated at 56°C for 30 min. Tris, sodium acetate, guanidium isothiocyanate, kalium chloride, magnesium chloride were obtained from Merck (Rathway, NY, U.S.A.). Caesium chloride was from BRL (Gaithersburg, MD, U.S.A.). SDS was obtained from Pierce Chemical Co (Paris, France). Triton X-100, DNA molecular weight markers VI, desoxyribonucleotides triphosphate and SP6-T7 transcription kit were from Boehringer (Mannheim, Germany). Taq polymerase was purchased from Cetus (Norwalk, CT, U.S.A.). Polaroid film type 665 were from Kodak Company (Rochester, NY, U.S.A.). The oligonucleotides were synthesized by the Laboratoire de Génétique Moléculaire des Eucaryotes (Strasbourg, France); a monoclonal antibody based ELISA-kit against human TM was a kind gift of Dr Amiral, Serbio, Paris, France.

Statistical analysis

The variations of tissue factor and thrombomodulin activities were compared by variance analysis followed by Duncan's multiple comparison test. *P* values less than 0.05 were considered to be statistically significant.

Results

Effects of forskolin

Forskolin (10^{-6} to 10^{-4} M) caused a statistically significant concentration-dependent decrease of tissue factor activity induced by IL-1 (Figure 1a). IL-1 (0.2 u ml^{-1}) induced tissue factor activity from 7.7 ± 1.6 (basal value) to 59 ± 12 pM generated factor Xa min^{-1} per 10^4 cells (mean ± s.e.mean, $n = 3$); 10^{-4} M forskolin caused 80 ± 5% inhibition of this activity after 4 h of incubation. Thrombomodulin activity, under basal conditions, was enhanced by 26 ± 8% after 4 h of incubation with 10^{-4} M forskolin (Figure 1b). One u ml^{-1} IL-1 caused a 15% decrease of basal thrombomodulin

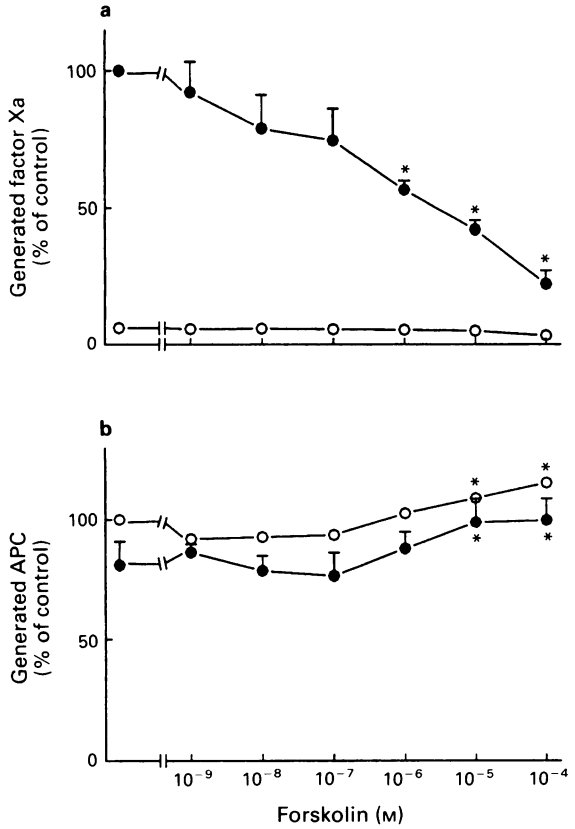


Figure 1 Concentration-effect relationship for forskolin on the expression of tissue factor and thrombomodulin activities at the surface of HSVEC. Confluent monolayers of HSVEC were exposed to the concentrations of forskolin indicated for 4 h in the presence of either interleukin-1 (IL-1) (●) or control buffer (○) tissue factor and thrombomodulin activities were measured as described in Methods. Points correspond to the mean + s.e.mean of three different experiments; each condition was performed in 6 wells. (a) Represents tissue factor activities: results are expressed as % of control value for tissue factor activity at the surface of HSVEC stimulated with 0.2 u ml^{-1} IL-1 in the absence of forskolin. (b) Represents thrombomodulin activities: results are expressed as % of control value of thrombomodulin activity at the surface of unstimulated HSVEC in the absence of forskolin. One u ml^{-1} IL-1 was used to decrease thrombomodulin activity. $^*P<0.05$ vs control (i.e. no forskolin) under the same experimental conditions.

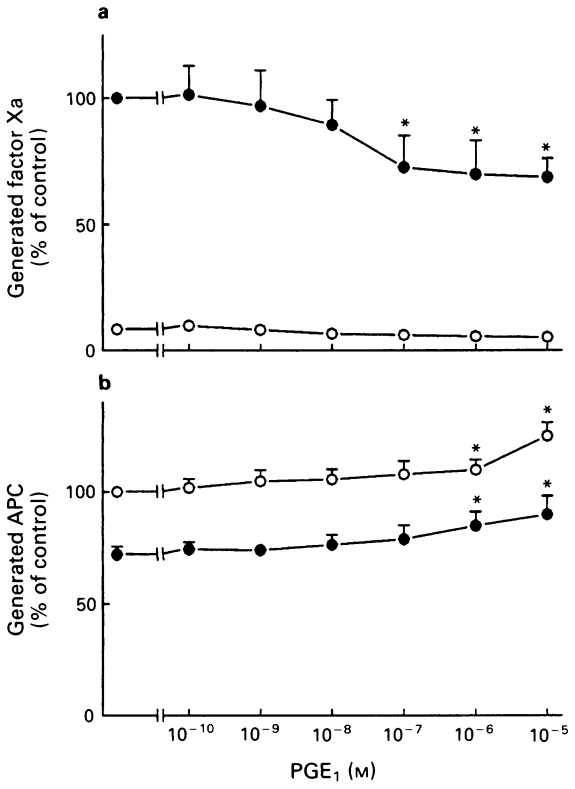


Figure 2 Concentration-effect relationship for prostaglandin E_1 (PGE_1) on the expression of tissue factor and thrombomodulin activities at the surface of HSVEC. Confluent monolayers of HSVEC were exposed to the concentrations of PGE_1 indicated for 4 h in the presence of either interleukin-1 (IL-1) (●) or control buffer (○). Tissue factor and thrombomodulin activities were measured as described in Methods. Points correspond to the mean + s.e.mean of four different experiments; each condition was performed in 6 wells. (a) Represents tissue factor activities: results are expressed as % of control value for tissue factor activity at the surface of HSVEC stimulated with 0.2 u ml^{-1} IL-1 in the absence of PGE_1. (b) Represents thrombomodulin activities: results are expressed as % of control value for thrombomodulin activity at the surface of unstimulated HSVEC in the absence of PGE_1. IL-1 (1 u ml^{-1}) was used to decrease thrombomodulin activity. $^*P<0.05$ vs control under the same experimental conditions.

activity from 22 ± 5 to 18.6 ± 7 pM generated APC min^{-1} per 10^4 cells (mean ± s.e.mean, $n = 3$). Forskolin caused an increase of thrombomodulin activity also in the IL-1 treated cells (Figure 1b). When forskolin at 10^{-4} and 10^{-5} M was added with IL-1, thrombomodulin activity recovered to the basal level of untreated cells.

Effects of prostaglandin E_1

PGE_1 caused a concentration-dependent decrease of induced tissue factor activity. The maximal decrease caused by 10^{-6} and 10^{-5} M PGE_1 reached, respectively, 30 ± 13% and 32 ± 7% after 4 h (Figure 2a). PGE_1 10^{-5} M caused a 25 ± 6% increase of thrombomodulin activity under basal conditions (Figure 2b). IL-1, 1 u mol^{-1} caused a 33 ± 3% decrease of basal thrombomodulin activity. Thrombomodulin activity was not significantly different from basal when PGE_1 10^{-5} M was added at the same time as IL-1.

Effects of 3-isobutyl-1-methylxanthine

IBMX caused a concentration-dependent decrease of induced tissue factor activity, the maximal decrease caused by 5 × 10^{-4} M IBMX reached 56 ± 3% (Figure 3a). IBMX caused a slight but not significant increase of basal thrombomodulin activity and of thrombomodulin activity decreased by IL-1 (Figure 3b).

Effects of dbcyclic AMP

dbCyclic AMP caused a concentration-dependent decrease of induced tissue factor activity and a simultaneous increase in thrombomodulin activity (Figure 4a and b).

dbCyclic AMP (10^{-3} M) decreased tissue factor activity induced by thrombin (to 45 ± 1%), by IL-1 (to 35 ± 12%) or by TNF (to 46 ± 13%) of controls. The same concentration of dbcyclic AMP increased basal thrombomodulin activity by 28 ± 3%. Thrombin alone did not alter constitutive thrombomodulin expression. dbCyclic AMP added with thrombin increased thrombomodulin activity more than dbcylic AMP alone, this increase reaching 45 ± 18% with thrombin plus 10^{-3} M dbcyclic AMP and 35 ± 12% with thrombin plus 5 × 10^{-4} M dbcyclic AMP. dbCyclic AMP, 10^{-3} M, only partially reversed the inhibition caused by IL-1 or TNF.

Concentration-effect studies of dbcyclicGMP were performed on the same cell population as those used to investigate the effect of dbcyclic AMP. In contrast to dbcyclic AMP, dbcyclicGMP (10^{-5} M to 10^{-3} M) did not modulate tissue factor or thrombomodulin activities (Figures 4c and d),

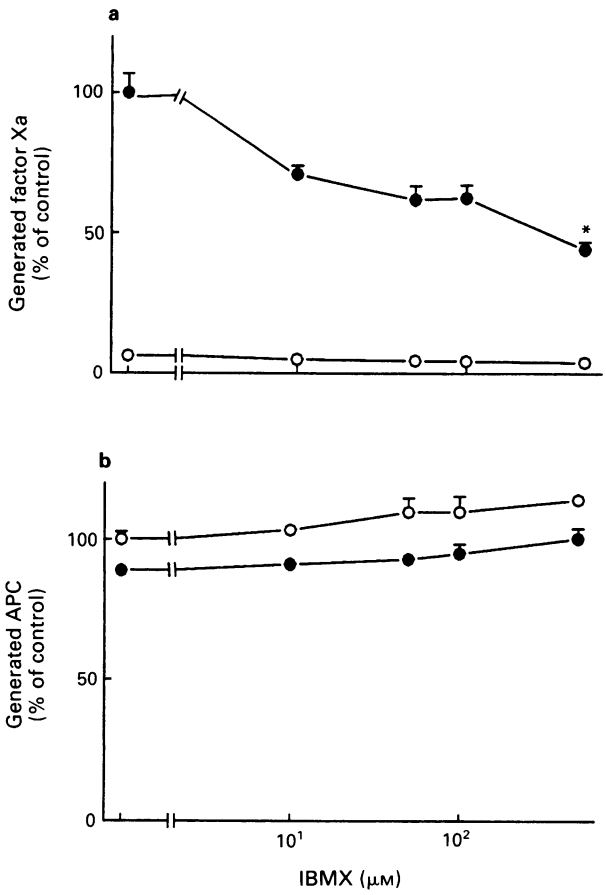


Figure 3 Concentration-effect relationship for 3-isobutyl-1-methyl xanthine (IBMX) on the expression of tissue factor and thrombomodulin activities at the surface of HSVEC. Confluent monolayers of HSVEC were exposed to the concentrations of IBMX indicated for 4 h in the presence of either interleukin-1 (IL-1) (●) or control buffer (○). Tissue factor and thrombomodulin activities were measured as described in Methods. Points correspond to the mean + s.e.mean of three different experiments; each condition was performed in 6 wells. (a) Represents tissue factor activities: results are expressed as % of control value for tissue factor activity at the surface of HSVEC stimulated with 0.2 u ml^{-1} IL-1 in the absence of forskolin. (b) Represents thrombomodulin activities: results are expressed as % of control value for thrombomodulin activity at the surface of unstimulated HSVEC in the absence of forskolin. IL-1 (1 u ml^{-1}) was used to decrease thrombomodulin activity. *$P<0.05$ vs control under the same experimental conditions.

the variations of these activities shown in the figures not being statistically significant.

Kinetic studies of the action of forskolin, IBMX and dbcyclic AMP

Forskolin (10^{-5} M), IBMX (10^{-4} M) or dbcyclic AMP (5×10^{-4} M) (final concentrations) were added to the cells for 4 to 48 h before measurement of tissue factor or thrombomodulin activities. At each time point, forskolin significantly decreased IL-1 induced tissue factor activity (from 69 ± 3% after 4 h to 64 ± 2% of control after 48 h of incubation) (Table 1A). The decrease caused by IBMX was maximal after 4 h (40 ± 18%) and was weaker and not statistically significant at the other incubation times. The effects of forskolin and IBMX added together were additive; the decrease of induced tissue factor activity in the presence of both agents reached 80% at each incubation time. Similar to forskolin, dbcyclic AMP inhibited induced tissue factor activity at each time point and by approximately the same extent. Forskolin and dbcyclic AMP increased thrombomodulin expression at each incubation time (Table 1b). The effect of forskolin was little different from 12 to 48 h (from 26 ± 7% increase at 12 h to 30 ± 9% at 48 h), but this increase was significant. IBMX alone did not influence basal thrombomodulin activity. However, addition of IBMX enhanced the increase of thrombomodulin expression induced by forskolin. At 4 h, thrombomodulin stimulated with forskolin was 108 ± 14% as compared to control; while in the presence of 10^{-4} M IBMX, this activity reached 129 ± 5%. At 24 h and 48 h, addition of IBMX also enhanced the increase induced by forskolin. This additive effect of forskolin was statistically significant after 4 and 48 h of incubation. Dibutyryl cyclic AMP exerted a similar effect to that of forskolin: dbcyclic AMP increased thrombomodulin activity with a maximal effect between 12 and 24 h. When thrombomodulin activity was decreased by IL-1, the presence of forskolin or dbcyclic AMP totally reversed this inhibition at 12 and 24 h. At 48 h only forskolin still exerted this effect; forskolin in combination with IBMX induced a 100% recovery of the basal activity after 4 or 12 h and a significant increase over basal values of thrombomodulin activity after 24 or 48 h, levels reaching respectively 121 ± 10% and 126 ± 16% of controls.

Thrombomodulin and tissue factor mRNA levels

The level of mRNA for tissue factor or thrombomodulin was assayed in endothelial cells using the reverse transcriptase-polymerase chain reaction method (RT-PCR). The amount of mRNA was normalized to that of the mRNA for GAPDH, a housekeeping gene which is not affected by the different agents used in the present study.

Resting endothelial cells showed no detectable expression of tissue factor mRNA. Incubation with 1 u ml^{-1} IL-1 for 2 h induced the appearance of tissue factor mRNA, while coincubation with 10^{-4} M forskolin inhibited by 1.5 fold this appearance of tissue factor mRNA (Figure 5). There was constitutive expression of thrombomodulin mRNA in endothelial cells and preincubation of the cells with 10^{-4} M forskolin induced a 2.5 fold increase in thrombomodulin mRNA levels. One u ml^{-1} IL-1 did not change the thrombomodulin mRNA level after 2 h of incubation; forskolin coincubated with IL-1 increased mRNA above the control level.

Ultrastructural localization of thrombomodulin

Immunoelectronmicroscopy was used to localize more precisely thrombomodulin in intact endothelial cells monolayers under basal conditions and after forskolin treatment. Under basal conditions, a thin labelling appeared on the plasma membrane (Figure 6). In forskolin-stimulated cells, this labelling was more dense and there was intense intracellular labelling, especially in the endoplasmic reticulum.

Effects of forskolin and PGE_1 on cyclic AMP levels

In an attempt to correlate changes in expression of thrombomodulin and tissue factor with actual rather than inferred changes in cyclic AMP levels, we carried out a small series of experiments in which intracellular cyclic AMP was measured following exposure of cells to either forskolin or PGE_1 (see Methods). As shown in Table 2, after a 15 min incubation, 10^{-4} M forskolin caused a 2.6 fold increase in total intracellular cyclic AMP levels, 10^{-5} M forskolin a 1.7 fold increase and 10^{-5} M PGE_1 a 1.4 fold increase compared with controls.

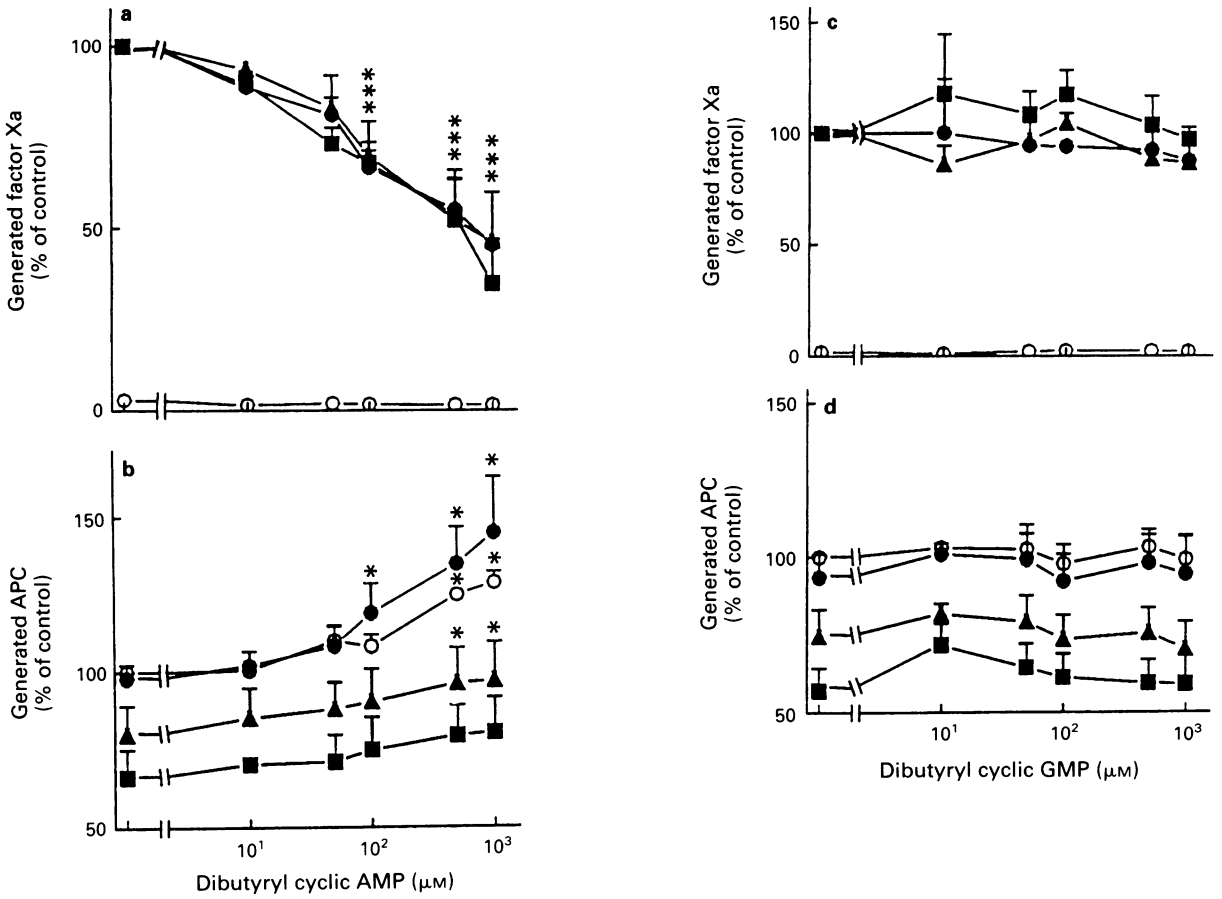


Figure 4 Concentration-effect relationship for dibutyryl cyclic AMP (dbcyclic AMP) and dbcyclic GMP on the expression of tissue factor and thrombomodulin activities at the surface of HSVEC, stimulated by either thrombin (1 u ml^{-1}), interleukin-1 (IL-1) (1 u ml^{-1}) or tumour necrosis factor (TNF) (1 ng ml^{-1}). Confluent monolayers of HSVEC were exposed to the concentrations indicated of dbcyclic AMP (a,b) or dbcyclic GMP (c,d) with either thrombin (●), IL-1 (■), TNF (▲) or control buffer (○) for 4 h. Tissue factor and thrombomodulin activities were measured as described in Methods. Points correspond to the mean + s.e.mean of five experiments; each condition was performed in triplicate. (a) and (c) represent tissue factor activities: results are expressed as % of control value for tissue factor activity at the surface of stimulated HSVEC. (b) and (d) represent thrombomodulin activities: results are expressed as % of control value for thrombomodulin activity at the surface of unstimulated HSVEC. *$P<0.05$ vs control under the same experimental conditions.

Table 1 Time course of the effect of forskolin (10^{-5} M), 3-isobutyl-1-methyl xanthine (IBMX) (10^{-4} M) and dbcyclic AMP (5×10^{-4} M) on tissue factor and thrombomodulin activities

A *Tissue factor activity* (% inhibition)

	4 h	12 h	24 h	48 h
Control	0	0	0	0
Forskolin	69 ± 3 *	54 ± 12*	63 ± 8 *	64 ± 2 *
IBMX	40 ± 18*	22 ± 5	15 ± 27	25 ± 12
Forskolin + IBMX	80 ± 2 *	68 ± 12*	78 ± 4 *	77 ± 4 *
dbcyclic AMP	30 ± 28*	45 ± 17*	55 ± 28*	47 ± 17*

Tissue factor activities were measured at the surface of HSVEC after stimulation during 4 h by 0.2 u ml^{-1} IL-1.
Results are the mean ± s.e.mean of three separate experiments.
*$P<0.05$ for significant difference from the control at the same time point.

B *Thrombomodulin activity* (% of control)

	IL-1 (u ml^{-1})	4 h	12 h	24 h	48 h
Control	0	100 ± 14	100 ± 5	100 ± 10	100 ± 12
	1	86 ± 14	89 ± 10	82 ± 8	82 ± 7
Forskolin	0	108 ± 16	126 ± 7 *	124 ± 9 *	130 ± 9 *
	1	91 ± 14	103 ± 9 *	102 ± 13*	112 ± 10*
IBMX	0	99 ± 14	99 ± 2	98 ± 4	106 ± 11
	1	81 ± 11	80 ± 8	78 ± 3	91 ± 5
Forskolin + IBMX	0	129 ± 5 *	126 ± 6 *	147 ± 19*	153 ± 12*
	1	105 ± 12*	103 ± 11*	121 ± 10*	126 ± 16*
dbcyclic AMP	0	112 ± 16	131 ± 5 *	128 ± 4 *	115 ± 9
	1	93 ± 5	106 ± 8	107 ± 8	94 ± 2

Thrombomodulin activities were measured at the surface of stimulated for 4 h with 1 u ml^{-1} IL-1 or unstimulated HSVEC.
Results are the mean ± s.e.mean of three separate experiments.
*$P<0.05$ for significant difference from the control at the same time point.

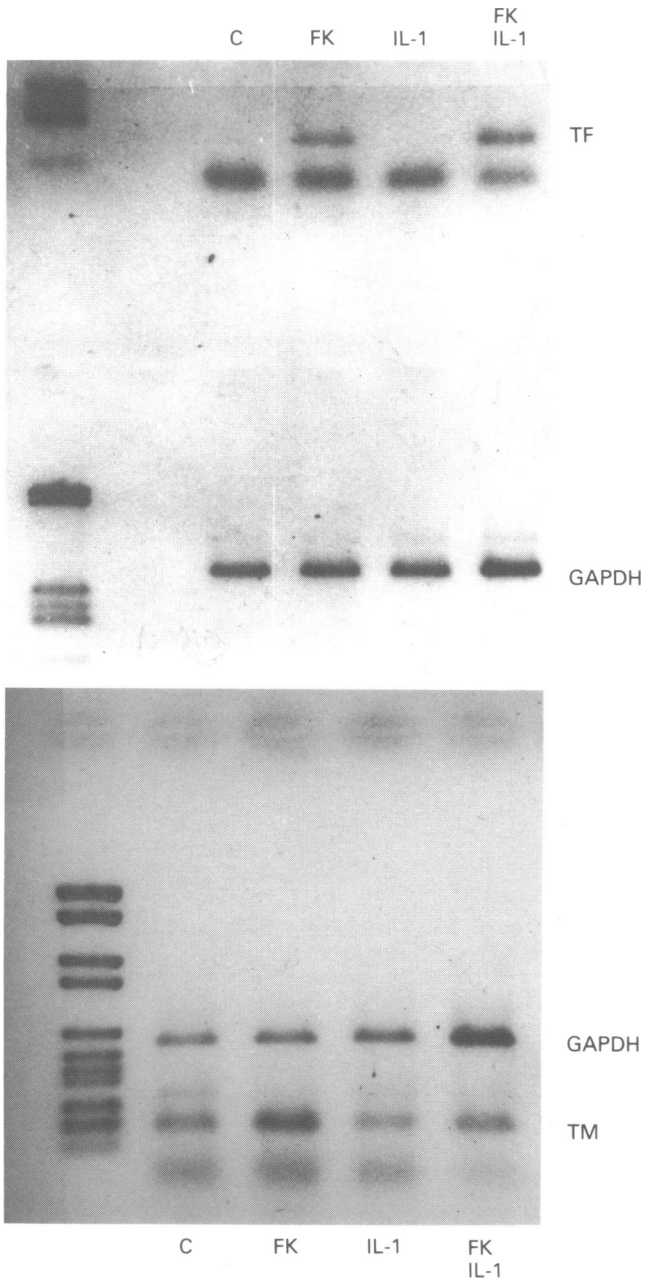


Figure 5 Effect of forskolin on thrombomodulin (TM) and tissue factor (TF) mRNA level in endothelial cells. Confluent monolayers of HSVEC were incubated for 2 h, at 37°C with interleukin-1 (IL-1) or without (C: control) 1 u ml^{-1} IL-1 and with or without 10^{-4} M forskolin (FK). Total cellular RNA was prepared and 100 ng was used for RT-PCR as described in Methods. GAPDH = glyceraldehyde phosphodehydrogenase.

Discussion

The aims of this study were to investigate if substances which are known to cause a rise in intracellular cyclic AMP levels could affect procoagulant and anticoagulant properties of endothelial cells. We found through the use of such agents, in this case forskolin and PGE_1, that it was possible to manipulate both tissue factor and thrombomodulin activities on HSVEC in a way that would be expected to preserve the antithrombotic potential of these cells. More specifically, we provide evidence that a rise in endothelial cell cyclic AMP levels caused an inhibition of tissue factor expression, enhanced thrombomodulin activity and reversed the decrease of thrombomodulin activity caused by cytokines.

Galdal *et al.* (1984, 1985) demonstrated an inhibition of the expression of tissue factor on human umbilical vein endothelial cells (HUVEC) by agents known to increase intracellular cyclic AMP levels. This effect has also been described for other cell types such as mouse trophoblasts (Dalaker & Prydz, 1986) or human monocytes (Lyberg, 1983). An increase of thrombomodulin activity in response to experimental manipulations has only recently been demonstrated. Foetomodulin, a surface protein marker of foetal development, was shown to be modulated by cyclic AMP (Imada *et al.*, 1987) and was later identified with thrombomodulin (Imada *et al.*, 1990). During the course of our study, other authors have described upregulation of thrombomodulin by cyclic AMP elevating agents in two human megakaryoblastic leukaemia cell lines (Ito *et al.*, 1990), in human umbilical vein endothelial cells (Ishii *et al.*, 1990; Hirokawa & Aoki, 1990; 1991; Maruyama *et al.*, 1991; Horie *et al.*, 1992), in mouse haemangioma cells (Maruyama *et al.*, 1991) and in F9 embryonal carcinoma cells (Weiler-Guettler *et al.*, 1992).

In contrast to the above mentioned studies, our experiments concerning the effects of cyclic AMP on tissue factor and thrombomodulin activities and mRNA levels were performed using the same cell population. This is of key importance for meaningful comparison of these results, since it has been shown that cell origin or cell culture conditions have major effects on the expression of tissue factor and thrombomodulin (Archipoff *et al.*, 1991).

IBMX reduced IL-1-induced tissue factor activity, while the coincubation of IBMX with forskolin showed an additive but not synergistic effect on the decrease of induced tissue factor activity. A synergistic effect would have been expected when combining a phosphodiesterase (PDE) inhibitor with an adenylate cyclase activator (Sutherland *et al.*, 1968). IBMX inhibits endothelial cell PDE (Lugnier & Schini, 1990), but also has other effects not mediated by cyclic AMP. Dasarathy & Fanburg (1988) have demonstrated that IBMX elevates angiotensin converting enzyme activity in endothelial cells by a calcium-calmodulin mediated process. The importance of calcium and calmodulin in the regulation of tissue factor expression in HUVEC has been stressed previously (Galdal *et al.*, 1984; 1985; Dalaker & Prydz, 1986; Callahan *et al.*, 1990; Bach & Rifkin, 1990). It is thus possible that IBMX does not regulate tissue factor expression on endothelial cells solely through inhibition of PDE, but also through other mechanisms that could involve interaction with calmodulin or other proteins.

Forskolin and PGE_1 decreased induced tissue factor activity and simultaneously increased thrombomodulin activity in a concentration-dependent manner. The down regulation of tissue factor and upregulation of thrombomodulin caused by forskolin (10^{-4} and 10^{-5} M) were statistically significant after 4 h of incubation. PGE_1 (10^{-5} M) was less effective. The difference in the effect of forskolin and PGE_1 may be due to the mechanisms through which these two drugs act on the cells; forskolin acts directly on the catalytic subunit of adenylate cyclase and desensitization to forskolin does not occur (Seamon & Daly, 1986), while PGE_1 may act through specific receptors on endothelial cells. We suggest that desensitization could explain the lower activity of PGE_1 compared to forskolin. A similar desensitization has been described in platelets (Ashby, 1989): activation of platelet adenylate cyclase by PGE_1 could be followed by a slow reversible transition of adenylate cyclase to an inactive form, through a distinct inhibitory receptor for PGE_1, leading to desensitization. Our results also showed no variation with time in the response to forskolin, while there was a time-dependent decrease in the effect of PGE_1 (data not shown). This adds support to the suggestion that the lower effect of PGE_1 is due to receptor desensitization more than to alteration of intracellular responses to cyclic AMP.

An increase of cyclic GMP *in vivo* in endothelial cells can be caused by EDRF/nitric oxide (NO) or by NO containing factors liberated by the cells themselves (Boulanger *et al.*,

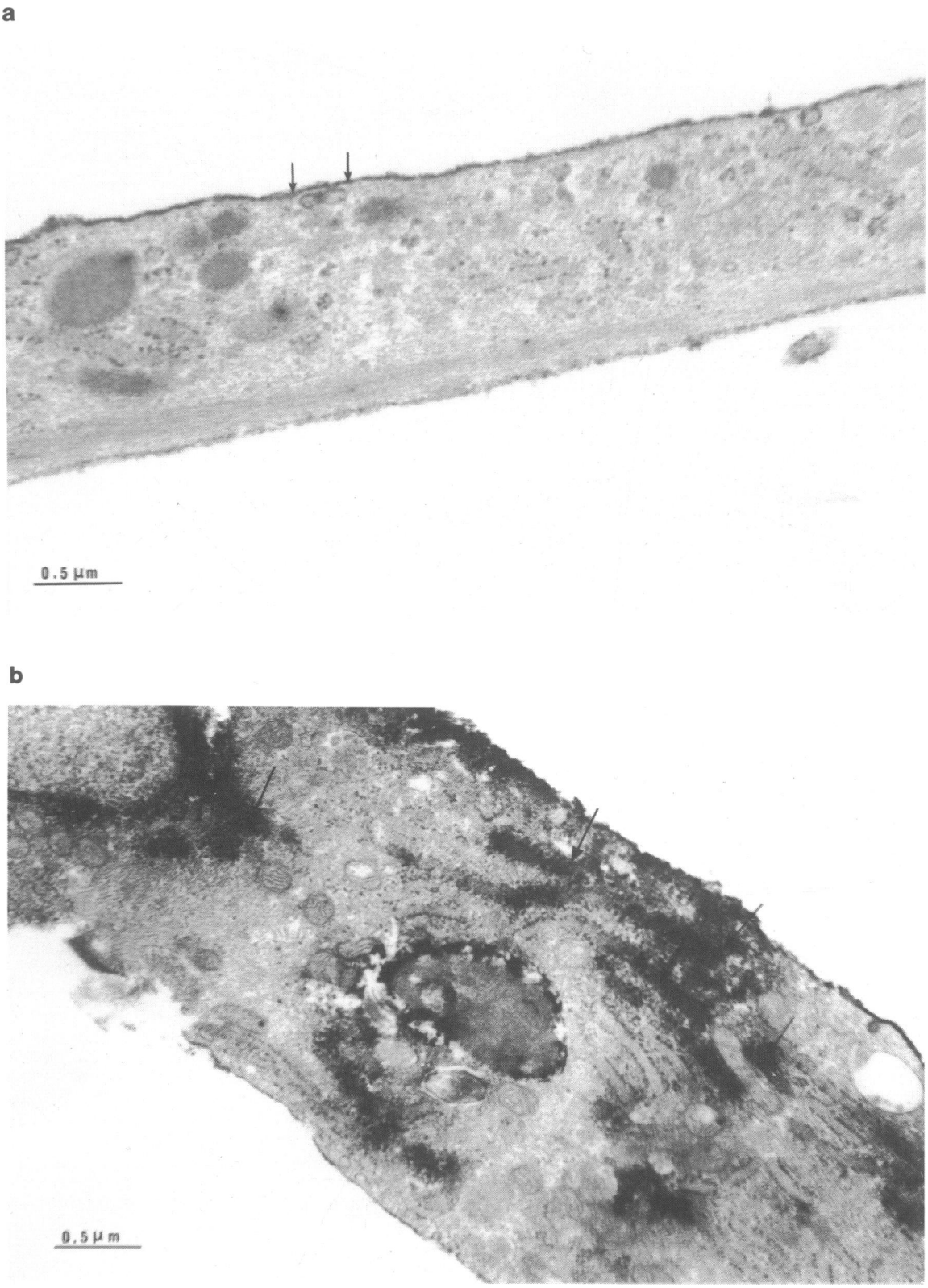


Figure 6 Immunoperoxidase staining of HSVEC under baseline and forskolin-stimulated conditions. HSVEC were permeabilized and incubated with antithrombomodulin antibodies. Unstimulated cells (a) show labelling on the apical plasma membrane, whereas forskolin (b)-stimulated cells contain dense staining in the reticulum in continuity with the plasma membrane.

1990). NO is known to induce vasodilatation of vessels and to have platelet antiaggregatory properties. Thus, it contributes to the antithrombotic properties of endothelium. In our experiments, the cyclic GMP at concentrations of up to 10^{-3} M had no significant effect on thrombomodulin and tissue factor activities (Figure 5c and d). Other compounds have been shown to increase NO and intracellular cyclic GMP in endothelial cells, for example, bradykinin, atriopeptin II or SIN-1 (Marin & Sanchez-Ferrer, 1990; Boulanger *et al.*, 1990; Needleman *et al.*, 1989; Gerzer *et al.*, 1988). However, although not reported here, we found that bradykinin (5×10^{-9}–5×10^{-7} M), atriopeptin II (5×10^{-8}–5×10^{-6} M)

Table 2 Effect of forskolin and prostaglandin E_1 (PGE_1) on cyclic AMP levels in HSVEC

	Cyclic AMP (pmol/10^6 cells)
Control	1.9 ± 0.16
Forskolin 10^{-4} M	5.0 ± 0.6*
Forskolin 10^{-5} M	3.2 ± 0.1
PGE_1 10^{-5} M	2.7 ± 0.2

HSVEC were incubated 15 min with forskolin, PGE_1 or control buffer and assayed for cyclic AMP by radioimmunoassay. Results are the mean ± s.e.mean of five separate experiments.
*$P < 0.05$ vs control.

or SIN-1 (10^{-8}–10^{-3} M) did not influence constitutive thrombomodulin or tissue factor induced by IL-1, thrombin or phorbol myristate acetate (Archipoff *et al.*, unpublished). Thus our results suggest that NO/EDRF does not play a major role in the regulation of tissue factor and thrombomodulin activities through stimulation of guanylate cyclase.

Sutherland *et al.* (1968) proposed four criteria to determine if cyclic AMP was involved in particular physiological responses: (i) drugs used should stimulate the formation of cyclic AMP, (ii) phosphodiesterase inhibitors should mimic or potentiate the action of the drug, (iii) cyclic AMP derivatives should cause the same effect as the drug, (iv) increased phosphorylation of protein(s) should be observed. In summary, our results confirm three of the four conditions proposed by Sutherland *et al.* (1968) to implicate cyclic AMP in a pharmacological regulation: (i) forskolin and PGE_1 caused an increase of intracellular cyclic AMP levels in HSVEC, (ii) IBMX enhanced the effects of forskolin and (iii) the addition of dbcyclic AMP, the permeant membrane analogue of cyclic AMP, caused the same effect on tissue factor and thrombomodulin activities as the stimulation of adenylate cyclase by forskolin and PGE_1. The increase of protein phosphorylation remains to be verified. We show here that the modulation of thrombomodulin or tissue factor activities is already observed at concentrations of PGE_1 and forskolin that have no significant effect on total cyclic AMP levels. A similar situation has often been described in other cell systems. For example, PDE inhibitors are potent inhibitors of platelet aggregation at concentrations that do not increase platelet cyclic AMP levels measured by a radioimmunoassay (Tsien *et al.*, 1982). Increases in cyclic AMP are usually observed at concentrations above those necessary for inhibiting platelet function. This could be caused by the existence of subcellular compartments for the cyclic nucleotide and/or lack of sensitivity of the assay (Ashida *et al.*, 1992). Also, correlation between cyclic AMP elevation and inotropic effects of PDE III has been equivocal. However it is widely accepted that these PDE inhibitors act through an increase in cyclic AMP, the discrepancy being explained in terms of compartmentation of cyclic AMP or cyclic AMP-dependent kinases (Nicholson *et al.*, 1991). Finally, only marginal increases of cyclic AMP were found at concentrations of forskolin that inhibit the permeability of endothelial cells monolayer (Langeler *et al.*, 1991); these authors suggest that this observation can be explained by local enhancements of cyclic AMP level by forskolin in endothelial cells. Our results add to the hypothesis that subcompartments for cyclic AMP exist in endothelial cells.

In conclusion, we have demonstrated that the enhancement of cell-surface thrombomodulin activity in HSVEC by agents which are known to increase intracellular cyclic AMP was associated with an increase in thrombomodulin mRNA. The localization of thrombomodulin by electron microscopy showed that newly synthesized protein was bound to the membrane structure. The discrepancy of the effect of forskolin on thrombomodulin activity (25% increase as compared to control) and on thrombomodulin mRNA level (250% increase as compared to control), could be explained by the fact that the newly synthesized thrombomodulin was stored in the reticulum and just partially exposed on the surface of the cell membrane. To confirm this hypothesis, we have performed preliminary experiments in order to evaluate thrombomodulin antigen levels in cell lysates using monoclonal antibody-based ELISA (Amiral *et al.*, 1991; Bartha *et al.*, 1993). Compared to the control conditions, addition of 10^{-4} M forskolin increased 1.6 times the thrombomodulin-antigen content of HSVEC lysates (from 266 ± 45 ng ml^{-1} thrombomodulin for control to 441 ± 18 ng ml^{-1} for forskolin, $n = 2$), while it did not influence the thrombomodulin-antigen level of conditioned medium (control = 10.8 ± 1.8 ng ml^{-1} thrombomodulin, forskolin = 9.9 ± 0.3 ng ml^{-1} thrombomodulin, $n = 2$). Although preliminary, these results are in accordance with the conclusions of the electron microscopy studies. Incubation of the cells with 1 u ml^{-1} IL-1 induced a decrease in thrombomodulin activity with no significant change in thrombomodulin mRNA levels (Figure 6). This suggests that IL-1 downregulates thrombomodulin activity in these conditions mainly through internalization and degradation, rather than through regulation of gene expression. However, it cannot be excluded that in other experimental settings, IL-1 can also influence thrombomodulin expression directly (Hirokawa & Aoki 1991). We also demonstrated that the inhibition of IL-1 induced tissue factor activity was associated with a decrease in tissue factor mRNA. This suggests that an increase in intracellular cyclic AMP: (i) enhances transcription of thrombomodulin mRNA and synthesis of the protein with subsequent increase of thrombomodulin on the cell surface: (ii) plays role in regulating transcription and/or degradation of tissue factor mRNA. Therefore, a rise in endothelial cyclic AMP levels has two complementary effects that could lead to decrease in the generation of thrombin at the endothelial cell surface during inflammatory or thrombotic episodes. Moreover, previous studies have shown that increased intracellular cyclic AMP levels in endothelial cells: (i) induce a reduction in plasminogen activator inhibitor-1 secretion coupled to a potentiation of tissue type plasminogen activator release (Santell & Levin, 1988), (ii) contribute to the regulation of permeability and maintenance of endothelial integrity (Yamada *et al.*, 1990) and provide protection against thrombin-induced permeability increases (Casnocha *et al.*, 1989) and (iii) increase 13-HODE synthesis, thus limiting endothelial cell reactivity towards platelets (Haas *et al.*, 1990). All these effects of cyclic AMP contribute to the antithrombotic properties of endothelial cells. Studies of the major PDE isoenzymes have shown that the characteristics of the enzymes isolated from endothelial cells differ from those of smooth muscle cells (Lugnier & Schini, 1990; Souness *et al.*, 1990; Kishi *et al.*, 1992). Since PDE types II and IV are the only isoenzymes present in endothelial cells, specific inhibition of these PDE forms could lead to an endothelium-specific increase in cyclic AMP. Although no data are yet available on the PDE isoforms of human endothelial cells, these data suggest that the use of such specific PDE inhibitors could ultimately lead to an enhancement of the antithrombotic capacity of the endothelium with only limited side effects.

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Ca^{2+}-dependent aggregation of rabbit platelets induced by maitotoxin, a potent marine toxin, isolated from a dinoflagellate

Akiko Watanabe, *Yukisato Ishida, *Hiromi Honda, *Masaki Kobayashi & [1]Yasushi Ohizumi

Department of Pharmaceutical Molecular Biology, Pharmaceutical Institute, Tohoku University, Aoba, Aoba-ku, Sendai, Miyagi 980 and *Mitsubishi Kasei Institute of Life Sciences, Machida, Tokyo 194, Japan

1 Administration of maitotoxin (MTX), a dinoflagellate toxin, caused aggregation of rabbit washed platelets. The cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), measured by fura-2 fluorescence technique, was also increased by the presence of MTX. Rates of aggregation response and $[Ca^{2+}]_i$-increase were dependent on tested concentrations (3–100 ng ml^{-1}) of the toxin.

2 The MTX-induced platelet aggregation and $[Ca^{2+}]_i$-increase were totally abolished in a Ca^{2+}-free solution. The successive administration of Ca^{2+} in the presence of MTX elicited the aggregation and increase in $[Ca^{2+}]_i$.

3 Ba^{2+} was capable of substituting for Ca^{2+} in the MTX-induced platelet aggregation. In the presence of external Ca^{2+}, transition metals, Co^{2+}, Cd^{2+} and Ni^{2+}, inhibited the aggregation response to MTX.

4 Organic calcium antagonists (verapamil and nifedipine) as well as a cyclo-oxygenase-inhibitor (aspirin) did not apparently inhibit the aggregation response to MTX, except for a high concentration (10^{-5} M) of verapamil, while procaine (10 mM) reduced the rate of platelet aggregation.

5 MTX also elicited a release of ATP from platelets, which was abolished in the absence of external Ca^{2+}.

6 In contrast, thrombin 0.5 unit ml^{-1} could elicit platelet shape change, $[Ca^{2+}]_i$-increase and ATP-release in the absence of external Ca^{2+}.

7 These results suggest that the MTX-induced platelet activation is caused by an enhanced Ca^{2+}-influx presumably through voltage-independent Ca^{2+} channels on the plasma membrane.

Keywords: Maitotoxin; aggregation; rabbit platelets; cytosolic Ca^{2+} concentration; divalent cation

Introduction

Maitotoxin (MTX) is a water-soluble toxin isolated from the dinoflagellate *Gambierdiscus toxicus* which causes a seafood poisoning in tropical regions (Yasumoto *et al.*, 1979). The minimum lethal dose of the toxin is 0.13 μg kg^{-1} i.p. in mice, being approximately 50 times more potent than tetrodotoxin. The chemical structure of MTX has partly been elucidated with approximate Mr 3500 by mass spectroscopy (Yokoyama *et al.*, 1988): the molecule characteristically contains sulphate esters besides many hydroxy groups and ester rings along carbonyl or acetal/ketal structure.

Since we reported for the first time that MTX causes a Ca^{2+}-dependent release of noradrenaline from rat phaeochromocytoma cells (Takahashi *et al.*, 1982; 1983) and a Ca^{2+}-dependent contraction of smooth muscles (Ohizumi *et al.*, 1983; Ohizumi & Yasumoto, 1983a,b), various stimulatory effects of MTX have been revealed to be highly dependent on the external Ca^{2+} in smooth muscles (Berta *et al.*, 1988; Sladeczek *et al.*, 1988), insect skeletal muscle (Gomi *et al.*, 1984; Miyamoto *et al.*, 1984), neuroblastoma cells (Freedman *et al.*, 1984; Yoshii *et al.*, 1987), anterior pituitary cells (Login *et al.*, 1987), synaptosomes (Ueda *et al.*, 1986a,b), pancreatic islets (Niki *et al.*, 1986) and mussel spermatozoa (Nishiyama *et al.*, 1986). Most MTX-induced responses described above were sensitive to organic calcium antagonists and divalent cations such as verapamil and Co^{2+}. Thus, it has been suggested that MTX activates some Ca^{2+} channels on the plasma membrane. Recently, MTX has been reported to stimulate phosphoinositide breakdown in many cell types (Gusovsky & Daly, 1990). The MTX-induced stimulation of phospholipid metabolism was abolished in the absence of external Ca^{2+} (Sladeczek *et al.*, 1988; Gusovsky *et al.*, 1989).

In cardiac muscle of the rat ventricle, MTX has been reported to prolong the action potential plateau (Legrand & Bagnis, 1984). Further, MTX elicited arrythmogenic effects as well as positive inotropic effects in cardiac tissues and isolated cells (Kobayashi *et al.*, 1985a,b). The arrythmogenic changes were associated with a morphological damage of cardiac cells (Kobayashi *et al.*, 1986). Applying patch-clump techniques to the guinea-pig myocytes, we have demonstrated that the Co^{2+}-sensitive Ca^{2+}-channels activated by MTX are quite different from normal voltage-gated Ca^{2+}-channels with respect to the current-voltage relationship, unitary conductance and mean open time of channels (Kobayashi *et al.*, 1987a,b). Recently, in rat ventricular myocytes, MTX has also been reported to elicit a transient activation of voltage-gated Ca^{2+} current and an increase in voltage-independent current carried by Ca^{2+} and Na^{+} (Faivre *et al.*, 1990).

Platelets are activated by many substances like thrombin, noradrenaline, etc (for a review, see Siess, 1989). However, the type and nature of Ca^{2+} channels in platelets have not been well clarified. Doyle & Rüegg (1985) reported that depolarization of platelets with 50 mM KCl did not induce Ca^{2+} entry into platelets and that a Ca^{2+} channel agonist Bay K 8644 did not affect the Ca^{2+} uptake or the cytosolic Ca^{2+} concentration of platelets. Further, a binding assay using [^{3}H]-nimodipine did not show any specific site for binding of the Ca^{2+}-channel antagonist (Grossmann *et al.*, 1992). These studies suggest that activation of platelets occurs through the operation of voltage-independent Ca^{2+} channels as well as the Ca^{2+} liberation from the cellular store. Thus, we have investigated how MTX affects Ca^{2+}

[1] Author for correspondence.

metabolism of platelets by measuring fura-2-fluorescence and aggregation of the rabbit platelets. We show here that MTX-induced activation of rabbit washed platelets is strictly dependent on the presence of external Ca^{2+} and that the toxin-induced activation is insensitive to organic calcium antagonists at their specific concentrations for the inhibition of voltage-gated Ca^{2+} channels. Part of this work was presented at the 64th Annual Meeting of the Japanese Pharmaceutical Society at Kobe, Japan (Watanabe *et al.*, 1991).

Methods

Preparation of platelets

Fresh blood was obtained from male rabbits (Japanese white rabbits weighing about 2–3 kg), collected into 77 mM EDTA, pH 7.4, and subsequently centrifuged at 950 *g* for 5 min to obtain platelet-rich plasma (PRP). PRP was centrifuged at 1800 *g* for 5 min. The pellet was washed twice by suspension and centrifugation (1800 *g*, 5 min). The resultant pellet was resuspended in the Ca^{2+}-free Tyrode-HEPES solution with a final density of approximately 2×10^5 cells μl^{-1}. The number of platelets was counted by use of a fluorescence microscope and haemocytometer. The first suspension solution for washing platelets contained: NaCl 140, 4-morpholinepropanesulphonic acid (MOPS) 12 and EDTA 1.2 (mM) at pH 7.4. The second solution did not contain EDTA. The Tyrode-HEPES solution was composed of NaCl 136.9, KCl 5.4, $MgCl_2$ 0.53, $NaHCO_3$ 11.9, NaH_2PO_4 0.33, glucose 11.0 and HEPES 5.0 (mM), pH 7.4.

Measurements of aggregation and cytosolic Ca^{2+} concentration of platelets

For loading fura-2 to platelets, the platelet pellet prepared as described above was resuspended in the Tyrode-HEPES solution containing 0.5 μM fura-2/AM and incubation for 10 min at 37°C and was subsequently washed with the Tyrode-HEPES solution to remove extracellular fura-2/AM. When platelets were exposed to fura-2/AM for more than 10 min, the rate of aggregation induced by agents was much smaller compared to those of non-loaded platelets, presumably due to the known buffering effect of fura-2 on $[Ca^{2+}]_i$ (Pollock & Rink, 1986). Fura-2-Ca^{2+} signal and aggregation were measured with a spectrofluorometer (CAF-100, Japan Spectroscopic, Tokyo, Japan), specially designed for the simultaneous measurement of fluorescence and light transmission. Platelets were exposed to excitation light having wavelengths of 340 and 380 nm and emission was measured at 500 nm. The platelet suspension (0.3 ml) in a siliconized cuvette was prewarmed at least for 1 min at 37°C in the spectrofluorometer. Then, Ca^{2+} at the final concentration of 1 mM was added. Five min after the Ca^{2+}-addition, MTX or thrombin was administered and platelet responses were monitored for a maximum of 30 min. Effects of Ca^{2+}-removal on platelet responses were examined without the addition of Ca^{2+}. At the end of experiments, the maximum and minimum fluorescence levels were determined with 0.2% Triton X-100 and 3 mM ethyleneglycol*bis*(β-aminoethyl)-N',N'-tetraacetic acid (EGTA) at pH 8.2, respectively, and the $[Ca^{2+}]_i$ was calculated by use of the fura-2-Ca^{2+} dissociation constant of 224 nM as reported by Pollock *et al.* (1986). Aggregation response was determined by a standard turbidimetric method and was expressed as % of light transmission. Attained levels and rates of aggregation and $[Ca^{2+}]_i$-increase were taken as parameters of the platelet responses. The rate of aggregation represents the maximum slope of the response determined with a ruler by eye and is expressed as % min^{-1}. The rate of $[Ca^{2+}]_i$-increase is also estimated by the maximum slope of the response and is expressed as the change in the fluorescence ratio excited by 340 to 380 nm per min. In some instances, the rate of $[Ca^{2+}]_i$-increase was expressed by $[Ca^{2+}]_i$-change min^{-1}.

In some experiments, the effect of divalent cations or various pharmacological blockers on platelet aggregation without loading fura-2 was examined by the turbidimetric technique using an aggregometer (PAM-6C, Mebanix). Various cations and blockers were added 4 min before administration of MTX. Ba^{2+}-substitution was performed by the addition of 1 mM Ba^{2+} instead of Ca^{2+} after 1 min equilibration of the platelet suspension.

Determination of ATP released from platelets

Purified luciferin-luciferase was added to a tube containing 0.3 ml washed platelet solution. The tube was placed in a dark chamber of a photon counter (Biocounter 2000, Lumac B.V./3M, The Netherlands). After equilibration for 10 min at 37°C, MTX was added and the yielded light was determined in the presence and absence of 1 mM Ca^{2+}. For standardization, known amounts of ATP (10 nmol–10 μmol) were added to the 0.3 ml medium with luciferin-luciferase. When luciferase was exposed to ATP, light intensity reached a peak in 1 s and it decayed only less than 10% of the peak intensity during 10 min observation time. The light intensity was linearly correlated to the amounts of ATP.

Drugs

The following drugs were used: verapamil hydrochloride (Sigma, St Louis, MO, U.S.A.), nifedipine (Sigma), procaine hydrochloride (Daiichi Seiyaku, Tokyo, Japan), fura-2/AM (Dojindo Laboratories, Kumamoto, Japan), luciferin-luciferase reagent (Lumac B.V./3M). MTX was a gift of Professor T. Yasumoto, Tohoku University.

Statistical comparison

Results of the experiments are expressed as mean ± s.e.mean. Student's *t* test and paired *t* test were used for stastistical analysis of the results.

Results

Effects of maitotoxin and thrombin on aggregation and $[Ca^{2+}]_i$

Aggregation responses and cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) changes of the fura-2-loaded washed platelets of the rabbit were monitored simultaneously. Administration of MTX (3–100 ng ml^{-1}) elicited aggregation of the platelets after a lag time (Figures 1 and 2a,b). During the lag time, light transmission often decreased transiently, presumably reflecting a shape change of platelets (Milton & Frojmovic, 1983). The lag time was dependent on MTX-concentrations tested. MTX, 100 ng ml^{-1}, elicited aggregation with a similar time course to 30 ng ml^{-1} of the toxin (data not shown). MTX at 1 ng ml^{-1} failed to cause aggregation during 30 min observation time. MTX at concentrations of 10–100 ng ml^{-1} elicited nearly maximum aggregation. The aggregation response to 3 ng ml^{-1} seemed to be submaximal in Figure 2a, but this might be due to the limitation of reaction time for 30 min because of the variance of turbidimetric response in time. At the termination of the reaction the aggregation response to 3 ng ml^{-1} MTX continued to increase as shown in Figure 1. Thus, it is suggested that, once MTX triggers aggregation, platelets aggregate completely. Compared to the finally attained level of aggregation, the rate of aggregation was dependent on the concentration of MTX.

Figure 1 (lower panel) shows that MTX (3–30 ng ml^{-1}) elicited a gradual increase in $[Ca^{2+}]_i$ of the fura-2-loaded platelets. The onset of $[Ca^{2+}]_i$-increase was prompt after administration of the toxin. The rate of $[Ca^{2+}]_i$-increase was

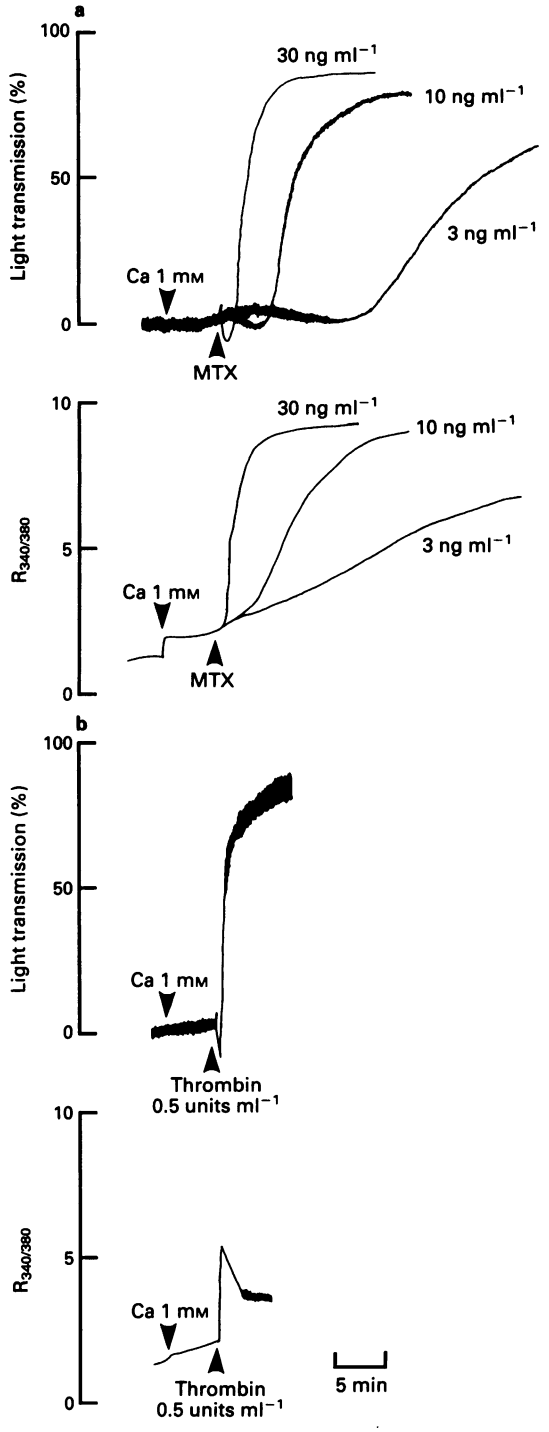


Figure 1 Simultaneous recordings of aggregation (upper panel) and fura-2 fluorescence (lower panel) of the rabbit washed platelets in response to maitotoxin (MTX) and thrombin. MTX 3–30 ng ml^{-1} (a) and thrombin 0.5 units ml^{-1} (b) were applied 5 min after addition of 1 mM $CaCl_2$ to the nominally Ca^{2+}-free solution. R340/380 in the ordinate of the lower panel represents the ratio of fluorescence intensity excited by 340 nm to 380 nm. Maximum and minimum values for R340/380 when platelets were exposed to Triton X-100 and EGTA were as follows: 9.33 and 0.66 in (a), and 11.78 and 0.80 in (b), respectively.

dependent on the MTX concentration rather than the finally raised level of $[Ca^{2+}]_i$ and it seemed to be associated with the rate of platelet aggregation rather than the lag period (Figure 2c vs 2b). As in the aggregation response, 100 ng ml^{-1} MTX elicited the $[Ca^{2+}]_i$-increase with a similar time course to that induced by 30 ng ml^{-1}, and 1 ng ml^{-1} failed to raised $[Ca^{2+}]_i$ (data not shown). The cytosolic Ca^{2+} concentrations before

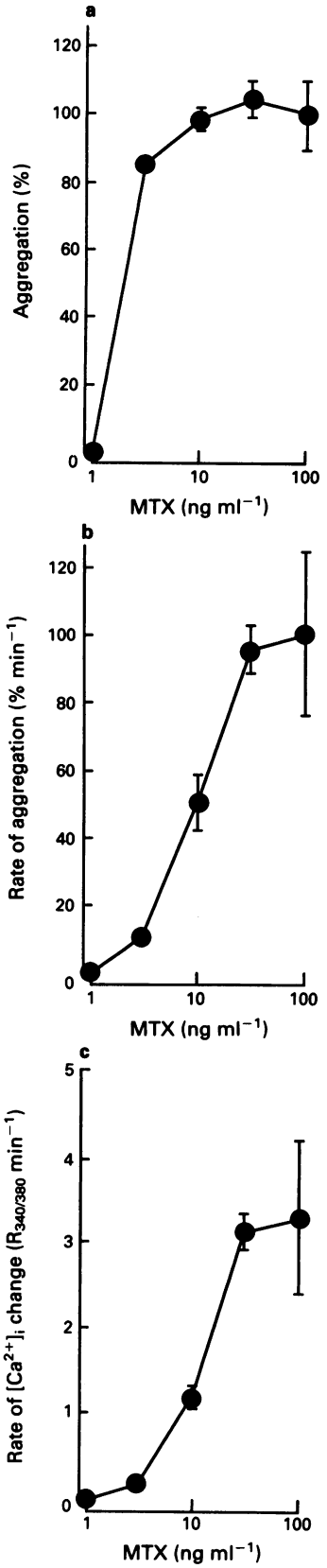


Figure 2 The concentration-response relationships for maitotoxin (MTX) on aggregation level (a), aggregation rate (b) and rate of fura-2-signal increase (c) in the rabbit platelets. Aggregation response was measured by turbidimetric method. In (a) and (b), the responses to 100 ng ml^{-1} MTX were taken as 100%. The maximum level of light transmission in the presence of 30 ng ml^{-1} MTX was 83.3 ± 2.64% (n = 4) and the rate of light transmission change after the administration of 30 ng ml^{-1} MTX was 40.5 ± 4.85% min^{-1} (n = 4). In (c), the rate of $[Ca^{2+}]_i$ change was given as the change of fura-2 fluorescence ratio (R340/380) per min. The rate of $[Ca^{2+}]_i$ change after administration of 30 ng ml^{-1} MTX was 272.6 ± 80.1 nM min^{-1} (n = 4). Values are mean with s.e.mean (n = 3–5).

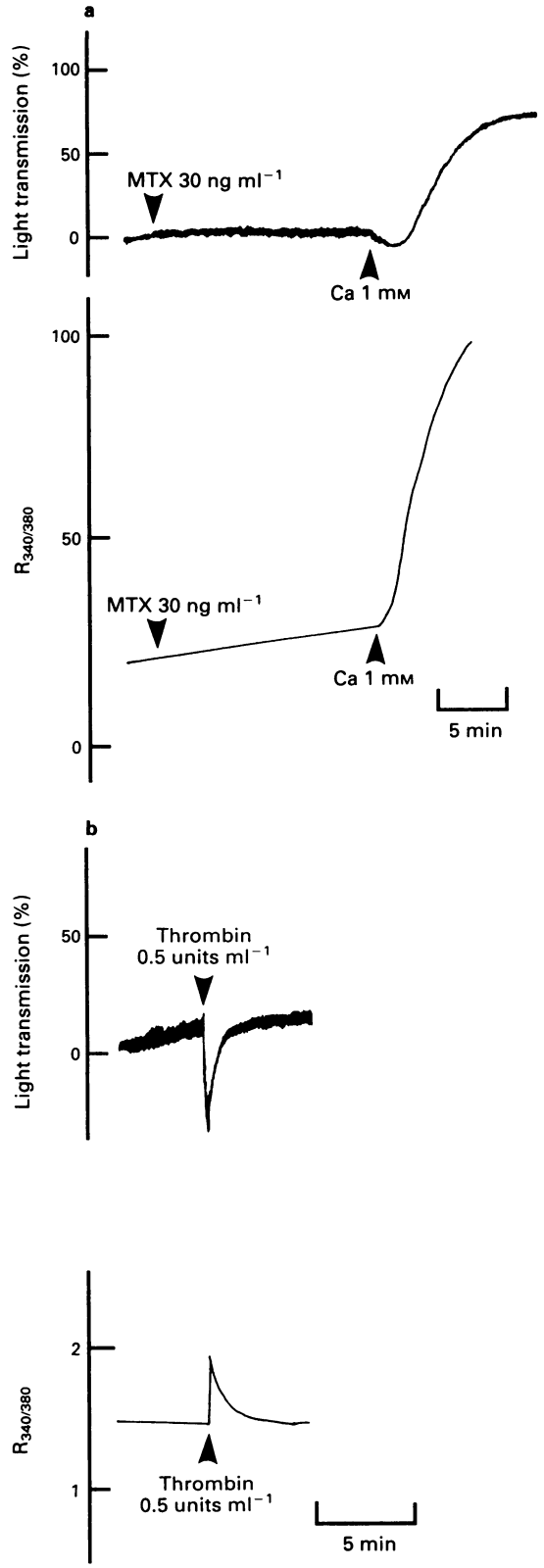


Figure 3 Effects of maitotoxin (MTX) 30 ng ml^{-1} (a) and thrombin 0.5 units ml^{-1} (b) on aggregation (upper panel) and fura-2-signal (lower panel) of the platelets in the absence of external Ca^{2+}. In (a) $CaCl_2$ (1 mM) was added 15 min after administration of 30 ng ml^{-1} MTX. Maximum and minimum values for R340/380 were as follows: 10.50 and 0.56 in (a), and 5.56 and 0.57 in (b), respectively.

stimulation and at 90% of the maximum level in the presence of 10–100 ng ml^{-1} MTX were 77.9 ± 11.6 (n = 12) and 2713.5 ± 130.5 nM (n = 12), respectively. The final level of $[Ca^{2+}]_i$ in the presence of MTX could not be estimated since the Ca^{2+}-fura-2 fluorescence signal in the presence of MTX was equivalent to that after application of Triton X-100.

For comparison of MTX-induced response with receptor-mediated response, we also investigated the effects of thrombin on aggregation and $[Ca^{2+}]_i$ of the rabbit washed platelets (Figure 1b). Thrombin 0.5 unit ml^{-1} elicited a transient decrease followed by a rapid increase in light transmission, indicating the shape change followed by aggregation of platelets. On the other hand, 0.5 unit ml^{-1} thrombin raised $[Ca^{2+}]_i$ with a rapid transient phase followed by a relatively small plateau phase: $[Ca^{2+}]_i$ was 298.0 ± 124.6 μM (n = 4) for the transient phase or 217.8 ± 124.6 μM (n = 4) for the plateau phase. These results indicate that MTX is much more effective in raising $[Ca^{2+}]_i$ of platelets than thrombin.

Effects of varying external Ca^2 concentration and Ba^{2+}-substitution for Ca^{2+} on platelet responses

When fura-2-load platelets were exposed to Ca^{2+}-free solution, administration of 30 ng ml^{-1} MTX elicited neither aggregation nor $[Ca^{2+}]_i$-increase (Figure 3). The subsequent addition of 1 mM Ca^{2+} elicited a transient decrease followed by a rapid increase in light transmission, suggesting the shape change followed by the aggregation of platelets (Figure 3). Fura-2-fluorescence signal showed that $[Ca^{2+}]_i$ of platelets started to increase promptly after the Ca^{2+} addition in the presence of MTX (Figure 3).

When external Ca^{2+} was replaced by Ba^{2+} (1 mM), administration of MTX 30 ng ml^{-1} elicited aggregation of the platelet. The rate of MTX-induced aggregation in the presence of Ba^{2+} was approximately 45% lower than in the presence of Ca^{2+}, while aggregation was nearly maximal (Table 1). This suggests that, like many other Ca^{2+}-dependent responses of a number of cells (review: Evans, 1988), Ba^{2+} is capable of substituting for Ca^{2+} in the MTX-induced aggregation response of the platelet.

Figure 4 shows concentration-response relationships for external Ca^{2+} in the MTX (30 ng ml^{-1})-induced aggregation and $[Ca^{2+}]_i$-increase of the fura-2-loaded platelets. Rates of aggregation were small at external Ca^{2+} concentrations less than 0.3 mM and dose-dependently increased at 1 and 3 mM Ca^{2+}. Similarly, the rates of $[Ca^{2+}]_i$-increase were small at external Ca^{2+} concentrations less than 0.3 mM and markedly increased at 1 and 3 mM Ca^{2+}. On the other hand, attained levels of aggregation seemed to be dose-dependent on Ca^{2+} concentrations less than 0.1 mM and reached nearly maximum at higher concentrations between 0.1 and 3 mM. Apparently, this Ca^{2+}-concentration-aggregation relationship is affected by the limited reaction time of 30 min, as in the case of MTX-induced response in normal media. From these results it appears that the platelet aggregation response to MTX is markedly accelerated by the presence of external Ca^{2+} concentrations over 0.3 mM.

In contrast to MTX, administration of thrombin 0.5 units ml^{-1} to the Ca^{2+}-free solution elicited a transient increase in $[Ca^{2+}]_i$ associated with a transient decrease in light transmis-

Table 1 Maitotoxin (MTX, 30 ng ml^{-1})-induced aggregation of the rabbit washed platelet in the presence of Ca^{2+} (1 mM) or Ba^{2+} (1 mM)

	Aggregation (%)	*Rate of aggregation* (%)
Ca^{2+}	100	100
Ba^{2+}	88.1 ± 0.4*	54.9 ± 8.7**

Platelet aggregation was measured by the turbidimetric method. To examine the effects of Ba^{2+} on platelet aggregation, Ba^{2+} was added to the medium instead of Ca^{2+}. Responses in the presence of Ca^{2+} were taken as 100% and the responses in the presence of Ba^{2+} were compared by paired experiments. Values are given as mean ± s.e.mean (n = 3 or 4).
Significantly different value from control (Ca^{2+}) by paired t test at *P < 0.01 and **P < 0.05, respectively.

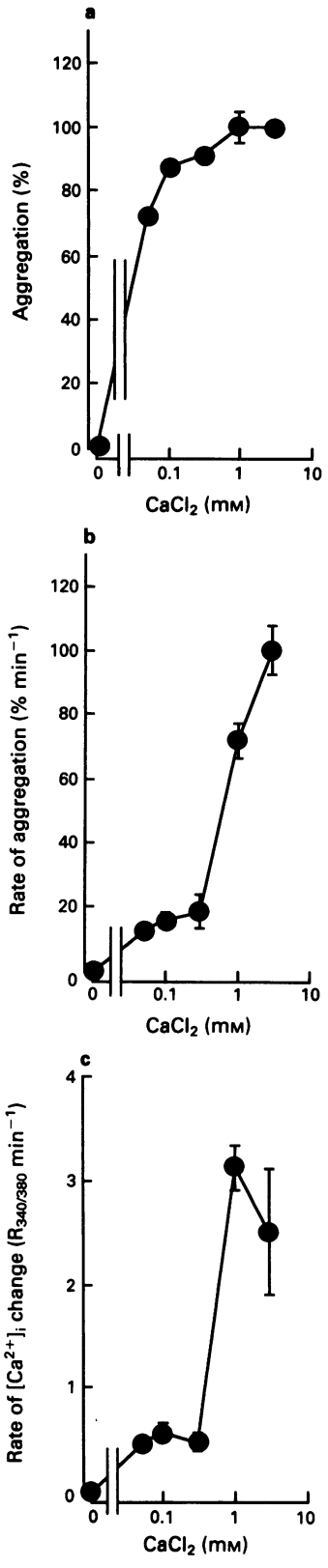


Figure 4 Effects of various external Ca^{2+} concentrations on aggregation level (a), aggregation rate (b) and rate of fura-2-signal increase (c) of the platelets in the presence of maitotoxin (MTX) 30 ng ml^{-1}. In (a) and (b), the aggregation responses to 3 mM Ca^{2+} were taken as 100%. Values are mean with s.e.mean. ($n = 3-6$). The maximum level of light transmission in the presence of 3 mM Ca^{2+} was 85.1 ± 1.79% ($n = 4$) and the rate of light transmission change after addition of 3 mM Ca^{2+} was 66.0 ± 5.00% min^{-1} ($n = 4$). In (c), the rate of $[Ca^{2+}]_i$ change was given as the change of fura-2 fluorescence ratio (R340/380) per min. The rate of $[Ca^{2+}]_i$ change after addition of 3 mM Ca^{2+} MTX was 536.4 ± 108.3 nM min^{-1} ($n = 4$).

sion (Figure 3). Similarly, Pollock & Rink (1986) reported the thrombin-induced responses of aggregation and $[Ca^{2+}]_i$ in a Ca^{2+}-free solution. They observed a small aggregation response in a Ca^{2+}-free solution. This difference may be due to the difference in cellular fura-2-concentrations or may be due to species differences, rabbit washed platelets in our studies and human platelets in theirs. These results suggest that thrombin, but not MTX, could activate the platelet aggregation process through the release of Ca^{2+} sequestered at some cellular sites.

Inhibitory effects of divalent ions and various blockers on platelet aggregation

Since Co^{2+}, Cd^{2+} and Ni^{2+} are known to interfere with a Ca^{2+}-influx through Ca^{2+} channels at the plasma membrane (Evans, 1988), we investigated effects of these divalent cations on MTX (30 ng ml^{-1})-induced platelet aggregation in the presence of 1 mM Ca^{2+} (Table 2). Co^{2+} and Cd^{2+} (both 0.3 mM) significantly inhibited the platelet aggregation by approximately 20–30% of control. The rate of aggregation was also inhibited by Co^{2+} and Cd^{2+}. In the presence of Ni^{2+} (1 mM), MTX apparently elicited a smaller aggregation at a slower rate than control. However, we could not examine effects of higher concentrations of Co^{2+} and Cd^{2+}, since their presence at 1 mM elicited aggregation by themselves (data not shown). These results suggest that the transition metals tested could interfere with the platelet aggregating response to MTX.

We further investigated effects of organic Ca^{2+} antagonists (verapamil and nifedipine) and a non-specific channel blocker (procaine) (review: Godfraind *et al.*, 1988) on MTX-induced platelet aggregation (Table 3). Pretreatments with verapamil and nifedipine (both 10^{-6} M) had no significant effects on the extent or rate of the aggregation response to 30 ng ml^{-1} MTX. However, 10^{-5} M verapamil inhibited the rate of ag-

Table 2 Effects of transition metals, Cd^{2+}, Co^{2+} and Ni^{2+}, on maitotoxin (MTX, 30 ng ml^{-1})-induced platelet aggregation

	Aggregation (%)	*Rate of aggregation* (%)
Control	100	100
Cd^{2+} (0.3 mM)	71.7 ± 3.0***	62.4 ± 13.3*
Co^{2+} (0.3 mM)	77.5 ± 3.4***	69.5 ± 20.5*
Ni^{2+} (1.0 mM)	62.4 ± 7.9***	22.2 ± 7.1***

Aggregation responses to MTX in the presence of 1 mM Ca^{2+} were taken as 100% (control). Transition metals were added in the presence of Ca^{2+} before administration of MTX. Values are given as mean ± s.e.mean ($n = 6$ or 8). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, by paired t test when compared to control.

Table 3 Effects of verapamil (10^{-6} and 10^{-5} M), nifedipine (10^{-6} M), procaine (10 mM) and aspirin (10^{-5} M) on maitotoxin (MTX, 30 ng ml^{-1})-induced platelet aggregation

	Aggregation (%)	*Rate of aggregation* (%)
Control	100	100
Verapamil (10^{-6} M)	96.2 ± 2.9	80.6 ± 7.6
Verapamil (10^{-5} M)	95.2 ± 5.7	44.1 ± 3.7*
Nifedipine (10^{-6} M)	100.3 ± 2.5	101.4 ± 7.5
Procaine (10 mM)	99.4 ± 2.7	19.8 ± 1.8*
Aspirin (10^{-5} M)	100.0 ± 2.5	91.6 ± 5.8

Tested agents were added in the presence of 1 mM Ca^{2+} before administration of MTX. Control responses in the medium without agents are expressed as 100%. Values are given as mean ± s.e.mean ($n = 6$ or 8).
*$P < 0.001$, by paired t test when compared to control.

gregation to approximately 45% of control, but did not significantly affect the maximum aggregation response to MTX. This verapamil-induced inhibition may be due to a non-specific action rather than the inhibition of voltage-gated Ca^{2+} channels, since its concentration exceeds the specific range (Godfraind *et al.*, 1986). Similarly, the rate of aggregation, but not the maximum aggregation, was markedly inhibited by the presence of 10 mM procaine.

We also investiated the effects of aspirin, a cyclo-oxygenase inhibitor (Smith & Willis, 1971; Hamberg *et al.*, 1974). However, pretreatment with 10^{-5} M aspirin did not affect the platelet aggregation responses to 30 ng ml^{-1} MTX (Table 3).

Effects of maitotoxin and thrombin on ATP release from platelets

When platelets are activated, several substances including ATP are released from dense granules (Holmsen *et al.*, 1969). Thus, effects of MTX on ATP release were investigated in comparison to the thrombin-induced response (Figure 5). MTX 3–100 ng ml^{-1} as well as thrombin 0.5 units ml^{-1} elicited a transient increase in external ATP concentration measured with luciferase, indicating that both agents release ATP from platelets. The rate of increase and the peak of released ATP concentration were dependent on MTX concentrations in a similar range to those causing aggregation and $[Ca^{2+}]_i$-increase of the platelets. In the Ca^{2+}-free solution, MTX failed to release ATP, whereas thrombin still released ATP (Figure 5) as reported by Grette (1962). These results suggest that both aggregation and secretion following MTX activation of platelets is highly dependent on the presence of external Ca^{2+}.

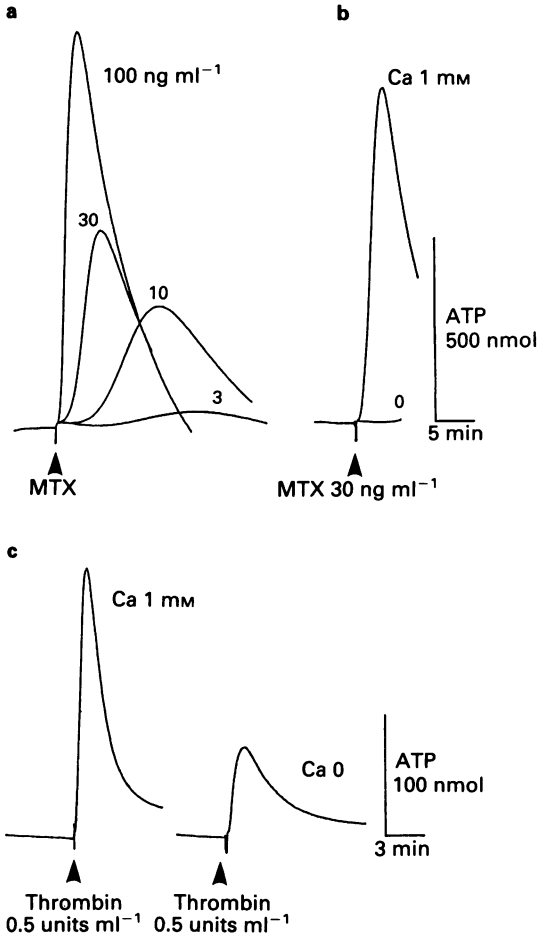


Figure 5 Effects of maitotoxin (MTX) (a and b) and thrombin (c) on ATP secretion of the rabbit platelets determined with luciferin-luciferase. In (a), various concentrations (3–100 ng ml^{-1}) of MTX were administered in the presence of 1 mM Ca^{2+}. (b) and (c) show a different set of experiments representing the effects of 30 ng ml^{-1} MTX and 0.5 units ml^{-1} thrombin on ATP secretion in the presence and absence of 1 mM Ca^{2+}, respectively.

Discussion

The present experiments demonstrate that MTX is capable of eliciting a shape change followed by aggregation as well as an ATP-secretion in the rabbit washed platelet. These stimulatory effects of MTX are specifically characterized by the very strict dependency on the presence of external Ca^{2+}. In the absence of external Ca^{2+}, MTX failed to cause any changes in the platelet function described above. While, many other stimulants such as thrombin, ADP, etc. could activate platelets through the release of intracellular Ca^{2+} (for reviews: see Siess, 1989; Rink & Sage, 1990). Ionophores like ionomycin and A23187 elicit an increase in $[Ca^{2+}]_i$ of platelets in the absence of external Ca^{2+} (Rink *et al.*, 1982; Thompson & Scrutton, 1985). However, MTX was reported to have no ionophore-like action on mitochondrial and liposomal membranes (Takahashi *et al.*, 1983). Thus, it is suggested that MTX activates platelets at the level of plasma membrane in a different manner from ionophore-induced activation. Further, Ca^{2+}-influx across the plasma membrane may be solely responsible for the MTX-induced activation of platelets.

By use of fura-2, MTX was shown to elicit a marked increase in $[Ca^{2+}]_i$ which was totally abolished in the absence of external Ca^{2+}. MTX at effective concentrations of 3–100 ng ml^{-1} raised the fluorescence signal induced by Ca^{2+}-fura-2 complex nearly to the maximum, suggesting that $[Ca^{2+}]_i$ reaches more than 3×10^{-6} M in the presence of MTX. For comparison, thrombin at the concentration which elicited the maximum aggregation raised the fluorescence signal to 50% of the maximum at the most effective case, i.e. $[Ca]_i < 10^{-6}$ M. Further, lower concentrations of MTX elicited slower increases in $[Ca^{2+}]_i$ and aggregation, but activated both to the maximum. These results indicate that once MTX activates platelets, the activation does not cease, resulting in the massive increase in $[Ca^{2+}]_i$ even at the lower concentrations. Presumably, such a long-lasting activation by MTX is consistent with the facts that MTX elicits the increase in Ca^{2+} content of smooth muscles (Ohizumi & Yasumoto, 1983a,b) and the marked prolongation of mean open time of Ca^{2+} channels in cardiac myocytes (Kobayashi *et al.*, 1987a).

The MTX-induced aggregation was inhibited by the presence of transition metals (Co^{2+}, Cd^{2+} and Ni^{2+}). When external Ca^{2+} was replaced by Ba^{2+}, MTX could cause the maximum aggregation. On the other hand, organic Ca^{2+} antagonists did not inhibit aggregation except at the highest concentration of verapamil. These results are comparable to the reported aggregation responses of platelets to agonists such as thrombin and ADP with respect to the transition metal-sensitivity, Ba^{2+}-substitution, and organic Ca^{2+} antagonist-ineffectiveness (Hallam & Rink, 1985; Blache *et al.*, 1985; Jy & Haynes, 1987; Sage & Rink, 1987; Avdonin *et al.*, 1988; Zschauer *et al.*, 1988). The fact that procaine significantly slowed the platelet aggregation response to MTX may be explained by its non-specific inhibition of Ca^{2+} influx through the plasma membrane (Godfraind *et al.*, 1986). Also, considering the previously reported facts that MTX could stimulate voltage-independent Ca^{2+} channels in cardiac muscle (Kobayashi *et al.*, 1987a,b; Faivre *et al.*, 1990) and that platelets may lack voltage-gated Ca^{2+} channels (reviews: Siess, 1989; Rink & Sage, 1990), it is suggested that MTX, like receptor agonists, is capable of activating voltage-independent Ca^{2+} channels on the plasma membrane of platelets.

In contrast, MTX-induced aggregation was not affected by the presence of aspirin. The irreversible platelet aggregation due to the stimulation of receptors by high concentrations of agonists is known to be amplified by a reaction of cyclo-oxygenase (Hamberg *et al.*, 1974; 1975), which is effectively blocked by the presence of aspirin (Smith & Willis, 1971; Hamberg *et al.*, 1974). These results suggest that if identical Ca^{2+} channels are responsible for both MTX and agonists, MTX acts more directly on the Ca^{2+} channels than the agonists do. Alternatively, MTX may activate other voltage-independent Ca^{2+} channels than those for agonists. Although the identification of specific Ca^{2+} channels for MTX awaits a molecular study, MTX may be a good tool for analysing the voltage-independent Ca^{2+} channels especially in platelets, since MTX did not affect the process of Ca^{2+} release from cellular stores and its effective concentration is very low (1–10 nM) based on the recently estimated molecular weight of 3500 (Yokoyama *et al.*, 1988).

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Effects of 5-HT-receptor and α_2-adrenoceptor ligands on the haemodynamic response to acute central hypovolaemia in conscious rabbits

[1]Roger G. Evans, *John M. Haynes & †John Ludbrook

Baker Medical Research Institute, Prahran, Victoria, Australia; *Department of Pharmacology, Monash University, Clayton, Victoria, Australia and †University of Melbourne Department of Surgery, Royal Melbourne Hospital, Parkville, Victoria, Australia

1 We set out to elucidate the pharmacological mechanisms by which α_2-adrenoceptor and 5-HT-receptor ligands affect the haemodynamic response to acute central hypovolaemia in conscious rabbits.

2 Acute central hypovolaemia was produced by inflating an inferior vena caval cuff so that cardiac output fell at a constant rate of ~8.5% of its baseline level per min.

3 Drugs were administered into the fourth cerebral ventricle in either 154 mM NaCl (saline) or 20% w/v 2-hydroxypropyl-β-cyclodextrin (β-CDX). After vehicle treatments, the haemodynamic response to acute central hypovolaemia had the usual two phases. During Phase I, systemic vascular conductance fell in proportion to cardiac output so that mean arterial pressure fell by only 8 mmHg. Phase II commenced when cardiac output had fallen to ~60% of its baseline level, when vascular conductance rose abruptly and arterial pressure fell to ≤ 40 mmHg. The haemodynamic response was not dependent on the vehicle used (saline or β-CDX).

4 Methysergide delayed the occurrence of Phase II in a dose-dependent manner, and prevented it at a dose of 30– 600 nmol (geometric mean = 186 nmol). The effects and potency of methysergide were not dependent on the vehicle used, indicating that β-CDX can be used as a vehicle for fourth ventricular administration of lipophilic drugs to conscious rabbits. Clonidine (10 nmol) reversed the effects of a critical dose of methysergide.

5 Phase II was also prevented by 8-hydroxy-2-(di-n-propylamino)tetralin (5-HT_{1A}-selective agonist, geometric mean critical dose (range) = 13.1 (10–30) nmol), sumatriptan (5-HT_{1D}-selective agonist, 72.1 (10–300) nmol), mesulergine (5-$HT_{2/1C}$-selective antagonist, 173 (30–1000) nmol), idazoxan (α_2-adrenoceptor-selective antagonist, 548 (100–3000) nmol), and mianserin (5-$HT_{2/1C}$-selective antagonist, 548 (100– 3000) nmol). It was not affected by MDL 72222 (5-HT_3-selective antagonist, 300 nmol) or ketanserin (5-$HT_{2/1C}$-selective antagonist, 3000 nmol).

6 To characterize the nature of α_2-adrenoceptors in rabbit brainstem, we examined the binding of [^{3}H]-rauwolscine to membrane homogenates of whole brainstem. [^{3}H]-rauwolscine bound to a population of sites with the characteristics of α_{2A}-adrenoceptors.

7 From these results we suggest that activation of 5-HT_{1A} receptors in the brainstem can prevent Phase II of the response to acute central hypovolaemia in conscious rabbits. Our results do not support the notion of an endogenous 5-hydroxytryptaminergic mechanism mediating Phase II. The mechanism by which the α_2-adrenoceptor antagonists yohimbine and idazoxan prevent Phase II remains to be elucidated. However, their potency relative to other 5-HT-receptor ligands indicates that an agonist action at 5-HT_{1A}-receptors is more likely than an antagonist action at α_2-adrenoceptors.

Keywords: β-Cyclodextrin; brainstem; cardiovascular system; haemorrhage; hypovolaemia; rabbit; radioligand binding; adrenoceptors; 5-HT receptors; vasodilatation

Introduction

The haemodynamic response to graded acute hypovolaemia in mammals consists of two distinct phases (Schadt & Ludbrook, 1991). During Phase I the fall in cardiac output is nearly matched by a reduction in systemic vascular conductance, so that arterial pressure is maintained at near normal levels. Heart rate rises during Phase I. In conscious dogs, rabbits and man this compensatory phase continues until cardiac output has fallen to about 50% of its baseline level. At this point a second, decompensatory, Phase II supervenes during which vascular conductance rises abruptly, arterial pressure plummets and heart rate falls.

The signal which triggers Phase II in rabbits is not known. Earlier reports implicated a signal from cardiac afferents, since Phase II was abolished when procaine was instilled into the pericardial sac (Burke & Dorward, 1988; Evans *et al.*, 1989a). A number of recent studies, however, have thrown doubt on this notion. Thus, Hayes *et al.* (1992) demonstrated that intrapericardial procaine has a number of confounding effects independent of its ability to block conduction in cardiac afferent nerves. Moreover, Phase II is not prevented in conscious rabbits by cervical vagotomy (Evans *et al.*, 1993). More specifically, the signal which triggers Phase II does not appear to involve the cardiopulmonary afferents subserving the 'Bezold-Jarisch-like' reflex resulting from right or left atrial administration of phenylbiguanide (Evans & Ludbrook, 1991).

We have recently provided evidence that, in conscious rabbits, Phase II of the response to acute hypovolaemia requires a δ-opioid receptor mechanism in the brainstem for its expression (Evans *et al.*, 1989a,b). Suprapontine centres are probably also important, though the neurotransmitter

[1] Author for correspondence: Baker Medical Research Institute, P.O. Box 348, Prahran, Victoria, 3181, Australia.

systems involved have yet to be identified (Evans *et al.*, 1991).

Drugs with high affinity for 5-hydroxytryptamine (5-HT)-receptors (methysergide and LY53857) and α_2-adrenoceptors (yohimbine), whether given intravenously or in lower doses into the fourth cerebral ventricle, prevent the occurrence of Phase II in conscious rabbits (Evans *et al.*, 1992a,b). These findings could be interpreted as indicating that both 5-HT-receptors and α_2-adrenoceptors are involved in the ponto-medullary pathways that subserve this reflex. However, there is a considerable body of evidence that α_2-adrenoceptor ligands such as yohimbine, rauwolscine and oxymetazoline also have high affinity for 5-HT_1 binding sites (Broadhurst *et al.*, 1988; Convents *et al.*, 1989a,b; Hoyer, 1989; McCall *et al.*, 1991; Schoeffter & Hoyer, 1991). There is also evidence that 5-HT-receptor ligands such as methysergide and 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) have high affinity for at least one putative α_2-adrenoceptor subtype (Crist & Suprenant, 1987; Brown *et al.*, 1990a,b). This raises the possibility that drugs that have been classified as selective 5-HT antagonists or partial agonists, and drugs that have been classified as α_2-adrenoceptor antagonists, could be acting at a single receptor site to prevent Phase II. On the evidence presented above, this single site could be an α_2-adrenoceptor, indicating that an endogenous noradrenergic or adrenergic mechanism is involved; or it could be a 5-HT_1-receptor, indicating that 5-HT is the neurotransmitter involved. However, since we have little information regarding the intrinsic efficacy of these compounds at 5-HT_1-receptors, an agonist action cannot be excluded. This possibility is supported by studies demonstrating 5-HT agonist activity of methysergide (Brazenor & Angus, 1981) and yohimbine (McCall *et al.*, 1991), and by our finding that treatment with the 5-hydroxytrptaminergic neurotoxins *p*-chlorophenylalanine and 5,7-dihydroxytryptamine failed to mimic the effects of methysergide and LY53857 (Evans *et al.*, 1992b).

In the present investigation we have attempted to clarify this issue by examining the effects on the haemodynamic response to acute central hypovolaemia of a range of ligands with differing affinities for 5-HT-receptor subtypes and α_2-adrenoceptors. Acute central hypovolaemia was produced by progressively inflating a cuff on the inferior vena cava to cause graded central hypovolaemia (simulated haemorrhage). This procedure results in similar haemodynamic and humoral responses to those produced by actual haemorrhage (Ludbrook *et al.*, 1988), but because it is reproducible both between and within study days (*see* Evans & Ludbrook, 1991; Evans *et al.*, 1991), it has the advantage that multiple doses of different drugs can be tested in the same rabbit. It was also necessary for us to test the validity of using 2-hydroxypropyl-β-cyclodextrin as a vehicle for fourth ventricular administration of drugs to conscious rabbits, since many 5-HT-receptor ligands are only sparingly soluble in water. We have complemented these *in vivo* experiments with receptor binding studies in order to determine the affinities of our test compounds for α_2-adrenoceptors in rabbit brainstem.

Methods

Female New Zealand White rabbits were used. The experiments were done in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (1990) and were approved in advance by the Animal Ethics Committee of the Royal Melbourne Hospital.

Twelve rabbits, weighing 2.18–2.86 kg (mean 2.50) at the time of the first haemodynamic study, were prepared surgically as set out below. Thirteen other rabbits, weighing 1.74–4.04 kg (mean 3.11), were killed by asphyxiation with CO_2 and the pons and medulla were removed after rapid craniectomy. Care was taken to ensure that little or no blood was included in the dissection, since α_2-adrenoceptors have been identified on platelets (Bylund *et al.*, 1988). Samples were stored in dry ice for no longer than 15 h before preparation of a crude membrane homogenate.

In vivo *studies*

Major surgical procedures These have been described in detail previously (Evans *et al.*, 1989a,b). They were performed under halothane anaesthesia after induction with i.v. thiopentone sodium (25 mg kg^{-1}) and endotracheal intubation. At a first operation, an inflatable cuff was placed around the inferior vena cava (caval cuff). Two to three weeks later an ultrasonic (transit time) flow probe (Transonic Systems Inc., Ithaca, New York, U.S.A., Type 6S) was placed around the ascending aorta. Three weeks later, a third operation was performed at which a 0.3 mm o.d. polyvinyl chloride catheter was introduced through the atlanto-occipital ligament so that its tip lay in the fourth cerebral ventricle, its 15 μl dead space being filled with 154 mM NaCl. We have found previously that dye injected through these catheters produces heavy staining of the floor of the fourth ventricle and the overlying surface of the cerebellum, with some spillover to the ventral surface of the pons and medulla (Evans *et al.*, 1992b). The first study was done 5–7 days later, at which time all rabbits were well and gaining weight.

Minor procedures on study days These were done under local analgesia with 2% procaine HCl (David Bull Laboratories). The rabbit was placed in a 15 × 40 cm box fitted with a wire mesh lid, 90 min before the beginning of the study. The connecting tubes for the caval cuff and fourth ventricular catheter, and the connecting plug for the flow probe, were retrieved from their subcutaneous positions. Catheters were inserted into a central ear artery and a marginal ear vein.

Recording of haemodynamic variables Arterial pressure was measured by connecting the ear artery catheter to a Statham P23Dc strain gauge which was placed 50 mm above the floor of the rabbit's box. The flow probe, pre-calibrated by a method described previously (Evans *et al.*, 1992a), was connected to a Transonic Systems Inc. (U.S.A.) small animal blood flow meter (model T206) to measure ascending aortic flow (cardiac output). Heart rate was measured by a tachometer that was actuated by the flow pulse.

The signals were amplified and recorded on a Grass Model 7 Polygraph, and sent to an Olivetti M24 computer equipped with an A-D converter which provided 10 s mean values for arterial pressure (MAP, mmHg), heart rate (MHR, beats min^{-1}) and cardiac output (MCO, ml min^{-1}). The computer also calculated 10 s means for cardiac index (MCI = MCO/body weight in kg) and systemic vascular conductance index (MSVCI = MCI/MAP × 10^2).

Haematocrit At the beginning of each study capillary tube haematocrit (Hct) was measured on a 0.2 ml blood sample taken from an ear vein. Between the first and last studies it fell from 37.8 ± 1.0% to 36.1 ± 1.1% ($F_{1,11} = 2.8$; $P = 0.13$).

Stimulated haemorrhage The caval cuff was inflated gradually by means of a micrometer-driven syringe so that MCI fell linearly at a rate of 8.6 ± 0.2% of its baseline level per min (Ludbrook *et al.*, 1988). The cuff was released when MAP had fallen to ~40 mmHg or when MCI had fallen to ~30% of its baseline level, depending on which occurred first. Simulated haemorrhage was repeated up to 6 times in one day at 90 min intervals so that the effects of various treatments could be tested. Phase II was deemed to have been abolished if systemic vascular conductance was still declining when MCI had fallen to ~35% of its baseline level.

Drug administration The compounds were dissolved in either 154 mM NaCl (saline) or a 20% w/v solution of β-

CDX. They were administered into the fourth ventricle 10 min before simulated haemorrhage started, as a loading dose in 15 μl over 1 min, followed by a slow infusion at 1/20th of the loading dose per min.

Experimental protocols Three separate experiments were performed, though some rabbits were used for more than one experiment.

(1) The aim of the first was to determine if β-CDX could be utilised in later experiments as a vehicle for fourth ventricular administration of highly lipophilic drugs. Each of the 4 rabbits underwent 2 studies, performed at 2–5 day intervals in a cross-over design. On any one day, methysergide (0, 10, 30, 100, 300 and in one case 600 nmol) was administered in either saline or β-CDX. The acute effects of the vehicles and methysergide, and their effects on the haemodynamic response to simulated haemorrhage, were tested.

(2) The aim of the second series of experiments was to examine the effects of a range of 5-HT-receptor and α_2-adrenoceptor ligands on the haemodynamic response to simulated haemorrhage. The goal was to determine the receptor type(s) at which methysergide and yohimbine act to affect the haemodynamic response to acute central hypovolaemia. Each of the 8 rabbits was studied up to 6 times, at 2–5 day intervals. On each study day, the effects of either idazoxan (0, 30, 100, 300, 1000 and 3000 nmol in saline), ketanserin (0, 100, 300, 1000 and 3000 nmol in β-CDX), mianserin (0, 30, 100, 300, 1000 and 3000 nmol in β-CDX), mesulergine (0, 30, 100, 300, 1000 and 3000 nmol in β-CDX), 8-OH-DPAT (0, 3, 10, 30, 100 and 300 nmol in saline), sumatriptan (0, 10, 30, 100, 300 and 1000 nmol in saline), or MDL 72222 (0, 3, 30, and 300 nmol in β-CDX) were tested. Each study was terminated if a dose was reached which prevented Phase II of simulated haemorrhage (critical dose). The order of the studies was randomized, though not all 7 drugs were studied in every rabbit. Some data from previous studies (Evans *et al.*, 1992a,b) were also used (see Table 1).

(3) The aim of the third experiment was to explore the interactions between methysergide and clonidine. Three of the rabbits used in the previous experiment were studied on 3 additional occasions, at 2–5 day intervals. In the first study a critical dose of methysergide was determined, by testing the effects on the response to simulated haemorrhage of ascending doses (0, 30, 100 and 300 nmol in saline). In the second study, the effects of clonidine (0 and 10 nmol in saline) were tested. In the third study, the effects of a mixture of the critical dose of methysergide and 10 nmol of clonidine were tested.

At the end of the last study the rabbits were killed with an overdose of pentobarbitone sodium (300 mg).

Analysis of results We analysed the data in a similar fashion to that used previously (Evans *et al.*, 1989a,b; Evans & Ludbrook, 1990; Evans & Ludbrook, 1991; Evans *et al.*, 1991).

Regression analysis confirmed that MCI fell linearly with time during simulated haemorrhage ($r = 0.977 \pm 0.003$). As we have found previously (Evans *et al.*, 1989a,b), there was a near linear relationship of MAP, MHR and MSVCI to MCI during simulated haemorrhage, up to the point that the abrupt rise of MSVCI occurred.

The levels of the haemodynamic variables and the slope of the regression between MSVCI and MCI during Phase II of the response to simulated haemorrhage, were subjected to analysis of variance (ANOVA) to distinguish main effects and interactions of such factors as rabbits, vehicles, drug treatments, and before vs after drug administration.

The effects of drug treatments on the responses of MSVCI, MAP and MHR to simulated haemorrhage were analysed graphically by the method we have used previously (Evans *et al.*, 1989a,b; Evans & Ludbrook, 1991; Evans *et al.*, 1991). The relationships between the dependent variables MAP, MHR and MSVCI and the independent variable MCI were characterized by three sets of coordinates: (a) post-treatment levels, immediately before the onset of simulated haemorrhage; (b) the point at which MSVCI reached a minimum, before it rose abruptly (or its interpolated equivalent); and (c) the final observation before the caval cuff was deflated.

Levels of haemodynamic variables are expressed throughout as between-rabbit means ± 1 s.e.mean.

Receptor binding studies

Preparation of crude membrane homogenate Each frozen brain sample was rinsed with 10 ml of ice cold Krebs phosphate buffer (KPB, of composition mM: NaCl 120, KCl 4.69, $MgSO_4$ 1.42, NaH_2PO_4 1.30, Na_2HPO_4, 8.7, $CaCl_2$ 2.5; glucose 11.66) at pH 7.4 It was then homogenized (Kinematica PT 10–35) in 10 ml of KPB for 10 s. The homogenate was centrifuged at approximately 45000 *g* for 15 min at 4°C (Sorvall model RC5C). The supernatant was decanted and the pellet resuspended in a further 10 ml of ice cold KPB. This homogenate was then incubated at 37°C for 15 min to promote breakdown of endogenous monoamines. It was then centrifuged (as above), the supernatant decanted, and the pellet resuspended in 80 ml g^{-1} wet weight KPB.

Saturation studies For saturation analysis each assay tube contained 50 μl of [^{3}H]-rauwolscine, 50 μl of buffer or idazoxan (50 μM), and 150 μl of the brainstem homogenate. Twelve points over the range 0.02–20 nM of [^{3}H]-rauwolscine were run in triplicate in each experiment.

Competition studies Each assay tube contained 50 μl of [^{3}H]-rauwolscine, 50 μl of the competing ligand, and 150 μl of membrane homogenate. The final concentration of [^{3}H]-rauwolscine was approximately its K_D value (see results). Competing ligand concentrations (14 points) were between 100 pM and 0.3 mM.

The binding reaction All incubations were performed at 37°C in a shaking water bath. Preliminary kinetic studies demonstrated that at this temperature [^{3}H]-rauwolscine binding reached equilibrium within 5 min and maintained equilibrium for at least a further 85 min. All saturation and competition studies were therefore performed with an incubation time of 10 min. The reaction was stopped with 4 ml ice cold KPB, and the incubate immediately drawn through pre-soaked filter papers (Gelman A/E) in a cell harvester (Brandel model M-24). The filter papers were then washed, dried, and placed in 6 ml plastic scintillation vials. Five ml of scintillation fluid (Amersham, Phase Combining System) was then added to each vial, and after vigorous shaking they were placed in a liquid scintillation counter (United Technologies Packard Tri-Carb 4000 series). Counts per min (c.p.m.) were determined over a 5 min period.

Protein assay Protein concentrations in tissue homogenates were determined by the method of Schacterle & Pollack (1973). Bovine serum albumin (Commonwealth Serum Laboratories, Australia) was used as the protein standard.

Analysis of results Initial estimates of binding parameters ($1/K_D$ and B_{max}) were obtained by use of the computer software EBDA (McPherson, 1983). The software LIGAND (Munson & Robard, 1980) was used to obtain final parameter estimates. The significance (or goodness of fit) of the final parameters estimated for one- and two-site binding models, from saturation and displacement studies, was determined by the *F*-test (Munson & Robard, 1980). A single-site model was assumed unless a two-site model had a significantly lower sum of squares ($P < 0.05$).

Throughout the text, K_I and K_D values are expressed as means ± 1 s.e.mean.

Drugs

The following drugs were used in this study: yohimbine HCl, clonidine HCl, and prazosin HCl (Sigma Chemical Company, U.S.A.); idazoxan HCl, (±)-8-hydroxy-2-(di-n-propylamino) tetralin HCl (8-OH-DPAT), rauwolscine HCl, ritanserin HCl, (±)-mianserin HCl, ketanserin tartrate, (±)-3-tropanyl-3,5-dichlorobenzoate (MDL 72222), and 2-hydroxypropyl-β-cyclodextrin (β-CDX) (Research Biochemicals Incorporated, U.S.A.); mesulergine and methysergide maleate (gifts from Sandoz, Australia); 4-isopropyl-7-methyl-9-(2-hydroxy-1-methylpropoxycarbonyl) 4, 6, 6a, 7, 8, 9, 10, 10a-octahydroindolo [4,3-fg]quinoline maleate (LY53857) (a gift from Eli Lilly, U.S.A.); sumatriptan succinate (GR 43175C) (a gift from Glaxo Group Research Ltd, U.K.).

[^{3}H]-rauwolscine (75.0 Ci mmol^{-1}) was obtained from Amersham (Australia) Pty Ltd.

Results

In vivo studies

General At the beginning of each study the levels of the haemodynamic variables in the 12 rabbits were within the normal range for our laboratory (Evans *et al.*, 1989a,b). The mean levels and compound standard errors across rabbits and studies were: MCI, 182 ± 5 ml kg^{-1} min^{-1}; MAP, 80 ± 3 mmHg; MHR, 202 ± 5 beats min^{-1}; MSVCI, 231 ± 10 ml kg^{-1} min^{-1} mmHg^{-1} × 10^2.

After vehicle treatments, the haemodynamic response to simulated haemorrhage was biphasic. During Phase I MSVCI fell progressively and MHR rose, while MAP fell by only 8 ± 1 mmHg. Phase II began when MCI had fallen to 61 ± 3% of its baseline level, at which stage MSVCI rose

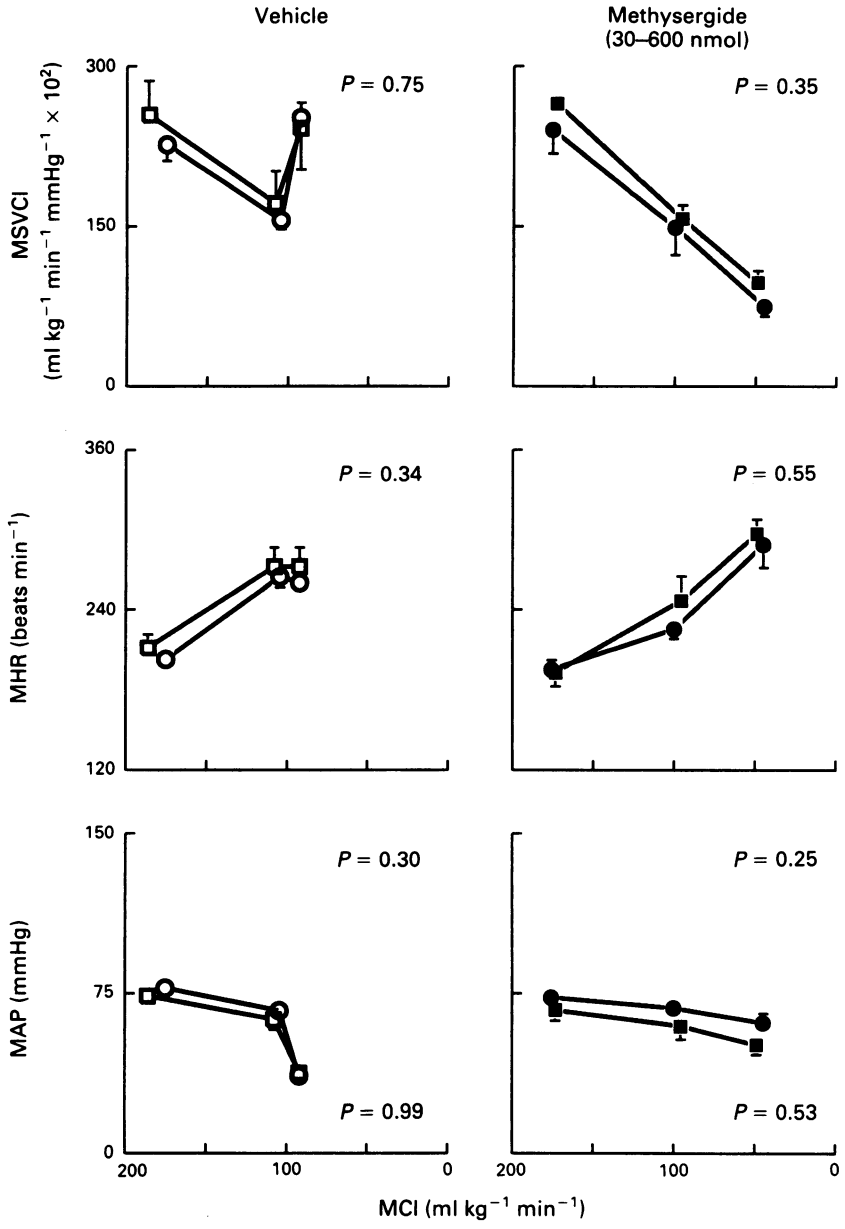


Figure 1 Haemodynamic changes during simulated haemorrhage after 4th ventricular administration of vehicle (154 mM NaCl (saline) or 20% w/v 2-hydroxypropyl-β-cyclodextrin (β-CDX)) or methysergide (in saline or β-CDX). Symbols are average coordinates for 4 rabbits with 1 s.e.mean shown unless this lies within the symbol. MCI, mean cardiac index. MSVCI, mean systemic vascular conductance index. MHR, mean heart rate. MAP, mean arterial pressure. The treatments were: saline vehicle (○); β-CDX vehicle (□); lowest dose of methysergide in saline which prevented Phase II (critical dose, 100–300 nmol) (●); lowest dose of methysergide in β-CDX which prevented Phase II (critical dose, 30–600 nmol) (■). In the top right hand corners of each panel are *P* values derived from $F_{1,3}$ (ANOVA) for influence of vehicle (saline vs β-CDX) on MSVCI, MHR and MAP just prior to deflation of the caval cuff and, bottom right, on MCI at this point.

abruptly, MAP fell precipitately and MHR fell slowly. The caval cuff was deflated when MAP had fallen to 37 ± 1 mmHg, at which stage MCI was $51 \pm 2\%$ of its baseline level (Figures 1–4).

(1) *Comparison of effects of methysergide in saline or β-CDX* There were no differences between the baseline haemodynamic variables at the beginning of the 2 studies ($F_{4,48} \leqslant 1.3$; $P \geqslant 0.3$). Over the course of each study, baseline MAP and MHR gradually declined and baseline MSVCI gradually rose ($F_{4,48} \geqslant 3.3$; $P \leqslant 0.02$), while MCI did not change ($F_{4,48} = 0.9$; $P = 0.5$). However, there were no acute effects of the vehicles or methysergide on any of the baseline haemodynamic variables ($F_{4,48} \leqslant 0.6$; $P \geqslant 0.6$).

As we have found previously (Evans *et al.*, 1992b), methysergide (10–300 nmol) had dose-related effects on both phases of the haemodynamic response to simulated haemorrhage. It increased the slope (b) of the relationship between MSVCI and MCI during Phase 1 in a dose-dependent fashion and delayed Phase II ($F_{1,6} \geqslant 18.3$; $P \leqslant 0.005$). At a critical dose, Phase II did not occur. None of these effects was dependent on the vehicle used ($F_{4,24} \leqslant 2.6$; $P \geqslant 0.07$). In

Table 1 Threshold (critical) doses (nmol), of 5-HT-receptor and α_2-adrenoceptor ligands, that prevent Phase II of the response to simulated haemorrhage after 4th ventricular administration

Drug	n	*Range (nmol)*	*Geometric mean* (nmol)	$-log_{10}$ *geometric mean*
8-OH-DPAT	4	10–30	13.1	7.88
Sumatriptan	4	10–300	72.1	7.14
LY53857†	6	100–300	173	6.76
Mesulergine	4	30–1000	173	6.76
Methysergide†	16	30–600	206	6.69
Yohimbine*	5	300–600	344	6.46
Idazoxan	4	100–3000	548	6.26
Mianserin	4	100–3000	548	6.26
MDL 72222	2	>300	>300	>6.52
Ketanserin	4	>3000	>3000	>5.52

This table includes data from Evans *et al.* (1992a)* and Evans *et al.* (1992b)†.

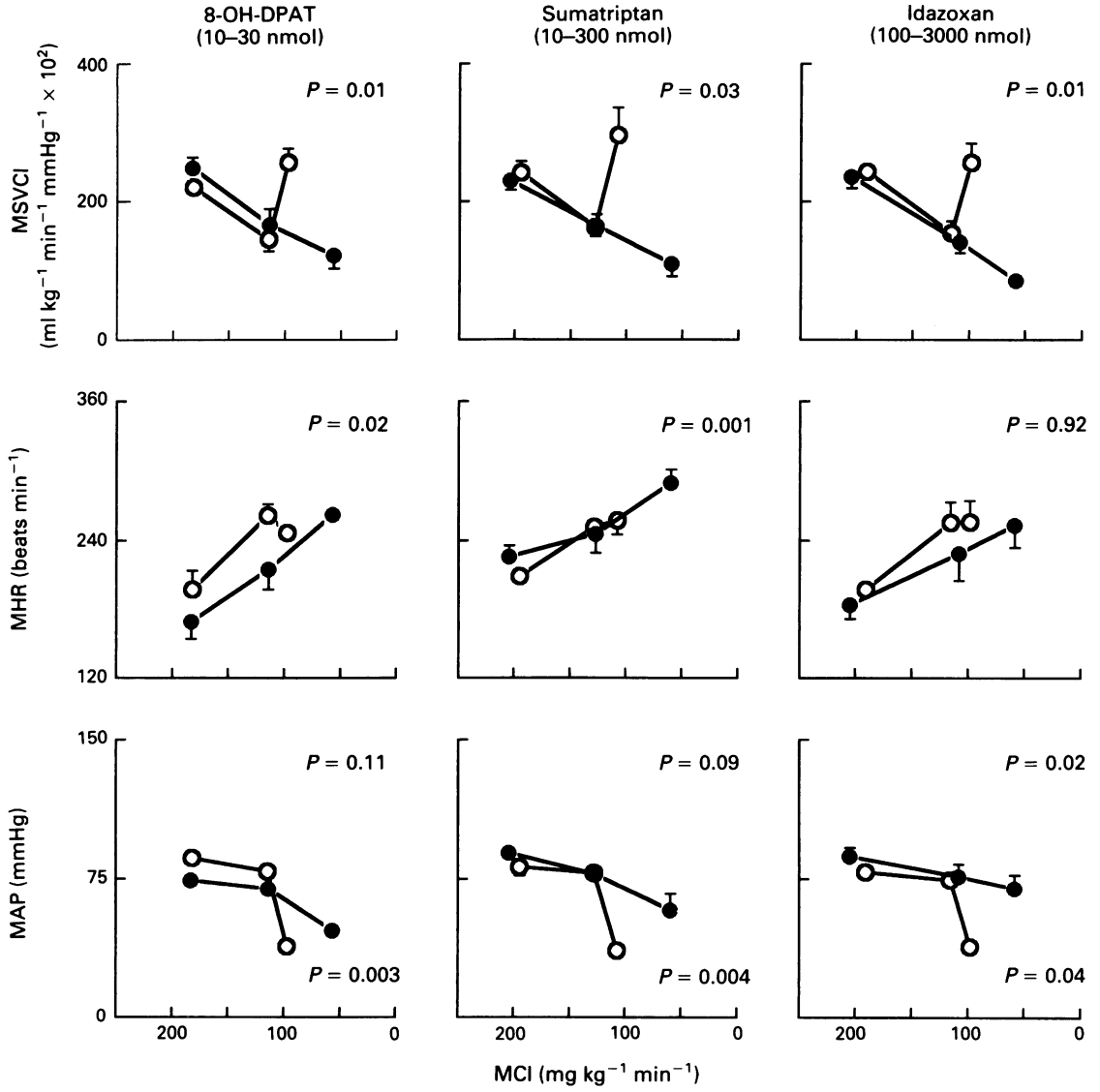


Figure 2 Haemodynamic changes during simulated haemorrhage after 4th ventricular administration of vehicle, or critical doses of 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT), sumatriptan, and idazoxan. Symbols are average coordinates for 4 rabbits with 1 s.e.mean shown unless this lies within the symbol. Haemodynamic variables as for Figure 1. The treatments were: vehicle (○); lowest dose which prevented Phase II (critical dose, see Table 1) (●). In the top right-hand corners of each panel are *P* values derived from $F_{1,3}$ for effects of critical doses on MSVCI, MHR and MAP just prior to deflation of the caval cuff and, bottom right, on MCI at this point.

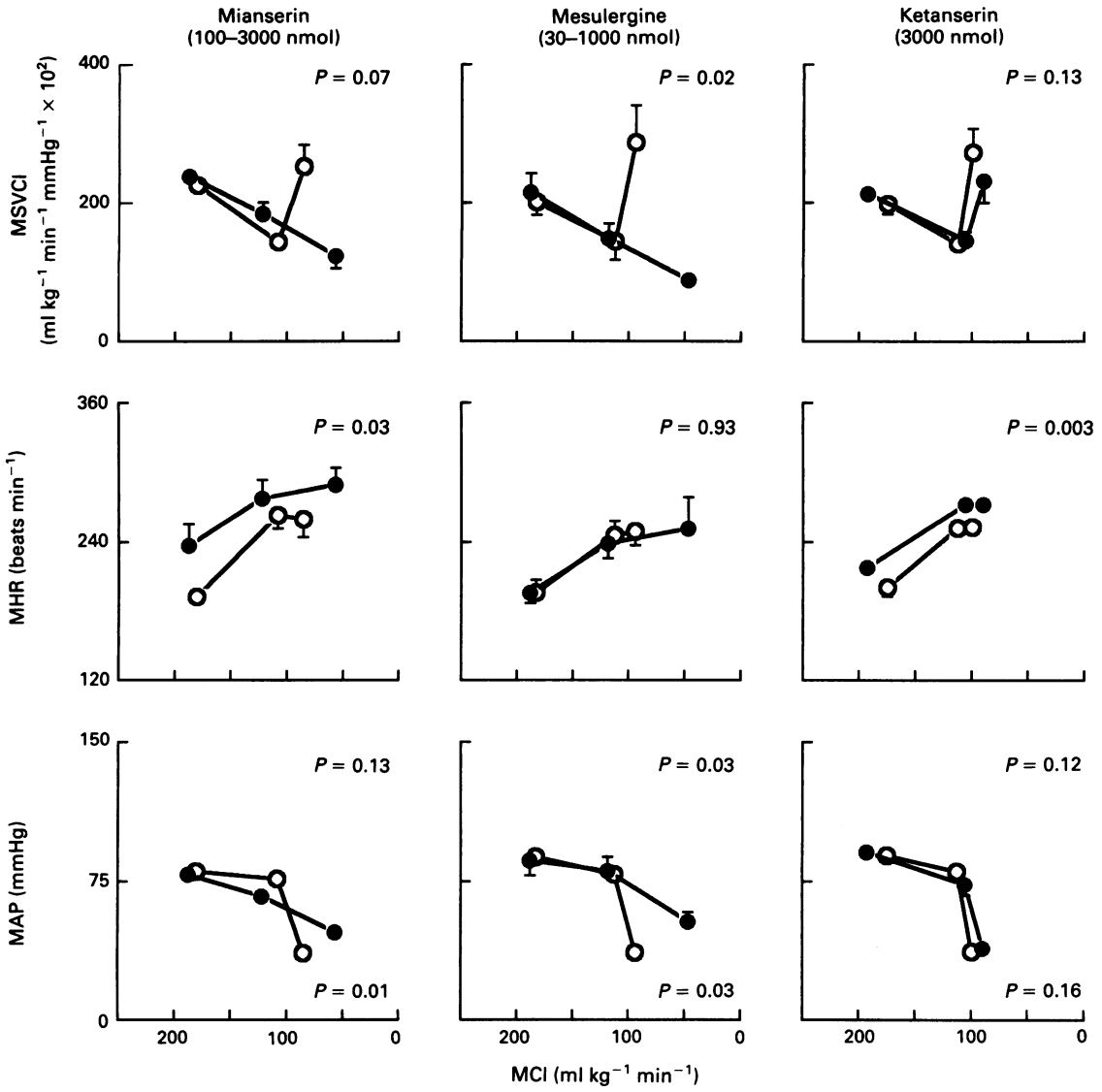


Figure 3 Haemodynamic changes during simulated haemorrhage after 4th ventricular administration of vehicle, or mianserin, mesulergine or ketanserin. Symbols are average coordinates for 4 rabbits with 1 s.e.mean shown unless this lies within the symbol. Haemodynamic variables as for Figure 1. The treatments were: vehicle (○); lowest dose which prevented Phase II (critical dose, see Table 1), or in the case of ketanserin, the maximum dose tested (●). In the top right-hand corners of each panel are P values derived from $F_{1,3}$ for effects of critical doses on MSVCI, MHR and MAP just prior to deflation of the caval cuff and, bottom right, on MCI at this point.

the 4 rabbits, the critical doses were 100, 300, 300 and 300 nmol (geometric mean = 228 nmol) in saline vehicle, and 100, 300, 30 and 600 nmol respectively (geometic mean = 152 nmol) in β-CDX vehicle (Figure 1).

(2) *Effects of other 5-HT-receptor and α_2-adrenoceptor ligands* The baseline haemodynamic variables were not affected by sumatriptan, idazoxan, mianserin, mesulergine, or ketanserin at any dose tested ($F_{1,3} \leqslant 5.5$; $P \geqslant 0.1$). In the case of MDL 72222, no changes in resting haemodynamic variables were observed in the 2 rabbits in which it was studied. In the case of 8-OH-DPAT, on the other hand, baseline MAP was 12 ± 2 mmHg lower and MSVCI 28 ± 7 ml kg^{-1} min^{-1} mmHg^{-1} × 10^2 higher after the critical dose compared with after vehicle administration.

For all of the test compounds except ketanserin and MDL 72222, a threshold (critical) dose was found which prevented the occurrence of Phase II in each rabbit (Table 1; Figures 2–3). In the case of ketanserin (Figure 3) and MDL 72222 (data not shown), the maximum doses of 3000 and 300 nmol respectively had no effects on the haemodynamic response to simulated haemorrhage (Table 1). None of the treatments affected the slope of the relationship between MSVCI and MCI during Phase I ($F_{1,3} \leqslant 7.8$; $P \geqslant 0.07$).

(3) *Interactions between methysergide and clonidine* In the 3 rabbits studies, the critical dose of methysergide ranged from 300 and 600 nmol (Figure 4). As we have found previously (Evans *et al.*, 1992a), fourth ventricular administration of 10 nmol of clonidine caused Phase II to occur prematurely (at a higher level of MCI), and blunted Phase I so that the slope of the relationship between MSVCI and MCI during the compensatory phase was reduced (Figure 4). When these 2 treatments were combined, the haemodynamic response to simulated haemorrhage was biphasic, and similar to that seen after saline vehicle administration (Figure 4).

Receptor binding studies

Scatchard analysis demonstrated that in KPB [^{3}H]-rauwolscine bound to a single site in homogenates of rabbit brainstem (Hill coefficient = 0.97 ± 0.11, $P = 0.3$ for a 2 site fit), with a B_{max} of 36.3 ± 6.4 fmol mg^{-1} protein and a pK_D of 8.44 ± 0.11.

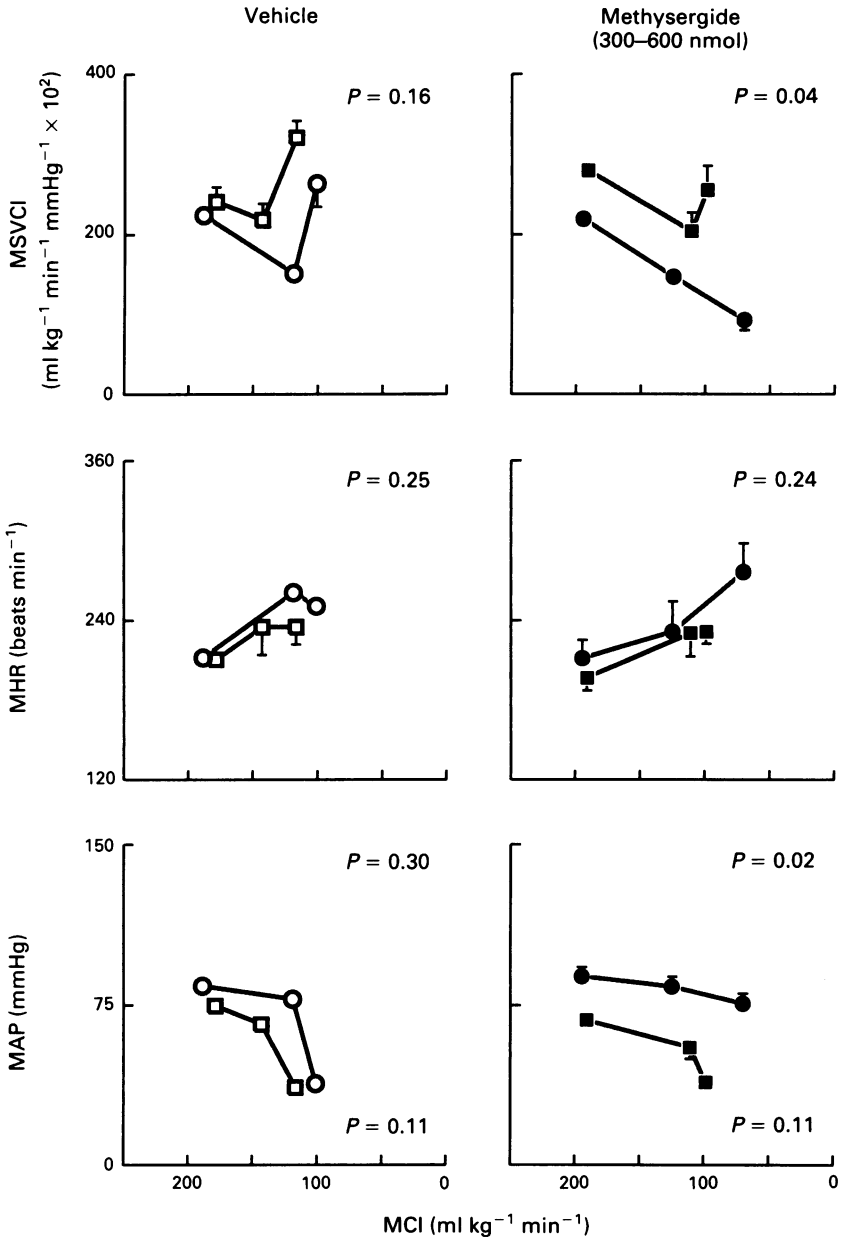


Figure 4 Haemodynamic changes during simulated haemorrhage after 4th ventricular administration of saline, methysergide, clonidine or mixtures of methysergide and clonidine. Symbols are average coordinates for 3 rabbits with 1 s.e.mean shown unless this lies within the symbol. Haemodynamic variables as for Figure 1. The treatments were: saline vehicle (○); 10 nmol clonidine (□); lowest dose of methysergide which prevented Phase II (critical dose, 300–600 nmol) (●); the critical dose of methysergide administered with 10 nmol clonidine (■). In the top right-hand corners of each panel are P values derived from $F_{1,2}$ for effects of clonidine on MSVCI, MHR and MAP just prior to deflation of the caval cuff and, bottom right, on MCI at this point.

The displacement profiles, for [^{3}H]-rauwolscine binding sites, of a whole range of reference compounds, including those whose effects were tested *in vivo*, are shown in Table 2.

Discussion

Our major conclusion is that Phase II of the haemodynamic response to acute central hypovolaemia in conscious rabbits can be prevented through activation of brainstem 5-HT_{1A}-receptors by 8-OH-DPAT. It is also possible that the prevention of Phase II by other 5-HT-receptor ligands such as methysergide and LY53857, and that by α_2-adrenoceptor antagonists such as yohimbine and idazoxan, is due to an agonist action at 5-HT_{1A}-receptors in the brainstem. In the course of these studies we have also provided information regarding the nature of α_2-adrenoceptor binding sites in rabbit brainstem and on the validity of using β-CDX as a vehicle for administration of lipophilic drugs into the cerebrospinal fluid.

Involvement of brainstem 5-HT-receptors in the haemodynamic response to hypovolaemia

We can exclude the possibility that endogenous activation of brainstem 5-HT_2- or 5-HT_3-receptors is required for expression of Phase II of the haemodynamic response to simulated haemorrhage, since neither ketanserin (Figure 3) nor MDL 72222 (data not shown) prevented it (Table 1). We found that the 5-HT_{1A}-receptor-selective agonist, 8-OH-DPAT, potently prevented Phase II (Figure 2, Table 1). This provides strong evidence for a role (albeit not a physiological one) of 5-HT_{1A}-receptors in the neural pathways subserving Phase II. We suggest that the other 5-HT receptor ligands

which prevented Phase II (sumatriptan, LY53857, mesulergine, methysergide and mianserin) might also be acting as 5-HT_{1A}-receptor agonists. This supposition is based on our finding of a positive correlation between their potency in preventing Phase II and their affinity for 5-HT_{1A}-receptors, but not for 5-HT_{1C}-, 5-HT_{1D}-, and 5-HT_2-receptors, or α_2-adrenoceptors (Figure 5). In the case of methysergide there is reasonable evidence that this might be so, since it does have 5-HT-receptor agonist activity *in vitro* (Brazenor & Angus, 1981) and, like 8-OH-DPAT, it lowers arterial pressure and heart rate in anaesthetized rabbits after intravenous administration (Evans, 1981; Shepheard *et al.*, 1990). Similarly, though sumatriptan is regarded as a selective agonist for 5-HT_{1D}-receptors, there have been reports that it has only moderate (about 4 fold) 5-HT_{1D}/5-HT_{1A}-selectivity (*see* Van Wijngaarden *et al.*, 1990). In the cases of LY53857, mesulergine and mianserin we are on shakier ground, since we are aware of no previous reports that they have agonist actions at 5-HT_{1A}-receptors. There has been one report, however, which proposed an antagonist action of LY53857 at 5-HT_{1A}-receptors (Pérgola & Alper, 1992).

We can find no data in the literature regarding the existence or location of 5-HT_{1A}-receptors within the rabbit brainstem, though they have been demonstrated in the rabbit caudate nucleus (Xiong & Nelson, 1989). In rats, 5-HT_{1A}-binding sites have been identified in the dorsal horn of the spinal cord (Marlier *et al.*, 1991), the raphe nuclei (Vergé *et al.*, 1985; Weissman-Nanopoulos *et al.*, 1985), nucleus tractus solitarii, trigeminal nucleus and area postrema (Thor *et al.*, 1992a,b). Evidence for a role of these binding sites in the integration of visceral afferent input is provided by the observation that the density of 5-HT_{1A}-binding sites is reduced after cervical vagotomy or nodose ganglionectomy (Thor *et al.*, 1992a). In man, 5-HT_{1A}-binding sites have been identified in the nucleus tractus solitarii, nucleus spinalis nervi trigemini and nucleus raphe obscurus (Pazos *et al.*, 1987). 5-HT_{1A}-receptors have also been identified in the brainstem by functional studies. Thus, injection of 8-OH-DPAT into areas of

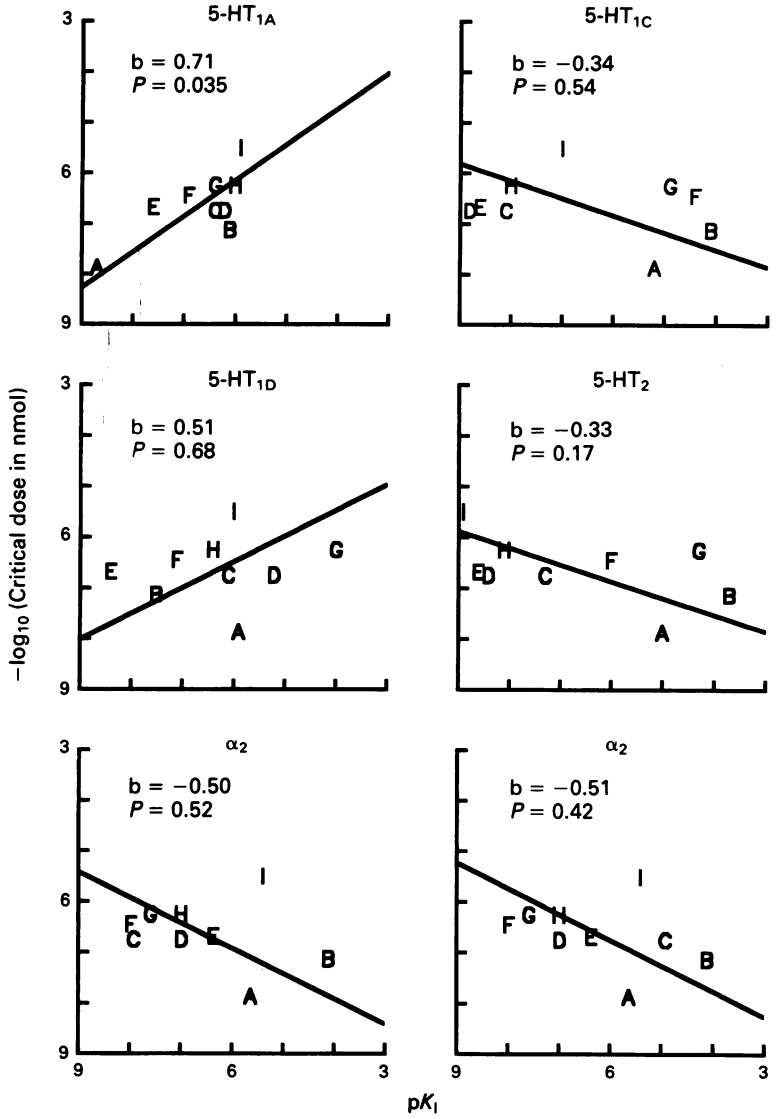


Figure 5 Correlation of the affinities (pK_I) of 8-hydroxy-2-(di-n-propylamino tetralin) (A), sumatriptan (B), LY53857 (C), mesulergine (D), methysergide (E), yohimbine (F), idazoxan (G), mianserin (H), and ketanserin (I), for 5-HT_{1A}-, 5-HT_{1C}-, 5-HT_{1D}- and 5-HT_2-receptors (Table 3), and α_2-adrenoceptors (Table 2), with their geometric mean critical doses after 4th ventricular administration (p(critical dose)) (Table 1). Note that two plots are shown for α_2-adrenoceptor binding sites, since LY53857 bound to two [^{3}H]-rauwolscine-binding sites (Table 2), either or both of which might be α_2-adrenoceptors (see Discussion). The plot on the left includes the high affinity site only, while the plot on the right includes the low affinity site only. Each number represents the coordinates for 1 drug (code given above). The lines of best fit (lines of symmetry) were calculated using model II regression (Brace, 1977). The calculated slope (b) and the *P* value for deviation of the slope from zero are given for each plot.

the brainstem of anaesthetized rats (Itoh & Bunag, 1991; nucleus tractus solitarius) and cats (Mandal *et al.*, 1990; rostral ventrolateral medulla) results in a fall in arterial pressure. There is also evidence that 5-HT_{1A}-receptors participate in the control of sympathetic outflow to the heart, and in the control of central respiratory drive, in a variety of species including rabbits (Shepheard *et al.*, 1990; Sporton *et al.*, 1991). It has been proposed than an endogenous 5-HT_{1A}-receptor mechanism in the brainstem is involved in the bradycardia evoked by intravenous administration of phenylbiguanide in rats (Bogle *et al.*, 1990).

Involvement of brainstem α_2-adrenoceptors in the haemodynamic response to hypovolaemia

So far as we can ascertain, this is the first account of the pharmacological characteristics of α_2-adrenoceptors in rabbit brainstem. Our data indicate that rabbit brainstem α_2-adrenoceptors are of the same type as those found in rabbit cerebral cortex (Limberger *et al.*, 1991), and correspond to the α_{2A}-subtype according to the proposed classification of Bylund (1985).

Our evidence that rabbit brainstem contains a pure population of α_{2A}-adrenoceptor-binding sites rests chiefly on the finding that prazosin displaced [^{3}H]-rauwolscine binding with extremely low affinity compared with yohimbine (Table 2). Prazosin displaced [^{3}H]-rauwolscine binding to 2 sites, but the lower affinity site ($pK_I = 2.81$) is unlikely to be of pharmacological significance since it is in the mM range. The site with higher affinity ($pK_I = 4.75$) is likely to present true α_2-adrenoceptor binding, and the affinity ratio between yohimbine and prazosin at this site is 1660, which is far closer to that found in the α_{2A}-binding sites in human platelets and HT-29 cells (267–618) (Bylund *et al.*, 1988) than in the α_{2B}-binding sites found in rat lung and NG108-15 cells (5.4–5.5) (Bylund *et al.*, 1988), the α_{2C}-binding sites found in opossum kidney and the OK cell line (80–90) (Blaxall *et al.*, 1991), or the putative α_{2D}-binding site in bovine pineal gland (29) (Simonneaux *et al.*, 1991). Our conclusion is also supported by the relatively low affinity of 8-OH-DPAT and methysergide for these binding sites (Table 2). The ratio of their K_I values to that of yohimbine were 209 and 40.7 respectively. These ratios are far closer to those reported in human platelet α_{2A}-binding sites (275 and 112 respectively) than in rat cortex α_{2B}-binding sites (28 and 5 respectively) (Brown *et al.*, 1990a,b).

A number of our test compounds did, however, displace [^{3}H]-rauwolscine binding in a complex manner that indicated displacement from two binding sites (Table 2). In the cases of prazosin, rauwolscine and mianserin, the higher affinity sites have the characteristics of α_2-adrenoceptors, since the pK_I values are in close agreement to those in the literature (see Bylund *et al.*, 1988). The pharmacological significance of the lower affinity sites is uncertain, though they do not correspond to any of the proposed subtypes of α_2-adrenoceptors (see Bylund *et al.*, 1988; Blaxall *et al.*, 1991; Simonneaux *et al.*, 1991). Moreover, they cannot be 5-HT-receptor binding sites, since neither 8-OH-DPAT nor sumatriptan displaced [^{3}H]-rauwolscine binding with sub-micromolar affinity (Table 2). These ligands possess high affinity for 5-HT_{1A} and 5-HT_{1D} binding sites (Table 3), the only 5-HT-receptor types for which rauwolscine has high affinity (Hoyer, 1989). We can find no data in the literature on the affinity of LY53857 for α_{2A}-adrenoceptor binding sites. We can make no comment, therefore, as to which of these binding sites represents the rabbit brainstem α_2-adrenoceptor.

There is little information in the literature regarding the functional significance or neuroanatomy of rabbit brainstem α_2-adrenoceptors. In the rabbit brain cortex, $\alpha_{2A\text{-like}}$-adrenoceptors have been demonstrated to have a presynaptic autoreceptor function (Limberger *et al.*, 1991). There appear to have been no autoradiographic studies of α_2-adrenoceptors in rabbit brainstem. In man, the highest densities are found in the dorsal motor nucleus of the Xth nerve, nucleus tractus solitius, locus coeruleus and substantia grisea centralis (Probst *et al.*, 1985). We have no information regarding the pharmacological nature of these receptors, though both α_{2A}- and α_{2B}-adrenoceptor binding sites have been identified in more rostral human brain regions (Petrash & Bylund, 1986). In the rat, the predominant α_2-adrenoceptor mRNA species in the brainstem is the 2A type (Zeng & Lynch, 1991).

Are these α_2-adrenoceptor binding sites in the rabbit brainstem involved in the haemodynamic response to acute central hypovolaemia? On this our data are equivocal. We found the potency of yohimbine and idazoxan in preventing Phase II to correlate well with their affinities for 5-HT_{1A}-receptors (Figure 5). Thus, it is plausible that they are acting, like 8-OH-DPAT, through activation of 5-HT_{1A}-receptors.

Table 2 Displacement of [^{3}H]-rauwolscine by a range of 5-HT-receptor and α_2-adrenoceptor ligands in rabbit brainstem

Drug	*Slope*	P	pK_I	B_{max} (*fmol mg^{-1} protein*)
Drugs tested in vivo				
8-OH-DPAT	0.70 ± 0.15	0.07	5.63 ± 0.12	47.5 ± 3.1
Sumatriptan	0.92 ± 0.28	0.44	4.10 ± 0.10	73.5 ± 5.3
LY53857	0.49 ± 0.14	0.008	7.92 ± 0.28	26.7 ± 3.5
			4.92 ± 0.57	11.9 ± 3.5
Mesulergine	0.66 ± 0.33	0.28	6.97 ± 0.23	25.4 ± 2.8
Methysergide	0.49 ± 0.14	0.26	6.36 ± 0.23	33.7 ± 3.4
Yohimbine	0.67 ± 0.26	1.00	7.97 ± 0.27	14.3 ± 1.8
Idazoxan	0.94 ± 0.38	0.52	7.59 ± 0.16	39.0 ± 2.7
Mianserin	1.81 ± 0.66	<0.001	6.99 ± 0.13	41.2 ± 2.7
			3.52 ± 0.40	25.8 ± 8.6
MDL 72222	not tested, but reported in other tissues to have a pK_I of <5 (Van Wijngaarden *et al.*, 1990)			
Ketanserin	0.79 ± 0.28	0.1	5.40 ± 0.13	45.9 ± 2.8
Drugs not tested in vivo				
Rauwolscine	2.57 ± 1.43	<0.001	7.85 ± 0.35	19.3 ± 3.2
			4.07 ± 0.30	29.0 ± 4.8
Prazosin	0.80 ± 0.41	0.032	4.75 ± 0.24	32.6 ± 11.2
			2.81 ± 1.9	34.9 ± 3.82
Clonidine	0.93 ± 0.55	0.31	6.95 ± 0.16	39.2 ± 2.8
Ritanserin	0.44 ± 0.18	0.053	6.50 ± 0.15	18.4 ± 1.4

Values are the mean ± s.e.mean of 3–5 observations. Slope = slope factor of displacement curve. *P* is probability that a 2 site fit is better than a 1 site fit. $pK_I = \log_{10}$ (equilibrium dissociation constant). B_{max} = estimated number of receptors.

Table 3 Affinity (pK_I) for 5-HT_1- and 5-HT_2-receptors, of the 5-HT-receptor and α_2-adrenoceptor ligands that were tested *in vivo*

Drug	*5-HT$_{1A}$*	*5-HT$_{1C}$*	*5-HT$_{1D}$*	*5-HT$_2$*
8-OH-DPAT	8.7	5.2	5.9	5.0
Sumatriptan	6.1	4.1	7.5	3.7
LY53857	6.4	8.1	6.1	7.3
Mesulergine	6.2	8.8	5.2	8.4
Methysergide	7.6	8.6	8.4	8.6
Yohimbine	6.9	4.4	7.1	6.0
Idazoxan	6.4	4.9	4.0	4.3
Mianserin	6.0	8.0	6.4	8.1
MDL 72222	<5	<5	<5	<5
Ketanserin	5.9	7.0	6.0	8.9

Data from Hoyer (1989), Van Wijngaarden *et al.* (1990), Hoyer & Schoeffter (1991) and Hoyer (personal communication).

This hypothesis is supported by a study by McCall *et al.* (1991) which demonstrated an agonist action of yohimbine at 5-HT_{1A}-receptors in the cat brainstem. We cannot, however, exclude the possibility that these drugs are acting here as α_2-adrenoceptor antagonists, which would imply there is an endogenous catecholaminergic link in the neuronal pathways subserving Phase II of the response to acute central hypovolaemia. This hypothesis can only be tested satisfactorily when α_2-adrenoceptor antagonists are developed that lack affinity for 5-HT_{1A}-receptors. The complex interaction between methysergide (putative 5-HT_{1A} agonist) and clonidine (α_2-adrenoceptor agonist), whereby co-administration of clonidine nullifies the effects of methysergide (Figure 4), supports the hypothesis that both 5-HT_{1A} receptors and α_2-adrenoceptors in the brainstem can modulate the response to hypovolaemia, and that their effects are opposing. This is consistent with the notion that brainstem 5-hydroxytryptaminergic and noradrenergic neurones have opposing functions (sympathoexcitatory and sympathoinhibitory respectively) (see Head, 1992).

β-CDX as a vehicle for fourth ventricular administration of lipophilic substances

In the course of this experiment, it was necessary for us to test the effects of a number of highly lipophilic drugs such as ketanserin. These drugs could not be administered in saline vehicle, since they are only sparingly soluble in it. Recently, much interest has surrounded the potential use of cyclodextrins as drug solubilizers (see Pitha *et al.*, 1988). These are ringed polymers of D-(+)-glucopyranose units that have a slightly apolar internal cavity and a polar external surface. This structure makes them readily soluble in water, yet they form inclusion complexes with small lipophilic compounds such as alkaloids. A recent report demonstrated that a range of drugs have comparable effects and potency, when dissolved in saline or in a 20% solution of β-CDX, after lateral ventricular and spinal administration to rats (Yaksh *et al.*, 1991).

We compared the effects of ascending doses of methysergide on the haemodynamic response to acute central hypovolaemia, when administered in saline or β-CDX. Neither saline- nor β-CDX-vehicle affected baseline haemodynamic variables or the haemodynamic response to acute central hypovolaemia. Moreover, we found no differences between the effects and potency of methysergide in saline or β-CDX vehicle.

Though our results are not directly applicable to drugs other than methysergide, a number of generalizations can be made. As discussed by Yaksh *et al.* (1991), two factors will influence drug bioavailability in β-CDX vehicle, at any particular β-CDX/drugs molar ratio. These are the molecular dimensions and conformation of the drug, and its lipid partition coefficient. Thus, it would be expected that drugs with similar physiochemical properties to methysergide should also display comparable bioavailability in β-CDX and saline vehicles.

In conclusion our data indicate that an endogenous 5-hydroxytryptaminergic mechanism is not a necessary component in the neuronal pathways subserving Phase II of the response to acute central hypovolaemia in conscious rabbits. Rather, it seems that drugs such as methysergide act to prevent Phase II via an agonist action at brainstem 5-HT_{1A}-receptors. At present, it is not possible to test this hypothesis with antagonists, since even the most recently developed 5-HT_{1A}-receptor antagonists MDL 73005EF, NAN-190, and BMY 7378 have some intrinsic efficacy (Gartside *et al.*, 1990; Greuel & Glaser, 1992). Though the effects of yohimbine and idazoxan can be satisfactorily explained in terms of agonist effects at 5-HT_{1A}-receptors, we cannot discount the possibility that an endogenous catecholaminergic mechanism in the brainstem is a necessary component in the neuronal pathways subserving Phase II of the response to acute central hypovolaemia in conscious rabbits. The testing of this hypothesis awaits the development of α_2-adrenoceptor antagonists that lack affinity for 5-HT_{1A}-receptors.

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Withdrawal contractures of guinea-pig isolated ileum after acute activation of κ-opioid receptors

L.A. Morrone, L. Romanelli, M.C. Amico & [1]P. Valeri

Institute of Pharmacology and Pharmacognosy, University of Rome 'La Sapienza', 00185 Rome, Italy

1 The present study was undertaken to investigate firstly whether a brief exposure for 5 min of guinea-pig isolated ileum to the κ-opioid agonist, U-50,488H produced a withdrawal contracture on addition of naloxone and secondly to ascertain whether the response was due to the activation of κ-opioid receptors.

2 Naloxone (10^{-6} M) did not elicit a response in preparations exposed to U-50,488H (5×10^{-7} M–2×10^{-6} M). However, after exposure to U-50,488H (5×10^{-7} M), naloxone (10^{-6} M) produced a strong contracture if the agonist was washed out 1 min before the addition of the antagonist.

3 The addition of naloxone (10^{-6} M) to the ileum preparation exposed to U-50,488H (10^{-7} M or lower) caused a response of similar intensity irrespective of whether the agonist had been washed out.

4 The selective κ-opioid antagonist, nor-binaltorphimine (2.7×10^{-9} M and 2.7×10^{-7} M), injected before the opioid agonists, prevented the naloxone-induced contracture after exposure to U-50,488H (8×10^{-8} M) but did not affect the contracture after exposure to morphine (5×10^{-7} M).

5 Nor-binaltorphimine (2.7×10^{-9} M) caused a contraction of the ileum preparation when injected 5 min after exposure to U-50,488H (8×10^{-8} M) but not after morphine (5×10^{-7} M).

6 The α_2-adrenoceptor agonist, clonidine (3×10^{-8} M) and the calcium channel blocker, nifedipine (3×10^{-8} M), injected 1 min before naloxone, blocked the ileum contraction to naloxone after exposure to U-50,488H (8×10^{-8} M). The results demonstrate that the stimulation of κ-opioid receptors can induce a similar dependence in guinea-pig ileum to that produced by activation of μ receptors.

Keywords: Guinea-pig ileum; withdrawal; U-50,488H; nor-binaltorphimine; morphine; naloxone; κ-opioid receptors

Introduction

Many biochemical and pharmacological studies clearly demonstrate that in peripheral tissues and in the central nervous system opioid receptors can be distinguished as at least three distinct types, μ, δ- and κ-receptors (Goldstein & James, 1984; Martin, 1984).

The attempts made to study the role of the various opioid receptors in the development of opiate dependence seem to indicate that a major role is played by μ-opioid receptors (DeLander *et al.*, 1984; Gmerek & Woods, 1985). The involvement of δ- and κ-opioid receptors in the development of opiate dependence is not well documented, essentially because until a few years ago agonists and antagonists specifically acting at the κ- and δ-type opioid receptors were not available. However, from recent observations, both *in vivo* and *in vitro*, δ- and κ- as well as μ-opioid receptors, appear to be involved in the development of opiate physical dependence (Gmerek *et al.*, 1987; Valeri *et al.*, 1990b; Abdelhamid *et al.*, 1991; Valeri *et al.*, 1992).

Several studies on the role of κ-receptors in dependence development have been carried out *in vitro*, mainly in guinea-pig ileum preparations as this tissue contains populations of both μ-and κ-opioid receptors (Hutchinson *et al.*, 1975) and the preparation is currently used to study the molecular mechanisms involved in dependence phenomena. The withdrawal contractions induced by naloxone in this isolated preparation after exposure to morphine show very similar characteristics to the withdrawal signs in the intact animal (Lujan & Rodriguez, 1981; Alfaro *et al.*, 1990; Valeri *et al.*, 1990a).

The results of these studies are not unequivocal. Chahl (1986, 1990) reports that the ileum preparations exposed to dynorphin exhibit a withdrawal contraction when challenged with naloxone, but those exposed to κ-opioid agonists, such as MR2043 or U-50,488H, do not respond to naloxone. On the other hand, other studies show that a withdrawal contraction to naloxone is obtained after contact of the isolated preparations with the same κ-agonists (Coupar & Quinn, 1987; Valeri *et al.*, 1990b). These latest results, however, do not provide evidence that specific stimulation of κ-opioid receptors induces dependence as the possibility could not be ruled out that these κ-opioid agonists may activate μ-receptors in the guinea-pig ileum.

The aim of the present work was to extend these previous studies and to ascertain if the acute activation of κ-opioid receptors produced dependence in guinea-pig ileum, as revealed by naloxone-induced contractions; isolated tissues have been incubated with the selective κ-opioid agonist, U-50,488H, alone or combined with the selective κ-antagonist nor-binaltorphimine (Portoghese *et al.*, 1987). In addition, the characteristics of the naloxone-induced contractions after acute κ-opioid stimulation are compared with those obtained, in the same tissue, after exposure to morphine (Valeri *et al.*, 1990a).

Methods

The experimental procedure was essentially that used by Valeri *et al.* (1990a), to study the characteristics of ileum dependence to morphine. Adult male guinea-pigs purchased from Morini (Italy) were killed by a blow to the head and bled. Two to four segments of ileum, 2–3 cm long, from the same animal were suspended under 1 g tension in a 5 ml organ bath containing Tyrode solution at 37°C. Tension changes were recorded by an isotonic force transducer connected with a pen recorder (Ugo Basile, 7006 and 7050 Italy) and calibrated before each experiment. At the start of each experiment a maximum response to acetylcholine (ACh, 10^{-6} M) was obtained in each tissue to check its suitability and to express the responses to naloxone as percentages of the ACh maximum. The opioid antagonists were added to

[1] Author for correspondence.

the baths after a 5 min contact with the agonists. Each experiment was performed in at least 4 to 6 isolated preparations from different animals.

Experimental conditions to obtain a reproducible contracture to naloxone after U-50,488H contact

In preliminary experiments the concentrations of U-50,488H (trans-(±)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl) cyclohexyl] benzene-acetamide, methane sulphonate, hydrate) applied to the organ baths varied from 5×10^{-7} M to 2×10^{-6} M and the time of contact with the tissue, before the addition of naloxone (10^{-6} M), from 5 to 30 min. In some experiments the κ-opioid agonist was washed out 1 min before the naloxone injection.

On the basis of the results of these pilot experiments, the concentrations of U-50,488H subsequently used were below 10^{-7} M and the time of exposure 5 min. After the injection of naloxone, the ilea preparations were washed, so that the opioid antagonist was in contact with the tissue for no more than 2–3 min, whether or not a contractile response had been obtained. The same preparation was used for different tests after allowing the tissue to rest, after washout, for a period of 25–30 min or longer, as specified in the text, until it gave reproducible contractile responses.

Effects of nor-binaltorphimine added 5 min before or after U-50,488H

The effect of the selective κ-opioid antagonist, nor-binaltorphimine (Portoghese *et al.*, 1987) on the withdrawal response to naloxone was investigated in guinea-pig ilea exposed to U-50,488H or to morphine by injecting it into the bath 5 min before the agonists. The guinea-pig behaviour to the addition of nor-binaltorphimine was also tested after 5 min exposure of the ilea to the κ-agonist or morphine. The time was chosen to reproduce the same experimental conditions as with nalaxone.

Effect of clonidine and nifedipine

The α_2-adrenoceptor agonist, clonidine, and the calcium channel blocker, nifedipine were placed in the bath 1 min before the addition of naloxone. The concentration used for these drugs was 5×10^{-8} M as at this concentration both drugs blocked almost completely the contraction to naloxone of guinea-pig ilea previously exposed to morphine (Valeri *et al.*, 1990a).

Results

Effect of different concentrations of U-50,488H and naloxone on withdrawal response

In pilot experiments the addition of naloxone (10^{-6} M) to isolated preparations of ilea after a 5 min exposure to morphine 5×10^{-7} M induced a strong contracture, but did not elicit any contraction after exposure to the same concentration of U-50,488H (Figure 1). Only very few preparations (4 out of the 27 tested) elicited a response which, however, was not reproducible and of variable intensity. The same behaviour was observed also in the ilea in which the concentrations of the κ-agonist were increased up to 2×10^{-6} M and the time of contact to 30 min.

The isolated preparations exposed to U-50,488H (5×10^{-7} M), which were unresponsive to naloxone, yielded a sustained contraction if the κ-opioid agonist was washed out 1 min before the addition of the antagonist (Figure 1). After washout and a 25–30 min interval between tests, the same procedure previously used with morphine (Valeri *et al.*, 1990a), a further 5 min exposure to the same concentration of the κ-opioid agonist elicited responses of the same intensity only when the ileum preparations were washed 1 min before the injection of naloxone. The height of the contracture was 75 ± 8,5% of the maximum to ACh (22 preparations from 9 different animals).

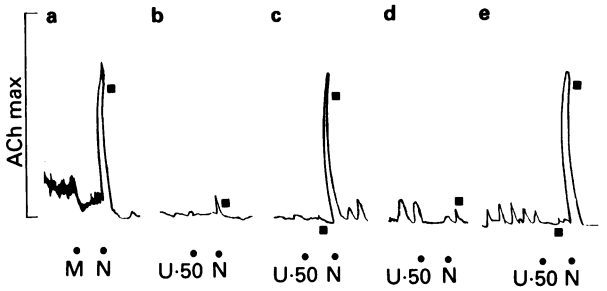


Figure 1 Guinea-pig ileum responses to naloxone (N, 10^{-6} M) after a 5 min exposure of the tissue to morphine (M) and U-50,488H (U-50). N (10^{-6} M) elicited a contractile response after exposure to M (5×10^{-7} M) (a). N did not elicit a response after exposure to U-50 (5×10^{-7} M) (b,d). A reproducible N-induced contracture was obtained after exposure to the same U-50 concentration when a washout was carried out 1 min before N administration (c,e). All tracings shown in figure were obtained from the same tissue preparation with a 25–30 min resting interval between tests. ACh max: acetylcholine (10^{-6} M); (■) washout. The contractile response to N started as soon as the drug was injected into the bath.

As shown in a typical tracing in Figure 2, with lower U-50,488H concentration (10^{-7} M) the addition of naloxone caused a response of the same intensity, whether or not the tissues had been washed 1 min before the injection of the antagonist (only very few preparations exhibited a higher response with the washout). According to this observation, in the following experiments we tested the ileum withdrawal to naloxone without washing and used concentrations of U-50,488H lower than 10^{-7} M. The same ileum preparation exposed for 5 min to a U-50,488H concentration of 8×10^{-8} M gave contractures, on challenge with naloxone 10^{-6} M, of the same amplitude as those obtained with the previous U-50,488H concentration (5×10^{-7} M), indicating that the responses were reproducible and maximal. The responses to naloxone were blocked by atropine (2×10^{-6} M), injected into the bath 1 min before the opioid antagonist. The blocking effect of atropine was maintained, in the same tissue preparation, after several tests with U-50,488H and naloxone, indicating that ACh mediated contractions both at the beginning of the experiment and after repeated exposure to the κ-opioid agonist. Following a resting period of 30 min

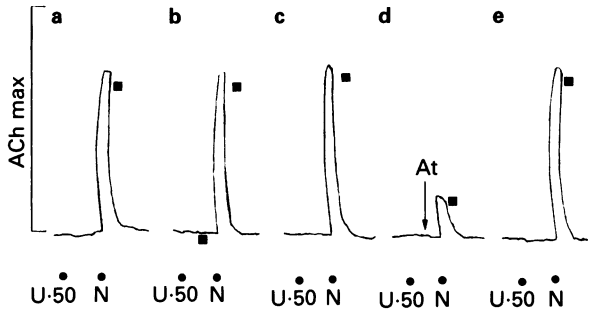


Figure 2 Reproducible responses of guinea-pig ileum to naloxone (N, 10^{-6} M) after a 5 min exposure to U-50,488H (U-50) at concentrations lower than in Figure 1 and inhibitory effect of atropine (At) on N-induced contracture. N elicited a contracture of the same amplitude after exposure to U-50 (10^{-7} M) both without (a) and with (b) a washout carried out 1 min before N administration. When the U-50 concentration was further lowered to 8×10^{-8} M, N elicited a contractile response of the same amplitude as with the higher concentration. At (2×10^{-6} M) inhibited the response to N. All tracings shown in figure were obtained from the same tissue preparation with 25–30 min resting interval between tests. ACh max: acetylcholine (10^{-6} M); (■) washout. The contractile response to N started as soon as the drug was injected into the bath.

the ileum responded again to naloxone, with the same intensity, after further exposures to U-50,488H.

When a constant dose of naloxone was applied to the ileum following different concentrations of U-50,488H ($2-8 \times 10^{-8}$ M) or morphine (2.5 and 5×10^{-7} M), contractions to the opioid antagonist appeared to be dependent upon the concentration of the κ- and μ-opioid agonists (Figure 3). The maximal response was of the same intensity for the two agonists even though the concentration needed to obtain it was much lower for U-50,488H in all tested preparations, despite the variation in sensitivity of ilea from different animals.

The selective κ-opioid antagonist, nor-binaltorphimine (2.7×10^{-9} M), injected into the bath 5 min before the opioid agonists, completely prevented the naloxone-induced contracture of the ileum after exposure to U-50,488H (8×10^{-8} M) but did not affect the contracture after exposure to morphine (5×10^{-7} M) even when the concentration of nor-binaltorphine was increased 100 fold (Figure 4). In these experiments the time interval between tests was 40–50 min because administration of the κ-antagonist 25–30 min after the previous test produced a sustained contracture, indicating that the dependence of the tissue had still not faded. In several tissues, a small contracture was observed even after this longer time interval, as shown in Figure 4. However, we never observed a contracture to the κ-opioid antagonist in naive tissues.

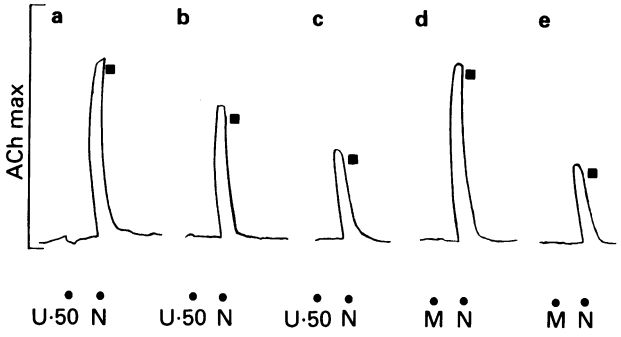


Figure 3 Guinea-pig ileum responses to naloxone (N, 10^{-6} M) after a 5 min exposure of the tissue to decreasing doses of U-50,488H (U-50) and morphine (M). N-induced contracture is dose-related to the concentration of U-50 (a: 8×10^{-8} M; b: 4×10^{-8} M; c: 2×10^{-8} M) and M (d: 5×10^{-7} M; e: 2.5×10^{-7} M). All tracings shown in figure were obtained from the same tissue preparation with a 25–30 min resting interval between tests. ACh max: acetylcholine (10^{-6} M); (■) washout. The contractile response to N started as soon as the drug was injected into the bath.

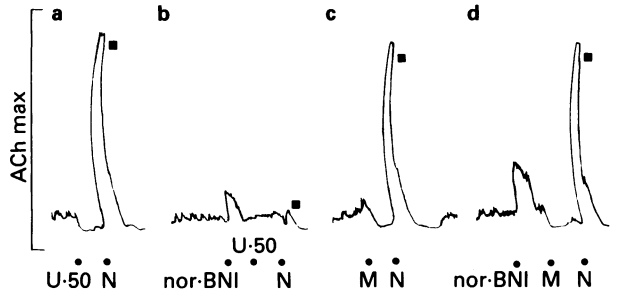


Figure 4 Effect of nor-binaltorphimine (nor-BNI), a κ-antagonist, on the guinea-pig ileum responses to naloxone (N, 10^{-6} M) after a 5 min exposure of the tissue to U-50,488H (U-50, 8×10^{-8} M) and morphine (M, 5×10^{-7} M). Nor-BNI, injected into the bath 5 min before the agonists, completely blocks the N-induced contracture after exposure to U-50 (b, nor-BNI concentration: 2.7×10^{-9} M) but not after exposure to M, even though the antagonist concentration is 100 fold higher (d; nor-BNI concentration: 2.7×10^{-7} M). All tracings shown in figure were obtained from the same tissue preparation with a 40–45 min resting interval between tests. ACh max: acetylcholine (10^{-6} M); (■) washout. The contractile response to N started as soon as the drug was injected into the bath.

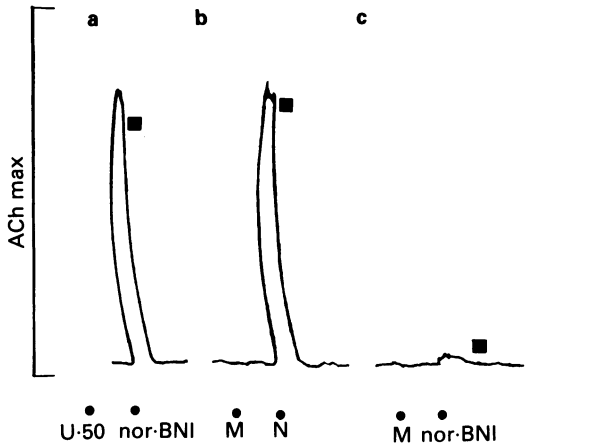


Figure 5 Guinea-pig ileum responses to nor-binaltorphimine (nor-BNI), a κ-antagonist, after exposure of the tissue to U-50,488H (U-50) and morphine (M). Nor-BNI (2.7×10^{-9} M) elicited a contractile response after a 5 min exposure to U-50 (8×10^{-8} M) (a). After a 5 min exposure to M (5×10^{-7} M) a contractile response is elicited by naloxone (N, 10^{-6} M) (b) but not by nor-BNI (2.7×10^{-9} M) (c). All tracings shown in figure were obtained from the same tissue preparation with a 40–45 min resting interval between tests. ACh max: acetylcholine (10^{-6} M); (■) washout. The contractile response to N and nor-BNI started as soon as the drugs were injected into the bath.

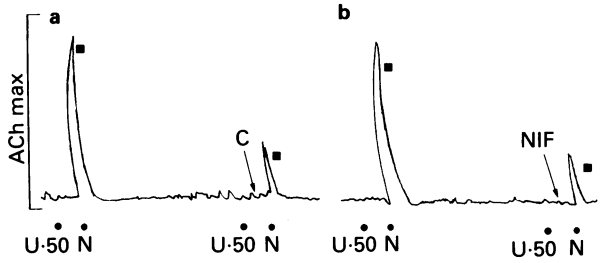


Figure 6 Inhibitory effect of clonidine (C) and nifedipine (Nif) on the guinea-pig ileum responses to naloxone (N, 10^{-6} M) after exposure of the tissue to U-50,488H (U.50). C (3×10^{-8} M) and Nif (3×10^{-8} M) block almost completely the N-induced contracture after a 5 min exposure to U-50 (8.8×10^{-8} M). All tracings shown in figure were obtained from the same tissue preparation with a 25–30 min resting interval between tests. ACh max: acetylcholine (10^{-6} M); (■) washout. The contractile response to N started as soon as the drug was injected into the bath.

Nor-binaltorphimine (2.7×10^{-9} M) produced a sustained contracture when injected after exposure to the κ-agonist but not after exposure to morphine (Figure 5). The α_2-adrenoceptor agonist clonidine (3×10^{-8} M) and the calcium channel blocker nifedipine (3×10^{-8} M), injected 1 min before naloxone, blocked almost completely the ileum contraction to naloxone after exposure of the tissue to U-50,488H (Figure 6).

Discussion

The present study demonstrates that following a brief period of contact (5 min) with the selective κ-opioid agonist, U-50,488H, the guinea-pig ileum exhibits a withdrawal response precipitated by the addition of naloxone. The results confirm previous observations with another selective κ-agonist, MR 2034 (Coupar & Quinn, 1988) and may explain the negative results obtained by Chahl (1986, 1991) with some κ-opioid agonists; when concentrations of U-50,488H as high as those used by the latter author, are employed, naloxone elicits a withdrawal response only after the removal of the κ-opioid agonist from the bath. However, with agonist concentrations lower than 10^{-7} M, naloxone elicits the same

contractile response either with or without a previous washout. The lack of withdrawal responses of the ilea after exposure to some κ-opioid agonists (Chahl, 1986, 1991) and in our experiments carried out without removing U-50,488H, when the concentration of the κ-agonist was as high as 10^{-6} M, is probably to be attributed to an inadequate displacement of the agonist by naloxone 10^{-6} M. Higher concentrations of naloxone were not tested as it has been reported that this antagonist is not specific at higher doses (Chahl, 1983). The fact that a withdrawal contraction is obtained, when challenging the ilea with naloxone, from the tissues previously exposed to morphine 10^{-6} M but not from the tissues exposed to the same concentration of U-50,488H may be explained by the higher antagonist potency of naloxone against morphine than against U-50,488H (Miller *et al.*, 1986); the pA_2 value for naloxone antagonism of morphine is indeed much higher than that against U-50,488H (Von Voigtlander *et al.*, 1983).

The availability of a highly selective κ-opioid receptor agonist and antagonist enabled us to demonstrate that κ-receptors are directly involved in the withdrawal contracture of guinea-pig ileum, in a similar manner to μ-receptors. The selective κ-opioid receptor antagonist, nor-binaltorphimine, when administered at very low concentration before the agonists, blocked the naloxone-precipitated contracture of the isolated preparation to the κ-agonist but not to morphine. The withdrawal response to morphine is unchanged even at a hundred fold higher concentration of the selective κ-opioid antagonist, indicating that the inhibitory effect of nor-binaltorphimine on the development of the dependence to U-50,488H is due only to antagonism at κ-opioid receptors. On the other hand, nor-binaltorphimine, like naloxone, elicited a contracture after acute exposure of the preparation to the κ-opioid agonist, but not after exposure to morphine. If the assumption is made that the withdrawal contracture of the isolated preparation is an index of dependence, these results suggest that, in the experimental conditions used in this study, the specific activation of μ- or κ-opioid receptor may lead to the development of dependence. The observation that the stimulation of the μ- and κ-opioid receptors, acting independently, can induce dependence in guinea-pig ileum is in agreement with the results obtained in the whole animal. Withdrawal behaviours induced by U-50,488H are qualitatively different from those induced by morphine and are suppressed by κ-agonist but not by morphine and *vice versa* (Gmerek *et al.*, 1987; Fukagawa *et al.*, 1989). The convergent results from *in vivo* and *in vitro* studies stress again the importance of the isolated preparations as useful and simple means of examining the mechanisms involved in the adaptational changes induced in the body by the opiates.

The guinea-pig ileum adaptive changes induced by opioids, revealed by the antagonist withdrawal contraction, seem to be independent from the intracellular biochemical mechanism mediating the inhibitory actions of opioids on the myenteric neurones; μ-opioid agonists increase potassium conductance whereas κ-opioid agonists reduce calcium conductance (North, 1986); the characteristics of the guinea-pig ileum withdrawal contracture induced after exposure to U-50,488H are very similar to those obtained with morphine (Valeri *et al.*, 1990a). These contractures are reversible and reproducible, a very useful property for pharmacological studies, and are elicited at lower concentrations than with morphine. The acute adaptational changes induced by U-50,488H, in addition, are almost completely blocked by clonidine and nifedipine, two drugs known to be effective in blocking the opiate withdrawal signs in the whole animal and in man (Gold *et al.*, 1982; Bongianni *et al.*, 1986; Valeri *et al.*, 1989). However, although it appears that dependence to κ-opioid agonists is mainly due to the excitation of the cholinergic neurone, as with μ-agonists, the present data do not allow us to ascertain whether these two opioid agonists activate the same neurones and if the chain of biochemical and neuronal events (Valeri *et al.*, 1990c) leading to the development of dependence and its symptoms is different for the two agonists.

In conclusion, this study has shown that a withdrawal response following brief exposure to U-50,488H can be elicited in the guinea-pig ileum. The previously reported negative results with U-50,488H and other κ-opioid agonists are probably due to the high concentrations used, not being adequately displaced by naloxone. The ability of the highly selective κ-antagonist, nor-binaltorphimine to prevent the development of dependence and to induce a withdrawal contracture in ileum preparations treated with U-50,488H but not in those treated with morphine, suggests that κ-opioid receptors mediate these effects without the involvement of μ-receptors. The characteristics of the precipitated withdrawal contracture to U-50,488H, considered in this study, are similar to those of the withdrawal contracture to morphine.

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Functional characterization of muscarinic receptors in murine airways

[1]*†J. Garssen, †H. Van Loveren, *C.M. Gierveld, *H. Van Der Vliet & *F.P. Nijkamp

*Department of Pharmacology, Faculty of Pharmacy, University of Utrecht, PO Box 80.082, 3508 TB Utrecht, The Netherlands and †National Institute of Public Health and Environmental Protection, PO Box 1, 3720 BA Bilthoven, The Netherlands

1 The effects of muscarinic receptor antagonists considered to be selective for M_1 receptors (pirenzepine; PZ), M_2 receptors (AFDX-116), and for M_3 receptors (4-diphenyl acetoxy N-methyl-piperidine (4-DAMP)) were used to investigate the existence of muscarinic receptor subtypes in murine airways. Atropine was used as a nonselective antagonist. The effects of these antagonists were studied upon tracheal contractions induced either by EFS (electric field stimulation) or by application of an exogenous cholinoceptor agonist (arecoline).

2 The muscarinic receptor antagonists tested inhibited arecoline-induced tracheal contractions with the following rank order of potency: 4-DAMP = atropine > pirenzepine = AFDX-116. The rank order of potency of the muscarinic antagonists used in inhibiting EFS-induced tracheal contractions was: 4-DAMP = atropine > PZ > AFDX-116. The pA_2 values for these antagonists were similar when compared to the pA_2 values determined in guinea-pig and bovine airway smooth muscle.

3 In addition to *in vitro* studies, the effects of inhalation of the different muscarinic antagonists on lung function parameters *in vivo* were investigated. Inhalation of 4-DAMP induced a decrease in airway resistance and an increase in lung compliance. In contrast, inhalation of AFDX-116 induced an increase in airway resistance and almost no change in lung compliance. Apart from some minor effects of atropine on airway resistance, atropine, PZ, and pilocarpine failed to induce changes in lung mechanics as determined by *in vivo* lung function measurements.

4 The results provide evidence for the existence of M_3 receptors on murine tracheae that are involved in the contraction of tracheal smooth muscle. This is in agreement with other animal species such as the guinea-pig and bovine. *In vivo* experiments also demonstrated that in the mouse, M_3 receptors play an important role in bronchial smooth muscle contraction and thus in bronchoconstriction. Interestingly we have also demonstrated that M_2 receptors can play a role in bronchodilatation. Inhalation of an M_2 receptor antagonist induced an increase in airway resistance whereas inhalation of an M_3 receptor antagonist induced a decrease in airway resistance. It is therefore likely that an M_3/M_2 receptor balance plays an important role in the regulation of airway function.

Keywords: Muscarinic receptors; murine airways; trachea

Introduction

The parasympathetic nervous system forms the predominant neural pathway in the airways and plays an important role in airway obstruction (Nadel, 1980; Partanen *et al.*, 1982; Sheppard *et al.*, 1983; Barnes, 1984; 1986; 1987; Nadel & Barnes, 1984; De Kock & Brandt, 1985). Receptors of this system can be divided into several subtypes. At the beginning of this century it was demonstrated that the effects of acetycholine could be divided into an action at 2 subtypes of receptors: those stimulated by nicotine and those stimulated by muscarine (Dale, 1914). These 2 different receptor subtypes formed the first division in the receptors of the parasympathetic nervous system. Latterly the existence of at least three functional muscarinic receptors has been postulated (Birdsall & Hulme, 1983; Eglen & Whiting, 1986; Doods *et al.*, 1987). However more recently, the discovery of more agonists and antagonists that show preferential selectivity for muscarinic receptors in different tissues, receptor isolation, and cloning techniques have provided evidence for the existence of 5 subtypes of muscarinic receptors (m_1–m_5) (Kerlavage *et al.*, 1987).

It is possible to differentiate muscarinic receptors for which the antagonist pirenzepine (PZ) has a high affinity (denoted as M_1 receptors), those for which AFDX-116, gallamine, and methoctramine have a high affinity (denoted as M_2 receptors), and those for which 4-diphenyl acetoxy N-methyl piperidine (4-DAMP) and hexahydrosiladiphenidol have a high affinity (denoted as M_3 receptors) (Birdsall & Hulme, 1983; Eglen & Whiting, 1986; Doods *et al.*, 1987). M_1 receptors are present in parasympathetic ganglia where they appear to facilitate ganglionic neurotransmission. M_2 receptors seem to be located presynaptically on cholinergic nerve endings where they function as autoreceptors, inhibiting acetylcholine release, and M_3 receptors appear to be located on effector cells of smooth muscle and mucus secreting glands (Minette & Barnes, 1990).

Previously, we described a method to measure tracheal smooth muscle responses in mice (Garssen *et al.*, 1990). In that study the effect of *in vitro* sympathetic and parasympathetic stimulation was studied. Muscarinic receptor subtypes were not analysed. This paper describes studies in murine isolated tracheae in which the effect of a range of selective muscarinic antagonists on contraction induced either by a cholinoceptor agonist or electrical field stimulation (EFS) were investigated. These studies were carried out in order to determine the muscarinic receptor subtypes mediating and/or modulating contractile responses in this tissue. In addition, the effects of inhalation of the antagonists used on lung function parameters such as airway resistance and lung compliance were investigated *in vivo*.

[1] Author for correspondence at: National Institute of Public Health and Environmental Protection, PO Box 1, 3720 BA Bilthoven, The Netherlands.

Methods

Animals

SPF BALB/c mice (male), 5–8 weeks of age, weighing 20–25 g were obtained from the breeding colony at the National Institute of Public Health and Environmental Protection, Bilthoven, the Netherlands. After delivery, the animals were rested for at least 1 week before use.

Isometric measurement of bronchial reactivity

Mice were killed by intraperitoneal (i.p.) injection of 0.25 ml nembutal (pentobarbitone sodium 60 mg ml^{-1}, Abbott Laboratories, North Chicago, IL, U.S.A.). The tracheae, resected *in toto*, were transferred to a Petri dish containing a modified oxygenated Krebs bicarbonate solution (composition mmol l^{-1}: NaCl 118.1, KCl 4.7, $CaCl_2.6H_2O$ 2.5, $MgCl_2.6H_2O$ 0.5, $NaHCO_3$ 25.0, $NaH_2PO_4.H_2O$ 1.0 and glucose 11.1). The tracheae were prepared free of excess tissue by use of a binocular preparation microscope and pieces of approximately 6 mm length (9 trachea rings just beneath the larynx) were taken. The weights of the tracheal preparations were similar. Thus, in each experiment similar tracheal sections were used (Garssen *et al.*, 1990).

The tracheae were mounted directly on two supports in an organ bath, one of which was connected to an isometric transducer and the other to a plastic holder. This procedure did not lead to tissue damage, as determined histologically. The organ baths were filled with 20 ml of Krebs solution. The solution was aerated continuously with a mixture of oxygen (95%) and carbon dioxide (5%). Temperature was maintained at 37°C with a constant temperature circulating unit (Thermomix 1460; Braun Melsungen, Germany).

Isometric measurements were made with a force displacement transducer (isometric transducer, Harvard Bioscience, Boston, U.S.A.) and a two-channel recorder (Servogor type SE-120) and were expressed as changes in force measured in grams. Optimal basic force for the mouse trachea was 1 g (Hooker *et al.*, 1977; Bartell & Busse, 1980; Garssen *et al.*, 1990). The tracheae were allowed to equilibrate for at least 1 h before drug effects were elicited. During the equilibration the bath fluid was exchanged every 15 min. Drugs were prepared in Krebs solution and kept on ice for the duration of the experiment.

Effect of muscarinic antagonists on arecoline-induced smooth muscle contraction

Cumulative contractile concentration-response curves were determined for arecoline. After the determination of this curve the bath fluid was exchanged 4 times over a period of 30 min. The basal tone (i.e. 1 g) returned during this washing procedure. Subsequently the muscarinic antagonist under test was added to the organ bath; 30 min later a second arecoline concentration-response curve was determined. At least three concentrations of each muscarinic antagonist were tested (a separate trachea was used for every concentration of antagonist). In control experiments it was demonstrated that if a second arecoline curve was constructed without antagonist incubation, that this curve was similar to the first arecoline curve (data not shown).

Effect of muscarinic antagonists on EFS (electric field stimulation)-induced smooth muscle contraction

The plastic holders, to which the tissues were attached, were equipped with platinum electrodes to facilitate EFS of tissues using a Grass S88 stimulator (Grass Medical Instruments, Quincy, MA, U.S.A.) connected to the electrodes. Prior experiments showed that an impulse voltage of 40 V, 1 ms, repeated every 100 s, was optimal, i.e. gave reproducible constant twitch contractions. Frequency-response curves were constructed using 5 to 160 Hz. From these frequency studies 20 Hz was chosen for subsequent studies. At this frequency it was possible to demonstrate increases and decreases of EFS-induced contraction.

Prior to the experiments, tracheae were equilibrated as mentioned above (1 h) and subsequently stimulated with EFS for 30 min (40 V, 20 Hz, 1 ms.). After this conditioning period the twitch contractions were stable. Thereafter cumulative concentration-response curves were determined for different muscarinic receptor antagonists. Each antagonist addition was followed by 5 stimulation periods. Cumulative concentration-response curves were finalised with the concentration of antagonist that inhibited the EFS-induced contraction totally.

Effect of muscarinic antagonist inhalation on lung function parameters

BALB/c mice were anaesthetized with pentobarbitone sodium (Abbott Laboratories, North Chicago, IL, U.S.A.) (50 mg kg^{-1} body weight, i.p.) An anaesthesia-induced fall in body temperature was avoided by placing the mice in a heated chamber that kept their body temperature at around 37°C. Pulmonary function parameters were measured in these anaesthetized and spontaneously breathing mice using a modification of previously published technique for lung function measurements in guinea-pigs (Folkerts & Nijkamp, 1985; Folkerts *et al.*, 1988). The animals were prepared for measurement of airway resistance (R_L) and dynamic lung compliance (C_{dyn}) as described elsewhere (Folkerts & Nijkamp, 1985; Folkerts *et al.*, 1988). Briefly, airflow ($\dot{V}$) and tidal volume (V_t) were determined by cannulating and connecting the trachea with a flow head to a Gould Godart Pneumotachograph (Bilthoven, The Netherlands). A Validyne MP45-2 (Validyne Corp., Northridge, CA, U.S.A.) pressure transducer measured the transpulmonary pressure (P_{TP}) by determining pressure differences between the tracheal cannula and a cannula filled with saline inserted in the oesophagus.

Lung compliance (C_{dyn}) is the amount of lung expansion (volume change) per unit of pleural pressure change when the pressure and volume changes are measured at moments of zero flow. During breathing an additional pleural pressure change is needed to drive air in or out of the lungs. The ratio between this pleural pressure change and airflow rate is termed airway resistances (R_L). To eliminate the influence of lung expansion on the calibre of airways, R_L was determined from the pressure (ΔP) and flow differences ($\Delta \dot{V}$) at points of equal lung expansion during inspiration and expiration respectively. R_L and C_{dyn} were determined breath by breath by a modified method of Amdur & Mead (1958) using a computerized respiratory analyzer (Folkerts & Nijkamp, 1985). Briefly: dividing ΔP_L and $\Delta \dot{V}$ at isovolume points yielded the R_L ($\Delta P_L : \Delta \dot{V} = R_L$), and dividing ΔV_t and ΔP_{TP} between points of zero flow yielded the C_{dyn} ($\Delta V_t : \Delta P_{TP} = C_{dyn}$). The lung function parameters of the spontaneously breathing mice were determined 30 min after preparation of the animals.

Effects of inhalation of muscarinic antagonists on respiratory functions were determined as follows: muscarinic antagonists or control solution were nebulized with an ultrasonic nebulizer. Each concentration of antagonist or control solution was given by inhalation for 20 breaths leading to a constant reproducible effect. The procedure started with an inhalation of the nebulized control solution (PBS) followed by the lowest concentration of the antagonist 15 min later. This procedure was continued up to the highest concentration of the antagonist tested. Between each different concentration of the antagonist tested, murine ventilation was stable for at least 15 min. Changes in airway resistance and lung compliance were calculated and expressed as Δ airway resistance or Δ lung compliance. Negative Δ values indicate a decrease and positive Δ values an increase of the parameter

as compared to the lung function measured after inhalation of the nebulized control solution.

Data analysis

Competitive antagonism in the arecoline-induced contraction experiments was assessed according to the method of Arunlakshana & Schild (1959). pA_2 values (affinity) were determined by 2 methods: (1) pA_2 values were calculated for each concentration of the antagonists used according to $pA_2 = -\log \{[\text{antagonist}]/(DR-1)\}$ and the mean value (± s.e.mean) was calculated; (2) pA_2 values were denoted as the X-intercept of the Schild plot (Mackay, 1978). Schild plots were determined with a computer programme (Lotus) in which $P<0.05$ was chosen as the limit for fitting the Schild plot. The slope and correlation of the Arunlakshana-Schild plot-line were also calculated. When the correlation (r) is reliable ($P<0.05$) and the slope of the Schild plot equals unity, the interaction between antagonist and receptor is competitive. In this case the pA_2 value could be obtained directly from the X-intercept.

Inhibition of EFS-induced contraction of murine tracheae was expressed as a percentage of tracheal contraction prior to the addition of the muscarinic antagonist (i.e. 100%). The EFS-induced contraction was expressed as the mean effect of 5 stimulation-periods (± s.e.mean). From these cumulative inhibition curves, IC_{50} values (concentration of antagonist necessary to inhibit the EFS-induced contraction by 50%) were automatically calculated by computer.

Changes in lung function parameters were analysed statistically by the unpaired Student's t test. A P value of $<0.05\%$ was taken as being statistically significant.

Source of materials

Arecoline was obtained from de Onderlinge Pharmaceutische Groothandel (OPG), Utrecht, The Netherlands. Atropine was obtained from Sigma Chemical Company, St. Louis, U.S.A. The muscarinic receptor antagonists pirenzepine (PZ), 4-diphenyl acetoxy N-methyl piperidine (4-DAMP), and 11-[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyridol[2,3-b][1,4]benzodiazepin-6-one (AFDX-116)) were gifts from Dr H.N. Doods, Dr Karl Thomae, GmbH, Div. of Molecular Pharmacology, Postfach 1755, D7950, Biberach, Germany. Pilocarpine and gallamine were gifts from Dr W. Vleeming, University of Utrecht, P.O. Box 80.082, 3508 TB, Utrecht, The Netherlands. All reagents used to make up the Krebs solution were of Analar quality.

Results

Effects of muscarinic receptor antagonists on arecoline-induced tracheal contractions

All antagonists used in the arecoline-induced tracheal contraction experiments shifted the arecoline concentration-response curves to the right in a concentration-dependent fashion and over a large concentration-range (Figure 1). None of the antagonists used depressed the maximum contraction significantly, even at the highest concentrations (Table 1). Figure 2 presents Schild plots for the 4 different antagonists used and Table 2 gives affinity (pA_2) values, slope factors, and correlation values. Affinities were highest for atropine ($pA_2 = 8.61 \pm 0.24$; mean ± s.e. mean) and 4-DAMP ($pA_2 = 8.68 \pm 0.26$; mean ± s.e. mean). The muscarinic receptor antagonists suppressed the arecoline-induced tracheal contractions with the following rank order of potency: atropine = 4-DAMP > PZ = AFDX-116. pA_2 values of PZ and AFDX-116 were both significantly ($P<0.05$) lower as compared to the pA_2 values of atropine and 4-DAMP.

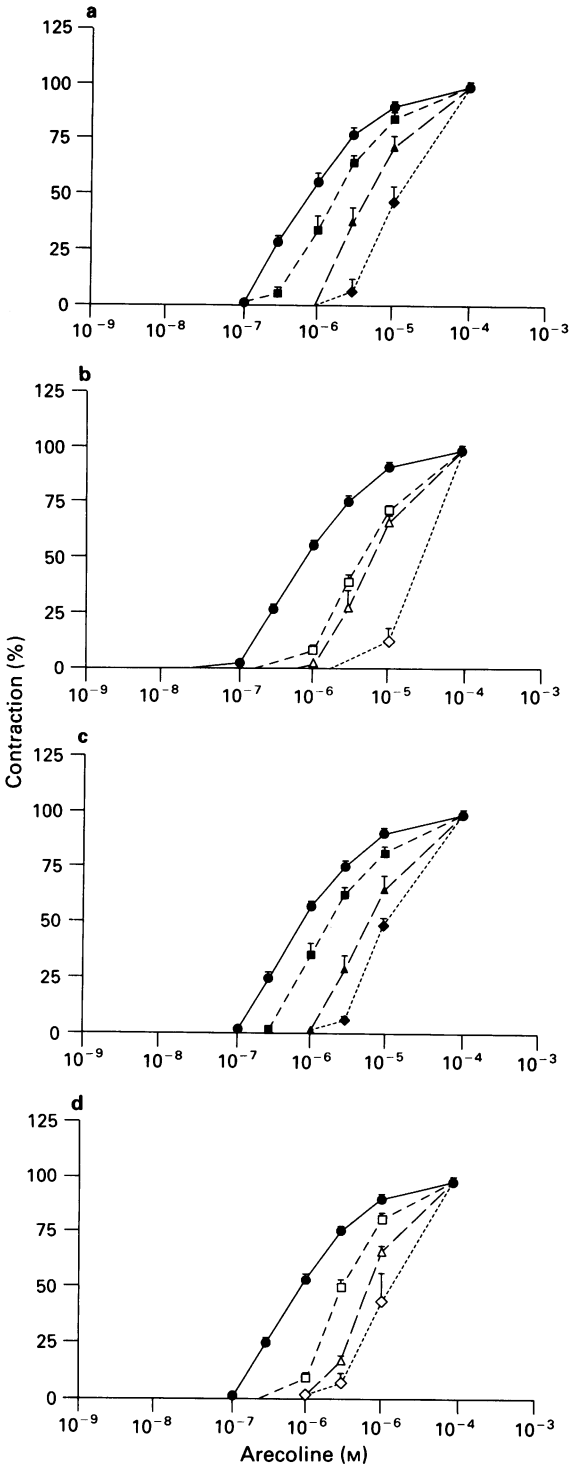


Figure 1 Effects of selective competitive muscarinic receptor antagonists on concentration-response curves for arecoline-induced contraction of murine isolated tracheae. An initial concentration-effect curve was performed for each experiment before the addition of each antagonist. The initial curves are shown as controls. Then, a second concentration-response curve was performed on the same tissues in the presence of antagonists, added 30 min earlier. Each concentration of antagonist (3 different concentrations) was tested on separate tracheae. Values are means (% of maximal contraction) + s.e. mean. $n = 5$ for each concentration of antagonist. (a) Effect of atropine; (b) effect of pirenzepine; (c) effect of 4-diphenyl acetoxy N-methyl piperidine (4-DAMP); (d) effect of AFDX-116. (●) Control; (■) 3×10^{-9} M; (▲) 10^{-8} M; (◆) 3×10^{-8} M; (□) 10^{-6} M; (△) 3×10^{-6} M; (◇) 10^{-5} M antagonist.

Table 1 Maximal contraction (E_{max}) of murine tracheae induced by 10^{-4} M arecoline in the absence or presence of maximal concentrations of antagonists

	E_{max} ± s.e. mean (mg)
Control	1602 (413)
+ Atropine (3×10^{-8} M)	1326 (372)
Control	1557 (326)
+ Pirenzepine (10^{-5} M)	1068 (234)
Control	1437 (190)
+ 4-DAMP (3×10^{-8} M)	1184 (182)
Control	1069 (169)
+ AFDX-116 (10^{-5} M)	933 (221)

$n = 5$ for each group.

Effects of muscarinic receptor antagonists on EFS-induced tracheal contractions

EFS-induced tracheal contraction was frequency-dependent (Figure 3). From these experiments 20 Hz was chosen as the frequency for subsequent experiments investigating the inhibition of EFS-induced contractions by muscarinic antagonists. The basal EFS-induced contraction, prior to antagonist addition, was characterized as the 100% effect (469.36 ± 63.40 mg). Figure 4 shows an example of a trace showing the inhibition of EFS-induced contractions by cumulative addition of PZ. All antagonists tested inhibited the EFS-induced contraction in a concentration-dependent manner (Figure 5). IC_{50} values for the antagonists used are given in Table 3. The muscarinic receptor antagonists used suppressed the EFS-induced tracheal contractions with the following rank order of potency: 4-DAMP = atropine > PZ > AFDX-116.

Effects of muscarinic receptor antagonists on lung function parameters

In the absence of any treatment with inhaled antagonist the values of airway resistance and compliance were 0.51 ± 0.12 $cmH_2O\ ml^{-1}\ s$ and 0.08 $ml\ cmH_2O^{-1}$ respectively. Inhalation of 4-DAMP induced a significant decrease in airway resistance and an increase in pulmonary compliance (Figure 6). In contrast to the results from *in vitro* experiments AFDX-116 induced bronchoconstriction, seen as a significant increase in airway resistance. At the concentrations tested, AFDX-116 did not however change pulmonary compliance (Figure 7).

PZ and pilocarpine did not significantly affect pulmonary function changes at the concentrations tested (10^{-10}–10^{-4} M). this was also true for atropine except for some minor decreases in airway resistance after inhalation of some lower concentrations (data not shown).

Discussion

Knowledge about cholinoceptors and especially muscarinic receptor subtypes in airway smooth muscle is available in many animal species such as guinea-pig, ox, dog, rat, rabbit and man, but not in mice (Fryer & Mclagan, 1984; Blaber *et al.*, 1985; Van Koppen *et al.*, 1985; Faulkner *et al.*, 1986; Eglen & Whiting, 1986; O'Rourke *et al.*, 1987; Madison *et al.*, 1987; Beck *et al.*, 1987; Bloom *et al.*, 1987; 1988; Roffel *et al.*, 1988; Ito & Yoshitomi, 1988; Minette & Barnes, 1988; Minette *et al.*, 1988; Maclagan & Faulkner, 1989). From results described previously it was concluded that carbachol and arecoline, both recognized as selective cholinoceptor agonists, can contract murine tracheal smooth muscle, indicating the presence of cholinoceptors on murine tracheae

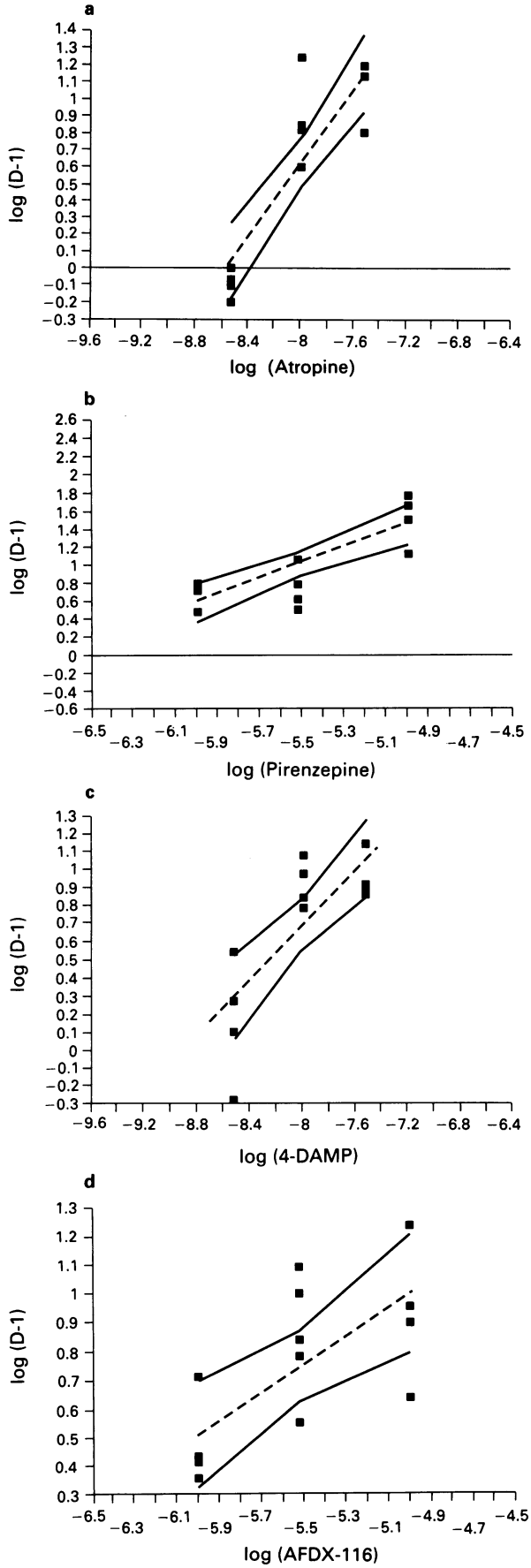


Figure 2 Arunlakshana-Schild plots for the antagonism of the arecoline-induced contraction of murine isolated tracheae by atropine (a), pirenzepine (b), 4-diphenyl acetoxy N-methyl piperidine, (4-DAMP) (c), and AFDX-116 (d). Individual data points (■) are presented (5 data points per concentration of antagonist). Regression lines were calculated with a reliability of $P < 0.05$. In the figures the Schild plot is demonstrated as the computerized line of best fit between upper ($P < 0.025$) and lower ($P < 0.025$) confidence limits.

Table 2 Inhibition of arecoline-induced contraction of murine tracheae (*in vitro*) by muscarinic receptor antagonists

Antagonist	*Mean* pA_2	pA_2 (Schild plot)	*Slope*	*Correlation* (r)
Atropine	8.61 (0.24)	8.56	1.09 (0.16)	0.88
Pirenzepine	6.52 (0.25)	6.68	0.86 (0.16)	0.83
4-DAMP	8.68 (0.26)	8.90	0.76 (0.16)	0.80
AFDX-116	6.28 (0.28)	7.04	0.49* (0.14)	0.71

Values are mean ± s.e. mean ($n = 15$ for each antagonist; $n = 5$ for each concentration). *Significantly different from unity; ($P < 0.05$).

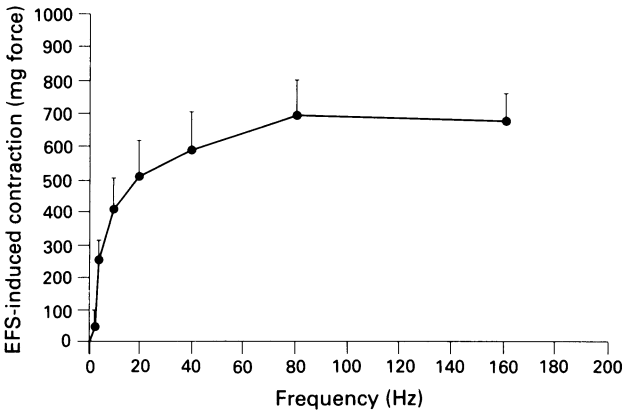


Figure 3 The effect of electric field stimulation (40 V, 1 ms, repeated every 100 s) at various frequencies from 0 to 160 Hz on murine isolated tracheae. Responses are shown as mean mg + s.e. mean ($n = 5$).

(Garssen *et al.*, 1990). In addition, we recently demonstrated the EFS can induce contraction of murine isolated tracheal smooth muscle (Van Oosterhout *et al.*, 1991).

In vitro studies in guinea-pig, ox and dog airway tissue showed that smooth muscle contraction to cholinoceptor agonists could be blocked strongly by 4-DAMP and less so by PZ. In addition, radioligand binding studies on bovine and human airway tissue confirm the low affinity of PZ for the muscarinic receptors present and the high affinity of 4-DAMP. Thus the muscarinic receptors on airway smooth muscle of most species investigated are predominantly M_3 receptors (Van Koppen *et al.*, 1985; Eglen & Whiting, 1986; O'Rourke *et al.*, 1987; Madison *et al.*, 1987; Roffel *et al.*, 1988). However, Roffel *et al.* (1988) demonstrated the existence of both M_2 and M_3 receptors in bovine tracheae using radioligand binding studies. By use of functional assays M_2 antagonists were shown to have a low affinity and M_3 antagonists a high affinity in this tissue. Bronchoconstriction in rabbits and dogs, induced by vagus nerve stimulation, can be blocked by PZ (Beck *et al.*, 1987; Bloom *et al.*, 1987; 1988). PZ has a low affinity for receptors on airway smooth muscle (Eglen & Whiting, 1986; O'Rourke *et al.*, 1987; Roffel *et al.*, 1988), thus excitatory M_1 receptors are most probably located on parasympathetic ganglia (Bloom *et al.*, 1988). M_2 muscarinic receptors are characterized as presynaptic autoreceptors. These prejunctional receptors inhibit the release of acetylcholine from cholinergic nerves. This has been found to be true for cats, dogs, guinea-pigs, and man (Fryer & Mclagan, 1984; Blaber *et al.*, 1985; Faulkner, 1986; Ito & Yoshitomi, 1988; Minette & Barnes, 1988; Minette *et al.*, 1988).

Because it is thought that different muscarinic receptor subtypes may play a role in airway diseases (Barnes, 1986; 1989) and because mice fulfil the requirements for an immunopharmacological model to study airway reactivity, in the present study the potencies of the different selective muscarinic antagonists were determined. All the muscarinic receptor antagonists suppressed the arecoline-induced contraction of murine tracheae concentration-dependently.

Although the maximal contraction induced by arecoline (10^{-4} M), was not significantly suppressed by the antagonists tested, there was a tendency for suppression of the maximal contraction at the highest concentration of the antagonist used. Such a tendency has also been found for rats and guinea-pigs (Gies *et al.*, 1989). The arecoline concentration-response curves were shifted to the right in a parallel and concentration-dependent fashion indicating the competitive nature of the antagonists used. Atropine and 4-DAMP exhibited the highest potencies. Thus muscarinic receptors on murine tracheal smooth muscle responsible for contraction may be characterized as M_3 receptors. This is in agreement

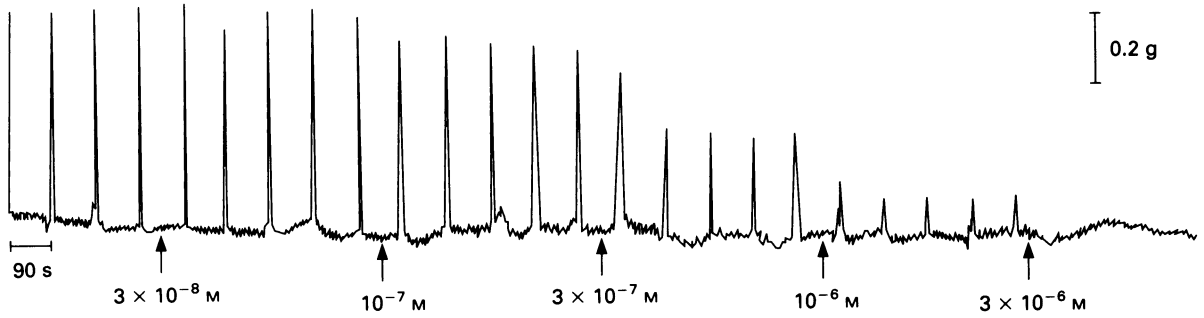


Figure 4 Representative tracing showing the effect of cumulative pirenzepine (PZ) addition on EFS (40 V, 20 Hz, 1 ms)-induced contraction of murine tracheae *in vitro*. Successive concentrations of PZ were added after each 5 stimulation periods (marked with an arrow).

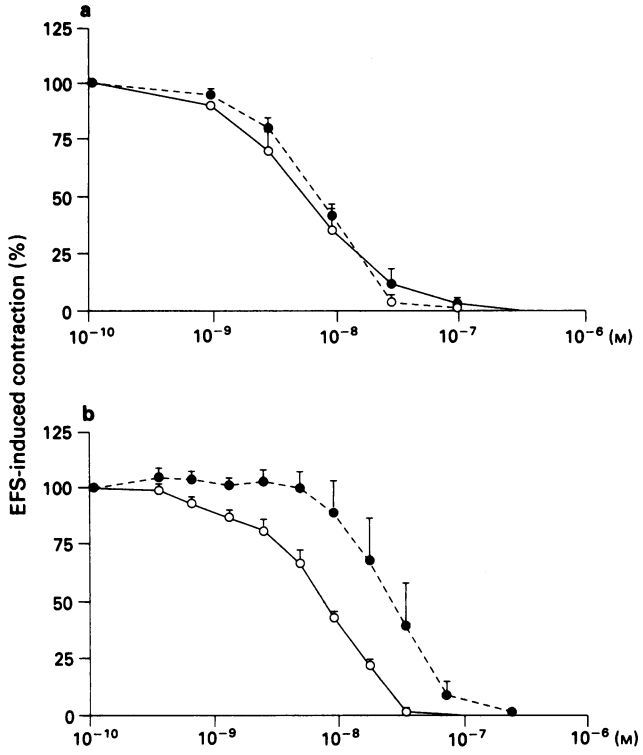


Figure 5 Inhibition of EFS (40 V, 20 Hz, 1 ms repeated every 110 s)-induced contraction of murine isolated tracheae by different muscarinic antagonists. Inhibition of EFS-induced contraction of murine tracheae is expressed as a percentage of tracheal contraction prior to the addition of the muscarinic antagonist (i.e. 100%). The EFS-induced contraction is expressed as the mean + s.e. mean of 5 successive stimulation-intervals (± s.e. mean. (a) Effect of atropine (●) and 4-diphenyl acetoxy N-methyl piperidine (○); (b) effect of pirenzepine (○) and AFDX-116 (●).

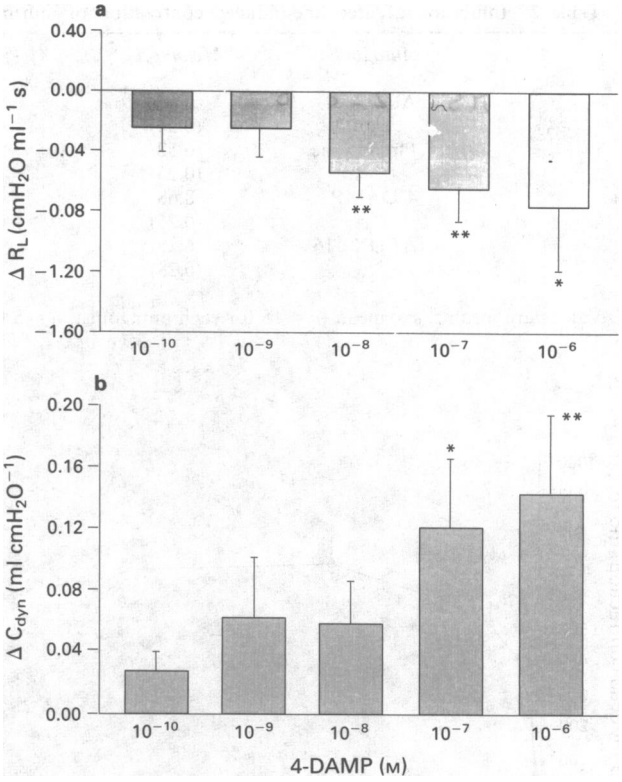


Figure 6 Effects of 4-diphenyl acetoxy N-methyl piperidine (4-DAMP) inhalation on respiratory function parameters in spontaneously breathing Balb/c mice. Each concentration of 4-DAMP or control solution was given by inhalation for 20 breaths. Changes in airway resistance and compliance were calculated and expressed as Δ airway resistance (R_L; a) or Δ lung compliance (C_{dyn}; b). Negative Δ values indicate a decrease and positive Δ values an increase of the parameter as compared to the lung function measured after inhalation of the nebulized control solution (PBS). Values are presented as the mean of 6 independent experiments + s.e. mean. $*P<0.05$; $**P<0.01$.

Table 3 Inhibition of EFS (40 V, 20 Hz, 1 ms, repeated every 100 s)-induced contraction of murine trachea by muscarinic antagonists (IC_{50} values)

Antagonist	IC_{50} (± s.e. mean)	$-\log IC_{50}$
Atropine	8.2×10^{-9} (1.2×10^{-9})	8.09
Pirenzepine	2.2×10^{-7} (3.1×10^{-8})	6.66
4-DAMP	7.5×10^{-9} (2.9×10^{-9})	8.13
AFDX-116	2.7×10^{-6} (1.0×10^{-6})	5.57

$n = 5$ for each antagonist.

with results in other animal species (Van Koppen *et al.*, 1985; O'Rourke *et al.*, 1987; Madison *et al.*, 1987; Roffel *et al.*, 1988). The potencies of AFDX-116 and pirenzepine were low as compared to both other antagonists. The pA_2 values that were found for murine tracheae agree very well with those reported for smooth muscle preparations from other animal species such as bovine and guinea-pig tracheal smooth muscle (Eglen & Whiting, 1986; Roffel *et al.*, 1988; Minette & Barnes, 1988). pA_2 values determined from the X-intercept of the Schild plots were similar except for a higher pA_2 value for AFDX-116. In this particular case the slope was also significantly different from unity ($P<0.05$). This could point to allosteric behaviour in murine tracheal smooth muscle or a lower selectivity of AFDX-116. In addition, the correlation-coefficient of 0.71 demonstrated that the X-intercept value for AFDX-116 was not very reliable in contrast to the good correlation values for the other three antagonists used, being higher than 0.80 (and thus significant at $P<0.05$). In summary, using arecoline-precontracted tracheae, we have demonstrated that the muscarinic receptor on murine smooth muscle cells can be characterized as predominantly M_3 receptors. For the study of receptors on other compartments such as nerves and/or ganglia, tracheae were precontracted by EFS. EFS induces release of endogeneous acetylcholine from nerve endings leading to smooth muscle contraction (Van Oosterhout *et al.*, 1991).

In prior studies we demonstrated that hexamethonium, which antagonizes ganglionic transmission, does not affect EFS-induced contractions. Thus in the EFS-model, ganglion stimulation does not play a role (Van Oosterhout *et al.*, 1991). Atropine and 4-DAMP inhibit EFS-induced contractions with a high efficacy. $-\log IC_{50}$ of AFDX-116 and PZ were low and the $-\log IC_{50}$ values for atropine and 4-DAMP were high. This is in agreement with data from the experiments with arecoline-induced tracheal smooth muscle contraction indicating the presence of M_3 receptors on murine trachea. In the EFS experiments the smooth muscle contraction is also indirectly M_3-dependent. Therefore M_3 receptors are probably located on smooth muscle cells.

M_2 autoreceptors could only be demonstrated in EFS-induced smooth muscle contraction experiments, because these receptors are located presynaptically on nerves. Pilocarpine, a selective agonist of M_2 receptors, inhibits cholinergic nerve-induced contraction of bronchi elicited by electric field stimulation, whereas it does not affect contraction induced directly by acetylcholine (Minette & Barnes, 1988). This

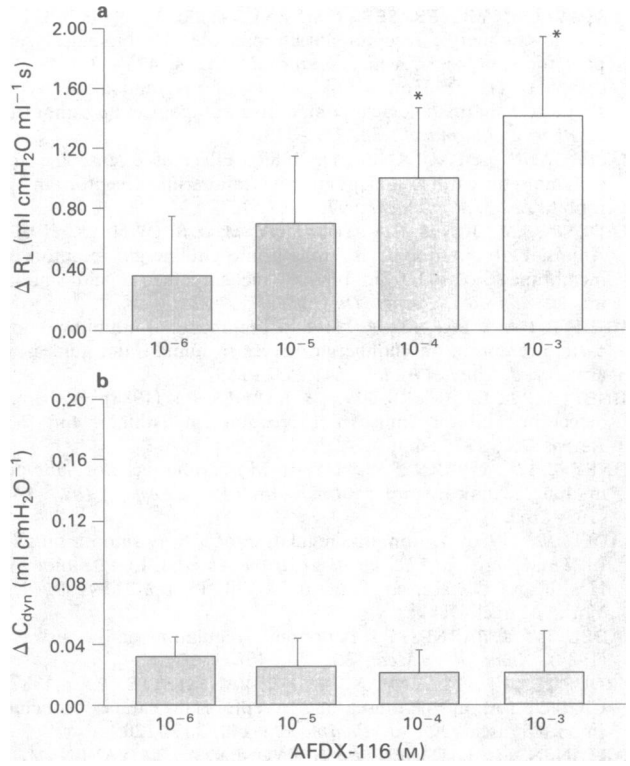


Figure 7 Effects of AFDX-116 inhalation on respiratory function parameters in spontaneously breathing Balb/c mice. Each concentration of AFDX-116 or control solution was given by inhalation for 20 breaths. Changes in airway resistance and compliance were calculated and expressed as Δ airway resistance (R_L; a) or Δ lung compliance (C_{dyn}; b). Negative Δ values indicate a decrease and positive 4 values an increase of the parameter as compared to the lung function measured after inhalation of the nebulized control solution (PBS). Values are presented as the mean of 6 independent experiments + s.e. mean. $*P<0.05$.

inhibitory effect can be blocked by gallamine (a non-competitive M_2 antagonist), confirming that the inhibition is mediated by M_2 receptors, which are presumably localized to postganglionic cholinergic nerves (Minette & Barnes, 1988). However in our experiments AFDX-116, a new selective M_2 receptor antagonist, did not induce inhibition of M_2 receptor function leading to an increase of EFS-induced contraction of murine tracheae. However, Figure 5 shows that small doses of AFDX-116 can have a tendency to increase EFS-induced contraction. In addition, AFDX-116 was less capable of inhibiting EFS-induced contractions than inhibiting arecoline-induced contractions, in which M_2 autoreceptors do not play a role. In rats there is also a controversy about the presence of M_2 receptors. Aas & Maclagan (1990) and Aas & Fonnum (1986) obtained evidence for muscarinic autoreceptors in pulmonary cholinergic nerves in albino rats. However, such receptors could not be detected in anaesthetized rats *in vivo* with gallamine as an antagonist (Fryer & Maclagan, 1984).

In contrast to the *in vitro* studies, AFDX-116 did change lung function measured *in vivo*, in that it increased pulmonary resistance significantly. This fits very well with the idea that M_2 receptors serve as autoreceptors responsible for the feed-back regulation of effects mediated by M_3 receptors that are directly responsible for smooth muscle contraction. Interestingly, inhalation of AFDX-116 had no effect on lung compliance. Because lung compliance depends in a major part on the elasticity of the alveolar walls, it can be suggested that M_2 receptors are located at sites higher in the airways such as the upper bronchi but not on the trachea, since in the *in vitro* trachea experiments it was not possible to increase EFS-induced tracheal smooth muscle contraction. Pilot experiments with gallamine demonstrated also that gallamine did not influence EFS-induced tracheal contraction. Inhalation of an M_2 agonist (pilocarpine) failed to decrease airway resistance. This may be due to the lower selectivity of this agonist as compared to the high selectivity of AFDX-116 for M_2 receptors. Pilocarpine has also some action at M_3 receptors. Interestingly 4-DAMP decreased airway resistance and increased lung compliance. This is in agreement with the theory that M_3 receptors, located on smooth muscle cells, are responsible for smooth muscle contraction. Inhibition of effects induced by M_3 receptor activation was detectable *in vivo* using the two lung function parameters, indicating the presence of M_3 receptors in lower and higher airways (upper bronchi/main bronchi) including the trachea as found in the *in vitro* trachea experiments. Effects of the other two antagonists tested, i.e. PZ and atropine, were almost nil except for some minor changes of airway resistance after atropine inhalation. These minor changes were characterized by a decrease in airway resistance at lower doses of atropine. The results with the two latter antagonists could be due to their lower selectivity. It could be argued that inhibition and stimulation of different receptor-mediated mechanisms negated their effects.

In summary it may be concluded that in murine airway smooth muscle, contraction can be induced by activation of muscarinic M_3 receptors. This phenomenon can be inhibited (feed-back regulation) by the activation of autoreceptors such as M_2 receptors. Using functional studies we have demonstrated the presence of M_3 receptors on murine tracheae and on other parts of higher (main bronchi) and lower murine airways. In contrast M_2 receptors could only be demonstrated in lung function studies *in vivo*. These studies demonstrated the presence of M_2 receptors at higher sites of murine airway such as the upper/main bronchi but taking the *in vitro* results into account, not on the trachea. Finally it may be concluded that a balance between M_3 and M_2 receptors plays an important role in the regulation of airway calibre in mice.

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Role of the endothelium in the response to cholinoceptor stimulation of rabbit ear and femoral arteries during cooling

L. Monge, A.L. García-Villalón, J.J. Montoya, J.L. García, N. Fernández, B. Gómez & [1]G. Diéguez

Departamento de Fisiología, Facultad de Medicina, Universidad Autónoma, 28029 Madrid, Spain

1 The role of the endothelium in the effects of cooling on the response to cholinoceptor stimulation of the rabbit central ear (cutaneous) and femoral (non-cutaneous) arteries was studied using 2 mm long cylindrical segments.

2 Concentration-response curves for acetylcholine (10^{-9}–10^{-5} M), methacholine (10^{-9}–10^{-5} M) and sodium nitroprusside (10^{-9}–10^{-4} M) were isometrically recorded in arteries under conditions, with and without endothelium or following pretreatment with the nitric oxide-synthesis inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 10^{-6}–3×10^{-4} M) at 37°C and at 24°C (cooling).

3 Ear and femoral arteries showed endothelium-dependent relaxation to acetylcholine and methacholine at 37°C and 24°C. The extent of relaxation of the control ear arteries, but not of the control femoral arteries, to acetylcholine and methacholine increased during cooling.

4 L-NAME (10^{-6}–3×10^{-4} M) reduced in a concentration-dependent way the response of ear arteries to acetylcholine at both 37°C and 24°C, this reduction being more potent at 37°C. L-Arginine (10^{-5}–10^{-3} M) reversed in a concentration-dependent manner the inhibitor effects of 10^{-5} M L-NAME at both temperatures.

5 Sodium nitroprusside caused a concentration-dependent relaxation in both arteries that was endothelium-independent. However, the extent of relaxation to this nitrovasodilator in ear and femoral arteries was lower at 24°C.

6 These results suggest that cooling augments the reactivity of cutaneous (ear) arteries, but not that of non-cutaneous (femoral) arteries to cholinoceptor stimulation by endothelium-mediated mechanisms. Cooling could therefore facilitate the stimulated release of endothelial nitric oxide in cutaneous vessels.

Keywords: Skin arteries; temperature; nitric oxide; acetylcholine; sodium nitroprusside

Introduction

It appears that cooling affects cutaneous vascular responses (Vanhoutte, 1980) and also it is widely accepted that the endothelium plays a major role in the regulation of vascular tone by releasing relaxing and contracting factors (Furchgott & Vanhoutte, 1989). The endothelium can produce nitric oxide, the precursor of which is L-arginine (Palmer *et al.*, 1987; 1988; Ignarro *et al.*, 1987). The role of the endothelium in the effects of cooling on vascular reactivity has been studied very little (Karaki & Nagase, 1987; Bodelsson *et al.*, 1989) and it could be of interest, especially in cutaneous blood vessels which have a thermoregulatory function.

In our laboratory we have recently observed that in the rabbit ear artery, a cutaneous blood vessel, the response to endothelin-1 (Monge *et al.*, 1991) and to adrenergic activation (García-Villalón *et al.*, 1992) decreases during cooling by endothelium-dependent mechanisms, probably by increasing the availability of endothelial nitric oxide. To investigate further the endothelial mechanisms underlying the effects of cooling on the response of cutaneous blood vessels, the present study was designed to examine the response of the rabbit central ear artery to acetylcholine, methacholine and sodium nitroprusside in isolated arterial segments under isometric conditions at 37°C and during cooling (24°C). The experiments were performed in ear arteries with and without the endothelium or treated with an inhibitor of nitric oxide synthesis, and compared to those obtained in rabbit femoral (non-cutaneous) arteries. Cholinoceptor stimulation produces vascular relaxation by releasing endothelial nitric oxide (Aisaka *et al.*, 1989; Furchgott & Vanhoutte, 1989; Vallance *et al.*, 1989; Moore *et al.*, 1990; Rees *et al.*, 1990; Gardiner *et al.*, 1991) and sodium nitroprusside relaxes vascular smooth muscle by generating nitric oxide (Ignarro & Kadowitz, 1985). The ear artery was selected because this artery is easily accessible and used as a model of cutaneous blood vessels (Patton & Wallace, 1978; Roberts & Zygmunt, 1984; Harker & Vanhoutte, 1988).

Methods

Thirty-eight New Zealand White rabbits of either sex, weighing 2–2.5 kg, were killed by i.p. injection of sodium pentobarbitone (Sigma, 100 mg kg^{-1}). Central ear and femoral arteries were dissected free, cut into 2 mm-cylindrical segments, and prepared for isometric tension recording in a 6 ml organ bath containing modified Krebs-Henseleit solution with the following composition (mM): NaCl 115, KCl 4.6, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, glucose 11.1. The solution was equilibrated with 95% oxygen and 5% carbon dioxide to give a pH 0f 7.3–7.4. Two fine, stainless steel pins, 150 μm in diameter, were introduced through the lumen of the vascular segment. One pin was fixed to the organ bath wall while the other was connected to a strain gauge for isometric recording, thus permitting the application of passive tension in a plane perpendicular to the long axis of the vascular cylinder. The recording system included a Universal Transducing Cell UC3 (Statham Instruments, Inc.), a Statham Microscale Accessory UL5 (Statham Instruments, Inc.) and a Beckman Type RS Recorder (model R-411, Beckman Instruments, Inc.). A resting tension of 0.5 g was applied to the tissue and the segments were allowed to equilibrate for 60–90 min.

[1] Author for correspondence.

First, responses of the vascular segments under resting conditions to KCl (50 mM) were recorded in ear and femoral arteries with and without endothelium at both temperatures to test functional ability, and the solution was renewed by repeated wash-outs. Then, the relaxant responses to acetylcholine, methacholine and sodium nitroprusside were determined in the arteries precontracted with 5-hydroxytryptamine. One group of arteries were precontracted with 10^{-7} or 10^{-6} M 5-hydroxytryptamine, and with these doses the arteries developed an active tone that was lower in ear arteries than in femoral arteries, and in both types of arteries it was lower at 24°C than at 37°C. As these differences in active tone could interfere with the results, another group of ear arteries at 24°C were precontracted with higher concentrations of 5-hydroxytryptamine (10^{-5}–3×10^{-5} M) in order to achieve a similar tone to that obtained at 37°C with lower concentrations of this amine (10^{-7} or 10^{-6} M).

In the presence of the 5-hydroxytryptamine-induced tone, cumulative concentration-response curves for acetylcholine (10^{-9}–10^{-4} M), methacholine (10^{-9}–10^{-4} M) and sodium nitroprusside (10^{-9}–10^{-4} M) were obtained in ear and femoral arteries with (intact) and without endothelium at 37°C and 24°C. Also, the effects of acetylcholine were recorded in precontracted ear arteries in the presence of different concentrations of N^G-nitro-L-arginine methyl ester (L-NAME, 10^{-6}–3×10^{-4} M), an inhibitor of nitric oxide synthase (Moore *et al.*, 1990; Rees *et al.*, 1990). The action of an intermediate concentration of L-NAME (10^{-5} M) on the acetylcholine-induced response was tested in the presence of different concentrations of L-arginine (10^{-5}–10^{-3} M) at 37°C and 24°C.

Acetylcholine and methacholine were used as cholinoceptor agonists, and sodium nitroprusside as an exogenous donor of nitric oxide, and all of them were dissolved in physiological saline solution. Removal of the endothelium was accomplished by gently rubbing the lumen of the vessel with a roughened steel rod. The presence of the endothelium and the adequacy of endothelial removal was tested morphologically at the end of the experiments after silver staining (Caplan *et al.*, 1974).

L-NAME and L-arginine, dissolved in distilled water, were added to the bath 20–30 min before recording the response to acetylcholine. The effect of L-NAME and L-arginine on the relaxant response to acetylcholine was studied only in ear arteries since cooling affected the response of these arteries, but not that of femoral arteries.

Data analysis

The relaxation produced by the vasodilator drugs was expressed as a percentage of the active tone achieved with 5-hydroxytryptamine in each artery, and the concentration of drugs causing 50% of maximum effect (EC_{50}) was calculated from each individual concentration-response curve. The geometric mean of the EC_{50} and its 95% confidence interval were calculated for each group of experiments. The logarithms of the EC_{50} at every condition were compared statistically and the data were evaluated by Student's *t* test. To compare the inhibitory effects of L-NAME at 37°C and at 24°C, a two-way analysis of variance followed by the Dunnet's test was applied to the data of the relaxation of ear arteries to acetylcholine at both temperatures in the presence of different concentrations of L-NAME. In each case, $P<0.05$ was considered statistically significant.

Drugs

Materials used were: 5-hydroxytryptamine creatinine sulphate, acetylcholine chloride, methacholine chloride, sodium nitroprusside, N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME) and L-arginine (free base), all from Sigma.

Results

Effects of potassium chloride

Potassium chloride (50 mM) contracted both ear and femoral arteries and the response in both types of arteries was significantly lower at 24°C than at 37°C. At 37°C the contraction of ear arteries with and without endothelium was not significantly different, whereas at 24°C the ear arteries without endothelium showed a 40% higher contraction than those with endothelium. In femoral arteries at 37°C and 24°C no significant differences were observed between intact and endothelium-free arterial segments (Table 1).

Tone induced by 5-hydroxytryptamine

When 10^{-7} M or 10^{-6} M 5-hydroxytryptamine was applied to the arteries to achive active tone, the contraction of ear arteries was significantly lower than that of femoral arteries, and in both types of arteries the contraction at 24°C was significantly lower than at 37°C. Endothelium removal did not significantly modify the magnitude of contraction developed by both ear and femoral arteries at 24°C and 37°C to these doses of 5-hydroxytryptamine (10^{-6} or 10^{-7} M) respectively (Table 1). In another group of ear arteries (12 segments) higher concentrations of 5-hydroxytryptamine (10^{-5}–3×10^{-5} M) induced a contraction at 24°C (2.0 ± 0.14 g) that was similar to that developed by these arteries (13 segments) at 37°C with lower concentrations of this amine (10^{-6} M) (2.2 ± 0.17 g) ($P>0.05$).

Effects of cholinoceptor stimulation

At 37°C, acetylcholine (10^{-9}–10^{-4} M) produced concentration-dependent relaxations of the intact ear (30 segments) and femoral (10 segments) arteries precontracted with 5-hydroxytryptamine, and the maximal relaxation was about

Table 1 Contractile response (g) elicited by KCl (50 mM) or 5-hydroxytryptamine (5-HT, 10^{-6} or 10^{-7} M respectively) in rabbit central ear and femoral arteries at 37°C and 24°C

	Central ear artery		*Femoral artery*	
	37°C	*24°C*	*37°C*	*24°C*
KCl				
Control	1.95 ± 0.11	0.73 ± 0.07*	3.10 ± 0.27	2.03 ± 0.13*
Without endothelium	2.08 ± 0.16	1.02 ± 0.10*†	2.78 ± 0.29	1.72 ± 0.13*
5-HT				
Control	2.12 ± 0.19	1.65 ± 0.15*	2.46 ± 0.18	2.02 ± 0.16*
Without endothelium	2.09 ± 0.15	1.70 ± 0.13*	2.45 ± 0.17	1.97 ± 0.18*

Values are means ± s.e.mean from 22–27 animals. *Statistically significant differences ($P<0.01$) between 37°C and 24°C. †Statistically significant differences ($P<0.05$) between arteries with (control) and without endothelium at 24°C.

90–100% of the achieved active tone. Methacholine (10^{-9}–10^{-4} M) also induced concentration-dependent relaxations of these arteries (7 ear and 6 femoral arterial segments), and the response to this drug was comparable to that induced by acetylcholine in the corresponding arteries. After removal of the endothelium, ear and femoral arteries exhibited absence of, or a very little relaxation to acetylcholine (23 ear and 10 femoral arterial segments) and methacholine (7 ear and 6 femoral arterial segments) (Figures 1 and 2, and Table 2).

At 24°C precontracted, intact ear and femoral arteries also exhibited a concentration-dependent relaxation to acetylcholine (28 ear and 10 femoral arterial segments) and methacholine (7 ear and 5 femoral arterial segments), and the maximal relaxation to these agents was about 90–100% of the active tone. However, the extent of relaxation and sensitivity of intact ear arteries, but not of intact femoral arteries, to both acetylcholine and methacholine at 24°C was significantly higher than at 37°C. The increase in the relaxation of ear arteries induced with acetylcholine at 24°C was observed both when the arteries had a lower tone at 24°C (16

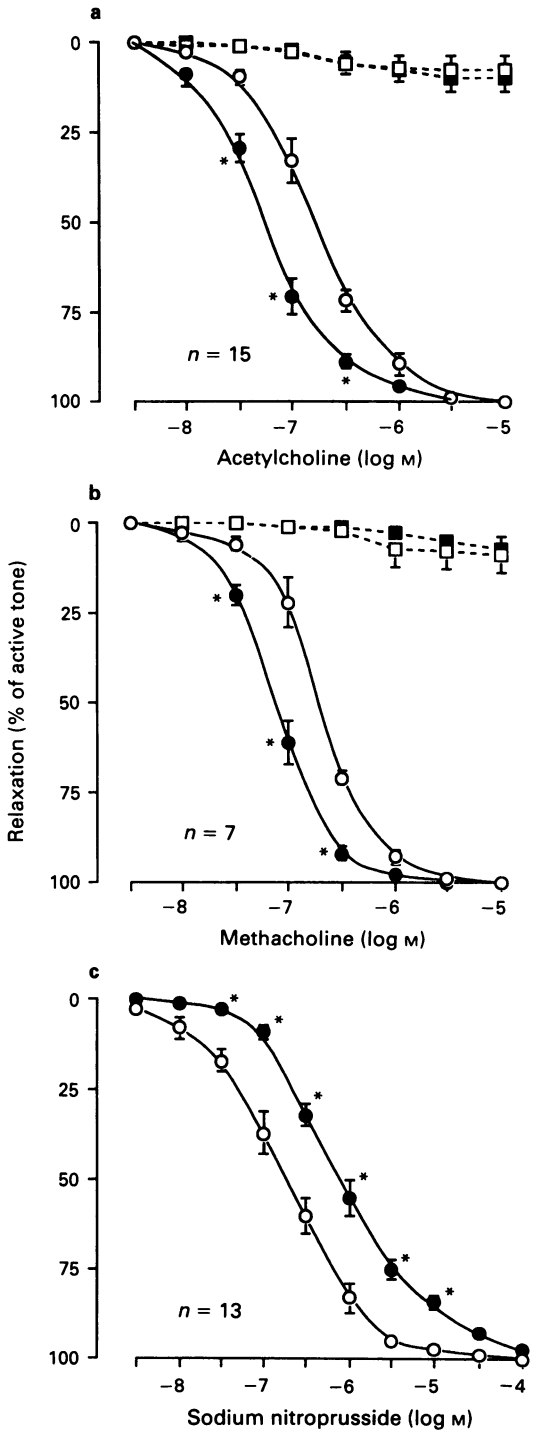


Figure 1 Relaxation of ear arteries with (○, ●) and without (□, ■) endothelium to (a) acetylcholine, (b) methacholine and (c) sodium nitroprusside obtained at 37°C (○, □) or 24°C (●, ■). Relaxation is expressed as a percentage of the active tone achieved with 5-hydroxytryptamine. Values are means with s.e.mean. *Statistically significant difference ($P<0.01$) between arteries with endothelium at 37°C and 24°C. n = number of animals.

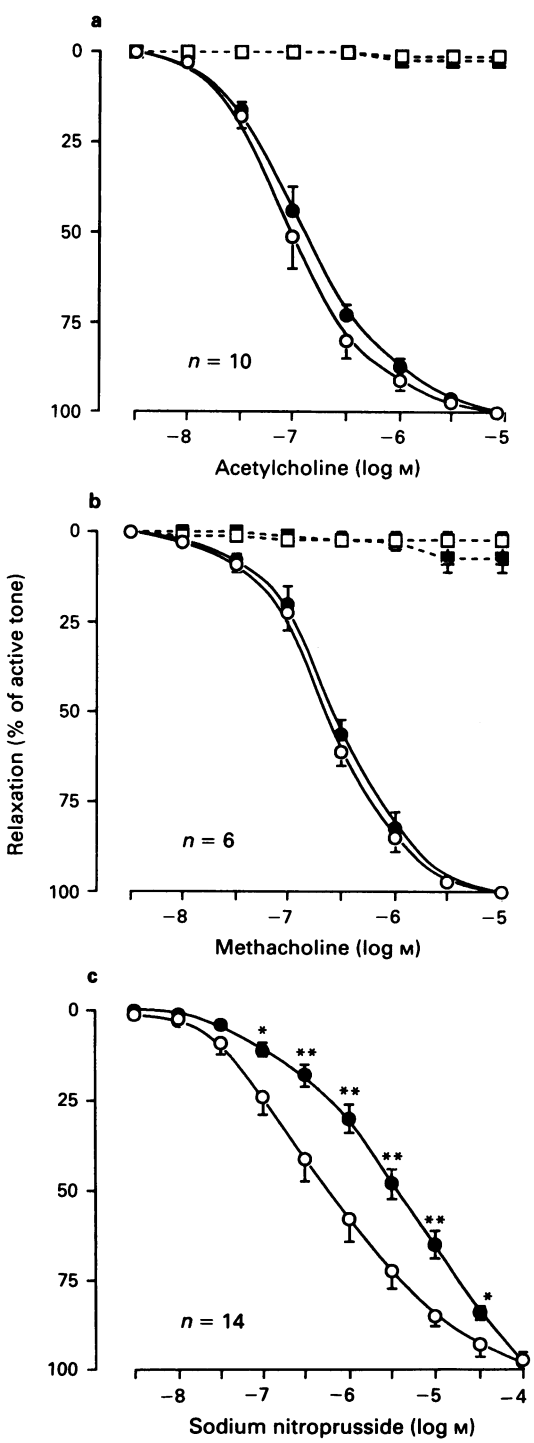


Figure 2 Relaxation of femoral arteries with (○, ●) and without (□, ■) endothelium to (a) acetylcholine, (b) methacholine and (c) sodium nitroprusside obtained at 37°C (○, □) or 24°C (●, ■). Relaxation is expressed as a percentage of the active tone achieved with 5-hydroxytryptamine. Values are means with s.e.mean. Statistically significant difference (*$P<0.05$; **$P<0.01$) between arteries with endothelium at 37°C and 24°C. n = number of animals.

Table 2 EC_{50} (M) values for the relaxation to acetylcholine and methacholine in rabbit central ear and femoral arteries precontracted with 5-hydroxytryptamine, at 37°C and 24°C

	Central ear artery		*Femoral artery*	
	37°C	*24°C*	*37°C*	*24°C*
Acetylcholine	1.4×10^{-7} (1.0×10^{-7}–2.0×10^{-7})	5.5×10^{-8}** (4.0×10^{-8}–7.6×10^{-7})	9.9×10^{-8} (6.3×10^{-8}–1.6×10^{-7})	1.2×10^{-7} (7.9×10^{-8}–1.7×10^{-7})
Methacholine	1.8×10^{-7} (1.2×10^{-7}–2.5×10^{-7})	7.7×10^{-8}* (5.6×10^{-8}–1.1×10^{-7})	2.1×10^{-7} (1.5×10^{-7}–3.1×10^{-7})	2.4×10^{-7} (1.6–10^{-7}–3.7×10^{-7})

Values are geometric means and, in parentheses, 95% confidence intervals from 6–10 animals. Statistically significant differences (*$P<0.01$; **$P<0.001$) between 37°C and 24°C.

segments) than at 37°C (17 segments), and when the concentration of 5-hydroxytryptamine was increased to ensure that the preparations had comparable tone at 24°C (12 segments) and at 37°C (13 segments). In both cases, the increase in the degree of relaxation at 24°C was of similar magnitude and the experiments on ear arteries with acetylcholine at each temperature was therefore pooled. In terms of EC_{50} values, the sensitivity of intact ear arteries to acetylcholine at 24°C was 2.5 times higher ($P<0.001$) than at 37°C, and the sensitivity to methacholine at 24°C was 2.3 times higher ($P<0.01$) than at 37°C. On the contrary, in intact femoral arteries, the sensitivity to acetylcholine as well as to methacholine was not significantly different at 24°C and 37°C ($P>0.05$). In both ear and femoral arteries without endothelium the relaxation to acetylcholine (17 ear and 10 femoral arterial segments) or methacholine (6 ear and 5 femoral arterial segments) was abolished or greatly reduced during cooling as occurred at 37°C (Figures 1 and 2, Table 2).

L-NAME (10^{-6}–3×10^{-4} M) reduced in a concentration-dependent way the relaxation of ear arteries to acetycholine, but these inhibitory effects of L-NAME were significantly more potent at 37°C than at 24°C (Figure 3). At both temperatures, 10^{-6} M or 3×10^{-6} M L-NAME did not affect significantly the relaxation of ear arteries to acetylcholine. At 37°C, 10^{-5} M L-NAME increased the EC_{50} values ($P<0.01$) and also decreased the maximal relaxation ($P<0.01$) to acetylcholine; these blocking effects of 10^{-5} M L-NAME were not significantly increased with the higher concentrations (3×10^{-5} and 10^{-4} M) of this substance. At 24°C, 10^{-5} M L-NAME increased the EC_{50} values ($P<0.01$) without affecting significantly the maximal relaxation to acetylcholine; the higher concentrations of L-NAME (3×10^{-5} and 10^{-4} M) did reduce the maximal relaxation to acetylcholine, but did not change significantly the EC_{50} values with regard to those obtained with 10^{-5} M L-NAME. By comparing the blocking effects of L-NAME on the acetylcholine-induced relaxation at 37°C and 24°C, we found that 10^{-5} M L-NAME was less effective in blocking the relaxation to acetylcholine at 24°C than at 37°C: the increment in the EC_{50} values and the reduction in the maximal relaxation induced with 10^{-5} M L-NAME on the response to acetylcholine were significantly ($P<0.003$) lower at 24°C than at 37°C.

The addition of L-arginine (10^{-5}–10^{-3} M) to the ear arteries previously treated with 10^{-5} M L-NAME reversed in a concentration-dependent manner the inhibitory effect of L-NAME on the relaxation to acetylcholine, and the reversion of this inhibition by L-arginine was not significantly different at 37°C and 24°C (Figure 4).

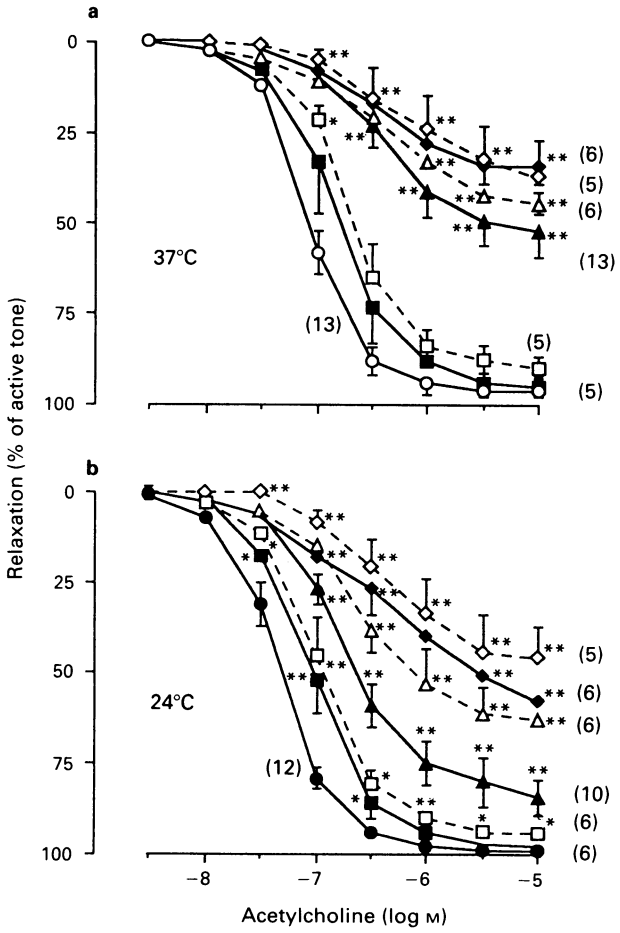


Figure 3 Relaxation of ear arteries from 5 rabbits to acetylcholine obtained at 37°C (a) or 24°C (b), under control conditions (○, ●) and in the presence of N^G-nitro L-arginine methyl ester at concentrations of 10^{-6} M (■), 3×10^{-6} M (□), 10^{-5} M (▲), 3×10^{-5} M (△), 10^{-4} M (◆) or 3×10^{-4} M (◇). Relaxation is expressed as a percentage of the active tone achieved with 5-hydroxytryptamine. Values are means with s.e. mean. Statistically significant difference (*$P<0.05$, **$P<0.01$) from control. In parentheses, number of segments.

Effect of sodium nitroprusside

At 37°C sodium nitroprusside (10^{-9}–10^{-4} M) produced a comparable concentration-dependent relaxation of precontracted ear (Figure 1) and femoral (Figure 2) arteries, and in both cases the maximal relaxant effect was 100% of the achieved tone. The sensitivity to sodium nitroprusside was not significantly different in ear arteries with (30 segments) and without (26 segments) endothelium, or in femoral arteries with (30 segments) and without (18 segments) endothelium.

During cooling, sodium nitroprusside also induced a concentration-dependent relaxation of ear and femoral arteries, the maximal relaxation being about 100% of the active tone. However, the sensitivity of the intact ear (30 segments) and femoral (31 segments) arteries to sodium nitroprusside, in terms of the EC_{50} values, was significantly lower at 24°C than at 37°C (Figure 1 and 2, Table 3). In intact ear arteries the sensitivity during cooling was 4.4 times lower than at 37°C, and in intact femoral arteries it was 6 times lower at 24°C

than at 37°C. As occurred at 37°C, the sensitivity to sodium nitroprusside at 24°C was not significantly different in ear and femoral arteries with (30 ear and 31 femoral arterial segments) and without (26 ear and 20 femoral arterial segments) endothelium.

Discussion

In the present work we have studied the effects of moderate cooling (24°C) on the response of central ear and femoral arteries from the rabbit to cholinoceptor stimulation. The ear artery is a cutaneous vessel that is likely to be exposed *in vivo* to the range of temperatures studied in the present *in vitro* work, and the results in this artery were compared with those obtained in the femoral artery, a deep blood vessel.

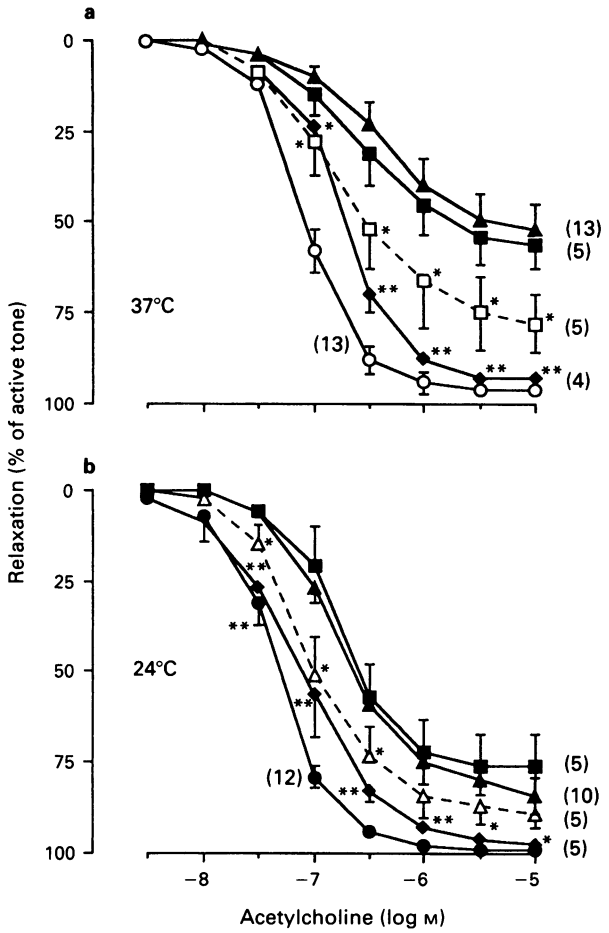


Figure 4 Relaxation of ear arteries from 4 rabbits to acetylcholine obtained at 37°C (a) or 24°C (b), under control conditions (○, ●) and in the presence of N^G-nitro L-arginine methyl ester (L-NAME) 10^{-5} M alone (▲), and in the presence of L-NAME 10^{-5} M plus L-arginine at concentrations of 10^{-5} M (■), 3×10^{-5} M (□), 10^{-4} M (△), or 10^{-3} M (◆). Relaxation is expressed as a percentage of the active tone achieved with 5-hydroxytryptamine. Values are means with s.e. mean. Statistically significant difference (*$P<0.05$, **$P<0.01$) from L-NAME alone. In parentheses, number of segments.

At 37°C, both ear and femoral arteries showed a similar relaxation to acetylcholine and methacholine that was endothelium-dependent as occurs in many vascular preparations (Furchgott & Vanhoutte, 1989). The relaxation of ear arteries to acetylcholine was also reduced in a concentration-dependent manner by L-NAME, and this reduction was reversed in a concentration-dependent way by L-arginine. These findings agree with the idea that the vascular relaxation to cholinoceptor stimulation is mediated, at least in part, by endothelial nitric oxide (Aisaka *et al.*, 1989; Furchgott & Vanhoutte, 1989; Vallance *et al.*, 1989; Moore *et al.*, 1990; Rees *et al.*, 1990; Gardiner *et al.*, 1991).

During cooling, both ear and femoral arteries also exhibited endothelium-dependent relaxation to acetylcholine and methacholine. This relaxation of ear arteries to acetylcholine was concentration-dependently reduced by L-NAME, and this reduction was reversed by L-arginine. Thus endothelial nitric oxide could be also involved in the relaxation of ear arteries to cholinoceptor stimulation during cooling, as occurs at 37°C. However, the effects of cooling on the response to acetylcholine and methacholine differed between ear and femoral arteries. In ear arteries, cooling increased, whereas in femoral arteries it did not affect the relaxation to cholinoceptor stimulation. The increased relaxation of ear arteries to acetylcholine at 24°C was found when these arteries had a lower or similar active tone at 24°C than at 37°C, thus indicating that the facilitatory effects of cooling on the relaxation of ear arteries to cholinoceptor stimulation are not dependent on the level of active tone, but on specific action of cold on these particular arteries. In addition, our results show that cooling decreased the ability of L-NAME to block the acetylcholine-induced relaxation of ear arteries.

Sodium nitroprusside has been used as a donor of nitric oxide and produces vascular relaxation in a similar way to nitric oxide by stimulating the guanylate cyclase of vascular smooth musculature (Rapoport & Murad, 1983; Ignarro & Kadowitz, 1985). On the other hand, evidence that the endothelium modulates the effect of nitrovasodilators is controversial and ranges from findings of potentiation (Shirasaki & Su, 1985; Gardiner *et al.*, 1991; Moncada *et al.*, 1991; García *et al.*, 1992) to attenuation (White *et al.*, 1986) and no response whatsoever (Furchgott *et al.*, 1991; Rapoport & Murad, 1983). In our experiments we found that sodium nitroprusside induced endothelium-independent relaxation of ear and femoral arteries, and that this relaxation was reduced in both types of arteries during cooling as compared to 37°C. The decreased relaxation to sodium nitroprusside clearly contrasts with the increased relaxation to cholinoceptor stimulation in ear arteries during cooling.

Although an explanation for these differences is at present not available, some considerations can be made on the basis that both sodium nitroprusside and stimulation of the endothelium with cholinoceptor agonists lead to formation of nitric oxide and a reduced sensitivity of arterial smooth

Table 3 EC_{50} (M) values for the relaxation to sodium nitroprusside in rabbit central ear and femoral arteries precontracted with 5-hydroxytryptamine, at 37°C and 24°C

	Central ear artery		*Femoral artery*	
	37°C	*24°C*	*37°C*	*24°C*
Control	1.8×10^{-7} (1×10^{-7}–3×10^{-7})	7.7×10^{-7}* (5.3×10^{-7}–1.1×10^{-6})	5.3×10^{-7} (2.6×10^{-7}–1.1×10^{-6})	3.2×10^{-6}* (1.6×10^{-6}–6.6×10^{-6})
Without endothelium	1.2×10^{-7} (8×10^{-8}–1.9×10^{-7})	4.8×10^{-7}* (3.2×10^{-7}–7.2×10^{-7})	2.9×10^{-7} (1.7×10^{-7}–4.9×10^{-7})	8.8–10^{-7}* (3.7×10^{-7}–2.1×10^{-6})

Values are geometric means and, in parentheses, 95% confidence intervals from 13–14 animals. Statistically significant differences (*$P<0.01$) between 37°C and 24°C.

musculature to nitric oxide could occur during cooling. The possibility that an increased stability of nitric oxide and a higher rate of its reaction with -SH groups to form S-nitrosothiols accounts for the facilitatory effects of cooling on cholinoceptor-induced relaxation in ear arteries can also reasonably be excluded, because if that were the case, these facilitatory effects of cooling should also be present in femoral arteries, as well as in both types of arteries, when exposed to sodium nitroprusside. Thus, a more probable explanation is that an increased release of endothelial nitric oxide might occur in ear arteries, but not in femoral arteries when stimulated with acetylcholine or methacholine during cooling. This might be because cooling increases the interaction of the cholinoceptor agonists with the muscarinic receptors or the signal transduction mechanisms in the endothelial cells, or to cooling directly stimulating the production of nitric oxide in ear artery, a feature that could be specific for cutaneous arteries.

The reduced relaxation of both ear and femoral arteries to sodium nitroprusside might be related not only to a decreased sensitivity of arterial smooth musculature but also to a decreased formation of nitric oxide from sodium nitroprusside during cooling. The process that leads to nitrocompounds providing nitric oxide in the organ bath might be altered at a low temperature. It has been found that the ability of sodium nitroprusside to release nitric oxide and to form S-nitrosothiols varies with the medium conditions (Ignarro *et al.*, 1981).

L-NAME was able to block the relaxation of ear arteries to acetylcholine in a concentration-dependent way at both temperatures. However, higher concentrations of L-NAME were needed at 24°C than at 37°C to block the relaxation to acetylcholine. Also, the concentration of L-NAME that caused the maximal blockade to acetylcholine at 37°C, was less efective at 24°C than at 37°C in reducing the relaxation of ear arteries to cholinoceptor stimulation. These results with L-NAME suggest that the biochemical reaction involved in the conversion of L-arginine to nitric oxide in the endothelium of ear arteries is less sensitive to inhibition during cooling. With our data we cannot suggest which mechanisms underlie this lower sensitivity to inhibition by L-NAME during cooling, but our results support the hypothesis that the formation of endothelial nitric oxide in stimulated ear arteries increases during cooling.

We also found that cooling reduced the contraction to potassium chloride in ear and femoral arteries, a feature that is known to occur in cutaneous and non-cutaneous blood vessels (Vanhoutte, 1980). In our experiments we observed that endothelium removal did not affect the contracton of either ear or femoral arteries to potassium chloride at 37°C. However, at 24°C the ear arteries without endothelium showed a higher contraction than the arteries with endothelium to potassium chloride, a phenomenon that did not occur in the femoral arteries. This suggests that during cooling the endothelium also exerts an inhibitory effect on the contractile response of cutaneous arteries to stimuli considered as direct activators of smooth musculature. We have recently observed that the endothelium inhibits the contraction of underlying smooth musculature of cutaneous arteries to endothelin-1 (Monge *et al.*, 1991) and to adrenergic stimulation (García-Villalón *et al.*, 1992) at low temperature, probably by an increased availability of endothelial nitric oxide. These two studies (Monge *et al.*, 1991; García-Villalón *et al.*, 1992) and the present results suggest that cooling could non-specifically facilitate the release of nitric oxide from the endothelium of cutaneous vessels when it is activated by different types of vasoactive stimuli.

In summary, the present results suggest that cooling facilitates the stimulated release of nitric oxide from the endothelium in cutaneous, but not in deep vessels. If the results in the ear artery could be extended to other cutaneous vessels, the endothelium function may be of relevance to thermoregulatory function of the cutaneous circulation, as well as to the pathophysiology of some pathological situations. The endothelium is thought to be of some importance in the pathophysiology of Raynaud's phenomenon (Yang *et al.*, 1990), which is in part characterized by intense cold-induced cutaneous vasospasm (Harper & Le Roy, 1987). From our results we can speculate that during cooling the increased endothelium function would limit or avoid cutaneous vasospasm, and, in consequence, a damage of the cutaneous vascular endothelium could lead to uncover or increase the cold-induced vasoconstriction.

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Endothelin (16-21): biphasic effect and no desensitization on the guinea-pig isolated ileum

[1]N. Miasiro, C.R. Nakaie & A.C.M. Paiva

Department of Biophysics, Escola Paulista de Medicina, C.P. 20388, 04034 São Paulo, SP, Brazil

1 In the guinea-pig ileum the C-terminal hexapeptide of the endothelins, endothelin (16-21), induced a biphasic effect (relaxation followed by contraction) qualitatively similar to that seen in the responses to endothelins 1 and 3. Both components of the response were concentration-dependent in the range studied (2–100 μM).

2 The response induced by endothelin (16-21) was inhibited in low-sodium (80 mM) medium.

3 Repeated administration of endothelin (16-21) induced no desensitization of the preparation, contrasting with the tachyphylaxis induced by endothelin-1 and endothelin-3 in the guinea-pig ileum.

4 Tissues rendered tachyphylatic to endothelin-1 or endothelin-3 responded normally to endothelin (16-21).

5 The results suggest that the C-terminal tail of the endothelins contains the message for the biphasic response, whereas the N-terminal domain may be responsible for the strong binding to the receptor and for the tachyphylactic properties of endothelin-1 and endothelin-3, in the guinea-pig isolated ileum. However, the possibility that endothelin (16-21) may be acting on a site other than the endothelin receptor cannot be ruled out.

Keywords: Endothelin (16-21); endothelin-1; endothelin-3; guinea-pig ileum; desensitization; sodium ions

Introduction

The known members of the endothelin family comprise four distinct peptides: endothelin-1, endothelin-2, endothelin-3 and vasoactive intestinal contractor (VIC). These isopeptides from different species are characterized by an N-terminal cyclic moiety with two disulphide bonds and a C-terminal hexapeptide tail rich in hydrophobic amino acid residues (Yanagisawa *et al.*, 1988; Saida *et al.*, 1989). The C-terminal moiety has the same amino acid sequence in all four isopeptides, the differences between them residing in the cyclic portion of the molecule. The sarafotoxins, a group of four cardiotoxic isotoxins, are another family of 21-residue isopeptides structurally related to the endothelins (Kloog *et al.*, 1988; Bdolah *et al.*, 1989).

We have investigated the effect of endothelin-1 (Miasiro & Paiva, 1990) and endothelin-3 (Miasiro & Paiva, 1992) on the guinea-pig ileum and observed that both peptides induced a direct biphasic effect (relaxation followed by contraction). In this tissue, the responses to endothelin-1 and endothelin-3 were characterized by a homologous desensitization (tachyphylaxis), and were markedly dependent on the Na^+ gradient across the smooth muscle cell membrane (Miasiro & Paiva, 1990; 1992). Wollberg *et al.* (1991) have also reported a relaxant component in the response of the guinea-pig to endothelins and observed evidence for desensitization, but did not investigate this property further.

The C-terminal hexapeptide, with the common sequence endothelin (16-21), has been shown to be a full contractile agonist in the guinea-pig bronchus, rat vas deferens and rabbit pulmonary artery, but to have little if any activity in several other smooth muscle preparations (Maggi *et al.*, 1989a). On electrically field-stimulated guinea-pig ileum, several isopeptides of the endothelin/sarafotoxin family produced a concentration-dependent inhibition of the nerve-mediated contractions. However, endothelin (16-21) had no significant inhibitory effect and had instead, at high concentrations, a contractile effect which enhanced the nerve-mediated contractions (Maggi *et al.*, 1989a).

We have further investigated the effect of endothelin (16-21) on the guinea-pig isolated ileum and found that the hexapeptide is able to elicit responses that are qualitatively similar to those of endothelins 1 and 3. In the present paper we describe the results of an attempt to correlate the effect of endothelin (16-21) with those related to activation of endothelin receptors as induced by endothelin-1 and endothelin-3.

Methods

Guinea-pigs of either sex (200–250 g) were stunned by a blow to the head and bled. A 20 cm portion of the terminal ileum was removed and washed with Tyrode solution at room temperature. Segments of the ileum (3.5–4.0 cm) were suspended in a 5 ml organ chamber which contained Tyrode solution maintained at 37°C and was bubbled with a constant stream of O_2. The isometric contractions were recorded through a Narco BioSystems force transducer, model F-60, and an ECB model 102-B recorder. Resting tension was adjusted to 1 g.

Unless otherwise noted, the agonists were left in contact with the preparations for 3 min. At the beginning of each experiment, 60 mM KCl was always applied to elicit maximal control responses.

Analysis of the results

The results are presented as mean ± s.e.mean. Statistical analysis of the data was made by means of Student's *t* test or analysis of variance followed by Tukey's test when applicable. *P* values less than 0.05 were considered significant.

Solutions and drugs

The Tyrode solution had the following composition (in mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.36, $MgCl_2$ 0.49, $NaHCO_3$ 11.9, NaH_2PO_4 0.36 and glucose 5.1. The sodium-deficient solutions were obtained by isosmotic replacement of the NaCl with *D*-glucose to give a solution containing 80 mM Na^+.

[1] Author for correspondence.

Glucose was chosen as a Na^+ substitute because, in contrast to sucrose, it did not affect the ileum response to the addition of hyperosmotic KCl (Shimuta *et al.*, 1982). The pH of the solutions, when equilibrated with air, was 8.0 ± 0.1.

The inorganic salts were from Merck, Darmstadt, Germany. All agonists (except for the hexapeptide which was dissolved in 0.1% acetic acid) were prepared in aqueous concentrated solutions and were diluted with saline solution (9 g/l NaCl) before addition to the bath. The maximal volume used for the addition of the peptides to the bath was 200 µl.

Peptides

Synthetic endothelin-3 and endothelin-1 were from the Peptide Research Institute (Osaka, Japan). Endothelin (16-21) and angiotensin II were synthesized on a Biosearch 9500 peptide synthesizer by solid phase protocols (Paiva *et al.*, 1974; Barany & Merrifield, 1980) on Trp(For) and Phe-Pam resins, respectively. Amino acid analysis, analytical high performance liquid chromatography (h.p.l.c.), paper electrophoresis and thin layer chromatography (t.l.c.) were employed to assess the purity of both peptides which was higher than 95%.

Results

Effect of endothelin (16-21) in normal and low-Na^+ medium

Endothelin (16-21) induced a biphasic effect (relaxation followed by contraction) in the guinea-pig isolated ileum (Figure 1c). This pattern of response was also observed with endothelin-1 and endothelin-3 (Figures 1a, 1b). The initial relaxant phase of the responses induced by 50 µM endothelin (16-21) and by 250 nM endothelin-3 was more pronounced than that caused by 50 nM endothelin-1, which induced a rapid sharp relaxation. The contractions induced by the three peptides were transient, but that due to endothelin (16-21) (Figure 1c) was more sustained than the contraction induced by endothelin-3 (Figure 1b). For all three peptides, washout of the agonist was followed by slow relaxation of the preparation and a new resting level was reached (Figure 1).

Figure 2 shows the concentration-response curves for endothelin (16-21), endothelin-1 and endothelin-3. For endothelin (16-21) at low concentration (2 µM) only a small contraction of the preparation was observed (Figure 2a), but as the concentration was increased a relaxant component appeared which was dose-dependent (Figure 2b), as was the contractile component (Figure 2a). Because of limitations in the solubility and availability of the peptide, the highest dose of endothelin (16-21) tested was 100 µM, which elicited only about 58% of the maximal contraction induced by 60 mM KCl. Apparently, the maximum contractile response to the peptide was not reached at this concentration. For endothelin-3, there was only the relaxant component in the dose range of 0.1–1 nM (Figure 2b) and as the concentration increased, the response became biphasic (Figure 2a, 2b). For endothelin-1, the biphasic effect was present in all tested concentrations (Figure 2a, b).

Since it was not possible to obtain a maximum response to endothelin-3 and endothelin (16-21), EC_{50KCl} values were determined. From the data of Figure 2, these concentrations were 10^{-8} M for endothelin-1, 10^{-7} for endothelin-3, and 8×10^{-4} M for the hexapeptide. Endothelin (16-21), therefore, is less active than endothelins 1 and 3 by 3–4 orders of magnitude.

Repeated administration of 40 µM endothelin (16-21), at 10 min intervals, caused no desensitization of the tissue, as evidenced by the unaltered response (relaxation and contraction always present) of the isometric responses (Figure 3). Isometric recordings showed that in low-Na^+ (80 mM) medium both components of the response induced by endothelin (16-21) were greatly reduced (Figure 4) as was previously observed with endothelin-1 (Figure 4; Miasiro & Paiva, 1990) and endothelin-3 (Miasiro & Paiva, 1992).

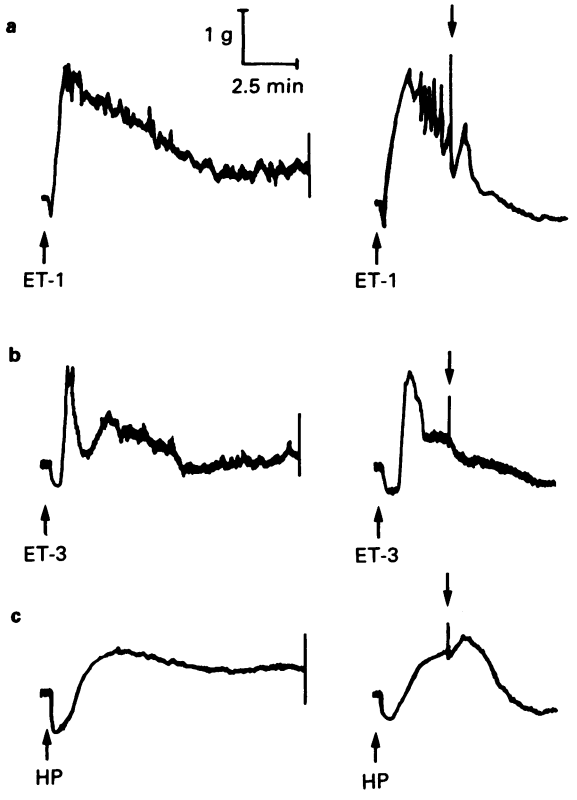


Figure 1 Isometric recordings of the responses of the guinea-pig isolated ileum induced by (a) 50 nM endothelin-1 (ET-1), (b) 250 nM endothelin-3 (ET-3) and 50 µM endothelin (16-21) (HP). Upward and downward arrows indicate addition to and removal of the agonists from the bath.

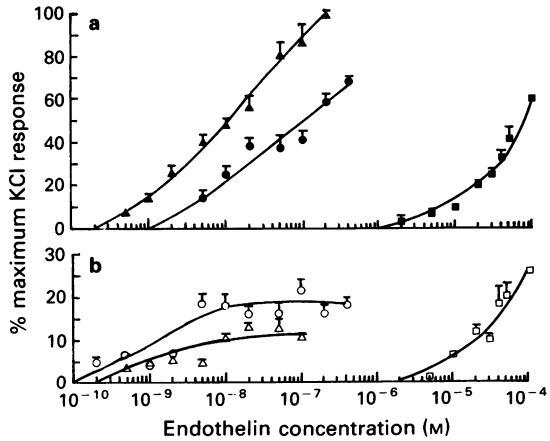


Figure 2 Dose-response relationships for the contractile (a) and the relaxant (b) components of the responses of the ileum to endothelin-1 (▲, △), endothelin-3 (●, ○) and endothelin (16-21) (■, □). Response amplitudes are expressed as percentages of the phasic response induced by 60 mM KCl. Each point represents the mean ± s.e.mean of 5–9 experiments. The contact time with the agonist was 3 min and the time interval between administrations was sufficient to avoid interference by desensitization. Responses to high concentrations were obtained in separate fresh preparations.

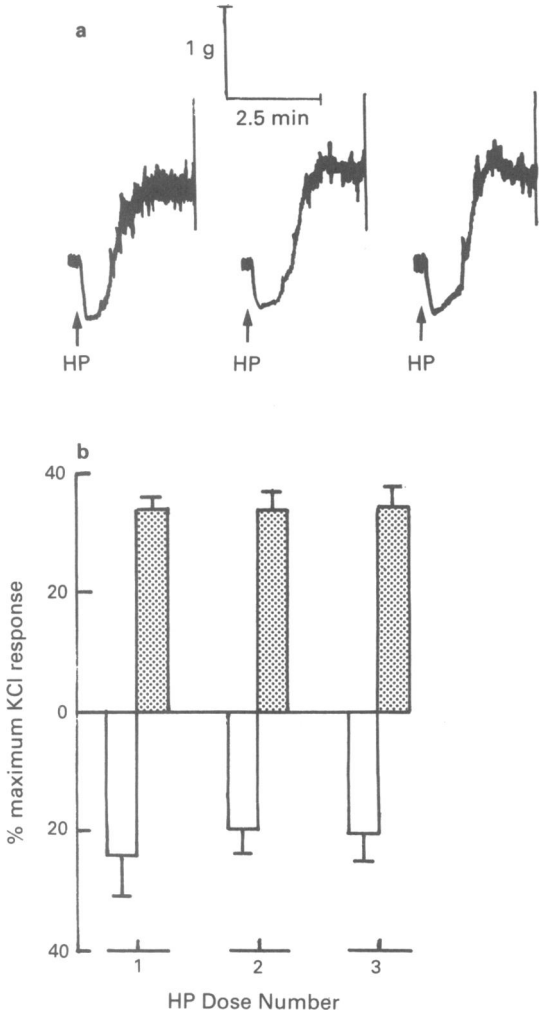


Figure 3 Responses induced by three successive administrations of 40 μM endothelin (16-21) (HP): (a) isometric recordings; (b) representation of mean ± s.e.mean of 3 experiments. The amplitude of the contractile component of the responses is represented by the stippled bars and of the relaxant component by the open columns. Response amplitudes of both components are expressed as percentages of the phasic response induced by 60 mM KCl given 15 min before addition of the peptides. Contact time with the agonist was 3 min and the time interval between doses was 10 min. Upward arrows indicate addition of the agonist.

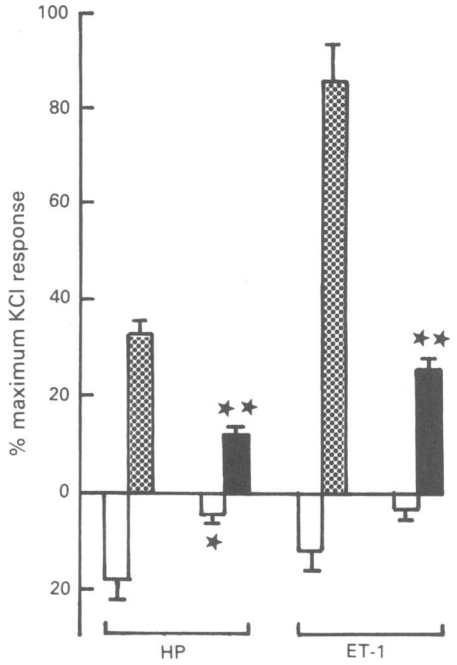


Figure 4 (a) Responses of the guinea-pig isolated ileum to 50 μM endothelin (16-21) (HP) and 100 nM endothelin-1 (ET-1) in normal Tyrode solution (hatched columns for the contractile component) and in low-Na^+ (80 mM) medium (solid columns for the contractile component). The relaxant components are represented by the open columns. Response amplitudes of both compounds are expressed as percentages of the phasic response induced by 60 mM KCl given 15 min before addition of the peptides ($n = 4$). * and ** significantly different ($P < 0.05$ and $P < 0.01$ respectively) from control.

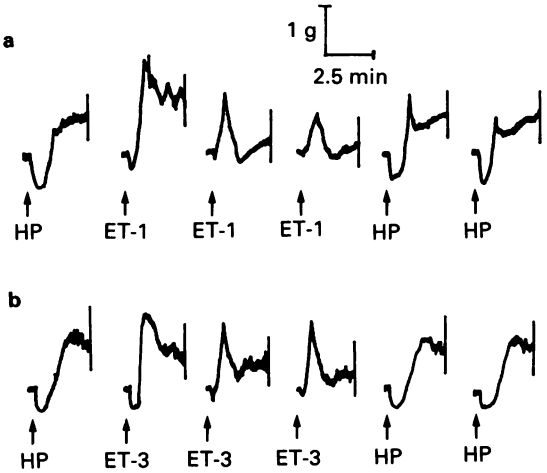


Figure 5 Isometric recordings of the response of the guinea-pig isolated ileum to (a) 40 μM endothelin (16-21) (HP) followed by three successive doses of 100 nM endothelin-1 (ET-1) and two other doses of endothelin (16-21); (b) 40 μM endothelin (16-21) followed by three successive doses of 100 nM endothelin-3 (ET-3) and two other doses of endothelin (16-21). In both experiments, the response to 60 mM KCl was recorded 15 min before the addition of the peptides (not shown). Upward arrows indicate addition of the agonists.

Effect of endothelin (16-21) on tissues desensitized to endothelin-1 and endothelin-3

We have previously observed that endothelin-1 and endothelin-3 induce specific desensitization of the guinea-pig ileum (Miasiro & Paiva, 1990; 1992). By studying cross-tachyphylaxis between these two isopeptides, it was concluded that there are at least two subtypes of endothelin receptors in the guinea-pig ileum: one selective for endothelin-1 and another one which is non-selective (Miasiro & Paiva, 1992). To investigate whether or not the responses to endothelin (16-21) could interact with one of these receptor subtypes, we studied the possibility of cross-tachyphylaxis between endothelin-1 or endothelin-3 and the hexapeptide.

Figure 5a shows that, after treatment with 40 μM endothelin (16-21), a normal response to 100 nM endothelin-1 was obtained, and that repeated application of 100 nM endothelin-1, at 10 min intervals, resulted in desensitization of the tissue as expected. The tissue thus desensitized, however, continued to respond normally to 40 μM endothelin (16-21), which elicited relaxation followed by contraction of the same amplitudes as before. This absence of cross-tachyphylaxis was also observed when the guinea-pig ileum was rendered tachyphylatic to endothelin-3 (Figure 5b).

Discussion

Endothelin (16-21), a hexapeptide containing the highly conserved sequence in the peptides of the endothelin family,

induced a biphasic response (relaxation followed by contraction) in the guinea-pig ileum, which is qualitatively similar to the responses induced by endothelin-1 and endothelin-3. Both components of the response induced by endothelin (16-21) were dose-dependent in the dose-range studied, contrasting with endothelin-1 and endothelin-3 (Figure 2). For the hexapeptide and endothelin-3, at all concentrations, the relaxant component was very pronounced. In contrast, for endothelin-1, the higher the concentration used, the shorter the duration of the relaxation (Miasiro & Paiva, 1990).

In our studies, endothelin (16-21) is less active than endothelins 1 and 3 by 3–4 orders of magnitude. This indicates that the C-terminal moiety of the endothelins is relatively less important for their effect on the guinea-pig ileum than that on the bronchus, where endothelin (16-21) is only *ca.* 30 fold less active than endothelin-1 (Maggi *et al.*, 1989b).

In view of the low activity of endothelin (16-21), the assumption that it acts on an endothelin receptor should be validated. The C-terminal hexapeptide of endothelin-1 has been shown to be important for endothelin receptor recognition and functional activity in selected tissues (Rovero *et al.*, 1990; Doherty *et al.*, 1991). Maggi *et al.* (1989b) reported that endothelin (16-21) is a full agonist on guinea-pig bronchial tissue and functional studies in this tissue have indicated that Trp^{21}, Asp^{18}, Leu^{17} and His^{16} are important for the biological activity of the hexapeptide (Rovero *et al.*, 1990). In contrast, endothelin (16-21) was devoid of any agonistic and antagonistic activities in other tissues (Kimura *et al.*, 1988; Maggi *et al.*, 1989a).

Tschirhart *et al.* (1991) demonstrated that endothelin (16-21), up to 10 μM, did not inhibit binding of ^{125}I-endothelin-1 to guinea-pig airway smooth muscle. Moreover, Maggi *et al.* (1991) observed effective competition with ^{125}I-endothelin-1 binding only when they used millimolar concentrations of the C-terminal hexapeptide in rabbit uterus. On the other hand, Davenport *et al.* (1990) showed that endothelin (15-21) competed for endothelin-1 receptors in the rat kidney. These results in various tissues would be consistent with different distributions of receptor subtypes.

In a functional biochemical assay measuring intracellular levels of second messengers (IP_3 accumulation) it was found that C-terminal endothelin peptides with D stereochemistry at position 16, are indeed endothelin receptor antagonists (Doherty *et al.*, 1992). One of these improved analogues, Ac-D-Bhg-L-Leu-L-Asp-L-Ile-L-Ile-L-Trp (PD145065), is a potent combined ET_A/ET_B receptor antagonist, having 2.6 and 19 nM affinity for the ET_A and ET_B receptor, respectively (Cody *et al.*, 1992). On the other hand, *cyclo*[D-Asp-L-Pro-D-Val-L-Leu-D-Trp] (BQ-123), one of a series of cyclic pentapeptides, is a potent and selective ET_A receptor antagonist (Ihara *et al.*, 1992). Both PD 145065 and BQ-123 are closely related to the endothelin C-terminal hexapeptide His-Leu-Asp-Ile-Ile-Trp, indicating that this portion of the molecule contains the information regarding the structural and conformational features important for receptor recognition and binding.

Our experiments with the guinea-pig ileum also yielded some results that suggest that endothelin (16-21) may be acting at an endothelin receptor: (1) In the guinea-pig ileum, the responses to the endothelins and sarafotoxins are characteristic, consisting of a relaxation followed by contraction, differing markedly from other known agonists. Endothelin (16-21) elicited the same unusual biphasic effect in spite of its low activity.

(2) The Na^+-dependence of the effect of endothelin (16-21) was similar to that observed for the cases of endothelin-1 (Miasiro & Paiva, 1990), endothelin-3 (Miasiro & Paiva, 1992) and sarafotoxins (unpublished data).

However, repeated application of endothelin (16-21) did not induce desensitization of the preparation, in contrast to the desensitization induced by endothelin-1 and endothelin-3, which was not reversed even after a 3 h resting period (Miasiro & Paiva, 1990; 1992). This suggests that endothelin (16-21) might interact with some site other than the endothelin receptor, which would give rise to an endothelin-like response in the guinea-pig ileum. On the other hand, if we assume that endothelin (16-21) acts on the endothelin receptor, our results may indicate that different domains are involved in the agonistic and tachyphylactic activity of endothelins in the guinea-pig ileum. The C-terminal tail, which is common to all the members of the endothelin family, contains the information needed to elicit the response, but not that responsible for the desensitization. In this case, the cyclic N-terminal moiety would be important for the affinity, and also for the induction of desensitization. Indeed, besides Ile^{20} and Trp^{21}, there are two other 'binding sites' important for receptor binding which are Asp^8 and Tyr^{13}, present at the N-terminal portion of the molecule (Hunt *et al.*, 1991).

Both the cyclic N-terminal region and the C-terminal tail of the endothelins are known to be important for activity. Thus, no activity on porcine coronary artery was detected in endothelin (1-16) (Kimura *et al.*, 1988; Hirata *et al.*, 1989), while endothelin (16-21) had little or no activity in different smooth muscle preparations (Hirata *et al.*, 1989; Maggi *et al.*, 1989a). One might speculate that the qualitative characteristics of the response are encoded in the C-terminal tail, while the binding energy needed to form the fully effective peptide-receptor complex comes from interactions involving groups at the N-terminal domain. The absence of cross-tachyphylaxis between endothelin-1 or endothelin-3 and endothelin (16-21) might be thought to indicate different receptors, but it is also possible that the receptors desensitized by some reaction with the N-terminal moiety of the endothelin molecule would still be able to interact productively with the C-terminal hexapeptide. The presence of distinct relaxant and contractile components in the responses to endothelin (16-21), suggests that both components may be induced by the same endothelin moiety. However, the possibility that endothelin (16-21) may be acting on a site other than the endothelin receptor cannot be ruled out.

In the case of blood vessels, it was suggested that different receptors mediate vasodilatation and vasoconstriction (Le Monnier de Gouville *et al.*, 1990; Yanagisawa & Masaki, 1989). The ET_A receptor, present on smooth muscle cells, is probably linked to the vasoconstrictor effect of the endothelins, whereas the effect of ET_B receptor activation, presumably located on endothelial cells, seems to be responsible for vasodilatation (Hemsén, 1991). In the guinea-pig ileum, the biphasic response might be due to the existence of different receptors, although a single binding site was demonstrated in the guinea-pig ileum smooth muscle membranes (Wollberg *et al.*, 1991).

Definite pharmacological analysis of the agonistic and desensitizing properties of the endothelin isopeptides awaits the development and testing of selective agonists and antagonists with different structural requirements to induce or inhibit the characteristic responses.

Note added in proof

ET(16–21) acts on an endothelin receptor, since BQ-123 a specific ET_A) receptor antagonist, interferes with the contractile action induced by the hexapeptide in the guinea-pig ileum (unpublished observation).

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L-Arginine exerts a dual role in nociceptive processing in the brain: involvement of the kyotorphin-Met-enkephalin pathway and NO-cyclic GMP pathway

Atsufumi Kawabata, Nahoko Umeda & [1]Hiroshi Takagi

Department of Pharmacology, Faculty of Pharmaceutical Sciences, Kinki University, 3-4-1 Kowakae, Higashi-Osaka 577, Japan

1 Intracerebroventricular (i.c.v.) administration of L-arginine (L-Arg), at 10–100 μg per mouse, produced antinociception in mice, as assessed by the tail flick test; this antinociception was reversed by pretreatment (s.c.) with naltrindole (NTI), a δ-selective opioid antagonist, and by co-administered L-leucyl-L-arginine (Leu-Arg), a kyotorphin (endogenous Met-enkephalin releaser) receptor antagonist.

2 L-N^G-nitroarginine methyl ester (L-NAME), a NO synthase inhibitor, but not D-N^G-nitroarginine methyl ester, given i.c.v. at 3–10 μg per mouse, exhibited antinociceptive activity that was resistant to naloxone (s.c.), NTI (s.c.) and Leu-Arg (i.c.v.).

3 The L-NAME (i.c.v.)-induced antinociception was not reversed by L-Arg (i.c.v.), which was antinociceptive by itself, but was abolished by combined injection of L-Arg plus Leu-Arg (i.c.v.) or by L-Arg (i.c.v.) after NTI (s.c.).

4 Methylene blue (MB), a soluble guanylate cyclase inhibitor, at 0.1–1 μg per mouse, produced antinociception by i.c.v. administration. The antinociception induced by MB (i.c.v.) or L-NAME (i.c.v.) was reversed by co-administered dibutyryl cyclic GMP.

5 These findings suggest that L-Arg plays a dual role in nociceptive processing in the brain, being antinociceptive via the kyotorphin-Met-enkephalin pathway and nociceptive via the NO-cyclic GMP pathway.

Keywords: L-Arginine; L-N^G-nitroarginine; methylene blue; kyotorphin; enkephalin; nitric oxide; cyclic GMP; nociceptive modulation; D-N^G-nitroarginine

Introduction

L-Arginine, a constituent amino acid of kyotorphin (L-tyrosyl-L-arginine), an endogenous Met-enkephalin releaser in the brain and spinal cord (Takagi *et al.*, 1979a,b), is considered to be an effective kyotorphin precursor, since the K_m value for L-arginine of kyotorphin synthetase, a specific enzyme forming kyotorphin from L-tyrosine and L-arginine, is much higher than its physiological concentration in the brain (Ueda *et al.*, 1987). L-Arginine administered systemically inhibits carrageenin-induced mechanical hyperalgesia in the rat and mouse, the effect being reversed by naloxone, naltrindole (a δ-opioid receptor antagonist), and L-leucyl-L-arginine (a kyotorphin receptor antagonist), thereby suggesting the involvement of the kyotorphin-Met-enkephalin linked system (Kawabata *et al.*, 1992a,b). Furthermore, L-arginine, when given centrally, produces potent antinociception even in intact mice, both in mechano- and thermo-nociceptive tests (Kawabata *et al.*, 1992b). Clinical studies have also demonstrated a naloxone-reversible persistent analgesia brought about by L-arginine in patients with various types of chronic pain (Takagi *et al.*, 1990; Harima *et al.*, 1991), suggesting its potential for therapeutic application.

L-Arginine is also a source of nitric oxide (NO), which was first identified as an endothelium-derived relaxing factor (For review, see Moncada *et al.*, 1988), and, most recently, as a new type of neuronal messenger with various neurotransmitter-like functions (for review, see Vincent & Hope, 1992; Bredt & Snyder, 1992). Recent studies have suggested the involvement of the L-arginine-NO pathway in nociceptive transmission and/or modulation both in the CNS and in the peripheral region, although the results reported have not necessarily been consistent. The peripheral mechanism is complex, both inhibitory and promotive roles having been reported for this pathway in nociceptive events (Meller *et al.*, 1990; Duarte *et al.*, 1990; 1992; Ferreira *et al.*, 1992; Haley *et al.*, 1992; Kawabata *et al.*, 1992a). Evidence for the spinal nociceptive action of this pathway has come from findings that spinal injections of a NO synthase (NOS) inhibitor, L-N^G-nitroarginine methyl ester (L-NAME), reduced neural activity in the dorsal horn in response to formalin injected into the peripheral receptive field (Haley *et al.*, 1992), and from findings that intrathecal L-arginine and N-methyl-D-aspartate (NMDA) produced a rapid and transient facilitation of the nociceptive tail-flick reflex, the latter effect being reversed by L-NAME and by methylene blue, a guanylate cyclase (a known NO target) inhibitor (Meller *et al.*, 1992). A histochemical study has suggested that NO may act as a messenger from sensory neurones in dorsal root ganglia to their satellite cells, where guanosine 3′:5′-cyclic monophosphate (cyclic GMP) levels increase in response to NO (Morris *et al.*, 1992). Evidence for a supraspinal role of NO in nociception is the study of Moore *et al.* (1991), which found that L-NAME, given i.c.v., dose-dependently reduced formalin-induced paw licking responses in the mouse. These observations predict that L-arginine may act as a nociceptive promoter through enhancement in NO production at spinal and supraspinal levels, being opposite to its antinociceptive action, which is exerted through the kyotorphin-enkephalin pathway.

The aim of this study was to clarify the role of L-arginine in supraspinal nociceptive processing, with special reference to the kyotorphin-Met-enkephalin and NO-cyclic GMP systems. The results described here strongly suggest that L-arginine plays a dual role in the brain, exerting antinociceptive effects via the former system and nociceptive effects via the latter, under certain conditions.

[1] Author for correspondence.

Methods

Animals

Male ddY mice, weighing 20–30 g (Japan SLC. Inc.), which were used only once, received free access to food and water for at least 1 day before experiments.

Tail-flick test

To determine thermal nociception, mice were tested for responsiveness to radiant heat, with a tail-flick analgesia meter (MK-330, Muromachi Kikai Co. Ltd., Japan). The tail of each mouse was exposed to a focused light beam, and the latency to remove the tail from the path of the stimulus was recorded automatically via a photoelectric cell. The stimulus intensity was adjusted to obtain base-line latencies of 2–2.5 s. A cut-off latency of 8 s was used to avoid damage to the tail. The tail-flick latency of each mouse was measured 4–5 times at 20-min intervals, and the basal latency was defined as the mean of the last 3 stable latencies. Results were expressed as Δ latencies, obtained by subtracting the basal latency from the latency.

Experimental procedure

Antinociceptive effects of intracerebroventricular (i.c.v.) administration of L-arginine, L-N^G-nitroarginine methyl ester, and methylene blue: L-Arginine (L-Arg), at 1–300 μg per mouse, L-N^G-nitroarginine methyl ester (L-NAME), a NO synthase (NOS) inhibitor, at 0.1–30 μg per mouse, D-N^G-nitroarginine methyl ester (D-NAME), at 10 μg per mouse, and methylene blue (MB), a soluble guanylate cyclase inhibitor, at 0.001–1 μg per mouse, were administered i.c.v. (Haley & McCormick, 1957) in a volume of 5 μl, and the tail-flick assay was conducted 5, 10, 20, 40 and 60 min later.

Effects of naloxone, naltrindole and L-leucyl-L-arginine on antinociception induced by L-arginine or L-N^G-nitroarginine methyl ester: Naltrindole (NTI), a δ-selective opioid receptor antagonist, was administered s.c., at 0.1 mg kg^{-1}, 5 min before i.c.v. injections of L-Arg (300 μg per mouse), or L-

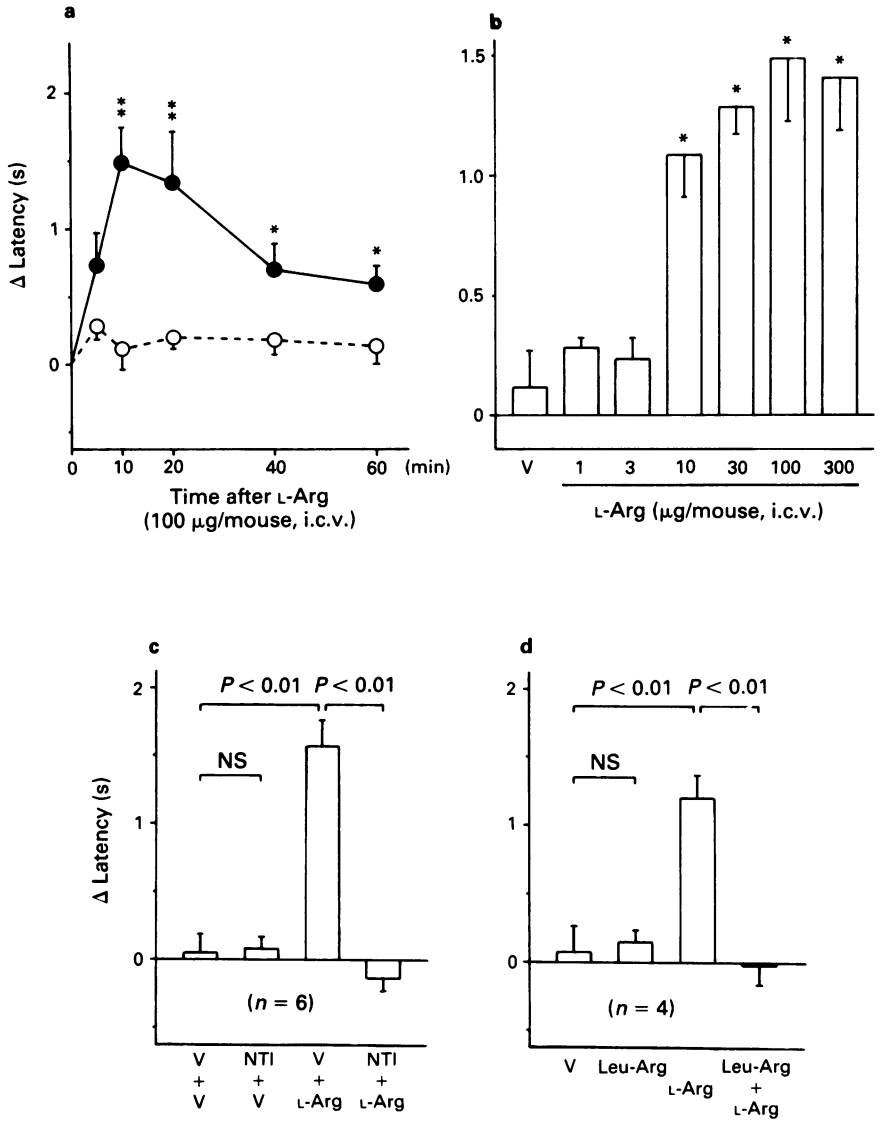


Figure 1 (a) Time-related effects of L-arginine (L-Arg, i.c.v.) on tail-flick latency in the mouse: (○) vehicle; (●) L-Arg (100 μg per mouse). $*P<0.05$, $**P<0.01$ vs vehicle $n = 6$. (b) Dose-related antinociceptive effects of L-arginine (i.c.v.) in the mouse. Data indicate Δ latencies 10 min after i.c.v. injection. $*P<0.05$ vs vehicle (V) $n = 6$. (c) and (d) Naltrindole (NTI)- (c) and L-leucyl-L-arginine (Leu-Arg) (d) antagonism of L-arginine-induced antinociception in the mouse. NTI (0.1 mg kg^{-1}) was administered s.c. 5 min before L-Arg (300 μg per mouse, i.c.v.), and Leu-Arg (3 μg per mouse) was co-administered i.c.v. with L-Arg (300 μg per mouse). Data indicate Δ latencies 10 min after i.c.v. injection. V, vehicle; NS, not significant.

NAME (10 μg per mouse). Similarly, naloxone (Nlx; 1 mg kg^{-1}) was given s.c. 5 min before L-NAME (10 μg per mouse, i.c.v.). L-Leucyl-L-arginine (Leu-Arg), a kyotorphin receptor antagonist (3 μg per mouse), was co-administered i.c.v. with L-Arg (300 μg per mouse) or L-NAME (10 μg per mouse).

Interaction between L-arginine and L-N^G-nitroarginine methyl ester: In general, the physiological actions of L-NAME are believed to be reversed by L-Arg. To examine whether L-Arg (i.c.v.), which has been reported to produce antinociception by itself (Kawabata *et al.*, 1991b), antagonizes the antinociceptive effect of L-NAME (i.c.v.), we designed the following three experiments: (A) L-Arg (30 μg per mouse) and L-NAME (10 μg per mouse) were co-administered i.c.v.; (B) L-Arg (30 μg per mouse) plus Leu-Arg (3 μg per mouse) were co-administered i.c.v. with L-NAME (10 μg per mouse); (C) NTI (0.1 mg kg^{-1}) was administered s.c. 5 min before i.c.v. co-administration of L-Arg (30 μg per mouse) and L-NAME (10 μg per mouse). In experiments (B) and (C), it was expected that the antinociceptive potency of L-Arg itself would be abolished by Leu-Arg and by NTI, respectively.

Effects of dibutyryl-cyclic GMP on antinociception induced by methylene blue or L-N^G-nitroarginine methyl ester: Dibutyryl-cyclic GMP (db-cyclic GMP; 1 μg per mouse) was injected i.c.v. in combination with MB (0.1 μg per mouse) or L-NAME (10 μg per mouse).

Drugs

Drugs used were: L-arginine hydrochloride (Nacalai Tesque, Japan), L-N^G-nitroarginine methyl ester hydrochloride, naloxone hydrochloride, naltrindole hydrochloride, L-leucyl-L-arginine acetate, dibutyryl cyclic guanosine monophosphate sodium salt (Sigma, U.S.A.), D-N^G-nitroarginine methyl ester hydrochloride (Bachem, Switzerland), and methylene blue (Wako Pure Chem., Japan). All chemicals were dissolved in saline.

Statistics

The results were expressed as means and s.e.mean, and were statistically analysed by Student's *t* test for unpaired data or by Newman-Keuls' multiple comparison test. The difference

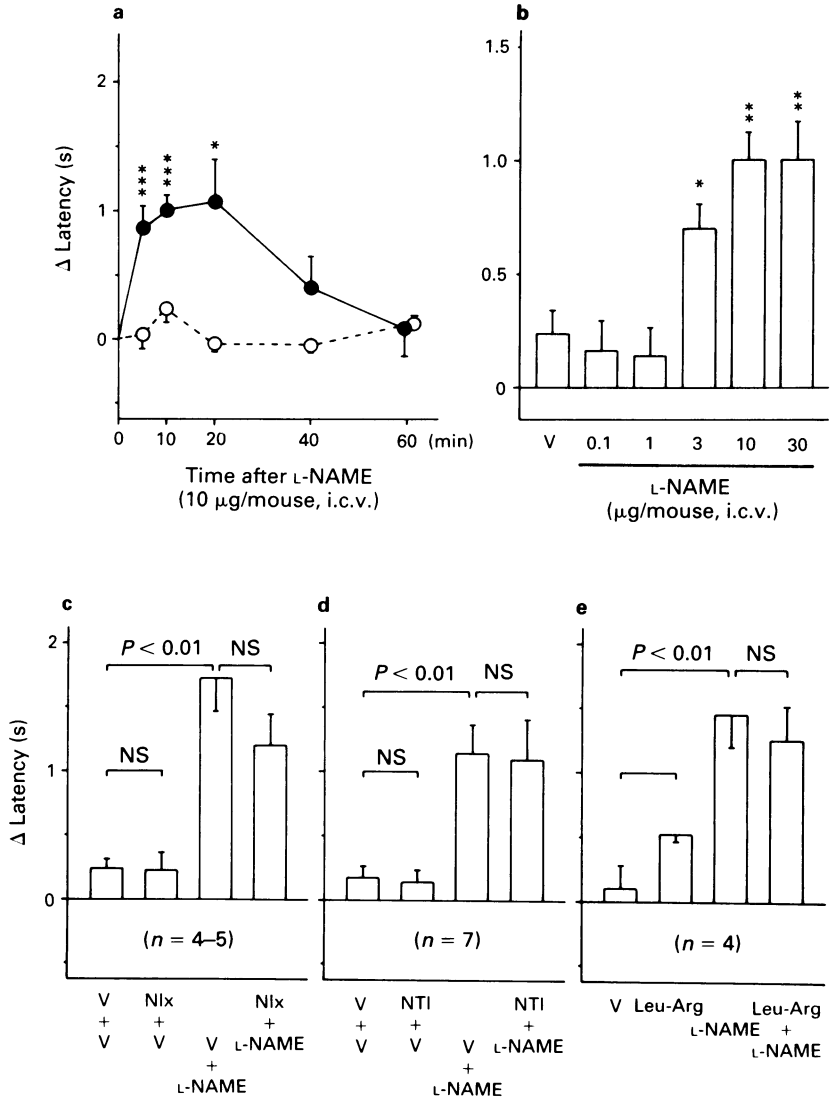


Figure 2 (a) Time-related effects of L-N^G-nitroarginine methyl ester (L-NAME, i.c.v.) on tail-flick latency in the mouse: (○) vehicle ($n = 6$); (●) L-NAME (10 μg per mouse) ($n = 5$). $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs vehicle. (b) Dose-related antinociceptive effects of L-NAME in the mouse. Data indicate Δ latencies 10 min after i.c.v. injection. $^*P<0.05$, $^{**}P<0.01$ vs vehicle (V) $n = 4$–6. (c), (d) and (e) Lack of antagonism by naloxone (Nlx) (c), naltrindole (NTI) (d), and L-leucyl-L-arginine (Leu-Arg) (e) of antinociception induced by L-NAME (i.c.v.). Nlx (1 mg kg^{-1}) and NTI (0.1 mg kg^{-1}) were administered s.c. 5 min before L-NAME (10 μg per mouse, i.c.v.), and Leu-Arg (3 μg per mouse) was co-administered i.c.v. with L-NAME (10 μg per mouse). Data indicate Δ latencies 10 min after i.c.v. injection. V, vehicle; NS, not significant.

between groups was considered statistically significant when $P<0.05$.

Results

Antinociception induced by centrally-administered L-arginine

Intracerebroventricular administration of L-Arg (100 μg per mouse) gradually prolonged the tail-flick latency in mice, which reached a maximum at 10 min, without producing any sedative behaviour. Thereafter, the prolonged latency slowly recovered, but the difference in latency from baseline still remained significant 60 min after i.c.v. injection (Figure 1a). L-Arg, at a dose-range of 10–100 μg per mouse, produced significant antinociception, at a peak time of 10 min after i.c.v. injection, in a dose-dependent manner, although at the maximum dose (300 μg per mouse) employed, it failed to elicit additional effects (Figure 1b).

NTI, when pre-administered s.c. at a dose of 0.1 mg kg^{-1}, completely antagonized the antinociception induced by i.c.v. L-Arg (300 μg per mouse), although it alone did not affect the latency (Figure 1c). Similarly, when Leu-Arg, at 3 μg per mouse, was co-administered i.c.v. with L-Arg (300 μg per mouse), L-Arg failed to elicit any antinociception, although Leu-Arg itself was without significant effect on the latency (Figure 1d).

Antinociceptive effects of i.c.v. administration of L-N^G-nitroarginine methyl ester

L-NAME, given i.c.v. at 10 μg per mouse, caused a significant prolongation of the tail-flick latency from 5 min after administration, without producing any sedative behaviour; this prolongation was maintained until 20 min, when it was followed by recovery (Figure 2a). The antinociceptive effects of L-NAME were dose-dependent, at a dose-range of 3–10 μg per mouse, 10 min after administration, although this agent produced no effect at 0.1–1 μg per mouse. The maximum dose of L-NAME (30 μg per mouse) did not elicit further effects (Figure 2b). These results were obtained from May to June, whereas the results from experiments performed in December indicated much stronger effects of L-NAME; for instance, mice treated with L-NAME (i.c.v.), even at 3 μg per mouse, showed mean Δ latency of 2.66 s ($n = 5$). These facts suggest the presence of seasonal fluctuation in the effect of L-NAME, although all experiments in the present study were carried out in a temperature controlled room (22–24°C).

The antinociception induced by L-NAME (10 μg per mouse; i.c.v.) was resistant to s.c. preadministration of Nlx (1 mg kg^{-1}), and NTI (0.1 mg kg^{-1}) (Figure 2c,d). Similarly, L-NAME-induced antinociception was not blocked by Leu-Arg (3 μg per mouse), co-administered i.c.v. with L-NAME at 10 μg per mouse (Figure 2e).

By contrast, D-NAME given i.c.v., at 10 μg per mouse, failed to produce significant antinociception; Δ latencies at 10 min after administration in groups treated with vehicle and D-NAME were 0.11 ± 0.12 and 0.17 ± 0.11 s (not significantly different from vehicle), respectively.

Interaction between L-arginine and L-N^G-nitroarginine methyl ester in the brain

L-Arg (30 μg per mouse; i.c.v.)-induced antinociception was not influenced by co-administration of an antinociceptive dose (10 μg per mouse) of L-NAME. In other words, L-NAME-induced antinociception was not changed by co-administration of an antinociceptive dose of L-Arg (Figure 3a).

Combined i.c.v. administration of L-Arg (30 μg per mouse) and Leu-Arg (3 μg per mouse) had no significant effects on the tail-flick latency (Figure 3b), corresponding to the results in Figure 1d. L-NAME (10 μg per mouse)-induced antinociception was abolished when L-NAME was given i.c.v. (10 μg per mouse) in combination with L-Arg (30 μg per mouse) plus Leu-Arg (3 μg per mouse) (Figure 3b). In other words, L-Arg, when its antinociceptive potency was blocked by Leu-Arg, inhibited L-NAME-induced antinociception.

Similarly, L-Arg (30 μg per mouse; i.c.v.), when its antinociceptive potency was blocked by pretreatment with NTI (0.1 mg kg^{-1}, s.c.), inhibited the antinociception induced by L-NAME (10 μg per mouse; i.c.v.) (Figure 3c).

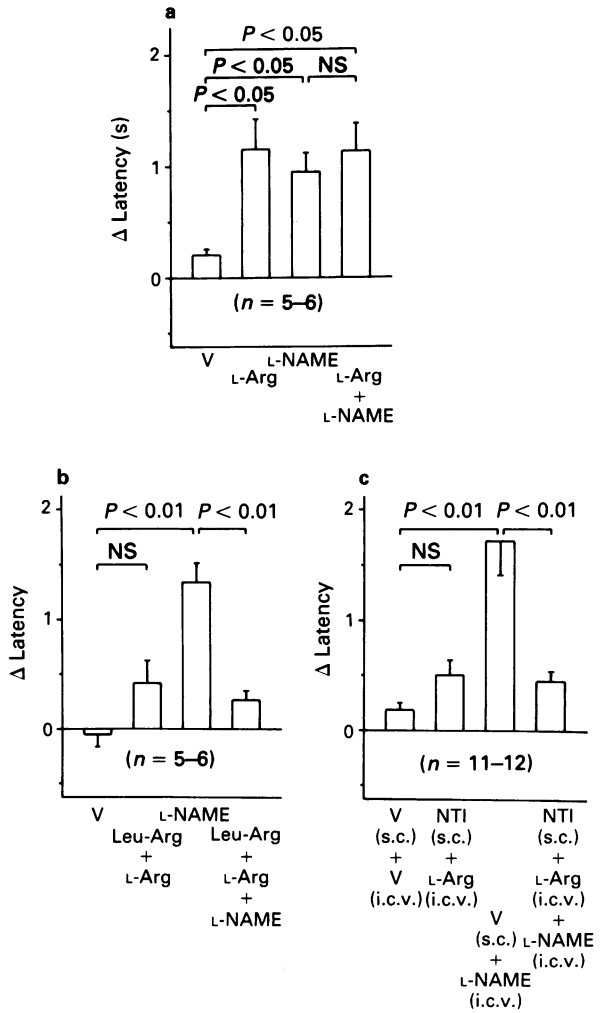


Figure 3 (a) Lack of L-arginine (L-Arg, i.c.v.) antagonism of antinociception induced by L-N^G-nitroarginine methyl ester (L-NAME, i.c.v.). L-NAME (10 μg per mouse) and L-Arg (30 μg per mouse) were co-administered i.c.v. Data indicate Δ latencies 10 min after i.c.v. injection. V, vehicle; NS, not significant. (b) and (c) Antagonism of L-NAME (i.c.v.)-induced antinociception by L-arginine in combination with L-leucyl-L-arginine (Leu-Arg) (b) or naltrindole (NTI) (c) in the mouse. L-Arg (30 μg per mouse) plus Leu-Arg (3 μg per mouse) was co-administered i.c.v. with L-NAME (10 μg per mouse), and NTI (0.1 mg kg^{-1}) was administered s.c. 5 min before combined i.c.v. administration of L-Arg (30 μg per mouse) and L-NAME (10 μg per mouse). Data indicate Δ latencies 10 min after i.c.v. injection. V, vehicle; NS, not significant.

Antinociceptive activity of methylene blue and reversibility by dibutyryl-cyclic GMP

Intracerebroventricular administration of MB, at 1 μg per mouse, caused a prolongation of the tail-flick latency, which peaked 10 min after i.c.v. injection, and remained prolonged thereafter for at least 60 min (Figure 4a). The antinociceptive

effects of MB at peak time (10 min after administration) were dose-dependent at a dose-range of 0.001–1 μg per mouse (Figure 4b).

The MB (0.1 μg per mouse; i.c.v.)-induced prolongation of tail-flick latency was reversed by co-administered db-cyclic GMP (1 μg per mouse), which exerted no effect by itself (Figure 4c). Similarly, db-cyclic GMP (1 μg per mouse), when administered i.c.v. in combination with L-NAME (10 μg per mouse), significantly attenuated the L-NAME-induced antinociceptive activity (Figure 4d).

Discussion

The present study indicates that L-Arg (i.c.v.)-induced antinociception in mice is mediated by kyotorphin and δ-opioid receptors, and that L-NAME (i.c.v.) elicits antinociception by inhibiting the NO-cyclic GMP pathway.

On the basis of results from experiments in hyperalgesic mice, using the paw pressure test (a mechano-nociceptive test), we have previously postulated that L-Arg, given exogenously (s.c.), facilitates the synthesis of kyotorphin (L-tyrosyl-L-arginine) in kyotorphin neurones by acting as an effective precursor, and that kyotorphin released in response to nociceptive stimuli enhances Met-enkephalin release, which is then followed by the activation of δ-receptor, and, in part, μ-receptor, resulting in antinociception (Kawabata *et al.*, 1992b). The antagonism of L-Arg (i.c.v.)-induced antinociception exhibited by NTI and by Leu-Arg in intact mice, as assessed by the tail-flick test (a thermo-nociceptive assay), further supports a modulatory role of the kyotorphin-Met-enkephalin pathway in the brain in nociceptive processing.

The finding that L-NAME (i.c.v.) produced significant antinociception in the tail-flick test – antinociception which was resistant to Nlx (s.c.), NTI (s.c.) and Leu-Arg (i.c.v.) – is

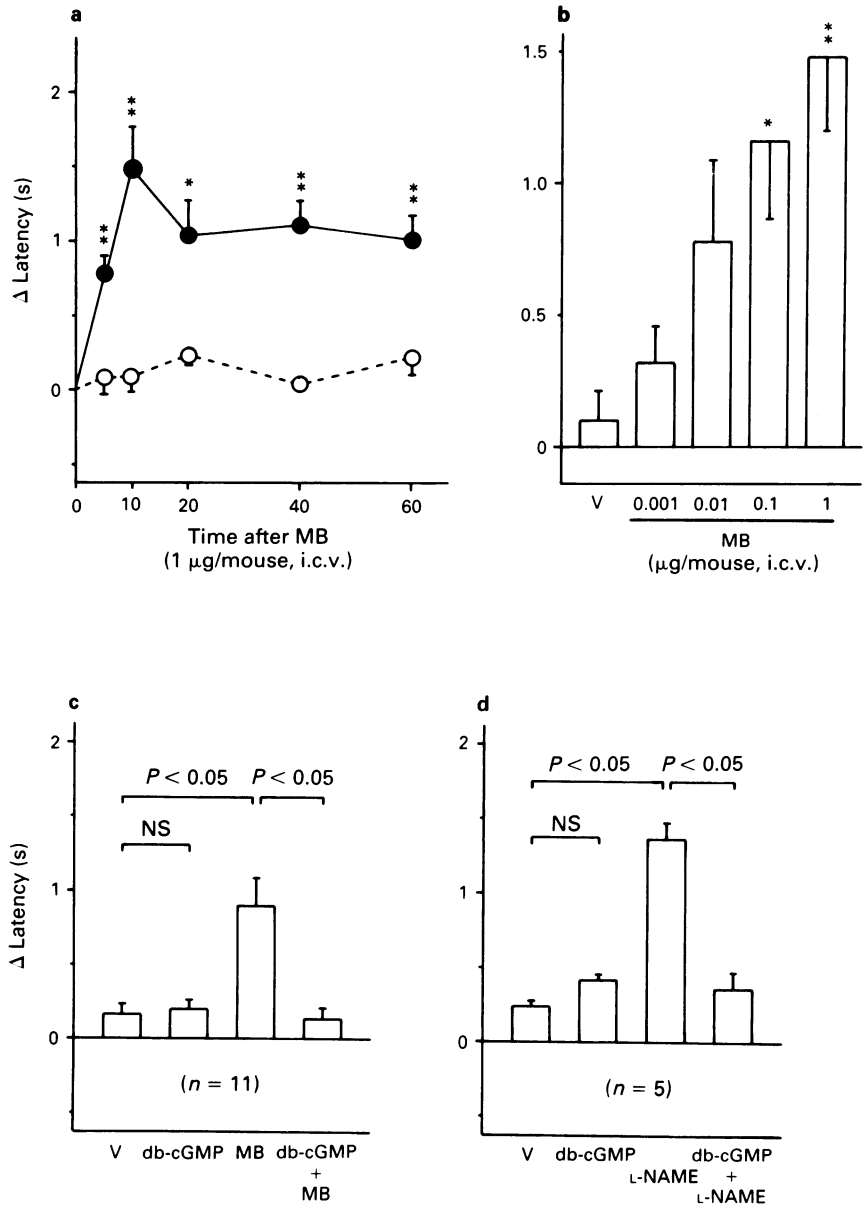


Figure 4 (a) Time-related effects of methylene blue (MB, i.c.v.) on tail-flick latency in the mouse: (○) vehicle; (●) MB (1 μg per mouse). $*P<0.05$, $**P<0.01$ vs vehicle $n = 5$. (b) Dose-related antinociceptive effects of methylene blue (i.c.v.) in the mouse. Data indicate Δ latencies 10 min after i.c.v. injection. $*P<0.05$, $**P<0.01$ vs vehicle (V) $n = 5$. (c) and (d) Dibutyryl cyclic GMP (db cGMP, i.c.v.) antagonism of antinociception induced by i.c.v. methylene blue (c) and by i.c.v. L-N^G-nitroarginine methyl ester (L-NAME) (d). MB (0.1 μg per mouse) or L-NAME (10 μg per mouse) was co-administered i.c.v. with db-cGMP (1 μg per mouse). Data indicate Δ latencies 10 min after i.c.v. injection. V, vehicle; NS, not significant.

consistent with the report of Moore et al. (1981) that systemic L-NAME exhibits an opioid-independent and potent antinociceptive activity in the formalin-induced nociceptive test, hot plate test, and acetic acid-induced writhing test, most probably by a central effect.

To our knowledge, although its role in spinal nociceptive mechanisms has already been shown (Meller et al., 1992; Morris et al., 1992), this study is the first to demonstrate the involvement of soluble guanylate cyclase in nociceptive processing at the supraspinal level. That MB (i.c.v.) produced dose-dependent potent antinociception which was reversed by a low dose (1 μg per mouse) of db-cyclic GMP (i.c.v.), suggests that cyclic GMP production by soluble guanylate cyclase is involved in physiological nociceptive processing at the supraspinal level. Furthermore, the finding that L-NAME (i.c.v.)-induced antinociception was abolished by db-cyclic GMP (i.c.v.) implies the functional coupling of endogenous NO and soluble guanylate cyclase. Although db-cyclic GMP (i.c.v.) at extremely high doses, above 50 μg per mouse, has been reported to prolong the tail-flick latency in the mouse (Vocci et al., 1978), its physiological significance is questionable. NO has also been shown to act on an adenosine diphosphate-ribosyltransferase (Brune & Lapetina, 1989; Dimmeler & Brune, 1991). This enzyme is present in the brain, but its functional role is unknown. At present, therefore, it appears that the L-Arg-NO-cyclic GMP pathway in the brain, like that in the spinal cord, participates in nociceptive processing.

At least two distinct types of NOS have been identified: a constitutive Ca^{2+}-calmodulin-dependent enzyme which is present in the brain and vascular endothelium (Moncada et al., 1989; Palmer & Moncada, 1989; Bredt & Snyder, 1990; Bredt et al., 1991), and an inducible Ca^{2+}-independent enzyme that can be expressed by macrophages (Kwon et al., 1990; Yui et al., 1991). Constitutive NOS purified from rat cerebella has high affinity for L-Arg, with a K_m value of 1.5 μM (Bredt & Snyder, 1990), implying that brain NOS may be saturated by L-Arg under physiological conditions. On the other hand, the K_m value (926 μM) of kyotorphin synthetase for L-Arg has been found to be much higher than its physiological concentration (100 μM) in the brain (Ueda et al., 1987). Therefore, L-Arg given exogenously may activate kyotorphin synthesis, but not NO synthesis. This notion would explain the finding that L-Arg (i.c.v.) produced antinociception, but did not elicit any facilitation of pain responses (tail-flick responses), even when kyotorphin-Met-enkephalin pathway was blocked by NTI or by Leu-Arg (see Figure 1c,d).

Most physiological actions of L-NAME are believed to be reversed by L-Arg (Bohme et al., 1991; Dawson et al., 1991). Moore et al. (1991) have reported that, in the formalin-induced nociceptive test, systemic L-NAME produces antinociception, i.e. suppression of both first- and second-phase responses, which is blocked by systemic L-Arg, although L-Arg, by itself, produces small but significant second-phase antinociception. The mechanism responsible for the L-Arg-induced second-phase antinociception remains to be elucidated. The antagonism by systemic L-Arg of systemic L-NAME-induced antinociception does not suggest their interaction exclusively within the brain, since L-NAME also exhibits antinociceptive activity via peripheral mechanisms (Haley et al., 1992). The antinociception induced by spinal administration of L-NAME has been found to be resistant to the co-administration of L-Arg, which is antinociceptive by itself (Haley et al., 1992), these results being similar to our present findings. In experiments with L-Arg in combination with Leu-Arg or NTI, however, L-NAME (i.c.v.)-induced antinociception was reversed by L-Arg (i.c.v.), which itself was incapable of producing antinociception due to the inhibition of the kyotorphin-Met-enkephalin pathway brought about by co-administered Leu-Arg or preadministered NTI. This finding suggests that L-Arg, without Leu-Arg or NTI, antagonizes L-NAME-induced antinociception, but produces antinociception by itself via the kyotorphin-Met-enkephalin pathway, apparently resulting in neither antagonism nor synergism. In other words, L-Arg may play a nociceptive role under conditions where NOS activity is inhibited by L-NAME and either kyotorphin or δ-receptors are blocked. The discovery of kyotorphin synthetase inhibitors will dramatically demonstrate this proposed possibility.

In conclusion, L-Arg plays a dual role in nociceptive processing in the brain, i.e. it is antinociceptive via the kyotorphin-Met-enkephalin pathway, but nociceptive via the NO-cyclic GMP pathway under certain conditions. Based

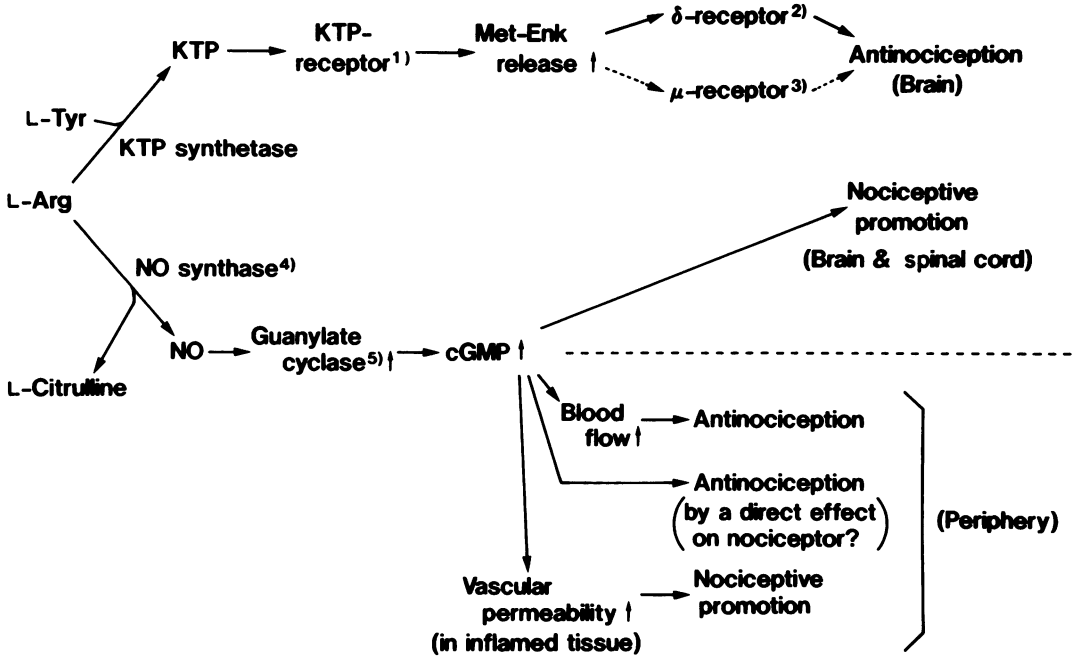


Figure 5 Possible mechanisms for L-arginine nociceptive modulation in the CNS and periphery, and points of action of the inhibitors and blockers employed. KTP, kyotorphin; Met-Enk, methionine-enkephalin. Points of action of inhibitors and blockers used: (1) L-leucyl-L-arginine; (2) naltrindole and naloxone; (3) naloxone; (4) L-N^G-nitroarginine methyl ester; (5) methylene blue.

upon the above evidence and recent reports regarding the spinal and peripheral mechanism of the L-Arg-NO-cyclic GMP pathway (Duarte *et al.*, 1990; 1992; Meller *et al.*, 1990; 1992; Ferreira *et al.*, 1992; Haley *et al.*, 1992; Ialenti *et al.*, 1992; Kawabata *et al.*, 1992a; Morris *et al.*, 1992), we show a hypothetical scheme that explains the role of L-Arg in nociceptive events in the CNS and periphery, and that indicates the points of action of the inhibitors and blockers used (Figure 5).

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Investigation of the subtypes of α_1-adrenoceptor mediating contractions of rat aorta, vas deferens and spleen

R. Aboud, M. Shafii & [1]J.R. Docherty

Department of Physiology, Royal College of Surgeons in Ireland, 123 St. Stephen's Green, Dublin 2, Ireland

1 The subtypes of α_1-adrenoceptor mediating contractions to exogenous noradrenaline (NA) or phenylephrine in rat vas deferens, spleen and aorta, and mediating contractions to endogenous NA in rat vas deferens have been examined.

2 In rat vas deferens, the competitive antagonists prazosin, WB 4101, benoxathian and 5-methyl-urapidil inhibited contractions to NA with pA_2 values of 9.26, 9.54, 9.02 and 8.43, respectively. The irreversible antagonist chloroethylclonidine (CEC) (100 μM) failed to affect contractions to NA.

3 In rat vas deferens in the presence of nifedipine (10 μM), contractions to NA were significantly attenuated and under these conditions, CEC (100 μM) significantly reduced the maximum response to NA.

4 In rat spleen, the competitive antagonists prazosin, WB 4101 and benoxathian inhibited contractions to phenylephrine with pA_2 values of 9.56, 8.85 and 7.60, respectively, and 5-methyl-urapidil had a K_B of 6.62. CEC (100 μM) significantly reduced the maximum contraction to phenylephrine.

5 In rat aorta, the competitive antagonists, prazosin, WB 4101, benoxathian and 5-methyl-urapidil inhibited contractions to NA with pA_2 values of 9.45, 9.21, 8.55 and 8.12, respectively. CEC (100 μM) produced an approximately parallel shift in the potency of NA, without significantly reducing the maximum response.

6 In epididymal portions of rat vas deferens in the presence of nifedipine (10 μM), the isometric contraction to a single electrical pulse was significantly reduced by CEC (100 μM), and by the competitive antagonists prazosin, WB 4101, benoxathian and 5-methyl-urapidil at concentrations of 1 nM.

7 In prostatic portions of rat vas deferens, the α_1-adrenoceptor agonist, amidephrine, produced concentration-dependent increases in the isometric contraction to a single electrical stimulus and the maximum increase in the evoked response produced by amidephrine was unaffected by CEC (100 μM).

8 Contractions of rat vas deferens produced by NA (and amidephrine) are mediated predominantly by α_{1A}-adrenoceptors as shown by the high potency of α_{1A}-adrenoceptor selective antagonists and the lack of effect of CEC. A small CEC-sensitive response, particularly in epididymal portions, was revealed in the presence of nifedipine. Contractions of rat spleen are mediated by α_{1B}-adrenoceptors since α_{1A}-selective antagonists showed low potency and CEC significantly reduced the maximum contraction to phenylephrine. Contractions of rat aorta to NA are mediated by non-α_{1A}, non-α_{1B}-adrenoceptors, due to the high potency of the α_{1A}-selective antagonists and sensitivity to CEC.

9 The noradrenergic contraction of epididymal portions of rat vas deferens in the presence of nifedipine is CEC-sensitive, but the α_{1A}-selective antagonists showed high potency, suggesting that this response is mediated by non-α_{1A}, non-α_{1B}-adrenoceptors.

10 In conclusion, at least three subtypes of functional α_1-adrenoceptors have been demonstrated in these studies.

Keywords: Rat vas deferens; rat spleen; rat aorta; α_{1A}-adrenoceptors; α_{1B}-adrenoceptors; prazosin; WB 4101; benoxathian; 5-methyl-urapidil

Introduction

α_1-Adrenoceptors have been subdivided into α_{1A}- and α_{1B}-adrenoceptor subtypes based on affinities of a series of ligands for binding sites in rat cortex, hippocampus, vas deferens and spleen (Morrow & Creese, 1986; Han *et al.*,1987; Gross *et al.*,1988; see Docherty, 1989), and based on the ability of the alkylating agent chloroethylclonidine (CEC) to inactivate the α_{1B} but not the α_{1A}-subtype. Based on this classification, it has been suggested that contractions of rat vas deferens to exogenous noradrenaline (NA) or adrenaline are mediated predominantly by α_{1A}-adrenoceptors (Han *et al.*, 1987; Hanft & Gross, 1989), whereas contractions to NA in rat spleen are mediated predominantly by α_{1B}-adrenoceptors (Han *et al.*, 1987).

Molecular cloning techniques have revealed the existence of at least 3 genes coding for α_1-adrenoceptors, α_{1A} (rat: Lomasney *et al.*, 1991), α_{1B} (hamster: Cotecchia *et al.*, 1988; rat: Voigt *et al.*, 1990), α_{1C} (bovine: Schwinn *et al.*, 1990), although controversy exists as to whether the clone termed α_{1A} is indeed an α_{1A} or a novel α_{1D}-subtype (rat: Perez *et al.*, 1992).

However, although contractions of the rat spleen have been used as a model for α_{1B}-adrenoceptors and contractions of the rat vas deferens to NA have been used as a model for α_{1A}-adrenoceptors, there is evidence for the presence of an additional subtype of α_1-adrenoceptor involved in contractions to nerve stimulation in rat vas deferens. Mallard *et al.* (1992) have demonstrated that the α_1-adrenoceptor response to a single stimulus is sensitive to the alkylating agent CEC, and these authors have suggested that an α_{1B}-adrenoceptor mediates this response.

The subtype of α_1-adrenoceptor present in the rat aorta has been variously classified as α_{1B} (Han *et al.*, 1990) both α_{1A} and α_{1B} (Tian *et al.*, 1990; Piascik *et al.*, 1991) or atypical (Muramatsu *et al.*, 1991; Aboud & Docherty, 1992; Oriowo & Ruffolo, 1992). In the latter four studies, CEC failed to

[1] Author for correspondence.

reduce the maximum response to NA, but caused a parallel shift in the concentration-response curve.

The objects of this study were to re-examine the α_1-adrenoceptor subtypes present in rat aorta, spleen and vas deferens in the light of current knowledge of α_1-adrenoceptor subtypes.

Some of these results have been published in abstract form (Aboud & Docherty, 1992) or in tabular form in a review article (Docherty, 1989).

Methods

Male Wistar rats (200–300 g) were obtained from Trinity College Dublin, and aorta, spleen and vas deferens were employed as outlined below.

Rat aorta

Aortic rings of 3–5 mm in length were gently rubbed to remove the endothelium, and attached to myograph transducers under 1 g tension in organ baths at 37°C in Krebs-Henseleit solution of the following composition: (mM): NaCl 119, $NaHCO_3$ 25, D-glucose 11.1, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.0, EDTA 0.03, ascorbic acid 0.28. Additionally, cocaine (3 μM), propranolol (3 μM) and indomethacin (10 μM) were present.

Tissues were contracted with NA (10 μM), exposed to acetylcholine (ACh, 10 μM) to test for absence of endothelium-dependent relaxations (EDR), and washed. Bathing fluid was then changed every 15 min for the next hour. Tissues were then contracted with NA administered cumulatively in 0.5 log unit increments beginning with 1 nM. Once a maximum response to NA had been obtained, tissues were washed and bathing fluid was changed every 15 min for 120 min, for the last 60 min of which tissues were incubated with various concentrations of competitive antagonists (prazosin, WB 4101, 5-methyl-urapidil or benoxathian) or vehicle (one tissue from each animal received vehicle). Tissues were then contracted with NA in the continuing presence of antagonist or vehicle. Antagonist potency was expressed as the dissociation constant K_B from the equation $K_B = [B]/(DR-1)$, where [B] is the concentration of antagonist and DR is the agonist dose-ratio produced by the antagonist, or as a pA_2 value. Antagonist pA_2 values were obtained from the x-intercept of the plot of log (agonist DR − 1) against log antagonist concentration, where the slope was not significantly different from negative unity (Arunlakshana & Schild, 1959).

In another series of experiments, tissues were incubated with CEC (10 or 100 μM) for 30 min, beginning 60 min after wash-out of the first NA concentration-response curve. After washing for another 45 min, a concentration-response curve to NA was repeated.

Rat spleen

Spleens were bisected transversely into two portions, and experiments were carried out as for the rat aorta except that phenylephrine was used as the contractile agent (except for a few experiments employing NA, as outlined in the results section).

Rat vas deferens

Whole vas deferens, or prostatic or epididymal portions were obtained.

In investigations of contractions produced by exogenous agonists, experiments were carried out as described for rat aorta, employing NA as agonist, except for the following differences. Tissues were pre-exposed to NA (10 μM), and following 60 min exposure to antagonist or vehicle, a single agonist concentration-response curve was obtained per tissue. In experiments carried out in the presence of nifedipine (10 μM), nifedipine was added during the 45 min washout after a 30 min exposure to CEC (100 μM).

In investigations of contractions produced by nerve stimulation, cocaine was omitted from the Krebs-Henseleit solution and tissues were placed between platinum electrodes and stimulated every 5 min with a single stimulus (0.5 ms pulses, supramaximal pulses) to produce isometric contractions. In investigations of the ability of the α_1-adrenoceptor agonist, amidephrine, to increase the isometric twitch response of prostatic portions, tissues were stimulated with a single electrical pulse every 5 min. When consistant control twitches had been obtained in tissues pre-exposed to CEC (100 μM) or vehicle for 30 min followed by 45 min wash, amidephrine was added in 0.5 log unit increments and a stimulus was obtained after 5 min exposure to each amidephrine dose.

In experiments investigating the ability of competitive antagonists to inhibit the isometric twitch in epididymal portions, nifedipine (10 μM) was present to block the non-noradrenergic component of the twitch, and antagonists were added in three cumulative concentrations at 15 min intervals. An isometric twitch was obtained following 15 min exposure to each antagonist concentration, or following exposure to the vehicle.

Drugs

The following drugs were used: acetylcholine chloride (Sigma, Poole, U.K.): amidephrine hydrochloride (gift: Bristol-Myers Squibb, Wallingford, CT, U.S.A.); benoxathian hydrochloride (Research Biochemicals, Natick, MA, U.S.A.); chloroethylchlonidine (Research Biochemicals); cocaine hydrochloride (Sigma); corticosterone (Sigma); 5-methyl-urapidil (gift: Byk Guilden, Konstanz); nifedipine (Sigma); noradrenaline bitartrate (Sigma); phenylephrine hydrochloride (Sigma); prazosin hydrochloride (gift; Pfizer, Sandwich, U.K.); propranolol hydrochloride (Sigma); WB 4101 (2-(2,6-dimethoxyphenoxyethyl) aminomethyl-1,4-benzodioxane hydrochloride: Research Biochemicals).

Drugs were dissolved in distilled water, except for corticosterone (100% ethanol).

Statistics

Values are arithmetic mean ± s.e.mean, or geometric mean and 95% confidence limits. The 95% confidence limits were calculated from the standard deviation, except in the case of pA_2 values (95% limits of the intercept of Schild plot). Antagonist EC_{50} K_B or pA_2 values were compared between tissues, and were compared with the effects of vehicle by Student's t test for unpaired or paired data, where appropriate, and by ANOVA analysis. Slopes and elevation of Schild regressions were compared by covariance analysis (see Snedecor & Cochran, 1980).

Results

Rat aorta

NA produced isometric contractions with a pD_2 of 7.50 ± 0.15 (mean and 95% confidence limits, −log M) and a maximum contraction of 1.02 ± 0.18 g ($n = 15$). Prazosin, WB 4101, benoxathian and 5-methyl-urapidil produced concentration-dependent shifts in the potency of NA without reducing the maximum response. Schild plots were constructed from the effects of a range of concentrations of each antagonist, and since the slope of these regressions did not differ significantly from negative unity, pA_2 values were calculated (see Figure 1 and Table 1).

The irreversible antagonist CEC (10 and 100 μM) failed to reduce significantly the maximum response to NA but produced parallel shifts in the potency of NA (Figure 2). The

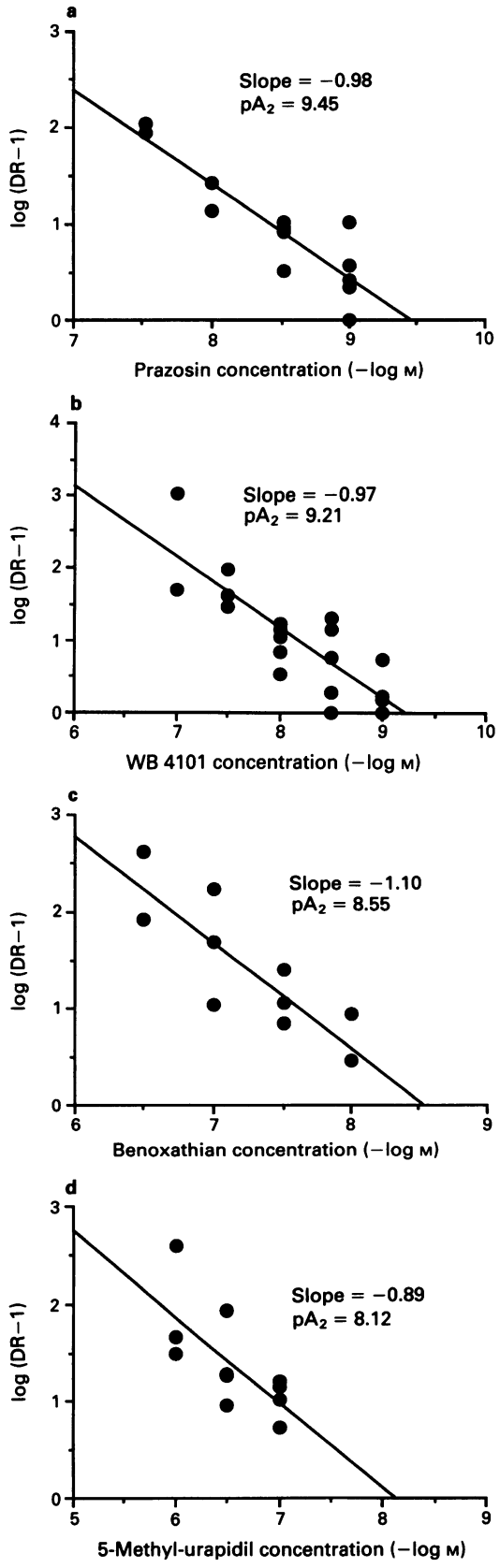


Figure 1 pA_2 values obtained for the competitive antagonists prazosin (a), WB 4101 (b), benoxathian (c) and 5-methyl-urapidil (d) against contractions to noradrenaline in rat aorta. Each point represents an individual experiment. Full details of pA_2 values are shown in Table 1.

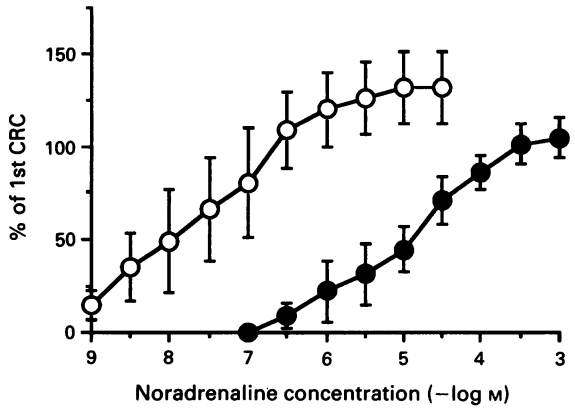


Figure 2 Effects of chloroethylclonidine (CEC, 100 μM) or vehicle on contractions to noradrenaline in rat aorta. Responses in the presence of CEC (●) or vehicle (○) are expressed as a percentage of the maximum response obtained in the first (control) concentration-response curve. Vertical bars represent s.e. of mean from at least 4 experiments.

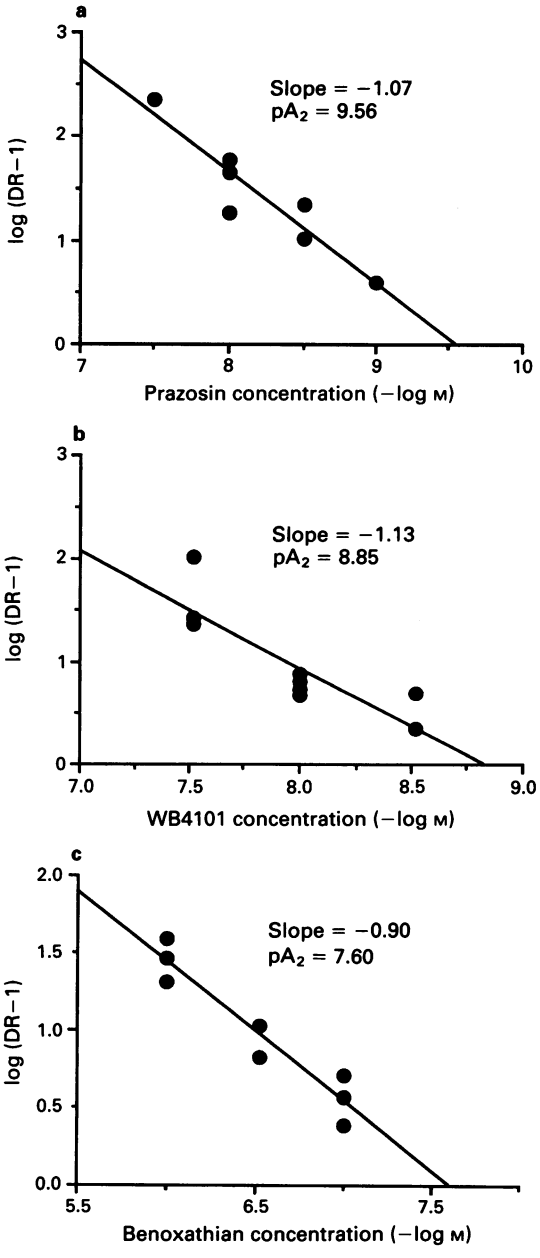


Figure 3 pA_2 values obtained for the competitive antagonists prazosin (a), WB 4101 (b) and benoxathian (c) against contractions to phenylephrine in rat spleen. Each point represents an individual experiment. Full details of pA_2 values are shown in Table 1.

Table 1 Potencies of antagonists against contractions to noradrenaline (NA) in rat vas deferens and aorta, and to phenylephrine in rat spleen

	Prazosin	*WB 4101*	*Benox*	*5-MU*	*CEC*
Vas deferens	9.26	9.54	9.02	8.43	no effect
(α_{1A})	(8.17–11.88)	(8.82–11.70)	(8.35–9.97)	(7.74–9.32)	
Spleen	9.56	8.85	7.60	$6.62 \pm 0.50^{+}$	effective
(α_{1B})	(9.02–10.49)	(8.21–10.13)	(7.27–8.05)		
Aorta	9.45	9.21	8.55	8.12	effective
(non-α_{1A}/α_{1B})	(8.90–10.12)	(8.44–10.16)	(7.86–9.58)	(7.20–10.99)	

Potencies are expressed as pA_2 values (and 95% confidence limits) from the Schild plots shown in Figures 1, 3 and 6, or as a K_B ($\pm$ 95% confidence limits) from the effects of a fixed concentration of antagonist (5-methyl-urapidil in rat spleen). The effects of chloroethylclonidine (CEC, 100 μM) on agonist responses are also listed.
Abbreviations: Benox., benoxathian; 5-MU, 5-methylurapidil

shifts in potency of noradrenaline produced by CEC (10 and 100 μM) were 26.1 ± 11.0 times ($n = 5$) and 464 ± 174 times ($n = 4$), respectively.

Rat spleen

NA and phenylephrine produced isometric contractions with pD_2 values of 5.80 ± 0.21 and 5.37 ± 0.40, and maximum contractions of 0.68 ± 0.10 g ($n = 6$) and 0.18 ± 0.01 g (n = 28). Prazosin, WB 4101, benoxathian and 5-methyl-urapidil produced shifts in the potency of NA and phenylephrine without reducing the maximum response. Schild plots were constructed from the effects of a range of concentrations of each antagonist, and where the slope of these regressions did not differ significantly from negative unity, pA_2 values were calculated (see Figure 3 and Table 1). However, slopes of Schild plots differed from negative unity for all antagonists against NA, so that effects against NA were not quantified. For 5-methyl-urapidil, the slope of the Schild plot against phenylephrine was also significantly different from negative unity, so that potency was expressed as a K_B from the lowest concentration (1 μM) to produce a significant shift in potency of phenylephrine.

The irreversible antagonist CEC (100 μM) significantly reduced the response to phenylephrine (1 mM) (Figure 4).

Whole vas deferens: responses to exogenous agonists

In vehicle-treated whole vas deferens, NA produced contractions consisting of intermittent spikes superimposed on a tonic contraction with a pD_2 of 6.36 ± 0.50 and a maximum contraction (measured as the maximum height of intermittent spikes) of 1.76 ± 0.20 g (mean ± s.e. of mean, $n = 5$). In tis-

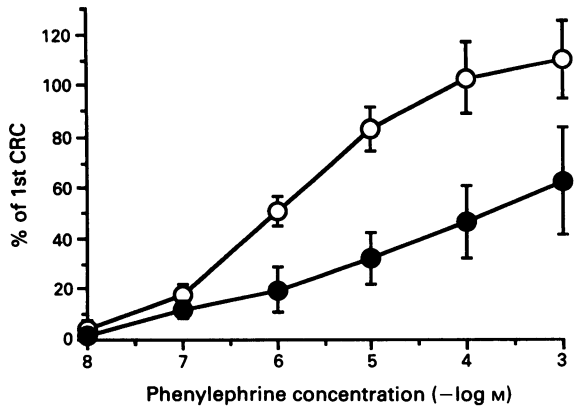


Figure 4 Effects of chloroethylclonidine (CEC, 100 μM) or vehicle on contractions to phenylephrine in rat spleen. Responses in the presence of CEC (●) or vehicle (○) are expressed as a percentage of the maximum response obtained in the first (control) concentration-response curve. Vertical bars represent s.e. of mean from at least 4 experiments.

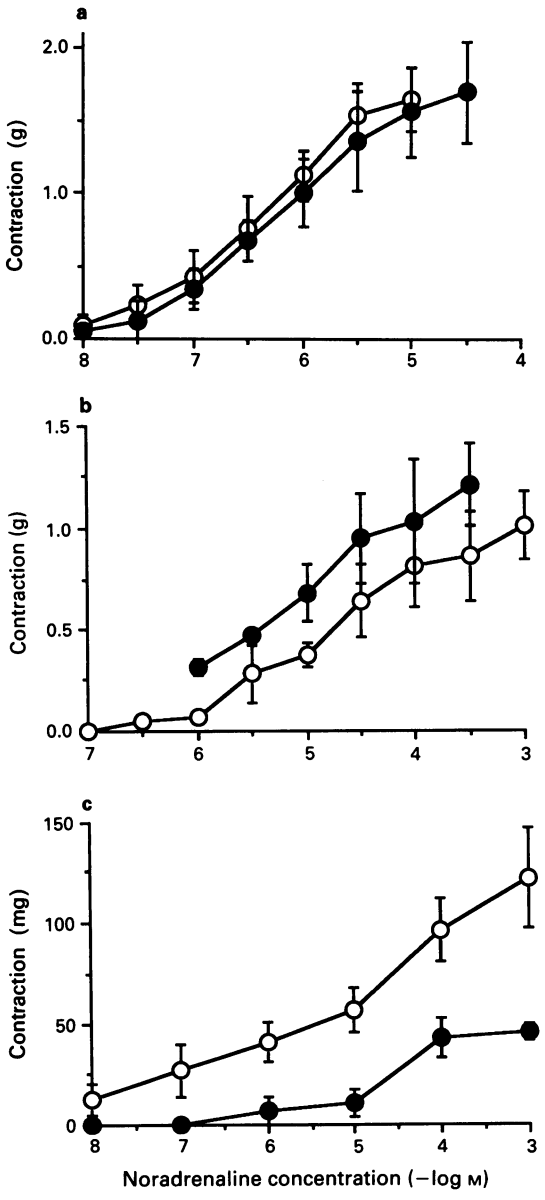


Figure 5 Effects of chloroethylclonidine (CEC) on contractions to noradrenaline (NA) in rat whole vas deferens in the presence of cocaine (a), in the absence of cocaine (b) and in the presence of cocaine and nifedipine (10 μM) (c). Responses are expressed as contraction (g or mg) to NA produced in a single concentration-response curve following exposure to CEC (10 μM) (a), CEC (100 μM) (b,c) (●) or vehicle (○). Vertical bars represent s.e. of mean from at least 4 experiments.

sues pretreated with CEC (10 μM), NA had a pD_2 of 6.38 ± 0.36, and a maximum contraction of 1.62 ± 0.28 g ($n = 5$) (no significant difference from controls) (see Figure 5a). In the absence of cocaine the potency of NA was reduced, with pD_2 values of 4.67 ± 0.94 and 4.57 ± 0.92 and a maximum contraction of 1.01 ± 0.17 g ($n = 3$) and 1.25 ± 0.20 g ($n = 3$) in vehicle-treated and CEC (100 μM)-treated tissues, respectively (no significant difference) (see Figure 5b).

Prazosin, WB 4101, benoxathian and 5-methyl-urapidil produced concentration-dependent shifts in the potency of NA, and behaved competitively in that they did not decrease the maximum contraction to NA, and slopes of Schild plots were not significantly different from negative unity, so that pA_2 values could be calculated (Figure 6).

In the presence of nifedipine (10 μM), NA no longer produced intermittent spikes but produced small tonic contractions with a maximum contraction of 0.12 ± 0.02 g ($n = 5$). In the presence of CEC (100 μM), this contraction was reduced to 0.05 ± 0.01 g ($n = 5$) (significant reduction; $P < 0.05$, Student's t test) (Figure 5c).

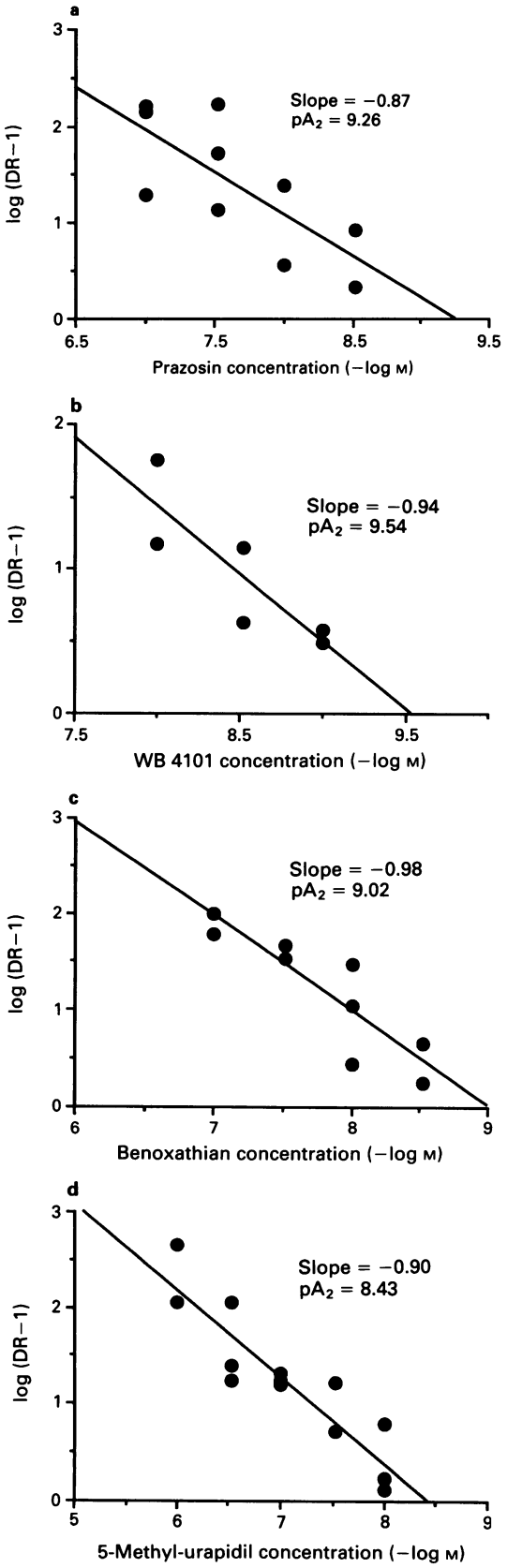


Figure 6 pA_2 values obtained for the competitive antagonists prazosin (a), WB 4101 (b), benoxathian (c) and 5-methyl-urapidil (d) against contractions to noradrenaline in rat vas deferens. Each point represents an individual experiment. Full details of pA_2 values are shown in Table 1.

Prostatic portions of rat vas deferens: responses to exogenous agonists

In prostatic portions of rat vas deferens, NA produced transient contractions with a pD_2 of 5.29 ± 0.11 and a maximum contraction of 1.19 ± 0.18 g ($n = 3$), and this contraction was virtually abolished by nifedipine (10 μM) (reduced to 0.04 ± 0.01 g, $n = 5$). Following nifedipine and CEC (100 μM), the contraction to NA was 0.03 ± 0.01 g ($n = 3$) (no significant difference).

Prostatic portions of rat vas deferens: nerve-mediated responses

In prostatic portions, single pulse electrical stimulation produced a biphasic contraction, consisting mainly of the first non-noradrenergic phase. Amidephrine produced a concentration-dependent increase in the stimulation-evoked contraction, increasing the first phase to 269.2 ± 25.4% of control ($n = 4$) with a pD_2 of 5.82 ± 0.41 in vehicle-treated animals (Figure 7).

In prostatic portions exposed to CEC 100 μM for 30 min, the small second (noradrenergic) phase of the contraction to a single stimulus was abolished and the first (nonadrenergic) phase was reduced to 44.1 ± 6.0% of the response prior to CEC ($n = 4$). Exposure to vehicle for 30 min did not significantly alter the response to a single stimulus (first phase: 102.0 ± 5.4% of response prior to vehicle, $n = 4$). Following exposure to CEC (100 μM), amidephrine produced a concentration-dependent increase in the first phase of the response to a single stimulus, with a maximum increase to 533.1 ± 100.0% of control and a pD_2 of 6.59 ± 1.08 (Figure 7).

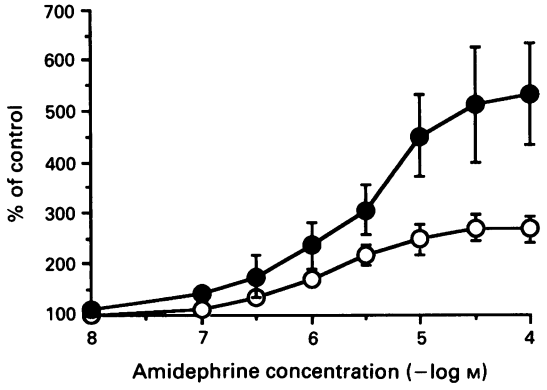


Figure 7 Effects of amidephrine on the isometric contraction produced by single pulse electrical stimulation in prostatic portions of rat vas deferens. Responses in the presence of amidephrine are expressed as a percentage of the response in the absence of amidephrine. Symbols: vehicle-treated (○), chloroethylclonidine (100 μM)-treated (●). Vertical bars represent s.e. of mean from at least 4 experiments.

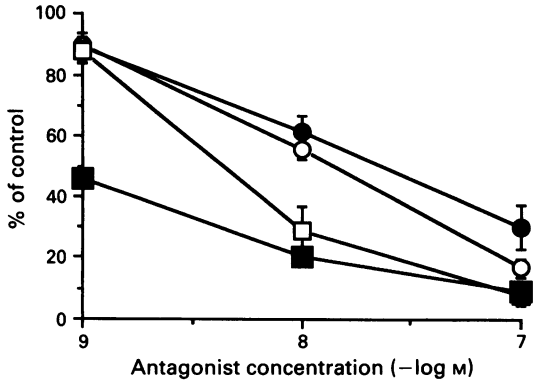


Figure 8 Effects of prazosin (●), WB 4101 (□), benoxathian (○ and 5-methyl-urapidil (■) on isometric contractions produced by single pulse electrical stimulation in epididymal portions of rat vas deferens in the presence of nifedipine (10 μM). Effects of all concentrations of all antagonists shown were significantly different from the effects of vehicle (Student's t test, $P<0.05$). Vertical bars represent s.e.mean from at least 4 experiments.

Although the maximum increase in the evoked response produced by amidephrine was much greater in tissues exposed to CEC, this can be explained entirely by the significant reduction in the evoked contraction produced by CEC. When expressed as a percentage of the initial (prior to exposure to CEC or vehicle) first phase contraction to a single electrical stimulus, amidephrine produced a maximum increase in the evoked contraction to 274.9 ± 31.0% and 226.4 ± 39.9% of control, in vehicle- and CEC-treated tissues, respectively (no significant difference). In experiments in which vehicle replaced amidephrine, there was no significant increase in the twitch response over the same time period.

Epididymal portions of rat vas deferens: nerve-mediated responses

In epididymal portions of rat vas deferens, the initial biphasic twitch to a single electrical stimulus was reduced to a monophasic response by nifedipine (10 μM), which eliminates the first (non-noradrenergic) phase, leaving the second (noradrenergic) phase. Under these conditions, the twitch to a single stimulus was 0.99 ± 0.14 g ($n = 10$), and this was reduced to 4.2 ± 3.7% of control ($n = 5$) by exposure to CEC (10 μM) for 30 min, as compared to 81.8 ± 11.8% of control ($n = 5$) when exposed to vehicle ($P<0.05$).

The competitive antagonists prazosin, benoxathian, WB 4101 and 5-methyl-urapidil produced concentration-dependent inhibitions of the isometric twitch to a single electrical stimulus, with an order of potency 5-methyl-urapidil ≥ WB4101 > benoxathian = prazosin (Figure 8). For all 4 antagonists, nerve-mediated contractions were significantly reduced by a concentration of 1 nM ($P<0.05$ from effects of vehicle).

Discussion

In this study, we have looked at subtypes of α_1-adrenoceptors mediating contractions of the rat aorta, spleen and vas deferens to exogenous agonists (NA, phenylephrine or amidephrine) and contractions of the vas deferens to endogenous NA. These will be discussed separately, beginning with contractions to exogenous agonists.

Responses to exogenous agonists

In whole vas deferens, NA produced concentration-dependent contractions which consisted of intermittent spikes superimposed on a small tonic contraction. Contractions to NA were unaffected by CEC, but potently inhibited by WB 4101, benoxathian and 5-methyl-urapidil. Insensitivity to CEC, which irreversibly inactivates α_{1B}-adrenoceptors, would suggest that the receptors involved in contractions to NA are of the α_{1A}-subtype, and the high potency of WB 4101, benoxathian and 5-methyl-urapidil would tend to confirm this identification. The pA_2 values of WB 4101 (9.54), benoxathian (9.02) and 5-methyl-urapidil (8.48) found in this study compare well with pA_2 values of 9.40, 8.75 and 8.47, respectively, obtained in functional studies of rat vas deferens (Han *et al.*, 1987; Hanft & Gross, 1989), and with ligand binding affinities for α_{1A}-sites in rat hippocampus (9.53, 9.04 and 9.07, respectively: Han *et al.*, 1987; Gross *et al.*, 1988). Since WB 4101, benoxathian and 5-methyl-urapidil have lower affinities for α_{1B}-ligand binding sites (8.57, 7.30 and 7.17, respectively: Han *et al.*, 1987; Gross *et al.*, 1988), our results would confirm the identification of the receptor mediating contractions of rat vas deferens to NA as an α_{1A}-adrenoceptor.

In the presence of the calcium entry blocker nifedipine, the intermittent spikes produced by exogenous NA in whole vas deferens were abolished, and a small tonic contraction remained. Pre-exposure to CEC significantly reduced this contraction, suggesting that the residual contraction is mediated by a non-α_{1A}-adrenoceptor. Mallard *et al.* (1992). have demonstrated that contractions to NA are resistant to CEC in rat vas deferens, but failed to find a component of the response to exogenous NA which was resistant to nifedipine, although Martinotti *et al.* (1991) found a component following calcium entry block which could be blocked by CEC. Various authors have suggested that α_{1B}-adrenoceptors mediate contractions which are resistant to calcium entry blockers, employing calcium stores (Han *et al.*, 1987). Interestingly, in prostatic portions of rat vas deferens, almost no contractile response was left following nifedipine, and what little remained was unaffected by CEC. Hence, the CEC-sensitive component of the response to NA is found mainly in the epididymal portion of the rat vas deferens, where a CEC-sensitive α-adrenoceptor-mediated response to nerve stimulation is present (present results and Mallard *et al.*, 1992), so that this action of higher concentrations of NA may be mediated by receptors in the synaptic region. Since the response to NA in the presence of nifedipine was so small, even in epididymal portions, the interaction with competitive antagonists was not examined, but the CEC-sensitive α-adrenoceptor response to nerve stimulation was examined in more detail (see below).

In rat spleen, it proved impossible to obtain competitive interactions between NA and the antagonists prazosin, WB 4101, and benoxathian. This may be due to the involvement of α_2- as well as α_1-adrenoceptors in this response (Kenakin & Novak, 1988). When phenylephrine was used as a selective α_1-adrenoceptor agonist, contractions were inhibited in a competitive manner by the above antagonists and pA_2 values from Schild plots could be obtained for all antagonists except 5-methyl-urapidil, for which a K_B was calculated. The low potencies of the competitive antagonists benoxathian, WB 4101 and 5-methyl-urapidil, and the effectiveness of CEC all indicated that the receptors involved in noradrenergic contractions of rat spleen are α_{1B}-adrenoceptors, as previously suggested (Han *et al.*, 1987).

The receptor mediating contractions of the rat aorta produced by NA resembled the α_{1A}-adrenoceptor producing contractions of rat vas deferens in that benoxathian, WB 4101 and 5-methyl-urapidil all showed high potency, but resembled the α_{1B}-adrenoceptor of rat spleen in that responses were susceptible to blockade by CEC. Interestingly, CEC did not reduce the maximum response to NA in rat aorta, but produced a parallel shift in the potency of NA. Other authors have reported that CEC causes a parallel shift in the potency of NA in rat aorta (Muramatsu *et al.*, 1991; Oriowo & Ruffolo, 1992). This may suggest either the presence of 2 receptor subtypes, 1 of which is sensitive to CEC, or that

CEC interacts in a complex way with the α_1-adrenoceptor of rat aorta. Piascik *et al.* (1990) found that CEC (100 μM) made the concentration-response curve to phenylephrine in rat aorta biphasic, indicating that CEC inactivated 1 of 2 subtypes of α_1-adrenoceptor. In our studies, the sensitivity to CEC and the high potency of the competitive antagonists suggest that this receptor is a non-α_{1A}-, non α_{1B}-receptor, and may represent a functional α_{1D}-site which is expressed in rat aorta (Perez *et al.*, 1991).

Responses to endogenous noradrenaline: rat vas deferens

In rat whole vas deferens, the electrical stimulation-evoked contraction to a single stimulus consists of a biphasic response, the first phase of which is non-adrenoceptor mediated and predominates in prostatic portions, and the second phase of which is α-adrenoceptor mediated, and predominates in the epididymal portion (see Brown *et al.*, 1983). The second α-noradrenergic phase can be examined in isolation in the epididymal portion in the presence of nifedipine which eliminates the second non-noradrenergic response. Since α_{1A}-adrenoceptors are thought to produce responses via entry of extracellular calcium, this might suggest that the nerve response is mediated by a non-α_{1A}-adrenoceptor. Indeed, CEC abolished the α-noradrenergic contraction, and this has led to the suggestion that the response is mainly α_{1B}-adrenoceptor-mediated (Mallard *et al.*, 1991). However, the high potencies of 5-methyl-urapidil, WB 4101 and benoxathian in inhibiting the twitch response are not consistent with an α_{1B}-site, at which these agents have low potency, so that the receptor mediating nerve stimulation-evoked contractions in the presence of nifedipine may be a non-α_{1A}, non-α_{1B}-site, and may belong to either the α_{1C} or α_{1D}-sites which are CEC-sensitive, but at which 5-methyl-urapidil and WB 4101 show high potency (Perez *et al.*, 1991). Since the α_{1D}-receptor is reported to be expressed in rat vas deferens (Perez *et al.*, 1991), the above receptor may represent a functional α_{1D}-adrenoceptor.

In prostatic portions, the twitch response to a single stimulus is mainly nonadrenergic, but can be increased in a concentration-dependent manner by α_1-adrenoceptor agonists. CEC significantly reduced the first (non-noradrenergic) phase of the contraction but did not affect the ability of the α_1-agonist amidephrine to increase the evoked contraction, confirming that contractions to exogenous α_1-adrenoceptor agonists, be it NA or amidephrine, are mediated predominantly by CEC-insensitive receptors, presumably α_{1A}-adrenoceptors. Furthermore, it is likely that the increase in stimulation-evoked contractions produced by amidephrine is mediated by the same population of receptors as mediate contractions to NA.

The fact that CEC (100 μM) did not affect contractions to exogenous NA but significantly reduced the non-noradrenergic contraction to single pulse electrical stimulation demonstrates that, although CEC may show high selectivity for α_{1B} over α_{1A}-adrenoceptors, it appears to have major effects at other receptors.

It is clear from this study that it is at present difficult to subclassify definitively functional α_1-adrenoceptors with the tools available. However, 3 subtypes can be identified: α_{1A} (resistant to CEC); α_{1B} (sensitive to CEC, at which 5-methyl-urapidil, WB 4101 and benoxathian show low potency), non-α_{1A}, non-α_{1B} (sensitive to CEC but at which the latter antagonists show high potency). Under this classification, contractions of the rat vas deferens to NA are mediated predominantly by α_{1A}-adrenoceptors, but in the presence of nifedipine a second CEC-sensitive component can be identified, particularly in the epididymal portion. The isometric twitch response of the epididymal portion of rat vas deferens in the presence of nifedipine, appears to be mediated by a CEC-sensitive, non-α_{1A}-, non-α_{1B}-adrenoceptor, which may also mediate the CEC-sensitive component of the response to exogenous NA. Isometric contractions of rat spleen to phenylephrine are mediated predominantly by CEC-sensitive α_{1B}-adrenoceptors. Contractions of rat aorta to NA appear to be mediated by CEC sensitive non-α_{1A}, non-α_{1B}-adrenoceptors. Hence, at least 3 subtypes of α_1-adrenoceptor can be identified functionally.

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Effects of pyrogenic immunomodulators on the release of corticotrophin-releasing factor-41 and prostaglandin E_2 from the intact rat hypothalamus *in vitro*

[1]N.G.N. Milton, C.H. Self & E.W. Hillhouse

Department of Clinical Biochemistry, University of Newcastle upon Tyne, Framlington Place, Newcastle upon Tyne, NE2 4HH

1 The actions of the following pyrogens: lipopolysaccharide (LPS), polyinosinic:polycytidylic acid (Poly-I:C), human interleukin (IL)-1α and IL-1β, human IL-6 and rat interferon (INF) on corticotrophin-releasing factor-41 (CRF-41) and prostanglandin E_2 (PGE_2) release from the intact rat hypothalamus *in vitro* have been studied.

2 Rat hypothalami were incubated *in vitro* in an artificial cerebrospinal fluid. Immunoreactive (ir)-CFR-41 and PGE_2 released into the medium were measured by two-site enzyme amplified immunometric assay (EAIA) and radioimmunoassay (RIA) respectively.

3 Human IL-6 (1 to 10,000 iu ml^{-1}) caused a dose-dependent release of irCRF-41, rising to a maximal 3–4 fold increase over basal at the highest dose tested. Human IL-1α (1 to 1000 iu ml^{-1}), human IL-1β (1 to 1000 iu ml^{-1}), poly-I:C (10 pg ml^{-1} to 100 μg ml^{-1}) and rat INF (1 to 10,000 IRu ml^{-1}) all failed to alter irCRF-41 release.

4 LPS (1 mg ml^{-1}) caused a 35% decrease in irCRF-41 release; however, over the dose-range of 0.1 μg ml^{-1} to 100 μg ml^{-1}, LPS failed to alter irCRF-41 release. The decreased irCRF-41 release in response to LPS (1 mg ml^{-1}) was accompanied by a decrease in the subsequent 56 mM KCl stimulation of irCRF-41.

5 Human IL-1α and IL-1β (1000 iu ml^{-1}) were able to stimulate the release of $irPGE_2$ from intact hypothalami, causing a 2 fold increase over basal release. Poly-I:C (100 μg ml^{-1}), LPS (0.1 μg ml^{-1} to 1 mg ml^{-1}), rat INF (10,000 IRu ml^{-1}) and human IL-6 (1 to 10,000 iu ml^{-1}) all failed to alter $irPGE_2$ release.

6 In conclusion, these results suggest that the *in vitro* release of CRF-41 and PGE_2, in response to pyrogens, are mediated via different cytokines. In view of this it is possible that different cytokines may mediate the temperature, prostaglandin and hypothalamo-pituitary-adrenocortical axis activation seen during pyrogenic stimulation *in vivo*.

Keywords: Prostaglandin; corticotrophin releasing factor; pyrogen

Introduction

A number of observations have led to the hypothesis that the fever inducing properties of both endogenous and exogenous pyrogens are mediated via the action of prostaglandins. Firstly, cyclo-oxygenase inhibitors prevent fever in response to pyrogens (Vane, 1971). Secondly, prostglandin E_2 (PGE_2) causes fever when injected into the third ventricle of cats and rabbits (Milton & Wendlandt, 1971). Thirdly, peripherally administered pyrogens cause an increase in both blood (Rotondo *et al.*, 1988) and cerebrospinal fluid (Feldberg & Gupta, 1973; Feldberg *et al.*, 1973. Harvey *et al.*, 1975) concentrations of prostaglandins. Furthermore, many pyrogens have been shown to activate the hypothalamo-pituitary-adrenal (HPA) axis (Yasuda & Greer, 1978; Woloski *et al.*, 1985; Uehara *et al.*, 1987; Tsagarakis *et al.*, 1989; Gwosdan *et al.*, 1990; Milton *et al.*, 1992b). For both interleukin-1 (IL-1) and polyinosinic:polycytidylic acid (Poly-I:C) such activation has been shown to occur via a corticotrophin-releasing factor-41 (CRF-41)-dependent mechanism (Uehara *et al.*, 1987; Milton *et al.*, 1992b). However, there is much controversy over the site of action of both the fever-inducing and HPA axis activating properties of pyrogens.

Some pyrogens demonstrate a distinct lag phase before inducing fever or HPA axis activation (Rotondo *et al.*, 1988; Milton *et al.*, 1992b). This argues either for a delay in reaching the sites of action or a requirement for the generation of other endogenous mediators. In the case of peripherally administered IL-1, the pyrogenic and adrenocorticotrophic hormone (ACTH) inducing properties may not be mediated via a direct action on the hypothalamus since radiolabelled IL-1 is unable to cross the blood brain barrier (Dinarello *et al.*, 1978). However, it is possible that certain pyrogenic substances may penetrate the blood brain barrier during fever or may by synthesised in the brain itself. Certainly there is evidence for the existence of central IL-1 and IL-6 neuronal networks and binding sites (Farrar *et al.*, 1987; Spangelo & MacLeod, 1990). Recent studies using *in vitro* methods have suggested that the endogenous pyrogens IL-1 and IL-6 stimulate the release of hypothalamic CRF-41 via a prostaglandin-dependent mechanism (Navarra *et al.*, 1991). The rises in blood levels of PGE_2, unlike those in $PGF_{2\alpha}$ mirror the increases in body temperature (Milton *et al.*, 1993) and PGE_2 is the most potent of the prostaglandins which activate body temperature increases (Milton & Wendlandt, 1971). In view of this we examined the effect of a number of endogenous and exogenous pyrogens on the secretion of immunoreactive (ir) CRF-41 and PGE_2 from the rat hypothalamus *in vitro*.

Methods

Rat hypothalamus in vitro

The incubation of rat hypothalami was performed *in vitro* by the well established technique of Bradbury *et al.* (1974).

[1] Author for correspondence.

Adult male Wistar derived rats were decapitated, the skull cap removed and the exposed brain deflected backwards after the optic nerves had been cut. The hypothalamus was then dissected out and transferred to a tube containing 1 ml of artificial cerebrospinal fluid (composition mM: NaCl 126, KCl 6, Na_2HPO_4 1, $MgSO_4.7H_2O$ 0.877, $NaHCO_3$ 22, $CaCl_2$ 1.45, D-glucose 10 and 0.1% w/v BSA; pH 7.4) in a shaking water bath at 37°C with an atmosphere of 95% O_2:5% CO_2. After a 60 min pre-incubation period, during which the medium was changed three times, tissue was incubated for 20 min to establish basal release. Tissue was then incubated for a further 20 min in the presence of test substances. The basal and test incubations were then repeated and after a further 20 min incubation with basal medium alone, the tissue was exposed to a depolarizing stimulus with medium containing 56 mM KCl. The Na^+ concentration was reduced from 126 mM to 76 mM to maintain osmolarity. The release of irCRF-41 and $irPGE_2$ into the medium was measured after extraction and concentration of the samples on Sep-Pak C_{18} cartridges.

Test substances

LPS (from *E.coli* serotype 055:B5), Poly-I:C and rat interferon (2.7×10^5 IRu mg^{-1} protein) were obtained from Sigma Chemical Co, Poole, Dorset UK. Human IL-1α (100,000 iu μg^{-1} protein), human IL-1β (100,000 iu μg^{-1} protein) and human IL-6 (5,000 iu μg^{-1} protein) were obtained from the National Institute for Biological Standards and Controls, Mill Hill, UK.

Extraction of irCRF-41 and $irPGE_2$

(i) *irCRF-41* Sep-Pak C_{18} cartridges were pre-wetted with methanol followed by water. Acidified samples were applied, unbound material washed off with acetic acid, and irCRF-41 eluted with 2 ml acetonitrile (75%). Supernatants were dried down under a stream of nitrogen prior to assay. Recoveries for synthetic irCRF-41 added to samples were 86 ± 9%.
(ii) *$irPGE_2$* Sep-Pak C_{18} cartridges were similarly prepared and samples applied. Unbound material was washed off with 10 ml H_2O followed by 0.5 ml methanol. Bound PGE_2 was eluted with 1.5 ml methanol. Recoveries of PGE_2 added to extracts were 94 ± 5%.

Enzyme amplified immunometric assay for rat CRF-41

IrCRF-41 was measured with an ultra-sensitive, highly specific enzyme amplified immunometric assay (Milton *et al.*, 1990a) modified with a monoclonal antibody (KCHMB003) to rat CRF-41 (Milton *et al.*, 1990b). A commercial enzyme-amplified substrate system-AMPAK, (Dako Diagnostics, Cambridge, UK) was used to develop the colour.

NUNC 96 well Immunoplates were coated with 100 µl/well of rC70 antiserum (1:20,000) in carbonate buffer (pH 9.6) and incubated overnight at 4°C. The plates were washed to remove unbound antiserum and incubated with 250 µl/well of 5% lactose (w/v) and 0.2% casein (w/v) in carbonate buffer for 1 h at room temperature. Plates were then washed, air dried and sealed with acetate sealer before storage at 4°C in a desiccator.

Synthetic rat CRF-41 standard solutions (prepared by serial doubling dilutions in assay buffer (50 mM Tris 0.1% BSA; 0.1% Triton X-100; pH 7.4) to cover the range 0.4 to 200 pM) or samples were added to pre-coated plates (100 µl/well) and incubated overnight at 4°C. The plates were washed four times with washing buffer prior to addition of 100 µl/well anti-rat CRF-41 monoclonal antibody (KCHMB003)-alkaline phosphatase conjugate (1:1,000) and incubation for 2 h at room temperature. The plates were again washed four times with washing buffer, AMPAK substrate (100 µl/well) added and incubated for 15 min at room temperature. The AMPAK amplifier (100 µl/well) was then added. The plates were again incubated at room temperature and after sufficient colour had developed the reaction was stopped by addition of 0.3 M sulphuric acid (50 µl/well). The absorbance of each well at 495 nm was measured and irCRF-41 levels in samples calculated by interpolation from the standard curve.

The assay had a sensitivity of 1 fmol ml^{-1} and showed cross-reactivity with oxidised CRF-41 (79%). Serial dilutions of synthetic CRF-41, Sep-Pak C_{18} purified hypothalamic extracts and Sep-Park C_{18} extracts of medium from hypothalamic incubations all showed parallel curves.

Radioimmunossay of prostaglandin E_2

$IrPGE_2$ concentrations were determined by RIA with a kit purchased from Du Pont Inc. This assay had a sensitivity of 0.7 fmol ml^{-1} and showed cross-reactivity with PGE_1, (3.2%). Serial dilutions of standard PGE_2 and Sep-Pak C_{18} extracts of medium from hypothalamic incubations showed parallel curves.

Statistics

Results were calculated as means ± s.e.mean from at least five determinations and the degree of significance tested by one-way analysis of variance. For all studies a P value of <0.05 was considered significant.

Results

Basal irCRF-41 and $irPGE_2$ release

Considerable variation in the mean basal release was observed between different experiments. The mean (± s.e.mean) for irCRF-41 ranged from 3.0 ± 1.0 fmol/hypothalamus per 20 min ($n = 9$) to 12.3 ± 3.1 fmol/hypothalamus per 20 min ($n = 9$). The mean (± s.e.mean) for $irPGE_2$ ranged from 88 ± 20 fmol/hypothalamus per 20 min ($n = 6$) to 403 ± 66 fmol/hypothalamus per 20 min. Basal release in successive samples taken from the same group of hypothalami showed no statistically significant variation (Table 1). To account for within-group variations in irCRF-41 or $irPGE_2$ release, all data from each individual hypothalamus were expressed as a percentage of its own basal release during the preceding incubation period. Comparisons were made between mean % basal release from groups of at least 5 hypothalami treated with each test substance.

Effects of pyrogenic immunomodulators

ir CRF-41 release The addition of human IL-6 over a dose-range of 1 to 10,000 iu ml^{-1} caused a dose-dependent release of irCRF-41 from intact rat hypothalami incubated *in vitro* (Figure 1). The threshold dose was 100 iu ml^{-1}, with 1,000 iu ml^{-1} causing a 2–3 fold increase and 10,000 iu ml^{-1} causing a 3–4 fold increase, relative to the basal release. Basal release after the first stimulation with human IL-6 was not significantly different from the first basal and the tissue was able to respond to a subsequent dose of IL-6 with a comparable release of irCRF-41.

The addition of LPS over a dose range of 0.1 µg ml^{-1} to 100 µg ml^{-1} failed to alter irCRF-41 release from intact rat hypothalami incubated *in vitro*, relative to the basal release (Figure 2). Addition of a depolarized stimulus of 56 mM KCl to tissues treated with LPS resulted in a 2 fold increase in irCRF-41 release. The release of irCRF-41 in response to 56 mM KCl did not differ between groups, given different doses of LPS. Basal release after the first stimulation with LPS was not affected and irCRF-41 release was not altered during a subsequent incubation with LPS.

However, LPS in a dose of 1 mg ml^{-1} caused a 35%

Table 1 Variability in immunoreactive corticotrophin releasing factor-41 (irCRF-41) and ir prostaglandin E_2 (irPGE$_2$) release from the rat hypothalamus *in vitro*

Incubation Period (20 min)	*irCRF-41* (fmol/hypothalamus per 20 min)	*irPGE$_2$* (fmol/hypothalamus per 20 min)
1	8.1 ± 1.4	99 ± 20
2	7.5 ± 1.0	117 ± 31
3	7.9 ± 1.4	105 ± 28
4	7.0 ± 0.9	88 ± 20

Results are shown as mean (± s.e.mean) irCRF-41 or irPGE$_2$ release from at least five observations per group.

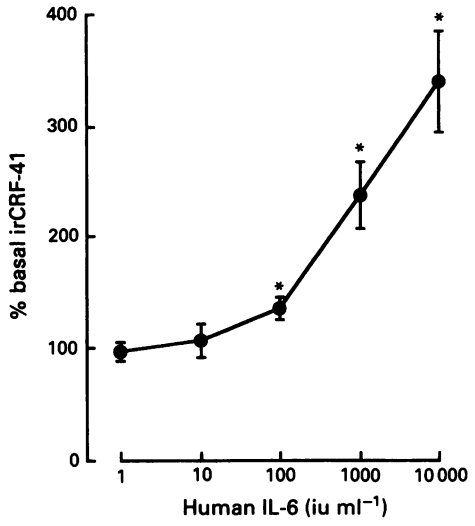


Figure 1 Effect of human interleukin-6 (human IL-6) on the release of immunoreactive corticotrophin releasing factor-41 (irCRF-41) from the rat hypothalamus *in vitro*. Results expressed as % basal release, are shown as mean ± s.e.mean from at least five observations. *$P<0.05$ by one way analysis of variance compared to unstimulated controls.

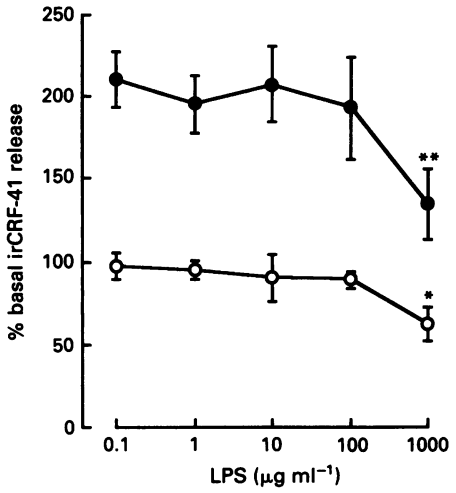


Figure 2 Effect of lipopolysaccharide (LPS) (*E.coli* 0.55:B5) on release of immunoreactive corticotrophin releasing factor-41 (irCRF-41) from the rat hypothalamus *in vitro* (○) and on the 56 mM KCl stimulated release of irCRF-41 (●). Hypothalami were stimulated with LPS before challenge with 56 mM KCl. Results, expressed as % basal release, are shown as mean ± s.e.mean from at least five observations. *$P<0.05$ compared with unstimulated controls; **$P<0.05$ compared with controls treated with media alone followed by 56 mM KCl by one-way analysis of variance.

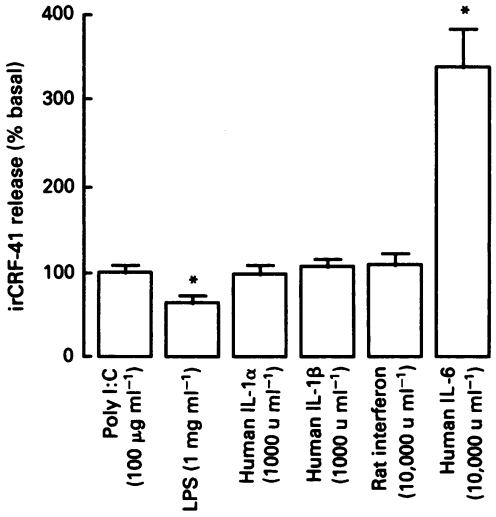


Figure 3 Effect of pyrogenic immunomodulators (for abbreviations see text) on the release of immunoreactive corticotrophin releasing factor-41 (irCRF-41) from the rat hypothalamus *in vitro*. Results, expressed as % basal release, are shown as mean ± s.e.mean from at least five observations. *$P<0.05$ by one way analysis of variance compared to unstimulated controls.

decrease in irCRF-41 release which was observed in both the first and second incubation periods. The release of irCRF-41 was non-significantly decreased in the intervening basal period. The 56 mM KCl stimulation evoked a 2 fold increase in irCRF-41 relative to basal release in control groups, which was reduced by 30% in hypothalami preincubated with 1 mg ml^{-1} LPS (Figure 2).

The addition of poly-I:C (10 pg ml^{-1} to 100 μg ml^{-1}), human IL-1α (10 to 1,000 iu ml^{-1}), human IL-1β (10 to 1,000 iu ml^{-1}) or rat interferon (INF) (1 to 10,000 IRu ml^{-1}) failed to alter irCRF-41 release from intact rat hypothalami incubated *in vitro*, relative to the basal release (Figure 3). Addition of a depolarizing stimulus of 56 mM KCl to tissues treated with these pyrogens resulted in a 2–4 fold increase in irCRF-41 release. The release of irCRF-41 in response to 56 mM KCl did not differ between groups given different doses of these pyrogens. Basal release after the first stimulation with pyrogen was not affected and neither was the irCRF-41 release during a subsequent incubation with pyrogen.

When tissues were incubated with acetylcholine (10 nM) a 2.5 fold increase in the release of irCRF-41 was observed. This response was not affected by the presence of poly-I:C (1 μg ml^{-1}) in the incubation medium (Table 2).

irPGE$_2$ release Poly-I:C (1 μg ml^{-1}), LPS (0.1 μg ml^{-1} to 1 mg ml^{-1}), rat INF (10,000 IRu ml^{-1}) and human IL-6 (1 to 10,000 iu ml^{-1}) all failed to alter irPGE$_2$ release from intact rat hypothalami incubated *in vitro*, relative to the basal release (Figure 4). Basal release after the first stimulation with pyrogen was unaffected and irPGE$_2$ release was not altered during a subsequent incubation with any of these pyrogens.

Both human IL-1α and IL-1β at a dose of 1,000 iu ml^{-1} caused a 2 fold increase in irPGE$_2$ release from intact rat hypothalami incubated *in vitro*, relative to the basal release (Figure 4). Basal release after the first stimulation with IL-1α or IL-1β was not significantly different from the first basal

Table 2 Effect of acetylcholine and polyinosinic: polycytidilic acid (poly-I:C) on immunoreactive corticotrophin releasing factor-41 (irCRF-41) from the rat hypothalamus *in vitro*

	irCRF-41 (% basal)
Poly-I:C (1 $\mu g\ ml^{-1}$)	107 ± 15
Acetylcholine (10^{-9} M)	237 ± 30*
Poly-I:C + acetylcholine	252 ± 41*

Results, expressed as % basal release, are shown as mean ± s.e.mean from at least five observations. *$P<0.05$ by one way analysis of variance compared to unstimulated controls.

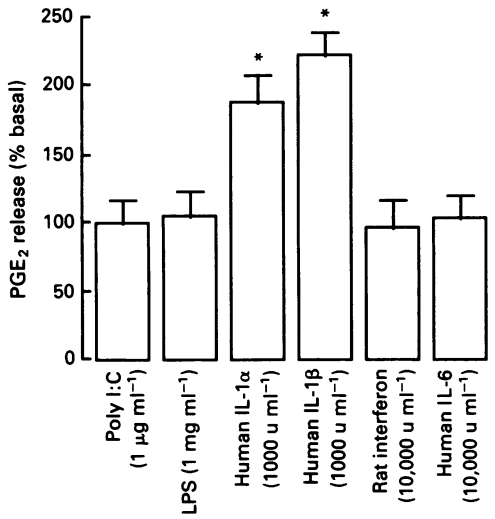


Figure 4 Effect of pyrogenic immunomodulators (for abbreviations, see text) on the release of immunoreactive prostaglandin E_2 ($irPGE_2$) from the rat hypothalamus *in vitro*. Results expressed as % basal release, are shown as mean ± s.e.mean from at least five observations. *$P<0.05$ by one way analysis of variance compared to unstimulated controls.

samples and $irPGE_2$ release was similarly elevated during a subsequent incubation with IL-1α or IL-1β.

Discussion

The rat hypothalamus *in vitro* technique has previously been well validated (Bradbury *et al.*, 1974; Hillhouse 1975; Buckingham & Hodges 1977; Hillhouse & Milton 1989a,b) for the study of regulators of CRF bioactivity and immunoreactivity. The experimental design was such that all tissue samples, subjected to incubations with test substances, were exposed to a depolarizing 56 mM KCl stimulus at the end of the experiment. This test confirmed the viability of each tissue and enabled any adverse or prolonged effects of test substances on irCRF-41 release to be detected. Previous studies have confirmed that the hypothalamus incubated in this manner remains viable for up to 3 h (Hillhouse, 1975) and none of the experimental procedures involved incubations of longer duration. The variability in the basal release of irCRF-41 and $irPGE_2$ between groups may reflect differences in experimental animal groups or procedures. However, within group variation in basal release was sufficiently small to allow comparison of consecutive samples. The tissues were taken at the same time (between 09 h 00 min and 10 h 00 min) to eliminate contributions from hormonal circadian rhythms. The variability in the basal release, however, prevented comparison of actual irCRF41 levels stimulated by test substances in different experiments. The problems of variability in basal release were overcome by expression of irCRF-41 or $irPGE_2$ release from each individual hypothalamus as a percentage of its own basal release during the preceding incubation period, so making meaningful comparisons possible. However, since the underlying causes of the differences in basal release may also influence the responsiveness of the tissue to a range of secretagogues only qualitative comparisons between individual experiments can be made.

The hypothalamus has been shown to mediate the stimulatory effect of LPS on ACTH secretion in rats (Yasuda & Greer 1978) and poly-I:C activates the HPA axis in rabbits via a CRF-41 dependent mechanism (Milton *et al.*, 1992b). In this study neither of these exogenous pyrogens stimulated the secretion of either irCRF-41 or $irPGE_2$ from the rat hypothalamus *in vitro*. Indeed at the highest dose tested (1 $mg\ ml^{-1}$) LPS caused a significant inhibition of irCRF-41 release which was long lasting and probably reflects a non-specific toxic effect. This strongly suggests that, at least in rats, neither of these exogenous pyrogens activate CRF-41 containing neurones via a direct effect at the hypothalamic level.

The failure of poly-I:C either to stimulate or to potentiate irCRF-41 secretion from the rat hypothalamus is interesting in view of our recent observations that peripheral CRF-41 plays a role in the febrile and prostaglandin responses to poly-I:C in rabbits (Milton *et al.*, 1992a). In these studies there was a lag phase of 30–60 min before an increase in circulating ir-cortisol was observed in response to poly-I:C (Milton *et al.*, 1992b). This suggests either a delay in poly-I:C reaching its site of action or the contribution of another endogenous activator of the HPA axis. Two such potential mediators are interferon and IL-1, both of which are activated by poly-I:C (Stringfellow, 1984; Souvannavong & Adam, 1990). In rats, injection of human γ-INF into the third cerebral ventricle, stimulates ACTH secretion (Gonzalez *et al.*, 1990) and IL-1 activates the HPA axis via a CRF-41-dependent mechanism (Uehara *et al.*, 1987). However, in the present study neither of these compounds had any effect on CRF-41 secretion from the rat hypothalamus *in vitro*. The rat interferon preparation used was chosen because it was obtained from poly-I:C stimulated cells and because of the suggested species specificity for some of the actions of this cytokine. Our results suggest that in the rat the main site of action of IL-1 and interferon is not the hypothalamic CRF-41 neurones.

The failure of IL-1 to stimulate CRF-41 release from the intact hypothalamus is in direct contrast to the observations of other groups using a similar preparation (Loxley *et al.*, 1992; Hagan *et al.*, 1992). However, effects due to endogenous glucocorticoids, which act to suppress hypothalamic CRF-41 release in response to cytokines (Loxley *et al.*, 1992) may account for these observed differences. The observations of an increase in the hypothalamic tissue content of CRF-41 in response to IL-1 (Hagan *et al.*, 1992) could be due to the presence of amino acids in the incubation medium and the observed release may well be a result of such synthesis rather than CRF-41 release in direct response to IL-1. The results from this study also contrast with previous reports of IL-1 stimulation of CRF-41 release in bisected rat hypothalamic fragments *in vitro* (Tsagarakis *et al.*, 1989; Navarra *et al.*, 1991). In view of the differences in experimental design and the nature of the neurones exposed by such dissection, methodological differences may provide an explanation for the many contradictory results. The different assay techniques may also play a role in the observed results. In our study, we used an ultrasensitive immunometric assay which is both more sensitive and specific for rat CRF-41 than available radioimmunoassays (Milton *et al.*, 1990a). The basal release of irCRF-41 reported for the rat hypothalamic

fragment system corresponded to 8.6 ± 0.8 fmol/hypothalamus per 20 min (Tsagarakis *et al.*, 1988) and is within the range found in the present study. However, the release in response to a 56 mM KCl stimulation was greater, giving a ten fold increase over basal compared to the 2–4 fold increase observed in the present study. These results suggest that the dissection of the hypothalamus may expose more CRF-41 producing neurones to the test substance. The comparison of parallel incubations rather than serial samples from the same tissue may also be a source of differences in the results obtained.

A recent study using cyclo-oxygenase inhibitors has suggested that the IL-1 and IL-6 stimulated release of ir CRF-41 from rat hypothamic fragments is prostaglandin-dependent (Spangelo & MacLeod, 1990). The molecular weight of IL-1 (17,000) is sufficiently large to suggest that it is unlikely for the material to diffuse far into the hypothalamic blocks studied *in vitro*. The neurones stimulated are likely, therefore, to be close to the surface of the tissue. The majority of the CRF-41 perikarya are contained within the paraventricular nucleus (Sawchenko & Swanson, 1985; Palkovits *et al.*, 1985; Petrusz *et al.*, 1985) which is situated near the surface of the hypothalamic blocks used in this study (Bradbury *et al.*, 1974). In the hypothalamic fragments method, other hypothalamic neurones may also be exposed to test substances. The IL-1 activation of prostaglandins has previously been reported for rabbit hypothalamic fragments and provides the basis for a bioassay for IL-1 (Symans *et al.*, 1987). In this study we showed the ability of IL-1 to stimulate PGE_2 release from the hypothalamus *in vitro* without any concomitant increase in CRF-41 release. This argues against a PGE_2-mediated action of IL-1 on the hypothalamic CRF-41 neurones. The ability of intravenously administered IL-1 to activate the HPA axis and irCRF-41 release into the hypophyseal portal circulation *in vivo* (Sapolsky *et al.*, 1987) suggests that such activation is at the hypothalamic level. However, the failure of indomethacin implants to attenuate the IL-1 induced ACTH rises suggests that such *in vivo* activation is not prostaglandin-dependent (Sapolsky *et al.*, 1987). However, Rivier & Vale (1991) have suggested that IL-1β activation of ACTH may have a prostaglandin-dependent component. These observations are consistent with the view that the pyrogenic and ACTH-inducing actions of IL-1 are mediated via different mechanisms. The failure of IL-1 to cross the blood-brain barrier (Dinarello *et al.*, 1978; Coceani *et al.*, 1988) has led to the suggestion that peripherally generated IL-1 gains access to the brain via the circumventricular organs (Coceani *et al.*, 1988; Katsuura *et al.*, 1990). One such site is the organum vasculosum lamina terminalis which is thought to be the site whereby IL-1 activates hypothalamic CRF-41 containing neurones via a prostaglandin-dependent mechanism (Coceani *et al.*, 1988).

Another cytokine which is secreted by activated immune cells during the acute phase of an immune response is IL-6 (Spangelo & MacLeod, 1990). Like IL-1 it causes both fever and activation of the HPA axis. Both *in vitro* (Navarra *et al.*, 1991) and *in vivo* (Naitoh *et al.*, 1988) experiments suggest that such activation is mediated via the production of CRF-41. Our results confirm that IL-6 stimulates the secretion of CRF-41 from the rat hypothalamus *in vitro*. However, this was not accompanied by a concomitant release of PGE_2 suggesting that this prostaglandin is not involved in the activation of CRF-41 release by IL-6. This is in agreement with the results of Bernadini *et al.* (1989) who reported that $PGF_{2\alpha}$, but not PGE_2, can stimulate the release of CRF-41 from rat hypothalamic explants. Further evidence in favour of prostaglandins being involved in the IL-6 activation of CRF-41 secretion has been provided from experiments using inhibitors of prostaglandin synthesis in hypothalamic fragments (Navarra *et al.*, 1991).

In conclusion, our results suggest that in the rat: (i) the exogenous pyrogens, LPS and Poly-I:C, and the endogenous pyrogens, IL-1 and INF, do not activate the HPA axis via a direct action at the level of the hypothalamic CRF-41 containing neurones; (ii) the endogenous pyrogen, IL-6, can activate hypothalamic CRF-41 neurones via a PGE_2-independent mechanism; (iii) IL-1 stimulates PGE_2 secretion from the hypothalamus without any concomitant change in CRF-41 secretion.

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The Leu[13]-motilin (KW-5139)-evoked release of acetylcholine from enteric neurones in the rabbit duodenum

[1]Takio Kitazawa, [2]Akio Ishii & *Kohtaro Taniyama

Pharmaceutical Research Laboratories, Kyowa Hakko Kogyo. Co. Ltd., Shizuoka, 411 and *Department of Pharmacology II, Nagasaki University School of Medicine, Nagasaki 852, Japan.

1 Involvement of cholinergic mechanisms in the contractile response to Leu[13]-motilin (LMT, KW-5139) was investigated in rabbit duodenal segments, and longitudinal muscle-myenteric plexus (LM-MP) preparations preincubated with [^{3}H]-choline.

2 Contractile response to LMT (0.1 nM–1 μM) consisted of an initial rapid (phasic) contraction and a tonic contraction slowly fading to a sustained plateau. LMT caused a concentration-dependent phasic contraction of rabbit isolated duodenal segments. The EC_{50} value was 2.5 nM and the maximum amplitude of the contraction was 103% of the response induced by acetylcholine (ACh, 100 μM). Neither tetrodotoxin nor atropine changed the EC_{50} value or the maximum amplitude of the response to LMT.

3 Both atropine and tetrodotoxin decreased the amplitude and accelerated fading of the tonic contraction produced by LMT.

4 LMT (30 nM–3 μM) induced an increase of ^{3}H-outflow, in a concentration-dependent manner. The LMT-induced increase of ^{3}H-outflow was prevented by removal of external Ca^{2+} or by the presence of tetrodotoxin.

5 Porcine motilin (10 nM–1 μM) also stimulated the release of ^{3}H at a similar concentration-range to that seen with LMT.

6 Pretreatment with LMT (3 μM for 20 min) decreased LMT- and the porcine motilin-evoked release of ^{3}H but did not alter the high K^+-evoked release.

7 Our results suggest that LMT and porcine motilin stimulate the release of ACh from enteric neurones through the same receptor, and that the release of ACh plays a role in tonic components of contraction in the rabbit duodenum.

Keywords: Motilin; rabbit duodenum; acetylcholine release; myenteric plexus

Introduction

Motilin, a 22 amino acid polypeptide discovered in the mucosa of the upper small intestine of the hog (Brown *et al.*, 1971; 1973), was found to localize in both enterochromaffin cells and non-enterochromaffin cells (Helmstaedter *et al.*, 1979). *In vivo* and *in vitro* studies have indicated that motilin causes contraction of gastrointestinal muscle in several species (Strunz *et al.*, 1975; Itoh *et al.*, 1976; Adachi *et al.*, 1981; Lüdtke *et al.*, 1989), and initiates periods of contractile activity that are similar to the interdigestive migrating motor complex in the dog (Itoh *et al.*, 1976; Inatomi *et al.*, 1989) and man (Janssens *et al.*, 1983).

The mechanisms of the gastrointestinal motor stimulating action of motilin differ between species; in the dog, motilin applied through intravenous or intra-arterial routes produced a contraction of the stomach, duodenum and jejunum and this response was abolished by tetrodotoxin or atropine (Fox *et al.*, 1984; Hirning & Burks, 1986; Inatomi *et al.*, 1989). Therefore, motilin is considered to increase gastrointestinal motor activity by stimulating a cholinergic pathway. However, in rabbit and human gastrointestinal smooth muscle strips (stomach, pylorus and duodenum), motilin produces contractile responses which are resistant to blockade of cholinergic transmission (atropine), and of nerve excitation and propagation (tetrodotoxin, Strunz *et al.*, 1975; Adachi *et al.*, 1981; Lüdtke *et al.*, 1989; Satoh *et al.*, 1990). Thus, motilin may act directly on smooth muscle cells of the rabbit duodenum and jejunum, and human stomach and pylorus.

Previously we noted the gastrointestinal motor stimulating action of KW-5139 (Leu[13]-motilin, LMT), an analogue of porcine motilin, in anaesthetized rabbits. LMT stimulated the motor activity of the gastric antrum, ileum and colon simultaneously and because there was a decrease by atropine pretreatment, we suggested an involvement of a cholinergic pathway in the response to LMT (Ishii *et al.*, 1992). Thus, there is a discrepancy in the mechanism of the excitatory response to motilin and motilin analogues between *in vivo* and *in vitro* studies of the rabbit intestine.

In an attempt to elucidate the mechanisms underlying the stimulating action of LMT on rabbit intestinal motility, we investigated whether or not LMT would induce a release of acetylcholine (ACh) from enteric neurones and then compared this with the effect of porcine motilin (Met[13]-motilin), using a smooth muscle preparation of rabbit duodenum. Release of ACh was estimated in the absence of cholinesterase inhibition as outflow of ^{3}H from longitudinal muscle-myenteric plexus (LM-MP) preparations preincubated with [^{3}H]-choline.

Methods

Animals

White male rabbits weighing from 2.0–3.0 kg were anaesthetized with pentobarbitone Na (30 mg kg^{-1}, i.v.) and exsanguinated. The duodenum was immediately isolated from the intestine 10 cm distal to the gastric antrum sphincter and cut into segments (30 mm long) which were then immersed in ice cold Tyrode solution with the following composition (mM): NaCl 136.9, KCl 2.7, NaH_2PO_4 1.8, $CaCl_2$ 1.8, $MgCl_2$ 2.1, $NaHCO_3$ 11.9 and glucose 5.6.

[1] Present address: Department of Veterinary Pharmacology, Faculty of Dairy Science, Rakuno Gakuen University, Ebetsu 069, Japan.
[2] Author for correspondence.

Mechanical study

Two types of rabbit duodenal muscle preparation were used in this mechanical study. One was whole segment (30 mm long, 8 mm wide) for tissue bath experiments, and the other was an LM-MP preparation (20 mm long, 3 mm wide) for perfusion experiments. LM-MP was obtained by the methods of Ambache (1954). The segment (20 mm long) of the duodenum was slipped on to a glass rod which caused slight radial stretching. After drawing a slight cut on the surface of the intestinal wall with a razor, the longitudinal muscle was torn off gently by pulling with forceps.

Whole segments were suspended vertically in a bath (10 ml and 40 ml), containing warmed (37°C) Tyrode solution continuously bubbled with a gas mixture (95% O_2 + 5% CO_2). The segments were initially loaded with 4 g weights and the mechanical activity measured by means of isotonic transducers (TD-112S, Nihon Kohden) and recorded on an ink-writing recorder. The preparation was allowed to equilibrate for 60 min at which time a steady baseline and full spontaneous contractile activity were established. Prior to the addition of LMT, each strip was subjected to repeated stimulation with ACh (100 μM) until a reproducible response was obtained. The concentration-dependent contraction (relative to the standard contraction induced by 100 μM ACh) was determined by cumulative addition of LMT (0.1, 0.3, 1, 3, 10, 30, 100, 300 nM, 1 μM) in the bath at 2 min cycles in the absence, and presence of tetrodotoxin or atropine. ACh, 100 μM, was enough to produce the maximum contraction of duodenal segments. The contractile response to 1 μM LMT was observed over a 30 min period in the absence and presence of atropine and tetrodotoxin, to analyse whether or not these drugs affect the tonic contraction and change the time course of the response to LMT. The amplitudes of the contractile responses were calculated as mean ± s.e.mean and were analysed by a Student's *t* test. A *P* value of 0.05 or less was considered statistically significant.

In some experiments, isometric contraction of the LM-MP preparation was examined in the perfusion system used in the ^{3}H-outflow study described below. The LM-MP preparation was mounted in the apparatus (25 mm long, 4 mm wide) and superfused at a flow rate of 1.2 ml min^{-1} with Tyrode solution that was continuously gassed with 95% O_2 + 5% CO_2 and warmed at 37°C. Each concentration of LMT and porcine motilin (0.1, 1, 10, 100 nM, 1 μM) was superfused for 2 min at 30 min intervals, and the mechanical response in the LM-MP were measured with a force displacement transducer (TB-611T, Nihon Kohden) and recorded on a multichannel polygraph (Nihon Kohden). Amplitude of the response was expressed as a percentage of that induced by ACh (100 μM) applied for 2 min.

Release study

The effects of LMT and porcine motilin on the release of ACh were examined in the LM-MP preparations of the rabbit duodenum preloaded with [^{3}H]-choline. The methods of incubation and perfusion were as described by Tanaka & Taniyama (1985). The LM-MP preparations were incubated with 140 nM [^{3}H]-choline for 60 min in 1.3 ml warmed Tyrode solution (37°C), equilibrated with 95% O_2 + 5% CO_2. After washing in the fresh Tyrode solution (37°C, bubbled with gas mixture) for 30 min, the preparations were mounted in the perfusion apparatus and perfused at a flow rate of 1.2 ml min^{-1} with the Tyrode solution. Experiments were started 30 min after the spontaneous outflow of ^{3}H had approached a plateau level. The perfused Tyrode solution contained 10 μM hemicholinium-3 to prevent the reuptake of choline formed from breakdown of ACh. Superfusate from the preparations was collected at 1 or 2 min intervals.

To investigate the effect of LMT and porcine motilin on the ^{3}H outflow, each concentration of both peptides (1 nM −3 μM) was included for 2 min in the perfusion medium. The radioactivity in the superfusate was counted in a liquid scintillation counter. Ca^{2+}-free medium was Tyrode solution from which $CaCl_2$ was removed and which ethylene glycol *bis* (β-aminoethylether) *N*, *N*, *N'*, *N'*-tetraacetic acid (EGTA, 1 mM) was included. Isotonic high-K solution (50 mM) was prepared by replacement of NaCl with KCl.

At the end of each experiment, the tissue was dissolved in Soluene (500 μl) and the radioactivity was measured in a scintillation counter. The ^{3}H outflow was expressed as the fractional rate, in which the amount of radioactivity in the superfusate was divided by the total radioactivity present in the tissue, in the same collection period. The ^{3}H-content of the tissue at each period was calculated by adding cumulatively the amount of ^{3}H in each fraction to the ^{3}H-content of the tissue at the end of the experiments. From each of the release curves obtained by plotting the fractional rate of ^{3}H-outflow against time, the 4 fractions (1 min fraction) from application of LMT or porcine motilin were considered to be a stimulation-induced increase over the spontaneous outflow. Therefore, the increase of ^{3}H-outflow induced by stimulation with the peptides was calculated by the following equation. Stimulation-induced increase = 100 × average of ^{3}H amounts in the 4 fractions during and after stimulation/average of ^{3}H amounts in the 4 fractions before stimulation.

The concentration of peptide giving 50% increase of the spontaneous outflow was derived from each concentration-response curve, and this value was compared with the contracting activity (see mechanical study) of the respective motilin peptides, using the same perfusion apparatus.

Drugs and chemicals

Substances used in this experiment were as follows, [^{3}H]-choline (85.1 Ci mmol^{-1}, NEN Research Products), acetylcholine chloride (Ovisot, Daiichi Seiyaku), tetrodotoxin (Sigma), ethylene glycol *bis* (β-aminoethylether) *N*, *N*, *N'*, *N'*-tetraacetic acid (EGTA) and atropine sulphate (Nakarai), hemicholinium-3 (Aldrich), porcine-motilin (Met^{13}-motilin, Peninsula Laboratories Inc) and Leu^{13}-motilin (KW-5139), produced in high degree with low cost using recombinant DNA techniques (Miyashita *et al.*, 1988). Amino acid sequences of porcine motilin and Leu^{13}-motilin are shown in Figure 1. Other chemicals used were of reagent grade. The drugs and motilin peptides were dissolved in Tyrode solution immediately before use and then added to the bath medium or the perfusate.

Porcine-motilin

1 10

Phe-Val-Pro-Ile-Phe-Thr-Tyr-Gly-Glu-Leu-Gln-Arg-Met-Gln-Glu-Lys-

20 22

Glu-Arg-Asn-Lys-Gly-Gln

Leu^{13}-motilin (KW-5139)

1 10

Phe-Val-Pro-Ile-Phe-Thr-Tyr-Gly-Glu-Leu-Gln-Arg-Leu-Gln-Glu-Lys-

20 22

Glu-Arg-Asn-Lys-Gly-Gln

Figure 1 Amino acid sequence of porcine-motilin and Leu^{13}-motilin (KW-5139) used in the present study.

Results

Contractile response of duodenal segments to LMT

The rabbit isolated duodenal muscle segments showed a rhythmic spontaneous contraction superimposed on the baseline tonus. LMT (0.1 nM–1 μM) applied to the tissue bath caused a rapid, initial phasic contraction followed by a slowly fading contraction (tonic contraction, Figure 2a). The recovery of the effect of LMT was usually accomplished 15–20 min after one to three rinses with a fresh Tyrode solution. The EC_{50} value and the maximum amplitude of the phasic contraction were 2.5 ± 0.6 nM ($n = 10$) and 103 ± 2.4% ($n = 10$) of the response to 100 μM ACh. Atropine (1 μM) and tetrodotoxin (300 nM) did not decrease the phasic contraction of LMT (Figure 2b,c). The EC_{50} values and the maximum amplitudes were 2.6 ± 0.9 nM and 106 ± 7.4% ($n = 6$) in the presence of atropine, and 2.2 ± 0.4 nM and 107 ± 5.5% ($n = 5$) in the presence of tetrodotoxin, respectively.

Figures 2a and 3 represent the time course of the contractile response to LMT (1 μM) in the rabbit duodenal segments. The decline in the tonic contraction (to 40–50% of the peak phasic response) ceased 20–30 min after exposure to LMT. Pretreatment with atropine (1 μM) or tetrodotoxin (300 nM) did not alter the initial phasic component but did decrease the tonic one. Therefore, the tonic contraction declined more rapidly to a significantly lower sustained plateau (20–30% of the peak phasic response, Figure 3).

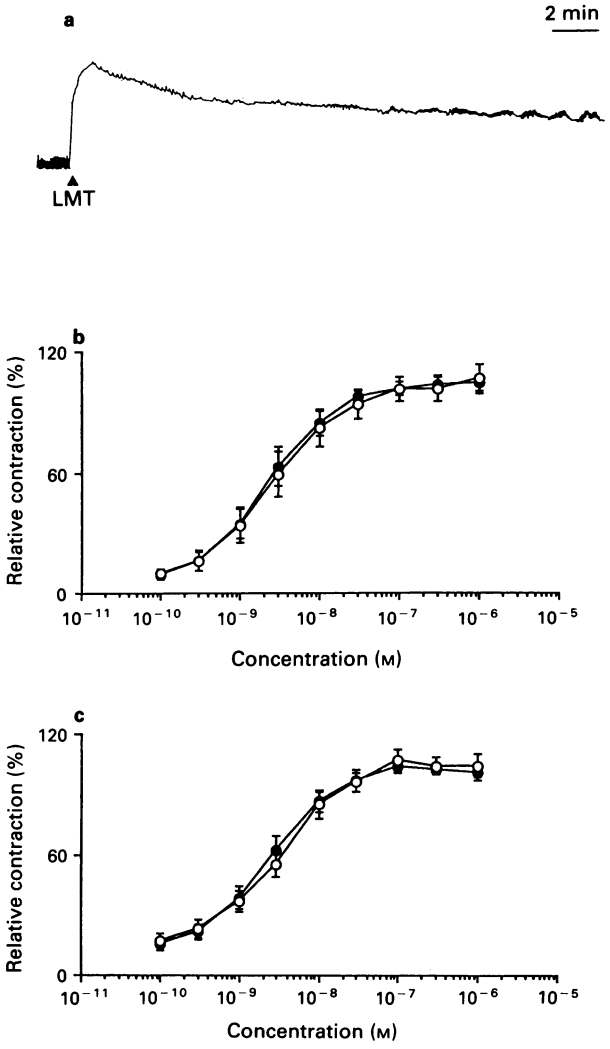


Figure 2 Contractile response to Leu[13]-motilin (LMT) of the rabbit duodenal segments. (a) Typical pattern of 1 μM Leu[13]-motilin-induced contraction. (b) Concentration-response curve for Leu[13]-motilin-induced phasic contraction in the absence (●) and presence (○) of atropine (1 μM). (c) Concentration-response curve for Leu[13]-motilin-induced phasic contraction in the absence (●) and presence (○) of tetrodotoxin (300 nM). Relative contraction was expressed as a % of contraction induced by acetylcholine (100 μM). Each point represents the mean of 5–6 segments ± s.e.mean.

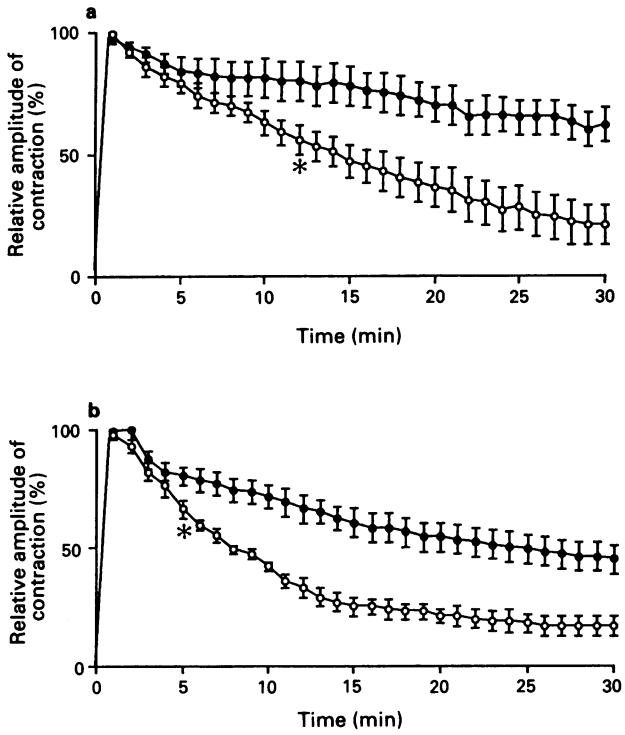


Figure 3 Alterations of time course of Leu[13]-motilin-induced tonic contraction of the rabbit duodenal segments by pretreatment with atropine and tetrodotoxin. Atropine (1 μM) or tetrodotoxin (300 nM) was added to the bath medium 20 min before addition of Leu[13]-motilin (1 μM). (a) In the absence (●) and presence (○) of atropine; (b) in the absence (●) and presence (○) of tetrodotoxin. The relative contraction was expressed as a % of the maximum amplitude of Leu[13]-motilin-induced phasic contraction in the absence, and presence of atropine or tetrodotoxin respectively. Each point represents the mean of 5–6 segments ± s.e.mean. *Indicates the point at which the contractions in the absence and presence of atropine or tetrodotoxin become significantly different with $P < 0.05$.

When either atropine (1 μM) or tetrodotoxin (300 nM) was added to the bath 10 min after exposure to 1 μM LMT, the tonic contraction, settled at approximately 70% of the phasic one, dropped suddenly to a significantly lower plateau than that of the control. However, tetrodotoxin did not inhibit the tonic responses induced by KCl (30 mM) or ACh (100 μM) and atropine did not alter the response to KCl, thereby indicating that tetrodotoxin and atropine did not directly affect myogenic activity (Figure 4).

Effects of LMT on ^{3}H-outflow from the LM-MP preparation

The spontaneous ^{3}H-outflow from the LM-MP preparations reached a fairly constant level after 30 min of superfusion with Tyrode solution at 1.2 ml min^{-1}, the fractional rate being 0.093 ± 0.0095% min^{-1} ($n = 33$). After 30 min of superfusion, we examined the effects of treatment with LMT (1 nM–3 μM) for 2 min on the spontaneous ^{3}H-outflow. Low concentrations (below 10 nM) of LMT did not change the spontaneous ^{3}H-outflow; however, concentrations of 30 nM and above produced a concentration-dependent increase in the ^{3}H-outflow (Figure 5). The concentration of LMT producing a 50% increase of the spontaneous outflow was 270 nM. Using the same apparatus, the effects of LMT on the

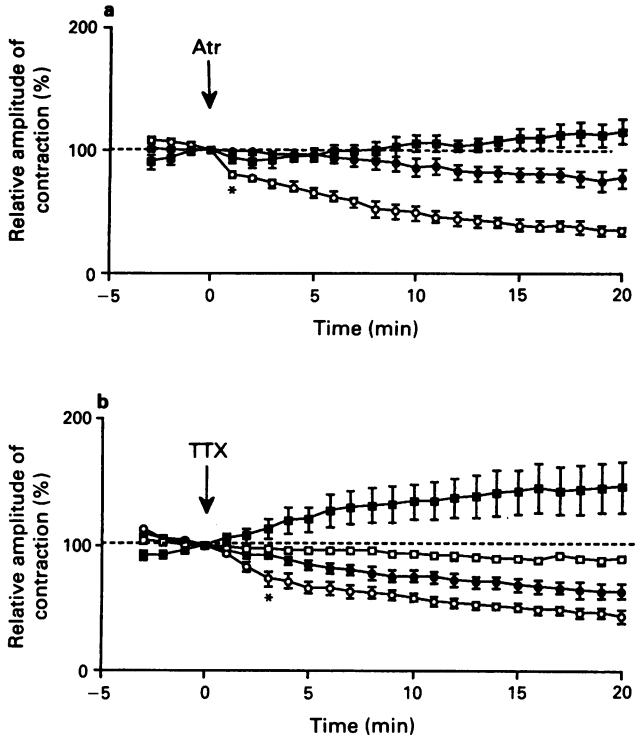


Figure 4 Alterations of Leu[13]-motilin-induced contraction of the rabbit duodenal segments by treatment with atropine and tetrodotoxin during tonic response. Atropine (1 μM) or tetrodotoxin (300 nM) was added to the bath medium 10 min after exposure to Leu[13]-motilin (1 μM), KCl (30 mM) or acetylcholine (100 μM). (a) Tonic contractions induced by Leu[13]-motilin of non-treated (●) and atropine-treated (○) segments, and induced by KCl in atropine-treated segments (■). (b) Tonic contractions induced by Leu[13]-motilin of non-treated (●) and tetrodotoxin-treated (○) segments, and induced by KCl (■) and acetylcholine (□) in tetrodotoxin-treated segments. The relative contraction was expressed as a % of tonic contraction immediately before applying atropine or tetrodotoxin. Each point represents the mean of 5–6 segments ± s.e.mean. *Indicates the point at which the contractions in the absence and presence of atropine or tetrodotoxin become significantly different with $P<0.05$.

contractility of LM-MP preparations were examined. LMT (0.1 nM–1 μM) included in the superfusion medium for 2 min produced contraction, in a concentration-dependent manner, as in the case of the tissue bath method (Figure 5). The concentration of LMT producing 50% of the contracile response to ACh (100 μM) was 2.8 nM, a value consistent with the EC_{50} value (2.5 nM) in the tissue bath study, described above.

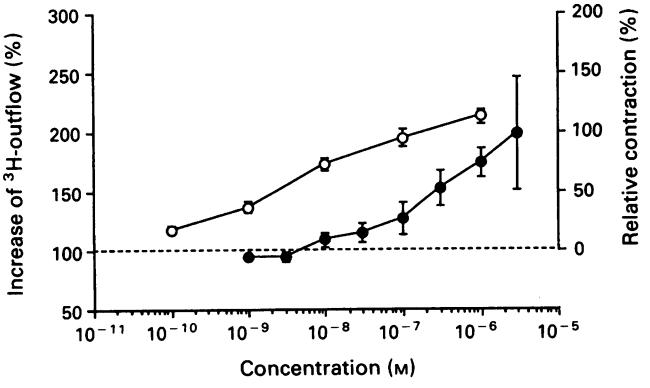


Figure 5 Concentration-response curves for Leu[13]-motilin-induced increase of ^{3}H-outflow and contractile response in longitudinal muscle with myenteric plexus preparation of the rabbit duodenum. Relative contraction was expressed as a % of contraction induced by acetylcholine (100 μM) (○). Increase of ^{3}H-outflow was expressed as a % increase of spontaneous outflow (●, see Methods). Each point represents the mean of 5–11 strips ± s.e.mean.

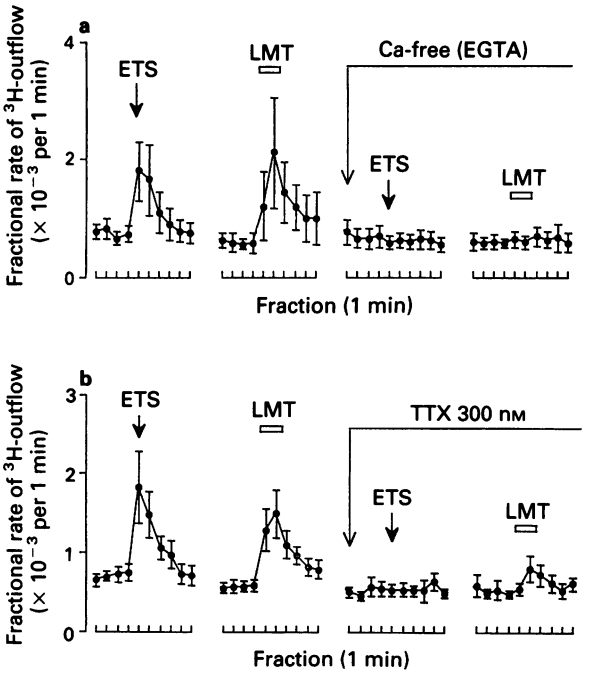


Figure 6 Effects of removal of external Ca^{2+} and tetrodotoxin on increase of ^{3}H-outflow induced by electrical transmural stimulation and Leu[13]-motilin. Ca^{2+}-free solution containing EGTA (1 mM, a) and tetrodotoxin (TTX, 300 nM) containing solution (b) were superfused 20 min before applying electrical transmural stimulation (ETS, 15 V, 1 ms duration, 10 Hz for 30 s) or Leu[13]-motilin (LMT, 1 μM, for 2 min). Each point represents the mean of 6 strips ± s.e.mean.

The LMT (1 μM)-induced increase of ^{3}H-outflow was abolished in the Ca^{2+}-free solution containing 1 mM EGTA, as was the increase in ^{3}H-outflow induced by electrical transmural stimulation (ETS, 15 V intensity, 1 ms duration, 10 Hz for 30 s, Figure 6a), thereby indicating that LMT evoked increase of ^{3}H-outflow is Ca^{2+}-dependent. Tetrodotoxin (300 nM) also abolished the ETS-induced increase of ^{3}H-outflow, however the toxin inhibited markedly, but not completely the LMT-induced increase (Figure 6b).

Effects of porcine motilin on the mechanical activity and ^{3}H-outflow from the LM-MP preparation

The contractile and ^{3}H-outflow increasing activities of the naturally occurring porcine motilin were examined and compared with those of LMT. Application of porcine motilin for 2 min also produced a contraction (0.1 nM–1 μM) and an increase of ^{3}H-outflow (10 nM–1 μM), in a concentration-dependent manner. The concentration of porcine motilin producing 50% increase of the spontaneous ^{3}H-outflow was 110 nM. Therefore, the potency of the releasing action of porcine motilin was slightly greater than that of LMT (EC_{50} = 270 nM, Figure 7a). On the other hand, the concentration of porcine motilin producing 50% of the contractile activity to ACh (100 μM) was 3.1 nM, comparable to that of LMT (2.5 nM). Hence a higher concentration of both porcine motilin and LMT was required to induce the increase of ^{3}H-outflow than to produce the contractile response.

Desensitization of ^{3}H-outflow increasing action

Desensitization of LMT was observed after a long exposure of the LM-MP preparation to LMT (3 μM for 20 min). As shown in Figure 7b, the addition of LMT (1 μM) now failed to induce any response, but the response to high K^+ (50 mM)

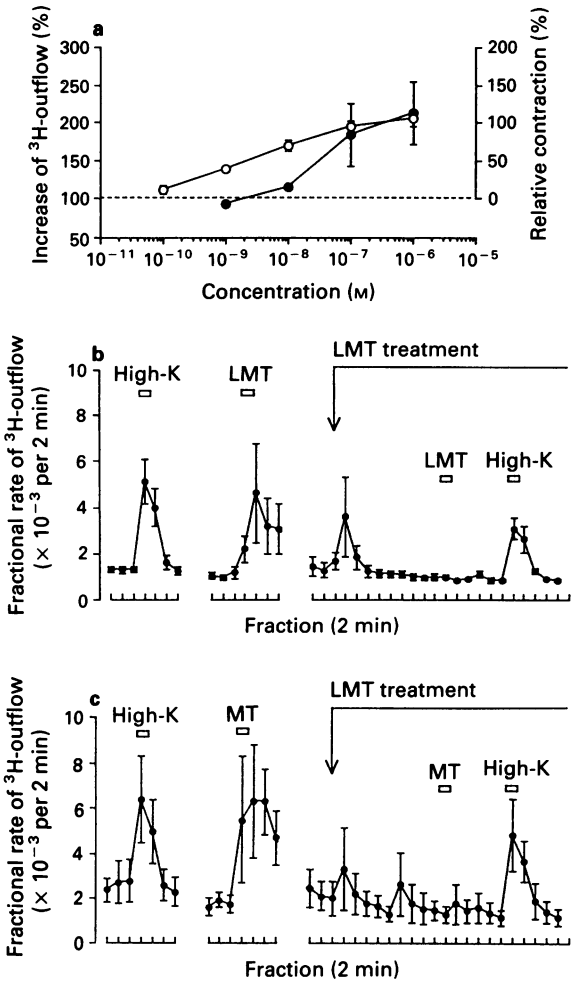


Figure 7 Porcine motilin-induced increase of ^{3}H-outflow from longitudinal muscle with myenteric plexus preparations through stimulation of the same receptor as Leu^{13}-motilin. (a) Concentration-response curves for porcine motilin-induced increase of ^{3}H-outflow and contractile response. Relative contraction was expressed as a % of contraction induced by acetylcholine (100 μM, ○). Increase of ^{3}H-outflow was expressed as a % increase of spontaneous outflow (●). (b), (c) Desensitization of Leu^{13}-motilin (LMT, 1 μM, b) or porcine motilin (MT, 1 μM, c)-induced increase of ^{3}H-outflow following continuous treatment with LMT (3 μM). High K^+ (50 mM) and LMT (1 μM) or porcine motilin (1 μM) were superfused for 2 min at 30 min intervals. During exposure to high concentration of LMT (3 μM) for 20 min, LMT (1 μM) or porcine motilin (1 μM), and then high K^+ (50 mM) were applied. Each point represents the mean of 6 strips ± s.e.mean.

remained. Similarly, the porcine motilin-induced increase of ^{3}H-outflow was also prevented in the presence of LMT (3 μM), without any apparent effect on the high K^+-induced release (Figure 7c).

Discussion

The contraction induced by LMT consisted of two components, an initial phasic contraction and a subsequent tonic contraction which slowly faded. The phasic contraction was not affected either by atropine or tetrodotoxin, thereby indicating that LMT acts directly through stimulation of smooth muscle. Others have also suggested that the contraction evoked by motilin is mediated through the motilin receptor present on smooth muscle cells and not through cholinergic transmission (Strunz *et al.*, 1975; Adachi *et al.*, 1981; Lüdtke *et al.*, 1989; Satoh *et al.*, 1990). This notion was given support in a receptor binding study using ^{125}I-labelled motilin (Bormans *et al.*, 1986) and a mechanical study using rabbit colonic myocytes (Hasler *et al.*, 1992).

It is noteworthy that the tonic contractile components induced by LMT were inhibited by either atropine or tetrodotoxin. Pretreatment with either of these substances accelerated fading of the tonic contractile responses, with no conspicuous effect on the phasic response, Application of either atropine or tetrodotoxin during the tonic response suddenly reduced the tonic contraction and accelerated the fade of the contraction, without affecting the ACh- and high K^+-induced responses. The findings led to the idea that LMT produces an increase of ACh release, which contributes the tonic response, by the stimulation of cholinergic neurones. Similar phenomena were observed in case of the substance P-induced contraction of the guinea-pig isolated intestine (Holzer & Lembeck, 1980; Holzer *et al.*, 1981), and it was then confirmed that the peptide induced the release of ACh from the guinea-pig intestinal myenteric neurones (Fosbraey *et al.*, 1984).

Measurement of ETS- and drug-induced ^{3}H-outflow from intestinal tissue preincubated with [^{3}H]-choline, which were decreased by Ca^{2+}-free solution or tetrodotoxin, appears to provide a satisfactory index for the release of ACh from cholinergic neurones (Kilbinger & Wessler, 1980; Tanaka & Taniyama, 1985; Koelbel *et al.*, 1989). In this study, LMT increased the ^{3}H-outflow at concentrations ranging from 30 nM to 3 μM and this increase was completely prevented in Ca^{2+}-free medium (containing 1 mM EGTA). These results suggest that ACh is released by LMT through the activation of the cholinergic synaptic process, since Ca^{2+} is necessary for the release of neurotransmitters from nerve terminals (Hubbard *et al.*, 1968). Tetrodotoxin markedly decreased the release of ACh induced by LMT, although a small part of the induced release remained. Since tetrodotoxin prevents neurotransmitter release induced by stimulation of soma-dendritic regions of the neurone (Vizi *et al.*, 1973), but not by direct depolarization of nerve terminals (Alberts *et al.*, 1982), the main site of action of LMT may be the soma-dendritic region of the enteric cholinergic neurones. Nevertheless, we cannot exclude the possibility that LMT acts on axon terminals of cholinergic neurones or on interneurones connecting with postganglionic cholinergic neurones. These results may, therefore, explain the observation that the gastrointestinal motor stimulating action of LMT could be inhibited by atropine in anaesthetized rabbits (Ishii *et al.*, 1992), and the tonic contractile response to LMT *in vitro* could be attenuated by atropine or tetrodotoxin (this study).

Porcine motilin also induced contraction of the rabbit duodenum (Adachi *et al.*, 1981) and the release of ACh, in a similar concentration-range to LMT (10 nM–1 μM). The binding of porcine motilin to the rabbit duodenum preparation was inhibited by LMT (Peeters *et al.*, 1988), thereby indicating involvement of the same receptor mechanisms in the contractile response to porcine motilin and LMT. At present, a motilin receptor antagonist is not available, and a long-lasting exposure to motilin was used as a pharmacological tool to block the response mediated by activation of the motilin receptor (Satoh *et al.*, 1990). Pretreatment with LMT attenuated the ACh releasing actions of LMT and porcine motilin, without affecting the response to high K^+. These auto- and cross-desensitizations of the response to LMT indicate that both LMT and porcine motilin induce a release of ACh through activation of the same motilin receptor located on myenteric neurones.

In summary, we found that LMT produced an initial phasic contraction mediated by stimulation of smooth muscle cells directly and the subsequent tonic contraction mediated by stimulation of cholinergic neurones as well as smooth muscle cells. LMT and porcine motilin may act on the same receptors located on these neurones and smooth muscle cells. However, the EC_{50} values of the contractile responses to LMT and porcine motilin were 100 times lower than those of the ACh releasing responses. Thus, the possibility that there are subtypes of the motilin receptor, a high affinity binding site on the smooth muscle cells and a low affinity binding site on the myenteric neurones, paves the way for future studies.

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Radioligand and functional estimates of the interaction of the 1,4-dihydropyridines, isradipine and lacidipine, with calcium channels in smooth muscle

Salvatore Salomone & [1]Théophile Godfraind

Laboratoire de Pharmacologie, Université Catholique de Louvain, UCL 7350, Avenue E. Mounier 73, B-1200 Bruxelles, Belgium

1 The present experiments were undertaken in order to characterize further the apparently irreversible inhibition of the contraction of depolarized rat aorta caused by lacidipine, a 1,4-dihydropyridine calcium antagonist.

2 We studied the effect of lacidipine on contraction evoked by 100 mM KCl solution in rat aorta, treated by N^{ω}-nitro-L-arginine (0.1 mM), an inhibitor of nitric oxide (NO) synthesis. We compared the effect of prolonged depolarization on lacidipine and (+)-isradipine inhibition and the reversal of this inhibition after washout in the absence of dihydropyridines. Assuming that the onset of lacidipine-evoked inhibition was a pseudo-first order association kinetics, we estimated the dissociation rate constant ($k_{-1} = 0.031\ min^{-1}$), the association rate constant ($k_1 = 2.70 \times 10^8\ M^{-1}\ min^{-1}$) and the dissociation constant ($K_D = k_{-1}/k_1 = 115$ pM) which was close to the IC_{50} value in steady-state conditions (160 pM).

3 The inhibitory effects of lacidipine and (+)-isradipine on rat aorta contraction were reversibly enhanced after preincubation with the drug in a 40 mM KCl-solution. Washout with drug-free 40 mM K^+-depolarizing solution reversed inhibition in the (+)-isradipine-treated preparations, but not in the lacidipine-treated ones.

4 Radioligand binding studies were performed with [^{3}H]-lacidipine and [^{3}H]-isradipine in microsomes from rat aorta and rat ileum. Both ligands bound to a homogeneous population of binding sites (for [^{3}H]-lacidipine: $K_D = 23 \pm 2.6$ pM, $B_{max} = 380 \pm 21$ fmol mg^{-1} protein in membranes from aorta; $K_D = 23 \pm 3.1$ pM, $B_{max} = 790 \pm 60$ fmol mg^{-1} protein in membranes from ileum; for [^{3}H]-isradipine: $K_D = 140 \pm 46$ pM, $B_{max} = 350 \pm 64$ fmol mg^{-1} protein in membrane from aorta; $K_D = 68 \pm 14$ pM, $B_{max} = 760 \pm 75$ fmol mg^{-1} protein in membranes from ileum). After isotopic dilution, [^{3}H]-lacidipine and [^{3}H]-isradipine dissociated according to a monoexponential kinetics. In membranes from ileum, the calculated dissociation rate constants (k_{-1}) were 0.0257 min^{-1} and 0.0595 min^{-1}, for [^{3}H]-lacidipine and [^{3}H]-isradipine, respectively.

5 The non specific binding of [^{3}H]-lacidipine and [^{3}H]-isradipine, was measured in intact rat aorta preparations incubated under the conditions of the functional experiments, in the presence of nifedipine (1 μM). After incubation with [^{3}H]-lacidipine 77.6 ± 1.9 pM for 2 h the concentration of drug in the tissue was 15.15 ± 1.18 fmol mg^{-1} w.wt. and still amounted to 7.24 ± 0.61 fmol mg^{-1} w.wt. after 3.5 h washout in drug-free solution. After incubation with [^{3}H]-isradipine 47.2 ± 0.4 pM for 2 h it was 2.26 ± 0.07 fmol mg^{-1} w.wt. and was undetectable after 3.5 h washout in a drug-free solution.

6 It is concluded that lacidipine interacts reversibly with dihydropyridine binding sites and that the apparent irreversible inhibition of contraction in depolarized preparations could be related to a non specific binding in a tissue compartment different from the plasma membrane.

Keywords: Lacidipine; isradipine; calcium antagonists; dihydropyridine binding sites; smooth muscle

Introduction

Calcium antagonists (L-type Ca^{2+} channel blockers) are widely used for the management of some major cardiovascular disorders, including hypertension and angina pectoris (Opie, 1990). It is generally accepted that the opening of voltage-dependent calcium channels allows the rise of cytosolic Ca^{2+} concentration that triggers contraction in both cardiac and smooth muscle tissues (Godfraind *et al.*, 1986). It has been shown that calcium antagonists inhibit the sustained contraction evoked by noradrenaline in vascular smooth muscle by interacting with specific binding sites on voltage-operated Ca^{2+} channels (Morel & Godfraind, 1991). The binding of the three main classes of calcium antagonists (the phenylalkylamines, benzothiazepines and 1,4-dihydropyridines) occur at three different binding sites that are distinct but allosterically linked (Glossmann *et al.*, 1985) on the α_1 subunit of L-type calcium channels (Striessnig *et al.*, 1990a,b; Nakayama *et al.*, 1991).

Lacidipine is a 1,4-dihydropyridine calcium antagonist that exerts a long-lasting inhibition of contraction on depolarized vascular preparations *in vitro*, and a long-lasting antihypertensive activity *in vivo* (Micheli *et al.*, 1990). We previously showed (Godfraind & Salomone, 1991) that inhibition by lacidipine of smooth muscle contraction increases after preincubation in a depolarizing medium and that such an increased inhibition is reversible after return to physiological solution. However, this increased inhibition persisted in depolarizing solution, even in the absence of the drug.

In the present study, functional and radioligand experiments were performed in order to characterize the kinetics of lacidipine interactions with voltage-dependent calcium channels. The functional effects and the binding of lacidipine were

[1] Author for correspondence.

compared to those of (+)-isradipine. The results show that the interaction of lacidipine with calcium channels is reversible and that the persistent inhibition in drug-free conditions is likely to reflect drug release from a cellular compartment different from the plasma membrane.

Methods

Tissue preparation

Wistar rats (250 g) were killed by decapitation and the aorta or ileum were removed. The aorta was cleaned from the surrounding connective tissue; the longitudinal smooth muscle of ileum was dissected as described by Godfraind & Burton (1967). The tissues were either used immediately to perform functional experiments or collected and stored at − 20°C to prepare membrane fractions.

Measurement of contractile responses

Aortic rings (2 mm wide) were suspended under a resting tension of 2 g in 50 ml organ baths filled with a physiological solution (composition, mM: NaCl 122, KCl 5.9, $NaHCO_3$ 15, $MgCl_2$ 1.25, $CaCl_2$ 1.25, glucose 11) bubbled with 95% O_2 and 5% CO_2 and maintained at 37°C. The rings were connected to an isometric lever with two strain gauges as part of a balanced bridge. The output was fed into an analog/digital converter, which was connected to a microcomputer. The programme allowed input from up to 8 force transducers simultaneously. After calibration, tension was displayed on the screen of the microcomputer, recorded on a printer, and stored on disk at predetermined time intervals. The preparations were allowed to equilibrate for 60 min in physiological solution, and tension was readjusted once after 30 min.

All contractile experiments were performed in the presence of N^{ω}-nitro-L-arginine (0.1 mM), an inhibitor of NO synthesis (Moore *et al.*, 1990), in order to avoid any influence of NO on vascular tone.

To analyse the kinetics of lacidipine interaction with calcium channels the experiment was carried out as follows: contractions were evoked by changing from physiological solution to a 100 mM K^+ solution (composition, mM: NaCl 27, KCl 100, $NaHCO_3$ 15, $MgCl_2$ 1.25, $CaCl_2$ 1.25, glucose 11); the first stimulation was used to check the sensitivity of the vascular preparations to the relaxing effect of acetylcholine (1 μM); the lack of relaxation confirmed the inhibition of NO synthesis by N^{ω}-nitro-L-arginine. After washout for 30 min in physiological solution, a control stimulation was recorded for 35 min; after washout and relaxation, the preparations were incubated for 90 min in physiological solution with or without various concentrations of lacidipine; a third contraction was then induced in the continuous presence of lacidipine. Results obtained in the presence of lacidipine were compared to controls (that were treated simultaneously without lacidipine) and corrected.

Radioligand binding experiments

Strips of ileal longitudinal smooth muscle (about 3 g wet wt.) were thawed, minced with scissors and homogenized in about 10 vol g^{-1} of a solution containing 250 mM sucrose, 5 mM Tris and 0.1 mM phenylmethylsulphonylfluoride, pH 7.4 at 2°C, using an all-glass Potter-Elvehjem-type grinder (Braun, Melsungen, Germany) kept at 2°C. Four passes of the pestle rotating at 1,500 rev min^{-1} were carried out to produce an homogenate. It was centrifuged at 1,000 *g* for 5 min, then the pellet was rehomogenized as above with 15 ml of the same solution and the resulting suspension centrifuged at 1,000 *g* for 5 min. The pellet resulting from the low-speed runs was suspended in 250 mM sucrose buffered at pH 7.4 with 5 mM Tris-HCl and designated as nuclear fraction (N fraction), whereas the supernatants were combined and centrifuged at 20,000 *g* for 30 min. The pellet of this latter centrifugation was resuspended and designated as mitochondrial fraction (M fraction), whereas the supernatant (including the gelatinous material covering the pellet) was spun at 200,000 *g* for 30 min. The pellet was then suspended in 250 mM sucrose buffered at pH 7.4 with 5 mM Tris-HCl and designated as total microsomal fraction. This suspension was loaded over Tris-buffered 31.5% (w/w) sucrose (density 1.14 at 0°C) and then spun at 200,000 *g* for 60 min. The material banding at the interface was collected, resuspended in 250 mM sucrose buffered at pH 7.4 with 5 mM Tris-HCl and designated as light microsomal fraction (L fraction) whereas the pellet was designated as heavy microsomal fraction (H fraction).

Aortae (about 3 g wet wt.) were thawed, minced with scissors and homogenized in about 20 vol g^{-1} as above. The homogenate was centrifuged at 1,000 *g* for 10 min; the pellet was rehomogenized as above with 15 ml of the same solution and the resulting suspension centrifuged at 1,000 *g* for 10 min. The cycle homogenization-low speed centrifugation was repeated four times. The supernatants from the low-speed runs were combined and submitted to the same protocol as that used for intestinal smooth muscles.

The amount of protein was determined according to Lowry *et al.* (1951).

In saturation experiments, samples from the different membrane fractions (10–25 μg of protein) were incubated, at 37°C for 60 min, with [^{3}H]-lacidipine or [^{3}H]-isradipine in 2.5 ml of a buffered salt solution of the following composition (mM): NaCl 145, KCl 5, $MgCl_2$ 1.25, $CaCl_2$ 2.5, Tris 20, pH 7.4 at 37°C. To determine non-specific binding, nifedipine was included in the incubation medium, at a final concentration of 1 μM. In dissociation experiments, isotopic dilution was performed, by adding nifedipine 1 μM, after 1 h incubation.

After incubations, the membrane suspensions were rapidly filtered on Whatman GF/F filters, that had been presoaked in an aqueous solution of polyethylenimine (3% w/w) for 30 min. Each tube was emptied and washed with 2.5 ml of ice-cold aqueous solution containing polyethylene glycol (5% w/w) and Tris 50 mM (pH 7.4 at 4°C); filters were further washed with two 10 ml portions of the same solution. The radioactivity retained on the filters was counted in 10 ml of scintillation cocktail (Pico-fluor 30/toluene, 1/4 v/v), with an efficiency of approximately 44%.

The non specific binding was also measured (in triplicate) for each membranal fraction, at seven different concentrations of [^{3}H]-lacidipine (20 pM–440 pM) and at six different concentrations of [^{3}H]-isradipine (40 pM–520 pM), by subtracting the radioactivity retained on the filters in the presence of nifedipine (1 μM) and in the absence of membranes (blank), from the radioactivity retained on the filters in the presence of nifedipine (1 μM) and membranes; therefore it represents the non specific binding to membranes.

The non specific binding of [^{3}H]-lacidipine and [^{3}H]-isradipine was evaluated in intact rat aortae, in the presence of nifedipine (1 μM). Aortic rings (0.7–2.5 mg wet wt.) were incubated in the presence of [^{3}H]-lacidipine or [^{3}H]-isradipine at 37°C for 60 min in physiological solution (for composition see above) followed by 60 min in a zero Ca^{2+} physiological solution (composition, mM: NaCl 122, KCl 5.9, $NaHCO_3$ 15, $MgCl_2$ 1.25, glucose 11) and by 30 min in a zero Ca^{2+} depolarizing solution (composition mM: NaCl 88, KCl 40, $NaHCO_3$ 15, $MgCl_2$ 1.25, glucose 11) bubbled with a mixture of 95% O_2:5% CO_2. At the end of this incubation time, a washout was performed for 3.5 h, still in the presence of nifedipine (1 μM) but in the absence of [^{3}H]-lacidipine or [^{3}H]-isradipine, in a zero Ca^{2+} depolarizing solution. Each half hour 4–8 rings were dried on filter paper, weighed and dissolved in 0.1 ml of a mixture of perchloric acid:H_2O_2 (1:1). Radioactivity bound to the tissue was counted in 4 ml of Pico-fluor 30 by liquid scintillation.

Drugs

Lacidipine {(E)-4-[2-[3-(1,1-dimethylethoxy)-3-oxo-1-propenyl]-phenyl]-1,4-dihydro-2,6-dimethyl-3,5-pyridine-dicarboxylic acid diethyl ester} (Glaxo) and nifedipine (Bayer) were dissolved in ethanol as stock solutions at 1 mM and further diluted in water as required before use. [^{3}H]-lacidipine (specific activity 21.6 Ci mmol^{-1}) and [^{3}H]-isradipine ([^{3}H]-(+)PN 200-110, specific activity 85.4 Ci mmol^{-1}) were obtained from New England Nuclear. All experiments were done under illumination of a sodium lamp only, to prevent dihydropyridine photoinactivation.

Data analysis

Results of functional experiments are expressed as means ± standard error (s.e.mean). Tests of significance have been made by use of paired or unpaired Student's *t* test, *P* values smaller than 0.05 being considered significant.

Binding measurements from two different light microsomes preparations were pooled and analysed by non-linear regression to obtain binding parameters; the non-linear regression was done by means of the software Multifit 2.0 (Day Computing, U.K.). Kinetic association and dissociation constants of lacidipine for the inactivated L-channels, were also derived from the analysis of the timecourse of inhibition on K^+-evoked contraction of rat aorta; pseudo-first order conditions were assumed according to Weiland & Molinoff (1981).

Results

The kinetics of lacidipine inhibition of K^+-evoked contraction of rat aorta

The inhibition of the K^+-contraction was studied in the presence of N$^\omega$-nitro-L-arginine (0.1 mM), an inhibitor of NO synthesis (Moore *et al.*, 1990), in order to avoid any influence of NO on vascular tone. Rat aortic preparations were preincubated for 90 min in physiological solution in the presence of lacidipine (80 pM–640 pM), and stimulated with a 100 mM KCl-depolarizing solution containing lacidipine. The contraction was monitored for up to 120 min and the response recorded in the presence of lacidipine was compared to the control response and corrected for untreated controls. As Figure 1 illustrates, lacidipine depressed concentration-dependently the response to K^+-depolarization, steady state being attained after about 1 h, a time much longer than with other dihydropyridines (Morel & Godfraind, 1987; 1988; Wibo *et al.*, 1988). At steady-state, the concentration inhibiting 50% of contraction (IC_{50}), was 160.0 pM (142.2 pM–180.1 pM).

Assuming that the time-course of lacidipine inhibition on K^+-evoked contraction of rat aorta is related to association with inactivated calcium channels as previously found for isradipine (Wibo *et al.*, 1988), the time-course of inhibition may be described as a pseudo-first order process (Figure 2) according to Weiland & Molinoff (1981):

$$\ln LR_e/(LR_e - LR_t) = k_{obs}\ t \qquad (1)$$

where LR_e, the steady-state concentration of drug-bound channels, is estimated from the maximum inhibition attained for a given concentration and LR_t from the level of inhibition at the time t of depolarization. The measurement of the slopes (k_{obs}) of pseudo-first order plots over a range of lacidipine concentrations (c) allowed the estimation of the association and dissociation rate constants from the equation:

$$k_{obs} = k_1\ c + k_{-1} \qquad (2)$$

which were found to be equal to 0.0310 min^{-1}, and 2.70×10^8 min^{-1} M^{-1} respectively. Therefore, K_D (k_{-1}/k_1) was equal to 115 pM.

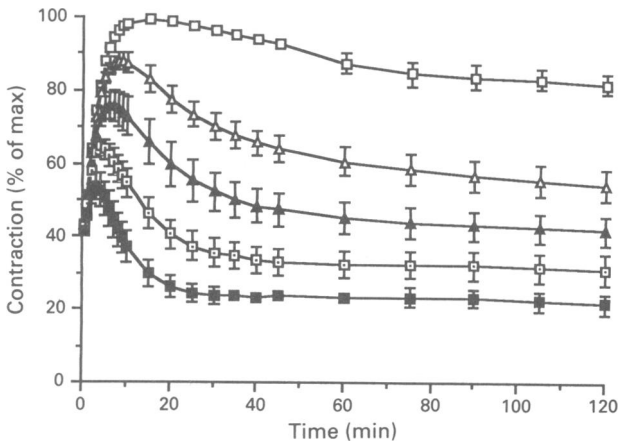


Figure 1 Time course of the contractile response of rat aorta to 100 mM K^+-solution in the absence (□) or in the presence of lacidipine 80 pM (△), 160 pM (▲), 320 pM (⊡) and 640 pM (■). Preparations were preincubated for 90 min in the absence or in the presence of lacidipine and then stimulated by 100 mM K^+-solution (third K^+-contraction; see Methods). Values are mean ± s.e.mean from 8 different preparations. Contraction is expressed as % of the maximal tension attained in the previous K^+-contraction in the absence of the drug.

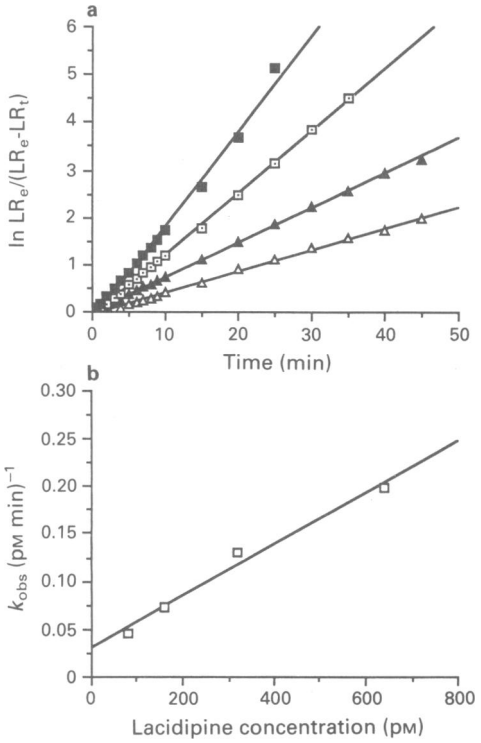


Figure 2 Pseudo-first order analysis of kinetics of lacidipine inhibition on 100 mM K^+-contraction of rat aorta (from data illustrated in Figure 1, see also text). (a) Time course of association of lacidipine 80 pM (△), 160 pM (▲), 320 pM (⊡) and 640 pM (■). Ordinate scale: ln $LR_e/(LR_e - LR_t)$, where LR_e represents the steady-state concentration of drug-bound channels, estimated from the maximum inhibition attained for a given concentration, and LR_t the concentration of drug-bound channels, estimated from the level of inhibition at the time t of depolarization, before equilibrium. The level of inhibition at each time was calculated from contractions observed before and after incubation in the presence of lacidipine and corrected according to the variations observed between two consecutive contractions recorded simultaneously in controls. From the equation $\ln LR_e/(LR_e - LR_t) = k_{obs}\ t$, the following k_{obs} values were calculated by linear regression: at 80 pM $k_{obs} = 0.0456$ (pM min)$^{-1}$; at 160 pM, $k_{obs} = 0.0734$ (pM min)$^{-1}$; at 320 pM, $k_{obs} = 0.1306$ (pM min)$^{-1}$; at 640 pM, $k_{obs} = 0.1980$ (pM min)$^{-1}$. (b) Pseudo-first order plot of k_{obs} over a range of lacidipine concentrations.

Voltage-dependency and reversibility of lacidipine and isradipine-induced inhibition of K^+-evoked contraction of rat aorta

In order to characterize the voltage-dependency of lacidipine and isradipine in rat aorta, preparations were incubated in the presence of lacidipine or isradipine first in physiological solution and then in a zero Ca^{2+} 40 mM K^+-depolarizing solution. At 30 min intervals the preparations were stimulated by short pulses with a solution containing 100 mM K^+ and 1.25 mM Ca^{2+}. Figure 3 shows that the inhibitory effect of both dihydropyridines on the contraction elicited by 100 mM K^+ was enhanced ($P<0.01$ for all concentrations of isradipine and lacidipine; paired t test) when the preparations had been pre-incubated in the depolarizing solution (40 mM K^+, zero Ca^{2+}). After drug washout from the zero Ca^{2+} 40 mM K^+ solution, isradipine-treated aortae (Figure 3a) recovered initial sensitivity, whereas lacidipine-treated ones did not (Figure 3b); the persistent inhibition of lacidipine pretreated preparations was related to the concentration of the drug before washout and a residual ED_{50} value of 164 pM could be estimated in such conditions. Moreover, when lacidipine pretreated preparations were repolarized by transfer to physiological solution, the residual inhibitory effect of lacidipine decreased significantly ($P<0.01$ at 240 pM; $P<0.05$ at 120 pM) (Figure 3b).

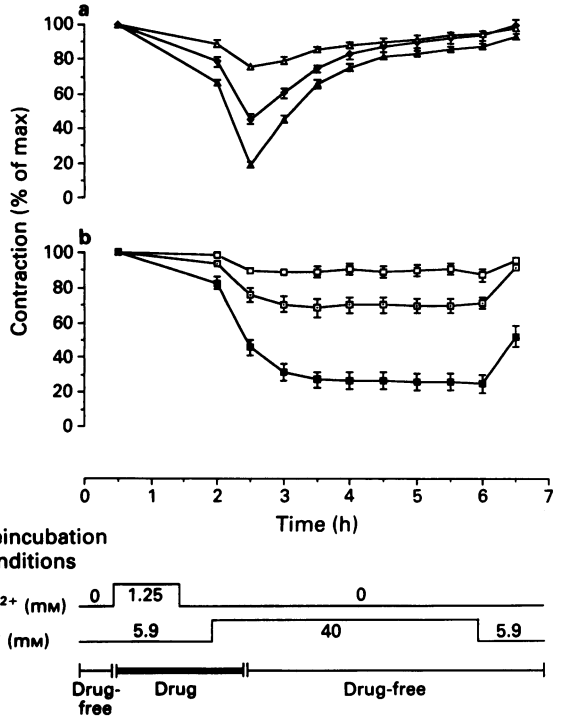


Figure 3 Reversal of (+)-isradipine (a) and lacidipine (b) inhibition of K^+-contraction of rat aorta, in drug-free Ca^{2+}-deprived solutions (see also text). Ordinate scale: response to 100 mM K^+-solution (containing 1.25 mM Ca^{2+}) pulse of 2 min expressed as % of controls; abscissa scale: experimental time (h). After recording of control contractions, aortic rings were preincubated for 60 min in the presence of (+)-isradipine [60 pM (△), 120 pM (◆) and 240 pM (▲)] or lacidipine [60 pM (□), 120 pM (⊡) and 240 pM (■)] in physiological solution. They were then preincubated for 30 min in zero Ca^{2+} physiological solution with calcium antagonists before contraction was elicited by exposure to a solution containing 100 mM KCl and 1.25 mM $CaCl_2$ for 2 min, in the continuous presence of calcium antagonists. Aortic rings were thereafter preincubated for 30 min in zero Ca^{2+} 40 mM KCl-depolarizing solution containing the same concentration of calcium antagonists before a second contraction was recorded. At the end of this contraction, a washout was performed in a drug-free zero Ca^{2+} 40 mM KCl-depolarizing solution, and a contraction was elicited each 30 min. The last contraction (time 6.5 h) was evoked after 30 min preincubation in drug-free zero Ca^{2+} physiological solution.

Binding of [^{3}H]-lacidipine and [^{3}H]-isradipine to membranes from rat aorta and ileum

The non specific and the specific binding of [^{3}H]-lacidipine and [^{3}H]-isradipine were measured in the different membrane fractions obtained from rat aorta. The non specific binding increased linearly with concentration (correlation coefficients 0.97–1.00), whereas the specific binding was saturable (see below). Table 1 shows the values of the non specific and the specific binding of [^{3}H]-lacidipine and [^{3}H]-isradipine, calculated for a free ligand concentration of 100 pM. The specific binding of [^{3}H]-isradipine was much higher, on a protein basis, in light microsomes (L), a fraction enriched in plasma membrane (Wibo *et al.*, 1980; Morel *et al.*, 1981), than in the other fractions. The specific binding of [^{3}H]-lacidipine was easily measurable in the L fraction, but was undetectable in the other fractions, presumably because the non specific binding of [^{3}H]-lacidipine was negligible in the L fraction, but much higher than that of [^{3}H]-isradipine in the other fractions (N, M and H). Indeed, at a concentration of 100 pM, the expected specific binding of [^{3}H]-lacidipine in N, M and H fractions was estimated to be 0.03–0.04 pmol mg^{-1} protein (twice the values calculated for [^{3}H]-isradipine), i.e. 2–6% of the non specific binding of [^{3}H]-lacidipine.

In light microsomes (L fraction) from both aorta and ileum, saturation experiments revealed a linear Scatchard plot for [^{3}H]-lacidipine as well as for [^{3}H]-isradipine specific binding, indicating, as expected, only one class of specific binding sites (Figure 4). Binding parameters calculated by non-linear regression are given in Table 2. They show that B_{max} values were similar for the two dihydropyridines and that the binding sites density was two fold higher in ileum.

In light microsomes from rat ileum, dissociation of specifically bound [^{3}H]-lacidipine and [^{3}H]-isradipine, induced by adding nifedipine 1 μM, followed monoexponential kinetics (Figure 5). The dissociation of [^{3}H]-lacidipine was slower than that of [^{3}H]-isradipine, the calculated dissociation rate constants being 0.0257 min^{-1} and 0.0595 min^{-1} respec-

Table 1 Non specific and specific binding of [^{3}H]-lacidipine and [^{3}H]-isradipine in different membrane fractions from rat aorta

	Non specific binding (pmol mg^{-1} protein)		*Specific binding* (pmol mg^{-1} protein)	
Fraction	*[^{3}H]-lacidipine*	*[^{3}H]-isradipine*	*[^{3}H]-lacidipine*	*[^{3}H]-isradipine*
Nuclear (N)	1.50	0.041	ND	0.016
Mitochondrial (M)	0.48	ND	ND	0.015
Heavy microsomes (H)	1.18	0.015	ND	0.021
Light microsomes (L)	ND	ND	0.31	0.15

Non specific and specific binding of [^{3}H]-lacidipine and [^{3}H]-isradipine were estimated at a free ligand concentration of 100 pM. The non specific binding was estimated by linear regression; the specific binding was estimated from the Michaelis-Menten function $B = (F \times B_{max})/(K_D + F)$. ND = not detectable.

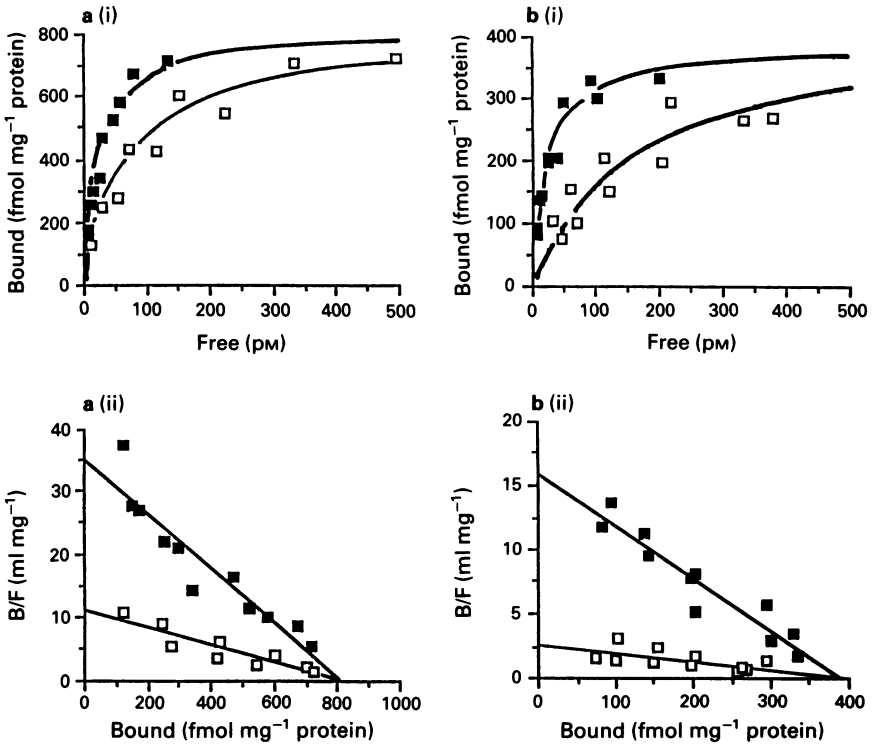


Figure 4 Saturation isotherms (i) and Scatchard representations (ii) of [^{3}H]-lacidipine (■) and [^{3}H]-isradipine (□) specific binding measured in light microsomes from rat ileum (a) and aorta (b). Each point is the mean from 3 determinations; data obtained from two different preparations were pooled. Non specific binding (not shown) was measured in the presence of nifedipine (1 μM) and increased linearly with the concentrations of both tritiated molecules. Solid lines represent the function $B = (F \times B_{max})/(K_D + F)$, where B is the specific binding and F is the free concentration of ligand. Binding parameters calculated by non-linear regression are given in Table 2.

Table 2 Binding parameters of [^{3}H]-lacidipine and [^{3}H]-isradipine in light microsomes from aorta and ileum of rat (from data illustrated in Figure 4)

Rat aorta	K_D (pM)	B_{max} (fmol mg^{-1})
[^{3}H]-lacidipine	23 ± 2.6	380 ± 21
[^{3}H]-isradipine	140 ± 46	350 ± 64
Rat ileum		
[^{3}H]-lacidipine	23 ± 3.1	790 ± 60
[^{3}H]-isradipine	68 ± 14	760 ± 75

tively; the corresponding $t_{1/2}$ values were 27.0 min and 11.7 min. In the aorta, because of the low yield of microsomal protein, we measured only the dissociation of [^{3}H]-lacidipine at 90 min. After incubation with [^{3}H]-lacidipine 71 pM and subsequent isotopic dilution, the specific binding fell from 276 ± 23 fmol mg^{-1} protein at time 0 (equilibrium) to 60 ± 21 fmol mg^{-1} protein at 90 min ($P < 0.01$).

Measurement of the non specific binding of [^{3}H]-lacidipine and [^{3}H]-isradipine in intact rat aorta

The non specific binding of [^{3}H]-lacidipine and [^{3}H]-isradipine was measured in intact rat aorta. The preparations were incubated in the presence of nifedipine (1 μM) and either [^{3}H]-lacidipine (77.6 ± 1.9 pM) or [^{3}H]-isradipine (47.2 ± 0.4 pM) for 120 min as shown in Figure 6. The experimental protocol was similar to that followed in the functional experiments summarized above. After incubation with [^{3}H]-lacidipine, the non specific binding was 15.15 ± 1.18 fmol mg^{-1} wet wt. and was still 7.24 ± 0.61 fmol mg^{-1} wet wt. after 3.5 h washout in drug-free solution. After incubation with [^{3}H]-isradipine the non specific binding was 2.26 ± 0.07 fmol mg^{-1} wet wt. and it was no longer detectable after 3.5 h washout in a drug-free solution.

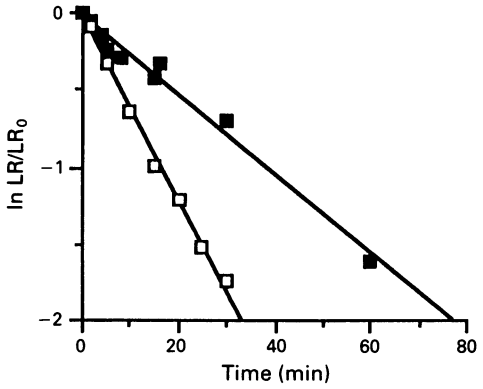


Figure 5 Semilogarithmic dissociation plots of [^{3}H]-lacidipine (■) and [^{3}H]-isradipine (□) in light microsomes from rat ileum. LR = complex ligand-receptor at a given time of the dissociation process; LR_0 = complex ligand-receptor at equilibrium, before starting the dissociation. After 1 h incubation with the tritiated ligand, the isotopic dilution (about 10,000 fold) was carried out by adding nifedipine (1 μM final concentration). Each point is the mean from 4–8 measurements. The calculated k_{-1} and $t_{1/2}$ values are given in the text.

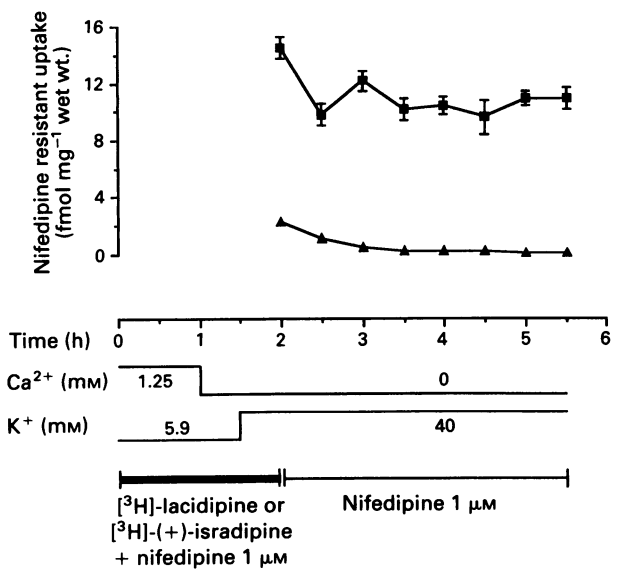


Figure 6 Washout of the non specific binding of [^{3}H]-lacidipine (■) and [^{3}H]-isradipine (▲) in intact rat aorta. Aortic rings were preincubated for 60 min in the presence of [^{3}H]-lacidipine (77.6 ± 1.9 pM) or [^{3}H]-isradipine (47.2 ± 0.4 pM) and nifedipine (1 μM) in physiological solution. They were then incubated for 30 min in zero Ca^{2+} physiological solution and for 30 min in zero Ca^{2+} 40 mM KCl-depolarizing solution containing the same concentration of tritiated calcium antagonists and nifedipine. At the end of this incubation time, some samples were taken from the incubation medium to measure the amount of bound radioactivity, while the others were submitted to a washout in a [^{3}H]-lacidipine- and [^{3}H]-isradipine-free zero Ca^{2+} 40 mM KCl-depolarizing solution, containing nifedipine (1 μM). Each 30 min a number of samples were taken out of incubation medium to measure the amount of bound radioactivity. Each point is the mean ± s.e.mean of 4–8 measurements.

Discussion

The mode of inhibition of K^+-evoked contraction by lacidipine appeared to be time-dependent, it increased slowly after the initiation of depolarization, despite a prolonged preincubation in physiological solution with the drug. Such a time-related increase in inhibition has already been observed by us for isradipine, nisoldipine and nitrendipine and has been related to an increase in the affinity of dihydropyridine binding, reflecting the inactivation of Ca^{2+} channels evoked by prolonged depolarization (Morel & Godfraind, 1987; 1988; Wibo *et al.*, 1988; Salomone *et al.*, 1991). Radioligand studies in intact arteries have shown that the binding affinity of (+)-isradipine and nisoldipine is increased by depolarization, whereas the binding of nifedipine is almost unaffected. When a given dihydropyridine is considered, the time-dependency of K^+-contraction depends on the ratio of its dissociation constants for inactivated (K_{Di}) and resting (K_{Dr}) L-type calcium channels (Morel & Godfraind, 1987; 1988). Different K_{Di} and K_{Dr} have also been described in patch clamp studies, with dihydropyridines such as nisoldipine (Sanguinetti & Kass, 1984), nifedipine (Uehara & Hume, 1985) and nitrendipine (Bean *et al.*, 1986), and the ratio K_{Dr}/K_{Di} may vary by two orders of magnitude according to the molecule considered. For nisoldipine the reported K_{Dr}/K_{Di} ratio is about 1500, whereas for nifedipine it is 10. Assuming that lacidipine binds with a much higher affinity to the inactivated than to the resting channels, we analysed the onset of inhibition of contraction of depolarized rat aorta according to a pseudo-first order association kinetics. This allowed us to estimate a dissociation rate constant of lacidipine from contraction experiments in rat aorta (0.0310 min^{-1}) similar to the value obtained in radioligand binding experiments on light microsomal fractions of rat ileum (0.0257 min^{-1}). As expected, the dissociation rate constant of [^{3}H]-isradipine in rat ileal membranes (0.0595 min^{-1}) was similar to the value observed previously in microsomes from rat aorta (0.052 min^{-1}; Wibo *et al.*, 1988).

The observation that inhibition by lacidipine was reduced upon repolarization further confirms that lacidipine could interact reversibly with dihydropyridine binding sites in intact depolarized aorta, despite the apparent irreversibility of its action during washout in drug-free solution. The interaction of lacidipine with calcium channels was reversible in plasma membrane preparations; therefore, the non-reversible inhibition observed after lacidipine-treatment of depolarized aorta could be due to the non specific binding observed in whole tissue and in the heavy membrane fractions. This non specific binding was related to the concentration of lacidipine in the preincubation solution (data not shown), as was the resulting persistent inhibition. The observed difference between IC_{50} measured in whole tissue and K_D of lacidipine estimated in membranes may be related to this non specific binding which could reduce the drug concentration in the vicinity of binding sites, a hypothesis consistent with the model proposed by Kenakin (1984), according to which the uptake of a drug in a tissue store may affect the functional interaction with its receptors, usually reducing its apparent affinity. Furthermore, the non specifically bound drug is likely to be responsible for the apparent irreversible effect of lacidipine after extensive rinsing. This hypothesis strengthens the interest of studies on the interaction of lacidipine with some components of cellular membranes. For instance, it has been shown by Herbette *et al.* (1992) that the lacidipine partition coefficient in bovine cardiac phosphatidylcholine membrane vesicles is 340,000, as compared with only 5,800 for isradipine (Mason *et al.*, 1992). Furthermore, the lacidipine partition coefficient decreases ten fold when the cholesterol:phospholipid molar ratio is increased from 0 to 0.6. It is noteworthy that, in the present study, the non specific binding of [^{3}H]-lacidipine was not detectable in light microsomes from rat aortic membranes, a fraction where plasma membrane fragments, which are especially rich in cholesterol, are appreciably purified (Wibo *et al.*, 1980; Morel *et al.*, 1981). Rhodes *et al.* (1985) have proposed that 1,4-dihydropyridines could reach their specific binding sites by a lipophilic 'membrane approach'. At first sight, this hypothesis does not seem to be consistent with the interaction of compounds with a quaternary ammonium group with 1,4-dihydropyridine binding sites (Kass *et al.*, 1991; Feron *et al.*, 1992). We have previously shown that the binding of 1,4-dihydropyridines may be displaced by pinaverium, a compound containing a quaternary ammonium group (Feron *et al.*, 1992). This indicates that binding sites could be accessible from the aqueous phase. This, however, does not preclude the possibility that lacidipine could also reach its specific binding sites by a lipophilic 'membrane approach'.

In conclusion, the apparently-irreversible inhibition observed after treatment of isolated arteries with lacidipine does not reflect an irreversible binding to L-type Ca channels, but is probably related to a non specific binding of lacidipine occurring in membranes poor in cholesterol and different from the plasma membrane.

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Effects of pH on the actions of dizocilpine at the N-methyl-D-aspartate receptor complex

Sunita Rajdev & [1]Ian J. Reynolds

Department of Pharmacology, University of Pittsburgh, Pittsburgh PA 15261, U.S.A.

1 The effects of varying pH from 6.5 to 7.4 and 8.0 on the actions of dizocilpine (MK801) on the N-methyl-D-aspartate (NMDA) receptor were investigated by use of a [^{3}H]-dizocilpine binding assay and NMDA-induced intracellular free Ca^{2+} ($[Ca^{2+}]_i$) increases in cultured forebrain neurones.

2 Increasing pH from 6.5 to 8.0 significantly increased the rate of association and dissociation of [^{3}H]-dizocilpine. The association process was better described by two rate constants under each condition, while only dissociation of [^{3}H]-dizocilpine at pH 8.0 required two rate constants adequately to describe the curve. Equilibrium affinity of [^{3}H]-dizocilpine was not altered by changing pH from 6.5 to 8.0.

3 NMDA and glycine together increased $[Ca^{2+}]_i$ measured by fura-2 microspectrofluorimetry in single cultured neurones from rat forebrain. Compared to control response measured at pH 7.4, the combined effects of NMDA and glycine were reduced to 38.9% of control values by lowering pH to 6.5 and increased to 148.9% by raising pH to 8.0.

4 Dizocilpine (200 nM) effectively reversed increases in $[Ca^{2+}]_i$ produced by NMDA together with glycine. The rate of reversal produced by this concentration of dizocilpine was considerably slower than that required for cells to recover to baseline following agonist removal. The rate at which dizocilpine reversed NMDA- and glycine-induced $[Ca^{2+}]_i$ increases was not altered by raising pH from 6.5 to 8.0.

5 These data support the hypothesis that the rates associated with [^{3}H]-dizocilpine binding are controlled by the level of activation of the receptor. In addition, these data confirm previous findings that NMDA responses are sensitive to small changes in pH. However, the lack of sensitivity to pH changes of dizocilpine inhibition of NMDA responses show that other factors are also important in controlling the action of channel-blocking NMDA receptor antagonists.

Keywords: NMDA receptor; pH; [^{3}H]-dizocilpine binding; intracellular Ca^{2+}; fura-2

Introduction

The N-methyl-D-aspartate (NMDA)-preferring glutamate receptor is a highly modulated ligand gated ion channel. Included in the list of possible endogenous regulators are Mg^{2+} (Nowak *et al.*, 1984; Mayer *et al.*, 1984), Zn^{2+} (Peters *et al.*, 1987; Westbrook & Mayer, 1987), glycine (Johnson & Ascher, 1987), polyamines (Ransom & Stec, 1988), oxygen radicals (Aizenman *et al.*, 1990), H^+ ions (Traynelis & Cull-Candy, 1990; Tang *et al.*, 1990) and neurosteroids (Wu *et al.*, 1991). In addition, a number of drugs bind to sites on the NMDA receptor that may be distinct from the endogenous modulator sites, such as the dissociative anaesthetics phencyclidine, ketamine and dizocilpine (Anis *et al.*, 1983; Honey *et al.*, 1985; Wong *et al.*, 1986).

Drugs that bind to the dissociative anaesthetic site are valuable biochemical tools for probing drug interactions with the NMDA receptor, as these agents have proved to be the most potent NMDA receptor-specific drugs. The dissociative anaesthetic site is believed to exist within the NMDA-activated ion channel (MacDonald & Nowak, 1990). An important consequence of this locus of action is that the binding of phencyclidine-like drugs requires the presence of agonists to activate the receptor (MacDonald *et al.*, 1987; Huettner & Bean, 1988). This use-dependence can be demonstrated both functionally with electrophysiological techniques (MacDonald *et al.*, 1987; Huettner & Bean, 1988), and also in receptor binding assays where the rate of binding and unbinding of, for example, [^{3}H]-dizocilpine ([^{3}H]-MK801) can be modified by the addition of NMDA and glycine site agonists (Loo *et al.*, 1986; Bonhaus & McNamara, 1988; Kloog *et al.*, 1988; Reynolds & Miller, 1988a; Javitt & Zukin, 1989). Indeed, results from ligand binding assays appear to correspond well with a model of ligand binding to transiently accessible binding sites proposed by Starmer and colleagues (Starmer *et al.*, 1987). According to this model, altering access to a ligand binding site changes the rate of association and dissociation but has no effect on equilibrium affinity of ligand binding.

Although a number of studies have demonstrated an increase in binding rates upon addition of agonists to [^{3}H]-dizocilpine binding assays, precise quantitation of rates has proved difficult in some circumstances because the rate of binding in the absence of agonists is very slow ($t/2 > 12$ h). Furthermore, in addition to investigating the mechanism of action of well-established NMDA receptor modulators like Zn^{2+} (Reynolds & Miller, 1988a), measuring the rate of [^{3}H]-dizocilpine binding has been used to propose a mechanism of action for novel, poorly understood modulators like the polyamines (Williams *et al.*, 1990; Reynolds & Miller, 1989). However, in these studies the effects of modulators are measured in the presence of maximal agonist concentrations and these studies have generally lacked positive controls in the form of clearly established channel modulation that is independent of changes in NMDA and/or glycine concentration.

To test the hypothesis critically that the rate of ligand binding to the dissociative anaesthetic site is controlled by the level of channel activation, we measured the effects of pH on the rate of dizocilpine binding. Several studies have shown that raising pH from 6.5 to 8.0 increases NMDA responses by increasing the amount of time the channel spends in the open state (Traynelis & Cull-Candy, 1990; 1991; Tang *et al.*, 1990). As the effect of pH is independent of the concentration of NMDA or glycine, this provides an ideal opportunity to test the hypothesis without resorting to excessive incubation times. To provide a functional correlate of the ligand

[1] Author for correspondence.

binding studies, we also measured the rate at which dizocilpine reversed NMDA-induced increases in $[Ca^{2+}]_i$ in single neurones.

Methods

Binding assays

Radioligand binding assays using [^{3}H]-dizocilpine (28 Ci mmol^{-1}, Du Pont/New England Nuclear, Boston, MA, U.S.A.) were performed with well washed membranes prepared from frozen rat brains as previously described (Reynolds & Palmer, 1991). Association rates were determined using a final concentration of [^{3}H]-dizocilpine of 3 nM (1 nM [^{3}H]-dizocilpine + 2 nM unlabelled ligand), about 150 μg membrane protein, 100 μM glutamate, 30 μM glycine in 1 ml HEPES with the pH adjusted to 6.5, 7.4 or 8.0 by use of NaOH at room temperature (20–22°C); 22 time points were measured between 0.5 and 240 min. Non-specific binding, defined by 30 μM dizocilpine, was determined at 0.5 min and 240 min and found to be the same, so the former was used. After incubation for the appropriate period of time, assays were terminated by vacuum filtration over glass fibre filters and radioactivity determined by liquid scintillation counting. For dissociation assays, tissue (about 4 mg ml^{-1}) was incubated with 4 nM [^{3}H]-dizocilpine for 2 h; 25 μl aliquots were then added to 3 ml buffer containing 100 μM glutamate, 30 μM glycine at the appropriate pH. Incubations were continued for 5–180 min before filtration and counting as described above. Saturation curves were performed with a single concentration of [^{3}H]-dizocilpine (0.05 nM) in 3 ml buffer of the appropriate pH that contained about 100 μg ml^{-1} membrane protein and 100 μM glutamate and 30 μM glycine, and unlabelled dizocilpine from 0.08–10 nM. Tubes were incubated for 4 h before filtration and scintillation counting.

$[Ca^{2+}]_i$ assays

$[Ca^{2+}]_i$ was determined in single neurones cultured from e17 foetal rat forebrain using the Ca^{2+}-sensitive fluorescent dye fura-2 (Grynkiewicz *et al.*, 1985) as previously described (Reynolds & Aizenman, 1992). Cells were loaded with 5 μM fura-2AM (Molecular Probes, Eugene, OR, U.S.A.) for about 60 min at 37°C. Coverslips containing neurones were then mounted in a recording chamber and perfused with buffer of the following composition (mM): NaCl 137, KCl 5, $MgSO_4$ 0.9, $CaCl_2$ 1.4, $NaHCO_3$ 3, Na_2HPO_4 0.6, KH_2PO_4 0.4, glucose 5.6, and HEPES 20, pH adjusted to 7.4 with NaOH, at a flow rate of 20 ml min^{-1}, which represents about 20 bath volume changes per min. NMDA and glycine were used at 30 μM and 1 μM respectively, throughout this study. To measure the effects of dizocilpine, NMDA and glycine were added and the response allowed to reach a stable maximum. Dizocilpine (200 nM) was then added and the response followed until a new plateau was reached. It should be noted that fura-2 is relatively insensitive to pH over the range used in the present study (Grynkiewicz *et al.*, 1985).

Data analysis

Data from association and dissociation experiments were fitted with a non-linear, least squares curve fitting routine (KINETIC, Elsevier, Biosoft, New York, NY, U.S.A.). Two phase fits are reported when they consistently provide a significantly better fit of the data ($P<0.05$, partial F test). Saturation data were fitted using EBDA and LIGAND (Elsevier Biosoft, New York, NY, U.S.A.). Rate constants for the reversal of NMDA- and glycine-induced $[Ca^{2+}]_i$ increases were determined by fitting $[Ca^{2+}]_i$ values obtained after the addition of dizocilpine and before the washout of agonists to a single exponential decay equation of the form $Y = P.e^{(-k.t)} + B$, where P is the peak $[Ca^{2+}]_i$ value and B is the basal $[Ca^{2+}]_i$ value at the point of full reversal. Typically, 300–500 time points were fitted to this equation using the InPlot package (GraphPad Software, San Diego, CA, U.S.A.). It was often possible to fit these data to two or more exponentials with a significantly better fit. Visual inspection of the curves (e.g. Figure 4) suggested that multiple site fits did not provide a markedly improved description of the inhibition process, so single site fits are reported here.

Results

We first examined the effects of pH on [^{3}H]-dizocilpine association kinetics (Figure 1). At pH 7.4, [^{3}H]-dizocilpine bound with biphasic association kinetics as we have previously noted (Reynolds, 1992), with approximately 20% of the total binding in the fast phase (Table 1). Increasing the pH to 8.0 significantly increased the slow association rate compared to pH 6.5 and 7.4, while having no significant effect on the fraction of binding in the fast phase. [^{3}H]-dizocilpine binding at pH 6.5 was clearly much slower than that observed at the higher pH values, and did not reach equilibrium within the 4 h time period used. For this reason it is necessary to interpret the increase in the fast association rate with caution.

Increasing the pH also produced marked effects on the dissociation of [^{3}H]-dizocilpine (Figure 2). At pH 6.5 and 7.4 the dissociation of [^{3}H]-dizocilpine was well described by a single exponential, as previously observed. Increasing pH from 6.5 to 7.4 produced a significant increase in the dissociation rate (Table 1). Raising the pH to 8.0 resulted in a biphasic dissociation curve. Both of the observed rates were apparently faster than those observed at the lower pH values but it is obviously inappropriate to make a statistical comparison of these rates. Nevertheless, the predominant effect of increasing the pH was to increase the observed rate constant and to increase the dissociation rate of [^{3}H]-dizocilpine.

We next examined the effects of pH on [^{3}H]-dizocilpine saturation parameters. Saturation data were well fitted to a single site. Increasing pH had no effect on the affinity of [^{3}H]-dizocilpine, or on the density of [^{3}H]-dizocilpine binding sites (Table 1). We have previously observed that combining the dissociation rate with the slow association rate gives a kinetically derived value for the dissociation constant that is similar to that obtained by saturation studies. This was also true in this study at pH 7.4, where the values were 0.91 and

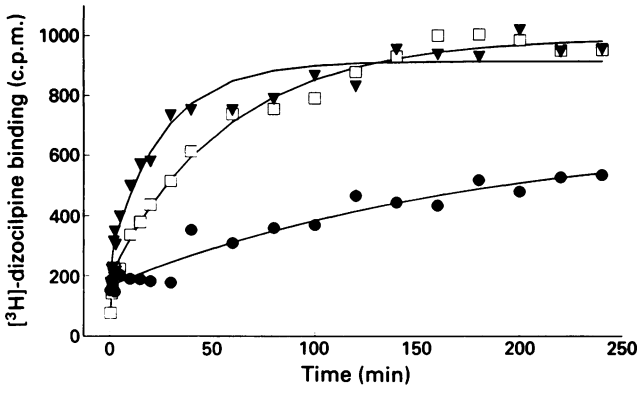


Figure 1 Increasing pH from 6.5 to 8 accelerated [^{3}H]-dizocilpine association. The binding of [^{3}H]-dizocilpine at pH 6.5 (●) was slow and did not reach equilibrium within 4 h. Increasing the pH to 7.4 (□) or 8.0 (▼) significantly increased the rate of binding without changing the level of binding at equilibrium. The association of [^{3}H]-dizocilpine was found to be best described by two rates, which are given in Table 1. The symbols represent data points, while the curves reflect the curve fitted data. The graph represents a typical experiment that was repeated 5 additional times with similar results.

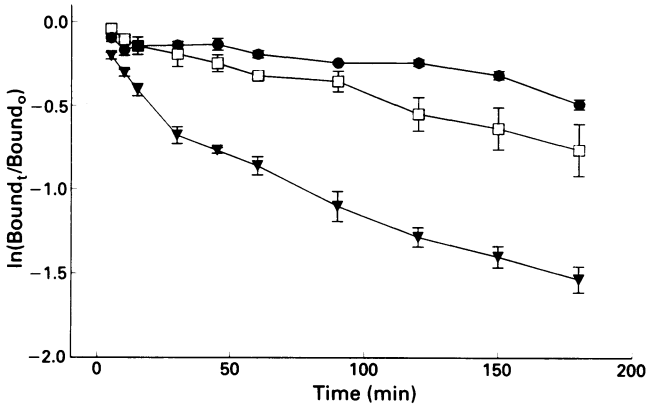


Figure 2 Increasing pH from 6.5 to 8 accelerated [^{3}H]-dizocilpine dissociation. The dissociation of [^{3}H]-dizocilpine was slowest at pH 6.5 (●) and was progressively accelerated by increasing the pH to 7.4 (□) and 8.0 (▼). The dissociation at pH 8 was better described by two exponentials in most experiments, while at pH 6.5 and 7.4 two exponentials did not provide a significantly better fit. These data represent the mean (± s.e.mean) of 5 experiments.

1.20 nM for kinetically and saturation-derived parameters respectively.

We next investigated the effects of pH on the ability of dizocilpine to inhibit NMDA- and glycine-induced $[Ca^{2+}]_i$ changes in cultured neurones. NMDA (30 μM) added together with 1 μM glycine produced prompt increases in $[Ca^{2+}]_i$ that were rapidly reversible (Figure 3) by superfusion with agonist-free buffer. After initial responses were obtained at pH 7.4, cells were superfused in buffer at pH 6.5 or 8.0. Reducing the pH from 7.4 to 6.5 decreased $[Ca^{2+}]_i$ changes from 475 ± 57 to 185 ± 23 nM ($P<0.05$, paired t test, $n=9$). Conversely, increasing the pH from 7.4 to 8.0 resulted in mean $[Ca^{2+}]_i$ changes increasing from 258 ± 33 to 384 ± 27 nM ($P<0.05$, paired t test, $n=10$).

Addition of NMDA and glycine for longer periods usually results in $[Ca^{2+}]_i$ response that reached a plateau level and remained stable. Addition of 200 nM dizocilpine to the stable response resulted in reversal of the $[Ca^{2+}]_i$ increase to near baseline concentrations (Figure 3). There was not a significant difference between the degree of inhibition of

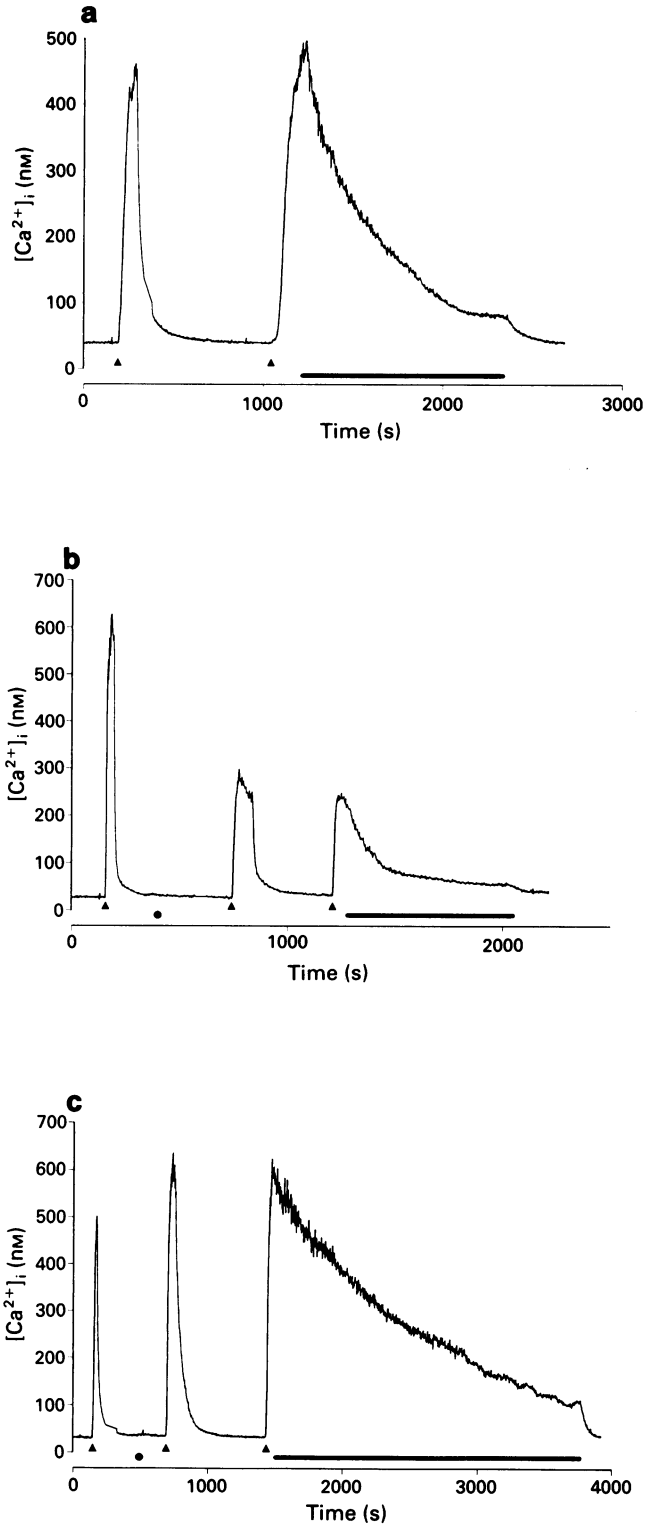


Figure 3 Effects of pH on the block of NMDA- and glycine-induced $[Ca^{2+}]_i$ increases by dizocilpine. In each trace the first response was measured at pH 7.4. (a) After obtaining a control response, a second prolonged application of NMDA and glycine was obtained, after which 200 nM dizocilpine was added, as indicated by the bar. The response was then followed until a new plateau was obtained. When the pH was changed to (b) 6.5 or (c) 8.0, as indicated by (●), a second transient response was obtained to measure the effects of pH on the initial response prior to monitoring the effects of dizocilpine, which was added as indicated by the solid bar. These records were obtained in single cells and are representative of results obtained in 8–9 additional cells at each pH value.

Table 1 Effects of pH on rates of [^{3}H]-dizocilpine binding and saturation parameters

pH	k_{-1} (× 10^3, min^{-1})	*% fast*	K_{obs} (min^{-1})	*% fast*	k_D (nM)	B_{max} (pmol mg^{-1} prot)
6.5 Fast	1.64 ± 0.17	–	2.77 ± 0.56	16.1 ± 3.3	3.62 ± 1.91	0.51 ± 0.24
Slow			0.0147 ± 0.006			
7.4 Fast	3.85 ± 0.22	–	1.08 ± 0.24*	20.6 ± 1.8	1.20 ± 0.08	0.52 ± 0.05
Slow			0.0165 ± 0.003			
8.0 Fast	64.3 ± 19.5	44.2 ± 4.7	1.33 ± 0.20	27.4 ± 3.4	1.48 ± 0.24	0.54 ± 0.11
Slow	5.00 ± 0.71		0.0352 ± 0.004*,**			

The data represent the mean (± s.e.mean) of 4–6 experiments. k_{-1} is the dissociation rate constant, while K_{obs} is the observed association rate of [^{3}H]-dizocilpine using 3 nM ligand.
Significantly different from pH 6.5 condition (*$P<0.05$, ANOVA with Bonferroni correction).
Significantly different from pH 7.4 condition (**$P<0.05$, t test)

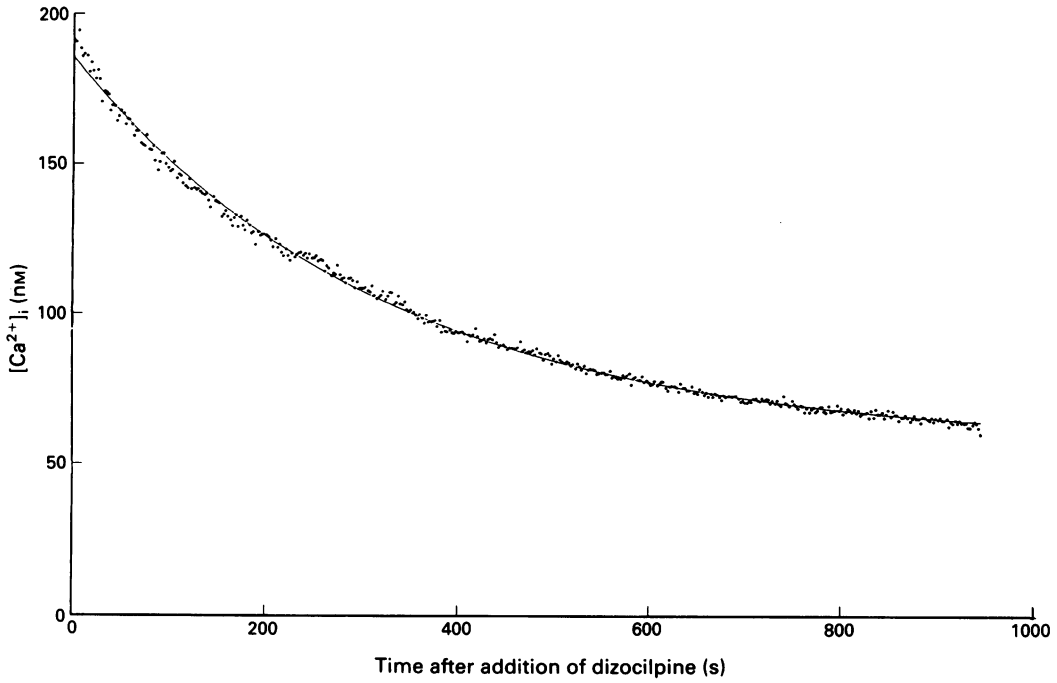


Figure 4 Curve fitting of the effects of dizocilpine on NMDA responses. The points represent the actual data, which are $[Ca^{2+}]_i$ values measured approximately 2 s apart. The zero time point represents the addition of 200 nM dizocilpine. The solid line is the curve fitted data. These data were generated in a neurone superfused with buffer at pH 7.4 and is representative of a total of 21 cells used at the three different pH values.

Table 2 Effects of pH on the inhibition of NMDA-induced increases in $[Ca^{2+}]_i$ by dizocilpine.

		Recovery rate (min^{-1})	
pH	*% control*	*Agonist washout*	*Dizocilpine* (200 nM)
6.5	16.5 ± 3.6	3.98 ± 0.94	0.283 ± 0.052
7.4	7.8 ± 2.1	3.77 ± 0.62	0.385 ± 0.176
8.0	17.1 ± 5.2	2.91 ± 0.45	0.197 ± 0.046

% control represents the difference between the peak $[Ca^{2+}]_i$ response measured at the time of addition of 200 nM dizocilpine, and the response remaining at the time the agonists were washed out. The data represent the mean (± s.e.mean) of 9–10 cells measured at each pH value.

NMDA response at the three pH values examined (Table 2). We also measured the rate at which dizocilpine reversed the response to NMDA and glycine (Table 2). Although the rate of inhibition by dizocilpine was considerably slower than that observed following agonist removal, indicating that Ca^{2+} homeostasis processes were not the rate limiting step, the rate at which dizocilpine blocked responses was not altered by changes in pH. An example of the curve fitting used is shown in Figure 4.

Discussion

In this series of experiments we have examined critically the hypothesis that the action of NMDA receptor antagonists that bind to a site located within the ion channel is dependent on the degree of channel activation. To test this hypothesis, we varied the pH, as this has been shown to alter the level of NMDA receptor activation in response to a given agonist concentration without major effects on other parameters (Traynelis & Cull-Candy, 1991). In this way, we were able to monitor effects on the rate of binding of dizocilpine without altering agonist concentrations, which has previously been shown to slow considerably the rates of association and dissociation (Bonhaus & McNamara, 1988; Kloog *et al.*, 1988; Reynolds & Miller, 1988a; Javitt & Zukin, 1989). Using this approach, we were able to demonstrate that, as predicted, decreasing receptor activation (by lowering pH) results in slower ligand association and dissociation, while increasing the amount of time the channel spends open (by increasing pH) results in an increase in the rate of association and dissociation. However, the alterations in rates of binding produced by changes in pH do not result in altered equilibrium affinity, which is consistent with a previously proposed model of ligand binding to this site (Bonhaus & McNamara, 1988).

The biphasic association process that we describe here has been described previously (Javitt & Zukin, 1989; Williams *et al.*, 1990; Reynolds, 1992). It has been suggested that the rapid phase of association corresponds to [^{3}H]-dizocilpine binding to the open channel, while the slow phase arises from a different route by which [^{3}H]-dizocilpine approaches its binding site (Javitt & Zukin, 1989). However, it is noteworthy that kinetically derived affinity constants determined from the slow phases of [^{3}H]-dizocilpine binding (association and dissociation) correspond very closely to those obtained from saturation analysis, whereas those generated from fast association rates usually give much higher affinities than are actually measured (Reynolds, 1992). This suggests that the pharmacologically well-characterized [^{3}H]-dizocilpine binding site, which appears to show the characteristics of the activated NMDA receptor (Foster & Wong, 1987), is the one that corresponds to the slow phase of binding. Conversely, we have previously noted that the fast association rate observed in [^{3}H]-dizocilpine binding studies is similar to that observed with electrophysiological approaches (Reynolds, 1992). Thus, it is difficult to provide a precise functional correlate to the observation that we have made in the present study that the slow observed association rate was accelerated somewhat by increasing pH.

It is interesting to compare the results obtained in the present study to those generated using polyamines to modify [^{3}H]-dizocilpine binding (Random & Stec, 1988). As the mechanism of action of polyamines is poorly understood, several laboratories have used the [^{3}H]-dizocilpine binding assay to gain insight into the functional effects that

polyamines may produce on the NMDA receptor (Reynolds & Miller, 1989; Williams *et al.*, 1990; Sacaan & Johnson, 1990). It appears that a principle effect of a selective polyamine agonist, namely 1,5-diethylaminopiperidine, on the association of [^{3}H]-dizocilpine is to increase the fraction of binding residing the fast phase (Reynolds, 1992). In addition, there was a small increase in the rate associated with the slow phase of binding. Most notable, however, was a decrease in the dissociation rate, and a corresponding increase in the equilibrium affinity. As both studies were performed under conditions that did not involve changing agonist concentrations it seems fair to compare the results with respect to drawing mechanistic conclusions. Thus, it seems unlikely that the principle, arcaine-sensitive effect of polyamines occurs through a simple increase in receptor activation, because the profile of effects of polyamines on the kinetics of [^{3}H]-dizocilpine are so different from the effects of altered pH. Spermidine has been shown to increase both the rate of association and dissociation of [^{3}H]-dizocilpine (Reynolds, 1990; Williams *et al.*, 1990). However, the actions of spermine and spermidine on the dissociation of [^{3}H]-dizocilpine were not blocked by arcaine, suggesting that this phenomenon is not mediated by the same site that increases [^{3}H]-dizocilpine binding (Reynolds, 1990).

To provide a correlate to the ligand binding studies, we monitored the effects of pH on the ability of dizocilpine to block NMDA-induced increases in $[Ca^{2+}]_i$, which, as the NMDA receptor is Ca^{2+} permeable (MacDermott *et al.*, 1986; Murphy *et al.*, 1987), should provide a direct indication of NMDA receptor activation. We were able to confirm previous findings that increasing pH between 6.5 and 8.0 enhances NMDA-induced $[Ca^{2+}]_i$ changes (Takadera *et al.*, 1992). Moreover, we did not find any evidence to suggest that changing extracellular pH had any effect on the ability of neurones to clear Ca^{2+} from the cytoplasm, as indicated by the identical rates of recovery of responses following washout of agonists (Table 2). Dizocilpine was an effective antagonist of NMDA responses at each pH value tested; this is in accord with the prediction that would have been made from the finding that the equilibrium affinity of [^{3}H]-dizocilpine was not changed by pH. However, we were surprised to find that the rate of block of NMDA responses by dizocilpine was completely insensitive to pH. Indeed, after accounting for the difference in concentrations of dizocilpine used in the two experimental approaches, the rate of inhibition of NMDA responses by dizocilpine was some 5–20 fold slower than would have been predicted from the ligand binding experiments.

There are obviously many differences in the conditions used for the receptor binding studies compared to the physiological assay. There is unlikely to be a membrane potential across the homogenized membrane fragments. However, although the reversal of the effects of dizocilpine is sensitive to membrane potential, this is apparently not true for the blocking effects (Huettner & Bean, 1988). There is also a substantial difference in the ionic composition of the buffers used for the two types of experiment. In the ligand binding assays low osmotic strength buffers are used, and divalent cations are specifically excluded from assays as they tend to inhibit binding at physiological concentrations (Reynolds & Miller, 1988b). It is difficult to control fully cation levels in the physiological experiments, however, because a certain amount of Ca^{2+} is necessary to produce responses and the inside of the cell was not readily accessible in these studies, making it difficult to control or exclude the effects of intracellular Mg^{2+}. It is noteworthy that both Ca^{2+} and Mg^{2+} at the concentrations likely to be present would both accelerate the binding process rather than slow it (Reynolds & Miller, 1988a; Williams *et al.*, 1990), which suggests that the slower binding rates seen in the fura-2 experiments are not the result of the effect of these cations. One can postulate either a tonic inhibitory effect by Zn^{2+} in the physiological assay, which is known to slow the binding of [^{3}H]-dizocilpine (Reynolds & Miller, 1988a), or else it is possible that [^{3}H]-dizocilpine might bind to a desensitized or otherwise distinct state of the NMDA receptor in the ligand binding assay compared to the normal physiological state. It has also been shown that the use-dependence of channel-blocking NMDA receptor antagonists is sensitive to temperature (Davies *et al.*, 1988). However, this is unlikely to be a significant factor in the present experiments as both studies were performed at the same temperature. Regardless of the precise difference between the two experimental conditions, the important therapeutic consequence of these findings is that dizocilpine should be equally effective as an NMDA receptor antagonist under normal and acidotic conditions, which is notable given the existence of an acidotic state following cerebral ischaemia.

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Calcium antagonistic and antiarrhythmic actions of CPU-23, a substituted tetrahydroisoquinoline

[1]Hui Dong, [2]Jian-Zhong Sheng, [3]Chi-Ming Lee & [4]Tak Ming Wong

Department of Physiology, Faculty of Medicine, University of Hong Kong, Sassoon Road, Hong Kong

1 The effects of CPU-23 (1-{1-[(6-methoxyl)-naphth-2-yl]}-propyl-2-(1-piperidine)-acetyl-6,7-dimethyoxy-1,2,3,4-tetra-hydroisoquinoline) were studied on mechanical and electrical activities, and intracellular free calcium ($[Ca^{2+}]_i$) of isolated cardiac tissues in order to investigate its spectrum and mechanisms of action in the heart. Its antiarrhythmic and haemodynamic effects in pentobarbitone-anaesthetized rats subjected to coronary artery ligation were also evaluated.

2 CPU-23 at 10^{-6}–10^{-4} M markedly inhibited slow action potential characteristics in guinea-pig papillary muscles and pace-maker action potential of rabbit sinoatrial node. It affected fast action potential only at 10^{-4} M. None of the effects of CPU-23 was reversed by washout for up to 2 h.

3 Like nifedipine and diltiazem, CPU-23 decreased the heart rate of the isolated perfused heart of the rat. However, in contrast to these two classical calcium antagonists which dose-dependently inhibited the force of contraction, CPU-23 inhibited and stimulated the force of contraction at 10^{-7}–3×10^{-6} M and 10^{-5} M, respectively.

4 CPU-23 at 10^{-6}–10^{-5} M inhibited the KCl-induced $[Ca^{2+}]_i$ increase in the Ca^{2+} medium, but did not affect the caffeine-induced $[Ca^{2+}]_i$ increase in the Ca^{2+}-free medium in isolated ventricular myocytes.

5 CPU-23 at 1–5 mg kg^{-1} reduced dose-dependently ventricular arrhythmias including ventricular ectopic beats, VT and VF as well as mortality during coronary artery ligation. At 2.5–5 mg kg^{-1} it even abolished VF, which was accompanied by 100% survival.

6 It is suggested that CPU-23 has calcium antagonistic properties in cardiac tissues. It selectively blocks the transmembrane influx of extracellular Ca^{2+} through Ca^{2+} channels, thus reducing the heart rate and developed tension, altering the slow action potential characteristics and producing antiarrhythmic effect against ischaemic arrhythmias.

Keywords: CPU-23; calcium; papillary muscle; sinoatrial node; isolated heart; arrhythmias; coronary artery ligation; heart rate; action potential; blood pressure

Introduction

Calcium antagonists are increasingly popular for treatment of various cardiovascular disorders including various cardiac arrhythmias in recent years. Their therapeutic use is based on their interference with voltage-dependent calcium channels in cardiac muscle and vascular smooth muscle (Fleckenstein, 1977; Conti *et al.*, 1985). The search for novel calcium antagonists with distinctive qualities has grown in the last few years and numerous new ones have been developed (Freedman & Waters, 1987; Ohtsuka *et al.*, 1989). With large numbers of calcium antagonists developed, several classifications have been proposed (Nayler, 1988), including one by a WHO committee (Vanhoutte & Paoletti, 1987). Many of these classifications require the use of electrophysiological or biochemical techniques to prove their ability to block specifically calcium entry into myocardial cells.

In a previous study, with a [^{3}H]-nitrendipine binding assay we screened a series of substituted tetrahydroisoquinolines in order to develop novel calcium antagonists based on the lead compound, tetrandrine, isolated from a Chinese medicinal herb, *Stephania tetrandra* (Huang *et al.*, 1990). CPU-23 (1-{1-[(6-methoxyl)-naphth-2-yl]}-propyl-2-(1-piperidine)-acetyl-6,7-dimethyoxy-1,2,3,4-tetra-hydroisoquinoline), the most potent compound identified in this series, was found to inhibit KCl-induced contraction of rat aorta and displace [^{3}H]-nitrendipine binding in rat cerebral cortical membranes with similar potency (Dong *et al.*, 1992). It was also found that CPU-23 behaves as a simple competitive inhibitor at the [^{3}H]-nitrendipine binding sites (Dong *et al.*, 1992). In addition, it induces hypotension and bradycardia in rats (Dong *et al.*, 1992). It was therefore suggested that CPU-23 may exert its cardiovascular effects in inhibiting calcium influx via specific calcium channels probably L-type calcium channels (Dong *et al.*, 1992). The purpose of the present study was to characterize further the calcium antagonistic actions of CPU-23 by studying its effects on heart rate and developed tension in rat isolated hearts, action potential characteristics of guinea-pig papillary muscles and rabbit sinoatrial node, and KCl-induced increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in isolated ventricular myocytes. We also investigated antiarrhythmic and haemodynamic actions of CPU-23 in anaesthetized Sprague Dawley rats subjected to coronary artery ligation.

Methods

Langendorff perfused rat heart

Hearts were obtained from male Sprague Dawley rats weighing 200–250 g. The animals were anaesthetized with pentobarbitone (75 mg kg^{-1}, i.p.) and injected with heparin (25 u, i.p.). They were killed by cervical dislocation 10 min later. The hearts were rapidly excised and perfused through the aortic stump with a normal Krebs-Henseleit solution aerated with 95% O_2 and 5% CO_2, pH 7.4, under a constant pressure (65 mmHg) and at 34°C. The spontaneously beating preparations were allowed to adapt to the new environmental conditions for 20 min. A pressure transducer (Gould p23 ID) was connected to one opening of a three-way stopcock, the other

Present address: [1] Department of Biophysics, Faculty of Basic Medical Sciences, Shanghai Medical University, Shanghai, China; [2] Department of Physiology, Zhejiang Medical University, Hangzhou, China; [3] Neuroscience Area, D47H, AP10, Abbott Laboratories, One Abbott Park, Illinois 60064, U.S.A.
[4] Author for correspondence.

two openings of which were connected to the aortic stump of the heart and the perfused solution, respectively. The heart rate and force of contraction of the isolated perfused heart were measured with a pressure transducer and recorded on a Grass polygraph (79 D). After 20 min equilibration, the test drugs were added into the perfusing solution starting from low to high concentrations. Preliminary studies showed that the effect of drugs reached a plateau at 5 min following drug administration and therefore measurement was made at 5 min following the addition of the test drug at each concentration. Each experiment was completed in about 30 min. In a preliminary experiment, it was shown that both the heart rate and force of contraction were stable during these periods in the normally perfused isolated heart.

Guinea-pig papillary muscles

Guinea-pigs (weighing 200–300 g) of either sex were anaesthetized with pentobarbitone (75 mg kg^{-1}, i.p.) and killed by a blow on the head and the hearts were quickly excised. Papillary muscles were isolated from the right ventricles, mounted in a 2 ml tissue chamber and superfused continuously at 35°C with an oxygenated (95% O_2 + 5% CO_2) Tyrode solution at pH 7.4. The preparations were electrically stimulated at 1 Hz with rectangular pulses (0.1 ms in duration, 1.5 times threshold). After the preparations had been stabilized for 1 h in normal Tyrode solution (flow rate 8–10 ml min^{-1}), the cell was penetrated by a glass microelectrode (3 M KCl, 10–20 MOh tip resistance) and allowed to equilibrate for 30 min more. By means of conventional microelectrode techniques, action potentials were derived from stable impalements, the maximum upstroke velocity (V_{max}) was obtained by electronic differentiation and both were displayed on a storage oscilloscope (COS5020-ST) for the evaluation of action potential amplitude (APA), resting potential (RP), overshoot (OS) and the action potential duration at 50% and 90% of repolarization (APD_{50} and APD_{90}). Slow action potentials were induced in papillary muscles as follows: after 1 h of stabilization in Tyrode solution, the preparations were superfused with a solution containing 22 mM KCl. The stimulation rate was then reduced to 0.2 Hz, the duration was increased to 1 ms and the amplitude of the stimulus intensity was quintuplicated. Addition of 10^{-5} M histamine induced the appearance of overshooting action potentials, the characteristics of which (low maximum diastolic potential and rate of depolarization) were typical of those of Ca-dependent action potentials (Ledda *et al.*, 1976; 1980). The following parameters were evaluated: APA, OS, V_{max}, APD_{90} and maximum diastolic potential (MDP). After the control period, superfusion with increasing concentration of CPU-23 started and the effect of the drug was determined after 30 min superfusion with each concentration. The characteristics of both fast and slow action potentials were stable in the absence of drug for up to 2 h.

Rabbit sinoatrial node

Rabbits (1.5–2 kg) of either sex were anaesthetized with pentobarbitone (30 mg kg^{-1}, i.v.) and the hearts were quickly excised. The region of the right atrium bounded by the crista terminalis, the superior and inferior vena cava, and the interatrial septum was dissected free from the adjacent tissue in Tyrode solution at room temperature. The preparation was fixed with fine pins to the Sylgard resin (Dow Corning, Midland, MI, U.S.A.) base of a perfusion chamber (2.5 ml) and allowed to equilibrate for 1 h. The tissue was perfused with Tyrode solution at a rate 8–10 ml min^{-1}. The region between the superior and inferior vena cava was explored with microelectrodes. Sinoatrial cells were identified based on the intracellular potential profile, which shows a pronounced diastolic depolarization merging smoothly into a fast upstroke, a maximum rate of fast depolarization not greater than 10 V s^{-1} and the small amplitude of the action potential (Opthof *et al.*, 1985; Mugelli *et al.*, 1987). CPU-23 was administered as described in the previous section.

Measurements of $[Ca^{2+}]_i$

Ventricular myocytes were prepared from 200–250 g male Sprague-Dawley rats according to the method described previously (Young & Scarpa, 1987; Huang & Wong, 1991) with slight modifications. In brief, the heart was mounted on a modified Langendorff perfusion apparatus and perfused at 10 ml min^{-1} with oxygenated Joklik modified Eagles medium supplemented with 1.25 mM $CaCl_2$ and 10 mM HEPES, pH 7.2, at 37°C for 5 min followed by 5 min in the same medium without calcium. Type I collagenase was added to this medium to a concentration of 125 u ml^{-1} with 0.1% BSA. After 35–45 min of collagenase digestion by perfusion, both atria were discarded and ventricular tissue was dissociated by shaking in a collagenase solution for 5 min under oxygen at 37°C (pH 7.2). Following a brief trituration, the residue was filtered through 250 μm mesh screens, sedimented by centrifugation at 100 *g* for 1 min and resuspended in fresh Joklik solution with 2% BSA. In the cell preparation, more than 70% of the cells were rod-shaped and were not trypan-blue permeant. Calcium concentration of the Joklik solution was increased gradually to 1.25 mM in 30 min. Yields were approximately $1-2 \times 10^6$ rod-shaped cells per heart in most experiments.

Myocytes were loaded with 5 μM fura 2/AM at 37°C for 40 min in Joklik solution supplemented with 1.25 mM $CaCl_2$, 10 mM HEPES and 2% BSA. For studying the action of the drugs on the KCl-induced increase in $[Ca^{2+}]_i$, $1-2 \times 10^4$ cells were washed and resuspended in a cuvette in 2 ml normal Krebs solution with HEPES 10 mM and 2% BSA (pH 7.4). For studying the action of the drugs on the caffeine-induced intracellular Ca release, cells were washed and resuspended in 2 ml of calcium-free Krebs solution. Fluorescence measurements were carried out under constant stirring and temperature (37°C). A Hitachi F-4000 Fluorescence Spectrophotometer was used. The excitation wavelength was established with a 340 nm interference filter with a 5 nm bandpass and the emitted light was collected through a 500 nm filter with a 10 nm bandpass. The response time was 2 s. $[Ca^{2+}]_i$ was calculated according to the formula: $[Ca^{2+}]_i = 224 \times (F\text{-}F_{min})/(F_{max}\text{-}F)$ nM (Grynkiewicz *et al.*, 1985). F_{max} was determined as the fluorescence of fura 2 after cell lysis with 33 μg ml^{-1} digitonin while F_{min} was the fluorescence after the addition of 5 mM EGTA and 50 mM Tris. Cells were used for fluorescence measurements for up to 5 h after cell isolation with little variation in the measured parameters. Under identical conditions, comparable stimulation of unloaded cells did not produce detectable fluorescence changes.

Coronary artery ligation in anaesthetized rats

Male Sprague-Dawley rats, weighing 350–450 g were used. Animals were anaesthetized with pentobarbitone sodium (75 mg kg^{-1}, i.p.). The systemic arterial blood pressure was measured from the left common carotid artery with a Statham pressure transducer (Gould P23 ID). Heart rate was recorded with a cardiac tachometer (Model 7P4) triggered by the arterial blood pressure pulse. Arterial blood pressure and heart rate were recorded continuously with a polygraph data recording system (Grass, model 79D). The limb II lead of an electrocardiogram (ECG) was monitored continuously on an oscilloscope (Gould, Type 1425) and recorded on a Cardisuny electrocardiograph (Fukuda, model 501D). A catheter was placed in a femoral vein for administration of drugs, and the trachea was cannulated to allow artifical ventilation. Rectal temperature was maintained at about 37°C. The chest was opened by left thoractomy at the fifth intercostal space and the fifth and fourth ribs were sectioned approximately 2 mm from the left margin of the sternum. Immediately after opening the chest, the animals were ventilated with room air

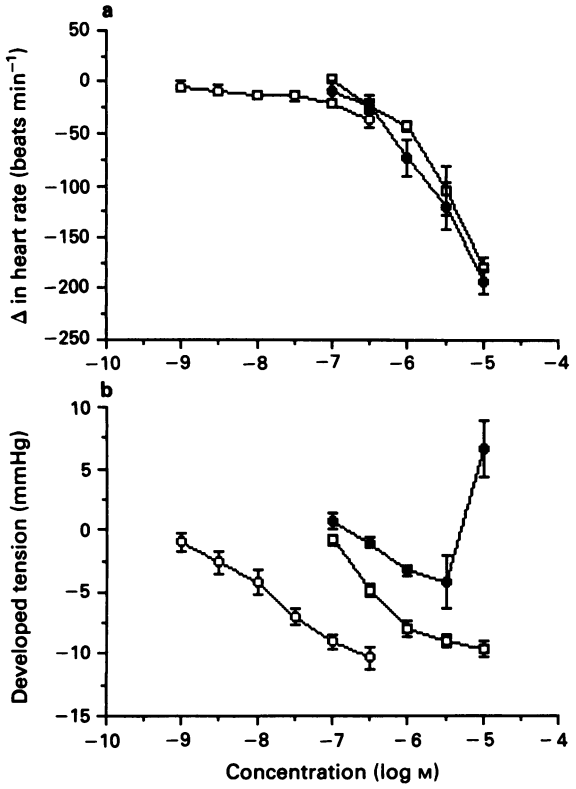


Figure 1 Effects of CPU-23, nifedipine and diltiazem on the heart rate (a) and force of contraction (b) in the isolated perfused heart of the rat. Values are mean ± s.e.mean of at least 5 hearts. (○) Nifedipine; (□) diltiazem; (●) CPU-23.

using a stroke volume of approximately 2 ml 100 g^{-1} and a rate of 54 stroke min^{-1}. These ventilation parameters maintained arterial Po_2, Pco_2 and pH within the normal range. After opening the pericardium the heart was exteriorised by gentle pressure on the chest wall, and a 6/0 braided silk suture attached to a 10 mm micro-point reverse cutting needle was placed under the left coronary artery as described by Selye *et al.* (1960) and Clark *et al.* (1980). The heart was replaced in the chest cavity, which was immediately closed. Any animal that exhibited arrhythmias or a sustained fall in mean arterial blood pressure (MAP) to less than 70 mmHg after the operation was discarded from further study. After an equilibration period of 15–30 min, the ligature was tied and the blood pressure and electrocardiogram were recorded for at least 30 min. When the animals died during the experiment or were killed by an overdose of pentobarbitone at the end of the experiment (4 h), the heart was removed rapidly for measurement of infarct size in order to determine if coronary ligation was successful. The hearts were rinsed under running water and the atria were discarded. The ventricles were sectioned into 3 slices from base to apex, and then stained by incubating for 15 min at 37°C in 20 ml of 0.1 M phosphate buffer, pH 7.6, containing 0.5% nitro blue tetrazolium. Infarct tissues were separated from the normal tissues, weighed, and then expressed as a percentage of the total ventricular weight. Animals with infarct size ranging from 20% to 45% were used and the infarct sizes did not differ from each other between the control and CPU-23 treated groups.

Animals were separated into 4 groups, namely, control and CPU-23-treated at 3 doses (1, 2.5 and 5 mg kg^{-1}). After a 15 min equilibration period, the animals in the control group were given saline solution and those in CPU-23-treated groups were given different doses of CPU-23 dissolved in saline solution. The saline solution was given at 1 ml kg^{-1}. The ligature was tied 15 min after saline or CPU-23 administration.

In the present study only results of early (0–30 min) post-ligation arrhythmias were presented as calcium antagonists have been shown to prevent early post-infarction ventricular arrhythmias (Fagbemi & Parratt, 1981; Curtis *et al.*, 1984; Curtis & Walker, 1986; 1988; Au *et al.*, 1987) and both mortality and arrhythmias occurred mainly during this period.

Drugs and chemicals

Nifedipine, histamine, fura 2/AM and Joklik modified Eagle's medium were purchased from Sigma Chemicals Co. (St. Louis, MO, U.S.A.). Type I collagenase was from Cooper Biomedical. CPU-23 was synthesized as previously described (Huang *et al.*, 1990) and generously provided by Professor S.X. Peng of the China Pharmaceutical University. All chemicals except nifedipine and fura 2/AM were dissolved in distilled water. Nifedipine and fura 2/AM were dissolved in dimethyl sulphoxide (DMSO). The choice of concentrations of the three calcium antagonists studied were based on previous findings in the literature and by trial and error. The highest concentrations in the experiments involving the measurement of heart rate and force of contraction in the isolated perfused heart were the concentrations that caused cardiac arrest. In the present study changes in heart rate and force of contraction in response to drugs at concentrations below the concentrations that caused cardiac arrest, were presented.

Statistical analysis

Data were expressed as mean ± standard error of the mean (s.e.mean). Statistical significance of differences between means was calculated by a paired or unpaired Student's *t* test where appropriate. A level of $P<0.05$ was considered significant. For evaluating the differences between the incidence of arrhythmias (VT and VF) and mortality, Chi-squared test was used.

Results

Effects on the heart rate and force of contraction of the Langendorff perfused rat heart

All three drugs, nifedipine, diltiazem and CPU-23 decreased the heart rate dose-dependently, the effect of nifedipine being less obvious compared with the other two drugs (Figure 1a). Nifedipine and diltiazem markedly inhibited the force of contraction in a dose-dependent manner (Figure 1b). On the other hand, the effect of CPU-23 was biphasic, inhibiting and

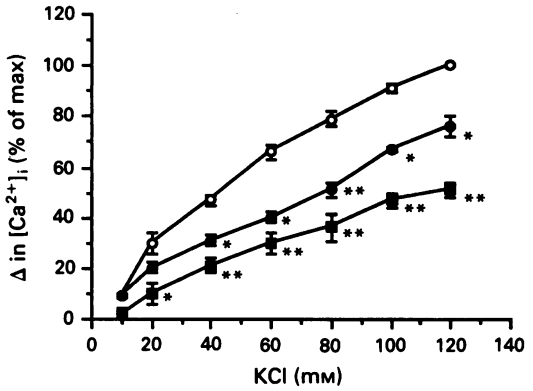


Figure 2 Inhibitory effects of CPU-23 on KCl-induced increase in $[Ca^{2+}]_i$ in isolated ventricular myocytes in 1.25 nM Ca^{2+} medium. Each point represents the mean of 5 separate experiments ± s.e.mean. $*P<0.05$; $**P<0.01$, when compared to the control. Baseline $[Ca^{2+}]_i$ level: 128 ± 11 nM. (○) Control; (●) CPU-23 1 μM; (■) CPU-23 10 μM.

Table 1 Effects of 10^{-4} M CPU-23 on fast action potential characteristics in guinea-pig papillary muscles

	RP (mV)	*APA* (mV)	*OS* (mV)	APD_{50} (ms)	APD_{90} (ms)	V_{max} (V s^{-1})
Control	89.3 ± 0.9	135.5 ± 2.2	46.3 ± 2.0	151.1 ± 13.6	200.8 ± 10.4	202.5 ± 1.8
CPU-23	88.8 ± 1.0	126.0 ± 1.4*	37.3 ± 1.9*	143.8 ± 12.6*	191.3 ± 10.2*	183.5 ± 8.6*

Data shown are the mean ± s.e.mean of 4 separate experiments.
RP: resting potential; APA; amplitude of action potential; OS: overshoot; APD_{50} and APD_{90}: action potential duration at 50 and 90% of repolarization, respectively; V_{max}: maximum upstroke velocity.
*$P<0.05$ vs control.

Table 2 Effects of CPU-23 on slow action potential characteristics in guinea-pig papillary muscles

	MDP (mV)	*APA* (mV)	*OS* (mV)	APD_{90} (ms)	V_{max} (V s^{-1})
Control	56.2 ± 5.4	94.6 ± 4.1	33.0 ± 3.1	177.0 ± 13.2	11.0 ± 0.5
CPU-23					
10^{-6} M	54.8 ± 5.7	84.4 ± 2.9*	28.8 ± 4.3*	168.6 ± 11.8*	10.2 ± 0.6*
10^{-5} M	53.0 ± 3.0	73.0 ± 2.6*	27.7 ± 3.9*	145.0 ± 3.0*	7.3 ± 0.3**
10^{-4} M	50.7 ± 3.1	67.0 ± 6.5**	17.7 ± 3.9**	84.3 ± 4.3**	4.3 ± 0.6**

Data shown are the mean ± s.e.mean of 5 separate experiments. MDP: maximum diastolic potential.
*$P<0.05$; **$P<0.01$ vs control.

Table 3 Effects of CPU-23 on maximum diastolic potential (MDP), amplitude of action potential (APA), spontaneous rate (SR) and slope of phase 4 spontaneous depolarization (*dV/dt*) of rabbit sinoatrial node tissue

	MDP (mV)	*APA* (mV)	*SR* (b.p.m.)	dV/dt (mV s^{-1})
Control	51.8 ± 3.1	38.5 ± 3.4	206 ± 10	53.0 ± 12.0
CPU-23				
10^{-7} M	52.4 ± 2.9	37.5 ± 3.1	180 ± 5*	42.0 ± 11.8*
10^{-6} M	48.0 ± 1.8	32.3 ± 4.4*	166 ± 10**	38.0 ± 10.7*
3×10^{-6} M	41.8 ± 4.0	25.0 ± 1.1*	97 ± 18**	26.8 ± 6.8*

Data shown are the mean ± s.e.mean of 5 separate experiments.
*$P<0.05$; **$P<0.01$ vs control.

Table 4 Effects of intravenous CPU-23 on early post-infarction arrhythmias and mortality in pentobarbitone-anaesthetized rats

Group	n	*Total number of ectopic beats*	*VT* *Duration* (s)	*VT* *Incidence* (%)	*VF* *Duration* (s)	*VF* *Incidence* (%)	*Mortality* (% in 30 min)
Saline	10	915 ± 197	93 ± 22	100	53 ± 27	70	20
CPU-23							
1 mg kg^{-1}	8	264 ± 130**	37 ± 26	63	2 ± 2**	13*	13
2.5 mg kg^{-1}	6	40 ± 18**	2 ± 2**	33*	0**	0**	0**
5 mg kg^{-1}	8	3 ± 1**	2 ± 2**	13*	0**	0**	0**

Values are means ± s.e.mean of (*n*) animals.
*$P<0.05$, **$P<0.01$ when compared with control.

increasing the developed tension at 10^{-7}–3×10^{-6} M and 10^{-5} M, respectively (Figure 1b). It is of interest to note that nifedipine caused cardiac arrest at concentrations higher than 3×10^{-7} M, whereas diltiazem and CPU-23 caused cardiac arrest only at concentrations higher than 3×10^{-5} M.

Effects on action potentials in guinea-pig papillary muscles

CPU-23 decreased APA and V_{max}, and shortened APD_{50} and APD_{90} only at a concentration of 10^{-4} M (Table 1). The effect usually occurred within 30 min after perfusion. It did not, however, affect RP and OS.

CPU-23 at 10^{-6}–10^{-4} M dose-dependently decreased APA, OS and V_{max}, and shortened APD_{90} of slow action potentials induced by 10^{-5} M histamine in a K^+-depolarized guinea-pig papillary muscle without affecting MDP (Table 2).

Effects on spontaneous action potentials of rabbit sinoatrial node

As shown in Table 3, CPU-23 decreased the APA, spontaneous rate (SR) and the slope of phase 4 depolarization (*dV/dt*) in a dose-dependent manner. However, it did not influence MDP significantly.

Recovery of the CPU-23 effects

The effects of CPU-23 on rat isolated heart, guinea-pig papillary muscles and rabbit sinoatrial node were not reversed by superfusing with Krebs Ringer for up to 2 h.

Effects on $[Ca^{2+}]_i$ in rat ventricular myocytes

As expected, KCl dose-dependently increased $[Ca^{2+}]_i$ only in a medium with calcium, and the effect was attenuated by

Table 5 Effects of CPU-23 (given 15 min before ligation) on heart rate (HR) and mean arterial blood pressure (MAP) in anaesthetized rats subjected to coronary artery occulsion (at 0 min)

					HR (beats min^{-1})						
					Time (min)						
Treatment	n	*−15*	*−10*	*−5*	*0*	*1*	*3*	*5*	*10*	*20*	*30*
Saline	10	454 ± 10	450 ± 10	452 ± 10	454 ± 10	454 ± 10	455 ± 9	456 ± 9	450 ± 9	459 ± 6	459 ± 6
CPU-23											
1 mg kg^{-1}	8	471 ± 11	403 ± 10**	403 ± 3**	409 ± 6**	423 ± 8*	423 ± 8*	423 ± 8*	433 ± 7	435 ± 7	435 ± 8
2.5 mg kg^{-1}	6	453 ± 12	288 ± 31**	313 ± 28**	323 ± 32*	323 ± 32*	348 ± 20**	357 ± 17**	357 ± 17**	365 ± 18**	386 ± 13**
5 mg kg^{-1}	8	430 ± 12	243 ± 23**	276 ± 22**	283 ± 22**	311 ± 23**	318 ± 25**	319 ± 25**	351 ± 13**	363 ± 17**	373 ± 15**
					MAP (mmHg)						
					Time (min)						
Treatment	n	*−15*	*−10*	*−5*	*0*	*1*	*3*	*5*	*10*	*20*	*30*
Saline	10	104 ± 9	104 ± 9	105 ± 9	104 ± 9	71 ± 7††	87 ± 9††	92 ± 9††	92 ± 9††	93 ± 8††	94 ± 8††
CPU-23											
1 mg kg^{-1}	8	106 ± 7	97 ± 5	101 ± 5	103 ± 7	76 ± 4††	72 ± 4††	81 ± 9†	89 ± 10	105 ± 9	109 ± 8
2.5 mg kg^{-1}	6	97 ± 4	71 ± 9**	78 ± 5*	79 ± 5*	70 ± 5	73 ± 4	79 ± 3	82 ± 2	86 ± 9	88 ± 8
5 mg kg^{-1}	8	99 ± 5	53 ± 4**	69 ± 5**	76 ± 6*	66 ± 5	71 ± 6	78 ± 7	83 ± 9	86 ± 9	88 ± 8

Values are mean ± s.e.mean of (*n*) animals.
* $P<0.05$; ** $P<0.01$ vs control and †$P<0.05$; ††$P<0.01$ vs 0 min.

nifedipine at 1 μM, whereas caffeine increased $[Ca^{2+}]_i$ even in the absence of calcium (data not shown). CPU-23 at 10^{-6}–10^{-5} M inhibited the KCl-induced increase in $[Ca^{2+}]_i$ in the calcium medium significantly (Figure 2), but had no significant effect on the caffeine-induced increase in $[Ca^{2+}]_i$ in the calcium-free medium (data not shown).

Effects on ventricular arrhythmias and mortality following coronary artery ligation

In agreement with the observations in a previous study (Clark *et al.*, 1980), there were mainly ventricular ectopic beats during the first 30 min post-ligation. Ventricular tachycardia (VT) and ventricular fibrillation (VF) also occurred. CPU-23 reduced dose-dependently the total number of ectopic beats, and both the duration and the incidence of VT and VF occurring in the first 30 min following coronary artery ligation (Table 4). The effects were statistically significant except for the duration and incidence of VT at 1 mg kg^{-1} of CPU-23. CPU-23 also reduced the mortality in a dose-dependent manner. At 2.5–5 mg kg^{-1} CPU-23 abolished completely VF, which was accompanied by 100% survival.

Effects on heart rate and mean arterial pressure following coronary artery ligation

CPU-23 dose-dependently reduced the heart rate and arterial blood pressure after its administration except at 1 mg kg^{-1} for mean arterial pressure (Table 5). Coronary artery ligation did not affect the heart rate in saline control and CPU-23-treated rats. On the other hand, it significantly lowered the arterial blood pressure in the saline control and transiently in the rats pretreated with CPU-23 of 1 mg kg^{-1}, the lowest dose used in the study, but not in the rats pretreated with CPU-23 at doses of 2.5 and 5 mg kg^{-1} (Table 5).

Discussion

In the present study we have further characterized the calcium antagonistic action of CPU-23 by studying the effects of the drug on (1) the heart rate and force of contraction in the isolated perfused rat heart; (2) slow action potential of the ventricular papillary muscle of guinea-pig and pace-maker potential of the sinoatrial node of rabbits, and (3) KCl-induced increase in $[Ca^{2+}]_i$ in isolated ventricular myocytes. It was found that CPU-23 dose-dependently produced effects, which are known to relate to a reduction in transsarcolemmal calcium influx. For example, the V_{max} of the slow action potential characteristics is an indirect index of slow inward current, I_{si} (Malecot & Trautwein, 1987), which may be chiefly responsible for the reduction in heart rate as observed in the present study. The reduction in force of contraction of the isolated perfused heart has been well established as resulting from inhibition of transmembrane calcium influx through the L-type calcium channels (Fleckenstein, 1977; Conti *et al.*, 1985). More importantly, CPU-23 also reduced the KCl-induced increase in $[Ca^{2+}]_i$ in isolated ventricular myocytes, providing direct evidence that the drug attenuated calcium influx. The findings render support for CPU-23 as a calcium antagonist.

Like other calcium antagonists such as nifedipine and verapamil, CPU-23 exerts antiarrhythmic action against ischaemic arrhythmias. The inhibitory effects of CPU-23 on the pace-maker action potentials in sinoatrial node, heart rate and developed tension in the rat isolated perfused heart, the slow action potential of the papillary muscle and more directly the KCl-induced increase in $[Ca^{2+}]_i$ in ventricular myocytes indicate that CPU-23 inhibits calcium influx in both pace maker and ventricular cells. In addition, CPU-23 was found to inhibit [^{3}H]nitrendipine binding sites and the potency was correlated with its ability to inhibit KCl-induced contraction, suggesting that the drug may inhibit influx of

calcium which is required for the initiation and maintenance of VF (Merillat *et al.*, 1990) by binding to dihydropyridine-sensitive calcium channels. In short the findings of the present study support the view that the antiarrhythmic action of CPU-23 may be due primarily to an inhibition of transmembrane calcium influx in the cardiac tissue as suggested by Curtis & Walker (1988) and Lydtin & Trenkwalder (1990).

CPU-23 has other effects which may not be related to reductions in calcium influx. At 10^{-4} M, a concentration approximately 500 times the IC_{50} for inhibiting KCl-induced contraction in the rat aorta (Dong *et al.*, 1992), CPU-23 affected the fast action potential characteristics of the ventricular papillary muscle of guinea-pigs, suggesting that at this concentration, it also interferes with the sodium channels. At 10^{-5} M, a concentration that inhibits KCl-induced increase of the inward calcium current, CPU-23 increased the developed tension in the rat isolated perfused heart. Unfortunately the present study provided no information on the mechanism of the effect and further study is needed. Many calcium antagonists, including nifedipine and verapamil, depress myocardial contractility, which is harmful to patients with cardiovascular disorders as a reduction in contractility of the heart may result in heart failure (Lydtin & Trenkwalder, 1990). The fact that CPU-23 has a propensity towards stimulating myocardial contractile force at the high concentration of 10^{-5} M distinguishes it from nifedipine and diltiazem and this may be beneficial when used in treating high blood pressure in patients with cardiovascular disorders.

Like some of the other classical calcium antagonists, CPU-23 induced bradycardia and hypotension in anaesthetized rats. The decrease in heart rate is beneficial to the ischaemic myocardium as it reduces myocardial oxygen demands (Smith *et al.*, 1975). CPU-23, at the dose exerting antiarrhythmic activity, induced transient hypotension, which is also beneficial to the heart as the afterload is reduced. Interestingly, while coronary artery ligation reduced signifcantly the mean arterial blood pressure in the saline controls, this effect was only transient (5 min) in rats pretreated with 1 mg kg^{-1} of CPU-23, a dose that did not itself lower arterial blood pressure. Coronary artery ligation did not further reduce the mean arterial blood pressure in rats pretreated with higher doses of CPU-23 which by themselves markedly lowered arterial blood pressure. That CPU-23 did not further reduce arterial blood pressure after coronary artery ligation at doses that themselves reduced arterial blood pressure and produced significant antiarrhythmic action is beneficial to patients with cardiac arrhythmias, as arterial blood pressure would not drop further with the use of the drug during myocardial ischaemia.

The ability of CPU-23 to displace the [^{3}H]-nitrendipine binding in the cerebral cortical membrane suggests that it may block calcium influx by acting as a dihydropyridine antagonist at the dihydropyridine-sensitive L-type calcium channel (Dong *et al.*, 1992). Results from the previous (Dong *et al.*, 1992) and present studies showed that in addition to the differences in efficacy in haemodynamic actions on blood pressure and heart rate, and binding affinity to the nitredipine binding sites in the cortical cerebral membranes, there are a number of differences between CPU-23 and the prototype dihydropyridine calcium antagonist, nifedipine. First, CPU-23 causes bradycardia whereas nifedipine leads to a reflex tachycardia. Second, at a high concentration of 10^{-5} M, CPU-23 increases the myocardial contractile force while nifedipine does not have such stimulatory action. Third, the effect of CPU-23 on heart rate is far more obvious than nifedpine. These observations suggest that CPU-23 may not have the same undesirable effect(s) as nifedipine when used in treating patients with high blood pressure. Further study is needed to determine the specificity of CPU-23 as a dihydropyridine calcium antagonist and explore the possibility of its use as a therapeutic agent in the treatment of cadiovascular disorders.

In summary, in the present study CPU-23 was found to inhibit slow action potential characteristics of the guinea-pig papillary muscle and the spontaneous action potential of the rabbit sinoatrial node; to decrease the heart rate and developed tension of the rat isolated perfused heart; and inhibit the KCl-induced increased calcium influx in isolated ventricular myocytes. All these effects relate to transmembrane influx of calcium. The observation render support for the suggestion that CPU-23 may be a calcium antagonist. In addition, CPU-23 also exerts antiarrhythmic actions against arrhythmias during the first 30 min post ligation of the coronary artery in a similar way to other prototypic calcium antagonists such as nifedipine and verapamil. It is suggested that CPU-23 selectively blocks calcium influx by blocking the L-type channels and thus inhibiting activities related to calcium influx such as heart rate, developed tension in the rat isolated perfused heart, slow action potential of the guinea-pig ventricular muscle and spontaneous action potential of the rabbit sinoatrial node, and arrhythmias in the anaesthetized rat subject to coronary artery ligation. In the present study it was also found that in addition to effects related to calcium influx, CPU-23 also had other effects, which may not be related to transmembrane influx of calcium. For example, at 10^{-4} M it affected the fast action potential characteristics, suggesting it may also block sodium channels. At 10^{-5} M, a concentration that inhibits KCl-induced calcium influx, it increased the developed tension in the rat isolated perfused heart. Further study is needed to delineate the mechanism of action of CPU-23 in addition to its calcium antagonistic action.

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The action of amlodipine on voltage-operated calcium channels in vascular smooth muscle

[1]A.D. Hughes & S. Wijetunge

Department of Clinical Pharmacology, St. Mary's Hospital Medical School, Imperial College of Science, Technology and Medicine, Norfolk Place, London W2 1NY

1 Amlodipine, a dihydropyridine derivative largely ionized at physiological pH, inhibited calcium channel currents in single vascular smooth muscle cells isolated from rabbit ear artery in a concentration-dependent manner.

2 Amlodipine inhibited the current-voltage relationship for calcium channel currents across the range of test potentials used. However, the effect of amlodipine was more marked on more depolarized test potentials. Amlodipine also shifted the steady-state inactivation curve for calcium channel currents in a hyperpolarized direction.

3 The potency of amlodipine as determined from the steady-state inhibition of calcium channel current induced by the drug was dependent on the holding potential of the cells. Use of a more depolarized holding potential increased the potency of amlodipine.

4 Onset of amlodipine-induced inhibition was relatively rapid at both − 60 mV and − 40 mV holding potential. The use of a more depolarized holding potential increased the rate of association of amlodipine. No recovery from amlodipine-induced inhibition was seen over a 20 min period following washout of the drug.

5 In addition to voltage-dependence, the action of amlodipine showed use-dependence, in that the effect of amlodipine was more marked when calcium channel currents were evoked frequently. Increasing the frequency of activation of calcium channel currents did not alter the apparent onset rate of amlodipine-induced inhibition, but increased the degree of inhibition achieved by the drug.

6 The electrophysiological properties of amlodipine, particularly its voltage-dependence are probably important determinants of its action *in vivo*.

Keywords: Vascular smooth muscle; calcium channels; dihydropyridine; voltage clamp; amlodipine

Introduction

Voltage-operated calcium channels represent a major route by which calcium enters vascular smooth muscle cells (Bolton, 1979; Tsien, 1983), and play an important part in excitation-contraction coupling (Van Breemen & Saida, 1989). Dihydropyridines block entry of calcium into the vascular smooth muscle cell by an action on voltage-operated calcium channels (Tsien, 1983). Dihydropyridines are believed to act by binding to a site, or sites, associated with the voltage-operated calcium channel, so causing an alteration in gating characteristics. This change in gating leads to reduced calcium entry, consequently the drugs are known as calcium antagonists or calcium channel blockers.

Amlodipine (2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl) -3-ethixycarbonyl-5-2-methoxycarbonyl-6-methyl-1,4dihydropyridine) is a novel dihydropyridine derivative. Although structurally related to other dihydropyridines such as nifedipine its chemical properties differ from typical dihydropyridines. Unlike the majority of dihydropyridines, amlodipine is relatively insoluble in organic solvents (partition coefficient in octanol/buffer (Kp_{oct}) = 29, cf. nimodipine Kp_{oct} = 145), and exists primarily in an ionized form at physiological pH (p*K*a = 9.02) (Mason *et al.*, 1989).

In vivo, the cardiovascular effects of amlodipine are largely due to vasodilatation, this action of amlodipine is relatively slow in onset and of long duration (Burgess & Dodd 1990). Similar vasorelaxant properties have been observed *in vitro*. In rat isolated aortic strips Burges *et al.* (1987) demonstrated that the onset and offset of amlodipine were much slower than nifedipine, and in radioligand binding studies, Rigby *et al.* (1988) reported that [^{3}H]-amlodipine displayed slow rates of association and dissociation with dihydropyridine binding sites in microsomes derived from dog ventricular myocytes.

Although it is a dihydropyridine, the action of amlodipine may also involve binding to non-dihydropyridine sites on the voltage-operated calcium channel; ligand binding studies indicate that amlodipine interacts not only with a dihydropyridine binding site but also with a site which binds diltiazem (Rigby *et al.*, 1988; Nayler & Gu, 1992) and another that binds phenylalkylamines such as (−)-D888 (Vaghy, 1992).

The object of these studies was to examine the effect of amlodipine on voltage-operated calcium channels in vascular smooth muscle cells.

Methods

Single smooth muscle cells were freshly dispersed from rabbit ear artery by a modification of the procedure of Benham & Bolton (1986). Short segments (1–2 mm) of artery were incubated for 50–60 min in a modified physiological salt solution containing 10 μM calcium, 2 mg ml^{-1} bovine serum albumin, 1 mg ml^{-1} collagenase (Worthington 130 u mg^{-1}), 0.5 mg ml^{-1} papain (Sigma 15 u mg^{-1}) and 5 mM dithiothreitol respectively. Cells were dispersed by mild agitation in this low-calcium physiological salt solution. After centrifugation the cells were resuspended in normal physiological salt solution (PSS) containing (mM): NaCl 130, KCl 6, $CaCl_2$ 1.7, $MgCl_2$ 1.2, glucose 14 and HEPES 10.7 buffered to pH 7.2 with NaOH. The cells were stored on cover slips at 4°C and used within 6 to 8 h. The experiments were performed using the whole-cell configuration of the patch-clamp technique

[1] Author for correspondence.

(Hamill *et al.*, 1981) by means of a List EPC-7 patch-clamp amplifier. Patch pipettes had resistances of 3 to 5MΩ. The internal (pipette) solution contained (mM): NaCl 126, $MgCl_2$ 1.2, EGTA 2, Mg_2 ATP 2, TEA 10, and HEPES 11 buffered to pH 7.2 with NaOH. The experiments were carried out in 'high-barium solution' containing (mM): $BaCl_2$ 110 and HEPES 10 buffered to pH 7.2 with TEA-OH to increase the size of the inward current elicited by depolarization and to minimise calcium-dependent inactivation of currents (Aaronson *et al.*, 1988).

An IBM compatible PC provided the voltage-clamp command pulses through a Labmaster A/D interface board using commercially available software (PClamp 5.5, Axon Instruments, CA, U.S.A.). Data were recorded on-line, or on digital audio tape using a DAT recorder (Biologic, France) and later analysed off-line after analogue-to-digital conversion at between 2.5–10 kHz depending on the voltage protocol used. The currents were digitally filtered at 2 kHz and leak currents were subtracted digitally using average values of steady leakage currents elicited by a 10 mV hyperpolarizing pulse (Aaronson *et al.*, 1988). All recordings were made at room temperature (22–25°C).

Drugs were applied by bath perfusion or by a concentration-jump technique previously described in detail (Hering *et al.*, 1987). This enabled the application of the drugs within less than 10 ms. Data are presented as means values ± s.e. mean of n observations. Concentration-response data were fitted to a logistic function:-

$$E = \frac{C}{E_{max} + \frac{C}{(K_D)^n}}$$

where E is effect of drug, E_{max} is maximum effect of drug, C is the concentration of drug, K_D is the apparent affinity and n is the pseudo-Hill slope, using nonlinear regression analysis and commercially available software on an IBM PC compatible computer (Inplot 3.1 and 4, GraphPAD Software, CA, U.S.A.).

Steady-state inactivation data similarly were fitted to a Boltzmann function:

$$I = \frac{I_{max}}{1 + \exp\frac{(V-V_h)}{k}} + C$$

where I is the normalized peak current at any potential, I_{max} is the maximum peak current evoked by such a step, V_h is the holding potential at which inactivating current is half inactivated, k is a slope factor and C is the fraction of non-inactivating current which is present in these cells (Hughes *et al.*, 1990).

Drugs

Amlodipine (a gift from Pfizer, U.K.) was prepared as a stock solution (10 mM) in ethanol. Subsequent dilutions were made in distilled water.

Results

Effect of amlodipine on inward currents

Amlodipine (10 pM–10 nM) induced a marked concentration-dependent inhibition of calcium channel current in single rabbit ear artery cells held under voltage clamp at − 60 mV and − 40 mV. Examination of the current-voltage relationships suggested that the inhibitory effects of amlodipine were more marked on more depolarized test potentials although amlodipine did induce inhibition of currents evoked by depolarization near to threshold (Figure 1). The potency of amlodipine as estimated from the concentration-response relationship was increased about 10 fold at the more depolarized holding potential of − 40 mV (Figure 2).

The rate of onset of amlodipine-induced inhibition was examined at two holding potentials, − 60 mV and − 40 mV. As indicated above, amlodipine is more potent at depolarized potentials so the rate of onset of amlodipine-induced inhibition was compared using concentrations of amlodipine chosen to produce around 30 and 75% inhibition of calcium channel currents (1 and 10 nM at − 60 mV, and 100 pM and 1 nM at − 40 mV). At − 60 mV, the onset of amlodipine-induced inhibition was relatively rapid; mean time constant of onset (τ) = 33.3 s (1 nM) and 7.6 s (10 nM) (n = 3-4). At − 40 mV the mean τ of amlodipine-induced inhibition was 25 s (100 pM) and 7.6 s (1 nM) (n = 3) (Figure 3). From these kinetic data it was possible to estimate the affinity of amlodipine for the voltage-operated calcium channel from the equation derived from Schwarz and colleagues (1973):

$$K_D = \frac{\tau' \times [\text{Amlodipine}'] - \tau \times [\text{Amlodipine}]}{\tau - \tau'}$$

Where τ is the mean time constant of onset with the lower concentration of amlodipine, and τ' is the onset time constant using the higher concentration of amlodipine.

Substituting the values obtained at − 60 mV and − 40 mV membrane potentials gave affinities of 286 pM and 29 pM respectively. These results compare favourably with the values of 240 pM and 34 pM for the IC_{50} derived from the concentration-response relationship.

Following washout of amlodipine no recovery from amlodipine-induced inhibition was seen at either holding potential during a 20 min recovery period.

Effect on availability

The above studies indicate that the potency of amlodipine is increased at more depolarized potentials and suggest that amlodipine may bind with higher affinity to an inactivated state of the voltage-operated calcium channel. This was further investigated by examining the effect of amlodipine (1 nM) on the availability of inward current following a 6 s conditioning pulse sufficient to induce steady-state inactivation of calcium channel current in these cells (Aaronson *et al.*, 1988). In the presence of amlodipine current was inhibited at all potentials (Figure 4) and amlodipine also shifted the mid-point of the inactivation curve to more negative potentials (control = − 26 ± 3 mV; amlodipine = − 47 ± 6 mV; n = 4 in each case) without significantly altering the slope factor (k) (control k = 7.1 ± 0.2; amlodipine k = 7.4 ± 1.0; n = 4 in each case). From the mean shift in the inactivation curve we calculated the dissociation constant for the inactivated state (K_i) according to the approach described by Bean and colleagues (1986) assuming one to one building to resting and inactivated states in a simple two state model. Using this model

$$\Delta V_h = \frac{k \ln (1 + [\text{Amlodipine}]/K_i)}{(1 + [\text{Amlodipine}]/K_r)}$$

where ΔV_h is the shift in the mid point of the inactivation curve, k is the slope factor, [Amlodipine] is the concentration of amlodipine used, and K_r is the IC_{50} of amlodipine measured at a holding potential of − 60 mV when there is negligible inactivation. With ΔV_h = 21 mV, k = 7 mV, K_r = 240 pM K_i was calculated to be 10 pM.

The effect of frequency of activation

In general, dihydropyridines do not shown use-dependence (Terada *et al.*, 1987; Honore *et al.*, 1989; Kuga *et al.*, 1990). However Kass & Arena (1989) reported that amlodipine did display use-dependence in isolated myocardial cells. We examined the effect of increasing the frequency of stimulation on the inhibition produced by amlodipine. Over the range

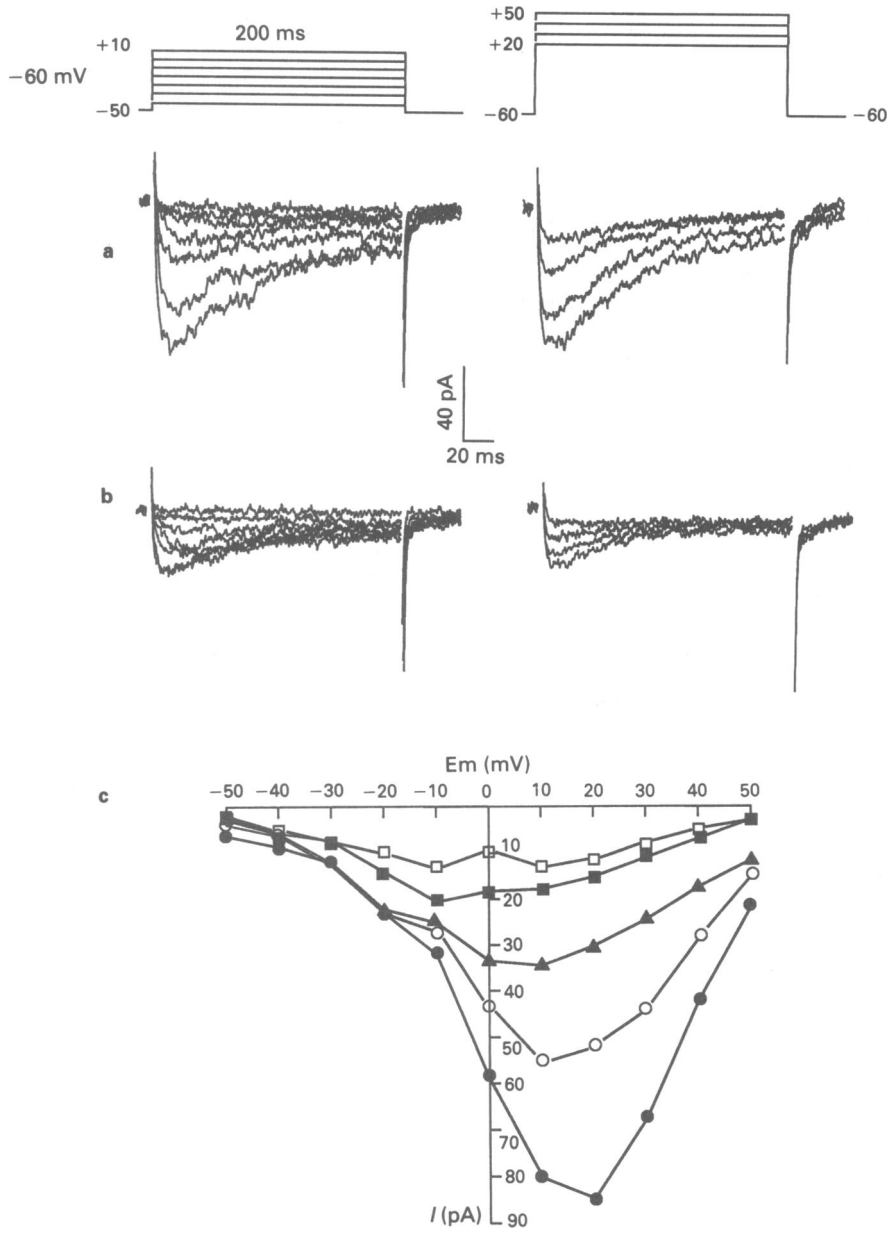


Figure 1 The effect of amlodipine on calcium channel currents in rabbit ear artery cells. Cells were bathed in 110 mM Ba^{2+} and voltage clamped at − 60 mV. (a) Control currents evoked by 200 ms depolarization steps from − 50 mV to + 50 mV in the absence of drug; (b) currents evoked by the same depolarization steps in the presence of amlodipine 1 nM. (c) The effect of amlodipine on the peak current-voltage relationship; (●) control; (○) amlodipine 100 nM; (▲) amlodipine 1 nM; (■) amlodipine 10 nM; (□) amlodipine 100 nM. The voltage protocol and scale bar are indicated.

0.03–1 Hz the magnitude of inhibition induced by 10 nM amlodipine was increased by increasing the frequency of stimulation, although inhibition of current did occur in the absence of activation (Figure 5). The rate of onset of amlodipine-induced inhibition was little altered when currents were elicited every 10 s; τ being 28 ± 4 s ($n = 4$) for 10 nM amlodipine at − 60 mV holding potential compared with 33 ± 3 s ($n = 4$) when currents were evoked at 1 Hz. In contrast the steady-state inhibition induced by 10 nM amlodipine when currents were evoked at 0.1 Hz was significantly less (53 ± 8%, $n = 4$) than when currents were evoked at 1 Hz (70 ± 7%, $n = 4$).

Discussion

Amlodipine, although a dihydropyridine, is unusual in that it is almost completely ionized at physiological pH; the conditions of this study. As has been previously reported for other dihydropyridines in vascular smooth muscle (Bean *et al.*, 1986; Nelson & Worley, 1989; Kuga *et al.*, 1990), we found that amlodipine-induced inhibition is affected by the resting membrane potential. Depolarization increases the potency of amlodipine. This effect of membrane potential appears to be, at least in part, due to an increase in the association rate constant at depolarized membrane potentials. In these studies we were unable to see any recovery from amlodipine-induced inhibition at any holding potential so it is unclear whether membrane potential can also influence the rate constant for drug-receptor dissociation. However in a previous study (Hering *et al.*, 1993) using another dihydropyridine, (−)-202 791, membrane potential had no effect on offset rate constant.

In addition to inhibiting calcium channel currents, amlodipine also caused the steady-state inactivation curve for calcium channel currents to be shifted to more polarized

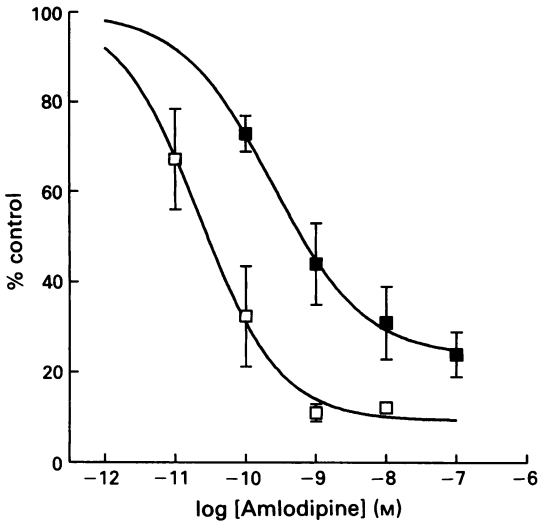


Figure 2 Concentration-response relationship for amlodipine-induced inhibition of peak calcium channel currents in cells voltage clamped at − 60 mV (■) and − 40 mV (□). Calcium channel currents were evoked every second using a 20 ms pulse to + 10 mV from a holding potential of − 60 mV. The average peak current was measured at each concentration of drug in each cell, once stable inhibition was achieved. Results are normalized with respect to the calcium channel current in the absence of any drug. Each point is the mean (with s.e.mean) of 4–5 observations in different cells.

potentials. Similar findings have previously been reported for other dihydropyridines and a common explanation is that the increased affinity at depolarized membrane potentials and the shift in the availability curve reflect a high affinity of the dihydropyridine for the inactivated state or states of the voltage-operated calcium channel (Bean, 1984; Sanguinetti & Kass, 1984). Using the model proposed by Bean *et al.* (1986) we calculated the affinity for the inactivated state of the calcium channel to be around 10 pM. This predicts that at − 40 mV (when from the availability curve around 20% of channels will be inactivated compared with <5% of channels at − 60 mV) the apparent affinity constant should fall from 200 pM to around 40 pM. This agrees reasonably well with the calculated IC_{50} for amlodipine at − 40 mV of 34 pM. In myocardial cells studied by whole cell voltage clamp, Kass & Arena (1989) reported that amlodipine induced little or no block of calcium channel currents in myocardial cells unless cells were depolarized to or below − 50 mV. This contrasts with our findings where block of calcium channel current can be seen in these cells at hyperpolarized membrane potentials when little inactivation is likely to occur (see Figure 4). However, compared with voltage-dependent calcium channels in myocardial cells, calcium channels in the resting state in vascular smooth muscle appear to have higher affinity for dihydropyridines (McCarthy & Cohen, 1989). The basis of this difference between the voltage-dependent calcium channel in cardiac and vascular smooth muscle is unclear, but has been reported previously (see Nelson & Worley, 1989).

The onset of amlodipine-induced inhibition of calcium

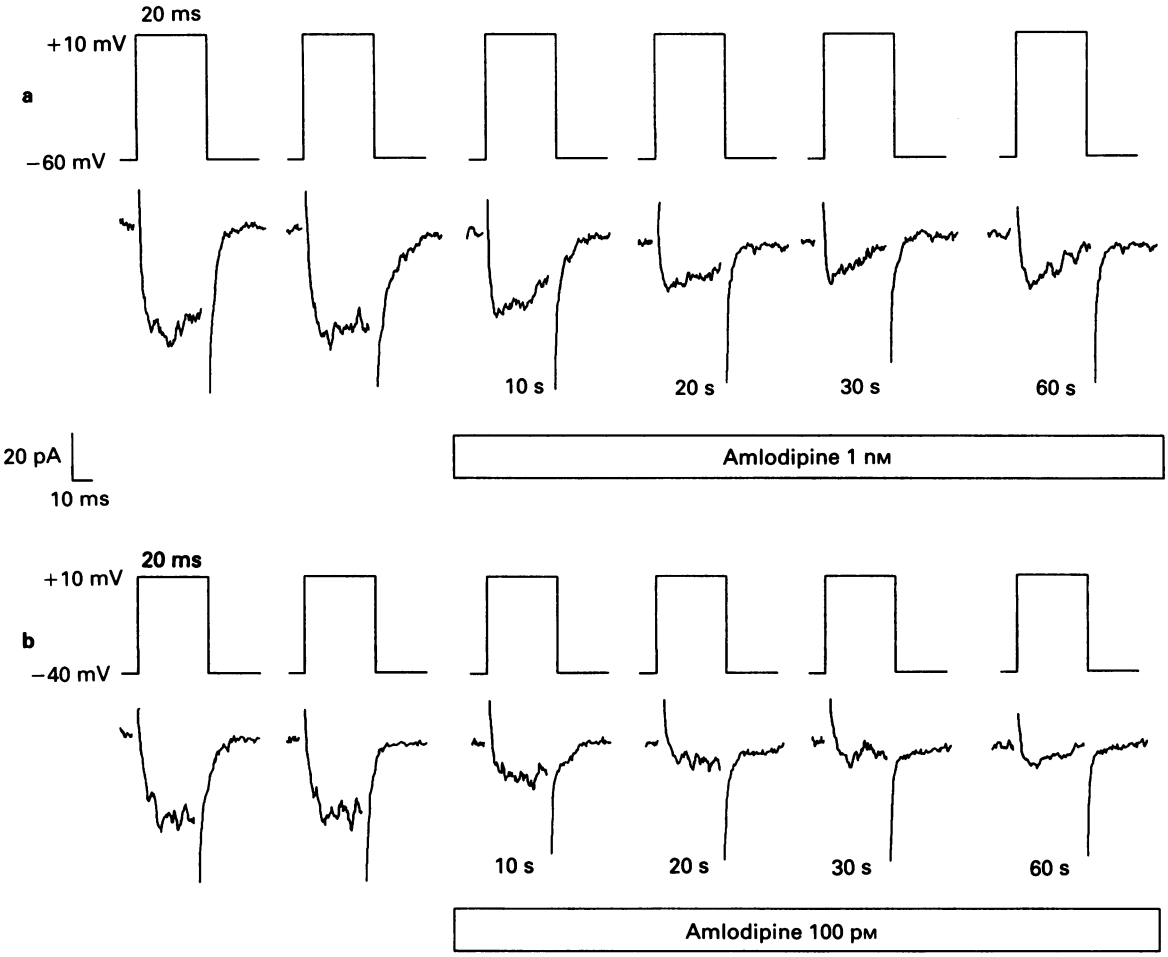


Figure 3 Onset of amlodipine-induced inhibition at (a) − 60 mV and (b) − 40 mV holding potential. Calcium channel currents were evoked at 1 Hz using a 20 ms pulse to + 10 mV from the holding potential. After a period in the absence of drug to ensure that currents were stable and did not 'run down', amlodipine was added to the chamber by a concentration-jump technique. Currents recorded at 10, 20, 30 and 60 s following addition of drug are shown.

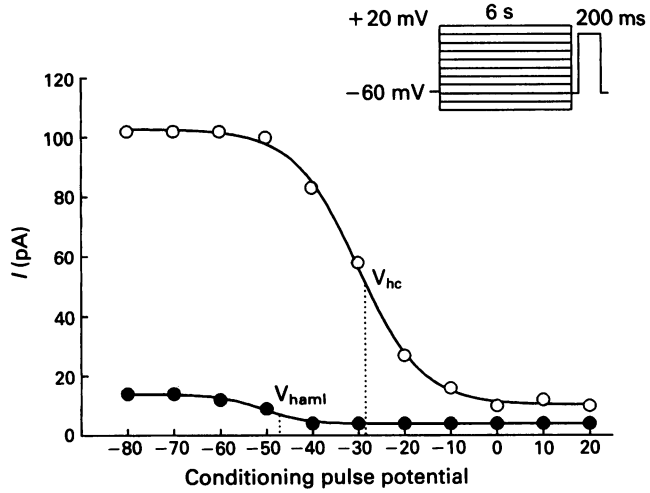


Figure 4 Effect of amlodipine on availability of calcium channel current in a single rabbit ear artery cell. Inactivation was induced by a 6 s prepulse. Data were fitted to a Boltzmann function as described in Methods. V_h indicates the potential at which current was inactivated by 50%, V_{hc} indicates this potential in the absence of drug, V_{haml} the potential in the presence of 1 nM amlodipine. Data are representative of 4 similar studies.

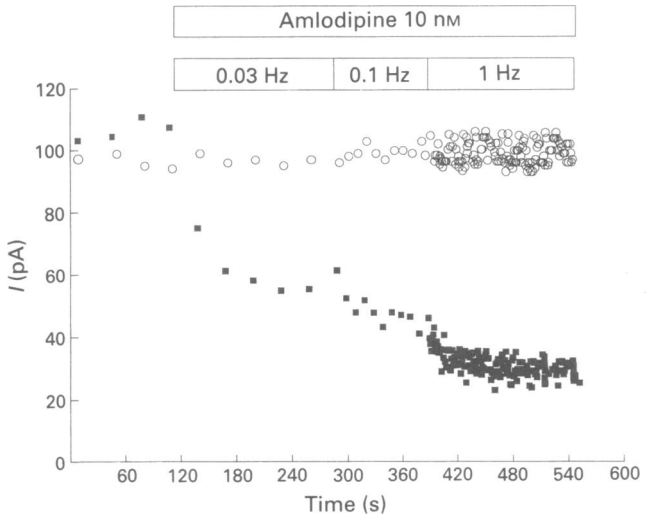


Figure 5 Effect of activation frequency on amlodipine-induced inhibition. Examples taken from single ear artery cells voltage clamped at −60 mV. Amlodipine 10 nM (■) or vehicle (○) was added to the cell at the time indicated by the upper bar. Current was elicited by a 20 ms step to + 10 mV at the rate indicated in the lower bar. The graph shows peak current plotted against time. The result for amlodipine is representative of 4 similar experiments.

channel currents in single vascular smooth muscle cells is rapid; near IC_{50} concentrations achieve stable inhibition in less than 1 min. Rapid onset of action of amlodipine has also been seen in studies in single myocardial cells (Kass & Arena, 1989). These findings contrast with studies of the onset of amlodipine-induced inhibition of contraction in isolated blood vessels, where amlodipine-induced inhibition is extremely slow (Burges *et al.*, 1987; Hughes *et al.*, 1991). Amlodipine is a charged compound relatively insoluble in organic solvents (Mason *et al.*, 1989), but several recent studies have shown that amlodipine readily partitions into lipid bilayers containing phospholipid and that insertion of amlodipine into the lipid bilayer occurs rapidly (Mason *et al.*, 1989; Seelig & Ganz, 1991).

The existence of a membrane pathway for the association of dihydropyridines with the calcium channel has been proposed previously (Rhodes *et al.*, 1985; Herbette *et al.*, 1986). In this model, access of the dihydropyridine molecule to the voltage-operated calcium channel is by lateral diffusion within the cell membrane. Such lateral diffusion within an essentially 2-dimensional structure decreases the time taken for drug and channel to interact (Rhodes *et al.*, 1985). Such a scheme may explain both the rapid onset of action of amlodipine seen in these studies as well as the apparent irreversibility of amlodipine-induced inhibition since amlodipine will tend not to diffuse out of the membrane (Mason *et al.*, 1989).

Although such a model can explain the rapid onset and slow offset of amlodipine seen in our studies it does not resolve the difference between the onset rates seen here and those observed in studies of isolated vessels. Bauerle & Seelig (1991) reported that both amlodipine and nimodipine had high mobility in lipid bilayers, so it seem unlikely that lateral diffusion would be restricted in the plasma membrane. We suggest that this difference reflects difficulty in amlodipine gaining access to multicellular preparations. Amlodipine may insert rapidly into the outer leaflet of the plasma membrane, but because of its polar side chain be unable to diffuse across the plasma membrane. Diffusion into the tissue could therefore not occur across cells and would be slowed in intact arteries. It is interesting to note that amlodipine is unable to block voltage-activated calcium channels when it is applied from the intracellular side (Kass & Kwan, 1992) suggesting that the ability of amlodipine to traverse the cell membrane is limited.

The finding that amlodipine displays use-dependence is of interest and similar findings were reported by Kass & Arena in myocardial cells (1989). Use-dependence is a well recognised feature of some calcium antagonists such as verapamil and diltiazem (Ehara & Kaufmann, 1978; Kuga *et al.*, 1990) but not dihydropyridines (Terada *et al.*, 1987; Honore *et al.*, 1989; Kuga *et al.*, 1990). The degree of use-dependence shown by amlodipine in these studies appears to be less than that shown by the phenylalkylamine, D600, in cells derived from the same tissue (Hering *et al.*, 1989). In the case of D600, almost no block of calcium channel current was seen following addition of D600 in the absence of channel activation. In contrast in this study amlodipine did cause block of calcium channel currents even in the absence of activation although frequency-dependent block of current by amlodipine was also clearly seen in these experiments. The molecular basis of use-dependence is uncertain but the most widely accepted explanation is that access of ionized drug to its receptor on the voltage-operated calcium channel is limited when the voltage-operated calcium channel is closed (i.e. resting or inactivated), so blocking and unblocking require open channels (Hille, 1984). It is therefore possible that this property of amlodipine may relate to the largely ionized nature of the drug under study conditions or alternatively reflect actions of amlodipine at non-dihydropyridine binding sites on the voltage-activated calcium channel (Rigby *et al.*, 1988; Nayler & Gu, 1992; Vaghy, 1992).

It is well known that vascular smooth muscle, particularly under vasoconstrictor influences such as those that exist *in vivo*, will be relatively depolarized (Nield, 1983). Moreover, there is evidence that experimental hypertension is associated with increased depolarization of vascular smooth muscle in resistance arteries (Harder & Hermsmeyer, 1983; Cheung, 1984; Stekiel *et al.*, 1986). Such a state of depolarization will make vascular smooth muscle much more susceptible to amlodipine-induced inhibition of voltage-operated calcium channels than other less depolarized tissues such as the heart. Indeed a similar explanation may underlie the apparent tissue selectivity of dihydropyridines towards different vascular beds (Towart *et al.*, 1985). Different regions of the circulation may be relatively more or less depolarized (Harder, 1983) and consequently the apparent potency of dihydropyridines may

differ between different vascular beds or even regions within a single vascular bed.

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Sensory nerve-mediated relaxation of guinea-pig isolated pulmonary artery: prejunctional modulation by α_2-adrenoceptor agonists but not sumatriptan

[1]A. Butler, S.P. Worton, C.T. O'Shaughnessy & H.E. Connor

Department of Neuropharmacology, Glaxo Group Research Ltd., Park Road, Ware, Herts SG12 0DP

1 Effects of the α_2-adrenoceptor agonists, UK14304 and clonidine, the 5-HT_1 receptor agonist, sumatriptan and the κ-opioid receptor agonist, GR103545, on sensory neurotransmission in histamine-contracted guinea-pig isolated pulmonary artery (GPPA) have been studied.

2 Electrical field stimulation (EFS) induced frequency-dependent relaxations of histamine-contracted GPPA, which were attenuated by tetrodotoxin and capsaicin pretreatment but not by the nitric oxide synthase inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME).

3 Substance P (0.3 μM) induced relaxations which were subject to rapid tachyphylaxis. Neither the NK_1 receptor antagonist, (±)-CP 96,345, nor desensitization to substance P had any effect of EFS-induced relaxations of histamine-contracted GPPA.

4 Calcitonin gene-related peptide (CGRP; 3 and 30 nM) induced concentration-dependent relaxations of histamine-contracted GPPA. The putative CGRP receptor antagonist, $CGRP_{8-37}$ (1 μM), markedly attenuated EFS-induced relaxations as well as relaxations induced by a low concentration of CGRP.

5 Sumatriptan (0.1 and 1 μM) and the selective κ-opioid receptor agonist, GR103545 (10 and 100 nM) had no effect on EFS-induced relaxations of histamine-contracted GPPA. In contrast, the α_2-adrenoceptor agonists UK14304 (1–100 nM) and clonidine (300 nM) attenuated responses to EFS, the attenuation of UK14304 (100 nM) being reversed by yohimbine (300 nM).

6 It is concluded that in GPPA, where a presynaptic inhibition of sensory neurotransmission by α_2-adrenoceptor activation could be shown, there was no evidence for such modulation by either sumatriptan-sensitive 5-HT_1 receptors or κ-opioid receptors.

Keywords: Sensory nerves; pulmonary artery; sumatriptan; calcitonin gene-related peptide (CGRP); κ agonists; α_2-adrenoceptors; presynaptic modulation

Introduction

It has been proposed that the anti-migraine activity of the novel 5-HT_1-receptor agonist, sumatriptan (GR43175C; Humphrey *et al.*, 1988) lies in its ability to constrict selectively cranial blood vessels (Humphrey & Feniuk, 1991). However, Buzzi & Moskowitz (1990) have shown that sumatriptan can inhibit neurogenically-mediated plasma protein extravasation from the dura of rats and guinea-pigs *in vivo* and propose that sumatriptan inhibits the release of neuropeptides via the activation of prejunctional 5-HT_1 receptors on perivascular sensory trigeminal nerve terminals.

The aim of this study was to investigate the effect of sumatriptan on sensory neurotransmission in isolated blood vessels. However, using electrical field stimulation we have been unable to reveal a sensory-nerve mediated response in isolated cerebral blood vessels: thus, using a variety of stimulation parameters, responses are either non-neuronally mediated (unpublished results) or are mediated by nitric oxide via activation of non-sensory nerves (Stubbs *et al.*, 1992). Recently, Maggi *et al.* (1990) have reported that electrical field stimulation (EFS) of guinea pig isolated pulmonary artery (GPPA), in the presence of atropine and guanethidine, causes marked relaxations which are both capsaicin- and tetrodotoxin-sensitive and therefore appear to be mediated by activation of sensory nerves. The present study was undertaken firstly to characterize further the neurogenically-mediated relaxations in GPPA in order to confirm their mediation by sensory nerves and secondly to investigate whether these relaxations are susceptible to prejunctional receptor modulation. Thus the ability of sumatriptan (via 5-HT_1 receptors), UK14304 (via α_2-adrenoceptors) and GR103545 (via κ-opioid receptors) to modulate release of neuropeptides in this tissue has been studied.

Preliminary results have been presented to the British Pharmacological Society (Butler *et al.*, 1992).

Methods

Male, Dunkin-Hartley guinea-pigs (250–300 g) were killed either by cervical dislocation or pentobarbitone overdose followed by exsanguination. Pulmonary artery trunks were carefully excised and any adherent connective tissue was removed. One ring was prepared from each artery. Rings were suspended between two L-shaped tissue holders, 200 μm diameter, in 15 ml organ baths containing Krebs-Henseleit solution maintained at 37°C and gassed with 95% O_2/5% CO_2. Atropine and guanethidine (both 3 μM) were present throughout the experiments to block cholinergic and sympathetic nerve-mediated responses respectively. Isometric recordings were made with Statham-Gould transducers attached to a Devices pen recorder. Tissues were placed under a resting tension of 0.5 g and allowed to equilibrate for 30 min. Tension was readjusted to maintain a resting tone of around 0.5 g.

Drugs were added directly to the bath in a volume not greater than 150 μl. Histamine was used to induce a contractile tone in the preparations. A cumulative concentration-contraction curve was constructed for histamine (1–100 μM) after which tissues were washed and left for approximately 30 min before being re-contracted to a single, submaximal

[1] Author for correspondence.

concentration (usually 10 μM) of histamine. In one series of experiments, tissues were also contracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 10 μM) after the initial histamine curve.

Electrical field stimulation (EFS) experiments

Rectangular wave electrical stimulations were applied to the tissue via two platinum electrodes placed parallel to and on opposite sides of the artery rings. Pulses of 0.1 ms duration, supermaximal (20 V) amplitude and 1 to 8 Hz frequency were generated with a Digitimer DS9 stimulator connected to a Digitimer D4030 pulse generator and were applied for 10 s every 15 min. Three electrical stimulations of submaximal (4 Hz) frequency were applied before the administration of any test agent in order to check the reproducibility of the responses. Test agents were added to the bath when the tone had returned to pre-stimulation levels and were allowed a contact time of 10 min. Electrical stimulations were then repeated. In experiments where the effect of capsaicin pretreatment on EFS-induced relaxations was studied, capsaicin was added to the baths and allowed a contact time of 10 min. The tissues were then washed several times and 30 min later were re-contracted with histamine and EFS-induced responses were re-established.

Effects of substance P and CGRP

Preliminary experiments showed that responses to substance P were susceptible to tachyphylaxis such that after application of a single, submaximal concentration of substance P, further applications had no relaxant effect unless preceeded by washing. For this reason, the antagonist effects of CP 96,345 were examined against a single concentration of substance P with a 15 min antagonist contact time.

In addition, experiments were performed to investigate the effects of substance P desensitization on responses to EFS. Control responses to electrical stimulation were established and when tone had returned to baseline, substance P was added directly to the bath and allowed a 15 min contact time. The tissues were then electrically stimulated in the presence of substance P.

Responses to human α-calcitonin gene-related peptide (CGRP) exhibited marked desensitization such that full concentration-response curves were not repeatable in the same tissue. Therefore, a two-point assay was employed in which submaximal concentrations (3 and 30 nM) of CGRP were used and an interval of 60 min allowed between successive curves. With this protocol, CGRP-induced responses were reproducible. In some experiments the effect of the putative CGRP antagonist, $CGRP_{8-37}$ (Chiba *et al.*, 1989), was studied. Responses to EFS or CGRP (3 and 30 nM) were established and the tissues were then washed. Tissues were re-contracted to histamine 60 min later and when the histamine-induced tone was stable, $CGRP_{8-37}$ (1 μM) was added to the bath. Responses to EFS or CGRP (3 and 30 nM) were re-established 15 min later.

Treatment of results

Responses to histamine are expressed as the increase in tone (g) above basal tone. Relaxations are expressed as a percentage reduction (mean ± s.e.mean) of the histamine-induced tone. Statistical analysis was performed by a two-tailed Student's *t* test which was paired when comparing values obtained in the same tissue.

Drugs and solutions

The composition of the Krebs-Henseleit solution was mM NaCl 118, $NaHCO_3$ 25, KCl 4.75, KH_2PO_4 1.2, $MgSO_4.7H_2O$ 0.6, glucose 11.1 and $CaCl_2$ 1.3. The following drugs were used: atropine methylbromide (Sigma), human α-calcitonin gene-related peptide (CGRP; Peninsular laboratories), $CGRP_{8-37}$ (Peninsular Laboratories), capsaicin (Sigma), clonidine hydrochloride (Boehringer), (±)-CP 96,345 ([cis-2-(diphenylmethyl)-N-[(2-methoxyphenyl)-methyl]-1-azabicyclo [2.2.2.] octan-3-amine; Glaxo Group Research, Ware), GR103545 ((**R**)-methyl 4-[(3,4-dichlorophenyl)acetyl)-3-(1-pyrrolidinyl-methyl)-1-piperazinecarboxylate fumarate; Glaxo Group Research, Ware), guanethidine sulphate (Ciba Laboratories), histamine diphosphate (Sigma), N^{ω}-nitro-L-arginine methyl ester (L-NAME; Sigma), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$ Lutalyse, Upjohn), substance P (Peninsula Laboratories), sumatriptan succinate (Glaxo Group Research, Ware), tetrodotoxin (TTX; Sigma), UK14304 (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline tartrate; Pfizer) and yohimbine hydrochloride (Sigma).

Capsaicin was dissolved in ethanol at a concentration of 100 mM. Substance P, CP 96,345, CGRP and $CGRP_{8-37}$ were dissolved in 1 mM acetic acid and frozen until required. All other drugs were dissolved in distilled water or Krebs. Dilutions were made in the Krebs-Henseleit medium.

Results

Histamine (1–100 μM) caused concentration-related contractions of GPPA. A submaximal dose (usually 10 μM) was used to produce a stable tone (1.0–1.5 g) in the artery rings (Figure 1a) which represented approximately 75% of the maximum contraction to histamine.

Electrical-stimulation experiments

Electrical field stimulation (EFS; 1–8 Hz) induced frequency-dependent relaxations of the histamine-contracted tissues. Responses to stimulation at frequencies up to 4 Hz were reproducible when an interval of 15 min between stimulations was used. After stimulation at 8 Hz, however, the tone did not usually return to pre-stimulation levels (Figure 1a). EFS (4 Hz)-induced relaxations were markedly attenuated by TTX (0.3 μM; Table 1). Capsaicin (1 μM) caused a marked relaxation of histamine-contracted GPPA (85.0 ± 3.8% of histamine tone; $n = 4$). The tone induced by histamine before and after capsaicin was similar (results not shown), but the relaxations to EFS (4 Hz) were markedly reduced after capsaicin treatment ($n = 4$; Table 1). In contrast, the nitric oxide synthase inhibitor, L-NAME (100 μM), did not modify EFS(4 Hz)-induced relaxations of GPPA (Table 1).

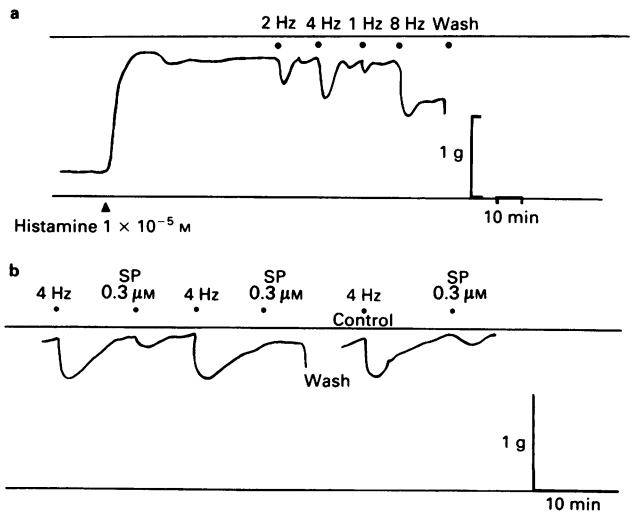


Figure 1 (a) Electrical field stimulation (EFS)-induced relaxations of guinea-pig isolated pulmonary artery (GPPA). Stimulation at stated frequency, 0.1 ms pulse duration, 20 V amplitude for 10 s every 15 min. (b) Effect of substance P (SP) desensitization on EFS-induced relaxations of GPPA.

Table 1 The effect of various agents on electrical field stimulation (EFS, 4Hz)-induced relaxations of guinea-pig isolated pulmonary artery

		% relaxation of histamine tone		
Treatment	*Conc. (μM)*	*Before treatment*	*After treatment*	n
TTX	0.3	35.4 ± 6.8	3.8 ± 1.8*	5
Capsaicin	1	41.5 ± 2.6	3.5 ± 3.5*	4
L-NAME	100	24.6 ± 10.6	26.2 ± 3.0	5
CP 96,345	0.3	24.3 ± 3.3	23.7 ± 3.0	3
$CGRP_{8-37}$	1	27.2 ± 6.7	9.0 ± 3.8*	4
Sumatriptan	0.1	28.4 ± 4.3	29.5 ± 4.4	5
Sumatriptan	1	30.3 ± 4.4	25.6 ± 4.1	5
UK14304	0.1	43.0 ± 5.5	12.3 ± 6.4*	3
Clonidine	0.3	31.4 ± 6.1	14.3 ± 6.0*	3

Values are mean ± s.e.mean of n tissues.
*$P<0.05$ (Student's t test).
For abbreviations, see text.

Substance P (0.3 μM) caused marked relaxations of histamine-contracted GPPA. These relaxations were short lasting such that tone had returned to basal levels within 5 min of substance P application. Responses to substance P were highly susceptible to tachyphylaxis; after a single application tissues were unresponsive to further applications unless preceded by washing (see Figure 1b). However, relaxations induced by EFS (4 Hz) were unaffected by desensitization of the tissue to substance P (Figure 1b). Additionally, EFS (4Hz)-induced relaxations were unaffected by the NK_1 receptor antagonist, CP 96,345 (Snider *et al.*, 1991) at a concentration that markedly attenuated responses to substance P (0.3 μM; Figure 2 and Table 1).

CGRP (1–100 nM) caused concentration-dependent relaxations of GPPA. A two-point, cumulative assay (3 nM and 30 nM CGRP) was used to obtain reproducible responses (see Methods). The putative CGRP receptor antagonist, $CGRP_{8-37}$ (1 μM), had no effect on histamine-induced tone. It significantly attenuated responses to the lower (3 nM) concentration of exogenously applied CGRP but had no effect on the relaxation induced by 30 nM CGRP (Figure 3a). In contrast, EFS-induced relaxations were markedly attenuated by $CGRP_{8-37}$ (1 μM) (the attenuation being statistically significant at the 4 Hz stimulation; Figure 3b).

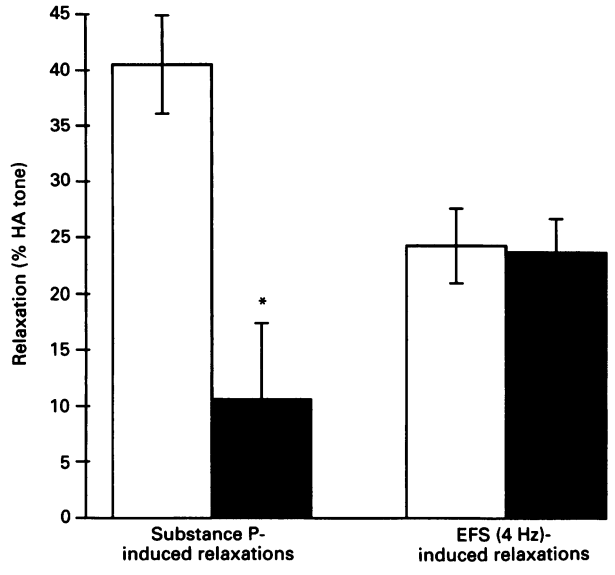


Figure 2 The effect of CP 96,345 (0.3 μM) on substance P (0.3 μM)-induced and EFS (4 Hz)-induced relaxations of guinea-pig isolated pulmonary artery. Open columns represent controls and filled columns responses in the presence of CP 96,345 (0.3 μM). Values are mean ± s.e.mean of 4 tissues. HA = histamine. *$P<0.05$; paired t test.

Effects of putative presynaptically acting agonists

Sumatriptan (0.1 μM) did not cause relaxations of histamine-contracted GPPA or attenuate EFS (4-Hz)-induced relaxations. Similarly at a higher concentration (1 μM) sumatriptan had no effect on the EFS-induced relaxations (Figure 4c) but did cause small transient relaxations of histamine-contracted GPPA (mean = 7.2 ± 0.6% of histamine tone, $n = 4$). However, when the tissues were contracted to $PGF_{2\alpha}$(10 μM), which induced the same degree of tone as histamine (10 μM), sumatriptan (1 μM) did not cause relaxations (results not shown). In contrast to sumatriptan, the α_2-adrenoceptor agonists, UK14304 (0.001–0.1 μM; Figure 4a) (Cambridge, 1981) and clonidine (0.3 μM; Figure 4b), markedly reduced the EFS-induced relaxations. The attenuation by UK14304 was reversed by the α_2-adrenoceptor antagonist, yohimbine at a concentration (0.3 μM; Figure 5) which alone had no effect on EFS-induced relaxations. However, UK14304 (0.1 μM) had no effect on relaxations induced by 5 nM CGRP (controls = 35 ± 7.7%, treated = 32 ± 8.4%). The selective κ-opioid receptor agonist, GR103545 (Hayes *et al.*, 1990; 10 and 100 nM) had no effect on EFS-induced relaxations of GPPA (Figure 4d).

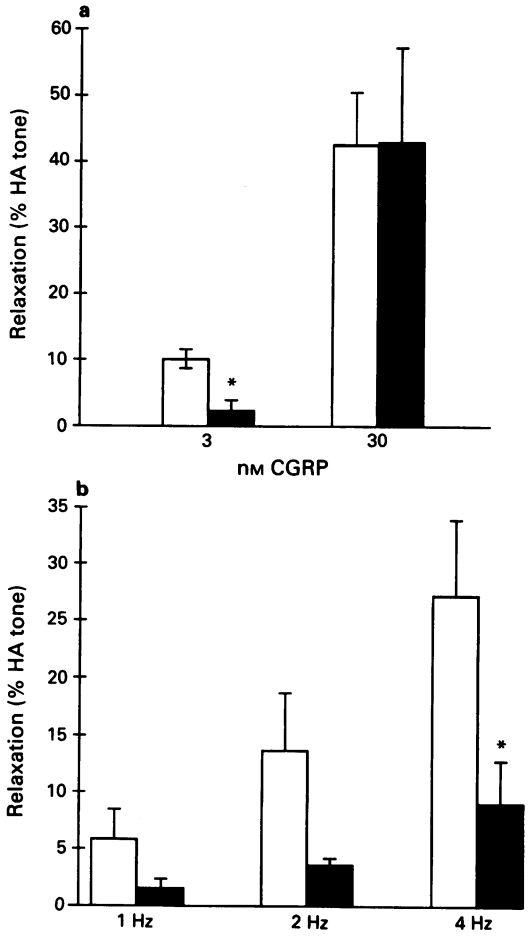


Figure 3 The effects of $CGRP_{8-37}$ (1 μM) on (a) calcitonin gene-related peptide (CGRP)-induced and (b) EFS-induced relaxations of guinea-pig isolated pulmonary artery. Open columns represent controls (first curves) and filled columns represent responses in the presence of $CGRP_{8-37}$ (1 μM). Responses to 3 and 30 nM CGRP were reproducible when an interval of 60 min was allowed between curves. Results are mean ± s.e.mean of 4 tissues. HA = histamine. *$P<0.05$; paired t test.

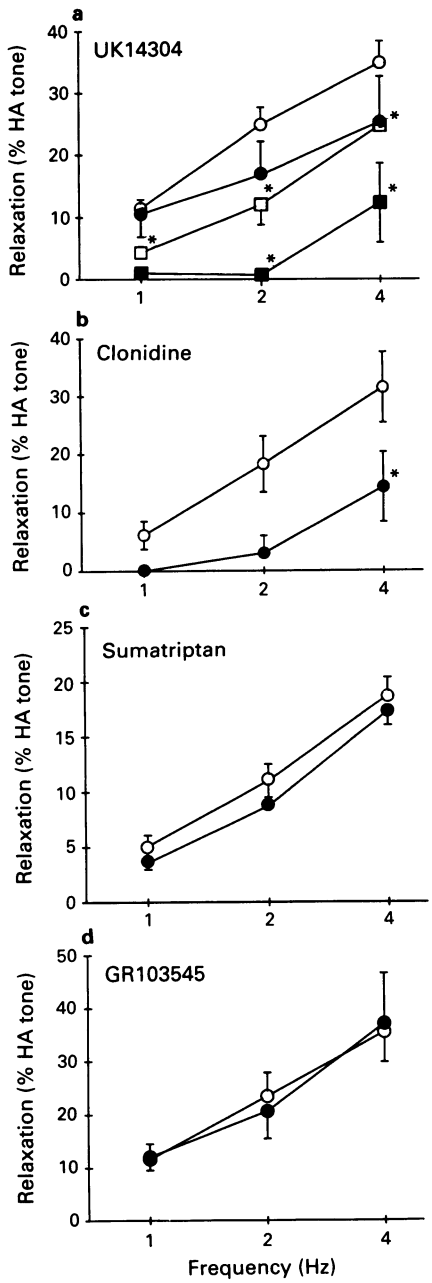


Figure 4 The effect of (a) UK14304 1 (●), 10 (□) and 100 (■) nM, (b) clonidine 300 nM(●), (c) sumatriptan 100 nM (●) and (d) GR103545 100 nM (●) on EFS-induced relaxations of guinea-pig isolated pulmonary artery. Control responses are shown by (○). Values are mean ± s.e.mean of 3–5 tissues. HA = histamine. $*P<0.05$; paired t test.

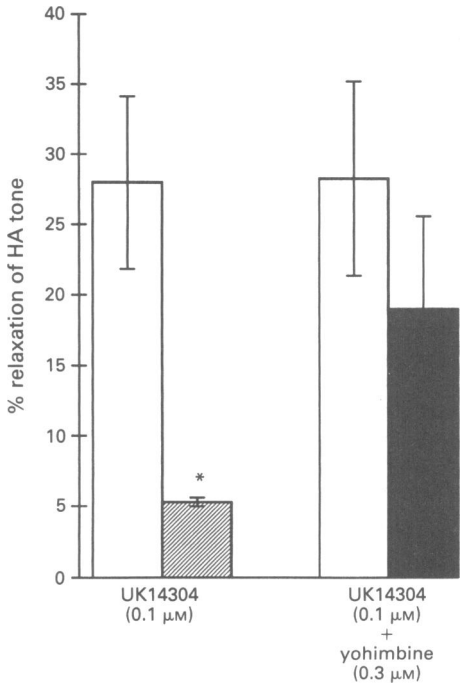


Figure 5 The effect of UK14304 and yohimbine on electrical field stimulation-induced (2 Hz) relaxations of guinea-pig isolated pulmonary artery (GPPA). Open columns represent control responses, hatched column represents responses in the presence of UK14304 (0.1 μM) and filled column represents responses in the presence of UK14304 (0.1 μM) and yohimbine (0.3 μM). Values are mean ± s.e.mean of 4 tissues. HA = histamine. $*P<0.05$; paired t test.

Discussion

The aims of the present study were two fold: firstly, to characterize further the electrical stimulation-induced relaxations of GPPA described by Maggi *et al.* (1990) and secondly to determine the effects of sumatriptan on these responses. We have confirmed that the EFS-induced relaxations of GPPA are both TTX and capsaicin-sensitive, strongly suggesting that these relaxations are mediated via activation of sensory nerves. Nitric oxide appears to be a mediator of non-adrenergic non-cholinergic neurogenic relaxations in some vascular preparations (Gonzalez & Estrada, 1991; Stubbs *et al.*, 1992). However, the lack of effect of the nitric oxide synthase inhibitor, L-NAME, on EFS-induced relaxations in GPPA indicates that nitric oxide does not have any significant role in neurogenically-mediated responses in this tissue.

Additionally, the present study suggests that substance P is not involved in EFS-induced relaxations of GPPA as such responses were not affected either by CP 96,345 or by desensitization to substance P. In addition, the lack of effect of L-NAME supports the hypothesis that endothelial NK_1 receptors are not likely to be involved. In contrast, responses to exogenously applied substance P were attenuated by both treatments. Maggi *et al.* (1990) showed that responses of GPPA to substance P were mimicked by the NK_1-receptor agonist, [Pro^9]-substance P sulfone and the ability of CP 96,345 to block substance P-induced relaxations provides further evidence for the existence of NK_1 receptors in this tissue. However, the reason for the lack of involvement of NK_1 receptors in the responses to EFS in GPPA is unclear. It may be that low levels of substance P are released, relative to CGRP, from sensory nerves in this tissue or that the released substance P cannot readily access endothelial NK_1 receptors which mediate relaxations.

CGRP does appear to be involved in EFS-induced relaxations of GPPA since, at each frequency examined, relaxations were attenuated by the CGRP receptor antagonist, $CGRP_{8-37}$. In addition, $CGRP_{8-37}$, at the same concentration (1 μM), attenuated relaxations induced by exogenously applied CGRP (3 nM). However, it appears that responses to electrical stimulation are more susceptible to blockade by $CGRP_{8-37}$ than those to exogenously applied CGRP since the relaxant effects of a higher concentration of CGRP (30 nM) were unaffected by the antagonist. It should be noted that the higher concentration of CGRP produced a relaxation of approximately twice the magnitude of the response obtained to EFS (4 Hz). It is possible that at these higher concentrations CGRP might be activating a different subtype of CGRP receptor which is less sensitive to blockade by

$CGRP_{8-37}$. This is not without precedent since Dennis *et al.* (1989) have suggested that such subtypes exist, primarily based on the differing antagonist potency of $CGRP_{8-37}$ in various functional models of CGRP receptor activation.

Buzzi & Moskowitz (1990) have proposed that sumatriptan can modulate neuropeptide release from sensory nerve terminals in the intracranial circulation *in vivo*. In the present study, sumatriptan did not modulate the EFS-induced relaxations at concentrations which are maximally effective at inhibiting sympathetically-mediated neuronal responses in dog isolated saphenous vein (Humphrey *et al.*, 1988) and it seems likely, therefore, that prejunctional 5-HT_1 receptors do not modulate sensory neuropeptide release in GPPA. Similarly the κ-opioid receptor agonist, GR103545, did not modulate EFS-induced responses at concentrations which were shown to be maximally effective at inhibiting twitch responses in the rabbit isolated vas deferens (Hayes *et al.*, 1990). In contrast, the α_2-adrenoceptor agonist, UK14304, inhibited EFS-induced relaxations in a concentration-dependent manner and this was reversed by the selective α_2-adrenoceptor antagonist, yohimbine. Since UK14304 had no effect on CGRP-induced relaxations, a direct effect on postjunctional CGRP receptors can be ruled out. This suggests that in this tissue, where a presynaptic modulation of sensory nerve-mediated responses by α_2-adrenoceptor agonists can be demonstrated, sumatriptan-sensitive 5-HT_1 receptors and κ-opioid receptors do not modulate neuropeptide release from sensory nerves.

In conclusion, we were unable to demonstrate modulation of sensory neurotransmission by sumatriptan in GPPA. This suggests, in this tissue at least, that 5-HT_1 receptors do not inhibit neuropeptide release from sensory nerves. However, 5-HT_1 receptors, may have a very localized distribution and therefore the results of the present study do not rule out the possibility that sumatriptan may modulate sensory neuropeptide release in intracranial blood vessels as suggested by Buzzi & Moskowitz (1990). Further studies are needed to investigate this possibility.

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Effect of passive sensitization on the mechanical activity of human isolated bronchial smooth muscle induced by substance P, neurokinin A and VIP

Abdellaziz Ben-Jebria, [1]Roger Marthan, Max Rossetti & Jean-Pierre Savineau

Laboratoire de Physiologie, Faculté de Médecine Victor Pachon, Université de Bordeaux II, 33076 Bordeaux Cedex-France

1 The effect of passive sensitization on the mechanical activity of human isolated bronchial smooth muscle induced by the following neuropeptides substance P (SP), neurokinin A (NKA) and vasoactive intestinal peptide (VIP) was studied both in the absence and in the presence of the neutral endopeptidase (NEP) inhibitor, phosphoramidon.

2 Cumulative concentration-response curves (CCRC) to these neuropeptides were constructed in human passively sensitized isolated bronchial rings and compared to those in paired controls. Passively sensitized human isolated bronchial rings were tissues incubated overnight in serum from asthmatic patients atopic to *Dermatophagoides pteronyssinus* and paired controls were tissues originating from the same lung specimens but incubated overnight in serum from healthy donors.

3 In the absence of phosphoramidon, passive sensitization significantly increased the amplitude of the contractile responses to SP and NKA including that to the maximal concentration given from 50 ± 5% to 76 ± 6% ($n = 5$, $P < 0.05$) and from 70 ± 7% to 101 ± 6% ($n = 5$, $P < 0.05$) of the maximal response to acetylcholine, respectively. Passive sensitization significantly shifted to the left the CCRC for both tachykinins as measured by the geometric means dose-ratios which were 8.5 (95% confidence limits (CL): 3.1–13.9) and 7.3 (95% CL: 4.2–10.3) for SP and NKA, respectively.

4 In the presence of phosphoramidon (10 μM), passive sensitization still increased significantly the amplitude of the contractile responses to SP and NKA including that to the maximal concentration given from 74 ± 4% to 115 ± 7% ($n = 5$, $P < 0.05$) and from 104 ± 9% to 146 ± 16% ($n = 5$, $P < 0.05$) of the maximal response to acetylcholine, respectively. Passive sensitization still significantly shifted to the left the CCRC for both tachykinins as measured by the dose-ratios which were 9.0 (95% CL: 4.3–13.6) and 5.4 (95% CL: 2.9–7.9) for SP and NKA, respectively.

5 The relaxant response to the maximal concentration of VIP given in tissues precontracted with histamine (0.5 mM) was significantly reduced by passive sensitization from 41 ± 4% to 25 ± 3% ($n = 5$, $P < 0.05$) of the amplitude of the precontraction in the absence of phosphoramidon and from 72 ± 1% to 49 ± 4% ($n = 5$, $P < 0.05$) in the presence of phosphoramidon (10 μM). Passive sensitization significantly shifted to the right the CCRC for VIP as measured by the dose-ratios which were 10.4 (95% CL: 6.6–14.1) and 6.4 (95% CL: 3.0–9.8) in the absence and in the presence of phosphoramidon, respectively.

6 We conclude that passive sensitization enhances the mechanical response to neuropeptides which contract human isolated bronchial smooth muscle and reduces that to a neuropeptide which relaxes it. The mechanism of passive sensitization-induced changes in the mechanical activity appears to be independent of a decrease in NEP activity since these changes persist in the presence of the NEP inhibitor, phosphoramidon.

Keywords: Substance P; neurokinin A; vasoactive intestinal peptide; phosphoramidon; human isolated bronchial smooth muscle; passive sensitization; asthma

Introduction

There is now accumulating evidence that many different neuropeptides such as substance P (SP), neurokinin A (NKA) and vasoactive intestinal peptide (VIP) may play an important role in the control of airway calibre. These peptides have been localized in airway tissues of several species, including man (Richardson & Beland, 1976; Barnes, 1987a). Tachykinins (SP and NKA) have potent contractile effects in airways both *in vivo* and *in vitro* (Advenier *et al.*, 1987; Ewans *et al.*, 1988; Shore *et al.*, 1988; Naline *et al.*, 1989) and several recent investigations have shown that, in human airways, NKA is considerably more potent than SP (Palmer & Barnes, 1987; Black *et al.*, 1988). VIP, a 28 amino acid peptide that was found in cholinergic efferent fibres, causes relaxation of bronchial smooth muscle (Diamond *et al.*, 1983; Barnes & Dixon, 1984; Palmer *et al.*, 1986).

Like neurotransmitters, neuropeptides are physiologically degraded by enzymes and mostly by neutral endopeptidase (NEP, also called 'enkephalinase') which is heavily concentrated within airway epithelium and smooth muscle (Johnson *et al.*, 1985). Removal of epithelium from tracheal or bronchial strips potentiates the contractile response to SP and NKA by inhibiting tachykinin cleavage and inactivation (Devillier *et al.*, 1988; Frossard *et al.*, 1989). In addition, pretreatment of airway tissue with phosphoramidon, a potent inhibitor of NEP, significantly enhances tachykinin-induced contraction of the smooth muscle (Black *et al.*, 1988; Lötvall *et al.*, 1991).

Although the alteration in the epithelial layer often seen *in vivo* in chronic severe asthma could likewise enhance tachykinin reactivity, to date, results about the potential contribution of neuropeptides to human airway obstruction are controversial. Indeed, Joos and coworkers (1986) demonstrated that inhalation of NKA induced bronchoconstriction in asthmatic subjects. A recent study of Cheung *et al.* (1991),

[1] Author for correspondence.

showing that inhalation of thiorphan (an inhibitor of NEP) potentiated tachykinin-induced bronchoconstriction in non-asthmatic subjects, supported the hypothesis that a decrease in NEP activity could contribute to bronchial hyperresponsiveness to NKA. Moreover, pharmacological evidence suggests that tachykinins could participate in the pathogenesis of airflow obstruction induced by isocapnic dry gas hyperpnoea in human subjects with asthma. Indeed, sodium cromoglycate which inhibits C-fibres discharge (Dixon *et al.*, 1980) and thus the release of tachykinins or the tachykinin receptor antagonist, SCH 37224 (Solway & Leff, 1991) blunt hyperpnoea-induced broncho-obstruction in asthma. Similarly, it has been suggested that VIP and related peptides released from airway cholinergic nerves may also play a role in asthma (Barnes, 1989). Joos and coworkers (1989), however, found a relatively small degree of tachykinin aerosol-induced bronchoconstriction in asthmatic patients. Moreover, no evidence for an impaired nonadrenergic noncholinergic (NANC) bronchodilator reflex has been observed in patients with mild asthma (Michoud *et al.*, 1988; Lammers *et al.*, 1989).

Passive sensitization produces hyperresponsiveness of human isolated airways (Black *et al.*, 1989; Marthan *et al.*, 1992) and provides the opportunity to study the interaction between allergic factors and smooth muscle behaviour. As far as we know, no studies have attempted to determine whether responses to neuropeptides are altered in human passively sensitized airway tissue. Therefore, we have examined the effect of passive sensitization on cumulative concentration-response curves to SP, NKA and VIP in the human isolated bronchus and, by the use of phosphoramidon, investigated the role of NEP activity in this effect.

Methods

Tissue preparation

Human lung was obtained at thoracotomy as previously described (Savineau *et al.*, 1991) from 34 patients. These patients (22 males and 12 females aged from 37 to 68 years) were current or ex-smokers and underwent resection for pulmonary carcinoma. As in previous studies (Marthan *et al.*, 1992; Villanove *et al.*, 1992), specimens were selected from patients whose lung function was within the normal range i.e. whose forced expiratory volume in one second (FEV_1) measured, before surgery, in our Lung Function Testing Laboratory in the Hospital was above 80% of predicted. Moreover, analysis of the medical record of the patients revealed that none of them had a history of atopy. After resection, the specimens were immediately transferred to the laboratory in an ice-cold oxygenated Krebs-Henseleit solution (composition in mM: NaCl 118.4, KCl 4.7, $CaCl_2.2H_2O$ 2.5, $MgSO_4.7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0, D-glucose 11.1). From a macroscopically tumour-free part of each of the specimens, segments of human bronchi (3rd and 4th generation) 30–40 mm in length and 3–5 mm in internal diameter were carefully dissected from surrounding parenchyma without damaging the airway membrane. After removal of adhering fat and connective tissue, bronchi were cut into rings measuring 4–5 mm in length.

Passive sensitization

Passive sensitization was carried out as previously described (Black *et al.*, 1989; Marthan *et al.*, 1992) once it was checked that each of the lung specimens was not spontaneously sensitized. This was tested by the fact that administration of 0.05 ml i.e. 300 units of two different allergens (house dust mite and grass pollen) to a single bronchial ring did not elicit a contractile response (see Mechanical recording). The remaining rings were then divided into two paired groups. One group was passively sensitized by incubation overnight at room temperature in a non-diluted serum pool from atopic asthmatic subjects whose concentration of both total IgE and specific IgE to *Dermatophagoides pteronyssinus* (*D. pter.*) was above 1000 International Units (iu) ml^{-1} and 17.5 PRU ml^{-1} (i.e. 4 + RAST titre), respectively. The other group of bronchial rings was similarly incubated overnight in a non-diluted serum pool from non-asthmatic non-atopic subjects whose total IgE concentration was below 10 iu ml^{-1}.

Mechanical recording

In the morning of the next day, the paired rings of bronchus were mounted in vertical organ baths of an automatic system (IOS_1 isolated organ system, Celaster, Lusignan, France) (Mahe *et al.*, 1989) filled with 20 ml of Krebs-Henseleit solution, bubbled with 95% O_2 in CO_2 at pH 7.4, and maintained at 37°C. Each ring was suspended by means of two stainless clips passed through the lumen. One clip was anchored to the bottom of the organ chamber and the other was connected to an isometric force transducer (Celaster). The resting tension was ajusted to a 1.5 g load. This load was selected on the basis of a separate series of experiments conducted on 4 different lung specimens in which we constructed length-tension curves using repeated administration of single doses of acetylcholine (ACh 1 mM). It was found that optimal length was achieved for these rings when they were stretched to equilibrate against a load of 1.5 g whether the contraction was expressed as raw value of force or force normalized to dry tissue weight (De Jongste *et al.*, 1985) (Figure 1).

Protocol

When stable tone had been established, for experiments using tachykinins (SP and NKA), a supramaximal dose of ACh (1 mM) was first administered to the bath to all of the rings and the contractile response (reference response) allowed to

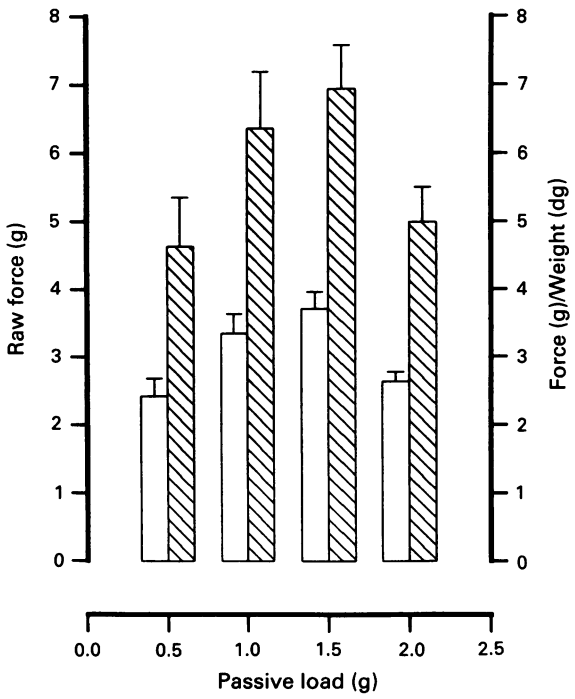


Figure 1 Optimal passive resting load in human isolated bronchial smooth muscle rings. Raw values of force (left axis, open column) as well as values of force normalized to dry tissue weight in response to iterative stimulation with a supramaximal concentration of acetylcholine (ACh, 1 mM bolus) are plotted versus passive preload values achieved by stretching the ring to increasing initial lengths. Data are means ± s.e.mean for 16 rings originating from 4 different lung specimens.

plateau (< 5 min) before the ACh was removed from the baths by repeated washing of the tissues. When stable resting tone returned to baseline, a cumulative concentration-response curve (CCRC) was obtained to the tachykinins in each ring either sensitized or non-sensitized. For experiments using VIP, tissues were precontracted with histamine (0.5 mM) and the CCRC for VIP was obtained at the plateau of the histamine-induced contraction. Only one peptide was tested in one patient and thus, this series of experiments was conducted on 10 sets of paired rings for each neuropeptide (i.e. 5 sets in the absence of phosphoramidon and 5 sets in the presence of phosphoramidon).

At the end of each experiment, when baseline tone had re-established on repeated washout of the tissue, 0.05 ml (300 u) of *D. pter.* was administered to all of the bronchial rings to verify that it induced a contractile response only in those that were passively sensitized. The mean (± s.d.) amplitude of antigen-induced contraction was 95 ± 11% of the maximal ACh-induced contraction in the passively sensitized tissues.

Data analysis

Data were acquired on-line with MOISE software and processed with the ANAMOISE programme (Mahe *et al.*, 1989). The contractile response in each ring to SP and to NKA was expressed as a percentage of the maximal contractile response to ACh in that ring and relaxation response to VIP was expressed as a percentage of the histamine-induced precontraction. Both ACh-induced contraction and histamine precontraction were always elicited in the reference solution i.e. in the absence of phosphoramidon. CCRC relating the response to the logarithm of the concentration of the neuropeptide were constructed for each tissue and for each peptide. From each of these curves, the concentration-ratio, so-called dose-ratio, was calculated from the concentrations of each neuropeptide that produced an arbitrarily determined response for each pair of tissues, i.e. sensitized and non-sensitized, which was on the linear parallel portion of the log concentration-response curves (Black *et al.*, 1981). Such a condition for paired CCRC in a single experiment was always achieved for a response in the range of 30 to 40% of the maximal response to ACh for SP and NKA in the absence and in the presence of phosphoramidon, respectively. Similarly, dose-ratios were calculated for a response in the range of 10 and 30% of histamine-induced precontraction for VIP in the absence and in the presence of phosphoramidon, respectively. Sensitization-induced changes in the potency of neuropeptides were assesed in this way because the true maximal response is difficult to obtain and requires very large concentrations of peptides. Since in each experiment, duplicate bronchial rings were studied for each peptide in both the passively sensitized and the non-sensitized tissues, a mean CCRC was calculated for each type of tissue (i.e. sensitized and non-sensitized) to be representative of the lung specimen. Overall mean CCRC were constructed for all experiments. Mechanical responses are given as means ± s.e.mean and dose-ratios as geometric means with 95% confidence limits (CL). Statistical analysis was carried out only between pairs of tissues dissected from the same lung specimens using both analysis of variance (ANOVA) and paired two-tailed Student's *t* test to compare results for sensitized and control (non-sensitized) tissues, either in the absence or in the presence of phosphoramidon. Dose-ratios were compared to unity which is the figure found when the potency of an agonist is the same whatever the two experimental conditions. Differences were considered as having statistical significance when a P value of less than 0.05 was found.

Drugs

The following drugs were used: substance P, neurokinin A, vasoactive intestinal peptide, acetylcholine, histamine acid phosphate, phosphoramidon (N-(α-L-rhamnopyranosyloxy-hydroxyphosphinyl)-L-leucyl-L-tryptophan) (from Sigma, St Louis, MO, U.S.A.) and house dust mite (*Dermatophagoides pteronyssinus*), grass pollen (*Phleum pratense*) 1:100 wt/vol, 10000 protein nitrogen units per milliliter (from Institut Pasteur, Paris, France). All drugs were dissolved in distilled water to make 1 ml aliquots of stock solutions which were stored frozen and further diluted in the Krebs-Henseleit solution on the day of experimentation. Since neuropeptides were to be used in these experiments, the automatic system IOS_1 was equipped with plastic organ baths.

Results

Effect of passive sensitization on responses to substance P

In the absence of phosphoramidon, contractile responses induced by SP were recorded over the concentration range 1 nM to 3 μM. In a set of experiments conducted on 5 different lung specimens, passive sensitization produced significant increases in the contractile response over the complete concentration-range tested (Figure 2a). Passive sensitization increased the response to the maximal concentration of SP given and shifted to the left the CCRC as measured by the mean dose-ratio (Table 1). The mean increase in the maximal isometric force ΔF_{max} i.e. the difference in the maximal response in sensitized and non-sensitized tissues expressed as a percentage of the maximal response in non-sensitized tissues was 53.2 ± 7.9%.

In the presence of phosphoramidon added to the organ

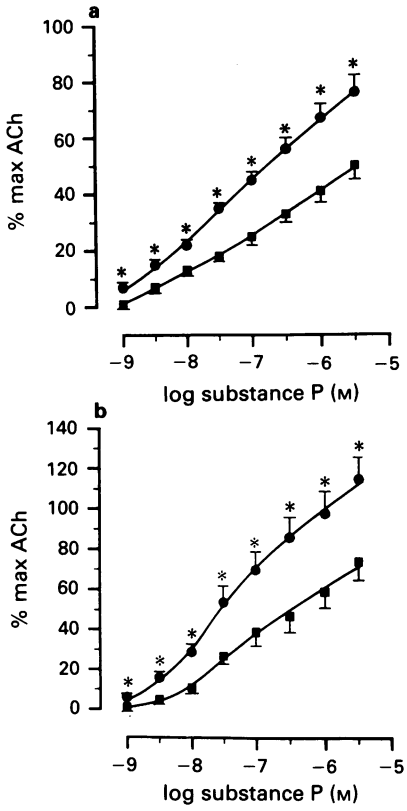


Figure 2 Mean cumulative concentration-response curves (CCRC) for substance P in paired non-sensitized (■) and passively sensitized (●) human isolated bronchial smooth muscle rings. (a) In the absence of phosphoramidon. Each symbol represents the mean value ± s.e.mean ($n = 5$ different lung specimens) of contractile force expressed as a percentage of the maximal contraction to acetylcholine (ACh). $^*P < 0.05$. (b) In the presence of phosphoramidon (10 μM) added to the bath 20 min prior to the CCRC. Legend as in (a).

Table 1 Effect of passive sensitization on responses to substance P (SP), neurokinin A (NKA) and vasoactive intestinal peptide (VIP) in human isolated bronchial smooth muscle rings in the absence of phosphoramidon

		Non-sensitized		*Passively-sensitized*
SP	F_{max} (%)	50 (5)*		76 (6)*
	Dose-ratio		8.5 (3.1–13.9)°	
NKA	F_{max} (%)	70 (7)*		101 (6)*
	Dose-ratio		7.3 (4.2–10.3)°	
VIP	R_{max} (%)	41 (4)*		25 (4)*
	Dose-ratio		10.4 (6.6–14.1)°	

F_{max}: maximal contractile response to the largest concentration of SP or NKA given expressed as a percentage of the maximal contraction to ACh; R_{max}: maximal relaxant response to the largest concentration of VIP given expressed as a percentage of the precontraction to histamine; figures in parentheses indicate s.e.mean for F_{max} or R_{max} and 95% confidence limits for dose-ratios.
Values of maximal response significantly different (*$P<0.05$) from each other; dose-ratio significantly (°$P<0.05$) different from 1.

bath 20 min before the CCRC at a concentration of 10 μM which inhibits NEP activity (Black *et al.*, 1988), contractile responses to SP were greater than those in the absence of phosphoramidon whatever the allergic status of the lung i.e. passively or non-sensitized. However, a direct statistical comparison was not made since experiments were conducted on a different set of 5 lung specimens in each case (see Data analysis). As was the case in the absence of phosphoramidon, passive sensitization significantly increased the contractile response over the complete concentration-range tested (Figure 2b). Passive sensitization increased the response to the maximal concentration of SP given and shifted to the left the CCRC as measured by the mean dose-ratio (Table 2). The mean increase in the maximal isometric force ΔF_{max} was 57.6 ± 11.5%.

Effect of passive sensitization on responses to neurokinin A

CCRC to NKA were obtained beteween 1 nM and 3 μM. In the absence of phosphoramidon, as was the case for SP, passive sensitization increased the amplitude of the contractile response to NKA and shifted to the left the CCRC (Figure 3a and Table 1). ΔF_{max} was 48.8 ± 8.9%.

Table 2 Effect of passive sensitization on responses to substance P (SP), neurokinin A (NKA) and vasoactive intestinal peptide (VIP) in human isolated bronchial smooth muscle rings in the presence of phosphoramidon

		Non-sensitized		*Passively-sensitized*
SP	F_{max} (%)	74 (4)*		115 (3)*
	Dose-ratio		9.0 (4.3–13.6)°	
NKA	F_{max} (%)	104 (9)*		146 (16)*
	Dose-ratio		5.4 (2.9–7.9)°	
VIP	R_{max} (%)	72 (1)*		49 (4)*
	Dose-ratio		6.4 (3.0–9.8)°	

F_{max}: maximal contractile response to the largest concentration of SP or NKA given expressed as a percentage of the maximal contraction to ACh; R_{max}: maximal relaxant response to the largest concentration of VIP given expressed as a percentage of the precontraction to histamine; figures in parentheses indicate s.e.mean for F_{max} or R_{max} and 95% confidence limits for dose-ratios.
Values of maximal response significantly different (*$P<0.05$) from each other; dose-ratio significantly (°$P<0.05$) different from 1.

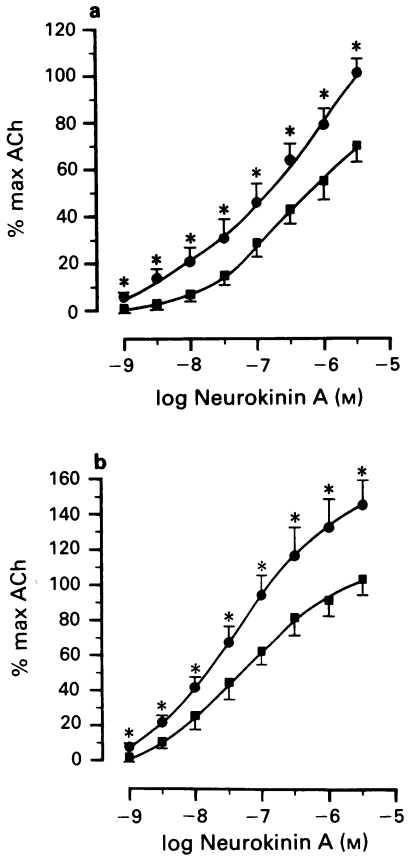


Figure 3 Mean CCRC for neurokinin A in paired non-sensitized (■) and passively sensitized (●) human isolated bronchial smooth muscle rings. (a) In the absence of phosphoramidon. Each symbol represents the mean value ± s.e.mean ($n = 5$ different lung specimens) of contractile force expressed as a percentage of the maximal contraction to acetylcholine (ACh). *$P<0.05$. (b) In the presence of phosphoramidon (10 μM) added to the bath 20 min prior to the CCRC. Legend as in (a).

Pretreatment with phosphoramidon performed on another set of 5 lung specimens led to modifications in the NKA-induced response that resembled those observed with SP: contractile responses appeared greater and passive sensitization still increased the amplitude of the contractile response to NKA and shifted to the left the CCRC (Figure 3b and Table 2). ΔF_{max} was 40.2 ± 6.3%.

Effect of passive sensitization on responses to vasoactive intestinal peptide

In order to measure relaxant responses in the absence of supramaximal tone, VIP-induced relaxation was studied in tissues precontracted with histamine at a concentration (0.5 mM) which is maximal but not supramaximal in human airway smooth muscle (Marthan *et al.*, 1987). When phosphoramidon was to be used, it was added to the plateau of the histamine precontraction, 20 min before the CCRC for VIP. Phosphoramidon induced no changes in the amplitude or maintenance of histamine-induced tone. Passive sensitization reduced the amplitude of the relaxation to VIP i.e. passively sensitized tissues relaxed less than did non-sensitized both in the absence of phosphoramidon (Figure 4a) ΔF_{max} being − 40.2 ± 6.5% and in the presence of phosphoramidon (Figure 4b) ΔF_{max} being − 31.2 ± 4.8%. Passive sensitization also shifted to the right the CCRC as assessed by the dose-ratios both in the absence (Table 1) and in the presence (Table 2) of the NEP inhibitor.

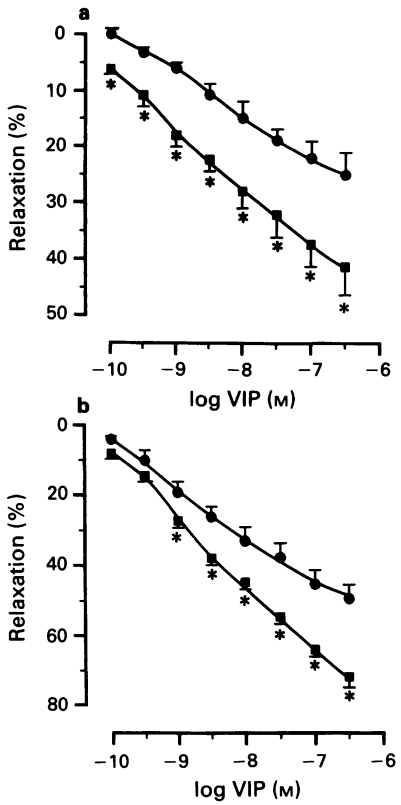


Figure 4 Mean CCRC for vasoactive intestinal peptide (VIP) in paired non-sensitized (■) and passively sensitized (●) human isolated bronchial smooth muscle rings. (a) In the absence of phosphoramidon. Each symbol represents the mean value ± s.e.mean ($n = 5$ different lung specimens) of relaxation response expressed as a percentage of the precontraction to histamine. $^*P < 0.05$. (b) In the presence of phosphoramidon (10 μM) added to the bath 20 min prior to the CCRC. Legend as in (a).

Discussion

This study shows that passive sensitization alters the mechanical activity in response to stimulation by neuropeptides in human isolated airways. It enhances the contractile properties of tachykinins (SP and NKA) and reduces relaxant responses to VIP. The mechanism of passive sensitization-induced changes in the mechanical activity appears to be independent of a decrease in NEP activity since these changes persist in the presence of the NEP inhibitor, phosphoramidon.

Since actual maximal responses to neuropeptides are difficult to obtain owing to the large concentrations required, the effect of passive sensitization had to be assessed in a manner different from the conventional one i.e. in terms of true maximal response and EC_{50} (the concentration producing 50% of the maximal response). This is why we used the dose-ratios to estimate the changes in the potency of these peptides induced by passive sensitization. For each experiment, in each pair of tissues, a dose-ratio was calculated from the two concentrations of the peptide which produced an arbitrarily determined response on the linear and parallel portion of the concentration-response curves (Black *et al.*, 1981).

Our data on the effect of neuropeptides in human non-sensitized airway smooth muscle can be compared to those of previous studies in this tissue. Overall, our results are in general agreement with those of Black *et al.* (1988) and Naline *et al.* (1989) for tachykinins, and those of Palmer *et al.* (1986) for VIP. Nevertheless, we found that the amplitude of the response to identical concentrations of SP was greater than that found by Black *et al.* (1988) and Naline *et al.* (1989). We also found that the amplitude of the reponse to identical concentrations of NKA was greater than that reported by Black *et al.* (1988) but similar to that reported by Naline *et al.* (1989). Using the same agonist to precontract human airways as that used by Palmer *et al.* (1986) (i.e. histamine) although at a higher concentration to standardize the level of tone, we found VIP to be as potent but less efficacious than in the latter investigation. These slight differences could be for several reasons: (i) responses to neuropeptides may vary according to the size and localization of the airway preparation in the respiratory tract (Palmer *et al.*, 1986); (ii) the biological activity of commercially available neuropeptides is batch-dependent (Palmer *et al.*, 1986); in the present study, each of the neuropeptides used was from the same batch; (iii) since our study was designed to assess the effect of passive sensitization, control tissues were processed in a different manner from that in the above cited studies in that they were incubated overnight in serum from healthy donors.

Previously we have reported that, as was the case for airway tissues from actively sensitized animals, passively sensitized bronchial preparations isolated from human lung specimens exhibited increased contractile responses to a variety of pharmacological agonists (Black *et al.*, 1989). Recently, we observed that passive sensitization also reduced responses to relaxant compounds (Villanove *et al.*, 1992). It decreased relaxation responses which depend upon modulation of ion channels (i.e. responses to calcium channel antagonists and to potassium channel openers) but not those which depend upon activation of β-adrenoceptors by isoprenaline. The results of the present study show that responses to neuropeptides are similarly altered i.e. passive sensitization increases the response to neuropeptides which contract human isolated airways and reduces that to a neuropeptide which relaxes it.

The question which then arises deals with the mechanism of the effect of passive sensitization on the response to neuropeptides. Since the airway mechanical activity induced by neuropeptides depends on their inactivation by NEP, we investigated the role of this enzyme in the effect of sensitization. The experiments performed in the presence of the NEP inhibitor, phosphoramidon, have revealed that passive sensitization-induced alterations in the mechanical activity were similar to those observed in the absence of phosphoramidon. Although a direct statistical comparison between experiments conducted with and without phosphoramidon cannot be made since they were performed on different sets of lung specimens in each case, both the increase in force induced by the maximal concentration of each of the neuropeptides given (ΔF_{max}) and the dose-ratio for each neuropeptide were similar whether phosphoramidon was present or not. Therefore, the effect of passive sensitization was independent of changes in NEP activity. Alternative mechanisms related to the coupling efficiency of neuropeptides should thus be evoked to account for the effect of sensitization. Complementary experiments investigating the coupling mechanisms of neuropeptides in human airway smooth muscle are required. Such information would help to clarify the cellular pathways via which passive sensitization alters responses to neuropeptides and to explain the differential effect of sensitization on SP and NKA on one hand and VIP on the other hand.

The results of the present study provide some *in vitro* evidence that responses to neuropeptides may be altered under conditions of sensitization of the airways in man. In this connection, our data lend some support to the hypothesis that neuropeptide release by axon reflexes account for, at least part of, the pathophysiological aspects of asthma (Barnes, 1986). However, one should be cautious in extrapolating these findings to the *in vivo* situation for patients suffering from bronchial hyperresponsiveness. In particular, although we found that hyperresponsiveness to neuropeptides occurred in the absence of alteration in NEP activity, such an alteration may play a role in the *in vivo* situation. Passive

sensitization does not alter the epithelium of human isolated airways, at least when examined histologically, whereas NEP activity may decrease in relation to the shedding of the epithelial layer in asthmatic bronchi (Barnes, 1987b).

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Blockade by calmodulin inhibitors of Ca^{2+} channels in smooth muscle from rat vas deferens

[1]Ken Nakazawa, Katsuya Higo, Kazuho Abe, Yoshio Tanaka, Hiroshi Saito & [2]Norio Matsuki

Department of Chemical Pharmacology, Faculty of Pharmaceutical Sciences, The University of Tokyo, Tokyo 158, Japan

1 Effects of three compounds which are used as calmodulin inhibitors (trifluoperazine, W-7 and calmidazolium) on Ca^{2+} channels were investigated in smooth muscle from rat vas deferens.

2 All three calmodulin inhibitors relaxed the smooth muscle precontracted by a high concentration of KCl (63.7 mM). The order of potency for the relaxation was trifluoperazine > W-7 > calmidazolium.

3 In binding studies using a microsomal fraction of vas deferens, all these calmodulin inhibitors displaced specific [^{3}H]-nimodipine binding. Trifluoperazine and W-7 inhibited the binding at concentrations that relaxed the smooth muscle whereas calmidazolium inhibited at concentrations much lower than those necessary for muscle relaxation.

4 Ba^{2+} current flowing through voltage-gated Ca^{2+} channels was measured under whole-cell voltage-clamp conditions in isolated smooth muscle cells. The Ba^{2+} current was suppressed by the three calmodulin inhibitors in the concentration-range where inhibition of [^{3}H]-nimodipine binding was observed. Neither voltage-dependence nor the inactivation time course of Ba^{2+} current were affected by these compounds.

5 The results suggest that the calmodulin inhibitors directly block Ca^{2+} channels in the smooth muscle cells. The channel inhibition by trifluoperazine and W-7, but perhaps not that by calmidazolium, may be responsible for the muscle relaxation observed with these compounds.

Keywords: Vas deferens; smooth muscle; calmodulin inhibitors; calcium channel; dihydropyridine binding; voltage-clamp

Introduction

Calmodulin is an intracellular Ca^{2+}-receptive protein that exhibits various functions (Cheung, 1980; Tanaka, 1988). Calmodulin also plays a role in smooth muscle contraction (Kreye *et al.*, 1983; Hidaka *et al.*, 1979; 1980; Tanaka, 1988). Agents that bind to calmodulin and block its functions have been developed (e.g., Gietzen *et al.*, 1981; Hidaka *et al.*, 1980; 1981; Tanaka, 1988). These calmodulin inhibitors have been widely used as pharmacological tools to test whether biological responses involve calmodulin-dependent mechanisms (e.g. Bkaily *et al.*, 1984; Campbell *et al.*, 1986; Koyama & Himori, 1988). However, it has been suggested that these compounds exert effects that are not related to calmodulin inhibition when applied extracellularly (e.g., Klöckner & Isenberg, 1987; Hori *et al.*, 1989). Inhibition of Ca^{2+} channels by these compounds was also investigated by electrophysiological techniques in cardiac cells (Klöckner & Isenberg, 1987) and in *Paramecium* (Ehrlich *et al.*, 1988). Klöckner & Isenberg (1987) reported that several calmodulin inhibitors reduced Ca^{2+} currents in smooth muscle cells from bovine portal vein. Effects of calmodulin inhibitors on Ca^{2+} channels in non-vascular smooth muscles were not reported. In addition, it is not known whether the channel inhibition leads to inhibition of smooth muscle contraction.

The present study was aimed to determine effects of calmodulin inhibitors on Ca^{2+} channels in smooth muscles from rat vas deferens. We found that three calmodulin inhibitors, trifluoperazine, W-7 and calmidazolium, relaxed the smooth muscle contracted by a high concentration of KCl, inhibited specific [^{3}H]-nimodipine binding, and Ba^{2+} current permeating through Ca^{2+} channels.

[1] Present address: Division of Pharmacology, National Institute of Hygienic Sciences, 1-18-1 Kamiyoga, Setagaya, Tokyo 158, Japan.
[2] Author for reprint requests at Department of Chemical Pharmacology address, above.

Methods

Measurement of muscle tension

Male Wistar strain rats (250 to 350 g) were killed by cervical dislocation and exsanguination. The vas deferens was dissected and surrounding blood vessels and adipose tissue were removed. Isometric tension of the longitudinal muscles was recorded under the conditions described previously (Higo *et al.*, 1988; Abe *et al.*, 1989). Briefly, the vas deferens was mounted in a 10 ml organ bath chamber filled with a solution of the following composition (in mM): NaCl 118, KCl 4.7, $CaCl_2$ 1.8, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25.0, glucose 11.1 (oxygenated with 95% O_2:5% CO_2; pH 7.4 at 37°C). The tissue was allowed to equilibrate for 60 min. After the equilibrium period, tonic contraction was produced by a solution containing 63.7 mM KCl (NaCl in the standard solution was replaced with equimolar KCl). Prazosin (10^{-6} M) was included in this solution to prevent any contribution of endogenus noradrenaline. Concentration-response curves for muscle relaxation were obtained by cumulative addition of calmodulin inhibitors. The tension was measured with a force-displacement transducer (Nihon Kohden TB-612T) and recorded on an ink-writing oscillograph (Nihon Kohden WI621-G).

Radioligand binding assay

Interaction of calmodulin inhibitors with dihydropyridine binding was determined according to Higo *et al.* (1989). Briefly, a microsomal fraction was prepared from rat vas deferens, and suspended in 0.2 mM of Tris-HCl buffer (pH 7.4). The protein content in the suspension was adjusted to 0.5–1.0 mg ml^{-1}. The suspension was incubated with 0.3 nM [^{3}H]-nimodipine in the presence of various concentrations of calmodulin inhibitors at 25°C for 20 min in a duplicate set of tubes. The incubation was terminated by filtration though Whatman GF/F filters. The radioactivity

remaining on the filters was counted with a liquid scintillation spectrometer (Aloka LSC-700). Specific binding of [^{3}H]-nimodipine was defined as the binding displaceable by 10 μM of unlabelled nimodipine. Data were fitted by curves according to a conventional drug-receptor theory (Tallarida & Jacob, 1979). The best fit was determined with a least squares method from the formula: $\ln[B/(B_{max}-B)] = -n_H \times [\ln A - \ln (IC_{50})]$, where A is a concentration of drugs, B is specific binding of [^{3}H]-nimodipine, and n_H is the Hill coefficient.

Membrane current recordings

Membrane currents were recorded from enzymatically dispersed single smooth muscle cells from rat vas deferens (Nakazawa *et al.*, 1987). Electrical recordings were made by the whole-cell voltage-clamp technique (Hamil *et al.*, 1981; Marty & Neher, 1983) under the conditions described elsewhere (Nakazawa *et al.*, 1987; 1988). The isolated cells were continuously superfused with an extracellular solution containing (in mM): NaCl 140, $BaCl_2$ 10.8, $MgCl_2$ 1.2, HEPES 10, glucose 11.1 (pH 7.4 with NaOH). We used 10.8 mM Ba^{2+} as a divalent charge carrier to obtain a large inward current through Ca^{2+} channels (Nakazawa *et al.*, 1988). Heat-polished patch pipettes had tip resistance of 2 to 4 Mohm when filled with an intracellular solution containing (in mM): CsCl 130, succinic acid 5, oxalacetic acid 5, pyruvic acid 5, HEPES 10, EGTA 2, $MgCl_2$ 1, $CaCl_2$ 0.18 (pH 7.2 with CsOH). The electrical signals were recorded with a patch-clamp amplifier (Nihon Kohden S-3666) and stored on magnetic tape for later analysis. The current traces were digitized and analysed on a personal computer (NEC PC9801). A linear component of leak current was estimated from a current response to a hyperpolarizing step from − 60 mV to − 100 mV and this component was subtracted from current traces obtained with depolarizing steps. Current measurements were carried out at room temperature (about 25°C).

Chemicals

Trifluoperazine dihydrochloride, W-7 (N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide) hydrochloride, calmidazolium (compound R24571) and nicardipine hydrochloride were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Agents used in the binding assays were [^{3}H]-nimodipine (specific activity: 138.5 Ci mmol^{-1}, Dupon-New England Nuclear, Boston, MA, U.S.A.) and nimodipine (Bayer, Lerverkusen, Germany). Trifluoperazine and W-7 were first dissolved in dimethyl sulphoxide (DMSO) and added to experimental solutions. Final concentrations of DMSO were less than 1%, which did not affect the muscle tension, [^{3}H]-nimodipine binding or Ca^{2+} current described here.

Results

Muscle relaxation induced by calmodulin inhibitors

Figure 1 shows effects of calmodulin inhibitors on smooth muscles contracted by 63.7 mM KCl. The tonic contraction was relaxed by cumulative application of the calmodulin inhibitors in a concentration-dependent manner. Trifluoperazine and W-7 completely relaxed the smooth muscle at the highest concentrations tested (Figure 1). The concentration-response curves suggests the order of potency was trifluoperazine > W-7. Calmidazolium also relaxed the muscles. The potency of calmidazolium was weaker than that of W-7; higher concentrations could not be tested.

Inhibition of [^{3}H]-nimodipine binding

We previously showed that [^{3}H]-nimodipine bound to a microsomal fraction prepared from smooth muscle of rat vas deferens in a saturable and reversible manner, and that other dihydropyridine Ca^{2+} channel antagonists inhibited the [^{3}H]-nimodipine binding (Higo *et al.*, 1988). A test was made to determine whether the calmodulin inhibitors affect [^{3}H]-nimodipine binding. As shown in Figure 2, all the calmodulin inhibitors inhibited [^{3}H]-nimodipine binding, trifluoperazine and W-7 inhibiting it at concentrations that relaxed the smooth muscles (Figure 1). IC_{50} values calculated were 0.40 μM for trifluoperazine and 12.6 μM for W-7. Low concentrations (10^{-7} to 10^{-5} M) of calmidazolium, which scarcely affected the smooth muscle tension (Figure 1), inhibited [^{3}H]-nimodipine binding (IC_{50} was 0.25 μM).

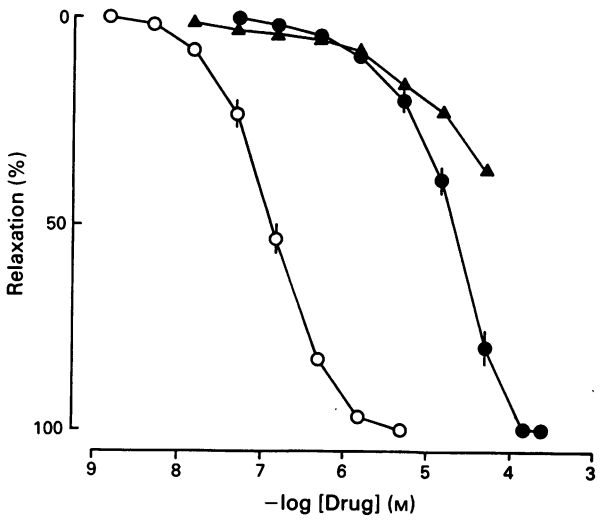


Figure 1 Relaxation by trifluoperazine, W-7 and calmidazolium of smooth muscles from rat vas deferens. The smooth muscles were contracted by a solution containing 63.7 mM KCl. W-7 (●), trifluoperazine (○) and calmidazolium (▲) were added during the tonic phase of contraction in a cumulative manner. Each point and vertical bar represent the mean and s.e.mean from five experiments.

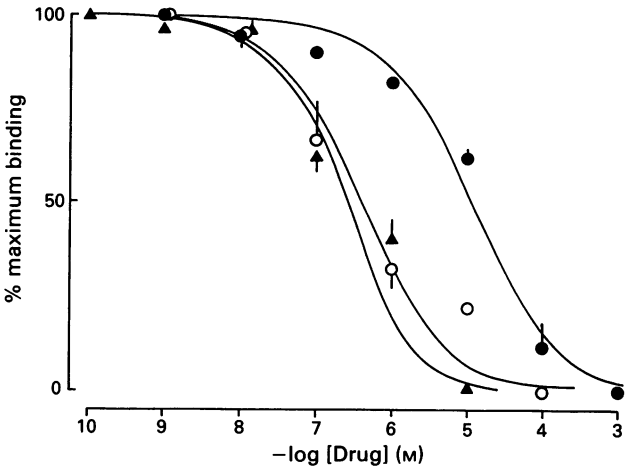


Figure 2 Inhibition of [^{3}H]-nimodipine binding by W-7 (●), trifluoperazine (○) and calmidazolium (▲). The drugs were incubated with the microsomal membranes of rat vas deferens in the presence of 0.3 nM [^{3}H]-nimodipine for 20 min at 25°C. Specific binding was determined as the fraction that was displaced by 10^{-5} M of unlabelled nimodipine. Each symbol represents the mean ± s.e.mean from 3–8 preparations. Smooth curves were best-fitted to the data as described in Methods using following values: n_H = 0.78 (W-7), 0.77 (trifluoperazine) or 0.78 (calmidazolium); IC_{50} = 12.6 (W-7), 0.4 (trifluoperazine) or 0.25 μM (calmidazolium).

Blockade of Ba^{2+} current flowing through Ca^{2+} channels

Ba^{2+} current flowing through voltage-gated Ca^{2+} channels was measured to determine whether the calmodulin inhibitors affect functional channels. Trifluorperazine and W-7 inhibited the Ba^{2+} current (Figures 3, 4 and 5) at concentrations that relaxed the smooth muscles (Figure 1) and inhibited the [^{3}H]-nimodipine binding (Figure 2). Calmidazolium suppressed the Ba^{2+} current (Figures 3 and 5) at concentrations (10^{-7} to 10^{-6} M) that scarcely affected the smooth muscle tension (Figure 1) and markedly inhibited [^{3}H]-nimodipine binding (Figure 2). The recovery from the inhibition was different among the calmodulin inhibitors (Figure 3). The Ba^{2+} current suppressed by trifluoperazine (10^{-6} M) was almost fully recovered after a 5 min rinse with a drug-free solution. The current inhibited by W-7 (10^{-4} M) or calmidazolium (10^{-7} M) was partially recovered after a 5 min rinse: the peak Ba^{2+} current recovered was about 70% (W-7) or 40% (calmidazolium) of the current before administration. Figure 4 shows the current-voltage relationship for the Ba^{2+} current. Trifluoperazine (10^{-7} M, Figure 4a), W-7 (3×10^{-5} M, Figure 4b) or calmidazolium (10^{-7} M, not shown) did not affect the voltage-dependence of the Ba^{2+} current.

Dihydropyridine Ca^{2+} channel antagonists are known to accelerate inactivation of L-type Ca^{2+} channels (Lee & Tsien, 1983). We previously reported that such acceleration was also observed with nicardipine in smooth muscle cells from rat vas deferens (Nakazawa *et al.*, 1988). To determine the effects of the calmodulin inhibitors on the inactivation kinetics of the Ba^{2+} current, we compared the amplitude of peak Ba^{2+} current and that of the current remaining after 400 ms (Figure 5). In this comparison, the Ba^{2+} current remaining after 400 ms was suppressed by nicardipine (10^{-7} M) to a significantly larger extent than the peak Ba^{2+} current (Figure 5). The Ba^{2+} current remaining after 400 ms was also somewhat more sensitive to any of three calmodulin inhibitors than the peak Ba^{2+} current, but the difference was not statistically significant (Student's t test, $P > 0.05$). The results suggest that the calmodulin inhibitors do not affect the inactivation kinetics as distinctly as dihydrophridine Ca^{2+} antagonists do.

Effects of preincubated calmidazolium on Ca^{2+}-induced contraction

As described above, [^{3}H]-nimodipine binding (Figure 2) and Ba^{2+} current permeating through Ca^{2+} channels (Figures 3 and 5) were markedly inhibited by calmidazolium at 10^{-7}–10^{-5} M whereas smooth muscle precontracted with 63.7 mM KCl was scarcely relaxed in this concentration-range (Figure 1). Spedding (1982) assessed effects of compounds that exert Ca^{2+} antagonistic activity in guinea-pig taenea caecum, and found that a subclass of such compounds fail to relax precontracted smooth muscle though they potentially inhibit Ca^{2+}-induced contraction when applied initially in Ca^{2+}-free media. We tested whether preincubation in Ca^{2+}-free media potentiates the inhibition by calmidazolium. In this experiment, smooth muscle was bathed in a Ca^{2+}-free solution containing 63.7 mM KCl, and contraction was achieved by addition of 1.8 mM Ca^{2+}. However, preincuba-

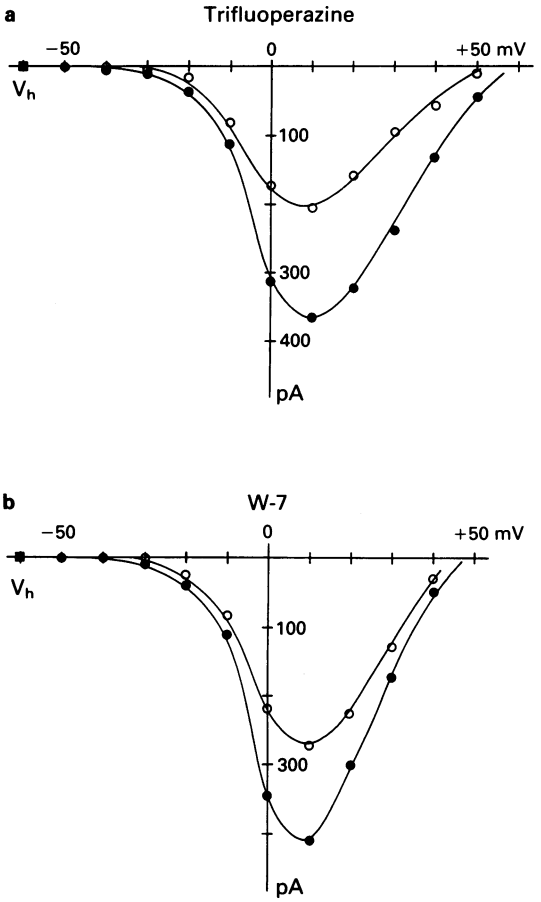


Figure 4 Current-voltage relationship for Ba^{2+} current flow through Ca^{2+} channels in the absence and presence of trifluoperazine (10^{-7} M, a) or W-7 (3×10^{-5} M, b). The cells were held at − 60 mV and 400 ms steps to various test potentials were applied every 10 s. Each symbol represents peak Ba^{2+} current just before (●) and 2 min after (○) application of drugs. (a) and (b) Were obtained from different cells.

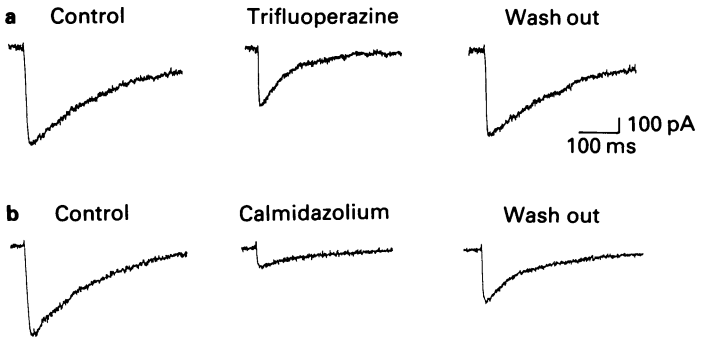


Figure 3 Blockade by trifluoperazine (10^{-6} M, a) or calmidazolium (10^{-7} M, b) of Ba^{2+} current flowing through voltage-gated Ca^{2+} channels in smooth muscle cells isolated from rat vas deferens. The cells were bathed in an extracellular solution containing 10.8 mM Ba^{2+} and held at − 60 mV. The Ba^{2+} current was activated by applying a voltage step to + 10 mV. Traces are the Ba^{2+} current just before (left) and 2 min after drug-application (middle), and the current after a 5 min rinse with a drug-free solution (right). (a) and (b) Were obtained from different cells.

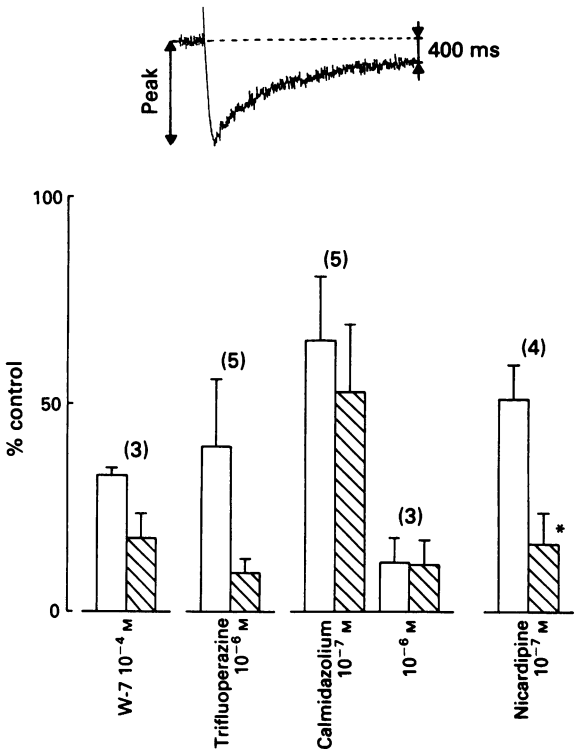


Figure 5 Blockade by trifluoperazine, W-7 and calmidazolium of peak Ba^{2+} current (open columns) and the Ba^{2+} current measured after 400 ms (hatched columns). The effects of nicardipine are also illustrated for comparison. The Ba^{2+} current was activated by a depolarizing step to + 10 mV from a holding potential of − 60 mV. The current amplitude 2 min after administration of drugs was normalized to the amplitude just before the administration. Each column represents the mean ± s.e.mean. The number of cells tested is indicated in parentheses. *Statistically significant difference from inhibition of the peak Ba^{2+} current (Student's t test, $P < 0.05$).

tion with calmidazolium (10^{-6} M) did not significantly inhibit smooth muscle contraction, the difference between the contraction in control condition and that with calmidazolium (10^{-6} M) being less than 10% ($n = 2$).

Discussion

We have investigated the effects of three compounds which have been used as calmodulin inhibitors on Ca^{2+} channels in smooth muscles from rat vas deferens. These compounds blocked the Ba^{2+} current flowing through Ca^{2+} channels (Figures 3, 4 and 5) at concentrations similar to those required for the inhibition of ionic currents through Ca^{2+} channels in other cells (Klöckner & Isenberg, 1987; Ehrlich *et al.*, 1988). Moreover, the calmodulin inhibitors inhibited [^{3}H]-nimodipine binding (Figure 2). Recent investigations have clarified that dihydropyridine binding sites exist on the pore-forming subunit of L-type Ca^{2+} channels ('α_1-subunit'; Miller, 1992). Thus, the inhibition of [^{3}H]-nimodipine binding suggests that the calmodulin inhibitors affect the Ca^{2+} channels themselves or compartments closely coupled with the channels, and may exclude the possibility that some second messenger such as calmodulin mediates the channel inhibition.

Trifluoperazine and W-7 relaxed the smooth muscle contracted by a high concentration of KCl (Figure 1). The contraction may be due to Ca^{2+}-influx through L-type Ca^{2+} channels because both the contraction (Higo *et al.*, 1988) and the current flowing through Ca^{2+} channels (Nakazawa *et al.*, 1987; 1988) are completely blocked by dihydropyridine Ca^{2+} channel antagonists. Thus, the relaxation by trifluoperazine or W-7 may also result from their inhibitory effect on the Ca^{2+} channels. Similarity between magnitude of the relaxation (Figure 1) and that of the current inhibition (Figure 5) may support this idea. In contrast, calmidazolium markedly inhibited the Ba^{2+} current (Figures 3, 4 and 5) without affecting the muscle tension (Figure 1) at concentrations lower than 10^{-7} M. This finding is rather paradoxical. Perhaps, the muscle relaxation which is expected from the blockade of the Ca^{2+} channels may be hampered by some unknown mechanisms. Protective effects of Ca^{2+} against Ca^{2+} antagonistic effects which have been reported for compounds such as flunarizine or cinnarizine (Spedding, 1982) may not contribute to the lack of significant blockade by calmidazolium because application of this compound to Ca^{2+}-free media did not markedly affect the contraction, either.

Thayer & Fairhurst (1983) reported that nitrendipine bound to calmodulin in rat brain. It may be argued that [^{3}H]-nimodipine binding in the present study comprises such binding to calmodulin. However, this may not be the case because the [^{3}H]-nitrendipine binding to calmodulin in rat brain was of low affinity and unsaturable (Thayer & Fairhurst, 1983) whereas the [^{3}H]-nimodipine binding in rat vas deferens was of a high affinity ($K_d = 0.31$ nM) and saturable (Higo *et al.*, 1988). The [^{3}H]-nimodipine binding sites in this paper may be best interpreted as L-type Ca^{2+} channels (Reynolds & Snyder, 1988).

We cannot conclude at present whether the blockade by the calmodulin inhibitors of [^{3}H]-nimodipine binding is due to displacement at the same binding site or some allosteric effect. Ehrlich *et al.* (1988) reported that W-7 inhibited 'T-type' Ca^{2+} channels isolated from *Paramecium*. Klöckner & Isenberg (1987) reported that trifluoperazine and calmidazolium inhibited Na^+ and K^+ channels in cardiac or vascular smooth muscle cells at concentrations about 10 fold higher than those necessary for Ca^{2+} channel inhibition. The blockade of these 'dihydropyridine-insensitive' channels may support the idea of an allosteric effect. One possibility is that the binding of the calmodulin antagonists induces a conformational change in the Ca^{2+} channels so that dihydropyridine binding as well as channel opening is inhibited.

Levin & Weiss (1979) and Itoh *et al.* (1986) studied the binding of calmodulin inhibitors to calmodulin. When K_i values were calculated from their data using the equation of Chang & Prusoff (1973), K_i values were 0.62 μM for trifluoperazine (Levin & Weiss, 1979) and 26 μM for W-7 (Itoh *et al.*, 1986). These values are comparable to IC_{50} values obained from the blockade of [^{3}H]-nimodipine binding (0.40 μM for trifluoperazine and 12.6 μM for W-7; Figure 2) and concentrations necessary for the muscle relaxation (Figure 1) or the inhibition of the Ba^{2+} current (Figure 3, 4 and 5). As for calmidazolium, the IC_{50} for inhibition of calmodulin-dependent ATPase was 0.35 μM (Gietzen *et al.*, 1981). This value is also comparable to the concentrations necessary for the blockade of [^{3}H]-nimodipine binding (Figure 2) or the Ba^{2+} current (Figures 3 and 5). Therefore, these compounds cannot inhibit calmodulin-mediated responses without affecting the Ca^{2+} channels when applied extracellularly.

In conclusion, we have demonstrated that three calmodulin inhibitors act as antagonists to L-type Ca^{2+} channels in smooth muscle cells from rat vas deferens, and that the channel inhibition by trifluoperazine and W-7, but not that by calmidazolium, can account for the relaxations of smooth muscle contracted with a high concentration of KCl. These compounds cannot be used as pure calmodulin inhibitors because the drugs directly inhibit Ca^{2+} channels.

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Modulation by histamine of the delayed outward potassium current in guinea-pig ventricular myocytes

Kazuto Yazawa & [1]Yasushi Abiko

Department of Pharmacology, Asahikawa Medical College, Asahikawa 078, Japan

1 Histamine receptor-mediated modulation of the delayed outward potassium current (I_K) was investigated in guinea-pig single ventricular cells by the whole-cell voltage clamp.

2 Histamine increased I_K in a dose- dependent manner with a half-maximum dose of 3.8×10^{-8} M. Histamine (10^{-6} M) increased I_K by a factor of 3.02 without a significant change in the current kinetics. The threshold dose of histamine for increasing I_K was 10^{-9} M and this value was similar to that for calcium current.

3 Cimetidine decreased I_K in the presence of histamine, by shifting the dose-response curve to histamine to the right. The pA_2 value of cimetidine against histamine was 6.38.

4 Forskolin did not increase I_K after application of 10^{-6} M histamine, and histamine scarcely increased I_K in the presence of a heat-stable inhibitor of cyclic AMP-dependent protein kinase (PKI).

5 We conclude that stimulation by histamine of I_K is mainly by way of the H_2-receptor, and is mediated by cyclic AMP-dependent phosphorylation.

Keywords: Potassium channels; histamine; cimetidine; chlorpheniramine; heart ventricle

Introduction

The delayed outward K^+ current (I_K) is activated during the plateau phase of the action potential and contributes to its duration. It is known that histamine increases I_K in ventricular cells (Hescheler *et al.*, 1987). It has been hypothesized that an underlying mechanism for this effect is phosphorylation of the channel by adenosine 3′:5′-cyclic monophosphate (cyclic AMP)-dependent protein kinase (PKA), by analogy with stimulation by histamine of Ca^{2+} current (I_{Ca}) in cardiac ventricular cells (Hescheler *et al.*, 1987).

It is also known that an agonist at the β-adrenoceptor increases I_K and I_{Ca} in ventricular cells. It has been hypothesized that this effect is mediated by phosphorylation of the channel by PKA. The threshold dose of isoprenaline for increasing I_K is near 10^{-9} M (Yazawa & Kameyama, 1990), and this value is similar to that for I_{Ca} (Kameyama *et al.*, 1985). Moreover, the maximum concentrations for increasing the two different types of current are nearly the same. From these results, we can hypothesize that the activation of the common phosphorylation pathway modulates both I_K and I_{Ca} at a physiological temperature.

However, it has been recently suggested that I_K and I_{Ca} may possess different sensitivities to protein phosphorylation mediated by cyclic AMP even in the same cell (Tanaka *et al.*, 1991). It has also been reported that I_K is under control of protein kinase C (Tohse *et al.*, 1987; Walsh & Kass, 1988). It is also possible that another cascade might participate in stimulation by histamine in addition to the cyclic AMP pathway.

To address these questions, we investigated the intracellular mechanism underlying stimulation by histamine of I_K in single ventricular cells by using the whole-cell patch clamp method.

Methods

Cell preparation

Single ventricular cells were obtained from the adult guinea-pig heart by an enzymatic dissociation method (Yazawa *et al.*, 1990). In brief, a dissected heart was mounted on a Langendorff apparatus and perfused with nominally Ca^{2+}-free Tyrode solution containing collagenase (Yakult, Japan; 0.16 mg ml^{-1}) at 37°C. After 8–15 min of the collagenase treatment, the enzyme solution was washed out with a storage solution (see below). The ventricle was cut into pieces, and then the dispersed cells were filtered through 210 μm mesh and kept in the storage solution. The cells were subsequently incubated with the storage solution containing both protease 0.04 mg ml^{-1} (Nagase, Japan, Alkaline protease) and deoxyribonuclease, 0.02 mg ml^{-1} (Tokyo Kasei, Japan) for 10–15 min. The cells were washed twice by centrifugation and stored at 4°C in the storage solution.

Solutions

The composition of the external solutions was as follows. Normal Tyrode solution (mM): NaCl 143, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, NaH_2PO_4 0.25, HEPES 5, glucose 5.6, pH to 7.4 with NaOH. The storage solution contained (mM): KOH 70, KCl 40, L-glutamic acid 50, taurine 20, KH_2PO_4 20, $MgCl_2$ 3, glucose 10, HEPES 10, EGTA 0.5, pH to 7.4 with KOH. The Na^+-, K^+ and/or Ca^{2+}-free solution (test solution) contained (mM): N-methyl-D-glucamine 149, $MgCl_2$ 5, HEPES 5, nisoldipine 0.003, pH to 7.4 with HCl; and/or $CaCl_2$ 1.8. The pipette solution contained (mM): KOH 110, KCl 20, $MgCl_2$ 1, K_2ATP 5, potassium creatine phosphate 5, HEPES 5, pH to 7.4 with aspartic acid. Thus the total K^+ concentration in the pipette solution was 150 mM. The concentration of Ca^{2+} in the internal solution was pCa 8 (10 mM EGTA + 1.43 mM Ca^{2+}), calculated by Fabiato & Fabiato's (1979) equations with the correction by Tsien & Rink (1980). The external solutions were warmed by a water jacket before entering the recording chamber. All the experiments were performed at 35–37°C.

Drugs

The following drugs were used: histamine (Nakarai, Japan), forskolin, chlorpheniramine (both from Research Biochemicals Inc. U.S.A.), cimetidine (Funakoshi, Japan) and a heat-stable inhibitor of cyclic AMP-dependent protein kinase (PKI_{5-24}) (American Peptide Company Inc. U.S.A.). Nisoldipine (generous gift from the Bayer Company, Germany)

[1] Author for correspondence.

was dissolved in dimethyl sulphoxide as a 10 mM stock solution.

Recording of the delayed outward potassium current (I_K)

The delayed outward potassium current (I_K) was recorded in Na^+-, K^+-, 3×10^{-6} M nisoldipine and/or Ca^{2+}-free solution, in which Na^+ current, Ca^{2+}, the inward rectifier K^+ current (I_{K1}), the electrogenic Na^+-Ca^{2+} exchange current and Na^+-K^+ pump current were eliminated (Tohse *et al.*, 1987). The concentration of intercellular Ca^{2+} was buffered at pCa 8 with 10 mM EGTA. Under these conditions, I_K was measured using a voltage clamp mode. The amplitude of I_K was not affected by 3×10^{-6} M nisoldipine.

The calcium current (I_{Ca}) was recorded in Na^+- and K^+-free solution. The composition of pipette solution was the same when I_K was measured.

The whole-cell clamp method was essentially the same as that described by Hamill *et al.* (1981). The resistance of the pipette filled with internal solution was 1.9–3MΩ. A tight seal was established with Tyrode solution. The perfusate was then changed to the test solution. The voltage drop across the pipette resistance was electrically compensated and voltage pulses were applied to the cell with an interval of 15 s. Current signals were filtered at 500 Hz, digitized at 1 kHz and stored in a computer (NEC 9801 RL) using an on-line data acquisition system.

Results

Effects of histamine on I_K

To examine the effect of histamine receptor stimulation on I_K, various concentrations of histamine were applied to the myocytes. Figure 1a shows current traces in response to various test potentials before and during the superfusion of 10^{-6} M histamine, with the concentration of Ca^{2+} in the pipette equivalent to pCa 8. The I_K was elicited by depolarizing pulses of 500 ms duration to the potentials between − 30 to 90 mV from the holding potential of − 50 mV.

Following the capacitive surge, I_K was activated with a clear delay at its onset and the tail current had two exponential components with time constants of approximately 100 and 300 ms at − 50 mV. Histamine increased I_K at all the test potentials examined. Consequently, the peak amplitude of I_K tail current was enhanced by histamine 3.02 fold when measured upon repolarization from 90 to − 50 mV. The fast and slow components of the I_K were increased almost equally. The two time constants of the tail current, however, were not changed significantly, suggesting that the kinetics of I_K were hardly affected. To examined a possible voltage-dependent action of histamine on I_K, the peak amplitude of the I_K tail current was plotted against the test potential in Figure 1b. Under control conditions, the maximum I_K tail current was at 90 mV, and the potential for a half-maximum amplitude ($V_{0.5}$) was 42 mV. In the presence of histamine, $V_{0.5}$ was shifted in a negative direction by about 10 mV.

The peak amplitude of the I_K tail current was measured upon repolarization from 90 to − 50 mV at various concentrations of histamine and was normalized by the control value. Although the effects of histamine were reversible and reproducible at low concentrations ($< 10^{-7}$ M), they diminished on repetitive application of higher concentrations of the drugs. Higher doses of histamine were therefore used only once in the same cell. The dose-response relationship thus obtained is shown in Figure 2.

The threshold dose of histamine for increasing I_K tail current was near 10^{-9} M and the maximum effect was obtained at about 10^{-6} M. The normalized response of I_K tail current to 10^{-6} M histamine was 3.02 ± 0.24 (mean ± s.e.mean, $n = 4$), and the half-maximum dose was 3.8×10^{-8} M. This value is an order-of-magnitude smaller than

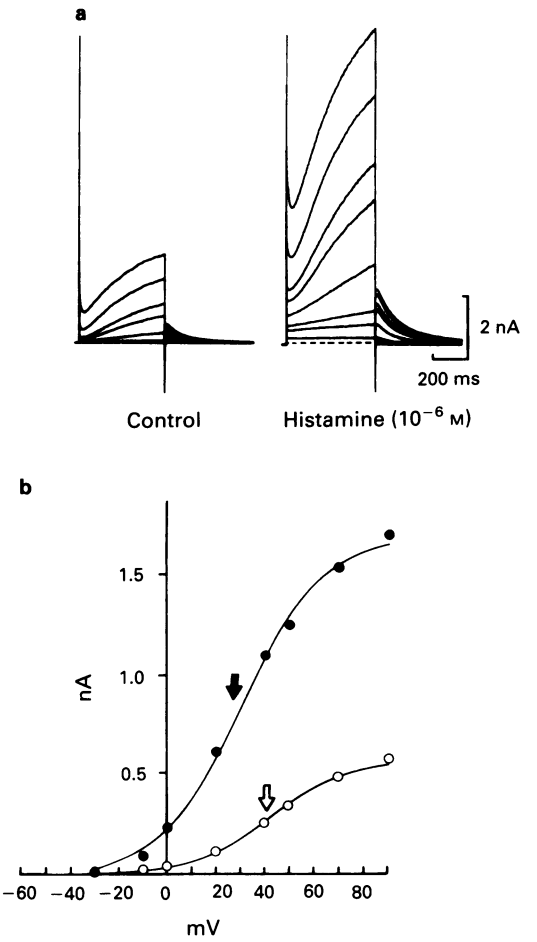


Figure 1 Effect of histamine on I_K. (a) Membrane currents were recorded at various test potentials of − 30, − 10, 0, 20, 40, 50, 70 and 90 mV from the holding potential of − 50 mV, with a duration of 500 ms. The currents on the left are the controls and on the right in the presence of 10^{-6} M histamine. Zero-current level is indicated by dashed lines. (b) I–V relations of I_K tail currents obtained from (a), for control (○) and histamine (●). The increased rate of I_K induced by histamine was greater at lower than at higher test potentials. Both open and closed arrows indicate the potentials for a half maximum amplitude ($V_{0.5}$).

that for histamine on I_K (Tanaka *et al.*, 1991) or on I_{Ca} (Hescheler *et al.*, 1987).

Effects of histamine-receptor blocking agents on I_K

To examine whether the effect of histamine on I_K is mediated by H_1-receptors or H_2-receptors, we investigated the effects of the H_1-antagonist chlorpheniramine and the H_2-antagonist cimetidine on I_K. First, we examined the effect of chlorpheniramine and cimetidine on I_K in the absence of histamine-stimulation (basal I_K). Application of either chlorpheniramine (10^{-7} and 10^{-6} M) or cimetidine (10^{-7}, 10^{-6} and 10^{-5} M) did not affect the basal I_K in four cells, respectively (not shown). We then applied 10^{-7} M or 10^{-6} M chlorpheniramine in the presence of histamine (Kondo *et al.*, 1985; Ahn & Barnett, 1986). Chlorpheniramine at these concentrations did not decrease the I_K tail current, suggesting that histamine stimulation of I_K was not through H_1-receptors.

To find out whether an antagonism occurs between cimetidine and histamine, we applied 10^{-7} M, 10^{-6} M or 10^{-5} M cimetidine in the presence of histamine (Figure 2). On application of 10^{-7} M cimetidine, the dose-response curve for histamine and the I_K value did not change. We then applied histamine in the presence of 10^{-6} M or 10^{-5} M cimetidine

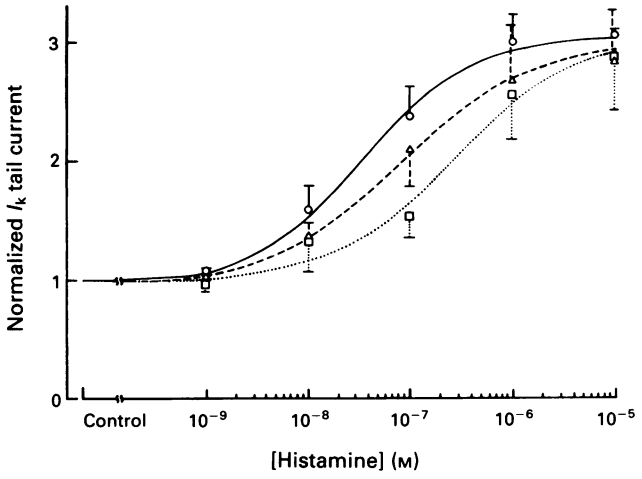


Figure 2 The relationship between histamine added to cimetidine and normalized amplitude of the I_K tail current: mean values (± s.e.mean) of histamine (○), 10^{-6} M cimetidine added to histamine (△) and 10^{-5} M cimetidine in the presence of histamine (□). Number of measurements at each concentration was 4–6. Cimetidine (10^{-7} M) does not displace the dose-response curve which is elicited by histamine only. In these agents, the continuous curves give half-maximum concentrations of 3.8×10^{-8} M, 7.2×10^{-8} M and 2.1×10^{-7} M, respectively.

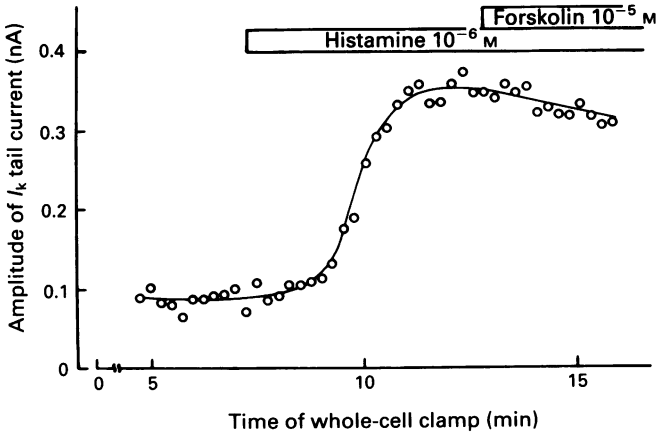


Figure 3 Time course of I_K change produced by forskolin in the presence of histamine. The amplitude of I_K tail current was measured upon repolarization from 20 to − 50 mV. At 1 min after application of 10^{-6} M histamine, I_K began to increase and reached a maximum after 3–4 min. I_K was not increased by the addition of 10^{-5} M forskolin to the histamine.

(Hescheler *et al.*, 1987). In the first case, the threshold dose of histamine for I_K tail current was near 10^{-9} M and the maximum effect was obtained at about 10^{-5} M. The normalized response of the I_K tail current to 10^{-5} M was 2.86 ± 0.63 (mean ± s.e.mean, $n = 4$), and the half-maximum dose was 7.2×10^{-8} M. In the second case, the threshold dose of histamine for I_K tail current was near 10^{-9} M and the maximum effect was obtained at about 10^{-5} M. The normalized response of the I_K tail current to 10^{-5} M was 2.89 ± 0.39 (mean ± s.e.mean, $n = 4$), and the half-maximum dose was 2.1×10^{-7} M. In these conditions, maximum responses of histamine-stimulated I_K tail currents were the same as the value in the absence of cimetidine. The two time constants of the I_K tail current were also uneffected by chlorpheniramine or cimetidine. Cimetidine only appeared to shift the dose-response curve to the right. These results suggest that histamine stimulation of I_K is mainly through H_2-receptors.

Furthermore, this shift shows that the effect of histamine vs cimetidine on I_K is a competitive antagonism. We obtained the pA_2 value from the data shown in Figure 2 by a Schild plot (Schild, 1947). The pA_2 value of cimetidine against histamine was 6.38. This value was similar in potency to cimetidine as an antagonist on the effects of histamine in the human temporal artery (Ottosson *et al.*, 1989) or in the renal artery (Tayo & Bevan, 1986).

Effects of histamine on I_K in the presence of either forskolin or PKI

To examine whether the effect of histamine on I_K is mediated by activation of adenylate cyclase, we investigated the relationship of histamine and forskolin, which is known to activate adenylate cyclase directly. In the experiment shown in Figure 3, we first applied 10^{-6} M histamine, which maximally increased the I_K tail current amplitude, obtained upon repolarization from + 20 to − 50 mV, from 0.086 to 0.355 nA.

After I_K reached the maximum level, 10^{-5} M forskolin, which increased the I_K tail current maximally, was added to the bath. Although I_K tended to decline because of run-down, no clear change in I_K by forskolin was observed. This result was confirmed in four other cells. Thus, the effects of histamine and forskolin are non-additive, suggesting that the effect of histamine is mediated by the activation of adenylate cyclase.

Some studies show that the increase of I_K is mediated by phosphorylation with not only PKA but also protein kinase C (PKC) (Tohse *et al.*, 1987; 1990; Yazawa & Kameyama, 1990; Walsh & Kass, 1988; 1991). To examine whether the effect of histamine on I_K is mainly mediated by PKA, we investigated the effect of histamine in the presence of $PKI_{(5-24)}$, which was known to inhibit PKA directly. In Figure 4, the whole-cell clamp was started with a pipette solution containing 10^{-5} M PKI, and after 11 min, 10^{-8} M histamine was added to the bath.

The time-dependent outward current was increased by a pulse of 500 ms duration to 20 mV from the holding potential of − 50 mV. On the other hand, although I_K tended to decline because of run-down, the amplitude of I_K and its tail was not significantly affected. This result was confirmed in four other cells. In the presence of 10^{-5} M PKI, the increase

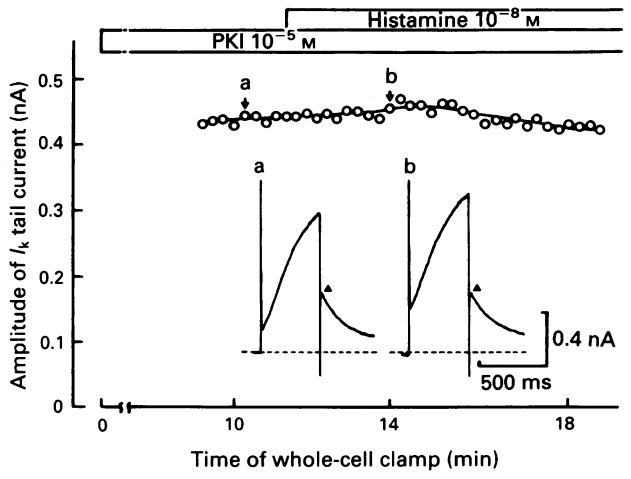


Figure 4 Effect of histamine on I_K in the presence of protein kinase inhibitor (PKI). Time course of the change in the I_K tail current produced by an internal application of 10^{-5} M PKI and 10^{-8} M histamine is illustrated. Test potential was 20 mV and holding potential was − 50 mV. At time zero, whole-cell clamp was established and dialysis of the cell with PKI was started. Inset shows examples of the current traces at the times indicated on the graph (a,b). Dashed lines indicate zero-current level.

measured at the peak of I_K tail current after the application of histamine upon repolarization from 20 to − 50 mV was 5 ± 3% ($n = 5$). By contrast, in the absence of PKI, 10^{-8} M histamine increased the amplitude of the I_K tail by 57 ± 21% ($n = 5$) compared to the control current level. These results suggest that histamine stimulation on I_K is mediated mainly by cyclic AMP-dependent phosphorylation.

Threshold of histamine on Ca^{2+} and K^+ currents

Recently, it has been reported that the threshold of histamine on I_K is different from that on I_{Ca} (Tanaka *et al.*,1991). They have shown that the threshold dose of histamine for increasing the I_K tail current is more than 10^{-8} M. But at this concentration, the previous study reported that I_{Ca} was significantly increased. However, our data showed that the threshold dose of histamine for increasing the I_K tail current was near 10^{-9} M. This dose is less than the value in the above study or the threshold concentration of histamine on I_{Ca} (Hescheler *et al.*, 1987). To examine the threshold dose of histamine on I_{Ca} and I_K, low concentrations ($< 10^{-7}$ M) of histamine were applied externally to the myocytes. In the experiment shown in Figure 5, we superfused the cell with histamine at 10^{-9} M, which is approximately the threshold concentration for affecting the I_K.

This dose increased both the I_K tail from 0.10 to 0.15 nA and the amplitude of I_{Ca} from 0.65 to 0.75 nA. The 10^{-8} M histamine was added to the bath and it increased the I_K tail current to 0.23 nA and I_{Ca} to 0.83 nA. At the concentrations, the time constants or increasing ratio of I_K tail were not significantly different in the external solution which contained 3×10^{-6} M nisoldipine in the absence of Ca^{2+}, 1.8 mM Ca^{2+} without nisoldipine, or 1.8 mM Ca^{2+} added to 3×10^{-6} M nisoldipine. These results exclude the idea that I_K was increased via elevation of the intracellular Ca^{2+} concentration followed by the activation of I_{Ca}. Histamine at 10^{-9} M increased the amplitude of both I_{Ca} and I_K by a factor of 1.37 ± 0.23 (mean ± s.e.mean, $n = 4$) and 1.28 ± 0.13 ($n = 4$) respectively, when they were measured upon depolarization from − 50 to 20 mV.

These results strongly suggest that the threshold doses of histamine for increasing the I_K tail current and the I_{Ca} are similar and the values are near 10^{-9} M.

Discussion

The present study shows that histamine increases I_K by a factor of 3.02. This agent does not affect the kinetics of I_K significantly. Histamine stimulation of I_K is mainly by way of H_2-receptors. Furthermore, the effect of forskolin on I_K is masked by the previous application of histamine, and I_K is little affected by histamine in the presence of internal PKI. These results indicate that the histamine-induced increase of I_K is mediated mainly by cyclic AMP and subsequent activation of PKA.

The threshold dose of histamine for increasing I_K tail current was near 10^{-9} M and the half-maximum dose was 3.8×10^{-8} M. This value is an order-of-magnitude smaller than that previously reported for histamine (Tanaka *et al.*, 1991). The difference in the value found in the previous work and our study may be concerned with both the holding potential and the compositions of the external solutions. Several possibilities are discussed below.

Firstly, several other currents were not eliminated in the previous study as Na^+ and K^+ were present in the test solution. Extracellular K^+ concentration is necessary to activate both inward rectifier K^+ (I_{K1}) and the Na^+-K^+ pump current. Furthermore, activating the Na^+-Ca^{2+} exchange current is dependent on extracellular Na^+. In our study, these other currents were ruled out during the measurement of the amplitude of the I_K tail.

Secondly, the holding potential is concerned with the activation of several currents. It is known that I_K is activated at a more positive than − 40 mV and does not have an inactivation phase. This shows that I_K is already activated when the holding potential is held at more positive than − 40 mV. It is suggested that I_K is already activated at − 40 mV and that in this condition, the holding current is contaminated by the current already activated. I_K is

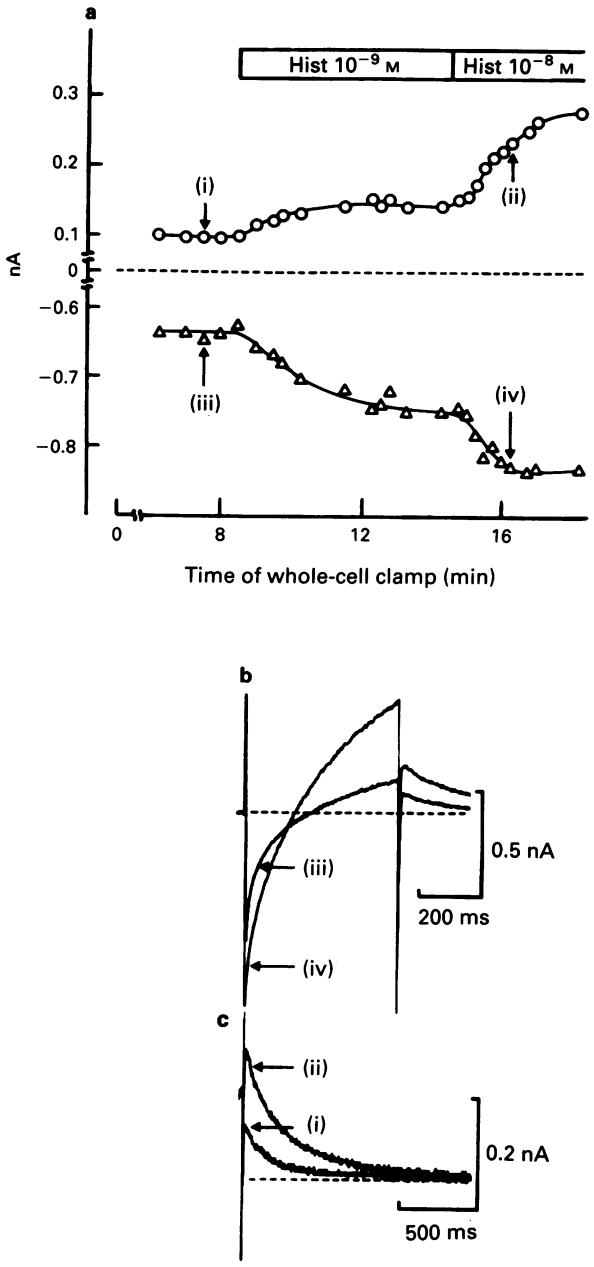


Figure 5 Effect of histamine on I_K and I_{Ca}. (a) Time course of the change in the I_K tail current and I_{Ca} during application of 10^{-9} M and 10^{-8} M histamine is illustrated from 6 min after the start of whole-cell clamp. Holding potential was − 50 mV and the test potential was 20 mV. Both I_K and I_{Ca} were increased in the presence of 10^{-9} M histamine. Dashed line indicates zero-current level. (b) The current traces (iii) in control, (iv) in the presence of 10^{-8} M histamine. When the amplitude of I_{Ca} was measured between peak inward current and the holding current, histamine increased I_{Ca} from 0.65 to 0.83 nA. Zero-current level is indicated by dashed lines. (c) The current traces (i) in control, (ii) in the presence of 10^{-8} M histamine. The amplitude of I_K tail current was measured upon repolarization from 20 to − 50 mV. Histamine increased I_K tail current from 0.10 to 0.23 nA. Zero-current level is indicated by dashed lines.

measured by the difference between the peak of I_K tail current and the holding current. Thus, with the holding potential of -40 mV, a small change in I_K, produced by histamine at near the threshold dose, might be underestimated. The Cl^- current has been recently investigated in guinea-pig ventricular cells (Bahinski *et al.*, 1989; Harvey & Hume, 1989; 1990; Matuoka *et al.*, 1990). The reversal potential for this current depends on the external and internal Cl^- concentrations. In our study, the reversal potential of Cl^- was about -50 mV as predicted by the Nernst equilibrium potential. Unless, the holding potential was around -50 mV, the I_K tail might be contaminated with the Cl^- current.

It has been shown that histamine increases the time-independent Cl^- current in cardiac myocytes (Harvey & Hume, 1990). In the present study, we also observed that a time-independent outward current was activated by a depolarizing pulse following capacitive surge. This current was increased by histamine at or more than 10^{-9} M. This result is consistent with the idea that a Cl^- current is stimulated by histamine with a threshold dose of near 10^{-9} M. However, the time-independent outward current was elicited in the presence of PKI, though I_K was not increased by histamine stimulation (inset of Figure 4). This suggests that the time-independent current might have a component that is not regulated by a cyclic AMP-PKA pathway. Possible mechanisms for this pathway might be a direct coupling of GTP-binding protein to the channel and activation of a protein kinase other than PKA (Yatani & Brown, 1989). It is also possible that the time-independent current is very sensitive to PKA which remains active even in the presence of PKI. Further studies are needed to solve this problem.

Walsh *et al.* (1988) have reported that both isoprenaline and forskolin enhance both I_{Ca} and I_K in guinea-pig ventricular myocytes at 28–32°C, while they increase only I_K at room temperature. In the frog atrial cell, I_K was not increased at room temperature by a concentration of isoprenaline that enhances I_{Ca} (Giles *et al.*, 1989). On the other hand, at a higher temperature (32–37°C), the threshold dose of β-adrenoceptor agents for increasing both I_{Ca} and I_K (I_X) is of a similar value in ventricular cells (Kameyama *et al.*, 1985; Yazawa & Kameyama, 1990), in calf cardiac Purkinje fibres (Kass & Wiegers, 1982). It has been suggested that an underlying mechanism for these effects is phosphorylation of the channel by PKA, although temperature modulates these currents. At a physiological temperature, the threshold dose of isoprenaline for increasing I_K is near 10^{-9} M (Yazawa & Kameyama, 1990), and this value is similar to that for I_{Ca} (Kameyama *et al.*, 1985). Furthermore, the maximum concentrations for increasing the two currents are also in the same range. These results strongly suggest that activation of the common phosphorylation pathway modulates both I_K and I_{Ca} with similar potency at physiological temperatures.

It is well known that histamine, like adrenoceptor stimulants, can produce arrhythmogenic effects on the heart (for review see Wolff & Levi, 1986). Histamine and adrenaline enhance not only I_{Ca} but also I_K, both of which contribute to the action potential duration (APD). When I_K is activated by histamine in working ventricular cells, APD becomes shorter, which may contribute to the ventricular premature conduction or tachycardia.

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Role of angiotensin AT_1 and AT_2 receptors in mediating the renal effects of angiotensin II in the anaesthetized dog

[1]Kenneth L. Clark, [2]Mark J. Robertson & G. Michael Drew

Department of Cardiovascular and Respiratory Pharmacology, Glaxo Group Research Limited, Ware, Hertfordshire

1 Experiments were performed using the selective AT_1 receptor antagonist, GR117289, and the selective AT_2 receptor antagonist, PD123177, to assess the relative importance of AT_1 versus AT_2 receptors in mediating the renal effects of angiotensin II (AII) *in vivo*, in salt-replete pentobarbitone-anaesthetized dogs.

2 The AT_1 receptor antagonist, GR117289 ($0.5\,mg\,kg^{-1} + 1\,\mu g\,kg^{-1}\,min^{-1}$, i.v.), caused renal vasodilatation, characterized by a mean increase of $21 \pm 5\%$ in renal blood flow, 45 min post-dose. GR117289 also caused a fall in mean blood pressure ($12 \pm 4\%$), but despite this, sodium and urine excretion were not reduced. Indeed, there was a tendency for urine output and sodium excretion to increase, although the changes were not statistically significant. GR117289 caused a reduction in plasma aldosterone levels ($-35 \pm 16\%$) 45 min post-dose, despite increasing plasma renin activity ($+173 \pm 42\%$). In contrast to GR117289, the AT_2 receptor antagonist, PD123177 ($20\,\mu g\,kg^{-1}\,min^{-1}$ intra-renal artery; i.r.a.) caused no significant change in blood pressure, renal blood flow, or sodium and urine excretion, indicating that the renal effects of endogenous AII in these salt-replete animals are mediated predominantly by AT_1 receptors.

3 Intra-renal artery infusion of AII ($1-300\,ng\,kg^{-1}\,min^{-1}$) caused dose-related renal vasoconstriction, and decreases in urine output, sodium excretion, fractional excretion of sodium, and glomerular filtration rate (GFR). The AT_1 receptor antagonist, GR117289 ($0.5\,mg\,kg^{-1} + 1\,\mu g\,kg^{-1}\,min^{-1}$, i.v.) antagonized these renal effects of AII, causing 15–38 fold rightward displacements of mean dose-response curves for these parameters. In contrast, PD123177 ($20\,\mu g\,kg^{-1}\,min^{-1}$, i.r.a.) failed to antagonize the renal haemodynamic and excretory effects of lower doses of AII ($1-10\,ng\,kg^{-1}\,min^{-1}$, i.r.a.). However, at higher doses of AII ($30-300\,ng\,kg^{-1}\,min^{-1}$, i.r.a.), while PD123177 still failed to antagonize the effects of the peptide on urine output, sodium excretion and GFR, it did cause a small, but significant, degree of inhibition of AII-induced renal vasoconstriction. In addition, at a higher dose ($50\,\mu g\,kg^{-1}\,min^{-1}$, i.r.a.), PD123177 caused a greater degree of antagonism of AII-induced renal vasoconstriction, while renal excretory responses to AII remained unaffected.

4 This study shows that the renal haemodynamic and excretory effects of AII in salt-replete anaesthetized dogs are mainly mediated by angiotensin AT_1 receptors. However, the inhibitory effect of PD123177 on renal vasoconstrictor responses to high doses of AII, raises the possibility that functionally important AT_2 receptors are present in the canine renal vasculature.

Keywords: Angiontensin II; AT_1 receptor antagonist; AT_2 receptor antagonist; GR117289; PD123177; renal function; anaesthetized dog

Introduction

The renin-angiotensin system plays an important role in the physiological control of blood pressure. However, it is now widely thought that overactivity of the system contributes to the pathology of essential hypertension, and angiotensin-converting enzyme (ACE) inhibitors, which prevent the formation of angiotensin II (AII), are effective antihypertensive agents (see Laragh, 1990). An alternative method of inhibiting the activity of the renin-angiotensin system is to block angiotensin receptors and this approach has led to the discovery and development of non-peptide angiotensin receptor antagonists, such as losartan (DuP 753; Chiu *et al.*, 1990) and GR117289 (Robertson *et al.*, 1992). These compounds are being investigated as potential novel treatments for hypertension and heart failure.

In addition to their potential therapeutic utility, non-peptide angiotensin receptor antagonists are proving valuable in the study of angiotensin receptor pharmacology, and their use in radioligand binding studies has already resulted in the identification of two clear subtypes of angiotensin receptors, termed AT_1 and AT_2 (Bumpus *et al.*, 1991). Angiotensin AT_1 receptors mediate the physiologically important effects of AII such as vasoconstriction and aldosterone release (Wong *et al.*, 1990), and selectively bind non-peptide antagonists such as losartan and GR117289 (Chiu *et al.*, 1989; Robertson *et al.*, 1992). Angiotensin AT_2 receptors selectively bind the non-peptide, PD123177 (Chiu *et al.*, 1989), and the peptide, CGP42112A (Whitebread *et al.*, 1989). In contrast to AT_1 receptors, the functional role of AT_2 receptors remains to be determined, although recently it has been reported that increases in guanosine 3′:5′-cyclic monophosphate (cyclic GMP) occur following stimulation of AT_2 receptors in the neuroblastoma cell line, NIE-115 (Zarahn *et al.*, 1992). The physiological relevance of AT_2 receptors, however, remains unclear (Wong *et al.*, 1990).

AII acts in the kidney to induce arteriolar vasoconstriction, and sodium and water retention. These effects are mediated by receptors at both vascular and proximal tubular sites (Navar *et al.*, 1991; Harris, 1992). In addition, AII stimulates the release of aldosterone which acts on the distal tubule and also stimulates sodium reabsorption. The effects of AII on renal function are pivotal in the role that the

[1] Author for correspondence.
[2] Present address: Department of Pharmacology, Fisons, p.l.c., Research and Development Labs., Pharmaceuticals Division, Bakewell Road, Loughborough LE11 0RH.

renin-angiotensin system plays in cardiovascular homeostasis. Autoradiographic studies have recently shown that both angiotensin AT_1 and AT_2 receptors are present in the renal cortex of monkeys (Chang & Lotti, 1991; Gibson *et al.*, 1991) and man (Grone *et al.*, 1992). Studies using the AT_1 receptor antagonist, losartan, indicate an important role for AT_1 receptors in mediating the renal haemodynamic effects of AII in rats and dogs (Batin *et al.*, 1991; Wong *et al.*, 1991a; Clark *et al.*, 1991). However, the importance, if any, of AT_2 receptors in the renal effects of AII remains unclear. Thus, using the selective AT_1 receptor antagonist, GR117289, and the selective AT_2 receptor antagonist, PD123177, the present study aimed to assess the role of AT_1 and AT_2 receptors in mediating the effects of AII on renal haemodynamic and excretory function *in vivo*. A preliminary account of these findings has been published in abstract form (Clark *et al.*, 1992).

Methods

Surgical preparation

Beagle dogs (10–12 kg) of either sex were anaesthetized with sodium pentobarbitone (30–40 mg kg^{-1} i.v.). Anaesthesia was maintained with a constant infusion of sodium pentobarbitone (5–10 mg kg^{-1} h^{-1}, i.v., in a volume of 5–10 ml h^{-1}) administered into the right cephalic vein. Dogs were intubated and artificially respired. Body temperature was maintained at 37 ± 1°C with a thermostatically controlled heating blanket. Dogs subjected to a lengthy period of abdominal surgery tend to develop metabolic acidosis. Consequently, physiological pH was maintained by giving a constant infusion of sodium bicarbonate (0.03 mmol kg^{-1} min^{-1}, in a volume of 0.03 ml kg^{-1} min^{-1}) via a cannula in the left femoral vein. Arterial blood pH, and O_2 and CO_2 tensions, were monitored with a Radiometer ABL30 acid-base analyser. Blood pressure was recorded from a cannula inserted into the abdominal aorta via the right femoral artery, using a Bell and Howell pressure transducer. Heart rate was derived electronically from the blood pressure signal.

A laparotomy was performed and both ureters were cannulated with polythene tubing (Portex, internal diameter = 0.06 mm), for the collection of urine. Urine output was measured gravimetrically. Left renal blood flow was measured with an electromagnetic flow probe (Statham 2.5 mm diameter). Renal vascular conductance was derived from the measurements of renal blood flow and mean blood pressure. A needle (Microlance 26G) connected to polythene tubing (Portex, internal diameter = 0.76 mm) was inserted into the left renal artery, and kept patent with an infusion of saline (0.5 ml min^{-1}). During the surgical period, from the point at which the laparotomy was performed, dogs were saline-loaded (30 ml kg^{-1} at 0.27 ml kg^{-1} min^{-1}) via a cannula in the right femoral vein. The dogs were then maintained in this saline-loaded state by lowering the infusion rate to 0.07 ml kg^{-1} min^{-1}. This saline load was given to ensure a steady state of urine production. Following the surgery, a bolus dose of creatinine (50 mg kg^{-1}. i.v., volume = 5 ml) was administered followed by a continuous infusion (0.75 mg kg^{-1} min^{-1}, i.v.) which was incorporated into the saline maintenance infusion described above.

Effects of saline alone

Following a post-surgical equilibration period of at least 60 min, a series of 10 min urine collections was made, while saline was continuously infused (0.5 ml min^{-1}) into the left renal artery. At the end of each 10 min period, readings of cardiovascular variables and urine output were made. Basal stability was achieved when urine output and cardiovascular parameters showed less than 10% variation over 3 consecutive collection periods. The intra-renal saline infusion was then continued for 10 consecutive 15 min urine collection periods. At the end of each of these periods, two separate 1 ml arterial blood samples were taken. These blood samples were immediately placed on ice in test tubes containing 0.1 ml EDTA (100 mg ml^{-1}), to prevent blood clotting and further generation of AI. Samples were then centrifuged (15 min, 2000 *g*) at 4°C, after which the plasma was removed and frozen until required for radioimmunoassay to determine plasma renin activity (PRA) or plasma aldosterone concentration. PRA and plasma aldosterone concentration were measured with commercially available radioimmunoassay kits from CIS (UK); SB-REN-1 and SB-ALDO-2, respectively. PRA values are expressed as ng AI formed per ml of plasma per hour at 37°C (ng AI ml^{-1} h^{-1}).

At the midpoint of each 15 min collection period, an arterial blood sample (1.5 ml) was collected into a lithium-heparin coated tube. Following the removal of an aliquot for haematocrit determination, the samples were centrifuged and the plasma removed. Plasma and urine samples were later analysed to assess sodium and potassium concentrations (Beckman electrolyte analyser E2A), and creatinine levels (Beckman creatinine analyser 2). In the dog, creatinine is filtered at the glomerulus, but not secreted or reabsorbed by the renal tubules (Levinsky & Levy, 1973) and hence, creatinine clearance can reliably be used in this species to estimate the glomerular filtration rate (GFR). Filtration fraction was obtained by dividing the GFR by the renal plasma flow (renal blood flow X 1 – haematocrit). For each collection period, sodium excretion (μmol min^{-1}) was calculated by multiplying the urine output (ml min^{-1}) by the urine sodium concentration (μmol ml^{-1}). Fractional sodium excretion (the fraction of filtered sodium which appears in the urine) was then determined by dividing the value obtained for sodium excretion, by the product of the plasma sodium concentration (μmol ml^{-1}) and the GFR (ml min^{-1}). Alterations in fractional sodium excretion provide an index of changes in renal tubular sodium reabsorption.

Effects of angiotensin II

The same protocol was followed as in control experiments with the exception that, after achieving baseline stability, the first dose of AII (1 ng kg^{-1} min^{-1}) was infused (0.5 ml min^{-1}) directly into the left renal artery (i.r.a.). After 15 min, measurements of urine output and cardiovascular parameters were taken and the dose of agonist was increased approximately three fold whilst keeping the infusion rate constant. This procedure was subsequently repeated with several higher doses of AII (3–300 ng kg^{-1} min^{-1}). Mean AII dose-response curves were then plotted.

Effects of AT_1 or AT_2 receptor blockade on basal renal function, and on the renal actions of exogenous angiotensin II

Following the attainment of baseline stability, either the selective angiotensin AT_1 receptor antagonist, GR117289, *or* the AT_2 receptor antagonist, PD123177, was administered. In no experiment was the effect of combined AT_1 and AT_2 receptor antagonism studied.

We have previously found that losartan can antagonize the renal effects of AII in the dog over a 2 h period when given at a dose of 5 mg kg^{-1}, i.v., along with a continuous infusion of 20 μg kg^{-1} min^{-1}, i.v. (Clark *et al.*, 1991). GR117289 has approximately 10–30 fold higher affinity than losartan for AT_1 receptors *in vitro* (Chiu *et al.*, 1990; Robertson *et al.*, 1992), and negligible affinity ($pK_i < 6$) for AT_2 sites (Robertson *et al.*, 1992). Thus, in the present study, GR117289 was administered at a dose of 0.5 mg kg^{-1}, i.v., plus a constant intravenous infusion of 1 μg kg^{-1} min^{-1}. In a separate group of dogs, since a limited quantity of PD123177 was available, the drug was infused locally at a dose of 20 μg kg^{-1} min^{-1},

i.r.a., for the duration of the experiment. An approximation of the concentration of PD123177 achieved in the renal circulation was made by dividing the drug infusion rate ($\sim 200\,\mu g\,min^{-1}$) by the average renal blood flow ($\sim 80\,ml\,min^{-1}$) to give a figure of $\sim 2.5\,\mu g\,ml^{-1}$ which equates to 4.5 μM. Since PD123177 has nanomolar affinity for AT_2 receptors (Chiu *et al.*, 1989), the dose given in the present study was anticipated to be high enough to block AT_2 receptors, even if there was high renal clearance of the drug. Conversely, we anticipated that the concentration of PD123177 achieved in the kidney would be unlikely to exceed 5 μM, and possibly become non-selective, unless significant renal concentration of the compound occurred. It has previously been reported that PD123177 does not inhibit functional responses mediated by AT_1 receptors at doses as high as 100 μM (Wong *et al.*, 1990). The effects of PD123177 were also studied in another group of dogs at the slightly higher dose of $50\,\mu g\,kg^{-1}\,min^{-1}$.

Following their administration, the effects of either GR117289, PD123177 or saline on renal haemodynamic and excretory function were monitored over three consecutive 15 min periods. AII ($1-1000\,ng\,kg^{-1}\,min^{-1}$) was then administered as described above. AII mean dose-response curves were plotted and compared with those obtained in dogs which received AII in the absence of antagonist. Where appropriate, the degree of rightward displacement of agonist curves caused by an antagonist was estimated from linear parts of the mean dose-response curves.

Expression of results and statistical analysis

Generally, drug-induced alterations in renal and cardiovascular variables have been expressed as mean percentage change ± s.e.mean from the experiment basal values. Data have been expressed in this manner to facilitate the comparison of results (especially dose-response relationships) obtained from different treatment groups. Changes in renal haemodynamics, excretory function, or glomerular filtration rate, refer, unless otherwise stated, to changes in the *left* kidney only.

Basal values In experiments in which the effects of saline alone, GR117289 or PD123177 were studied, the basal value for all parameters was taken as the mean of the readings obtained at the end of each of the three, 10 min collection periods, immediately prior to infusion of drug or saline.

Where the effects of AII were studied in the presence of GR117289 or PD123177, the basal value was taken as that obtained immediately prior to AII infusion (i.e. 45 min post-antagonist).

Statistical analysis was carried out using the RS1 package for data handling (BBN Software Products Corporation). Statistical significance was assumed when $P<0.05$.

Effects of GR117289 or PD123177 on basal renal function Changes in response to GR117289 or PD123177 were compared with those occurring over the corresponding time period in the saline controls. Unless otherwise stated, data for each individual parameter from the different treatment groups were examined by one way analysis of variance, followed by Dunnett's multiple comparison test. Dunnett's test is appropriate for comparing changes at several time points in two treatment groups (GR117289 and PD123177) with those observed in one control group.

Effects of angiotensin II in the absence and presence of antagonist In the case of AII dose-response relationships, for each parameter to be tested, mean percentage change over a range of AII doses (similar to area under the curve) was compared, by Student's *t* test for unpaired data, to that which occurred over the corresponding time period in the saline controls. Where warranted, the same type of analysis was used to compare differences between the effects of AII in the absence and presence of PD123177.

Drugs used

Drugs used were AII (Human sequence, Sigma); GR117289 (1-[[3-bromo-2-[1H-tetrazol-5-yl)phenyl]-5-benzofuranyl] methyl]-2-butyl-4-chloro-1H-imadazole-5-carboxylic acid), potassium salt (synthesized by Glaxo Group Research); PD123177 (1-[(4-amino-3-methylphenyl)methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imadazo[4,5-c] pyridine-6 carboxylic acid) hydrochloride (synthesized by Glaxo Group Research); pentobarbitone sodium (May & Baker). Drugs were dissolved and diluted in saline. Doses of drugs quoted in the text refer to the parent compound or peptide.

Table 1 Basal values prior to infusion of saline, GR117289, or PD123177

Parameter	*Experimental group*		
	Saline alone ($n = 4$)	*GR117289* ($n = 6$)	*PD123177* ($n = 5$)
Mean blood pressure (mmHg)	113.1 ± 7.4	109.4 ± 4.4	110.0 ± 1.4
Renal vascular conductance ($ml\,min^{-1}\,mmHg^{-1} \times 10^{-3}$)	791 ± 31	792 ± 79	837 ± 59
Renal blood flow ($ml\,min^{-1}$)	88.8 ± 3.3	86.8 ± 9.6	92.0 ± 6.0
Urine output ($ml\ 10\,min^{-1}$)	5.1 ± 0.5	4.5 ± 0.7	4.5 ± 0.8
Sodium excretion ($\mu mol\,min^{-1}$)	118.4 ± 9.6	104.6 ± 13.7	80.4 ± 8.5
Fractional sodium excretion (%)	3.0 ± 0.2	3.1 ± 0.4	2.3 ± 0.3
GFR ($ml\,min^{-1}$)	27.2 ± 1.0	23.7 ± 2.4	20.4 ± 2.6
Filtration fraction (%)	46.7 ± 1.6	45.9 ± 5.0	34.1 ± 3.2
Plasma aldosterone ($pg\,ml^{-1}$)	27.8 ± 7.8	101.0 ± 50.7	64.7 ± 5.4
Plasma renin activity ($ng\,AI\,ml^{-1}\,h^{-1}$)	1.5 ± 0.3	4.2 ± 1.1	5.1 ± 1.8

Values are arithmetic mean ± s.e.mean.

Results

Effects of AT_1 or AT_2 receptor blockade on basal renal function

The effects of the AT_1 receptor antagonist, GR117289 (0.5 mg kg^{-1} + 1 μg kg^{-1} min^{-1}, i.v.), or the AT_2 receptor antagonist, PD123177 (20 μg kg^{-1} min^{-1}, i.r.a.) were examined in separate groups of dogs. For ease of comparison, as explained above, drug effects on renal function have generally been expressed as % change from the experiment basal value. Basal values prior to administration of GR117289, PD123177, or saline are shown in Table 1. There was no significant difference between the antagonist-treated animals and the saline controls for any of the basal parameters shown in Table 1 (one way analysis of variance followed by Dunnett's multiple comparisons test).

Administration of the AT_1 receptor antagonist, GR117289, resulted in a fall in mean blood pressure which was maintained over the 45 min post-dose period (Figure 1a). In addition, GR117289 caused marked renal vasodilatation, characterized by sustained increases in renal vascular conductance and renal blood flow (Figure 1b and c). In contrast, Figure 1 shows that the AT_2 receptor antagonist, PD123177, caused no significant change in blood pressure or renal haemodynamics.

Despite the reduction in blood pressure, there was no decrease in sodium and urine excretion following GR117289; instead there was a tendency for urine output, sodium excretion (Figure 2a and b), and fractional sodium excretion (data not shown) to increase, although the changes failed to achieve statistical significance (analysis of variance/Dunnett's test). GR117289 caused no significant change in GFR (Figure 2c), and since renal blood flow was increased, there was a tendency for filtration fraction to decrease (mean fall of 15 ± 3%, 45 min post-dose) although this was not statistically significant. The AT_2 receptor antagonist, PD123177, caused no significant change in urine output, sodium excretion, fractional sodium excretion (data not shown), or GFR (Figure 2). There was a tendency for sodium excretion to increase, 45 min post-PD123177 (Figure 2b), but this was also not statistically significant. In addition, in four dogs where a slightly higher dose (50 μg kg^{-1} min^{-1}, i.r.a.) was used, PD123177 still failed to cause any significant change in blood pressure, renal haemodynamics, or sodium and urine excretion (data not shown).

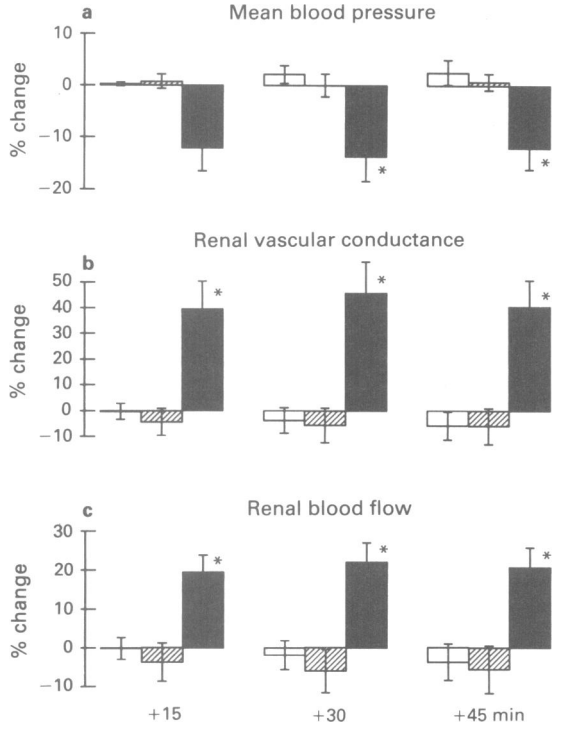


Figure 1 Effects of saline (open columns, $n = 4$), the angiotensin AT_1 receptor antagonist, GR117289, 0.5 mg kg^{-1} + 1 μg kg^{-1} min^{-1}, i.v. (solid columns, $n = 6$), or the AT_2 receptor antagonist, PD123177, 20 μg kg^{-1} min^{-1}, i.r.a. (hatched columns, $n = 5$) on (a) mean blood pressure, (b) renal vascular conductance, and (c) renal blood flow. Columns represent mean % change ± s.e.mean from basal values (see Table 1). *$P < 0.05$, Dunnett's test, drug treated groups vs saline controls.

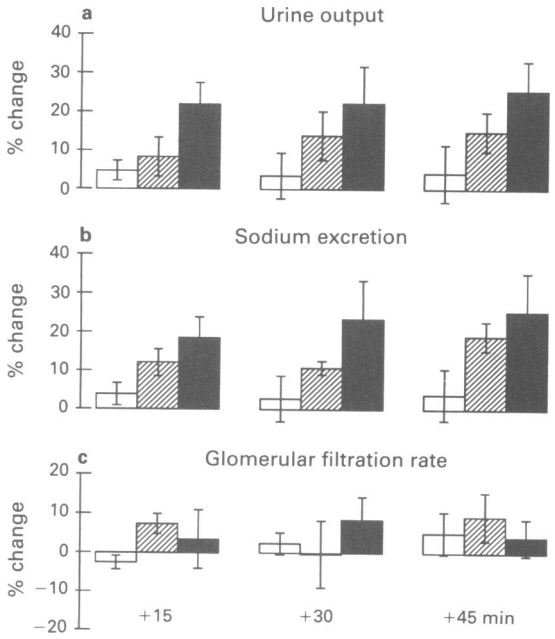


Figure 2 Effects of saline (open columns, $n = 4$), the angiotensin AT_1 receptor antagonist, GR117289, 0.5 mg kg^{-1} + 1 μg kg^{-1} min^{-1}, i.v. (solid columns, $n = 6$), or the AT_2 receptor antagonist, PD123177, 20 μg kg^{-1} min^{-1}, i.r.a. (hatched columns, $n = 5$) on (a) urine output, (b) sodium excretion, and (c) glomerular filtration rate. Columns represent mean % change ± s.e.mean from basal values (see Table 1).

The AT_1 receptor antagonist, GR117289, increased PRA 15, 30, and 45 min post-dose period (Figure 3a). Despite this increase in PRA, a significant reduction in plasma aldosterone concentration was observed 15 and 45 min post-dose (Figure 3b). PD123177 caused no significant change in PRA or plasma aldosterone levels at any time point. (Figure 3).

Effects of AT_1 or AT_2 receptor blockade on the renal actions of exogenous angiotensin II

Table 2 shows basal values measured prior to AII infusion in the different groups of dogs, and demonstrates that for most parameters basal values were not markedly different in the absence or presence of antagonist. Basal sodium excretion was significantly higher in dogs treated with GR117289 compared with antagonist-free dogs. This difference in basal sodium excretion should be borne in mind when comparing the effects of AII on this parameter between the two groups.

Renal haemodynamics

In antagonist-free animals, intra-renal artery infusion of AII (1–300 ng kg^{-1} min^{-1}) resulted in dose-related renal vasoconstriction, as indicated by decreases in renal vascular conductance and renal blood flow (Figure 4a and b). These effects of AII occurred in the absence of any change of blood pressure except at the highest dose (300 ng kg^{-1} min^{-1}), where a small pressor response was observed (Figure 4c). The AT_1 receptor antagonist, GR117289, antagonized the AII-induced renal vasoconstriction, causing approximately 12 and

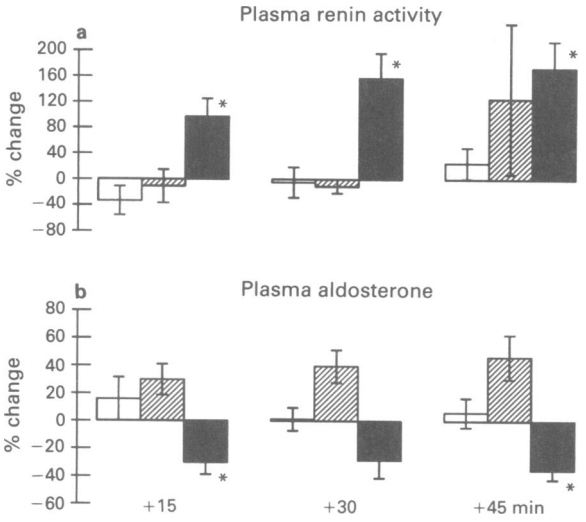


Figure 3 Effects of saline (open columns, $n = 4$), the angiotensin AT_1 receptor antagonist, GR117289, 0.5 mg kg^{-1} + 1 μg kg^{-1} min^{-1}, i.v. (solid columns, $n = 6$), or the AT_2 receptor antagonist, PD123177, 20 μg kg^{-1} min^{-1}, i.r.a. (hatched columns, $n = 5$) on (a) plasma renin activity, and (b) plasma aldosterone levels. Columns represent mean % change ± s.e.mean from basal values (see Table 1).
*$P < 0.05$, Dunnett's test, drug treated groups vs saline controls.

16 fold rightward displacements of the mean AII dose-response curves for renal vascular conductance (Figure 4a) and renal blood flow (Figure 4b), respectively. The AT_2 receptor antagonist, PD123177 (20 μg kg^{-1} min^{-1}), did not inhibit the renal vasoconstrictor effects of AII over the dose-range 1–10 ng kg^{-1} min^{-1}. Interestingly, however, PD123177 did cause inhibition of the vasoconstrictor responses to higher doses of AII (30–300 ng kg^{-1} min^{-1}), causing a small (~4 fold) rightward displacement of the AII curve for renal vascular conductance (Figure 4a), and decreasing the ability of AII to reduce renal blood flow (Figure 4b). Comparing mean % change over a range of agonist doses in the absence and presence of antagonist, AII (30–300 ng kg^{-1} min^{-1})-induced reductions in renal vascular conductance and renal blood flow were significantly inhibited by PD123177 (unpaired t test).

Renal excretory function

Changes in renal perfusion pressure can result in alterations in renal tubular function, and consequently, sodium and urine output. To avoid this complicating factor, the following results describe the effects of non-pressor doses of AII on renal function, in the absence or presence of antagonists.

AII (1–100 ng kg^{-1} min^{-1}) caused marked dose-related reductions in urine output and sodium excretion (Figure 5a and b) which occurred concomitantly with falls in fractional sodium excretion and GFR (Figure 5c and d). The AT_1 receptor antagonist, GR117289, antagonized the effects of AII on these parameters to a similar extent as it inhibited the renal vascular effects of AII. Mean AII dose-response curves for urine output, sodium excretion, fractional sodium excretion, and GFR (Figure 5) were displaced approximately 16, 20, 15, and 38 fold, respectively, to the right of those observed in the absence of antagonist. The AT_2 receptor antagonist, PD123177, caused no significant inhibition of the effects of AII on urine output, sodium excretion, or fractional sodium excretion (Figure 5). From Figure 5c, it could be deduced that PD123177 antagonized the ability of high doses of AII (30 and 100 ng kg^{-1} min^{-1}) to reduce GFR. However, this apparent effect of PD123177 was heavily influenced by the data from one animal in the group, and was not statistically significant.

Thus, the data indicate that the AT_2 receptor blocker, PD123177 (20 μg kg^{-1} min^{-1}, i.r.a.), did not antagonize the effects of AII on renal excretory function, but did cause a small, but significant, inhibition of the renal vasoconstrictor effects of high doses (30–300 ng kg^{-1} min^{-1}) of AII. The dose-dependency of this phenomenon was examined in a further group of four dogs where PD123177 was administered at the slightly higher dose of 50 μg kg^{-1} min^{-1}, i.r.a. At this higher dose, PD123177 caused a progressive antagonism of the renal vasoconstrictor effects of AII (Figure 6a and b), but still failed to inhibit the ability of AII to reduce urine output, sodium excretion (Figure 6c and d), fractional sodium excretion or GFR (data not shown).

Discussion

The present study investigated the role, and relative importance, of AT_1 versus AT_2 receptors in mediating the renal haemodynamic and excretory effects of AII *in vivo*. The drugs chosen to address this question were the AT_1 receptor antagonist, GR117289, and the AT_2 receptor antagonist, PD123177. Doses of these compounds were chosen which were predicted to be selective for AT_1 or AT_2 receptors, respectively (see Methods). Experiments were carried out in anaesthetized dogs, which provide stable conditions for leng-

Table 2 Basal values prior to infusion of angiotensin II (AII) alone, or AII in the presence of either GR117289 or PD123177

Parameter	*Experimental group*		
	AII alone ($n = 5$)	*AII in presence of GR117289* ($n = 5$)	*AII in presence of PD123177* ($n = 5$)
Mean blood pressure (mmHg)	116.6 ± 5.6	99.1 ± 8.9	110.8 ± 1.7
Renal vascular conductance (ml min^{-1} mmHg^{-1} × 10^{-3})	834 ± 152	954 ± 68	823 ± 96
Renal blood flow (ml min^{-1})	95.3 ± 14.2	93.9 ± 8.4	86.8 ± 7.7
Urine output (ml 10 min^{-1})	3.2 ± 0.5	6.3 ± 1.1	5.2 ± 1.0
Sodium excretion (μmol min^{-1})	89.6 ± 6.9	*135.2 ± 16.4	94.8 ± 8.1
Fractional sodium excretion (%)	2.6 ± 0.5	4.1 ± 0.7	2.4 ± 0.4
GFR (ml min^{-1})	24.3 ± 2.0	23.1 ± 2.6	22.4 ± 3.6

Values are arithmetic mean ± s.e.mean.
*$P < 0.05$, antagonist treated vs AII alone group. One way analysis of variance followed by Dunnett's multiple comparison test.

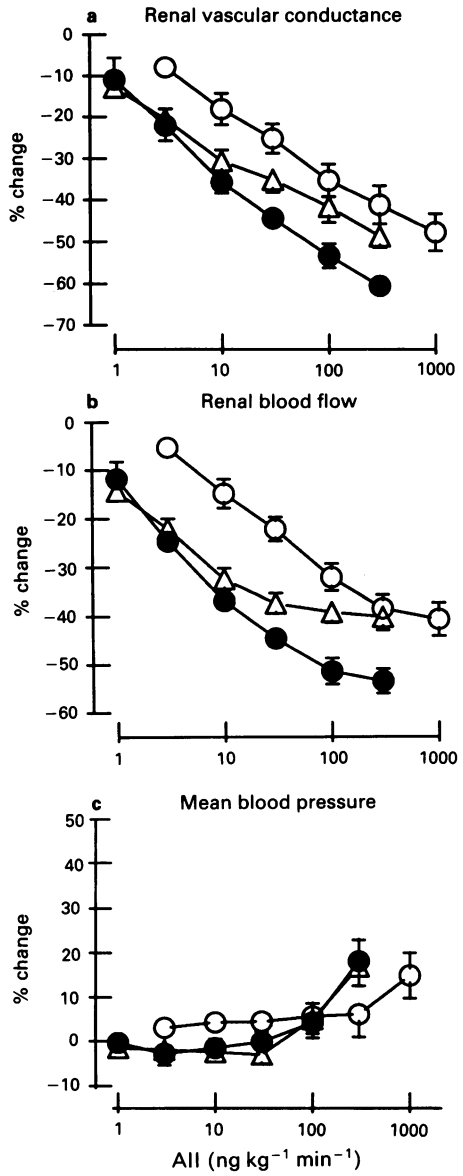


Figure 4 Effects of intra-renal artery infusion of angiotensin II (AII) on (a) renal vascular conductance, (b) renal blood flow, and (c) mean blood pressure in the absence (●, $n = 5$), and in separate groups of dogs, in the presence of the angiotensin AT_1 receptor antagonist, GR117289 (○, $n = 5$), or the AT_2 receptor antagonist, PD123177 (△, $n = 5$). See the text for antagonist doses. Values are mean % change ± s.e.mean from basal values (see Table 2).

thy renal function studies requiring the taking of multiple blood samples. In addition, preliminary radioligand binding data (unpublished observations) suggested that both AT_1 and AT_2 receptors are present in dog renal cortex.

The AT_1 receptor antagonist, GR117289, reduced blood pressure, and caused renal vasodilatation, characterized by a sustained increase in renal blood flow. GR117289 did not cause any significant change in GFR, and since renal blood flow was increased, there was a tendency for filtration fraction to decline. Urine output, sodium excretion, and fractional excretion of sodium all showed a tendency to increase following GR117289, although the changes were not statistically significant. Taken together, these effects of GR117289 on basal renal function are consistent with blockade of the renal effects of AII, and other workers have obtained similar results in anaesthetized dogs when studying the effects of ACE inhibitors (Schmidt *et al.*, 1989), or the AT_1 receptor antagonist, losartan (Wong *et al.*, 1991a). Additionally, since GR117289 has been shown to be a very specific angiotensin receptor antagonist (Robertson *et al.*, 1992), its effects are most likely due to blockade of AT_1 receptors. In contrast to GR117289, the AT_2 receptor antagonist, PD123177, caused no significant change in renal haemodynamics, or sodium and urine excretion. It could be argued that the lack of effect of PD123177 may reflect the fact that there were low background levels of AII. However, this is highly unlikely since basal PRA values in dogs which received PD123177 were similar to those observed in GR117289-treated animals. Thus, these data suggest that the effects of endogenous AII on basal renal function in the dog are primarily mediated by AT_1 receptors.

Interestingly, although pre-drug PRA levels were not particularly raised, basal filtration fractions were high, suggesting that the kidneys of these animals may have been under a rather high degree of tonic vasoconstriction. This was possibly due to the fact that the experiments were carried out under anaesthesia. It would be interesting to determine if AT_1 and AT_2 receptor blockade has similar effects on renal function in conscious dogs.

In agreement with Gibson *et al.* (1991), the present data also suggest that AII feedback inhibition of renin release is mediated predominantly by AT_1 receptors, since a marked increase in PRA was observed following GR117289, while PD123177 had no significant effect. It is probable that the fall in blood pressure observed after GR117289 also contributed to an increase in renin release; however, previous reports (Schmid, 1972) show that falls in renal perfusion pressure within the limits of renal autoregulation do not markedly change PRA in anaesthetized dogs. Despite causing an increase in PRA, GR117289 reduced plasma aldosterone levels. This was probably a consequence of antagonism of the effects of endogenous AII in the adrenal cortex. Previous reports (Wong *et al.*, 1990) indicate that functionally important angiotensin receptors in the adrenal gland are of the AT_1 subtype.

It is now established that angiotensin AT_1 receptors occur in the proximal tubule, particularly in the S1 segment (Cogan, 1990; Xie *et al.*, 1990; Harris, 1992), where they play a physiological role in mediating the stimulatory effect of AII on sodium reabsorption. Thus, we were initially surprised that in the current study, a more marked diuretic/natriuretic response was not observed following the AT_1 receptor antagonist, GR117289. However, on closer consideration the results are probably not surprising for the following reasons. Firstly, GR117289 caused a marked and sustained fall in blood pressure. It is well established that falls in blood pressure *per se* result in reduced sodium and urine excretion (Guyton *et al.*, 1980). Secondly, the anaesthetized dogs used in this study received a saline infusion throughout the experiment to help achieve steady urine excretion and to provide a background on which to also study the renal effects of exogenous AII. Under these conditions it is unlikely that the renin-angiotensin system was exerting a great degree of tonic influence over sodium reabsorption. Indeed, previous studies in salt-replete dogs using either ACE inhibitors (Schmidt *et al.*, 1989) or saralasin (Lohmeier *et al.*, 1977), have also found that when blood pressure is reduced, these agents do not cause a significant diuresis/natriuresis. Moreover, in anaesthetized salt-replete guinea-pigs (Amrani *et al.*, 1991), and volume-expanded rats (Fenoy *et al.*, 1991), the AT_1 receptor antagonist, losartan, reduces blood pressure but does not cause a significant diuresis.

Thus, while the effects of GR117289 or PD123177 on basal function suggest that the renal effects of endogenous AII in the dog are mediated by AT_1 receptors, the scope of the results is slightly limited, as discussed above, by the experimental conditions (i.e. the data only relate to the role of AT_1 and AT_2 receptors under conditions of limited activation of the renin-angiotensin system). Thus, the second part of this study examined the effects of AT_1 or AT_2 receptor

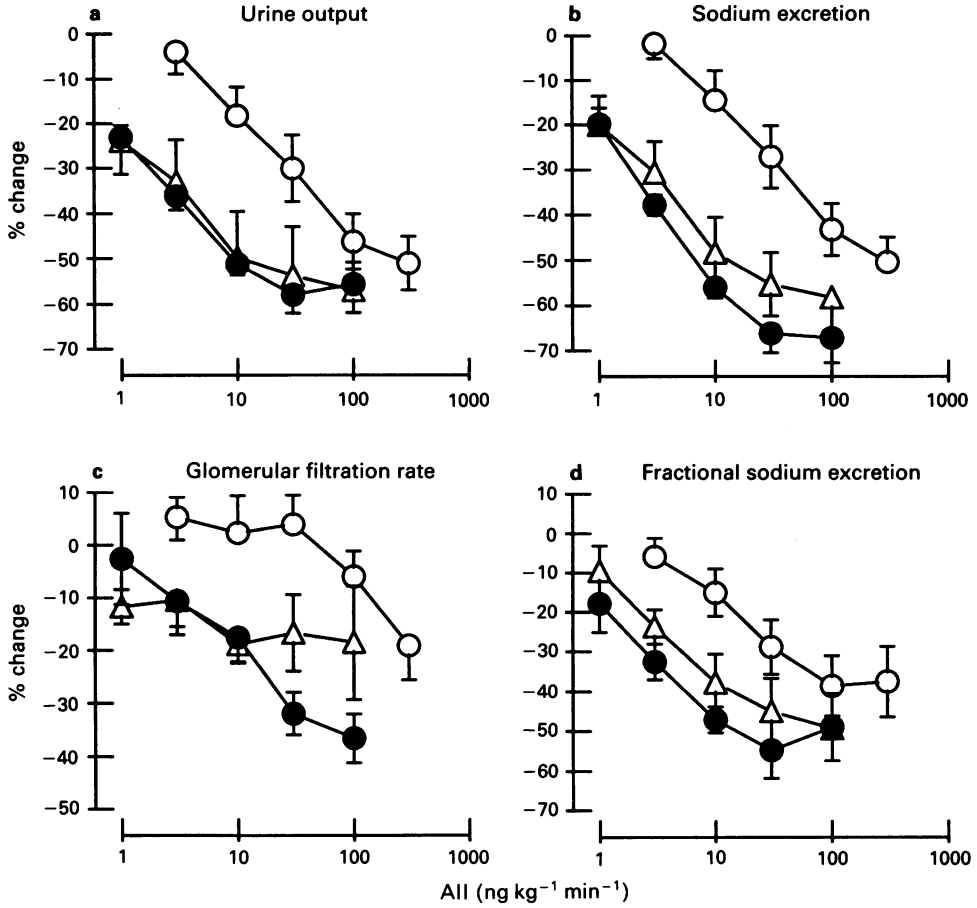


Figure 5 Effects of intra-renal artery infusion of angiotensin II (AII) on (a) urine output, (b) sodium excretion, (c) glomerular filtration rate, and (d) fractional sodium excretion, in the absence (●, $n = 5$), and in separate groups of dogs, in the presence of the angiotensin AT_1 receptor antagonist, GR117289 (○, $n = 5$), or the AT_2 receptor antagonist, PD123177 (△, $n = 5$). See the text for antagonist doses. Values are mean % change ± s.e.mean from basal values (see Table 2).

blockade on the renal haemodynamic and excretory effects of a wide dose-range of exogenous AII.

Local infusion of AII (1–300 ng kg^{-1} min^{-1}) into the renal artery resulted in dose-related renal vasoconstriction, characterized by decreased renal vascular conductance and blood flow. This represented a direct effect of the peptide on the renal vasculature since blood pressure was not increased, except at the highest dose where a small pressor response was observed. In addition to renal vasoconstriction, AII caused a dose-related reduction in urine output and sodium excretion. Since reductions in both GFR and fractional sodium excretion were observed, the AII-induced reduction in urine/sodium excretion was probably a consequence of both reduced filtered load and increased tubular sodium reabsorption. These well-established renal effects of AII are consistent with its role as a salt and water retaining hormone, and are almost certainly mediated by receptors at both vascular and proximal tubular sites (Navar *et al.*, 1991; Harris, 1992).

The AT_1 receptor antagonist, GR117289, markedly antagonized the effects of exogenous AII over the entire dose-range tested, on renal haemodynamics, sodium and urine excretion, fractional sodium excretion, and GFR. In contrast to GR117289, the AT_2 receptor blocker, PD123177 (20 μg kg^{-1} min^{-1}), failed to antagonize the renal effects of AII over the dose-range 1–10 ng kg^{-1} min^{-1}. This suggests that over the latter dose-range the effects of AII on renal function are mediated exclusively by AT_1 receptors. Interestingly, however, while PD123177 still failed to inhibit the ability of higher doses of AII (30–300 ng kg^{-1} min^{-1}) to reduce urine output, sodium excretion and GFR, it did cause a small, but significant, inhibition of renal vasoconstrictor responses to AII. At a higher dose (50 μg kg^{-1} min^{-1}), PD123177 exerted further blockade of the renal vasoconstrictor effects of AII, but still caused no antagonism of the effects of the peptide on urine/sodium excretion or GFR. It is worth noting that these results relate only to the effects of exogenous infusions of AII, which may not completely mimic the effects of high levels of endogenous renal angiotensins. It would be interesting to compare these results with the effects of AT_1 and AT_2 receptor antagonists in this model under conditions of raised endogenous renal AII levels; for example, following salt depletion.

Taken together, the present results suggest that the effects of infusions of exogenous AII on renal haemodynamic and excretory function are predominantly mediated by AT_1 receptors. However, could the small inhibitory effect of PD123177 on AII-induced renal vasoconstriction indicate some functional significance for AT_2 receptors in the renal vasculature? Certainly, it is unlikely that the antagonism observed is due to PD123177 binding to AT_1 receptors, since (a) at the doses used it is improbable that PD123177 reaches high enough concentrations in the renal circulation to have affinity for AT_1 receptors, and (b) the compound caused no antagonism of the effects of AII on absolute/fractional sodium excretion or urine output; actions which are inhibited by AT_1 receptor antagonists such as losartan (Clark *et al.*, 1991) or GR117289. It is an intriguing possibility that functionally important AT_2 receptors may be present in the renal resistance vasculature. This possibility was previously raised by Widdop and co-workers (1992), who found that PD123177 caused inhibition of renal vasoconstrictor responses to AII in conscious rats, but only when given 24 h after the AT_1

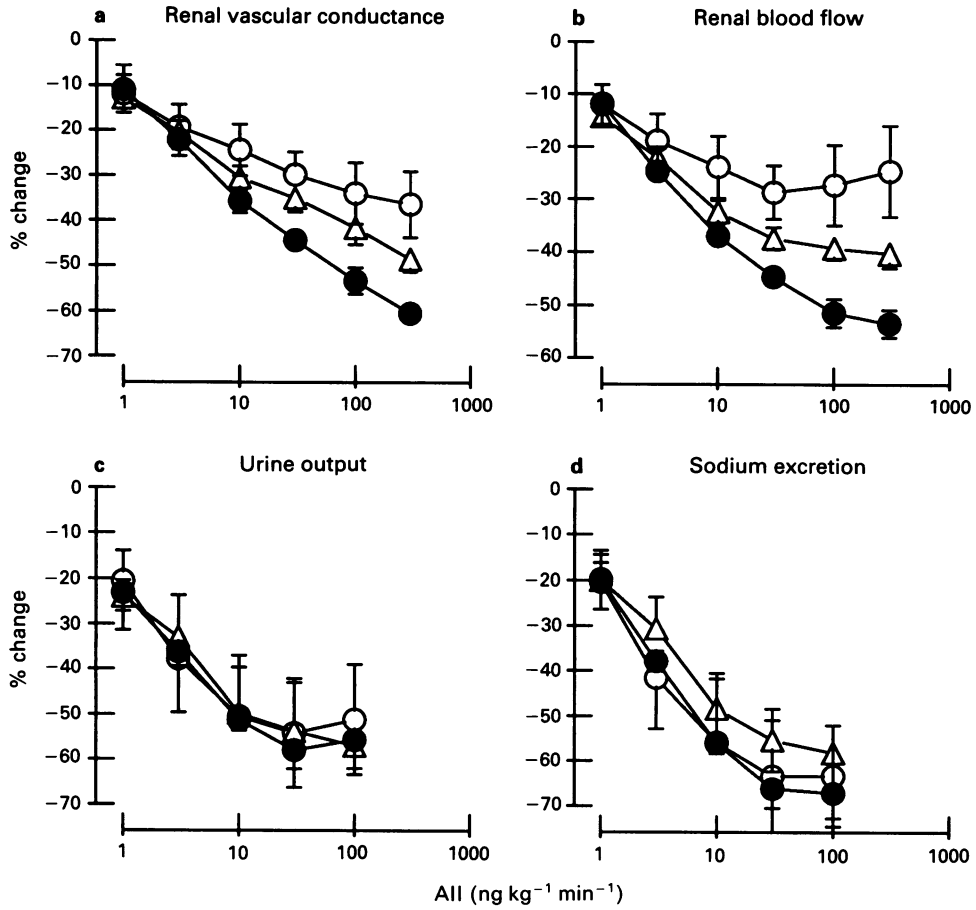


Figure 6 Effects of intra-renal artery infusion of angiotensin II (AII) on (a) renal vascular conductance, (b) renal blood flow, (c) urine output, and (d) sodium excretion, in the absence (●, $n = 5$), and in separate groups of dogs, in the presence of the angiotensin AT_2 receptor antagonist, PD123177 (Δ, 20 μg kg^{-1} min^{-1}, n = 5; ○, 50 μg kg^{-1} min^{-1}, $n = 4$). Values are mean % change ± s.e.mean from basal values.

receptor antagonist, EXP3174. Thus, these workers suggested that their results may have been due to displacement of EXP3174 by PD123177 from plasma protein binding sites, which does seem likely since binding studies (Edwards *et al.*, 1992; Sechi *et al.*, 1991) have failed to find a significant population of AT_2 receptors in rat kidney. In contrast, it is interesting to note that using autoradiography, Grone *et al.* (1992) have shown that AT_2 binding sites are present in the human pre-glomerular vasculature. As yet, there have been no reports on the distribution of AT_1 and AT_2 binding sites in the dog kidney, although preliminary observations (unpublished) within our own laboratory do suggest that AT_2 binding sites are present in dog renal cortex.

However, while it is possible that functionally important AT_2 receptors are present in the canine kidney, other explanations of the data deserve comment. Firstly, since few groups have been able to identify any antagonism by PD123177 of functional responses to AII, there has been little need to investigate the specificity of action of the drug. Thus, PD123177 could have had a non-specific spasmolytic action in the present study. This seems unlikely, however, since PD123177, at both doses tested, had no effect on basal blood pressure or renal vascular conductance. In addition, Wong and co-workers (1990), have shown that pressor responses to AII in pithed rats are unaffected by PD123177 at a dose of 100 mg kg^{-1}. Moreover, it has also been shown in anaesthetized dogs (Wong *et al.*, 1991b), that renal vasoconstrictor responses to noradrenaline were unaffected by PD123177 at doses up to 100 μg kg^{-1} min^{-1}; five times the dose used in the present study. Another explanation for the data is that more than two subtypes of angiotensin receptors are present in the kidney. In 1987, Douglas proposed two subtypes of renal angiotensin receptors; one subtype which primarily used adenylate cyclase as a transducer, while the other subtype is mainly coupled to phospholipase C. Since there is no evidence to suggest that AT_2 receptors are coupled via either of these transducers, it is possible that in addition to AT_2 receptors, there are AT_1 receptor subtypes, or atypical angiotensin receptors (neither AT_1 nor AT_2), present in the renal vasculature.

In conclusion, this study has shown that the renal effects of both endogenous and low doses of exogenous AII are mediated by angiotensin AT_1 receptors, since they are sensitive to blockade by the AT_1 antagonist, GR117289, but not the AT_2 receptor antagonist, PD123177. However, PD123177 did inhibit renal vasoconstrictor responses to high doses of AII. This observation could indicate heterogeneity of functionally important renal vascular angiotensin receptors and warrants further investigation.

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Effects of MS-551, a new class III antiarrhythmic drug, on action potential and membrane currents in rabbit ventricular myocytes

[1]Haruaki Nakaya, Noritsugu Tohse, Youji Takeda & Morio Kanno

Department of Pharmacology, Hokkaido University School of Medicine, Sapporo 060, Japan

1 Electrophysiological effects of MS-551, a new class III antiarrhythmic drug, were examined and compared with those of (+)-sotalol in rabbit ventricular cells.

2 In rabbit ventricular muscles stimulated at 1.0 Hz, MS-551 (0.1–10 μM) and (+)-sotalol (3–100 μM) prolonged action potential duration (APD) and effective refractory period without affecting the maximum upstroke velocity of phase 0 depolarization ($\dot{V}_{max}$). The class III effect of MS-551 was approximately 30 times more potent than that of (+)-sotalol.

3 Class III effects of MS-551 and (+)-sotalol showed reverse use-dependence, i.e., a greater prolongation of APD at a longer cycle length.

4 In rabbit isolated ventricular cells, 3 μM MS-551 and 100 μM sotalol inhibited the delayed rectifier potassium current (I_K) which was activated at more positive potentials than − 50 mV and saturated around + 20 mV.

5 MS-551 at a higher concentration of 10 μM decreased the transient outward current (I_{to}) and the inward rectifier potassium current (I_{K1}) although 100 μM sotalol failed to inhibit these currents.

6 MS-551 is a non-specific class III drug which can inhibit three voltage-gated K^+ channels in rabbit ventricular cells.

Keywords: Class III antiarrhythmic drugs; MS-551; (+)-sotalol; delayed rectifier K^+ current; transient outward current; inward rectifier K^+ current

Introduction

Class I antiarrhythmic drugs have been used for a long time in the treatment of life-threatening ventricular tachyarrhythmias. However, most currently used class I drugs may not adequately protect sudden cardiac death resulting from ventricular tachycardia and fibrillation (Friedman, 1984; CAST investigators, 1989). Such results provide further rationale for the continued search for alternative drug therapy to prevent or reduce likelihood of ventricular fibrillation. Recently much attention has been focused on class III antiarrhythmic drugs as the most promising candidates of antifibrillatory activity (Singh *et al.*, 1992). Several experimental studies have demonstrated that class III drugs such as (+)-sotalol (Lynch *et al.*, 1984; 1985), E-4031 (Lynch *et al.*, 1990; Katoh *et al.*, 1990; Chi *et al.*, 1991) and UK-68,798 (Black *et al.*, 1991; Zuanetti & Corr, 1991) prolonged ventricular effective refractory period (ERP) and showed anti-fibrillatory action in dogs with a previous myocardial infarction.

MS-551 is a new class III drug having a pirimidinedione structure (Figure 1), which is reported to prolong ventricular ERP and exert antifibrillatory action in dogs with myocardial infarction (Kamiya *et al.*, 1992). However, the underlying ionic mechanism(s) responsible for class III effect of MS-551 have not been evaluated. Accordingly, in the present study effects of MS-551 on ionic currents were examined and compared with those of (+)-sotalol, a specific blocker of I_K (Komeichi *et al.*, 1990), in rabbit ventricular myocytes. The rabbit ventricular cell may be a suitable preparation for the evaluation of class III effect because a variety of voltage-gated K^+ channels including I_{to} channels (Hiraoka & Kawano, 1989; Giles & Imaizumi, 1988) are known to exist in the cells as in human cardiac cells (Escande *et al.*, 1987).In addition, it has recently been reported that many class III drugs easily induce torsades de pointes in rabbits (Carlsson *et al.*, 1990). Therefore, in order to minimize the life-threatening proarrhythmias it is of importance to evaluate class III effects in rabbit ventricular cells.

Methods

Action potential study

New Zealand white rabbits weighing 2–4 kg were anaesthetized with pentobarbitone sodium (30 mg kg^{-1}, i.v.) and the hearts were removed rapidly. The heart was immersed in an oxygenated Tyrode solution and small papillary muscles were dissected from the right ventricle. The preparations were pinned to the bottom of a 5-ml tissue chamber and continuously superfused with the modified Tyrode solution equilibrated with 95% O_2 and 5% CO_2. The composition of the solution was (mM): NaCl 125, KCl 4, NaH_2PO_4 1.8, $MgCl_2$ 0.5, $CaCl_2$ 2.7, $NaHCO_3$ 25 and glucose 5.5. The bath temperature was kept constant at 36.0 ± 1.0°C.

Transmembrane potentials were recorded by standard microelectrode techniques, as previously described (Nakaya *et*

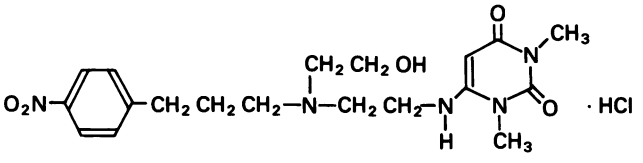


Figure 1 Chemical structure of MS-551.

[1] Author for correspondence at present address: Department of Pharmacology, School of Medicine, Chiba University, Chiba 260, Japan.

al., 1987). In brief, the preparation was electrically stimulated at 1.0 Hz with pulses of 1 ms duration at twice the diastolic threshold with a bipolar electrode. The stimuli were delivered by an electronic stimulator (Nihon Kohden SEN 6100, Tokyo, Japan) through an isolation unit (Nihon Kohden SS-302J). Transmembrane action potentials were recorded with glass microelectrodes filled with 3 M KCl, which had a tip resistance of 10–30 megohms. The microelectrode was coupled to the input stage of a high impedance amplifier with capacitance neutralization (Nihon Kohden MEZ 8201). The $\dot{V}_{max}$ of the action potential was measured with an electronic differentiator having a linearity up to 1000 Vs^{-1}. The amplified signals were displayed on a dual beam oscilloscope (Nihon Kohden VC-10), photographed on 35 mm film and recorded on a chart recorder (Watanabe Sokki WR 3101, Tokyo, Japan).

After an equilibration period of 2 h, a stable impalement was obtained and control recordings were made. Effective refractory period (ERP), defined as the longest interval at which the premature stimulation failed to generate an active response, was determined by delivering a single test stimulus at progressively earlier diastolic intervals after a 30-s pulse train with an interstimulus interval of 1000 ms. The preparations were then exposed to a solution containing the lowest concentration of a class III antiarrhythmic drug, and the concentration was increased in a stepwise fashion at intervals of 30 min. Preliminary experiments revealed that a 30 min superfusion period was sufficient for action potential changes to reach a steady state. The recording of the membrane potential and the determination of ERP were repeated at the end of drug superfusion period. Only experiments in which a stable impalement was maintained were used for data analysis.

In another set of experiments the rate-dependence of APD-prolonging effects were examined in preparations stimulated at basic cycle lengths of 500, 1000, 2000 and 5000 ms. At each cycle length sufficient time (about 5 min) was allowed for APD to reach a steady state before measurements were made. Relationships between APD and stimulation rate were determined before and after 30-min exposure to class III drugs or 4-aminopyridine.

Patch clamp study

Single ventricular myocytes were obtained from New Zealand white rabbits by enzymatic dispersion, as described previously (Tohse *et al.*, 1990). Briefly, the heart was removed from the open chest rabbits anaesthetized with pentobarbitone sodium, and mounted on a modified Langendorff perfusion system for retrograde perfusion of the coronary circulation with a normal HEPES-Tyrode solution. The perfused medium was then changed to a nominally Ca^{2+}-free Tyrode solution and then to a solution containing 0.01% w/v collagenase (Wako, Osaka, Japan). After digestion, the heart was perfused with a high K^+, low Cl^- solution (KB solution) (Isenberg & Klockner, 1982). Ventricular tissue was cut into small pieces in the KB solution and the cell suspension was stored in a refrigerator (4°C) for later use. The composition of the normal HEPES-Tyrode solution was (mM): NaCl 143, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, NaH_2PO_4 0.33, glucose 5.5 and HEPES-NaOH buffer (pH 7.4), 5.0. The composition of the KB solution was (mM): KOH 70, L-glutamic acid 50, KCl 40, taurine 20, KH_2PO_4 20, $MgCl_2$ 3, glucose 10, EGTA 1.0 and HEPES-KOH buffer (pH 7.4) 10.

Whole-cell membrane currents were recorded by the patch clamp method (Hamil *et al.*, 1981). Single ventricular cells were placed in a recording chamber (1 ml volume) attached to an inverted microscope (Olympus IMT-2, Tokyo, Japan) and superfused with the HEPES-Tyrode solution at a rate of 5 ml min^{-1}. The temperature of the external solution was kept constant at 36.0 ± 1.0°C. Glass patch pipettes with a diameter of 3–4 μm were filled with an internal solution composed of (mM): K-aspartate 110, KCl 20, $MgCl_2$ 1.0, ATP-K_2 5.0, phosphocreatinine-K_2 5.0, EGTA 0.5–10 and HEPES-KOH buffer (pH 7.4), 5.0. The resistance of the pipette filled with the internal solution was 1.5–2.5 megohms. After the gigaohm-seal between the tip of the electrode and the cell membrane was established, the membrane patch was disrupted by applying more negative pressure to make the whole cell voltage-clamp mode. The electrode was connected to the input stage of a current-voltage converter with a feedback resister of 100 megohms. The signals were displayed on a storage oscilloscope (Tektronics OP03, Beaverton, OR, U.S.A.) and were simultaneously fed to a data recording system, consisting of a converter system (SONY PCM-501 ES, Tokyo, Japan) and a video cassette recorder (National VF-F1, Tokyo, Japan) as a back up. The current and voltage signals, filtered at 2 kHz, were also stored on a personal computer (NEC PC98XA, Tokyo, Japan) equipped with a 20 Mbyte hard disc and an AD converter (Canopus Electronics ADX-98, Tokyo, Japan) for later analysis. A liquid junction potential between the internal solution and the bath solution of − 8 mV was corrected.

In some experiments the delayed rectifier potassium current (I_K) was isolated from other membrane currents by using Na^+- and K^+-free external solution and the Ca^{2+} channel blockers nisoldipine (1 μM), as described previously (Tohse *et al.*, 1987). The Na^+- and K^+-free solution was prepared by replacing NaCl and KCl with equimolar choline chloride. By use of this solution, we could block the inward rectifier potassium current (I_{K1}) the calcium current (I_{Ca}), the electrogenic Na^+/Ca^{2+} exchange current and the electrogenic pump current. In order to record the transient outward current (I_{to}), I_{Ca} was blocked by adding 1.8–2.0 mM Co^{2+} to the external solution. In a part of experiments for I_{to} recording, I_{Na} was incompletely blocked by adding 6–7 μM tetrodotoxin.

Drugs

The compounds used are as follows: MS-551 (Mitsui Pharmaceuticals, Tokyo, Japan), (+)-sotalol (Bristol-Myers Squibb Company, Wallingford, CT, U.S.A.), nisoldipine (Bayer AC, Leverkusen, Germany), tetrodotoxin (Sankyo, Tokyo, Japan) and 4-aminopyridine (Wako, Osaka, Japan). All drugs except for nisoldipine were dissolved in distilled water or suitable buffer solution. Nisoldipine was prepared as a stock solution (10 mM) in absolute ethanol.

Statistics

All values are presented in terms of mean ± s.e. Analysis by Student's *t* test was performed for paired or unpaired observations. *P* value of less than 0.05 was considered significant.

Results

Effects of MS-551 and (+)-sotalol on action potentials and ERP

Figure 2a illustrates representative changes in action potential configuration produced by MS-551 in rabbit papillary muscles stimulated at 1.0 Hz. MS-551 at 0.3–10 μM prolonged APD in a concentration-dependent manner with little effect on $\dot{V}_{max}$. The prolongation of APD induced by a high concentration of MS-551 was more marked at the late repolarization phase. In some preparations exposed to 10 μM MS-551, early afterdepolarizations appeared, as shown in Figure 2b. Similar changes in action potential configuration were also produced by (+)-sotalol although early afterdepolarizations were not evoked by the drug in concentrations examined (up to 100 μM).

Changes in action potential durations and ERP produced by MS-551 and (+)-sotalol are summarized in Table 1. The baseline characteristics of action potentials recorded from 12 preparations stimulated at 1.0 Hz were as follows: resting

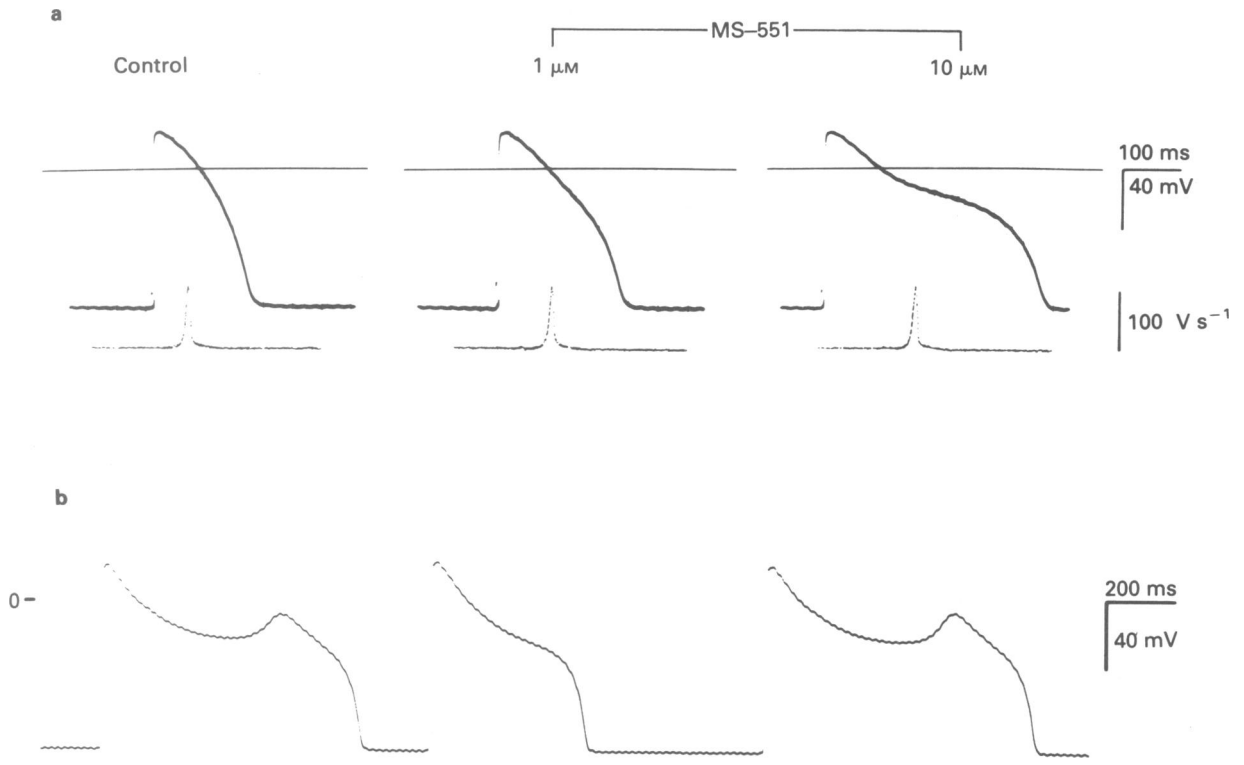


Figure 2 Effect of MS-551 on action potentials in rabbit papillary muscles driven at 1.0 Hz. (a) Concentration-dependent effects of MS-551 on action potentials. The upper, middle, and lower tracings in each record indicate zero potential, the transmembrane potential and dV/dt (on expanded scale), respectively. (b) Early afterdepolarizations induced by 10 μM MS-551 in a preparation.

Table 1 Effects of MS-551 and (+)-sotalol on action potential durations and effective refractory period in rabbit papillary muscles stimulated at 1.0 Hz

		APD_{20} (ms)	APD_{50} (ms)	APD_{90} (ms)	ERP (ms)
Control		63.3 ± 4.8	109.2 ± 8.1	147.5 ± 8.6	151.7 ± 9.5
MS-551	0.1 μM	61.7 ± 4.8	111.2 ± 9.0	154.2 ± 9.4	158.3 ± 10.5
	0.3	61.7 ± 5.3	119.2 ± 11.1	170.8 ± 17.9	171.7 ± 15.3*
	1	65.8 ± 5.5	148.3 ± 17.6*	196.2 ± 18.1*	205.8 ± 21.6*
	3	70.8 ± 5.1*	211.7 ± 40.2*	272.5 ± 42.7*	301.7 ± 59.1*
	10	72.0 ± 7.2*	246.0 ± 61.0	313.0 ± 64.5*	321.0 ± 73.1*
Control		61.5 ± 4.1	107.5 ± 5.4	149.2 ± 6.8	148.3 ± 8.9
(+)-Sotalol	3 μM	62.0 ± 5.0	110.3 ± 6.3	154.5 ± 9.3	155.0 ± 10.9
	10	61.8 ± 5.2	121.3 ± 7.8*	172.1 ± 12.1*	170.8 ± 14.6*
	30	62.0 ± 4.6	153.3 ± 19.4*	210.5 ± 24.2*	212.5 ± 29.3*
	100	62.8 ± 6.0	180.5 ± 32.0	248.7 ± 41.8*	249.2 ± 47.4

Values are mean ± s.e. of 5–6 experiments. APD_{20}, action potential duration at 20% repolarization level; APD_{50}, action potential duration at 50% repolarization level; APD_{90}, action potential duration at 90% repolarization level; ERP, effective refractory period. *$P < 0.05$ versus control value by paired t test.

membrane potential (RMP), − 92.8 ± 0.5 mV; action potential amplitude (APA), 119.8 ± 6.4 mV; $\dot{V}_{max}$, 158.5 ± 20.2 Vs^{-1}; APD at 20% repolarization level (APD_{20}), 62.4 ± 3.0 ms; APD at 50% repolarization level (APD_{50}), 108.3 ± 4.7 ms; APD at 90% repolarization level (APD_{90}), 148.3 ± 5.2 ms. There were no significant differences in any of the baseline values of action potential parameters between subgroups. APDs and ERP were increased by MS-551 and (+)-sotalol in a concentration-dependent fashion. The APD- and ERP-prolonging effects of MS-551 were approximately thirty times more potent than those of (+)-sotalol. Neither MS-551 nor (+)-sotalol significantly affected $\dot{V}_{max}$ in the concentrations examined. MS-551 did not produce any significant changes in RMP and APA in concentrations of 0.1–10 μM.

Rate-dependence of APD prolongation produced by MS-551 (3 μM) and (+)-sotalol (100 μM) were examined and compared with that of APD prolongation produced by 4-aminopyridine in another set of experiments. In drug-free condition, APDs at a stimulation rate of 0.2 Hz were slightly shorter than those at 2.0 Hz (Figure 3a). There was some difference in the rate-dependent effect on APD between these compounds. 4-Aminopyridine at a concentration of 2 mM produced the largest increase in APD_{90} at the longest cycle length and the smallest increase in APD at the shortest cycle length, showing a striking 'reverse use-dependence' (Figure

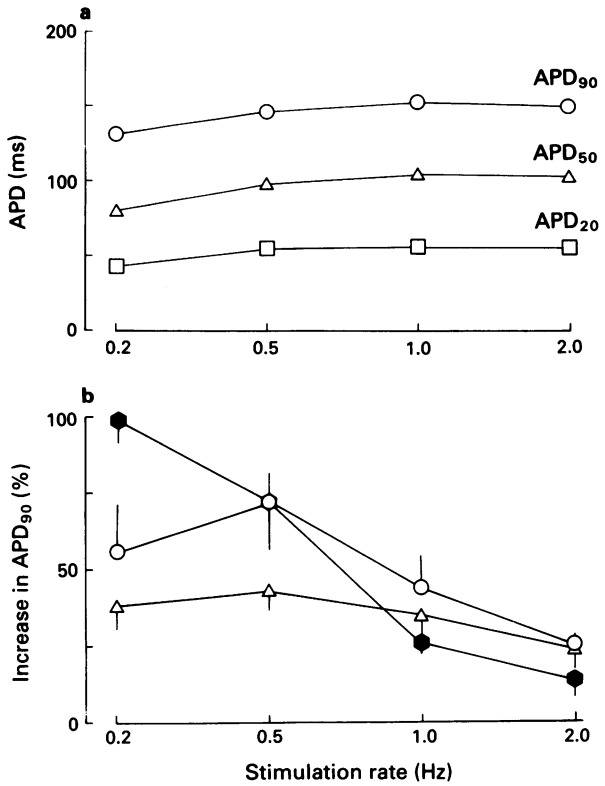


Figure 3 Rate-dependent effects of MS-551, (+)-sotalol and 4-aminopyridine (4-AP) on action potential duration (APD). (a) APD at 90% (APD_{90}, ○), 50% (APD_{50}, △) and 20% repolarization (APD_{20}, □) at various stimulation rates in drug-free condition. Absolute values of APDs are on the ordinate scale and stimulation rates are on the abscissa scale. Values are means of 15 preparations, and s.e. are smaller than the symbols. (b) Percentage increases in APD_{90} by MS-551 (○), (+)-sotalol (△) and 4-AP (●) at various stimulation rates. Values are expressed as mean ± s.e. of 5 preparations in each group.

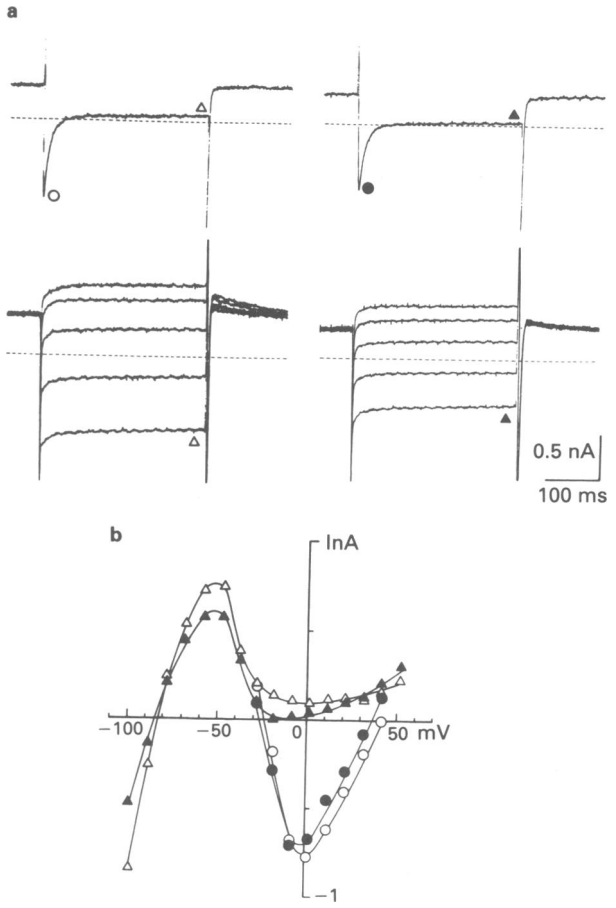


Figure 4 (a) Effects of MS-551 (10 μM) on membrane currents of a single ventricular cell. Current traces induced by a depolarizing pulse (2 mV) from a holding potential of − 38 mV (upper traces) and hyperpolarizing pulses (− 58 mV to − 98 mV) (middle traces) in the absence (left traces) and presence of 10 μM MS-551 (right traces). Broken lines indicate 0 current level. Current-voltage relations for the peak current (●,○) and the current at the end of a 300 ms test pulse (▲,△) in the absence (open symbols) and presence (closed symbols) of MS-551 are shown in (b). Note that MS-551 decreased the steady state outward current at − 38 mV to + 12 mV and the slopes of *I-V* relation at the level of resting potential.

3b). Although the reverse use-dependence of the class III effects of MS-551 and (+)-sotalol was not so marked as that with 4-aminopyridine, these drugs produced the largest increases in APD_{90} at 0.5 Hz and smaller increases in APD_{90} at shorter cycle lengths. The reverse use-dependence with MS-551 was more marked than that with (+)-sotalol.

Effects of MS-551 and (+)-sotalol on membrane currents in rabbit ventricular cells

Effects of MS-551 on membrane currents were examined and compared with those of (+)-sotalol in rabbit isolated ventricular myocytes. A representative example of the membrane ion currents of an isolated ventricular myocyte before and after application of 10 μM MS-551 is shown in Figure 4. Membrane currents were elicited by 300 ms test pulses to various potentials from a holding potential of − 38 mV at 0.1 Hz. MS-551 produced a small effect on the calcium current (I_{Ca}) that was induced by depolarizing pulses from the holding potential (Figure 4). MS-551 at concentrations of 3 and 10 μM slightly decreased an amplitude of I_{Ca} at 2 mV, which was obtained by subtracting the late current from the peak of the initial inward current, by 1.5 ± 12.7% ($n = 6$, NS) and 26.5 ± 5.7% ($n = 6$, $P < 0.01$), respectively. On hyperpolarization, the steady state outward and inward currents were recorded and designated as the inward rectifier potassium current (I_{K1}). MS-551 effectively reduced I_{K1} and the slopes of *I-V* relation at the level of resting potential, as shown in Figure 4. The decreases in I_{K1} at − 58 mV by 3 and 10 μM were 15.2 ± 6.6% (NS) and 30.1 ± 10.3% ($P < 0.05$), respectively. In addition, MS-551 decreased the steady state outward current at − 38 mV to + 12 mV, as shown in Figure 4. Similar changes in membrane currents were also elicited by 100 μM (+)-sotalol. (+)-Sotalol reduced I_{Ca} at 2 mV slightly but significantly ($n = 8$, 26.4 ± 8.0%, $P < 0.05$). The drug insignificantly reduced I_{K1} at − 58 mV by 8.7 ± 12.1%.

Effects of MS-551 and (+)-sotalol on the transient outward current (I_{to}) were examined in isolated ventricular myocytes. I_{to} was recorded by applying depolarizing pulses from a holding potential of − 68 mV in Co^{2+}-containing solution with or without tetrodotoxin. In these conditions, we could record I_{to} which was sensitive to 2 mM 4-aminopyridine. Although MS-551 at a concentration of 3 μM hardly affected I_{to}, the drug at a higher concentration of 10 μM inhibited the current, as shown in Figure 5a. MS-551 decreased the peak current with little influence on the steady state current at the end of the 300 ms pulse. The decrease in the amplitude of I_{to} at + 12 mV, which was designed as the difference between a peak current and the current at the end of the depolarizing pulse, after the exposure to 3 and 10 μM MS-551 were 5.7 ± 8.3% ($n = 5$, NS) and 30.8 ± 9.1% ($n = 5$, $P < 0.05$), respectively. In contrast with the high concentration of MS-551, (+)-sotalol at a concentration of 100 μM hardly affected I_{to} (Figure 5b). The decrease in the amplitude of I_{to} at + 12 mV after 100 μM (+)-sotalol was

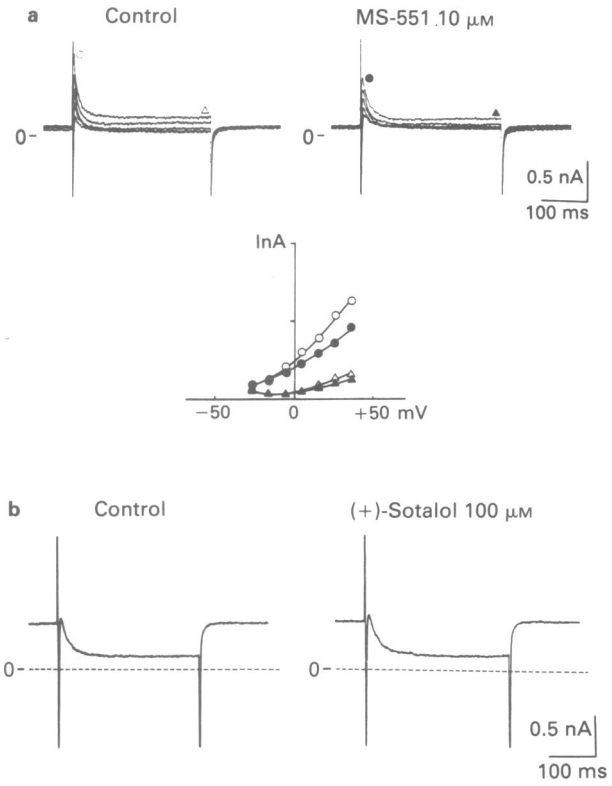


Figure 5 Effects of MS-551 (a) and (+)-sotalol (b) on the transient outward current (I_{to}). (a) I_{to} was elicited by depolarizing pulses of 300 ms duration to various potentials from a holding potential of − 68 mV in Co^{2+} (2 mM)-and tetrodotoxin (7 μM)-containing solution. MS-551 (10 μM) decreased peak currents (circles) with little effect on the steady state currents (triangles). *I-V* relationships before (open symbols) and after MS-551 (closed symbols) are also shown. (b) I_{to} was elicited by a depolarizing pulse to +12 mV from a holding potential of − 68 mV in Co^{2+} (2 mM)-containing solution. I_{to} was hardly affected by (+)-sotalol.

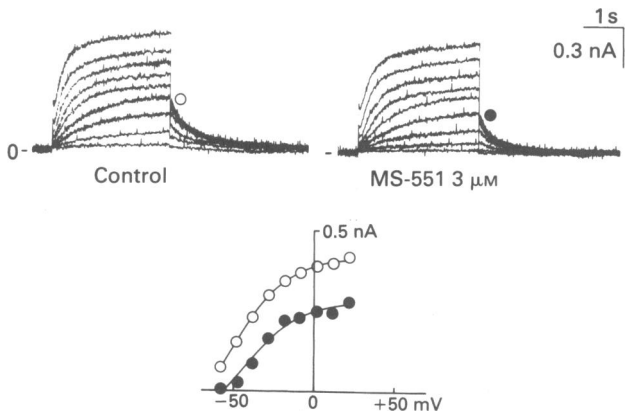


Figure 6 Effect of MS-551 on the isolated I_K. Current traces of I_K were recorded by using depolarizing pulses to nine different potentials with 10 mV steps between − 58 mV to + 22 mV before (left) and after 3 μM MS-551 (right) in a Na^+-free, K^+-free and nisoldipine-containing solution. The holding potential was − 68 mV and the duration of the pulse was 3 s. Current-voltage relations of the tail amplitude in the absence (○) and presence (●) of MS-551 are illustrated below.

− 5.3 ± 9.9% ($n = 4$, NS). Thus, MS-551 inhibited I_{to} especially at its high concentration but (+)-sotalol failed to inhibit the current up to the concentration of 100 μM.

The delayed rectifier potassium current (I_K) was recorded in Na^+-free, K^+-free and nisoldipine (1 μM)-containing external solution, in which I_{Ca}, I_{K1}, the electrogenic Na^+/Ca^{2+} exchange current and the electrogenic pump current were blocked. The membrane potential was held at − 68 mV and clamped to more positive potentials for 3 s and then repolarized to the holding potential. The isolated outward current showed the properties of delayed rectification and the sigmoidal time course in its activation process, as shown in Figure 6. The amplitude of the outward current increased progressively in response to progressively larger depolarizing test pulses and so did that of the outward tail current upon subsequent repolarization to − 68 mV. The outward tail current almost reached a saturation level around + 20 mV. MS-551 decreased I_K at all potentials, as shown in Figure 6. The amplitude of the tail current of I_K defined as the difference between the peak current of the tail and the holding current, after the test potential to + 2 mV was decreased by 29.9 ± 10.1% ($n = 5$, $P < 0.05$) and 39.8 ± 16.4% ($n = 3$) after the application of 3 μM and 10 μM MS-551, respectively. (+)-Sotalol also decreased I_K in the ventricular cells. The decrease in the amplitude of the tail current of I_K after the test potential of 2 mV was 36.4 and 24.3% after 100 μM sotalol in two cells.

It has been reported that the class Ia drug, quinidine, delayed the activation of I_K in guinea-pig ventricular cells (Roden *et al.*, 1988). Therefore, the effect of MS-551 on the time course of I_K activation was examined by generating envelopes of current tails in the present study. An example of control I_K tails following depolarizing pulses of variable duration to − 8 mV is shown in Figure 7a. The same envelope of current tails experiment was conducted after the application of 3 μM MS-551 (Figure 7b). The I_K tail amplitudes as a function of activation pulse duration are shown in Figure 7c. The reduction of I_K after shorter pulses (50–300 ms) was greater than that after longer pulses (2000–7000 ms). The effect of MS-551 on I_k tails was greater after shorter depolarizing pulses. In other words, MS-551 delayed the activation of I_K. Similar results were also observed in another preparation.

Discussion

The present electrophysiological study has demonstrated that MS-551 is a potent class III antiarrhythmic agent in rabbit ventricular muscles. The class III effect, i.e., APD- and ERP-prolonging effect, of MS-551 was approximately 30 times more potent than that of (+)-sotalol on a molar basis. MS-551 as well as (+)-sotalol prolonged APD, especially at late repolarization phase. In addition, MS-551 induced early afterdepolarizations in isolated rabbit ventricular muscles. Such early afterdepolarizations may potentially lead to torsades de pointes *in vitro*, as observed with other class III drugs such as clofilium, sematilide, UK-68,798, LY97119 and amperozide (Carlsson *et al.*, 1990).

It is well-established that at least three kinds of potassium outward currents, i.e., I_K, I_{to} and I_{K1}, are important for repolarization in cardiac cells. It has been reported that I_{to} is involved in the rate-dependent changes in APD (Hiraoka & Kawano, 1989) and regional variations in action potential configuration (Fedida & Giles, 1991) in the rabbit ventricle. In addition, it was demonstrated that I_{to} is present in human atrial cells and plays a role in the repolarization of the action potential (Escande *et al.*, 1987; Shibata *et al.*, 1989). In terms of I_{K1}, it has been recently reported that I_{K1} is mainly responsible for final repolarization in rabbit ventricular cells (Shimoni *et al.*, 1992). In guinea-pig ventricular cells, basic characteristics of I_K were extensively studied (Matsuura *et al.*, 1987; Tohse, 1990) and inhibitory action of class III drugs on I_K was demonstrated (Arena & Kass, 1988; Komeichi *et al.*, 1990; Sanguinetti & Jurkiewicz, 1990; Gwilt *et al.*, 1991). As far as we know, however, I_K in rabbit ventricular cells has not been characterized. In the present study I_K was isolated from other currents with a Na^+-free, K^+-free and nisoldi-

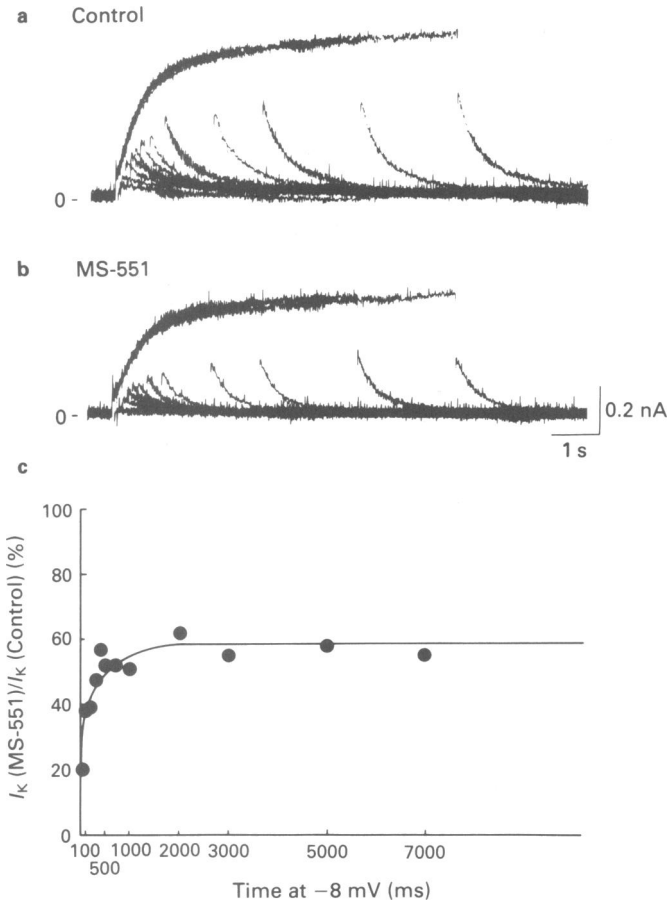


Figure 7 Envelopes of current tails before (a) and after exposure to 3 μM MS-551 (b). Holding potential was − 68 mV and depolarizing pulses to − 8 mV of various durations (50; 100; 200; 300; 400; 500; 700; 1000; 2000; 3000; 5000; and 7000 ms) were given. All twelve traces during the activating and deactivating sequences are shown superimposed. I_K tail amplitude (MS-551/Control as a %) as a function of activating pulse duration is shown in (c). Note that MS-551 delays I_K activation.

pine-containing solution. I_K of rabbit ventricular cells was activated at potentials more positive than − 50 mV and saturated around + 20 mV. These properties of rabbit ventricular I_K are clearly different from those of guinea-pig ventricular I_K, which is activated at potentials more positive than − 30 mV and saturated around + 50 mV (Komeichi *et al.*, 1990; Tohse, 1990). Such I_K, observed in rabbit ventricular myocytes, appeared to be in part similar to the I_K of rabbit sinoatrial and atrioventricular nodes (Shibasaki, 1987).

In the present study 3 μM MS-551 and 100 μM sotalol commonly inhibited the I_K in rabbit ventricular cells. The inhibition of I_K might be involved in the decrease in the steady-state outward current between − 40 mV and − 20 mV and the prolongation of APD at a late repolarization phase. In this context, Sanguinetti & Jurkiewicz (1990) reported that E-4031, a potent class III drug, and sotalol inhibited a rapid component of I_K (I_{Kr}) with small effect on a slow component (I_{Ks}) in guinea-pig ventricular cells. They showed that I_{Kr} was rapidly activated at − 30 mV to 0 mV and was effectively inhibited by class III drugs. However, in their study I_{Kr} was not completely separated from I_{K1}. Recently it has been reported that E-4031 can also block I_{K1} channels in guinea-pig ventricular cells (Koumi *et al.*, 1991). Therefore, I_{K1} was blocked by using K^+-free solution and effect of MS-551 on the envelopes of current tails of I_K was examined in rabbit ventricular cells. The envelope test revealed that MS-551 delayed the activation of I_K. Similar delay in the I_K activation was also observed with the class Ia drug, quinidine, in guinea-pig ventricular cells (Roden *et al.*, 1988). These findings might be interpreted as showing that binding of MS-551 to I_K channels is state- and/or voltage-dependent.

Sotalol at a concentration of 100 μM failed to inhibit I_{to} and I_{K1}. Although MS-551 at a concentration of 3 μM did not significantly inhibit I_{to} and I_{K1}, the drug at a concentration of 10 μM suppressed these currents. Therefore, MS-551 appears to nonspecifically inhibit potassium currents at high concentrations. It has been reported that tedisamil, a class III drug, blocks not only I_K but also I_{to} in rat and guinea-pig isolated ventricular myocytes (Dukes *et al.*, 1990). We have also reported that N-acetylprocainamide decreased both I_K and I_{K1} in guinea-pig ventricular myocytes (Komeichi *et al.*, 1990).

In the present study both MS-551 and (+)-sotalol had little effect on $\dot{V}_{max}$ of action potentials. Therefore, these drugs appear to be of little inhibitory action on the sodium channels. In isolated ventricular myocytes I_{Ca} was slightly but significantly decreased by 10 μM MS-551 and 100 μM (+)-sotalol. Therefore, in high concentrations these drugs may slightly inhibit I_{Ca}.

It is known that many class III drugs except for amiodarone exhibit reverse use-dependent effects on APD, i.e., greater prolongation of APD at a longer cycle length (Hondeghem & Snyder, 1990). The present study revealed that MS-551 and (+)-sotalol showed reverse use-dependence although it was not so marked as that produced by a specific I_{to} blocker, 4-aminopyridine. The reverse use-dependent class III effect may be undesirable, because these drugs may limit their efficacy during tachyarrhythmias and cause torsades de pointes at a slow heart rate. Recently it has been also reported that α-adrenoceptor stimulation causes QT prolongation and torsades de pointes potentially resulting from early afterdepolarization in animals receiving K^+ channel blockers (Carlsson *et al.*, 1990; Ben-David & Zipes, 1990). Although the underlying ionic mechanism(s) responsible for the α-adrenoceptor-mediated QT prolongation and early afterdepolarizations are not well defined, the α-adrenoceptor-mediated inhibition of I_{to} (Apkon & Nerbonne, 1988; Fedida *et al.*, 1989; Tohse *et al.*, 1990) and I_{K1} (Fedida *et al.*, 1991) may play an important role. In addition, hypokalaemia is known to prolong QT, which may be attributable to the inhibition of I_{K1} channels by extracellular hypokalaemia (Kameyama *et al.*, 1983). Therefore, class III drugs including MS-551 should be administered with a great caution to the patients with sinus bradycardia, hypokalaemia, and potentially high sympathetic tone.

In conclusion, MS-551 is a potent class III antiarrhythmic drug which prolongs APD reverse use-dependently. The class III effect of the drug may be ascribed to the inhibitory action on three voltage-gated K^+ channels in rabbit ventricular cells.

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Effects of cicletanine on whole-cell currents of single smooth muscle cells from the guinea-pig portal vein

[1]Thomas Noack & Petra Deitmer

Department of Physiology, Philipps University, Deutschhausstrasse 2, W-3550 Marburg, Germany

1 Smooth muscle cells of the guinea-pig portal vein were dispersed by enzymatic treatment and recordings of membrane currents were made in the whole-cell mode by the patch-clamp technique. The effects of extracellular application of cicletanine-hydrochloride on the whole-cell currents of isolated smooth muscle cells from the guinea-pig portal vein were studied in solutions containing a normal concentration of calcium (2.5 mM).

2 Cicletanine, 10 to 100 μM, reduced the voltage-dependent inward calcium current with an IC_{50} of 250 μM. These effects of cicletanine were reversible.

3 The action of cicletanine on calcium currents can be interpreted as a decrease of the availability of calcium channels but not by an alteration of the time course or voltage-dependency of inactivation.

3 The control calcium current was enhanced by application of Bay K 8644. On this enhanced inward current, cicletanine also exerted inhibitory effects which were not use-dependent.

5 Cicletanine, 1 to 100 μM, did not enhance outward potassium currents.

6 It is concluded that at least one component of the vasorelaxant effects of cicletanine is produced by inhibition of calcium currents.

Keywords: Cicletanine; vascular smooth muscle; voltage-clamp; calcium currents; potassium currents

Introduction

In rabbit aorta and rat portal vein the diuretic and antihypertensive drug, cicletanine, reduces the contraction induced by membrane depolarization (high K^+ concentration) (Baranes *et al.*, 1983). Cicletanine also antagonizes the contractions caused by noradrenaline of rabbit isolated iliac artery and guinea-pig portal vein (Noack, 1992) and relaxes human epigastric arteries precontracted with 25 mM or 80 mM potassium-rich solution (Ebeigbe & Cabanie, 1991). Furthermore in frog ventricular myocytes, cicletanine inhibits isoprenaline-enhanced inward calcium (Ca) currents (Gisbert *et al.*, 1988). Since Ca^{2+} ions play a determinant role in vascular contractility, these data suggest that a reduction of Ca^{2+} entry through Ca-channels could be one of the mechanisms by which cicletanine could counteract an increase in cytosolic free Ca^{2+} concentration.

In this paper we report that cicletanine inhibits the inward calcium current (I_{Ca}) in smooth muscle cells from guinea-pig portal vein and reduces the enhanced Ca-current generated by exposure to (-)-Bay K 8644. Some of the results were previously presented as an abstract (Noack *et al.*, 1991).

Methods

Cell preparation

All experiments were performed on single smooth muscle cells isolated from portal veins which were removed from male guinea-pigs, previously killed by stunning and bleeding. The portal vein (about 20 mm length) was carefully cleaned of fat and connective tissue with fine scissors and microscopic control. The tissue was incubated in a nominally Ca^{2+}-free physiological salt solution (PSS) for 30 min. Consecutive treatment of the tissue with 1 ml of a modified PSS (for details see Lammel *et al.*, 1991) containing purified collagenase (Sigma, Type VII, 100 units ml^{-1}) and the same solution containing papain (Sigma, 20 units ml^{-1}) and a collagenase inhibitor (phosphoramidon, Sigma, 200 μg ml^{-1}) provided isolated contractile cells. The cells were used for experiments within 12 h after isolation. During this time they were stored at 4°C in Kraftbrühe (KB-medium; Klöckner & Isenberg, 1985). During all experiments the organ bath temperature was held at 26°C.

Single-cell voltage-clamp

The whole-cell configuration of the patch-clamp technique (Hamill *et al.*, 1981) was employed to voltage-clamp the smooth muscle cells. Patch pipettes were pulled from Pyrex glass (Jencons H 15/10) and had resistances of 3–4 MΩ when filled with the intracellular solution.

Low-pass filtered signals (10 kHz) were digitized at 5 kHz with AXOLAB 1100 hardware and an IBM microcomputer in conjunction with pCLAMP 5.5 software (Axon Instruments). Currents were displayed on the computer monitor and hard copies were obtained with a plotter (Taxan). Calcium currents were assumed to be completely and specifically blocked by addition of 0.5 mM cadmium to the extracellular solution. Unless indicated, the cadmium (0.5 mM)-resistant current (leakage and background current) was not subtracted either from the current traces or from the current-voltage relationships presented in this paper. Only for calculation of the effect of cicletanine on the calcium current, was the current recorded in the presence of cadmium (0.5 mM) digitally subtracted from total current. Recordings were evaluated from a total of 25 cells and from each of these continuous measurements were made for at least 30 min.

Drugs and solutions

The PSS in the bath had the following composition (mM): NaCl 125, KCl 4.8, $MgCl_2$ 1.2, KH_2PO_4 1.2, glucose 11, $CaCl_2$ 2.5, HEPES 10, pH 7.3. The pipette (internal) solution contained (mM): NaCl 5, KCl 120, $MgCl_2$ 1.2, K_2HPO_4 1.2, glucose 11, HEPES 10, EGTA 1.2, oxalacetic acid 5, pyruvic acid 2, succinic acid 5, pH 7.3. In those experiments in which calcium currents were recorded, potassium in the pipette solution was replaced by equimolar amounts of caesium. The reagents used were (±)-cicletanine hydrochloride (IHB,

[1] Author for correspondence.

Paris, France), nifedipine and (-)-Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate, Bayer AG, Germany) and cadmium chloride (Sigma). To reduce any solvent effects on membrane currents, cicletanine was dissolved in dimethyl sulphoxide (DMSO) as a concentrated stock solution (2 M). From this, dilutions with water were prepared immediately before application to the cells to reduce the possibility of cicletanine precipitation.

Results

Interaction of cicletanine with net whole-cell currents

In those experiments in which the pipette solution did not contain caesium ions (potassium pipettes), the whole-cell currents were mainly carried by calcium and potassium ions. Representative examples of a whole set of current recordings are shown in Figure 1a which illustrates the typical size and time course of the whole-cell currents in a bathing solution containing 2.5 mM Ca^{2+}. The currents were elicited with 500 ms depolarizing voltage-clamp pulses from a holding potential of − 90 mV to test potentials in the range − 80 to + 50 mV in increments of 10 mV. At potentials positive to − 30 mV, the initial capacitive surge was followed by a transient net inward current that peaked in about 10 ms. This inward current then declined and reversed to become an outward current. The major part of outward current in these cells was also present in a calcium-free bathing solution (Noack *et al.*, 1990). The family of current traces shown in Figure 1a illustrates the general voltage-dependency of the inwardly- and outwardly-directed current in this tissue.

Within 3 min of adding cicletanine (10 μM) to the bathing solution, inwardly-directed currents became smaller than under control conditions (Figure 1b), whereas the outward currents elicited at each potential were little affected. A more detailed analysis of the effects of cicletanine in this vascular preparation was derived from the construction of the current-voltage relationships ($I-V$ curves) for the peak inwardly-directed (Figure 2a), the peak outwardly-directed (Figure 2b) and the current measured 500 ms after the depolarizing step (Figure 2c). As can be seen from Figures 2a–c the effects of cicletanine were exerted on the peak inwardly-directed currents and with essentially no effect on either the peak outwardly-directed currents or on the current at the end of the 500 ms depolarizing step.

The inhibitory effect of cicletanine on the peak inwardly-directed current could be due to inhibition of calcium channels. To evaluate this hypothesis, further experiments were carried out with pipettes in which the potassium ions were replaced by equimolar amounts of caesium ions.

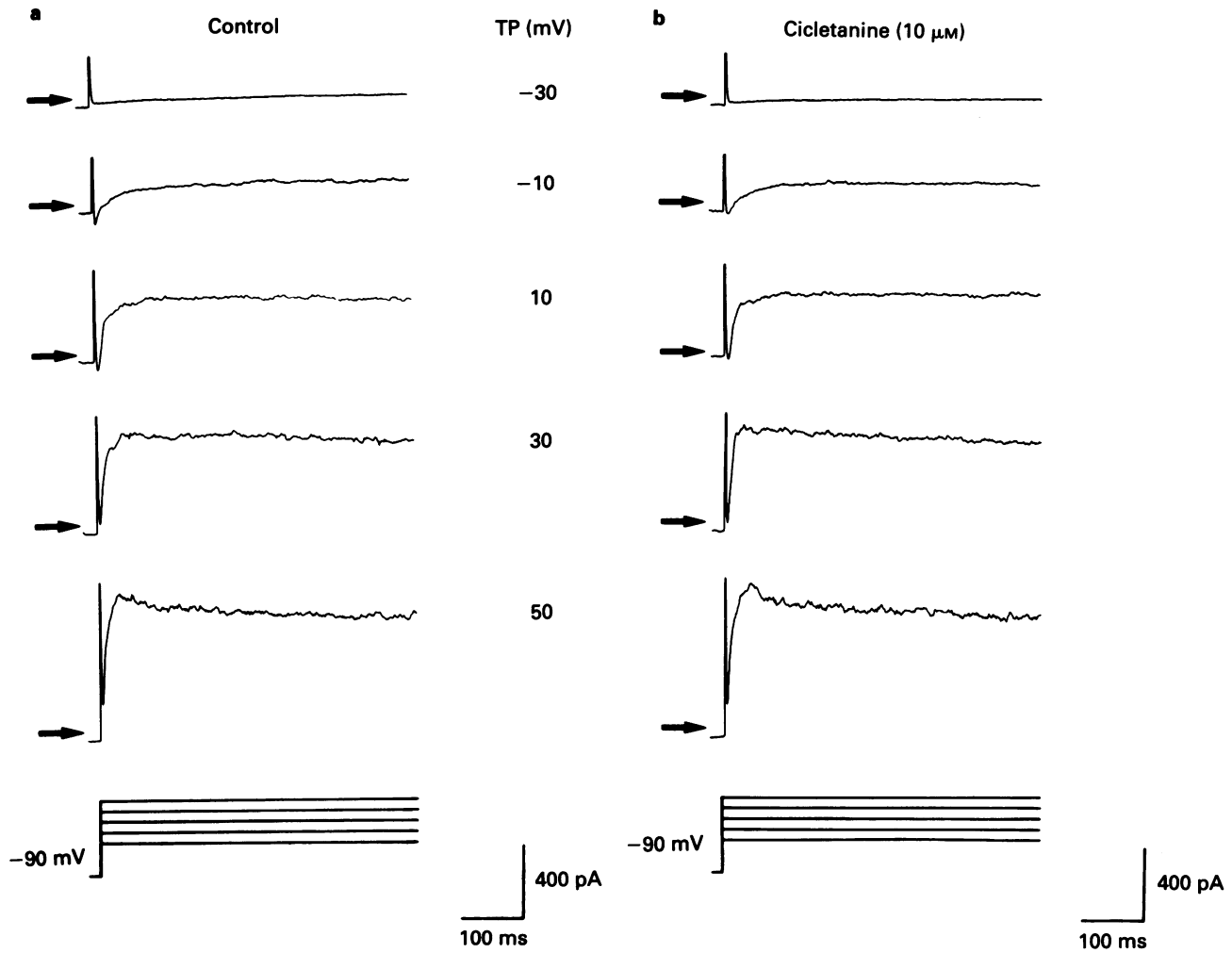


Figure 1 Examples of whole-cell currents (potassium pipette) generated by a smooth muscle cell from guinea-pig portal vein (a) and the effect of cicletanine (b). The cell was bathed in a physiological salt solution containing 2.5 mM calcium. It was held at − 90 mV and stepped for 500 ms to the test potentials (TP) as indicated. The arrows indicate zero current.

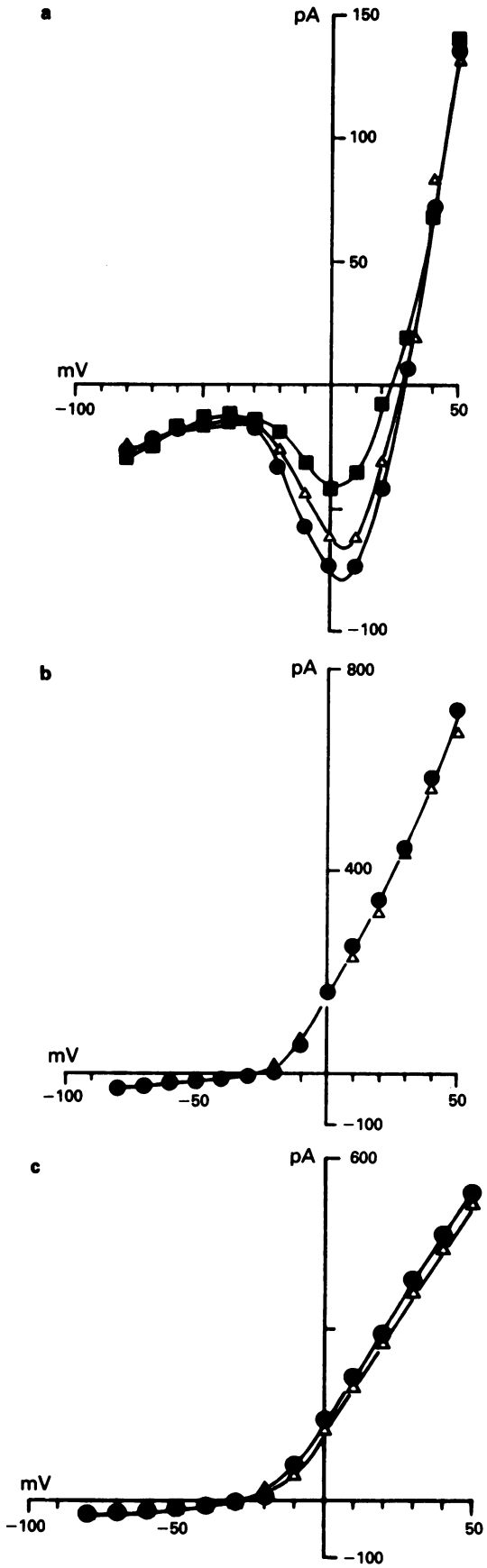


Figure 2 Current-voltage relationships for (a) peak inwardly-directed current, (b) peak outwardly-directed current and (c) current measured after 500 ms of the depolarizing voltage step. Mean values obtained with potassium pipettes and derived from 3 cells are shown. In (a) the control values (●), the effects of cicletanine 10 μM (Δ) and 50 μM (■) are given. In (b) and (c), the effects of cicletanine 50 μM were essentially the same as those for 10 μM and have been omitted from the diagrams for clarity.

Interaction of cicletanine with inward calcium currents

Representative records illustrating the size and time-course of the whole-cell currents in a bath solution containing 2.5 mM Ca^{2+} and with a caesium-loaded pipette are shown in Figure 3a. Note the four fold difference between the current calibration in Figures 3 and 1. The current traces were elicited with 500 ms depolarizing voltage-clamp pulses from a holding potential of − 90 mV to test potentials of − 80 to + 50 mV in increments of 10 mV. At potentials positive − 30 mV, the initial capacitive surge was followed by a transient net inward current that peaked in about 10 ms and then declined to a steady current level. This family of current traces illustrates the voltage-dependency of the inward current generated by this tissue and shows that the maximum inward current was obtained at test potentials between + 10 mV and + 20 mV. Within 3 min of adding cicletanine (10 μM) to the bathing solution, inwardly-directed currents were visibly reduced compared with control conditions (Figure 3b).

As can be seen from the current records shown in Figure 3a and b, the current at positive potentials became outward indicating a contamination of the net current by current components which were not carried by calcium ions. To identify the true calcium inward currents and to eliminate contaminating background currents from current taces, extracellular application of cadmium (0.5 mM) was used to produce complete block of calcium currents. The values of the peak inward currents under control conditions, and in the presence of cicletanine and of cadmium were transferred to a current-voltage relationship to allow the individual inhibitory effects of both cicletanine and of cadmium on inward currents to be determined (Figure 4). Cadmium inhibited the inward current throughout the voltage range allowing the $CdCl_2$-sensitive component of the net inward current to be isolated by subtracting the $CdCl_2$-insensitive current (background and leakage current) from the net current. Previous studies in various smooth muscle tissues have shown that the cadmium (0.5 mM)-sensitive current represents calcium current flow through voltage-gated calcium channels (Matsuda *et al.*, 1990; Noack *et al.*, 1992).

For calculation of the inhibitory effect of cicletanine on inward calcium currents, cadmium-insensitive currents were subtracted from current values. As illustrated in Figure 4, cicletanine inhibited the inward calcium current in a concentration-dependent manner, over the potential range from − 30 mV to + 50 mV. Although the peak current was reduced, the time-course of its inactivation was not greatly affected by cicletanine (Figure 3a and b). The reduction of inward current induced by cicletanine (10–100 μM) was marked but not complete. The concentration of cicletanine necessary to produce 50% inhibition of the peak inward current was estimated to be approximately 250 μM (Figure 5).

To determine whether cicletanine produced a change in the voltage-dependency of inactivation, three different holding potentials were chosen (− 90 mV, − 30 mV and − 10 mV) from which current traces were elicited with 500 ms depolarizing voltage-clamp pulses to test potentials of − 80 mV to + 50 mV. Inactivation of I_{Ca} occurred when the chosen holding potentials were − 30 mV and − 10 mV (Figure 6a–c). This experimental protocol was performed either under control conditions or in the presence of cicletanine (30 μM). When the peak inward current of each curve was plotted against each applied holding potential, the steady-state inactivation curves of I_{Ca} under both conditions could be constructed. Under control conditions the current was half available at $V_h = -30$ mV, and the slope, V_s, was 7.1 mV, when a Boltzmann equation of the form

$$I/I_{max} = (1 + \exp((V - V_h/V_s))^{-1}$$

was fitted to the inactivating component of the current. V is the holding potential which conditions the inactivation process. I/I_{max} is the fraction of current available relative to that available from − 90 mV. Cicletanine (30 μM) reduced the

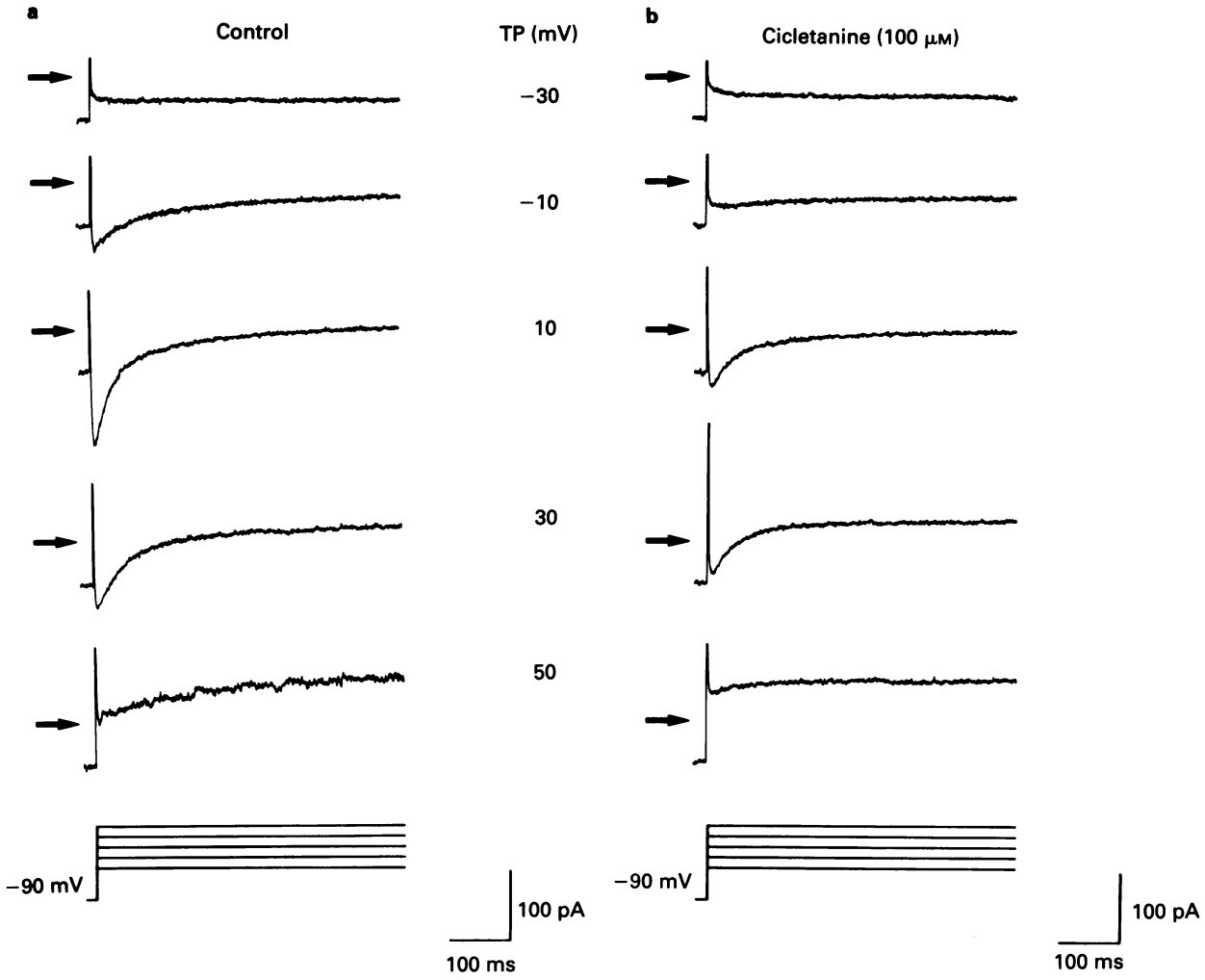


Figure 3 Examples of whole-cell currents (caesium pipette) of a smooth muscle cell from guinea-pig portal vein (a) and effect of cicletanine (b). The cell was bathed in a physiological salt solution containing 2.5 mM calcium. The cell was held at − 90 mV and stepped for 500 ms to the test potentials (TP) as indicated. The zero current is indicated by arrows.

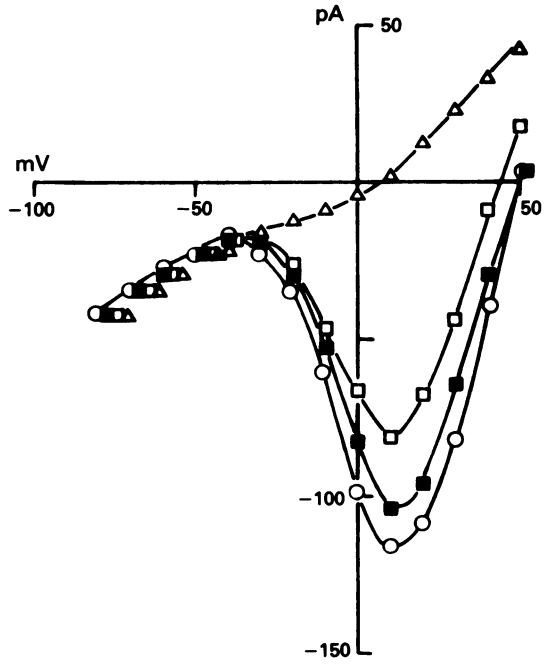


Figure 4 Current-voltage relationship for net peak inward current (caesium pipettes) activated from a holding potential of − 90 mV. Control (○), effect of cicletanine 3 μM (■) and 30 μM (□) and effect of cadmium 0.5 mM (△). Ordinate scale: peak inward current. Abscissa scale: test potential. Data points are means derived from four cells. The curves are fitted by eye.

peak current evoked at each potential by stepping from each of the three holding potentials, with little change in V_h (− 37 mV) or V_s (8.2 mV) (Figure 6d). This indicates that cicletanine exerts its inhibitory effects on I_{Ca} by reducing the availability of calcium channels and not by changing their inactivation parameters.

Interaction between cicletanine and Bay K 8644

In guinea-pig portal vein, L-type channels are the predominant type of calcium channel present. To test more directly whether cicletanine inhibits such channels, (-)-Bay K 8644, was used to increase calcium channel availability (Figure 7a). Peak inward currents increased two or three fold when (-)-Bay K 8644 (100 nM) was applied to the bathing solution. With a double pulse protocol used in the same cells, a pair of two inward currents was elicited every 15 s and the peak inward currents obtained at the two test potentials (0 mV and + 20 mV) were plotted against time (Figure 7b). When the double pulse protocol was applied to the cells, the inward current which developed under control conditions at + 20 mV was smaller than that seen at 0 mV, although the peak of the $I-V$ curve was near + 20 mV (Figure 7a). This indicates that the chosen time interval between the two depolarizing pulses was short enough to produce partial inactivation of I_{Ca} (50%).

When (-)-Bay K 8644 (100 nM) was added to the bathing solution, the inward current at both depolarizing pulses increased, although the increase associated with the first pulse was larger than that produced by the second stimulus (Figure

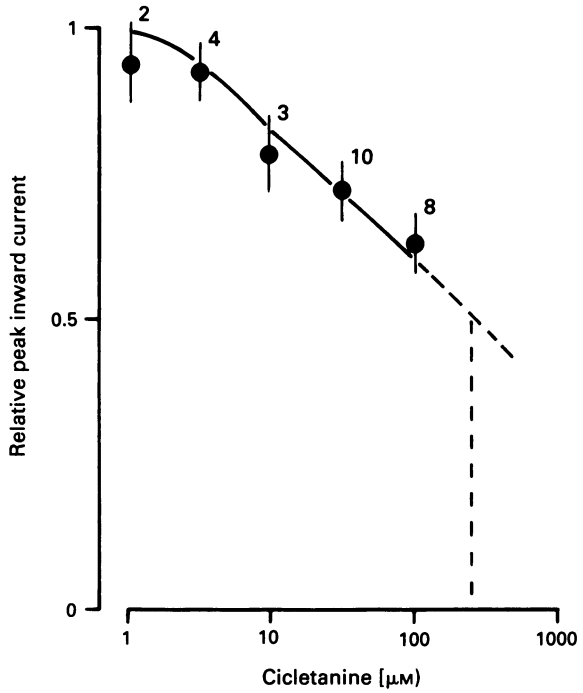


Figure 5 Inhibition by cicletanine of peak inward (cadmium-sensitive) current evoked at + 10 mV test potential when stepped from a holding potential of − 90 mV. Data points are mean values with s.e.mean. The dashed vertical line indicates the IC_{50} concentration when data points were fitted by eye and the curve was extrapolated. The numbers near data points indicate numbers of cells investigated.

7b). As expected, the inward current elicited by the first depolarizing pulse was of a similar magnitude to that obtained with the experimental protocol of Figure 7a. However, the current elicited by the second pulse was only approximately 50% of the magnitude expected from the *I–V* curve obtained with a single pulse protocol. This indicates that under control conditions and in the presence of Bay K 8644 the portion of channels being inactivated is similar and that Bay K 8644 neither changes the voltage-dependency of the steady-state inactivation nor the time for the recovery from inactivation at this membrane potential (− 60 mV) which was in both cases 730 ms. In the presence of Bay K 8644 cicletanine (30 μM to 100 μM) inhibited the Bay K-augmented inward calcium current. This result indicates that cicletanine interacts with dihydropyridine-sensitive L-type calcium channels. Furthermore inhibition of the partially-inactivated inward current by cicletanine indicates that there is some affinity of cicletanine for the inactivated state of the channels. An increase of block by repeatedly applying such depolarizing pulses (use-dependency) was not observed in the presence of cicletanine (Figure 7b).

Discussion

The results described in this paper indicate that cicletanine blocks the calcium currents in single isolated vascular smooth muscle cells. However, it does not seem to act as a vasodilator via the opening of potassium channels which was suggested by Ebeigbe & Cabanie (1991). The potency of cicletanine was 10–250 times lower than that of nifedipine, verapamil, diltiazem, flunarizine or pinaverium (Terada *et al.*, 1987a,b; Beech *et al.*, 1990).

Only a very small negative shift in the steady-state inactivation curve of inward current was observed with cicletanine. Under voltage-clamp conditions dihydropyridines like nifedipine cause a distinct negative shift of the steady-state inactivation curve (Noack *et al.*, 1992). Such a lack of

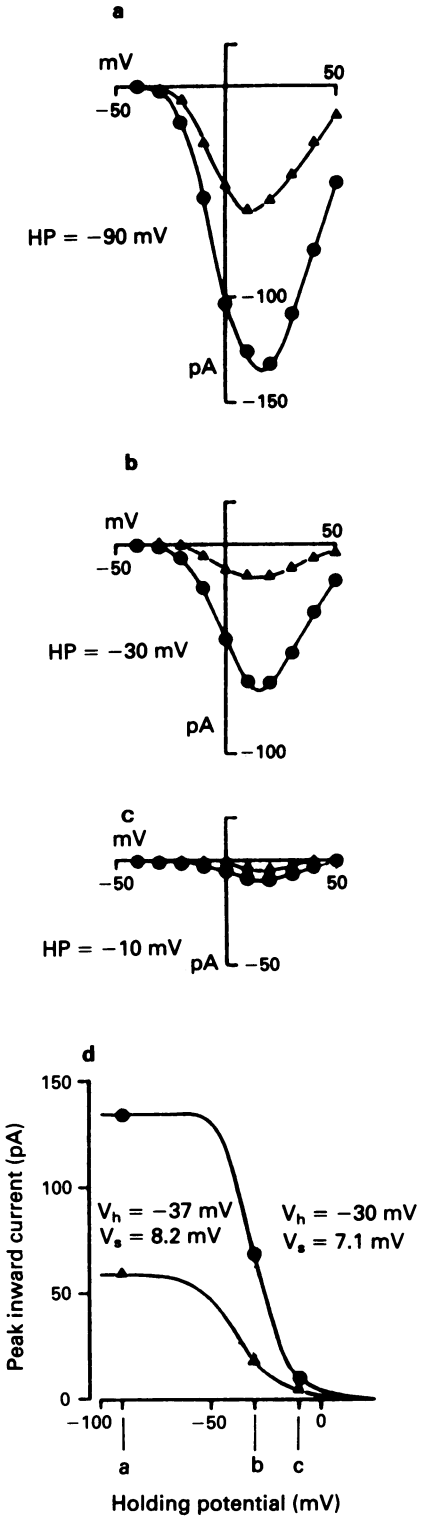


Figure 6 Effect of holding potential and of cicletanine (30 μM) on calcium current. (a–c) Current-voltage relationships for peak inward current elicited from the three holding potentials (HP: − 90 mV, − 30 mV and − 10 mV respectively) under control conditions (●) and in the presence of the drug (▲). Data points for these three plots represent mean values from three cells (s.e.mean in each case within the size of symbols). The calcium current was isolated from the total current by subtraction of the cadmium-resistant current. (d) Effects of cicletanine (30 μM) on the voltage-dependence of the steady-state inactivation of the calcium current. Peak values of currents were plotted against holding potentials. To each set of data points a Boltzmann equation was fitted (continuous curves). Voltages for half-inactivation (V_h) and slope factors (V_s) characterizing these equations are given at each curve.

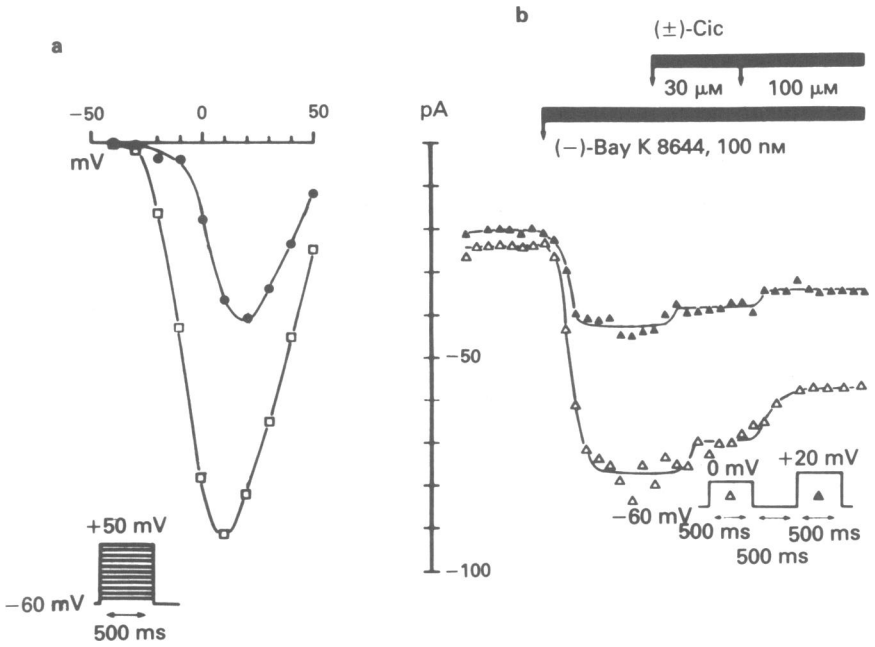


Figure 7 Effects of (a) (-)-Bay K 8644, 100 nM and (b) cicletanine in the presence of (-)-Bay K 8644 on whole-cell calcium currents in isolated cells from rat portal vein. Each point shows the mean value derived from two cells. (a) A current-voltage relationship for the peak inward current under control conditions (●) was determined using depolarising steps from a holding potential of − 60 mV to the different test potentials as indicated. (b) The cells were then stimulated every 15 s with a double pulse protocol (see insert for details) and the peak inward currents generated by the first (△) and the second pulse (▲) were determined. With this double pulse protocol, the currents elicited at each first test pulse were comparable in magnitude to those obtained with the experimental protocol of (a). However, the current elicited at each second pulse was partially inactivated. In the presence of (-)-Bay K 8644, a second current-voltage relationship was determined (a, □). The double pulse protocol was then used to determine the effects of cicletanine on (-)-Bay K 8644-enhanced calcium currents (b, △, ▲).

shift could indicate that cicletanine has similar affinities for both the closed, available state and for the inactivated state of the calcium channel. Holding the cell at a depolarized potential, which increases the fraction of channels in the inactivated state with a corresponding reduction of those in the closed state, did not increase the affinity for the channel. This is in contrast to the action of nifedipine on calcium current (Noack *et al.*, 1992). In the presence of nifedipine, an increasing affinity of the drug for the channel is characterized by an increasing reduction of current at depolarizing holding potentials. Such differences between drugs might be of great importance in their therapeutic use, in respect to the different patterns of electrical activity reported for a variety of smooth muscle organs.

In some respects, the actions of cicletanine on guinea-pig portal vein, are similar to those reported for pinaverium on the longitudinal muscle layer of the rabbit jejunum (Beech *et al.*, 1990). Both substances have little effect on the Ca-inactivation curve, neither is a dihydropyridine nor exhibits the use-dependency characteristic of D600, verapamil and diltiazem (Terada *et al.*, 1987b; Hering *et al.*, 1988).

In some smooth muscles two or more types of voltage-dependent calcium channel have been distinguished on the basis of their different unitary conductances and their modulation by several drugs (Bean *et al.*, 1986; Loirand *et al.*, 1986; Benham *et al.*, 1987; Ganitkevich & Isenberg, 1990). It has also been reported that different calcium current components can be identified by use of different holding potentials (Bean *et al.*, 1986). In the present study, however, distinct populations of calcium channel were not observed and no specific component of inward current was identified in the presence of cicletanine. From this and from the action of cicletanine in the presence of (-)-Bay K 8644 it seems reasonable to conclude that cicletanine acts on the L-type calcium channel.

The results of the present study indicate that cicletanine is a calcium channel blocking drug. In vascular smooth muscle, current flow through this channel is responsible for contractions initiated by spike discharges. Although cicletanine also inhibits nifedipine-resistant contractions of guinea-pig isolated portal veins, precontracted with noradrenaline (Deitmer *et al.*, 1991), the inhibitory action of cicletanine on L-type calcium channels observed in the present study seems likely to contribute to its vasorelaxant action previously described in several *in vitro* studies (Baranes *et al.*, 1983; Ebeigbe & Cabanie, 1991; Noack, 1992).

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A comparison of rat brain amino acid and monoamine content in diazepam withdrawal and after exposure to a phobic stimulus

Nick Andrews, [1]Nicholas M. Barnes, [1]Lucinda J. Steward, [1]Katie E. West, [2]Jo Cunningham, Pei-Ying Wu, Helio Zangrossi, Jnr & [3]Sandra E. File

Psychopharmacology Research Unit, UMDS Division of Pharmacology, Guy's Hospital, London SE1 9RT

1 The content of amino acids (taurine, glycine, glutamic acid, γ-aminobutyric acid (GABA) and aspartic acid) and monoamines (5-hydroxytryptamine (5-HT), 5-hydroxyindoleacetic acid (5-HIAA) and noradrenaline) in homogenates of rat cortical and hippocampal tissue were measured by high performance liquid chromatography (h.p.l.c.) with fluorescent and electrochemical detection respectively, after two anxiogenic treatments: exposure to a phobic stimulus (cat odour) and withdrawal from chronic diazepam treatment.

2 In neither of the two anxiogenic situations was there a significant change in any amino acid content, in either brain area.

3 In the group withdrawn from chronic diazepam, cortical 5-HT and 5-HIAA levels and hippocampal 5-HT levels were significantly increased. Noradrenaline content was significantly decreased in the hippocampus.

4 The changes in 5-HT and 5-HIAA levels following cat odour exposure were area-specific in that they decreased in the hippocampus, but increased in the cortex.

5 Following cat odour exposure, noradrenaline levels appeared not to change in either area studied. However during exposure to cat odour, it was found that half the animals avoided the odour source and half were indifferent. The animals showing marked avoidance had significantly higher cortical noradenaline content and this was significantly different from control, whereas hippocampal noradrenaline levels were not dependent upon the differences in avoidance of the odour source.

6 The results show clearly different neurochemical changes in the rat following exposure to a phobic stimulus and withdrawal from diazepam. It is hoped comparative studies such as this will enable better understanding of anxiety states in the rat which could parallel the different classes of anxiety recognised in the clinic

Keywords: Cat odour; diazepam withdrawal; noradrenaline (NA); 5-hydroxytryptamine (5-HT); amino acids

Introduction

Several different types of anxiety disorder are recognised clinically and these show differential sensitivity to pharmacological treatments. For example, generalised anxiety disorder is still mainly treated with benzodiazepines, whereas panic disorder responds best to tricyclic antidepressants, and simple phobias are often not effectively treated by drugs, although they sometimes respond to monoamine oxidase inhibitors. This differential diagnosis and pharmacological treatment suggests that the various anxiety disorders have different neurobiological substrates.

Blanchard *et al.* (1989) described the relative lack of effects of diazepam to the response of rats to the odour of a predator, and we have recently proposed (Zangrossi & File 1992a) that the responses of rats to cat odour could provide a model of phobic anxiety. In contrast, we believe that the anxiety produced by benzodiazepine receptor agonist withdrawal is closer to that of generalised anxiety disorder. The purpose of the present experiments was to compare the effects of these two manipulations on brain neurotransmitter content.

Methods

Animals

Male, hooded Lister rats (Olac, Bicester), approximately 250 g in weight were housed in groups of five, until five days before the experimental session at which time they were housed singly. Food and water were freely available at all times. The room was maintained at 22°C with lights on from 07 h 00 min to 19 h 00 min.

Drugs and chemicals

Diazepam (Roche U.K. Ltd.) was suspended (by use of a sonicating water bath) at a concentration of 1 mg ml^{-1} in distilled water containing 1 drop of Tween-20 per 20 ml of water. It was administered intraperitoneally (2 mg kg^{-1}, i.p.) once per day for 21 days. Controls received i.p. vehicle daily for 21 days. All injections were in a volume of 2 ml kg^{-1}.

Procedure

Diazepam withdrawal Eighteen animals were randomly allocated to either chronic vehicle ($n = 9$) or diazepam ($n = 9$) treatment for 21 days. On the test day, 24 h after the last chronic injection, all the animals received an i.p. injection of vehicle 30 min before they were killed.

Exposure to cat odour Seventeen rats were randomly allocated between neutral ($n = 8$) and cat ($n = 9$) odour conditions. The cat odour was obtained by rubbing a damp cloth

[1] Present address: Department of Pharmacology, The Medical School, University of Birmingham, Edgbaston, Birmingham B15 2TT
[2] UMDS Division of Pharmacology, St Thomas's Hospital, London SE1 7EH.
[3] Author for correspondence.

vigorously against the fur of a laboratory-housed domestic cat for 5 min. This procedure was carried out 1 h before the experimental session. The cat odour cloth was kept in a sealed plastic bag. Each cloth was used for 4 exposures only. Damp pieces from the same original cloth were used for the neutral odour.

All odour exposures took place in a separate small, dimly lit room and the neutral odour exposures always preceded the cat odour exposures in order to prevent any traces of cat odour influencing the neutral odour group. Before the first cat odour exposure, an impregnated cloth was left in the test room for 10 min. Each rat was carried to the exposure room in its home-cage which was placed next to an empty cage, with the odour cloth wedged between the cage tops, at the opposite end from the food and water containers. The duration of odour exposure was 5 min and the rats were videotaped for later scoring. A cloth contact was defined as a direct contact or sniffing $\leqslant 5$ cm from the cloth; sheltering was defined as and when the rat was underneath the food and water compartments. For further details see Zangrossi & File (1992b). Immediately after the exposure the animals were returned to the animal house and 30 min later they were killed. This time interval between odour exposure and death was selected because it was the time at which marked changes in behaviour in the social interaction and plus-maze tests were detected after exposure to cat odour (Zangrossi & File, 1992b).

Tissue preparation Animals were stunned and killed by cervical dislocation and the brains removed. Frontal cortex (approximately 25 mg) and hippocampus (approximately 100 mg) were dissected out on an ice-cooled petri-dish and immediately placed in 200 µl and 400 µl respectively of homogenizing solution (0.2 M perchloric acid containing 100 pg μl^{-1} dihydroxybenzylamine hydrobromide). The tissue was then sonicated using a soniprep soniprobe for 8 s. The resulting homogenate was then divided in half, with one aliquot for amino acid analysis and one aliquot for the monoamine analyses.

Amino acid analysis After storage at − 20°C for at least 2 h, and centrifugation for 1 min at 8,800 *g* in an Eppendorf bench top centrifuge (model 5413), the resulting supernatant was decanted and stored at − 20°C until assay. The samples were analysed for γ-aminobutyric acid (GABA), aspartic acid, glycine, glutamic acid and taurine on a Varian 5000 HPLC (high performance liquid chromatograph) with fluorescent detection following pre-column derivatisation with *o*-phthalaldehyde (Turnell & Cooper, 1981).

Indole- and catecholamine analysis The h.p.l.c.-electrochemical detection (e.c.d.) system comprised an isocratic pump (BAS PM-80, Solvent Delivery System) which was connected to an analytical column (Spherisorb 5 ODS; 150 × 4.6 mm; HPLC Technology) via a Rheodyne injector. The analytical column was protected by a C_{18} guard column. The effluent from the analytical column was passed into an electrochemical detector (BAS LC-4C Amperometric Detector; glassy carbon electrode potential + 700 mV versus silver/silver chloride reference electrode). The output from the electrode was monitored with a recording/plotting integrator (JCL6000 Jones Chromatography). The h.p.l.c.-e.c.d. system, with the exception of the integrator, was maintained at a constant temperature of 4°C inside a glass-fronted cool cabinet. The optimised mobile phase for the simultaneous detection of 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) in homogenates (methanol 11% v/v; disodium hydrogen orthophosphate 106 mM; citric acid 36 mM; tetraethylammonium bromide 2 mM; pH 6.2–6.3; slight adjustments to the pH and/or methanol concentration were made to overcome column to column variation) or noradrenlaine (NA) following alumina extraction (see Anton & Sayre, 1962, for detailed methodology) from the brain homogenate (methanol 11% v/v; citric acid 100 mM; sodium octylsulphate 0.225 mM; sodium EDTA 0.05 mM; pH 2.4–2.6; slight adjustments to the pH and/or methanol concentration were made to overcome column to column variation) were delivered to the analytical column at a rate of 1.4 ml min^{-1}. Injections of external standards were made in order to identify and calibrate the peaks resulting from the injection of the dialysates.

The results of all analyses were finally expressed in terms of the amount per mg protein in the tissue sample, as determind by the method of Lowry *et al.* (1951).

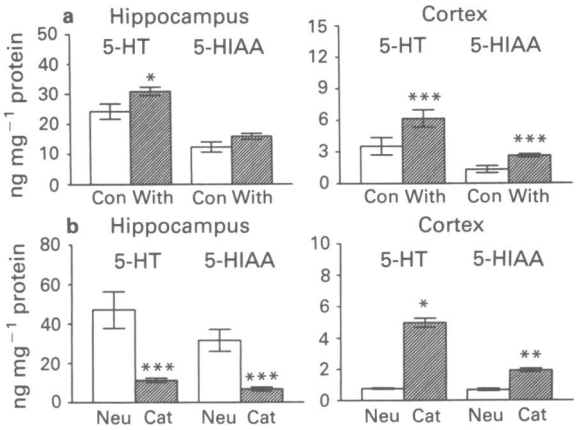


Figure 1 Mean (± s.e.mean) amounts (ng mg^{-1} protein) of 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) in hippocampus and cortex of rats following 24 h withdrawal from vehicle (21 days; Con) or diazepam (2 mg kg^{-1} daily for 21 days; With) (a) or 30 min following exposure to a neutral (Neu) or cat (Cat) odour (b). Group sizes were $n = 7–9$. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared with vehicle control group or with neutral odour group.

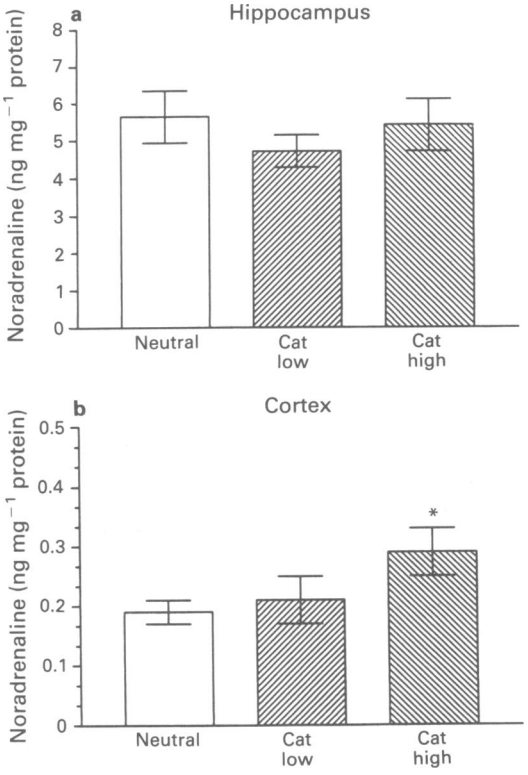


Figure 2 Mean (± s.e.mean) amounts (ng mg^{-1} protein) of noradrenaline in hippocampus (a) and cortex (b) of rats exposed to neutral or cat odour (with either low (Cat low) or high (Cat high) behavioural responses to the odour). Group sizes were $n = 7–9$. $^*P < 0.05$ compared with neutral odour.

Table 1 Amino-acid content ($\mu g\ mg^{-1}$ protein) in hippocampal and cortical tissue of rats either 24 h following withdrawal from chronic (21 days) vehicle (Control) or 2 $mg\ kg^{-1}$ diazepam (Withdrawal) or 30 min following exposure to neutral (Neutral) or cat (Cat) odour

	Aspartate	*Taurine*	*Glycine*	*Glutamate*	*GABA*
Hippocampus					
Control	1.23 ± 0.12	4.01 ± 0.37	0.66 ± 0.07	7.09 ± 0.67	0.90 ± 0.06
Withdrawal	1.21 ± 0.11	3.92 ± 0.32	0.64 ± 0.05	7.16 ± 0.33	0.93 ± 0.05
Neutral	1.67 ± 0.11	4.23 ± 0.48	0.68 ± 0.10	8.57 ± 0.78	1.00 ± 0.12
Cat	1.57 ± 0.11	3.84 ± 0.26	0.56 ± 0.04	7.85 ± 0.37	0.93 ± 0.06
Cortex					
Control	2.19 ± 0.24	4.84 ± 0.53	0.89 ± 0.08	10.41 ± 1.04	0.86 ± 0.07
Withdrawal	2.18 ± 0.19	4.60 ± 0.56	0.77 ± 0.06	10.91 ± 1.01	0.83 ± 0.08
Neutral	1.98 ± 0.26	4.32 ± 0.85	0.78 ± 0.09	9.80 ± 1.13	0.63 ± 0.13
Cat	2.11 ± 0.14	4.17 ± 0.38	0.77 ± 0.06	10.81 ± 0.69	0.64 ± 0.08

Values are mean ± s.e.mean. Group sizes were $n = 6$.

Statistics

The data from the diazepam withdrawn and cat odour exposed animals were compared with their own respective controls by independent t tests, and it is the significances of these that are shown in Figure 1 and Table 2. When the cat odour data were divided into low and high responder groups the results were analysed with analysis of variance, followed by Duncan's tests for significance of individual groups compared with the controls. It is the significance that is shown in Figure 2.

Table 2 Noradrenaline content ($ng\ mg^{-1}$ protein) of cortical and hippocampal tissue of rats 24 h following withdrawal from either chronic (21 day) vehicle (Control) or diazepam (Withdrawal) treatment

	Cortex	*Hippocampus*
Control	0.26 ± 0.05	6.00 ± 0.70
Withdrawal	0.19 ± 0.02	3.30 ± 0.40**

Values are mean ± s.e.mean. Each group contained 7–9 animals. Student's t test was used to analyse for statistically significant differences within groups.
**$P < 0.01$.

Results

Neurochemical consequences following diazepam withdrawal

There were no changes in amino acid content in either the hippocampal or cortical samples of rats withdrawn from chronic diazepam (see Table 1). However, Figure 1a shows the levels of 5-HT and 5-HIAA to be greater in the samples of cortex and hippocampus from withdrawn animals compared with control. The levels of noradrenaline were significantly lower in the hippocampus, but not the cortex of withdrawn animals, see Table 2.

Neurochemical consequences following cat odour exposure

There were no changes in amino acid content in either cortical or hippocampal homogenates in the animals exposed to cat compared with neutral odour (Table 1). However, a marked decrease in the levels of 5-HT and 5-HIAA was observed in the hippocampus of these animals compared with the animals exposed to neutral odour (see Figure 1b). Figure 1 also shows that in the cortex of rats exposed to cat odour, the levels of 5-HT and 5-HIAA were significantly increased.

The levels of noradrenaline were not significantly different between the two groups in either area investigated. However, some differences emerged when the behavioural scores of the individual rats were considered. Exposure to cat odour is characterized by the animals spending the majority of the exposure time sheltering under the food and water hoppers (Zangrossi & File, 1992b). In this particular experiment the animals formed two distinct groups based on their time spent sheltering. One group ($n = 5$) was highly responsive to the cat odour (mean of time sheltering = 219.8 ± 30.7 (s)) and the other ($n = 4$) was insensitive to the cat odour (mean of time sheltering = 52.3 ± 14.5 (s)), compared with the neutral odour exposed group (mean of time sheltering = 40.8 ± 14.4 (s)). When the noradrenaline levels of these two groups were compared with the levels of the animals exposed to neutral odour it was found that in the cortex, but not the hippocampus, the group highly responsive to cat odour showed a significant increase in the level of noradrenaline (Figure 2). The results of 5-HT, 5-HIAA and amino acid analyses were not changed by dividing the cat odour results in this way.

Discussion

The diazepam-withdrawn rats did not differ from their control in their cortical or hippocampal amino acid content. This suggests that amino acid transmitters do not play a major part in the withdrawal responses from this low dose of diazepam, and we found no change in GABA release and uptake in rats withdrawing from the same dose (Andrews *et al.*, 1992). However, the conclusion that amino acids are not important must be viewed with some caution since whole tissue levels do not always reflect alterations in extracellular levels. No changes in the levels of amino acids were found in either cortical or hippocampal tissue after exposure to cat odour. Yet we found increased [^{14}C]-GABA release and decreased uptake from both hippocampal and cortical slices from animals exposed to cat odour (File *et al.*, 1992). We do not at present know the reason for this discrepancy.

5-HT and 5-HIAA content were increased following diazepam withdrawal in both cortex and hippocampus. We have already suggested that the increased hippocampal 5-HT release underlies the increase in anxiety during diazepam withdrawal (Andrews & File, 1992), but we have no evidence to date of the behavioural changes that may be mediated by increased cortical 5-HT function.

Noradrenaline levels were significantly decreased only in the hippocampus following withdrawal from diazepam treatment. This finding agrees with that of Rastogi *et al.* (1976). It is not possible to say whether these changes reflect an increased or decreased noradrenergic function, but the present results suggest further investigations of noradrenaline release and uptake would be warranted.

Prior exposure to cat odour had differential effects on 5-HT and 5-HIAA according to the brain area analysed. Thus in the hippocampus, 5-HT and 5-HIAA levels were

lowered whereas in the cortex they were raised relative to levels of neutral odour exposed rats. Although there were significant changes in 5-HT and 5-HIAA, it is interesting that these were not dependent on the animal's actual behavioural response during the odour exposure.

In contrast the changes in noradrenaline content in the cortex were crucially dependent on the rat's response during the odour exposure. Thus animals showing high avoidance of the odour also had high noradrenaline content in cortical tissue. Gorman *et al.* (1989) have recently hypothesized that phobic avoidance frequently observed in panic patients may involve the activation of the pre-frontal cortex, while anticipatory anxiety and panic attacks involve activation of limbic and brain-stem structures respectively. The changes in noradrenaline content following exposure of rats to cat odour further suggests the involvement of the cortical noradrenergic system in the development of phobic avoidance.

In conclusion, this study has shown that the two treatments give rise to different changes in 5-HT and noradrenaline content. We also have evidence from release studies for differences between these two treatments. Hippocampal 5-HT release is increased during diazepam withdrawal (Andrews *et al.*, 1992), but is not altered following exposure to cat odour (File *et al.*, 1992). We have also found that drugs capable of inhibiting 5-HT function reverse the anxiogenic effects of diazepam withdrawal, but only a few studies have investigated the effects of 5-HT drugs on the behaviour of rats exposed to cat odour. Blanchard's group has shown that the 5-HT_2 receptor antagonist, ritanserin, and the 5-HT_{1A} receptor agonist, gepirone, failed to show reliable anxiolytic effects during the presentation of cat odour (Shepherd *et al.*, 1992; Blanchard *et al.*, 1990). More detailed behavioural pharmacology studies of the responses to cat odour are needed, and the results from this study would suggest that drugs acting on the noradrenergic system might be effective in modulating responses to phobic stimuli.

The results of the present study lend support to there being neurochemical differences between anxiety generated by a phobic stimulus and that induced by diazepam withdrawal.

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The effect of lifarizine (RS-87476), a novel sodium and calcium channel modulator, on ischaemic dopamine depletion in the corpus striatum of the gerbil

[1]C.M. Brown, C. Calder, B.J. Alps & M. Spedding

Syntex Research Centre, Research Park, Riccarton, Edinburgh EH14 4AP

1 Unilateral ligation of the right common carotid artery in the anaesthetized gerbil for 3 h caused a 62.7% decrease in ipsilateral dopamine in the corpus striatum from 1.40 ($\pm$0.13, $n = 27$) $\mu g\ g^{-1}$ in the non-ischaemic hemisphere to 0.47 ($\pm$0.07, $n = 27$) $\mu g\ g^{-1}$ in the ischaemic hemisphere (all results are expressed as mean $\pm$ s.e.mean). In sham-operated animals there were no differences in the dopamine levels ($1.31 \pm 0.14\ \mu g\ g^{-1}$, $n = 11$, left; $1.27 \pm 0.13\ \mu g\ g^{-1}$, $n = 11$ in the right hemisphere). Animals with intact communicating arteries in the circulus arteriosus were excluded.

2 Lifarizine (RS-87476; 250, 500, but not 50, $\mu g\ kg^{-1}$, i.p.) protected against this dopamine depletion showing only a 9.2% decrease at $250\ \mu g\ kg^{-1}$, i.p. ($P < 0.01$) and no decrease at $500\ \mu g\ kg^{-1}$, i.p. ($P < 0.01$).

3 Nicardipine ($250\ \mu g\ kg^{-1}$, p.o.) was effective when administered chronically once daily for 10 days (26.6% decrease, $P < 0.05$) but not when administered acutely at $50\ \mu g\ kg^{-1}$, i.p.

Keywords: Cerebral ischaemia; dopamine; gerbil; corpus striatum; lifarizine

Introduction

The basal ganglia have little collateral circulation and are especially susceptible to ischaemic damage following stroke involving the middle cerebral artery in man, with the risk of accompanying motor deficits (Caplan *et al.*, 1985; Saito *et al.*, 1987; Bozzao *et al.*, 1989). In animal models, cerebral ischaemia causes marked dopamine release and depletion from a variety of brain areas, particularly the striatum. Thus Zervas *et al.* (1974) and Brannan *et al.* (1987) showed that unilateral ligation of one common carotid artery caused ipsilateral dopamine depletion in the Mongolian gerbil. The gerbil has been widely used in studies of cerebral ischaemia because some of the animals lack communicating arteries in the circulus arteriosus, allowing a unilateral infarct; these animals can be readily identified by a low field microscopic examination of the circulus arteriosus post mortem.

Several approaches have been tried recently in an attempt to reduce the neurological deficit following stroke. Different types of calcium-antagonists have shown different abilities to protect against the deleterious effects of cerebral ischaemia in animal models and in man (Siesjo, 1981; 1988; Spedding & Middlemiss, 1985; Alps & Hass, 1987; Alps *et al.*, 1988; Spedding *et al.*, 1989) but selective L-type calcium channel antagonists may be insufficient. We have therefore developed lifarizine (RS-87476) a sodium and calcium channel modulator, which has only weak calcium-antagonistic and vasodilator effects, as a polyvalent modulator of ion channels, which is effective in a range of animal models of ischaemic stroke. This paper outlines the effects of lifarizine in a model of ischaemic stroke using the Mongolian gerbil where the right carotid artery was ligated for 3 h and dopamine depletion in the corpus striatum assessed.

Methods

Selection of stroke-prone animals

Only 30–50% of gerbils are susceptible to developing ipsilateral hemispheric ischaemia following unilateral carotid ligation. We defined stroke-prone animals as those with no or small branches between the two anterior cerebral arteries, where the critical point for effective anastomotic supply to the opposite anterior and middle cerebral arteries had been passed. These animals also lacked any communication between the anterior and posterior circulation. In stroke-resistant animals the anterior cerebral arteries fused to form a single vessel close to the optic chiasma. Often there was a good collateral supply and communication between the anterior and posterior circulation in these animals.

Male gerbils (60–80 g) were pretreated with lifarizine (50, 250 or $500\ \mu g\ kg^{-1}$, i.p.), nicardipine ($50\ \mu g\ kg^{-1}$, i.p. or $250\ \mu g\ kg^{-1}$, o.d. p.o. for 10 days) or vehicle, 30 min before being anaesthetized with 6 mg pentobarbitone (i.p.) and the right common carotid artery exposed in the paratracheal region. The artery was dissected free and double ligated. In sham-operated animals, the artery was exposed and the ligature put in place but not tied. The animals were kept on a heated blanket for 3 h to maintain body temperature (no effect of temperature was seen on gerbils in control experiments). After 3 h the animals were decapitated, the corpus striatum separated on ice into left and right hemispheres, homogenized in 2 ml 0.1 M perchloric acid and stored under liquid nitrogen. To 1 ml of sample, 3 mg dihydroxybenzylamine (DHB) was added as internal standard. Samples were centrifuged at 9,000 *g* for 5 min at room temperature and the catecholamines in the supernatant concentrated by alumina as described by Eriksson & Persson (1982). The elute ($100\ \mu l$) was injected onto a high performance liquid chromatography system coupled to a coulometric electrochemical detector (ESA) maintained at a carbon electrode voltage of +0.4 V. Separation was achieved by reversed-phase chromatography using an octadecyl polymer coated silica column (Ultrasphere ip, 250 × 4.5 mm id) and a short pre-column (60 × 5 mm id) packed with an octadecyl-coated pellicular support (CO: Pell ODS, Whatman). The mobile phase was composed of a water:methanol mixture (90:10 v/v) containing 0.1 mmol l^{-1} KH_2PO_4, 0.3 mmol l^{-1} sodium octyl sulphonate and 0.1 mmol l^{-1} EDTA, and the flow rate was maintained at 1 ml min^{-1}.

Statistics

Statistical analysis was carried out on the level of dopamine following drug treatment as compared with control animals

[1] Author for correspondence.

(sham-operated and ligated) by use of a Dunnett t test following one factor ANOVA using VIEWSTATS on the Apple Mac.

Drugs and solutions

Lifarizine (RS-87476 (1-(diphenylmethyl)-[4-methyl-[2-4-methylphenyl)4-methyl imidazol-5-yl]]piperazine trihydrochloride) (Syntex) and nicardipine (Syntex) were prepared as a 100 mg ml^{-1} solution, expressed in terms of base substance. Initially, the compounds were dissolved in 0.5% ethanol (100 μl) and made up to a volume of 50 ml in sterile saline (0.9% w/v NaCl) solution.

Results

A 3 h occlusion of the right carotid artery in pentobarbitone-anaesthetized gerbils caused a pronounced ipsilateral dopamine depletion from 1.27 ($\pm$0.13, $n = 11$) $\mu g\ g^{-1}$ in sham-operated animals to 0.47 ($\pm$0.07, $n = 27$) $\mu g\ g^{-1}$ in ligated animals (all results are expressed as mean $\pm$ s.e.mean). There were no effects on the contralateral dopamine levels (1.31 $\pm$ 0.14 $\mu g\ g^{-1}$, $n = 11$, sham-operated; 1.40 $\pm$ 0.13 $\mu g\ g^{-1}$, $n = 27$, ligated). This is in agreement with Zervas *et al.* (1974) and indicates dopamine release and subsequent depletion. There was no dopamine depletion in the animals with communicating arteries in the circulus arteriosus (ligated non-occluded hemisphere 1.34 $\pm$ 0.07 $\mu g\ g^{-1}$, occluded hemisphere 1.33 $\pm$ 0.07 $\mu g\ g^{-1}$, $n = 40$).

Pretreatment with lifarizine (250 and 500 $\mu g\ kg^{-1}$, i.p., 30 min prior to ligation) protected against the loss of dopamine in the ischaemic hemisphere (Figure 1). Lifarizine at 500 $\mu g\ kg^{-1}$ i.p. did not lower blood pressure nor modify heart rate over this time period in anaesthetised gerbils ($n = 4$, data not shown).

Nicardipine (250 $\mu g\ kg^{-1}$, p.o.) was effective when administered chronically for 10 days but not when administered acutely at 50 $\mu g\ kg^{-1}$, i.p.

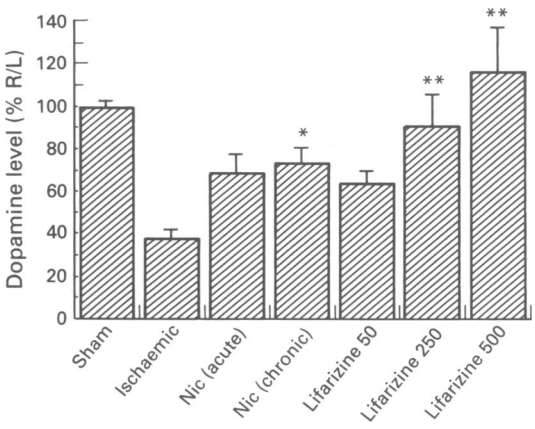


Figure 1 Dopamine depletion in the corpus striatum of the mongolian gerbil following 3 h ligation of the right carotid artery is expressed as the ratio of the dopamine levels in the ischaemic (right)/non-ischaemic (left) striata (the results are given as mean $\pm$ s.e. mean). There was no depletion in the sham-operated animals ($n = 11$), but marked depletion in the ischaemic controls ($n = 27$; $P < 0.01$ vs sham) and in the animals made ischaemic and pretreated for 30 min i.p. with nicardipine (Nic) (50 $\mu g\ kg^{-1}$, $n = 13$) or lifarizine (50 $\mu g\ kg^{-1}$, $n = 11$). In contrast, there was significant protection (**$P < 0.01$ vs ligated group) with lifarizine 250 $\mu g\ kg^{-1}$ ($n = 13$) and 500 $\mu g\ kg^{-1}$ ($n = 11$) and in animals pretreated for 10 days orally with nicardipine (250 $\mu g\ kg^{-1}$, p.o., $n = 10$; *$P < 0.05$ vs ligated group).

Discussion

Lifarizine has been shown to be a novel modulator of sodium and calcium channel function causing highly voltage-dependent inhibition of the sodium channel in NIE-115 cells (Sheridan *et al.*, 1991), inhibition of veratrine-induced contractures (Patmore *et al.*, 1991) and selective inhibition of the effects of calcium channel activators in smooth muscle (Fraser & Spedding, 1991). Thus the compound has time- and voltage-dependent effects on sodium channels. Sheridan *et al.* (1991) showed that the time course of the decay of the block after a depolarizing pulse had a time constant of 45 s. This implies that lifarizine will interact with sodium channels in cells which are depolarized for long periods of time (i.e. ischaemic cells) but will not affect ongoing action potentials. Sodium channel block may be a powerful anti-ischaemic mechanism because the turtle, one of the few animals able to withstand long periods of hypoxia, does so by down-regulating sodium channels (Perez-Pinon *et al.*, 1992). The allosteric interactions at the L-type calcium channel described by Fraser & Spedding (1991) show that low concentrations of lifarizine will block certain forms of L-channel activation without affecting voltage-dependent activation and consequently cardiovascular function. The drug has been shown to be highly effective in models of cerebral ischaemia. Thus lifarizine prevented delayed neuronal cell death following 10 min carotid ligation in the rat four vessel occlusion model (Alps *et al.*, 1991) and reduced infarct size caused by middle cerebral artery occlusion by 70–88% in the cat (Kucharczyk *et al.*, 1991). In the latter study, lifarizine reduced the development of oedema, as assessed by magnetic resonance imaging and spectroscopy and protected against striatal damage even though the striatum has little collateral blood flow.

Lifarizine prevented the ipsilateral dopamine depletion in the gerbil striatum, in a dose-dependent manner. Lifarizine does not inhibit dopamine release, it had no effect on striatal levels of dopamine and its metabolites at concentrations up to 30 mg kg^{-1}, p.o. in the rat (unpublished observations). The protective effects were not associated with hypotensive effects. Nicardipine showed weak protection following chronic dosing but not following single doses. In this respect, flunarizine and nicardipine but not nimodipine have been shown to be effective in preventing delayed neuronal cell death in the CA1 region of the hippocampus following 5 min bilateral occulsion of the common carotid arteries in the gerbil (Alps *et al.*, 1988), and the weak protective effects of nicardipine against dopamine depletion following chronic dosing support these earlier findings. Nicardipine has previously been shown to be effective in models of cerebral ischaemia and to have a high brain uptake (Grotta *et al.*, 1987; 1988; Hadani *et al.*, 1988). Lifarizine is the most effective agent in preventing neuronal cell death in the CA1 region of gerbil brain and rat brain following bilateral carotid occulsion (Alps *et al.*, unpublished observations) and the inhibition of dopamine depeletion is presumably consequent to a direct anti-ischaemic effect. It remains to be seen whether the drug will prove efficacious in preventing such deficits following stroke in man, but the compound has a novel mode of action and a unique protective spectrum of action.

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Effects of propafenone on calcium currents in single ventricular myocytes of guinea-pig

Lü Fei, [1]Jaswinder S. Gill, William J. McKenna, & A. John Camm

Department of Cardiological Sciences, St George's Hospital Medical School, Cranmer Terrace, London SW17 0RE

1 The effects of propafenone on the inward calcium current (I_{Ca}) were investigated in isolated single ventricular myocytes of the guinea-pig by the whole-cell clamp method. Propafenone inhibited I_{Ca} in a dose-dependent manner at concentrations of propafenone ranging from 1×10^{-8} to 1×10^{-3} M and half maximal block of I_{Ca} occurred at a propafenone concentration of 1.5×10^{-6} M. Propafenone did not change the current-voltage relation of I_{Ca} other than a reduction in amplitude and showed no clear use- or frequency-dependent effects upon I_{Ca} (stimulation frequencies from 0.03 to 2 Hz). Propafenone did not alter the steady-state inactivation process: the half maximal activation potentials were 18.5 ± 2.2 mV in the control state and 20.9 ± 5.0 mV in the presence of 1×10^{-6} M propafenone ($n = 12$, NS). Propafenone (1×10^{-6} M) increased the half-time of reactivation by 73.9% ($n = 6$, 212.3 ± 1.2 ms vs 369.2 ± 1.5 ms, $P < 0.05$).

2 We conclude that propafenone blocks I_{Ca} in a concentration-dependent and a channel state-, use- or frequency-independent manner. The I_{Ca} blockade elicited by propafenone at clinically therapeutic plasma concentration is significant and may be involved in its anti-arrhythmic effects.

Keywords: Propafenone; whole-cell clamp technique; calcium currents; cardiac myocytes; drug actions

Introduction

Calcium influx into the cardiac cells through voltage-dependent calcium channels is important in the initiation of both mechanical and electrical events (Hess, 1990). In well polarized tissues, the principal action of Class Ic anti-arrhythmic agents is the reduction of impulse propagation by inhibition of the fast inward sodium current (I_{Na}). However, in depolarized tissues, the availability of the fast sodium channel is reduced, and at levels more positive than -65 mV during ischaemia, premature beats and membrane potential oscillations relate to the inward calcium current (I_{Ca}) and the ascending phase of the action potential is markedly dependent on I_{Ca} (Sperelakis, 1984). It has been demonstrated that frog atrial and mammalian ventricular muscles develop slow oscillatory potentials in response to a depolarizing current which are strongly dependent on the external Ca^{2+} concentration and are antagonized by verapamil and quinidine (Ducouret, 1976; Grant & Katzung, 1976). Since the intracellular Ca^{2+} fluctuations appear to be responsible for the generation of arrhythmias directly and/or indirectly, the modulation of I_{Ca} by anti-arrhythmic drugs has become a matter of increasing interest (Sperelakis, 1984).

Propafenone is an effective anti-arrhythmic agent for the treatment of supraventricular and ventricular arrhythmias (Hammill *et al.*, 1987; Jonason *et al.*, 1988). It is a class I antiarrhythmic drug because of its potent effects upon the fast inward sodium currents (I_{Na}) with reductions of the maximal depolarization of the action potential (V_{max}) (Delgado *et al.*, 1985; Malfatto *et al.*, 1988). This block of I_{Na} demonstrates strong rate-dependent and voltage-dependent properties (Honjo *et al.*, 1989). There is indirect evidence (Ledda *et al.*, 1981; Dukes & Vaughn-Williams, 1984; Delgado *et al.*, 1985) that propafenone has an inhibitory effect on I_{Ca} and this may have an important role in its electrophysiological actions. However, the effects of propafenone on I_{Ca} in single cardiac myocytes remain to be adequately defined. We have therefore examined the effects of propafenone on I_{Ca} and the mechanisms of its calcium channel blockade in single ventricular myocytes of the guinea-pig using the whole-cell clamp technique.

[1] Author for correspondence.

Methods

Cell isolation

Isolated ventricular myocytes of the guinea-pig were prepared by a modification of the method of Trautwein & Kameyama (1987). Female guinea-pigs (weight 300–400 g) were killed by cervical dislocation (Schedule 1, Home Office Regulations). The heart was removed rapidly and transferred into a cold 'low calcium solution' of the following composition (mM): NaCl 120, KCl 5.4, $MgSO_4$ 5, pyruvate 5, glucose 20, taurine 20, HEPES 10; pH 6.96 with NaOH. The aorta was cannulated and the heart was retrogradely perfused on a Langendorff apparatus with the 'low calcium solution' at 37°C for approximately 4 min, then with protease solution (1.6 units ml^{-1}, Sigman type XXIV) for approximately 2 min. Collagenase (42.75 units ml^{-1}, Worthington class II) and hyaluronidase solutions (136 units ml^{-1}, Sigma) were then perfused for 2.5 min. The enzymatic solutions had the same composition as the low calcium solution but were adjusted with 100 mM $CaCl_2$ to give a free Ca^{2+} level of 200 μM. Finally, the heart was washed with a 'KB solution' (mM: glutamic acid 50, KOH 85, KCl 30, KH_2PO_4 30, $MgSO_4$ 3, taurine 20, EGTA 0.5, HEPES 10, glucose 10; pH 7.4 with KOH) for 1.5 min. The ventricles were cut into fresh KB solution, chopped and triturated briefly to release single myocytes. The KB solution containing single cells was filtered through a coarse mesh, centrifuged at 1000 r.p.m. for 1 min, and the pellet of cells resuspended in fresh KB solution and stored at room temperature for 30 min. The cells were then washed with 'recording solution' (mM: NaCl 112, $NaHCO_3$ 24, NaH_2PO_4 1, KCL 5.4, $CaCl_2$ 1, $MgCl_2$ 1, HEPES 5, glucose 10; pH 7.4 with NaOH) prior to transfer to the bath for studies.

Propafenone hydrochloride (Sigma Chemicals, Dorset) was dissolved in distilled water as 10^{-3} or 10^{-4} M solutions and then diluted to the different concentrations required in the recording solution immediately prior to use.

Electrophysiological experiments

Calcium currents were studied in these isolated myocytes by the whole-cell voltage clamp technique (Hamill *et al.*, 1981). The cell suspension was placed in a bath ($10 \times 25 \times 4$ mm)

which was constantly perfused with oxygenated recording solution with 95% O_2 and 5% CO_2 on the stage of an inverted microscope (STC-B1, Correct, Tokyo, Japan) at room temperature (19–25°C). Patch electrodes (1–4 MΩ) were pulled from glass micropipettes (GC150TF-10, 1.5 mm o.d.; 1.17 mm i.d., Clark Electromedical Instruments, England) on a micropipette puller (PB-7, Narishige Co, Ltd, Japan) and fire-polished. The electrodes were filled with the following internal solution (mM): CsCl 130, HEPES 15, $MgCl_2$ 1, Na_2ATP 2, EGTA 1; pH 7.2 with CsOH. After junction potential nulling, the electrodes were positioned onto the cells using a hydraulic micromanipulator (MO-203, Narishige Co, Ltd, Japan). Following the formation of a giga-ohm seal, the patch was disrupted by a suction to get the whole-cell clamp configuration, and the capacitance and series resistances were compensated.

Voltage-clamp protocols were applied to the myocytes through a patch clamp amplifier (RK 300, Biologic, France) from a programmable digital stimulus generator (Active 2, Intracel, England). The myocytes were clamped at a holding potential of − 80 mV and I_{Ca} was elicited by a 300 ms depolarization from − 40 mV to 0 mV at 0.18 Hz. Potassium and sodium currents were suppressed by intracellular caesium and a 100 ms pre-pulse from a holding potential of − 80 mV to − 40 mV, respectively.

Data were sampled at 5000 Hz by an analog-to-digital converter and saved on the hard disk of a microcomputer (Tandon PCA-sl) for off line analysis (Active 2, Intracel, England). Peak I_{Ca} was calculated as the difference between peak inward current and late current at the end of a 300 ms depolarization. All data were expressed as mean ± s.d. Paired or unpaired *t* test and linear regression analysis were used when appropriate. A *P* value less than 0.05 was considered to be statistically significant.

Results

The effects of propafenone on I_{Ca}

In the whole-cell configuration, 1 to 2 min was allowed to complete the exchange between intrapipette solution and intracellular liquid of the myocytes. The I_{Ca} which was stable at a stimualtion frequency of 0.18 Hz while the cell was being perfused with oxygenated recording solution was taken as the control value. I_{Ca} did not run-down for at least 30 min in these myocytes. After the measurements of I_{Ca} in the control condition, the propafenone solutions of concentrations varying from 10^{-8} M to 10^{-3} M were perfused at a speed of 1.5 ml min^{-1} while I_{Ca} was monitoring by use of the same depolarization protocol as above at a frequency of 0.03 Hz. The magnitude of the drug effect was defined as the differences between the amplitudes of I_{Ca} in the control state and in the presence of drug once I_{Ca} has reduced to a stable level and this was expressed as the percentage of its control value. About 15 (range 10 to 20) min were required to obtain a steady state of the inhibitory effect of propafenone on I_{Ca}, which could be reversed by washing with the recording solution (Figure 1a). In order to examine the frequency-dependency of the effect of propafenone on the I_{Ca}, cells were subject to depolarization frequencies varying from 0.03 to 2 Hz in the presence of the different concentrations of propafenone. Propafenone at the concentration from 1×10^{-7} to 10^{-3} M had no clear use- or frequency-dependent effects upon I_{Ca} within the depolarization frequency range of 0.3 to 2 Hz (Figure 1b).

Dose-response curve of propafenone on I_{Ca}

The dose-response curve of the effect of propafenone on I_{Ca} was measured at 10 different concentrations from 1×10^{-8} to 1×10^{-3} M, each of which was repeated in 3 to 11 (mean 5) cells from 2 to 4 hearts (Figure 1c). I_{Ca} was inhibited in a

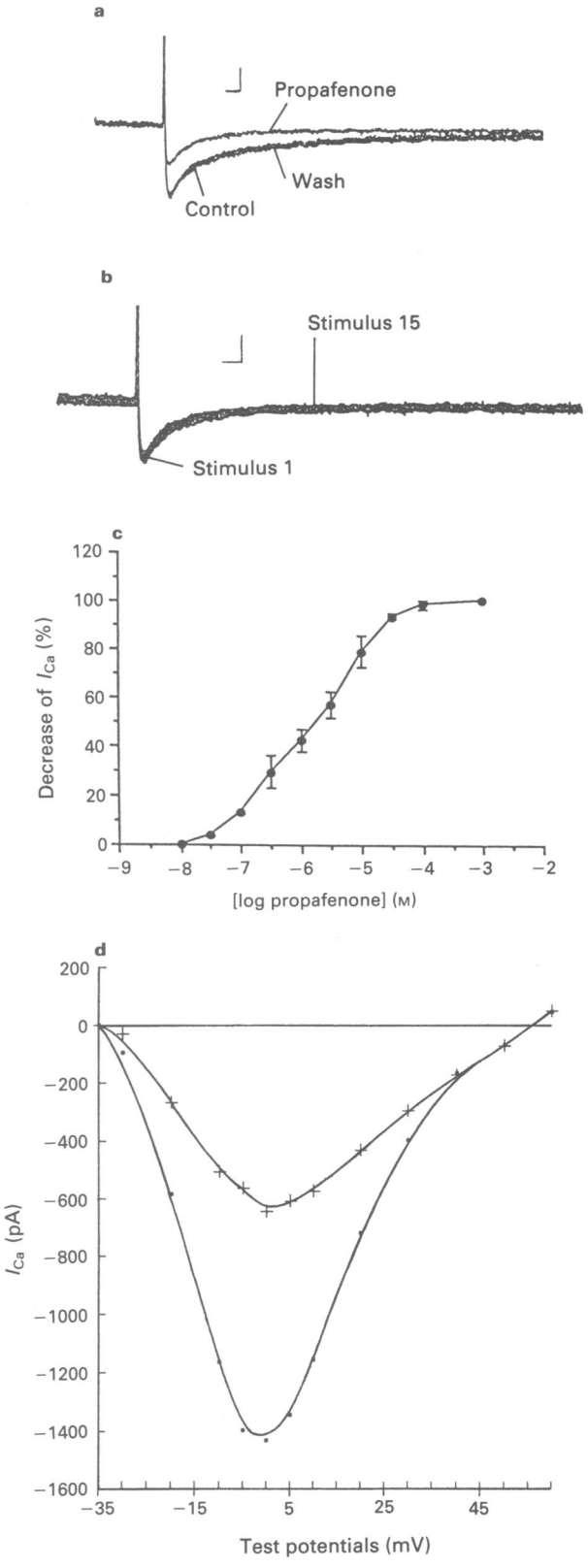


Figure 1 (a) The effect of propafenone (1×10^{-6} M) on I_{Ca}. This was reversed by washing with the recording solution. Scale bar: 200 pA (vertically) and 10 ms (horizontally). (b) Propafenone showed no distinct use-dependent blockade of I_{Ca}. The trace represents a stack of I_{Ca} elicited by 15 stimulations at 2 Hz in the presence of 1×10^{-6} M propafenone. Scale bar: 100 pA (vertically) and 10 ms (horizontally). (c) The dose-response curve of propafenone on I_{Ca}. Each dose level represents experiments of 3 to 5 cells (mean = 5) from 2 to 4 hearts. The half-maximal blockade of I_{Ca} occured at 1.5×10^{-6} M. (d) The current-voltage relationship of propafenone on I_{Ca} in the control state (●) and in the presence of 1×10^{-5} M propafenone (+).

dose-dependent manner at propafenone concentrations above 1×10^{-7} M with a near maximal blockade at the concentration of 1×10^{-4} M. Half maximal block of I_{Ca} occured at 1.5×10^{-6} M. In these experiments, one cell was used once for only one concentration of propafenone because of the duration of the experiments.

The effect of verapamil on I_{Ca}

Verapamil (1×10^{-6} M) showed use-dependent as well as tonic or rest inhibition of I_{Ca} as previously reported (Lee & Tsien, 1983). Tonic or rest block was defined by the first depolarization of a train of 15 stimulations. The further decrease in I_{Ca} on the last of the depolarizations gave the contribution of use-dependent block. Fifteen pulses were enough to produce steady state blockade of I_{Ca} (Uehara & Hume, 1985). Propafenone and verapamil at concentration of 1×10^{-6} M blocked I_{Ca} in the same experimental conditions by $42.1 \pm 4.7\%$ ($n = 11$) and $48.6 \pm 7.7\%$ ($n = 8$ including tonic and use-dependent block at 0.18 Hz), respectively ($P < 0.05$). In 5 cells propafenone (1×10^{-6} M) was applied after the application of verapamil (1×10^{-6} M). There was no increase in the degree of block of I_{Ca} in this situation ($46.2 \pm 8.2\%$ with verapamil; 48.8 ± 8.9 after further addition of propafenone).

The effect of propafenone on the current-voltage relationship and inactivation of I_{Ca}

Propafenone reduced the amplitude of I_{Ca}, but did not change the shape of the current-voltage relation and did not shift the position of its peak, which was 0 mV (Figure 1d). Steady-state inactivation of I_{Ca} was measured by a double-pulse protocol shown in Figure 2. A 300 ms pre-pulse from -40 mV to various potentials was followed by a 300 ms test-pulse to 0 mV separated by a 3 ms return to -40 mV. The amplitude of I_{Ca} evoked by the test pulse was expressed as the percentage of the I_{Ca} evoked by a step from -40 mV to 0 mV in the absence of a pre-pulse. Propafenone did not significantly alter the steady-state inactivation process (Figure 2a). The half maximal inactivation potentials were 18.5 ± 2.2 mV in the control and 20.9 ± 5.0 mV in the presence of 1 ± 10^{-6} M propafenone ($n = 12$, NS).

The effect of propafenone on the reactivation of I_{Ca}

We examined the effect of propafenone on the reactivation process of I_{Ca} in order to obtain more information about its use-dependent effect on I_{Ca}. Reactivation of I_{Ca} was measured by a double-pulse protocol with intervals between the pre-pulse and the test pulse varying from 32 to 4096 ms and expressed as the percentage of I_{Ca} elicited by the pre-pulse (Figure 2b). The half-times of reactivation ($t_{1/2}$, the time at which 50% of I_{Ca} had recovered) were 212.3 ± 1.2 ms in the control and 369.2 ± 1.5 ms in the presence of 1×10^{-6} M propafenone ($n = 6$, $P < 0.05$), an increase in the $t_{1/2}$ of 73.9%.

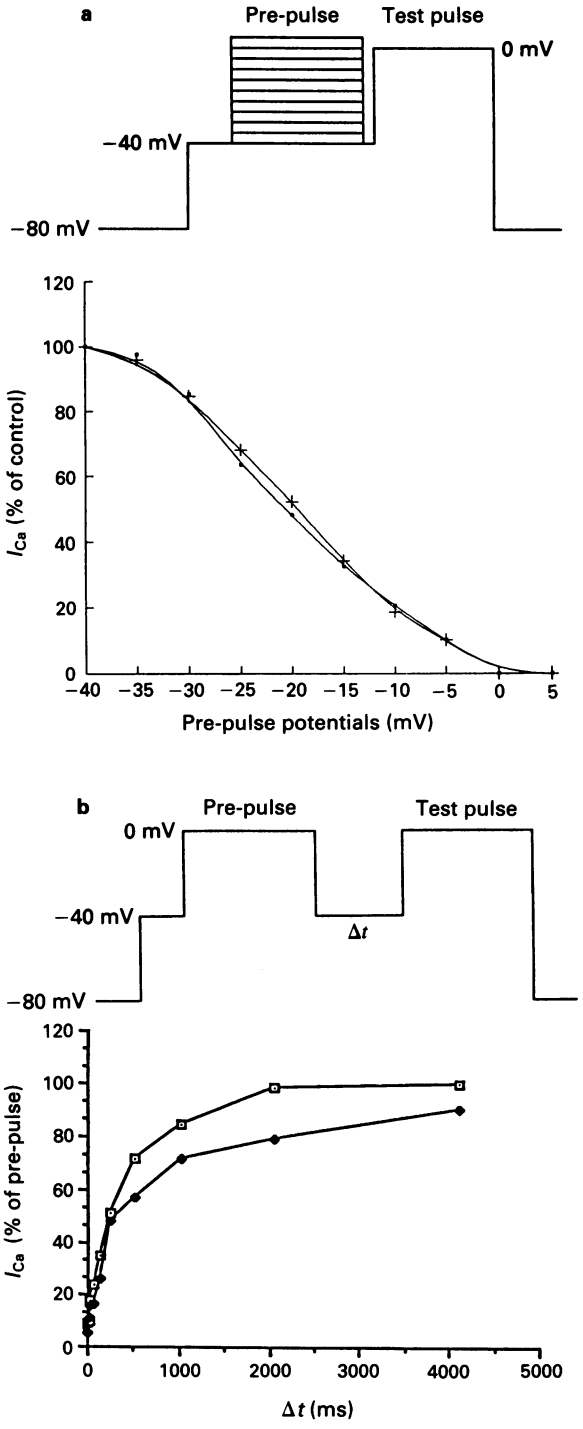


Figure 2 (a) The steady-state inactivation curve of I_{Ca}. Inactivation was measured by a double-pulse protocol. I_{Ca} was plotted as a percentage of the I_{Ca} elicited during a pulse from -40 mV to 0 mV in the absence of a pre-pulse. Propafenone did not alter the steady-state inactivation curve of I_{Ca}. (●) Control; (+) propafenone. (b) The effect of propafenone on reactivation of I_{Ca}. Reactivation was measured by a double-pulse protocol. Both pre- and test-pulse were 300 ms. I_{Ca} was plotted as the percentage of I_{Ca} evoked by the pre-pulse. (□) Control; (◆) propafenone.

Discussion

We have demonstrated in the present study that propafenone is a potent blocker of I_{Ca}. The I_{Ca} in this study can be assumed to be entirely the L-type current because T-type current was inactivated by the pre-pulse from -80 mV to -40 mV (Nilius *et al.*, 1985). The L-type I_{Ca} is the main inward current contributing to the maintenance of the action potential plateau, determining the rise rate of the slow action potentials and supplying the Ca^{2+} required for normal excitation-contraction coupling (Noble, 1984). Propafenone inhibits the I_{Ca} with a relatively slow kinetics. Its inhibitory effects on I_{Ca} reached the maximal level after being perfused for a relatively long time (approximately 15 min). This was consistent with the results reported by Campbell (1983) demonstrating that class Ic anti-arrhythmic agents had a 'slow' kinetics of rate-dependent effects on V_{max} of guinea-pig ventricular action potentials. There is also evidence that propafenone needs a long wash-out period for reversal of its electrophysiological effects (Schlepper, 1987), consistent with

our results and its slow kinetics of action (Rhodes *et al.*, 1985). Two distinct models have been postulated for the possible mechanisms for drugs to reach their acting sites (Rhodes *et al.*, 1985): the 'aqueous approach', the drug reaches the receptor by diffusion through the bulk solvent, and the 'membrane approach', the drug partitions into the membrane bilayer and then diffuses laterally to a specific receptor site. Whether the slow kinetics of its action and wash-out is attributed to its slow receptor kinetics or to another mechanism remains to be investigated. It is also not clear whether the binding site of the drugs is the calcium channel itself or an associated subunit for an adjacent receptor complex but our data suggest that propafenone may act at a site similar to verapamil.

Scamps *et al.* (1989) reported that the positive staircase effects of stimulation frequency on I_{Ca} were suppressed by quinidine and flecainide in frog cardiac cells. We confirmed that propafenone shared the same effect as quinidine and flecaininde in guinea-pig ventricular myocytes. We noted that the effect of propafenone on I_{Ca} was roughly parallel to its effect on the I_{Na} evoked by the pre-pulse. This suggests that both the effects of propafenone upon I_{Na} and I_{Ca} occur at concentrations within the clinically therapeutic concentration-range of propafenone.

Half maximal block of I_{Ca} occurred at 1.5×10^{-6} M (566.9 ng ml^{-1}), which is within the clinically therapeutic plasma concentration range of propafenone (300–1000 ng ml^{-1}) (Schlepper, 1987). We were surprised to find that 1×10^{-6} M propafenone achieved 87% of the blockage of I_{Ca} elicited by 1×10^{-6} M verapamil, suggesting the potent inhibitory effects of propafenone upon the calcium channel. Scamps *et al.* (1989) studied the dose-response curves of flecainide and quinidine on I_{Ca} in single frog myocytes. Comparing our results with the concentration of drugs required for half maximal blockade of I_{Ca} in their studies, propafenone was over 13 times and 7 times more potent in blockade of I_{Ca} than flecainide and quinidine, respectively. Although the differences in the results may be due to differences in the species of animal and the experimental conditions, these results suggest that the effects of propafenone on I_{Ca} at clinically therapeutic plasma levels are significant and may be involved in its antiarrhythmic effects directly or indirectly by changing the membrane calcium influx.

Propafenone did not show clear use-dependent inhibition of I_{Ca} at the concentrations used in this study. Propafenone did not change the shape of the current-voltage relationship or shift its peak. This suggests that the effects of propafenone on I_{Ca} are independent of the channel-state. Two models have been proposed to explain the effects of anti-arrhythmic agents on I_{Ca} the modulated receptor hypothesis, which was originally developed for local anaesthetic agents interaction with the sodium channel (Hondeghem & Katzung, 1984) and the guarded receptor hypothesis (Starmer & Grant, 1985). Propafenone and other class I drugs are known to block sodium current both in tonic and use-dependent ways, binding to the sodium channel during activated and inactivated states (Campbell, 1983; McLeod *et al.*, 1984; Clarkson & Hondeghem, 1985). In the present study, propafenone reduced I_{Ca} without changing the shape of the current-voltage relationship. This is similar to the block of I_{Ca} by flecainide, another class I anti-arrhythmic agent (Scamps *et al.*, 1989). The concentration of flecainide for the half maximal block of I_{Ca} was 20 μM and the $t_{1/2}$ increased by 71.3 ± 12.8% in the presence of 30 μM flecainide in their experiments. Our results are comparable: $t_{1/2}$ was increased by 73.9% in the presence of 1 μM propafenone. The mechanism of the blockade by propafenone upon I_{Ca} (channel state-independent) appears to differ from the inhibition of I_{Na} (channel state-dependent).

We conclude that propafenone blocks I_{Ca} in a concentration-dependent and in a channel state-, and use- or frequency-independent manner. The I_{Ca} blockade by propafenone at clinically therapeutic plasma concentrations is significant and may be involved in its anti-arrhythmic effects and its negative inotropic actions on the myocardium.

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Stimulation of insulin secretion and improvement of glucose tolerance in rat and dog by the P_{2y}-purinoceptor agonist, adenosine-5′-O-(2-thiodiphosphate)

[1]D. Hillaire-Buys, [2]G. Bertrand, J. Chapal, R. Puech, G. Ribes & M.M. Loubatières-Mariani

Faculté de Médecine, Laboratoire de Pharmacologie, URA 599 du CNRS, Montpellier, France

1 *In vivo* effect of a P_{2y}-purinoceptor agonist, adenosine-5′-O-(2-thiodiphosphate) (ADPβS), on insulin secretion and glycaemia were studied both in rats and dogs.

2 In anaesthetized rats, i.v. administered ADPβS (0.2 mg kg^{-1}) produced an insulin response dependent on the nutritional state of the animals, since we observed only a transient increase in overnight-fasted rats and a sustained insulin secretion followed by a reduction in plasma glucose levels in fed rats. During an i.v. glucose tolerance test, ADPβS enhanced insulin release and thus increased the glucose disappearance rate.

3 In anaesthetized fasted dogs, i.v. administered ADPβS (0.1 mg kg^{-1}) increased pancreaticoduodenal insulin output and slightly decreased blood glucose levels.

4 In conscious fasted dogs, orally administered ADPβS (0.1 mg kg^{-1}) transiently increased insulinemia and punctually reduced glycaemia. Furthermore, during an oral glucose tolerance test, orally administered ADPβS at the same dose markedly enhanced insulin secretion and consequently reduced the hyperglycaemia.

5 In conclusion, the P_{2y}-agonist, ADPβS, is a potent insulin secretagogue *in vivo*, improves glucose tolerance and is effective after oral administration. Thus, the P_{2y}-purinoceptors of the β cell may be a target for new antidiabetic drugs.

Keywords: P_{2y}-purinoceptors; adenosine-5′-O-(2-thiodiphosphate) (ADPβS); insulin secretion; glucose tolerance in rat, in dog

Introduction

The insulin stimulating effect of adenine nucleotides (ATP, ADP) and structural analogues has been previously well established on rat isolated perfused pancreas (Loubatières-Mariani *et al.*, 1979; Chapal & Loubatières-Mariani, 1981). These agents act on purinoceptors which have been characterized as P_2, according to Burnstock's classification (1978). By use of 2-methylthio ATP, these receptors have been shown to belong to the P_{2y} subtype (Bertrand *et al.*, 1987). Recently a specific and more stable P_{2y}-agonist, adenosine-5′-O-(2-thiodiphosphate) (ADPβS), was found to be one hundred times more potent than ATP, and effective in the nanomolar range in stimulating insulin secretion *in vitro* on perfused rat pancreas (Bertrand *et al.*, 1991).

The expected consequence of an insulin stimulation *in vivo* is a decrease in glycaemia. Such an effect has been previously observed in dogs with a P_{2y}-agonist, 2-methylthio ATP; however, because of the rapid metabolism of this drug, it was necessary to infuse it *in situ*, directly into the pancreaticoduodenal artery of the dog (Ribes *et al.*, 1988). The potential interest of P_{2y}-agonists in diabetes therapy is linked to their insulin-stimulating effect; this interest would be greatly enhanced by their ability to exert this effect after peripheral intravenous and, more interestingly, after oral administration. So, in this work we investigated the *in vivo* effects of the more stable P_{2y}-agonist, ADPβS, on insulin secretion and glycaemia in rats and dogs.

Methods

General experimental conditions

All the experiments were performed *in vivo*. Male Wistar rats weighing about 380–450 g and male mongrel dogs weighing 12–22 kg were used.

In vivo *experiments in anaesthetized animals*

Rats were anaesthetized with an i.p. injection of pentobarbitone (60 mg kg^{-1}) and maintained on a heated operating surface. A catheter, filled with heparin saline to prevent blood clotting, was inserted into each jugular vein. The test substances were injected through one catheter and blood samples were taken from the other one. In all the experiments, after a 30 min resting period, 3 samples (0.5 ml over 1 min) were taken to determine basal plasma insulin and glucose levels. In all cases the solution of ADPβS was injected slowly at time 0 over 1.30 min; the drug was diluted in 0.5 ml saline.

The effects of ADPβS (0.2 mg kg^{-1}) were tested both in overnight fasted and fed rats. Furthermore the effect of ADPβS on an intravenous glucose tolerance test (IVGTT) was performed in fed rats. For this purpose a mixture of glucose (0.5 g kg^{-1}) plus ADPβS (0.2 mg kg^{-1}) diluted in 0.5 ml saline was injected for 1.30 min. For each set of experiments, control experiments were performed with i.v. saline or glucose alone.

Adult dogs fasted for 16 h were anaesthetized with pentobarbitone (30 mg kg^{-1}, i.v.). The animals were given heparin intravenously (5 mg kg^{-1}) and provided with a venous pancreaticoduodenal by-pass. For this purpose, after a median laparotomy a T-shaped catheter was inserted into the superior pancreaticoduodenal vein just at its exit from the pancreas (Hillaire-Buys *et al.*, 1984). Blood was sampled from the pancreaticoduodenal vein in order to measure insulin concentration. For this, the clamp was taken off the perpendicular part of the venous T-shaped catheter, and the blood stream towards the portal vein was clamped; blood (5 ml) for pancreatic hormone evaluation was collected in a graduated tube and the time of sampling was measured. Thus, it was possible to determine the venous pancreaticoduodenal blood flow. Thereafter the blood stream towards the portal vein was re-established. Insulin output rate was determined by multiplying the concentration of the hormone

[1] Author for correspondence at: Faculté de Médecine, Laboratoire de Pharmacologie, Institut de Biologie, Boulevard Henri IV, 34060 Montpellier Cedex, France.

[2] Present address: Centre CNRS-INSERM de Pharmacologie UMR 6, Rue de la Cardonille, 34094 Montpellier Cedex, France.

in the plasma by the venous blood flow corrected by the haematocrit. Blood glucose concentration was continuously recorded from a peripheral vein with a Technicon autoanalyzer. Femoral arterial blood pressure was recorded with a Ludwig's manometer. After a 60 min equilibration period, ADPβS at a dose of 0.1 mg kg^{-1} was injected for 1 min into a saphenous vein.

In vivo *experiments in conscious dogs*

Two groups of experiments were performed in dogs fasted for 16 h: (1) ADPβS was administered by intragastric intubation at a dose of 0.1 mg kg^{-1} diluted in 10 ml saline; (2) oral glucose tolerance tests (OGTT) were performed. A mixture of glucose (1 g kg^{-1}) plus ADPβS (0.1 mg kg^{-1}) (in 100 ml saline) was administered for 1 min by intragastric intubation. In these experiments, blood samples were taken from a jugular vein in order to evaluate blood glucose and plasma insulin levels.

Assays

In rats, plasma glucose levels were immediately determined by an enzymatic method (Trinder, 1969). In dogs, blood glucose was measured with a Technicon autoanalyzer (Alric *et al.*, 1965).

Insulin concentrations were measured by the method of Herbert *et al.* (1965) using an antibody supplied by Miles Laboratories (Paris). [^{125}I]-insulin was obtained from International CIS (Gif-sur-Yvette, France); the standard used was rat insulin (Novo, Copenhagen, Denmark) the biological activity of which was 22.3 μu ng^{-1}. The intra- and inter-assay coefficients of variation were respectively 9% and 13.5%. The analytical sensitivity was 2.2 μu ml^{-1}.

Data analysis

Data are expressed as mean values ± s.e.mean. All results were submitted to analysis of variance followed by the multiple comparison test (Zar, 1974). For glucose tolerance tests the areas under the curves (AUC) were calculated.

Drug

Adenosine-5'-O-(2-thiodiphosphate) (ADPβS) was purchased from Boehringer Mannheim. It was the lithium salt.

Results

In vivo *effects of i.v. administered ADPβS to anaesthetized rats*

In overnight fasted rats, i.v. administration of ADPβS at the dose of 0.2 mg kg^{-1} induced an immediate but transient increase in insulin secretion (Figure 1). No significant effect on plasma glucose levels was recorded.

In fed rats, i.v. administration of ADPβS at the same dose (0.2 mg kg^{-1}) (Figure 2) evoked a biphasic insulin response: plasma insulin levels rose from 67 ± 10 to 158 ± 16 μu ml^{-1} at 3 min ($P<0.001$); after the first phase in a peak form, the insulin levels remained elevated throughout the 90 min experiment. This sustained insulin response induced a progressive decrease in plasma glucose levels statistically significant from 30 min ($P<0.01$). It should be noted that basal plasma glucose and insulin levels were significantly increased ($P<0.01$) as compared with those of overnight fasted rats (7.90 ± 0.22 versus 7.10 ± 0.17 mM and 67 ± 10 vs 41 ± 9 μu ml^{-1}) at − 2 min.

In fed rats, we tested the effects of ADPβS on the IVGTT (Figure 3). After the i.v. glucose injection alone (0.5 g kg^{-1}), the plasma glucose concentration at 3 min was 17.50 ± 0.77 mM; it then progressively returned to basal

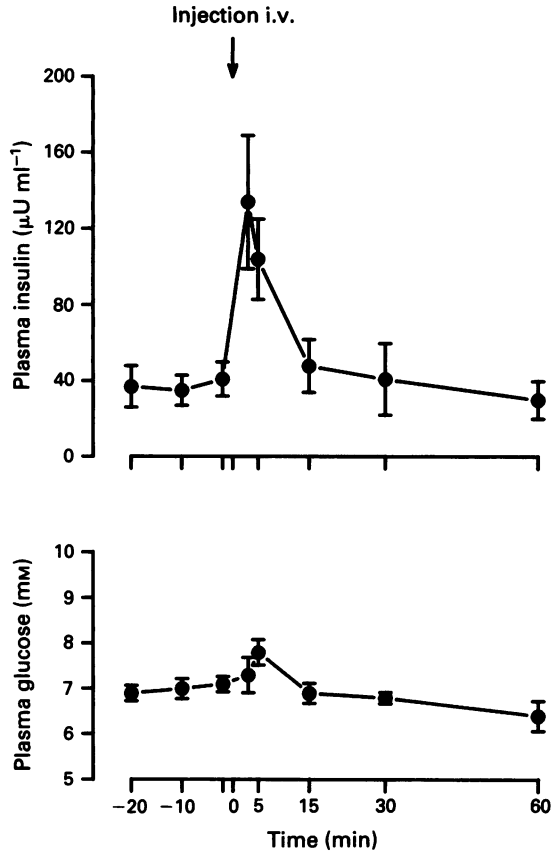


Figure 1 Effects of an intravenous injection of adenosine-5'-O-(2-thiodiphosphate) (ADPβS) 0.2 mg kg^{-1} on plasma insulin and glucose in anaesthetized rats that had been fasted overnight (●). Data are expressed as mean values ± s.e.mean as obtained from six different experiments.

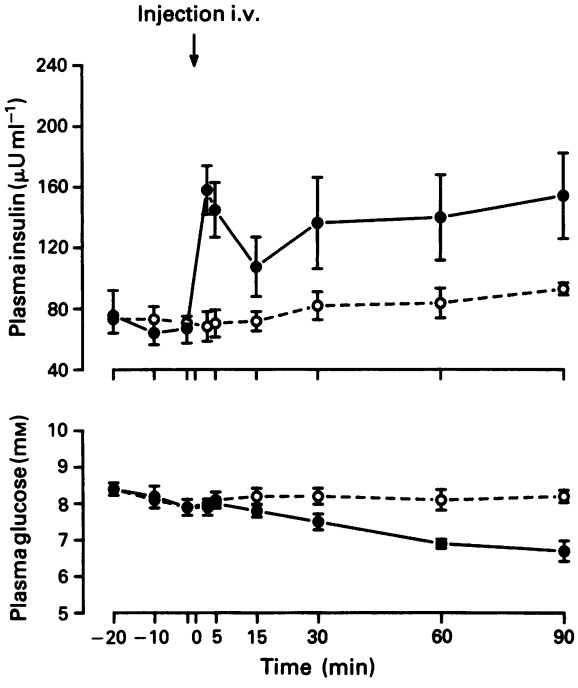


Figure 2 Effects of an intravenous injection of adenosine-5'-O-(2-thiodiphosphate) (ADPβS) 0.2 mg kg^{-1} on plasma insulin and glucose in anaesthetized fed rats (●). Control animals received i.v. saline (○). Data are expressed as mean values ± s.e.mean as obtained from six different experiments.

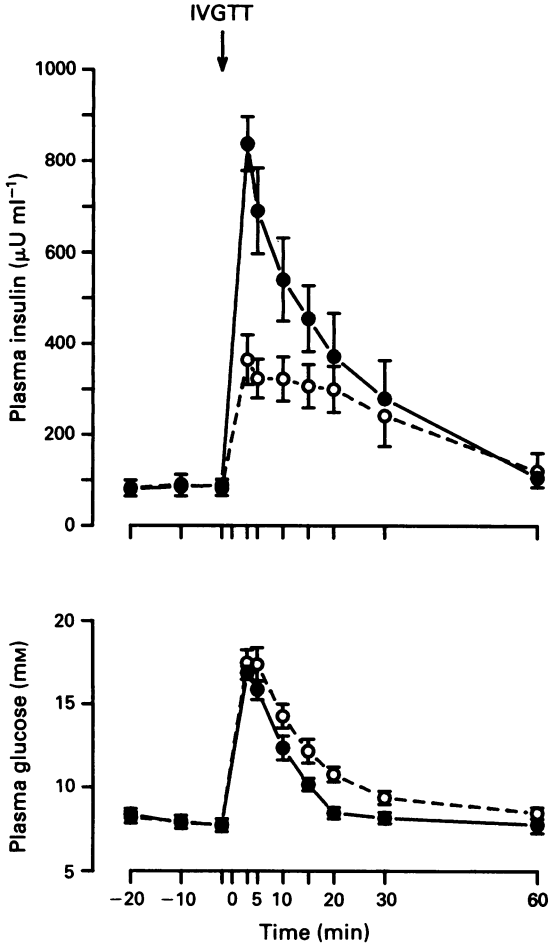


Figure 3 Effects of an intravenous injection of adenosine-5'-O-(2-thiodiphosphate) (ADPβS) (0.2 mg kg^{-1}) on the increases in plasma insulin and glucose levels in response to i.v. glucose injection (IVGTT) (0.5 g kg^{-1}) in fed rats (●). Control animals received i.v. glucose injection alone (○). Data are mean values ± s.e.mean as obtained from six different experiments.

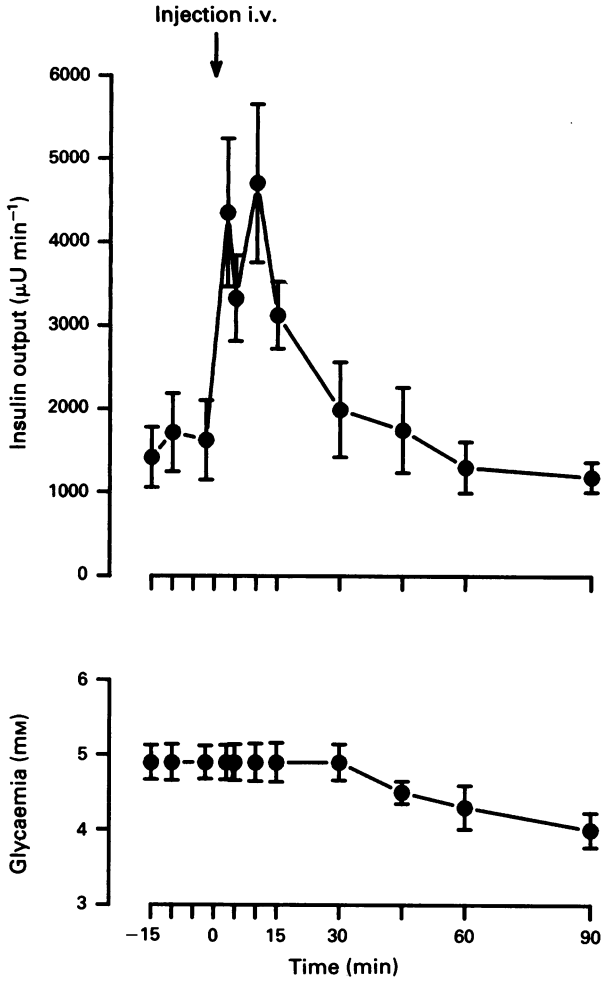


Figure 4 In anaesthetized fasted dogs, provided with a T shaped catheter in the pancreaticoduodenal vein, effects of an intravenous injection of adenosine-5'-O-(2-thiodiphosphate) (ADPβS) (0.1 mg kg^{-1}) on pancreatic insulin output and glycaemia. Data are expressed as mean values ± s.e.mean as obtained from five different experiments.

values which were reached at 60 min. The rise in glucose levels was associated with an increase in plasma insulin concentrations which rose to 366 ± 55 μu ml^{-1} at 3 min and remained significantly high up to 20 min. When ADPβS (0.2 mg kg^{-1}) was added, the increase in plasma insulin levels was significantly higher: 839 ± 59 μu ml^{-1} at min 3 ($P<0.001$ vs controls receiving glucose alone). The integrated insulin response (AUC) during the first 20 min was 8770 ± 1142 vs 4426 ± 656 μu ml^{-1} in controls ($P<0.01$). At 3 min the maximum hyperglycaemia was not significantly different from that obtained with glucose alone (16.90 ± 0.39 mM). However, the return to basal values was more rapid; these were already reached from 20 min. The integrated glucose response (AUC) during the first 20 min was 28% smaller than in controls ($P<0.01$). The glucose disappearance rate (K_G; slope of the logarithm of glucose levels between 3 and 20 min) was higher ($P<0.01$), in rats which received ADPβS (4.12 ± 0.02% min^{-1}) than in control rats (3.05 ± 0.23% min^{-1}).

In vivo *effects of i.v. administered ADPβS to anaesthetized fasted dogs*

Intravenous injection of ADPβS (0.1 mg kg^{-1}) induced an immediate and transient increase in pancreaticoduodenal insulin output (Figure 4). The increase was statistically significant from 3 min to 15 min. Then the insulin output returned towards the starting values. This increase in insulin secretion was followed by a progressive reduction in blood glucose level. In these anaesthetized dogs, glycaemia which was 4.90 ± 0.22 mM before ADPβS administration reached 4.00 ± 0.23 mM at 90 min ($P<0.01$). The injection of ADPβS produced a short lasting diminution in the arterial blood pressure (at 3 min, $P<0.001$) (Table 1); at 5 min the arterial blood pressure had almost returned to the starting values. ADPβS did not significantly influence the pancreaticoduodenal venous blood flow.

In vivo *effects of orally administered ADPβS to conscious dogs*

The administration of ADPβS (0.1 mg kg^{-1}) by intragastric intubation to fasted dogs induced an increase in plasma insulin levels (Figure 5). This hyperinsulinemia appeared 10 min after the intubation ($P<0.01$) and was also observed at 15 min; the plasma insulin then returned to the basal value which was reached at 45 min. At 15 min a reduction in glycaemia was also noted ($P<0.01$).

For the OGTT (1 g kg^{-1}) (Figure 6) the increment in glycaemia reached + 3.17 ± 0.39 mM within 30 min. This hyperglycaemia evoked an increase in insulinemia (+ 44 ± 7 μu ml^{-1} at 30 min). When ADPβS (0.1 mg kg^{-1}) was added to the glucose solution, increments in plasma insulin levels were significantly higher from 5 min (+ 90 ± 13 μu ml^{-1} at

Table 1 Effects of an intravenous injection of adenosine-5'-O-(2-thiodiphoshate) (ADPβS, 0.1 mg kg^{-1}) on arterial blood pressure (mmHg) and venous pancreaticoduodenal blood flow (ml min^{-1}) in anaesthetized, fasted dogs

Min	− 15	− 10	− 2	3	5	10	15	30	45	60	90	120	50	180
Arterial blood pressure (5)	129 ± 7	129 ± 7	129 ± 7	*88 ± 9	119 ± 9	118 ± 8	118 ± 8	123 ± 8	125 ± 8	123 ± 9	121 ± 12	116 ± 12	121 ± 13	120 ± 13
Blood flow in pancreatico-duodenal vein (5)	14.6 ± 1.8	15.1 ± 1.9	14.6 ± 1.5	13.6 ± 1.8	14.0 ± 1.6	13.7 ± 1.2	13.5 ± 1.4	13.6 ± 1.6	13.9 ± 1.7	14.3 ± 1.0	13.9 ± 1.2	13.8 ± 1.4	13.6 ± 1.3	12.9 ± 2.1

*Blood pressure reduced, compared to other time points, $P<0.001$. The number of animals is given in parentheses.

30 min, $P<0.01$). Thus the area under the curve (AUC) for hyperinsulinemia during the first 90 min was greater (5244 ± 1115 versus 2860 ± 563 μu ml^{-1} with glucose alone, $P<0.05$). The integrated glucose response during these first 90 min was significantly reduced ($P<0.05$) in ADPβS-treated dogs (93.3 ± 13.4 versus 183.4 ± 31.9 mM in controls).

Discussion

The main result of this study is that the P_{2y}-purinoceptor agonist, ADPβS, stimulates insulin secretion and improves glucose tolerance not only when administered intravenously but also orally.

In anaesthetized rats, ADPβS injected into a peripheral vein evoked an increase in plasma insulin levels. The insulin stimulating effect of ADPβS appears to be dependent on the nutritional state of the animals. Indeed in overnight fasted rats, ADPβS (0.2 mg kg^{-1}) elicited only a transient insulin response without modification of glycaemia. In contrast, in fed rats, in which plasma glucose levels were higher than in overnight fasted rats, the same dose of ADPβS evoked a sustained insulin response with a reduction of glycaemia. Furthermore, in fed rats, this effect seems to be dependent on the dose since a two fold lower dose of ADPβS (0.1 mg kg^{-1}) evoked a transient insulin response insufficient to induce a decrease of plasma glucose levels (data not shown). The lack of effect of ADPβS in fasted conditions could limit the risk of

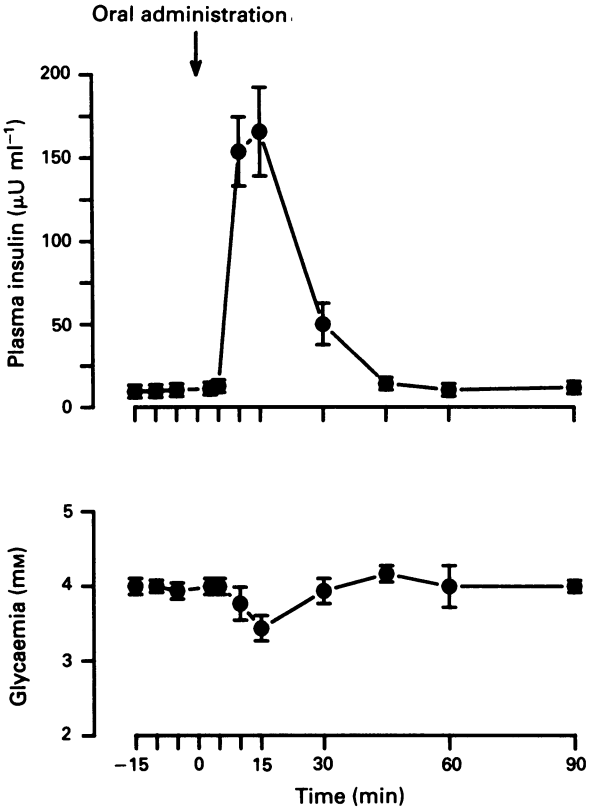


Figure 5 Effects of adenosine-5'-O-(2-thiodiphosphate) (ADPβS) at 0.1 mg kg^{-1} via intragastric intubation on plasma insulin and glycaemia in fasted conscious dogs. Data are expressed as mean values ± s.e.mean as obtained from five different experiments.

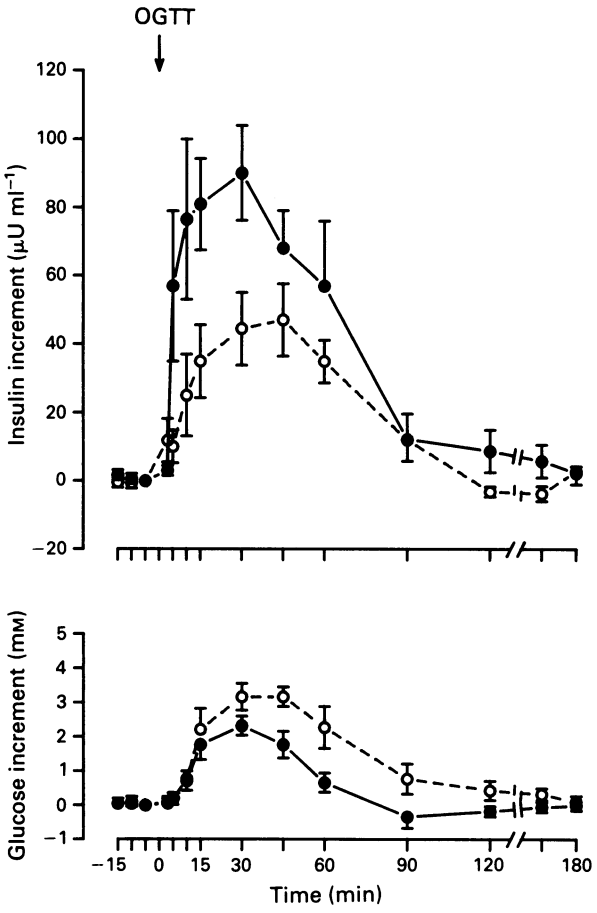


Figure 6 Increments in plasma insulin and glucose levels after oral glucose tolerance test (OGTT) in fasted conscious dogs, in response to glucose alone (1 g kg^{-1}) (○) or glucose (1 g kg^{-1}) + ADPβS (0.1 mg kg^{-1}) (●). Basal values of glycaemia and insulinemia (at min − 5) for dogs receiving glucose alone were 4.7 ± 0.2 mM and 10.1 ± 2.7 μu ml^{-1} respectively; they were 4.6 ± 0.1 mM and 9.7 ± 3.2 μu ml^{-1} respectively for dogs receiving glucose + ADPβS. Data are expressed as mean values ± s.e.mean as obtained from five different experiments.

hypoglycaemia. These *in vivo* findings are in agreement with previous *in vitro* experiments showing that the insulin stimulating effect of ADPβS was related to the glucose concentration (Bertrand *et al.*, 1991).

In anaesthetized fasted dogs, the peripheral intravenous injection of ADPβS induced an immediate but only transient increase in pancreaticoduodenal insulin which was followed by a slight reduction in blood glucose levels; these reached values comparable to basal glycaemia recorded in fasted conscious dogs. We have previously shown that another P_{2y}-agonist, 2-methylthio ATP, was able to induce insulin secretion and to reduce glycaemia slightly (Ribes *et al.*, 1988); however, because of its rapid degradation, this substance must be directly infused in the pancreaticoduodenal artery and thus is devoid of potential therapeutic interest. In contrast, ADPβS is a P_{2y}-agonist known to be resistant to ectonucleotidases (Welford *et al.*, 1986) and our results confirm the stability of this agent. It is known that purine nucleotides are metabolized in gut and largely converted to uric acid during passage across the intestinal mucosa (Stone & Simmonds, 1991) and therefore cannot exert systemic effects. In contrast, in the present study, the structural analogue, ADPβS, was effective after oral administration to conscious dogs, and, to our knowledge, it is the first time that such an effect has been observed. The ability of ADPβS to exert its effects after oral administration supports the potential interest of this P_{2y}-agonist as an insulin stimulating agent.

Another potential advantage could be the ability of ADPβS to improve glucose tolerance. Our results obtained both in anaesthetized rats and in conscious dogs show that this substance, administered either intravenously or orally, enhanced the rise in plasma insulin levels induced by a glucose load and reduced the hyperglycaemia.

From this study it can be concluded that: (1) ADPβS is a potent insulin stimulating agent *in vivo* in rats and dogs; (2) ADPβS administration improves glucose tolerance; (3) ADPβS is able to act after oral administration. Thus, the P_{2y}-purinoceptors of the β cell may be a target for new antidiabetic drugs.

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Support of renal blood flow after ischaemic-reperfusion injury by endogenous formation of nitric oxide and of cyclo-oxygenase vasodilator metabolites

J.P. Cristol, [1]C. Thiemermann, J.A. Mitchell, C. Walder & J.R. Vane

The William Harvey Research Institute, St. Bartholomew's Hospital Medical College, Charterhouse Square, London EC1M 6BQ

1 Ischaemia-reperfusion injury in the kidney is associated with a loss of autoregulation, an increase in renal vascular resistance (RVR), a decrease of renal blood flow (RBF) and ultimately acute renal failure. The aim of this study was to investigate the role of the release of endogenous nitric oxide (NO) in the recovery of RBF after ischaemic injury of the renal vascular bed.

2 Anaesthetized rats (thiopentone sodium; 120 mg kg^{-1}, i.p.) were submitted to acute renal ischaemia followed by 2 or 6 h of reperfusion (I/R). Reperfusion was associated with a significant reduction in RBF, an increase in RVR, and an impairment of the vasodilator effect of acetylcholine (ACh).

3 N^G-nitro-L-arginine methyl ester (L-NAME, 30 $\mu g\ kg^{-1}\ min^{-1}$, i.v., $n = 5$) significantly prevented the recovery of RBF after I/R injury. Similarly, inhibition of prostanoid formation with indomethacin (5 mg kg^{-1}, i.v., $n = 4$) significantly enhanced the rise in RVR associated with I/R injury.

4 Infusion of L-arginine (L-Arg; 1 or 3 mg $kg^{-1}\ min^{-1}$, i.v., $n = 5$ and 4, respectively) or D-Arg (1 mg $kg^{-1}\ min^{-1}$, i.v., $n = 6$), starting 30 min after occlusion, did not improve the recovery of RBF. Furthermore, infusion of L-Arg (20 mg $kg^{-1}\ min^{-1}$ for 15 min; $n = 4$) had no effect on the I/R-induced impairment of the vasodilator responses to ACh.

5 To elucidate the relative importance of the constitutive and inducible NO synthase isoforms for the formation of NO after I/R, calcium-dependent (constitutive) and calcium-independent (inducible) NO synthase activities were measured in kidney homogenates obtained from ischaemic or non-ischaemic kidneys. A calcium-independent NO synthase activity was not detectable in kidney homogenates obtained from either sham-operated control rats or from animals subjected to I/R. Moreover, dexamethasone (3 mg kg^{-1}, i.v., 60 min prior to I/R, $n = 6$), an inhibitor of the induction of NO synthase, had no effect on either RBF or RVR in rats subjected to I/R. In contrast to I/R, lipopolysaccaride (LPS, endotoxin; 5 mg kg^{-1}, i.p., $n = 3$) caused a significant induction of a calcium-independent NO synthase activity in the kidney.

6 These results confirm the importance of the release of vasodilator cyclo-oxygenase metabolites in the compromised renal circulation and indicate that the formation of NO derived from the constitutive, but not the inducible NO synthase, is also important for the maintenance of RBF after I/R injury of the renal vascular bed.

Keywords: Nitric oxide; renal ischaemia; endothelium; L-arginine; N^G-nitro-L-arginine methyl ester

Introduction

The formation of endothelium-derived nitric oxide (NO) from the guanidino nitrogen group of L-arginine (L-Arg) is important for the regulation of blood pressure and microcirculatory blood flow in a variety of species including man (see Moncada *et al.*, 1991; Thiemermann, 1991; Vane & Botting, 1992). There are several distinct enzymes responsible for the formation of NO (Förstermann *et al.*, 1991) including a constitutive, calcium-calmodulin-dependent NO synthase in endothelial (Pollock *et al.*, 1991) and neuronal cells (Bredt & Snyder, 1990). A calcium-independent NO synthase can be induced by cytokines in various cells including renal mesangial cells (Stuehr & Griffith, 1992). In macrophages, the biosynthetic pathway of NO synthesis from L-arginine includes an initial N-oxidation step resulting in the formation of N^G-hydroxy-L-arginine (Stuehr *et al.*, 1991). In addition, hydroxy-L-arginine is a substrate for NO synthase in cultured endothelial cells (Zembowitz *et al.*, 1991) and neuronal NO synthase from rat brain (Mitchell *et al.*, 1992). NO derived from the constitutive endothelial cell NO synthase regulates blood pressure and microcirculatory blood flow, while NO derived from the cytokine-inducible NO synthase is responsible for macrophage cytotoxicity (Marletta *et al.*, 1988; Moncada *et al.*, 1991), and contributes to the circulatory failure associated with endotoxic shock (Julou-Schaeffer *et al.*, 1990; Salter *et al.*, 1991; Szabo *et al.*, 1993).

A continuous basal release of NO within the renal vasculature is of major importance for the regulation of renal blood flow and function under physiological conditions in the anaesthetized (Tolins *et al.*, 1990; Walder *et al.*, 1991; Welch *et al.*, 1991; Zatz & De Nucci, 1991) or conscious (Baylis *et al.*, 1990) rat, as well as in the anaesthetized or conscious dog (Kiyomoto *et al.*, 1992). In addition, the substantial fall in renal blood flow (RBF) induced by inhibitors of NO formation, such as N^G-monomethyl-L-arginine (L-NMMA) or N^G-nitro-L-arginine methyl ester (L-NAME), in the anaesthetized rat can be prevented by both L-arginine and L-hydroxy-arginine, supporting the hypothesis that L-hydroxy-arginine is an intermediate in the formation of NO from L-arginine in the renal vasculature *in vivo* (Walder *et al.*, 1992). Moreover, perfusion of isolated kidneys of the rat with an L-arginine-free buffer solution is associated with a marked reduction in renal perfusion, suggesting that the supply of L-Arg may become rate-limiting, and that reduced supply of L-Arg may affect the regulation of RBF (Radermacher *et al.*, 1991). The vasodilator effect of acetylcholine in the isolated perfused kidney of the rat is dependent on the formation of NO (Radermacher *et al.*, 1990; Bhardwaj & Moore, 1989) resulting in an enhanced formation of guanosine 3':5'-cyclic monophosphate (cyclic GMP) by either vascular smooth muscle or renal mesangial cells or both (Burton *et al.*, 1990; Chevalier *et al.*, 1992).

Thus, under physiological conditions the basal release of

[1] Author for correspondence.

NO is important for the regulation of RBF and function but less is known about the role of NO after ischaemia- and reperfusion-induced (IR) injury. Inhibition of NO formation aggravates the acute renal failure associated with administration of radiocontrast dyes in the anaesthetized rat (Brezis *et al.*, 1991). In addition, I/R injury releases cytokines (Holtz & Goetz, 1992; Ascer *et al.*, 1992) which induce NO synthase in other cells. Here we investigate the role of NO in the maintenance of RBF after I/R injury of the kidney in the anaesthetized rat. The relative contributions of constitutive and cytokine-inducible NO synthase isoforms for the maintenance of RBF after I/R injury were evaluated by inhibition of both NO synthase isoforms with L-NAME (Moore *et al.*, 1990; Hecker *et al.*, 1990; Thiemermann *et al.*, 1990); inhibition of NO synthase induction by dexamethasone (Radomski *et al.*, 1990; Knowles *et al.*, 1990); and by measurement of calcium-dependent (constitutive) and calcium-independent (inducible) NO synthase activities in tissue homogenates from non-ischaemic (control) and I/R kidneys (Bredt & Snyder, 1990; Mitchell *et al.*, 1991; Salter *et al.*, 1991). In addition, the contribution of cyclo-oxygenase metabolites to the recovery and maintenance of RBF after I/R injury has been assessed.

Methods

Surgical procedure

Male Wistar rats (350 to 450 g) were anaesthetized with thiopentone sodium (120 mg kg^{-1}, i.p.). The trachea was cannulated to facilitate respiration and body temperature was maintained at 37°C by means of a rectal probe connected to a homeothermic blanket (Bioscience, Sheerness, Kent). The right carotid artery was cannulated and connected to a transamerica type 4-422-001 pressure transducer for the measurement of mean arterial blood pressure (MAP) on a Grass 7D polygraph (Grass Instruments, Quincy, Mass., U.S.A.). The left jugular vein was cannulated for the administration of drugs and the right femoral vein for the administration of saline (1.5 ml h^{-1}) to compensate for any fluid loss.

The left kidney was exposed via a mild-line laparotomy and the renal artery isolated. An ultrasonic flow probe (1RB, internal diameter = 1 mm), embedded in a silicone cuff to provide optimum alignment, was placed around the left renal artery for measurement of total renal blood flow (RBF) using a Transonic T206 Small Animal Flowmeter (Transonic Systems Inc., New York, U.S.A.). A small amount of acoustical couplant (100 mg Nalco 1181, mixed with 10 ml distilled water; Nalco Chemical Co., IL., U.S.A.) was deposited in the probe's acoustic window adjacent to the artery, to replace all air. This flowmeter system uses an ultrasonic transit time principle, which provides a continuous real-time measure of volume flow, expressed in ml min^{-1}. Ultrasound Doppler flowmetry was chosen to measure RBF, because other techniques (hippuran clearance) result in artefactual and, hence, invalid estimations of RBF in conditions of ischaemia-induced acute renal failure (Stein *et al.*, 1978). Renal vascular resistance (RVR) was calculated by dividing MAP by RBF. After surgery, all animals were allowed to stabilize for 30 to 45 min before occlusion of the renal artery. A vascular clamp was positioned around the left renal artery between its origin and the ultrasonic flow probe. The clamp was then tightened to stop RBF. After 60 min of occlusion, the clamp was reopened to allow reperfusion for 2 to 6 h. In addition, some animals were submitted to the same surgical procedure except occlusion of the renal artery (sham-operated animals, SOP, $n = 3$). Haemodynamic parameters were continuously monitored throughout the experimental period. At the end of the experiment, both kidneys were removed, weighed and frozen in liquid nitrogen and stored at − 70°C for the measurement of NO synthase activity.

Experimental protocol

To assess autoregulation in the kidney we injected pentobarbitone sodium (30 mg kg^{-1}, i.v. bolus, $n = 21$). This produced a transient fall in MAP ($\Delta MAP = 20 \pm 2$ mmHg), but no changes in RBF (-0.2 ± 0.2 ml min^{-1}) showing that the surgical procedures did not affect the autoregulatory adjustments of the renal circulation.

To assess the reponse of the renal vascular bed to endothelium-dependent vasodilators, acetylcholine (ACh, 10 μg kg^{-1} min^{-1}, i.v. for 5 min; infusion rate 0.1 ml min^{-1}; $n = 9$) was infused before and 1 h after occlusion of the renal artery. In some experiments, ACh was given before and after, an intravenous infusion of L-arginine (L-Arg, 20 mg kg^{-1} min^{-1} for 15 min; $n = 4$).

To investigate the role of basal release of NO in the regulation of RBF following ischaemic injury of the renal vascular bed, L-NAME (30 μg kg^{-1} min^{-1}, i.v., $n = 5$), an inhibitor of both constitutive and inducible isoforms of NO synthase (Moore *et al.*, 1990; Thiemermann *et al.*, 1990; Hecker *et al.*, 1990), was infused (at 1.5 ml h^{-1}) starting 30 min after the onset of ischaemia and continued throughout the reperfusion period. In a separate set of experiments, the effect of L-NAME (30 μg kg^{-1} min^{-1}, i.v., $n = 4$) was investigated in SOP-animals. The effects of L-Arg (1 or 3 mg kg^{-1} min^{-1}, $n = 5$ and $n = 4$, respectively) or D-Arg (1 mg kg^{-1} min^{-1}, $n = 6$) were measured on RBF and RVR by continuous infusions starting 30 min after the onset of ischaemia.

In other experiments, animals were pretreated with dexamethasone (3 mg kg^{-1}; i.v. after surgical procedure) to prevent induction of NO synthase. The concentration of dexamethasone used was sufficient to inhibit the synthesis of NO synthase in response to cytokines *in vitro* (Radomski *et al.*, 1990) and *in vivo* (Knowles *et al.*, 1990).

To investigate the potential contribution of prostaglandins to the maintenance of RBF after I/R, animals received indomethacin (5 mg kg^{-1}, i.v. bolus, $n = 3$) 15 min after surgical procedures and were allowed to stabilize for 30 min. Another group of animals ($n = 4$) were treated with indomethacin (above protocol) and L-NAME (30 μg kg^{-1} min^{-1}, i.v.) to investigate the relative contribution of prostanoids and NO to the maintenance of RBF after renal ischaemia.

Measurement of NO synthase activity

NO synthase activity was measured in both ischaemic and non-ischaemic kidneys obtained from control and sham-operated rats. To be sure that the measurements were sensitive enough to measure the induction of NO synthase (positive control), a separate group of animals was subjected to injection of lipopolysaccharide (5 mg kg^{-1}, i.p., $n = 3$), a well known inducer of NO synthase activity *in vitro* and *in vivo* (Moncada *et al.*, 1991; Thiemermann, 1991; Stuehr & Griffith, 1992). Six hours after endotoxaemia, the kidneys were removed, frozen in liquid nitrogen and stored at − 70°C.

The frozen kidneys were homogenized on ice in a buffer: Tris/HCl (50 mM); EDTA (0.1 mM); EGTA (0.1 mM); 2-mercaptoethanol (12 mM) and phenylmethylsulphonyl fluoride (1 mM, pH 7.4) with an Ultra-turrax T25 homogenizer. Conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline was measured in the homogenates (Bredt & Snyder, 1990). Briefly, 50 μl of tissue homogenate (approximately 100 μg protein) was incubated in the presence of L-arginine and [^{3}H]-L-arginine (10 mM, 5000 Bq/tube), NADPH (1 mM), calmodulin (30 mM), valine (50 mM), tetrahydrobiopterin (5 μM) and calcium (2 mM) for 20 min in HEPES buffer (pH 7.5). The reaction was stopped by the addition of HEPES buffer (pH 5.5) containing EGTA (2 mM) and EDTA (2 mM). The reaction mixture was applied to Dowex 50W (Na form) columns (Sigma Chemical Co., Poole, Dorset) and the eluted [^{3}H]-L-citrulline

activity was measured by scintillation counting (Beckman, 2 S 3801). Experiments performed in the absence of NADPH determined the extent of [^{3}H]-L-citrulline formation independent of a specific NO synthase activity. In addition, experiments in the presence of NADPH and calcium or in the presence of NADPH without calcium determined the calcium dependent and the calcium independent (i.e. total and inducible) NO synthase activity, respectively (Mitchell *et al.*, 1991; Salter *et al.*, 1991).

Materials

Trapanal (thiopentone sodium) was obtained from B.Y.K. Gulden (Konstanz, Germany) and Sagatal (pentobarbitone sodium) from RMB Animal Health Ltd. (Dagenham) acetylcholine chloride, dexamethasone 21-phosphate disodium salt, indomethacin, calcium, calmodulin, valine, L-arginine hydrochloride, D-arginine hydrochloride and N^G-nitro-L-arginine methyl ester were purchased from Sigma Chemical Co. (Poole, Dorset). All compounds were dissolved in 0.9% saline except for indomethacin which was prepared as a 5 mg kg^{-1} solution in 5% w/v sodium bicarbonate. [^{3}H]-L-arginine was obtained from Amersham (Buckinghamshire) and tetrahydrobiopterin from Dr B. Schircks Laboratories (Jona, Switzerland).

Statistical analysis

All values in the figures and text are expressed as mean ± s.e.mean of n observations. Statistical comparisons of differences within the same animal were made by Student's t test for paired determinations; comparisons of differences between groups of animals were made by Student's t test for unpaired determination. A P value of less than 0.05 was considered significant.

Results

Impairment of endothelium-dependent vasodilator responses after ischaemia-reperfusion injury of the renal vascular bed

After the stabilization period, baseline values were 12 ± 0.3 ml min^{-1} for RBF, 123 ± 2 mmHg for MAP and 11 ± 0.3 mmHg min ml^{-1} for RVR (n = 59). No significant changes in any of those haemodynamic parameters were observed in SOP animals (n = 4) during the 6 h experimental period (not shown for MAP and RVR; see Figure 1 for RBF). After 60 min of total cessation of RBF (ischaemic period), RBF recovered to 9 ± 1 ml min^{-1} (representing 69 ± 5% of the pre-occlusion value) within 30 min of reperfusion (n = 6). However, RBF remained significantly smaller than the pre-ischaemic level during the subsequent 2 or 6 h reperfusion period (Figure 1). RVR was significantly increased to 16 ± 2 mmHg min ml^{-1} after 30 min of reperfusion compared to the pre-ischaemic level, while no change was observed in MAP (MAP at 30, 60, 120 and 360 min of reperfusion: 130 ± 3, 128 ± 2, 126 ± 2, 127 ± 5 mmHg, respectively). Moreover, RVR remained significantly elevated when compared to either pre-occlusion values in the same animal or with SOP.

Before I/R injury, an infusion of ACh (10 μg kg^{-1} for 5 min) produced a significant (P<0.01) fall in MAP amounting to 13 ± 2 mmHg (10 ± 1%) (n = 9). In spite of this decrease of MAP, RBF was increased by 3 ± 0.3 ml min^{-1} (22 ± 2%; P<0.01) (Figure 2) and RVR was substantially decreased by 3 ± 0.5 mmHg min ml^{-1} (26 ± 2%; P<0.01).

After I/R injury, the fall in MAP in response to ACh was unchanged (ΔMAP: 13 ± 2 mmHg; 9 ± 1%, P<0.01). In contrast, the endothelium-dependent vasodilator responses to ACh in the renal vascular bed after I/R were impaired (ΔRBF: 1 ± 0.3 ml min^{-1}, 11 ± 3%, P<0.01; ΔRVR: 3 ± 0.3 mmHg min ml^{-1}, 18 ± 3%, P<0.01). When compared to pre-ischaemic control responses, the ACh-induced increase in RBF measured either as absolute value (3 ± 0.3 versus 1 ± 0.3 ml min^{-1}, P<0.01) or percentage change (22 ± 2 versus 9 ± 1%, P<0.01) was significantly reduced after I/R injury (Figure 2). When analysed as absolute value, the ACh-induced fall in RVR was not altered by I/R injury (3 ± 0.5 versus 3 ± 0.3 mmHg min ml^{-1}, P<0.05), whereas the percentage fall in RVR was significantly reduced by I/R injury (control: 26 ± 2%; I/R; 18 ± 3%, P<0.01).

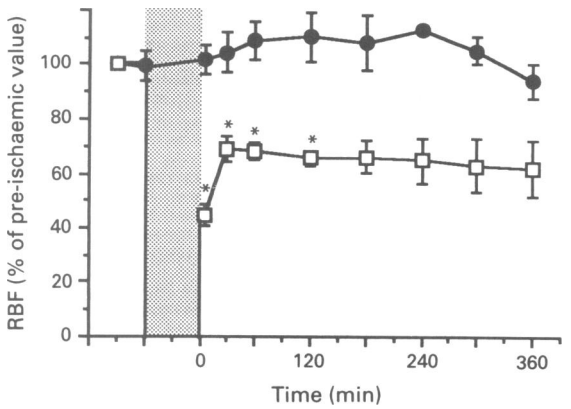


Figure 1 Changes in renal blood flow (RBF, percentage of pre-occlusion value) in sham-operated animals (SOP, ●, n = 4) and in animals submitted to 1 h ischaemia (stippled area) followed by 6 h of reperfusion (□, n = 6) of the renal vascular bed. Results are expressed as mean ± s.e.mean; *P<0.05 when compared with pre-occlusion value by Student's t test for paired determinations

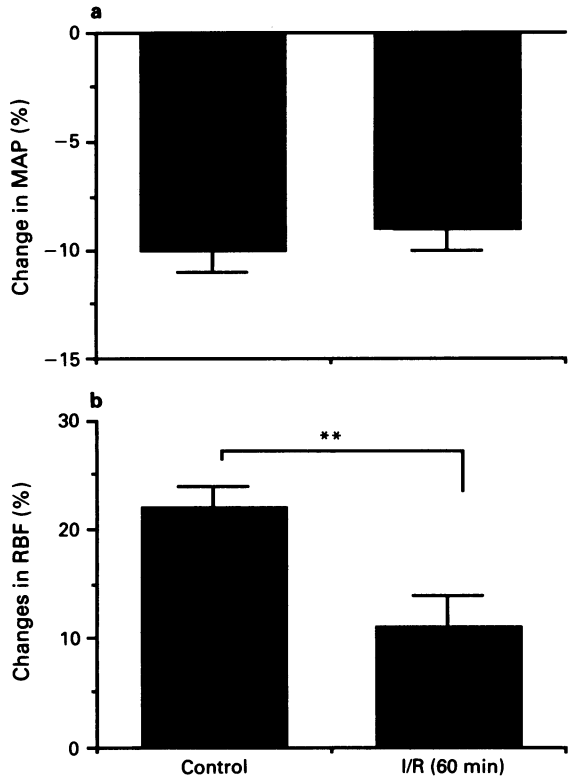


Figure 2 Ischaemia and reperfusion impairs the vasodilator responses to acetylcholine (ACh) in the renal vascular bed. Depicted are the effect of the endothelium-dependent vasodilator ACh on (a) mean arterial blood pressure (MAP) and (b) renal blood flow (RBF) before (control) and after 1 h of ischaemia followed by 60 min of reperfusion of the rat kidney *in vivo*. Data are expressed as mean ± s.e.mean of 9 observations. *P<0.05, **P<0.01 when compared by a Student's t test for paired determinations

Before renal artery occlusion, L-Arg (20 mg kg^{-1} min^{-1} for 15 min) produced a slight, but significant increase in RBF (1 ± 0.3 ml min^{-1}, 10 ± 1%, $P<0.05$). L-Arg had no effect on MAP, so that RVR decreased by 10 ± 1% ($P<0.05$). However, L-Arg did not affect either the systemic or renal vasodilator effect of ACh ($n=4$). After I/R injury, L-Arg produced the same increase in RBF (1 ± 0.2 ml min^{-1}, 10 ± 2%), but failed to restore the ischaemia-induced impairment of the vasodilator response to ACh (Figure 3).

Importance of the generation of NO from the constitutive, but not the inducible NO synthase for the maintenance of RBF after I/R injury of the renal vascular bed

Infusion of L-NAME (30 μg kg^{-1} min^{-1}) significantly attenuated the recovery of RBF after I/R. After 2 h of reperfusion, RBF was reduced to 3 ± 0.5 ml min^{-1} ($n=5$, $P<0.01$) representing 29 ± 3% of the pre-occlusion value (Figure 4a). L-NAME produced a significant increase in MAP (ΔMAP: 24 ± 6 mmHg) and MAP remained significantly elevated throughout the reperfusion period (Figure 4a). Both the systemic and renal effects of L-NAME resulted in a substantial increase in RVR to 46 ± 1 mmHg min ml^{-1} ($P<0.01$) at the end of experiment (Figure 4b). L-NAME produced a similar degree of fall in RBF from 11 ± 1 ml min^{-1} to 4 ± 1 ml min^{-1} after 2 h of infusion in SOP animals ($P<0.01$, Figure 4). When compared to L-NAME, indomethacin (5 mg kg^{-1}, i.v., 30 min before renal artery occlusion, $n=3$) had no effect on RBF but caused a significant rise in RVR after I/R injury (Table 1). However,

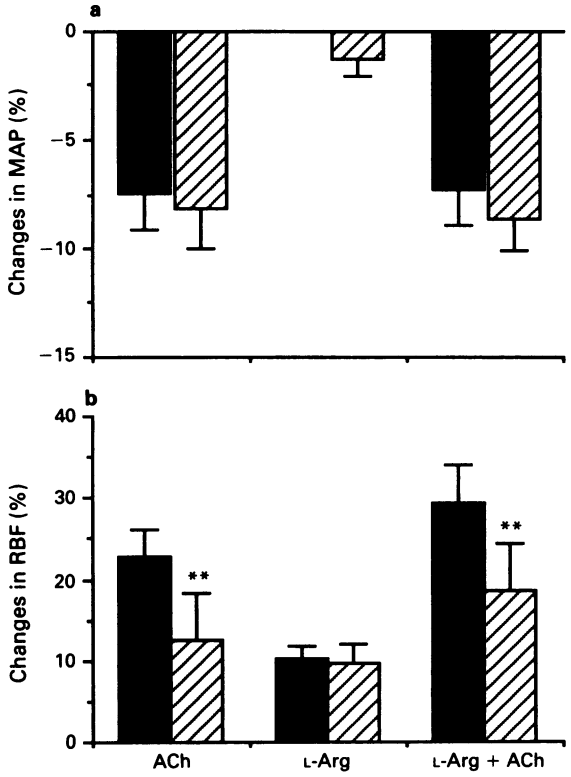


Figure 3 Effect of infusion of L-arginine (L-Arg) on the ischaemia-induced impairment of the vasodilator response to acetylcholine (ACh) in the rat. Depicted are changes in (a) mean arterial blood pressure (MAP) and (b) renal blood flow (RBF) induced by ACh (10 μg kg^{-1} min^{-1}, i.v. for 5 min; $n=4$), L-Arg (20 mg kg^{-1} min^{-1}, for 15 min; $n=4$) and L-Arg plus ACh before ischaemia (solid columns) and after 1 h of ischaemia followed by 1 h of reperfusion (hatched columns). Results are expressed as mean ± s.e.mean of 4 observations. *$P<0.05$ when compared by Student's t test for paired determinations

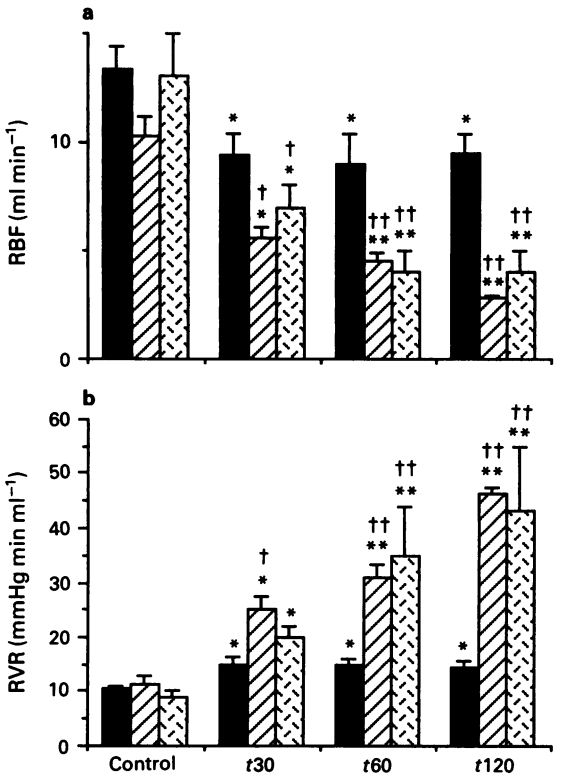


Figure 4 Inhibition of NO formation significantly reduced the recovery of RBF after ischaemia reperfusion injury of the renal vascular bed. Depicted are the changes in (a) renal blood flow (RBF) and (b) renal vascular resistance (RVR) in animals subjected to 60 min ischaemia followed by 120 min of reperfusion (t 30, t 60, t 120) in the absence (control, solid columns, $n=6$), or in the presence of N^{G}-nitro-L-arginine methyl ester (L-NAME, 30 μg kg^{-1} min^{-1}, i.v., hatched columns, $n=5$). In addition, sham-operated animals were treated with L-NAME (30 μg kg^{-1} min^{-1}, i.v., stippled columns, $n=5$). Data are expressed as mean ± s.e.mean. *$P<0.05$, **$P<0.01$ when compared by Student's t test for paired determination; †$P<0.05$, ††$P<0.01$ when compared by Student's t test for unpaired determinations

indomethacin did not affect the changes in RVR or RBF produced by L-NAME in rats subjected to I/R injury (Table 1).

Dexamethasone (3 mg kg^{-1}, i.v., 60 min prior to I/R), an inhibitor of the induction of NO synthase (Radomski *et al.*, 1990; Knowles *et al.*, 1990), had no effect on either RBF or RVR in rats subjected to ischaemia followed by either 2 ($n=6$) or 6 h ($n=3$) of reperfusion (Table 1). Moreover, no calcium-independent (inducible) NO synthase activity was detected in kidney homogenates obtained from either SOP rats or from animals subjected to I/R for 2 ($n=4$) or 6 h ($n=3$). However a calcium-independent NO synthesis (19 ± 2 pmol mg^{-1} 20 min^{-1}) occurred in the kidney homogenates obtained from rats treated with LPS (positive control, $n=3$).

L-Arg (1 or 3 mg kg^{-1} min^{-1}) or D-Arg (1 mg kg^{-1} min^{-1}) did not cause any change in MAP. Moreover, L-Arg or D-Arg had no effect on either the changes in RBF or RVR associated with I/R of the renal vascular bed (Table 1).

Discussion

This study demonstrates that formation of endogenous NO is important not only for the maintenance of normal RBF, but also for the recovery and maintenance of RBF after I/R injury of the renal vascular bed. The model of I/R injury of the kidney used in this study results in haemodynamic alterations within the renal vasculature including a rise in RVR, a

Table 1 Changes in (**A**) renal blood flow (RBF) and (**B**) renal vascular resistance (RVR) in rats subjected to 60 min ischaemia followed by 120 min of reperfusion

		Before	*30 min*	*60 min*	*120 min*
A	*RBF*	(ml min)			
	I/R	13 ± 1	9 ± 1*	9 ± 1*	9 ± 1*
	L-NAME	10 ± 1	6 ± 1*†	5 ± 1**††	3 ± 1**††
	Indo + L-NAME	10 ± 1	7 ± 1*	5 ± 1*†	4 ± 1**††
	Indo	10 ± 1	7 ± 1*	7 ± 1*	7 ± 1*
	Dexamethasone	13 ± 1	10 ± 1*	10 ± 1*	10 ± 1*
	L-Arg (1 mg)	11 ± 1	9 ± 1*	8 ± 1*	9 ± 1*
	L-Arg (3 mg)	11 ± 1	7 ± 1*	8 ± 1*	8 ± 1*
	D-Arg (1 mg)	13 ± 2	9 ± 1*	8 ± 1*	8 ± 1*
B	*RVR*	(mmHg min ml^{-1})			
	I/R	11 ± 0.5	16 ± 2*	15.7 ± 1*	15 ± 1*
	L-NAME	11.3 ± 1	25 ± 2*†	31 ± 2**††	46 ± 1**††
	Indo + L-NAME	9 ± 1	25 ± 9*	26 ± 6*†	37 ± 6*††
	Indo	12 ± 1	22 ± 2*†	21 ± 2*†	20 ± 2*†
	Dexamethasone	9 ± 1	13 ± 1*	13 ± 1*	13 ± 1*
	L-Arg (1 mg)	11 ± 1	14 ± 2*	14 ± 2*	14 ± 2*
	L-Arg (3 mg)	12 ± 1	20 ± 3*	19 ± 3*	18 ± 3*
	D-Arg (1 mg)	10 ± 1	16 ± 2*	16 ± 2*	16 ± 2*

Different groups of animals received vehicle (I/R, $n = 6$), (30 $\mu g\ kg^{-1}\ min^{-1}$, i.v., $n = 5$), indomethacin (Indo, 5 $mg\ kg^{-1}$, i.v. bolus, $n = 3$), indomethacin and N^G-nitro-L-arginine methyl ester (L-NAME) (Indo + L-NAME, $n = 4$), dexamethasone (3 $mg\ kg^{-1}$; i.v., $n = 6$), L-arginine (1 or 3 $mg\ kg^{-1}\ min^{-1}$), or D-arginine (1 $mg\ kg^{-1}$). Data are expressed as mean ± s.e.mean.
*$P<0.05$, **$P<0.01$ when compared by Student's t test for paired determinations; †$P<0.05$, ††$P<0.01$ when compared by Student's t test for unpaired determinations.

fall in RBF, a loss of autoregulation (Kashgarian *et al.*, 1976; Riley, 1978) and an impairment of the response to endothelium-dependent vasodilators (Conger *et al.*, 1991). In the isolated erythrocyte-perfused kidney of the rat, 25 min of ischaemia followed by reperfusion is associated with a marked increase in RVR (Lieberthal *et al.*, 1989). In this model, inhibition of NO formation with two non-selective inhibitors (methylene blue and gossypol) resulted in no further increase in RVR after I/R injury *in vitro* indicating an ischaemia-induced complete loss of the release of NO. However, we demonstrate here that in the anaesthetized rat *in vivo*, inhibition of NO formation with L-NAME, a selective and potent inhibitor of NO formation both *in vitro* and *in vivo* (Moore *et al.*, 1990; Thiemermann *et al.*, 1990; Hecker *et al.*, 1990) is associated with a marked reduction in RBF and a further enhancement of RVR (Figure 4), indicating that NO release contributes to the recovery of RBF after I/R. The reduction in RBF and the increase in RVR obtained after maximal inhibition of NO formation with L-NAME was not different in SOP-animals or rats subjected to I/R injury indicating that I/R is not associated with a substantial impairment of the basal release of NO.

The hypothesis that NO is released by the constitutive, but not inducible NO synthase, is supported by the findings that the recovery of RBF after I/R injury is largely inhibited by L-NAME (Figure 4), an inhibitor of both constitutive and inducible NO synthase, but is not affected by dexamethasone (Table 1), an inhibitor of the induction of NO synthase (Knowles *et al.*, 1990; Radomski *et al.*, 1990). Moreover, no inducible NO synthase was detected after 60 min of renal artery occlusion followed by either 2 or 6 h of reperfusion, even though cytokines, such as tumour-necrosis factor, are released by ischaemia reperfusion injury (Ascer *et al.*, 1992; Holtz & Goetz, 1992). The lack of activity of an inducible NO synthase after I/R was not due to a lack of sensitivity of the NO synthase assay employed, for i.p. injection of LPS, which causes induction of NO synthase, resulted in a significant increase in a calcium-independent NO synthase activity.

The hypothesis that I/R is associated with a reduced formation of NO is based on findings demonstrating an ischaemia-induced impairment of the response to the endothelium-dependent vasodilator ACh (Conger *et al.*, 1991; Sobey *et al.*, 1992). However, the present study demonstrates that an ischaemia-induced impairment of the vasodilator responses to ACh is not necessarily associated with a substantial reduction or even loss of the basal release of NO. Ischaemic injury of the myocardium is also associated with a reduced vasodilator response to ACh (Sobey *et al.*, 1992) despite a normal formation of NO (McMurdo *et al.*, 1992). Although hypoxia impairs endothelium-dependent relaxation to ACh in the pulmonary vascular bed (Warren *et al.*, 1989), the basal release of NO after unilateral hypoxia in the pulmonary vasculature of the rabbit is sufficient to oppose hypoxic vasoconstriction resulting in a mismatching of ventilation to perfusion (Sprague *et al.*, 1992). At present the mechanism of the ischaemia-induced impairment of the responses to ACh is unclear. However, a reduced availability of L-Arg (Radermacher *et al.*, 1991) is unlikely to account for this impairment, for infusion of L-Arg did not restore the responses to ACh. Interestingly, more evidence is emerging indicating that the mechanisms that regulate basal and receptor stimulated release under physiological conditions are different (Hecker *et al.*, 1992; MacArthur *et al.*, 1993).

In addition to NO, vasodilator prostanoids, presumably prostacyclin, contribute to the maintenance of RBF following I/R injury of the kidney, as indomethacin caused a rise in RVR which was significant throughout the 120 min reperfusion period. It is well known that cyclo-oxygenase inhibitors have little or no effect on normal RBF, but do reduce RBF when the renal circulation is compromised (Oliver *et al.*, 1981; Stoff & Clive, 1983). Thus, endogenous release of cyclo-oxygenase products is a reserve mechanism, not increasing normal RBF but helping to maintain a damaged circulation. Endogenous release of NO, however, is important in maintaining normal RBF, for synthesis inhibitors substantially reduce it. The fact that NO synthase inhibitors also reduce RBF when the blood flow has been compromised by I/R injury shows that the NO release mechanism is still functional, although there is no evidence that it has been increased.

What then accounts for the I/R-induced increase in RVR observed in this study? Clearly, I/R injury is associated with the formation of vasoconstrictor agents, such as angiotensin II (Stein *et al.*, 1978) or endothelin-1 (Kon & Badr, 1991). For instance, infusion of anti-endothelin-1 antibodies into the renal artery largely attentuates the rise in RVR following I/R in the anaesthetized rat (Kon & Badr, 1991). Hence, it is

possible that an increased formation of endogenous vasoconstrictors such as endothelin-1 contributes to the I/R-induced impairment of RBF, while the formation of endothelium-derived vasodilator autacoids serve as an endogenous defense mechanism to maintain a sufficient RBF.

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Characterization of the increase in vascular permeability induced by vascular permeability factor *in vivo*

[1]P.D. Collins, *D.T. Connolly & T.J. Williams

Department of Applied Pharmacology, National Heart and Lung Institute, Dovehouse Street, London SW3 6LY and *Monsanto Company, St Louis, Missouri, U.S.A.

1 Vascular permeability factor (VPF) is a protein secreted from a variety of human and rodent tumour and normal tissue cells. In addition to mediating angiogenesis and endothelial cell growth, VPF has been reported to be a potent mediator of increased microvascular permeability *in vivo*. In this study we have investigated these permeability changes *in vivo* using a quantitative model of local plasma leakage in rabbit skin.

2 Our results reveal that VPF is a potent mediator of plasma leakage which, in the rabbit, depends on a synergistic interaction with arteriolar vasodilators such as prostaglandin E_2. The requirement for an exogenous vasodilator further suggests that VPF does not act to increase blood flow in this model.

3 We show that this response does not require the presence of circulating neutrophils and in this respect is similar to direct-action permeability increasing mediators such as histamine and bradykinin. Similarly, the time course of plasma leakage induced by VPF resembles that of direct-action mediators, where the greatest response occurs over the first 30 min. In contrast, the neutrophil-dependent plasma leakage induced by the active component of zymosan-activated plasma, $C5a_{des\ arg}$, was maintained at a similar level over 2.5 h.

4 Further, using mediator antagonists and enzyme inhibitors we demonstrate that the mechanism of action of VPF is not via activation of histamine, kinin, or platelet-activating factor pathways.

Keywords: Vascular permeability factor; permeability; vascular endothelial growth factor

Introduction

The growth of tumours is often associated with plasma extravasation from 'leaky' tumour vasculature (O'Connor & Bale, 1984; Brown *et al.*, 1988). Investigation of this phenomenon has revealed that several animal and human tumour cell lines secrete a vascular permeability factor, VPF (Senger *et al.*, 1983; 1986; Connolly *et al.*, 1989b; Clauss *et al.*, 1990). VPF is a 34–42 kDa glycoprotein which has an identical amino acid sequence to vascular endothelial growth factor (VEGF), a product of bovine folliculostellate cells (Ferrara & Henzel, 1989; Leung *et al.*, 1989). In addition to its effects on vascular permeability, VPF is a mitogen for endothelial cells (Connolly *et al.*, 1989a,b) and is able to induce angiogenesis *in vivo* (Connolly *et al.*, 1989a). The mechanisms by which VPF exerts its effects on microvascular permeability are unknown; however, recent *in vitro* studies have demonstrated an interaction between VPF and endothelial cells via specific binding sites (Connolly *et al.*, 1989a; Vaisman *et al.*, 1990; Brock *et al.*, 1991; Olander *et al.*, 1991). *In vivo*, increases in permeability induced by intradermal VPF have been visualized by the local accumulation of intravenously injected Evans blue dye (Senger *et al.*, 1983; Connolly *et al.*, 1989a,b; Keck *et al.*, 1989). In these studies recombinant human and guinea-pig VPF was reported to be active at a concentration of 1–3 nmol making it 5,000 times more potent than histamine (Connolly *et al.*, 1989b). At present, however, the biological profile of VPF as a mediator of increased microvascular permeability has not been quantified nor has its possible interaction with inflammatory cells, such as neutrophils, been investigated.

In this study, therefore, we have carried out detailed experiments to investigate possible mechanisms of increased microvascular permeability induced by VPF *in vivo*. The role of circulating neutrophils and the synergy between VPF and vasodilators is also addressed.

[1] Author for correspondence.

Methods

Human recombinant vascular permeability factor

The 165 amino acid-containing isoform of recombinant human VPF (Leung *et al.*, 1989) was used in these studies. This protein was prepared from the serum-free conditioned medium of baby hamster kidney (BHK) cells which had been stably transfected with a cDNA for VPF. High level expression of VPF was controlled by the herpes virus immediate early promoter in conjunction with co-expression of the herpes virus transactivator, VP-16 (Paul Hippenmeyer and Maureen Highkin, unpublished observations). The VPF protein was purified to homogeneity by cation exchange chromatography and reverse-phase chromatography. Purified VPF was prepared for injection by dilution to the appropriate concentration in saline containing 0.1 mg ml^{-1} BSA.

Rabbit skin bioassay

The pro-inflammatory properties of VPF were investigated by use of an established model in the rabbit to determine changes in microvascular permeability (Wedmore & Williams, 1981). Male rabbits (2.5–3.0 kg) were anaesthetized (Sagatal; sodium pentobarbitone, 30 mg kg^{-1}, i.v.) and the dorsal fur removed. Agents under test were injected intradermally into the dorsal skin in 6 replicate sites (100 µl/skin site) according to a balanced site injection plan. [^{125}I]-labelled human serum albumin ([^{125}I]-HSA; 2.5 µCi/animal) and Evans blue dye (2.5% w/v in sterile saline, 0.5 ml kg^{-1}) were injected intravenously as quantitative and visual markers respectively of plasma protein leakage into the sites. At the end of the measurement period, blood was collected by cardiac puncture and the animal killed with an overdose of anaesthetic (Expiral; sodium pentobarbitone, 200 mg kg^{-1}, i.v.). The dorsal skin was removed and excess tissue and blood separated. Skin sites (17 mm diameter) were punched out and radioactivity measured in a multiwell gamma counter. By comparison with the radioactivity associated with 1 ml of plasma, the plasma leakage into the site was calculated as µl plasma leakage/skin site.

Bioassay protocols

Synergy between vascular permeability factor and the vasodilator prostaglandin E_2 In these experiments [^{125}I]-HSA and Evans blue dye was given 10 min prior to the intradermal injections. VPF, formyl-methionyl-leucyl-phenylalanine (FMLP), bradykinin (BK), and rabbit zymosan-activated plasma (ZAP; a source of $C5a_{des\ arg}$) were injected intradermally at various concentrations either alone or in the presence of prostaglandin E_2 (PGE_2; 3×10^{-10} mol 100 µl/site). The response was measured over a 30 min period.

Neutrophil-dependency of the inflammatory response induced by vascular permeability factor Rabbits were treated 3 days before vascular permeability studies with a single injection of mustine hydrochloride (1 mg kg^{-1}; 1.75 ml kg^{-1}, i.v.) to deplete circulating neutrophils by greater than 98%. On day four [^{125}I]-HSA and Evans blue dye was given 10 min before intradermal injections. VPF, BK, histamine (Hist), FMLP, human recombinant C5a (HrC5a), and rabbit ZAP were each co-injected intradermally with PGE_2 and the response measured over 30 min.

Time course of the vascular permeability factor-induced inflammatory response

In these experiments the kinetics of plasma protein leakage induced by VPF, ZAP, and BK were investigated. VPF, BK, and rabbit ZAP were injected intradermally at various times (−240, −120, −60, −30, 0 min) prior to superimposed intradermal injections of PGE_2 at time zero. Intravenous administration of [^{125}I]-HSA and Evans blue dye was carried out at −2 min. The inflammatory response was then measured over a 30 min period to give the rate of leakage over time windows of 0–30, 30–60, 60–90, 120–150, and 240–270 min.

Effect of mediator antagonists, enzyme inhibitors and anti-vascular permeability factor antibodies on plasma leakage

Radiolabel and blue dye were administered 10 min before intradermal injection of VPF together with PGE_2 and in the presence or absence of either a platelet-activating factor (PAF) receptor antagonist WEB 2086, a bradykinin receptor antagonist Hoe 140, a histamine receptor antagonist, mepyramine, an inhibitor of kinin formation, trasylol, or a specific guinea-pig anti-human VPF antibody. These antagonists and inhibitors were also co-injected with their respective agonists, PAF, bradykinin, histamine, kallikrein, and human VPF (at doses selected to give sub-maximal responses) to demonstrate inhibition of the plasma leakage induced by these agents. VPF is a cationic protein and it has previously been demonstrated that highly charged peptides can induce plasma leakage (Needham *et al.*, 1988). We therefore investigated this possibility by co-injecting VPF with heparin (heparin was also co-injected with the polycation poly-L-lysine to demonstrate the inhibitory activity of heparin). The response was measured over 30 min.

Materials

[^{125}I]-human serum albumin ([^{125}I]-HSA, 50 µCi ml^{-1} in sterile saline) was from Amersham International plc, Amersham, Bucks. Bovine serum albumin (BSA, less than 0.1 ng endotoxin per mg protein), bradykinin (BK) histamine acid phosphate (Hist), poly-L-lysine (mol wt. 305,700–370,500), kallikrein, and prostaglandin E_2 (PGE_2) were from Sigma Chemical Company, Poole, Dorset. Mepyramine was from Glaxo Ltd, Ware, Hertfordshire. Trasylol was from Bayer UK Ltd, Newbury, Berkshire. Heparin was from CP Pharmaceuticals Ltd, Wrexham, Clwyd. Evans blue dye was from Merck Ltd, Dagenham, Essex. Hoe 140 (D-Arg^0-Hyp^3-Thi^5-D-Tic^7-Oic^8-bradykinin) was a generous gift from Dr K.J. Wirth, Hoeschst AG, Frankfurt, Germany. WEB 2086 (3-[4-(2-chlorphenyl)-9-methyl-6H-thieno [3,2-f] [1,-2,4]-triazolo-[4,3,a][1,4]-diazepin-[2-yl]-1-(4-morpholinyl)-1-propanone) was a generous gift from Boehringer-Ingelheim KG, Ingelheim am Rhein, Frankfurt, Germany.

Statistical analysis

Statistical significance was assessed by two-way analysis of variance (ANOVA). Normality of experimental data was tested by the Shapiro-Francia test and when necessary (significance of non-normality $P<0.10$), as detailed in the figure legends, $\log_{10}$-transformed data were analysed. A P value of <0.05 was considered significant, however, in all cases significant differences were at a level <0.01 as indicated by an asterisk in the relevant figures.

Results

Synergy between vascular permeability factor and prostaglandin E_2

Figure 1 demonstrates that when injected into rabbit skin, VPF alone induces little microvascular plasma protein leakage. A similar lack of a response can be seen when the inflammatory mediators BK, FMLP, and rabbit ZAP are injected alone. However, when VPF is co-administered with a vasodilator dose of PGE_2 a marked synergy results leading to a significant dose-dependent plasma leakage into the tissue. Comparison with BK, FMLP, and rabbit ZAP co-injected with PGE_2 indicate that VPF is the most active mediator in this model. Subsequent experiments were carried out with all agonists in the presence of PGE_2.

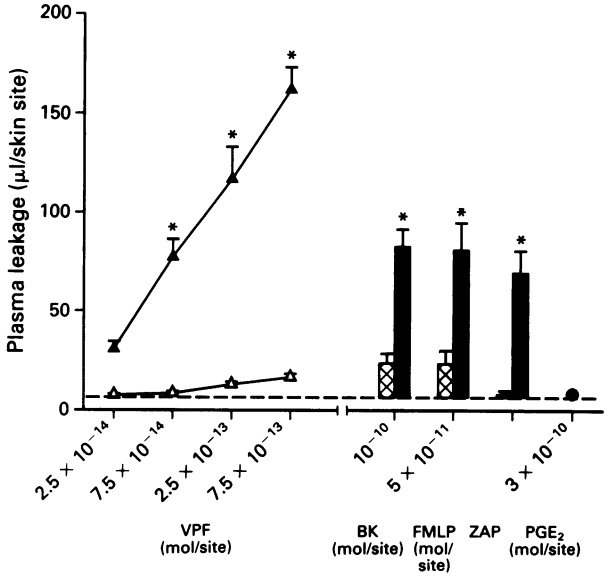


Figure 1 The synergistic interaction between vascular permeability factor (VPF) and prostaglandin E_2 (PGE_2); comparison with other mediators that increase microvascular permeability. VPF, bradykinin (BK), formyl-methionyl-leucyl-phenylalanine (FMLP), and zymosan-activated plasma (ZAP) were injected intradermally in the presence (▲, ■) and absence (△, ▩) of PGE_2 and plasma leakage measured over 30 min. The response to PGE_2 alone (●) and the basal response to intradermal saline (------) is also shown. Results are expressed as the mean plasma leakage ± s.e.mean from 5 separate experiments. Statistical analysis was carried out on untransformed data and * indicates a significant difference from the response to injection of saline alone ($P<0.01$).

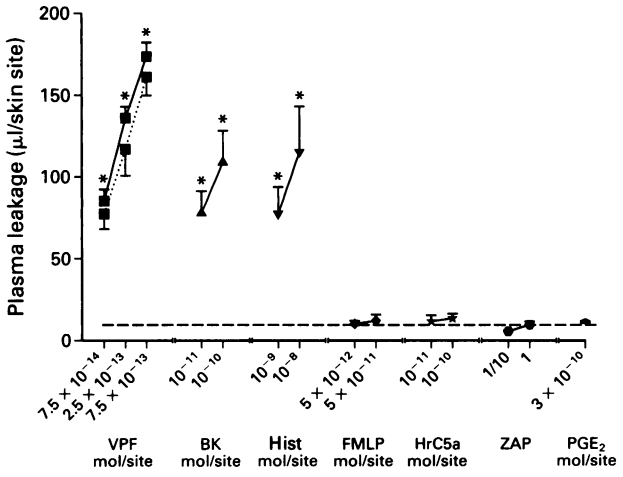


Figure 2 The effect of systemic neutrophil depletion on plasma leakage induced by vascular permeability factor (VPF). VPF (■), BK (▲), Hist (▼), FMLP (◆), HrC5a (★), and ZAP (● undiluted, 1; and diluted 10 fold in saline, 1/10) were injected intradermally in the presence of PGE_2 in animals depleted of circulating neutrophils. Hist = histamine; other abbreviations, see legend to Figure 1. Plasma leakage was measured over 30 min. For comparison the response to VPF + PGE_2 in normal animals is also shown (■····■) together with the response to PGE_2 alone (●) and saline (-----). The results are expressed as the mean plasma leakage/skin site ± s.e.mean from 4 separate experiments. Statistical analysis was carried out on $\log_{10}$-transformed data and * indicates a significant difference from the response to injection of PGE_2 alone ($P<0.01$).

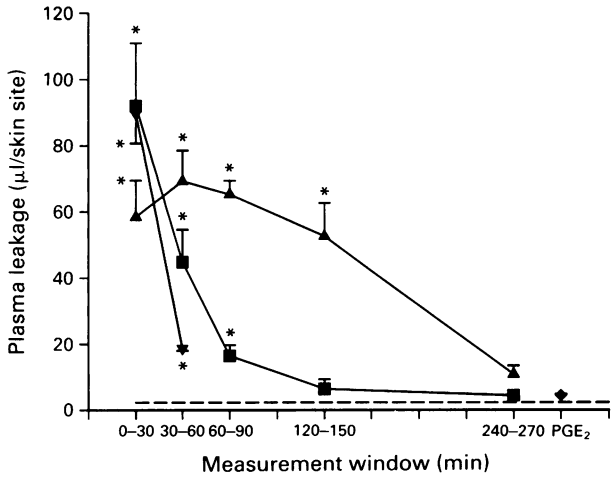


Figure 3 Kinetics of the plasma leakage induced by vascular permeability factor (VPF); comparison with rabbit zymosan-activated plasma (ZAP) and bradykinin (BK). VPF (■, 7.5×10^{-14} mol/site), ZAP (▲) and BK (▼, 5×10^{-10} mol/site) were injected intradermally at various times prior to a super-imposed injection of prostaglandin E_2 (PGE_2, 3×10^{-10} mol/site) at the beginning of the measurement period. Plasma leakage was measured over 30 min. The results are expressed as the mean plasma leakage/skin site ± s.e.mean from 5 separate experiments. The response to PGE_2 alone (◆) and saline (-----) is also shown. Statistical analysis was carried out on $\log_{10}$-transformed data and * indicates a significant difference from the response to injection of PGE_2 alone ($P<0.01$).

Neutrophil-dependency of plasma leakage induced by vascular permeability factor

The results in Figure 2 indicate that, despite depletion of circulating neutrophils, the plasma leakage in response to VPF is unaffected when compared to the response in normal animals. Similarly the increased permeability induced by the direct-action mediators BK and histamine is not inhibited. In contrast the chemoattractant mediators FMLP and rabbit ZAP, whilst active in normal animals (Figure 1), do not cause plasma leakage in these neutrophil-depleted rabbits (Figure 2).

Time course of the inflammatory response to vascular permeability factor

The time course of action of VPF as a mediator of increased microvascular permeability was compared with that of bradykinin, and the chemoattractant component of rabbit ZAP, C5a. Figure 3 shows that, like bradykinin, VPF induces a rapid plasma leakage which is maximal over the first 30 min. The VPF response then progressively declines so that at 2 h no response is observed. Comparison of the response to BK and VPF over 30–60 min indicates that BK has a shorter duration of action than VPF. In contrast, the leakage obtained in response to C5a has a rapid onset that is maintained for at least 2 h, returning to baseline by 4 h.

The effect of antagonists to bradykinin, PAF, and histamine on vascular permeability factor-induced plasma leakage

Co-injection of bradykinin, PAF, or histamine and their respective receptor antagonists (Hoe 140, WEB 2086, and mepyramine) inhibited the plasma leakage observed to the mediators alone (Figures 4, 5). In contrast the leakage induced by VPF was unaffected in the presence of the antagonists suggesting that none of these mediators or their receptors is involved in the plasma leakage response to VPF.

The effect of trasylol, heparin, and anti-human vascular permeability factor antibodies on the plasma leakage induced by VPF

The results displayed in Figure 5 demonstrate that trasylol, an inhibitor of kinin formation, totally inhibited the plasma leakage induced by kallikrein. In contrast, trasylol was with-

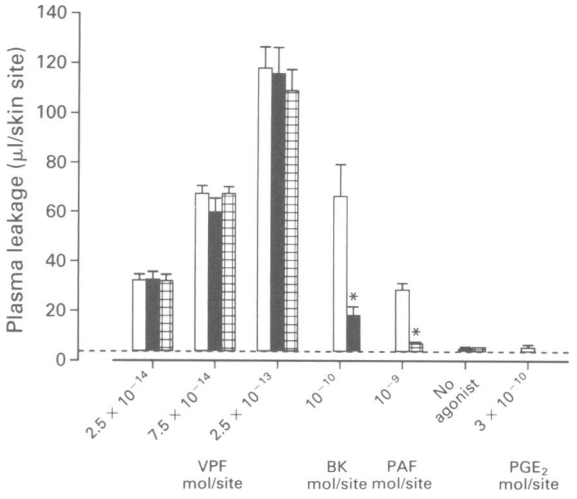


Figure 4 Effect of Hoe 140 and WEB 2086 on plasma leakage induced by vascular permeability factor (VPF). VPF was injected intradermally together with prostaglandin E_2 (PGE_2) alone (□) or in the presence of either the bradykinin antagonist Hoe 140 (■, 10^{-9} mol/site) or the platelet-activating factor (PAF) antagonist WEB 2086 (▦, 10^{-7}mol/site). Bradykinin and PAF were injected intradermally with PGE_2 in the presence (■, ▦) and absence (□) of their respective antagonists. Plasma leakage was measured over 30 min. The results are expressed as the mean plasma leakage/skin site ± s.e.mean from 3 separate experiments. The response to PGE_2 alone (□) and saline (-----) is also shown. Statistical analysis was carried out on untransformed data and * indicates a significant difference between the response to injection of agonist alone and agonist + antagonist ($P<0.01$).

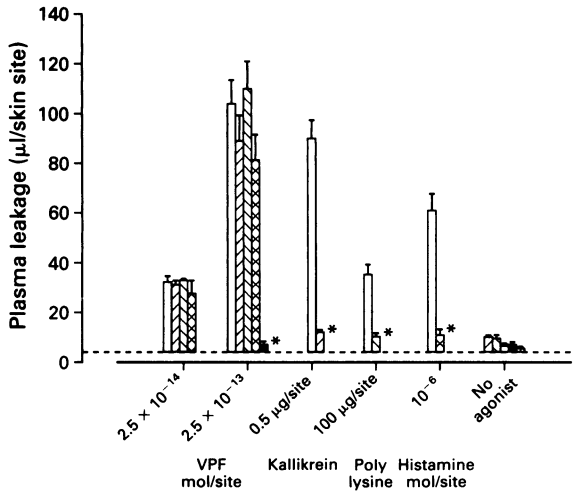


Figure 5 The effect of mepyramine, heparin, trasylol and anti-vascular permeability factor (VPF) antibody on plasma leakage induced by VPF. VPF was injected together with prostaglandin E_2 (PGE_2, 3×10^{-10} mol/site) alone (□) or in the presence of mepyramine (3×10^{-9} mol/site; ⊠), heparin (100 u/site; ▨), trasylol (100 µg/site; ▨), or an anti-VPF antibody (1/3000 dilution in saline; ■). Histamine, poly-L-lysine and kallikrein were injected with PGE_2 in the absence (□) or presence (⊠, ▨, ▨) of their respective antagonists or inhibitors. Plasma leakage was measured over 30 min. The response to PGE_2 alone (□) and saline (-----) is also shown. Results are expressed as the mean plasma leakage/skin site ± s.e.mean from 3–5 separate experiments. Statistical analysis was carried out on untransformed data and * indicates a significant difference between the response to the agonist alone and the agonist + appropriate antagonist ($P < 0.01$).

out effect on the response induced by VPF indicating that VPF does not function via the *in vivo* generation of kinins. Similarly heparin, whilst abolishing the response obtained to the poly-cation poly-L-lysine (average mol wt. = 341,000), had no inhibitory action on the response to VPF. This would indicate that the cationic nature of VPF is not responsible for the plasma leakage observed. The lack of effect of the inhibitors and antagonists was in marked contrast to the total inhibition of VPF-induced plasma leakage following co-injection with an anti-human VPF antibody (1/3000 dilution). In other studies this antibody did not inhibit the plasma leakage induced by histamine, bradykinin, or C5a at a 1/300 dilution (data not shown).

Discussion

Mediators of increased microvascular permeability have been classically divided into two distinct groups (Wedmore & Williams, 1981). This separation is based on the dependency of the response on the interaction between circulating neutrophils and the local post-capillary venular endothelium. Thus, chemoattractant mediators such as C5a, FMLP, and interleukin-8 form one group, whilst the direct-action mediators exemplified by bradykinin and histamine comprise the second group. Other early studies have demonstrated that the increased microvascular permeability induced by both neutrophil-dependent and direct-action mediators is potentiated via a synergistic interaction with agents that produce a local pre-capillary arteriolar vasodilatation. These vasodilators include prostaglandins (PGE_1, PGE_2, PGI_2) (Williams & Morley, 1973; Williams & Peck, 1977; Wedmore & Williams, 1981; Rampart & Williams, 1986) and neuropeptides (calcitonin gene-related peptide, vasoactive intestinal polypeptide) (Williams, 1982; Brain *et al.*, 1985; Brain & Williams, 1985). In this study we have demonstrated that human rVPF can be classed as a direct-action mediator since it induces plasma leakage in the absence of circulating neutrophils. Further evidence for this classification comes from studying the kinetics of the response. Unlike the neutrophil-dependent plasma leakage induced by $C5a_{des\ arg}$ (the active component of ZAP) which is similar over the first 2.5 h, the response to VPF is maximal in the first 30 min, decreasing to basal levels by 2 h. In this respect the profile of the response is similar to that of the direct-action mediator bradykinin which in the time course study also produces maximal plasma leakage over the first 30 min.

In addition, increased microvascular permeability in rabbit skin induced by VPF is entirely dependent on the presence of a vasodilator, in this case exogenous PGE_2. This would suggest that VPF itself does not increase local blood flow in this model, nor does it appear to induce the endogenous production of vasodilators from the local tissue. In contrast, induction of local plasma leakage in the guinea-pig skin does not require the presence of an exogenous vasodilator (Williams & Piper, 1980) due to higher basal blood flow. In this species it has been demonstrated that plasma leakage in response to VPF occurs at a similar concentration (2×10^{-13} mol/site) in the absence of any exogenous vasodilator (Senger *et al.*, 1990). In rabbit skin, at a VPF dose of 7.5×10^{-14} mol/site, a plasma leakage response was induced equivalent to that elicited by the direct-action mediator bradykinin and the neutrophil-dependent mediator FMLP at doses 1300 and 700 times greater respectively.

As part of the investigations into the mechanism of action of VPF the role of other endogenous mediators was studied. One possibility was that VPF caused the release of histamine, kinins, or PAF, or alternatively that VPF acted as an agonist at their receptors. Using specific antagonists we demonstrated almost complete inhibition of plasma leakage induced by sub-maximal doses of histamine, kallikrein, and PAF. There was, however, no effect on the response elicited by VPF, suggesting that neither these mediators nor their receptors are involved in the action of VPF. This would imply the presence of distinct binding sites for VPF *in vivo*, as has been demonstrated *in vitro* on capillary and large vessel endothelial cells from bovine and human tissues (Connolly *et al.*, 1989a; Vaisman *et al.*, 1990; Olander *et al.*, 1991; Gitay-Goren *et al.*, 1992). Indeed, activation of endothelial cells by VPF has been demonstrated by Brock *et al.* (1991) who showed that VPF stimulates a delayed and transient rise in $[Ca^{2+}]_i$, inositol triphosphate formation and von Willebrand factor release.

In addition to demonstrating that the actions of VPF did not appear to be associated with its cationic nature, we observed that co-injection of VPF with heparin did not modulate the leakage response. Gitay-Goren *et al.* (1992) found enhanced binding of VPF to endothelial cells in the presence of heparin and an inhibition of binding following treatment with heparinase suggesting a dependence on heparin for VPF binding. In our study it is possible that the lack of effect of heparin on the response is due to the presence of endogenous heparin associated with the VPF binding site in our *in vivo* model.

The presence of specific receptors for VPF on endothelial cells may suggest that this protein is released from non-tumour cells. It has recently been reported by Berse *et al.* (1992) using *in situ* hybridization techniques in guinea-pig tissue that in addition to secretion of VPF from tumour cells, VPF is expressed in epithelial cells within the pulmonary alveolar wall, the kidney glomerular loop and the outer cortex of the adrenal gland. In the heart, expression is associated with cardiac myocytes. VPF expression has also been detected in elicited guinea-pig peritoneal macrophages but not resident cells implying that activation of the cell is required for VPF generation.

The potency of VPF and the distribution of cells able to secrete this protein make it a candidate as a homeostatic regulator of microvascular permeability under physiological conditions. The release of VPF from activated macrophages may indicate an additional role as a component of the

inflammatory response, forming part of the host defence function of these cells.

Further studies are necessary to determine whether VPF has an important endogenous function and if the association between VPF and tumour cell growth represents a target for therapeutic intervention.

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Pharmacology of GABA ρ1 and GABA α/β receptors expressed in *Xenopus* oocytes and COS cells

T. Kusama, C.E. Spivak, ‡P. Whiting, V.L. Dawson, *J.C. Schaeffer & †[1]G.R. Uhl

Molecular Neurobiology, Addiction Research Center, National Institute on Drug Abuse, Baltimore, Maryland 21224 and †Departments of Neurology and Neuroscience, Johns Hopkins University School of Medicine, Baltimore, Maryland 21224, U.S.A.; ‡Neuroscience Research Centre, Merck Sharp & Dohme Research Laboratories, Harlow, Essex, England; *Department of Chemistry, California State University, Northridge, CA 91330, U.S.A.

1 The ρ1 protein, which we previously cloned from retina, assembles as a homooligomer that transduces the binding of γ-aminobutyric acid (GABA) into robust chloride currents. However, its insensitivity to bicuculline, pentobarbitone and benzodiazepines, all potent agents at typical $GABA_A$ receptors, suggested that it may react atypically to other GABA agonists and antagonists.

2 cDNAs for the ρ1 and the $\alpha_5\beta_1$ receptors for GABA were expressed as homo- and heterooligomers, respectively, in *Xenopus* oocytes. The selectivities of the respective receptors for various agonists were investigated using concentration-response experiments in voltage clamped cells.

3 The most potent agonists at the ρ1 receptor were *trans*-4-aminocrotonic acid (TACA) > GABA > muscimol; at the $\alpha_5\beta_1$ receptor the rank order was muscimol > GABA > 4,5,6,7-tetrahydroisoxazole[4,5-c]pyridine-3-ol (THIP). The most specific agonists were *cis*-(2-(aminomethyl)-cyclopropylcarboxylic acid (CAMP) and THIP for the ρ1 and the $\alpha_5\beta_1$ receptors, respectively.

4 Comparing GABA, TACA and *cis*-aminocrotonic acid (CACA) at ρ1 receptors expressed in COS cells gave results almost indistinguishable from those found at oocytes; the pharmacology of ρ1 seems independent of the expression system.

5 Agonists THIP, piperidine-4-sulphonic acid (P4S), and isoguvacine, whose C-C-C-N chains are constrained by rings into a folded conformation and were potent at the $\alpha_5\beta_1$ receptor, were among the weakest at the ρ1 receptor. However CACA and CAMP, which align better with the extended than the folded conformation, were weakest at the $\alpha_5\beta_1$ receptor but moderately potent at the ρ1 receptor. These findings suggest that the ρ1 receptor recognizes agonists in the extended conformation, in contrast to $GABA_A$ receptors, which are believed to recognize agonists in the partially folded conformation.

6 In contrast to the $\alpha_5\beta_1$ receptor, gradations in maximum responses were apparent in the ρ1 receptor, suggesting various degrees of partial agonism. In particular, imidazole-4-acetic acid (I4AA), whose maximum response was only 3% of GABA's maximum, had an apparent K_d for activating the ρ1 receptor of 16 μM; but it had an apparent K_d for competitively blocking the receptor of 0.64 μM. This difference suggests that steric constraints in the activated (open channel) receptor are tighter than in the resting receptor.

7 Hill coefficients approached 2 at the ρ1 receptor, but were closer to unity at the $\alpha_5\beta_1$ receptor. Thus, the ρ1 receptor displayed higher cooperativity.

8 Unlike typical $GABA_A$ receptors, the ρ1 receptor was insensitive to the competitive antagonists bicuculline, SR95531, securinine, and (+)-tubocurarine.

Keywords: GABA ρ1 receptor; $GABA_A$ receptor; oocyte; COS cells; GABA agonist; partial agonist

Introduction

The subunits that form receptors for the major brain inhibitory transmitter, γ-aminobutyric acid (GABA), can combine in a number of different fashions to form GABA-gated chloride channels with an array of distinct pharmacological and functional properties. Many GABA receptors are likely to be heterooligomeric, based on evidence from protein purification studies and from studies of the function of receptors reconstituted from individual cloned subunits. Receptors containing α subunits alone, for example, rarely express full function, but α/β heterooligomers can transduce the recognition of GABA into ion channel alterations yielding nearly-full functional choride currents (e.g. Sigel *et al.*, 1990; see reviews by DeLorey & Olsen, 1992; Olsen & Tobin, 1990; Burt & Kamatchi, 1991).

Homooligomers formed after expression of one of the newest members of the GABA receptor subunit family, ρ1, express robust GABA-mediated chloride conductance (Shimada *et al.*, 1991). Many of its properties are virtually identical to those exhibited by bicuculline-resistant GABA responses induced in *Xenopus* oocytes injected with mRNA extracted from a rich source of ρ1, the retina (Polenzani *et al.*, 1991). Thus: (1) many heterooligomeric GABA receptors with properties conferred by each of the different α, β, γ, and/or δ members of these different protein complexes could exist in brain and retina, and (2) GABA ρ1 homooligomers could exist *in vivo* and may provide the simplest *in vitro* system in which to test direct relationships between the structure of a single subunit and a GABA receptor's recognition of agonist, antagonist, and allosteric modulating drugs.

Different brain and retinal GABA receptors display distinct pharmacological profiles that are likely to be correlated with heterogeneous complements of GABA receptor subunits. One nomenclature that describes this heterogeneity subdivides GABA receptors into $GABA_A$ receptors, defined as encompassing GABA-gated ion channels, and $GABA_B$ receptors, defined as the 'metabotropic', G-protein linked receptors that are sensitive to baclofen (Bowery *et al.*, 1991). An alternative nomenclature defines $GABA_A$ receptors as those displaying bicuculline sensitivity, $GABA_B$ receptors as those that are insensitive to bicuculline but sensitive to baclofen, and $GABA_C$ receptors as those that are insensitive to both baclofen and bicuculline (Johnston, 1986). Selectivity

[1] Author for correspondence.

for *cis*-aminocrotonic acid (CACA) could constitute a second definition for $GABA_C$ receptors (Drew *et al.*, 1984).

Initial studies of the pharmacological properties of the GABA ρ1 homooligomer revealed its striking resistance to the competitive GABA antagonist, bicuculline, and suggested that the GABA recognition site for the ρ1 homooligomer might display GABA recognition substantially different from that of other GABA ligand-gated ion channel receptors. We have now characterized the potencies of a series of agents at GABA recognition sites displayed on ρ1 homooligomers and on $GABA_A$ α/β heterooligomeric receptors expressed in *Xenopus* oocytes. These results support the notion that the ρ1 homooligomeric receptor may recognize GABA and related agents in configurations corresponding to the 'extended' conformation of GABA, while agents that are constrained to mimic the 'partially folded' conformation of GABA show much lower ρ1 receptor potencies.

Methods

The cDNA for GABA ρ1 (Cutting *et al.*, 1991) was directionally cloned into the mammalian expression vector, pcDNA1 (Invitrogen, CA, U.S.A.) by standard methods, to produce pcDNA1-ρ1. The cDNAs encoding human α_2, α_5, β_1 and δ_{2L} cDNA were cloned into the expression vector pcDM8.

Adult female *Xenopus*, obtained from Nasco (Fort Atkinson, WI, U.S.A.) and Xenopus 1 (Ann Arbor, MI, U.S.A.), were anaesthetized with ice. The oocytes were removed and gently agitated for 1 h in Ca^{2+}-free ND96 solution (composition, mM: NaCl 96, KCl 2, $MgCl_2$ 1, N-(2-hydroxyethyl)-piperazine-N'-(2-ethanesulphonic acid) (HEPES) 5, pyruvate 2.5, pH 7.5) with 2 mg ml^{-1} collagenase (Clostridiopeptidase A; EC 3.4.24.3; from Sigma Chemical Co., Type IA·S). Stage 5 and 6 oocytes were manually defolliculated and injected intranuclearly with 10 nl of water, 10–50 ng of ρ1, or 10 ng of equimolar amounts of $GABA_A$ α_5 and β_1 expression vector dissolved in 10 nl of sterile water by use of a positive-displacement pipette (Drummond). The expression vector was injected just below the surface into the nucleus by piercing the oocyte at the pole of the animal hemisphere. Oocytes were incubated for 2–7 days at 19°C in ND96 medium (containing 1.8 mM $CaCl_2$, 50 u ml^{-1} penicillin, and 50 μg ml^{-1} streptomycin), which was changed daily (Uhl *et al.*, 1991). Oocyte responses to bath application of various drugs were recorded under two electrode voltage clamp at −70 mV, using an S-7000A amplifier (World Precision Instruments, Sarasota, FL, U.S.A.). Micropipettes were filled with 3 M KCl, as described by Shimada *et al.* (1991). ND96 with 1.8 mM $CaCl_2$ but without pyruvate perfused the clamped oocyte at 2 ml min^{-1}. Agonists, dissolved in this medium, were applied until the maximum current was attained. The oocyte was washed for between 5 and 30 min to permit complete recovery of the response to 10 μM GABA.

COS-7 cells (kidney cells from African green monkey, transformed by SV40; American Type Culture Collection, Rockville, MD, U.S.A.) were grown, transfected by electroporation, and allowed to express for 48 to 72 h as previously described (Schaeffer *et al.*, 1991). Transfected COS cells were tested in Dulbecco's phosphate buffered saline (Composition: mM: NaCl 137, KCl 2.68, KH_2PO_4 1.47, Na_2HPO_4 8.1, $MgCl_2$ 0.49, $CaCl_2$ 0.90, glucose 5.55) under whole cell voltage clamp (Hamill *et al.*, 1981) at −60 mV using a List EPC-7 amplifier. Recording micropipettes contained (in mM): CsCl 140, $CaCl_2$ 1.0, HEPES 1.0, ethylene glycol-*bis*(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA) 11.0, pH = 7.2. The 6–20 MΩ access resistance was compensated 50–90%. Concentrations of agonists were superfused in random order by means of a microcapillary attached to a U-tube (Murase *et al.*, 1989). Signals were recorded by an FM tape recorder and displayed by a strip chart recorder. All concentration-response data, except as noted, were fitted by nonlinear regression (MLAB, Civilized Software, Bethesda, MD, U.S.A.) to Equation 1,

$$I = \frac{I_m}{1 + \left(\frac{K_a}{[A]}\right)^n} \qquad \text{Equation 1}$$

in which $I \equiv$ measured current, $I_m \equiv$ maximum current, $K_a \equiv$ apparent dissociation constant for the agonist, $[A] \equiv$ agonist concentration, and $n \equiv$ Hill coefficient. Molecular graphics illustrations were constructed by use of the CAMSEQM programme (Weintraub Software Design Associates, Inc., Cincinnati, OH, U.S.A.).

Isonicotinic acid, isonipecotic acid, 3-aminobenzoic acid, and 5-amino-1,3-cyclohexadiene-1-carboxylic acid (gabaculine) were obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). 3-Aminopropylphosphonic acid, 3-amino-1-propanesulphonic acid, GABA, β-alanine, baclofen, bicuculline, 2,4-diaminobutyric acid (DABA), glycine, glutamic acid, homocarnosine, imidazole-4-acetic acid (I4AA), 4-hydroxybutyric acid (GHBA), muscimol, picrotoxin, securinine, taurine, and (+)-tubocurarine were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). Isoguvacine, 2-(3-carboxypropyl)-3-amino-6-(4-methoxyphenyl) pyridazinium bromide (SR-95531), guvacine, kojic amine, piperidine-4-sulphonic acid (P4S), and 4,5,6,7-tetrahydroisoxazole[4,5-c] pyridine-3-ol (THIP) were obtained from Research Biochemicals, Inc. (Natick, MA, U.S.A.). CACA, *trans*-4-aminocrotonic acid (TACA), and *cis*-(Z)-3-[(aminoiminomethyl)thio]prop-2-enoic acid (ZAPA) were obtained from Tocris Neuramin (Bristol, England).

Cis-(2-(aminomethyl)-cyclopropylcarboxylic acid (CAMP) was prepared from *cis*-1,2-cyclopropanedicarboxylic anhydride. The anhydride was converted to the corresponding lactone by sodium borohydride reduction. Treatment of the lactone with potassium phthalimide in dimethylformamide gave the phthalimide derivative. Hydrazinolysis of this compound gave a solid that, upon recrystallization from ethanol/water, gave pure CAMP: m.p. 202–204°C (dependent upon heating rate); t.l.c. (butanol/acetic acid/water, 25:4:10 on silica gel) gave a single ninhydrin positive spot, R_F 0.4; infrared spectrum: ν_{max} 1565 cm^{-1} (carboxylate). Proton n.m.r. spectrum (D_2O) δ 0.65–1.9 (4 H, m), 3.1 (2 H,d). Elemental analysis: C, 52.01; H, 7.65; N, 12.07%. $C_5H_9NO_2$ requires C, 52.16; H, 7.88; N, 12.17%.

Trans-(2-(aminomethyl)-cyclopropylcarboxylic acid (TAMP) was synthesized from *trans*-1,2-cyclopropanedicarboxylate. The dicarboxylate was converted to the half methyl ester by treatment with sodium in methanol. Methyl *trans*-2-carboxycyclopropanecarboxylate was reduced to the corresponding hydroxymethyl compound by treatment with ethyl chloroformate. This material was converted to the methanesulfonate derivative that was immediately reacted with potassium phthalimide in dimethylformamide to yield methyl *trans*-2-phthalimidomethylcyclopropanecarboxylate. Treatment of this compound with refluxing 4 M HCl for 16 h gave a crude product that was washed thoroughly with boiling acetone to yield TAMP●HCl: m.p. 161–163°C; t.l.c. (butanol/acetic acid/water, 25:4:10, on silica gel) gave a single ninhydrin positive spot, R_F 0.4. Infrared spectrum: ν_{max} 1690 cm^{-1} (carboxylate). Proton n.m.r. spectrum (D_2O, δ) 0.8–1.9 (4 H, m), 2.88 (2 H, d). Elemental analysis: C, 39.67; H, 6.75; N, 9.29%. $C_5H_{10}ClNO_2$ requires C, 39.61; H, 6.65; N, 9.24%.

The syntheses of CAMP and TAMP via different routes have been described previously (Allan *et al.*, 1980).

Results

Intranuclear injection of cDNAs for the ρ1 or the $\alpha_5\beta_1$ heterooligomer into oocytes yielded pharmacological charac-

teristics that were similar to those obtained after cytoplasmic injection of *in vitro* transcribed mRNAs encoded by each of these cDNAs (Cutting *et al.*, 1991; Shimada *et al.*, 1992). Intranuclear injection of ρ1 cDNA elicited GABA responses with an EC_{50} of 1.7 μM that were unaffected by either bicuculline or benzodiazepine at concentrations up to 100 μM. Conversely, coinjections of $\alpha_5\beta_1$ yielded dramatically different patterns of sensitivity, with potent responses to GABA, pentobarbitone, bicuculline, picrotoxin, and some responsiveness to diazepam (data not shown). Oocytes injected with water alone were unreactive.

As previously described, injection of ρ1 alone into oocytes yielded an expressed receptor with a unique pharmacological profile. A number of agents with GABA-like structures had full, or near full activity at the ρ1 homooligomeric receptor (Figure 1a and Table 1). These included GABA, TACA, CACA, CAMP, isoguvacine, and muscimol. Agents that lacked full intrinsic activity but displayed significant activities included, in approximately descending rank order, β-alanine, isonipecotic acid, TAMP, kojic amine, isonicotinic acid, homocarnosine, I4AA, and guvacine. There were only feeble or no responses (<2% of GABA's maximum) to 1 mM THIP, P4S, nipecotic acid, 3-aminobenzoic acid, gabaculine, DABA, GHBA, baclofen, glycine, glutamate, taurine, ZAPA, 3-amino-1-propanesulphonic acid, and 3-aminopropylphosphonic acid.

TACA reproducibly demonstrated the highest potency at this site, with an apparent K_d value of 0.62 ± 0.05 μM. Pairwise comparisons of the relative potencies of structurally similar geometric isomers revealed interesting results. The more extended TACA was more than two orders of magnitude more potent than the more folded CACA at the ρ1 homooligomeric receptor. Similarly, the more extended TAMP had a 3 fold higher potency than the more folded CAMP. Interestingly, the otherwise potent $GABA_A$ agonist, muscimol, displayed an affinity lower than that of GABA itself. THIP, with an even more rigidly constrained conformation that includes the core muscimol structure, displayed virtually no activity.

This profile contrasted with that obtained using $\alpha_5\beta_1$ heterooligomeric receptors (Figure 1b and Table 1). Potencies at these $GABA_A$ receptors followed the rank order muscimol->GABA>TACA. CACA displayed very low potency. THIP, P4S, and isoguvacine each had potencies in the same range of GABA itself. Interestingly, in this system, TAMP was more potent than CAMP, which was virtually inert.

This relative rank order of potencies: GABA>TACA> CACA was preserved in other α, β and γ subunit combinations. Combined injection of cDNA for α_5, β_1 and γ_{2L} showed TACA to be as potent as GABA, with CACA less than 5% as potent (data not shown). Similarly, at expressed α_2, β_1 and

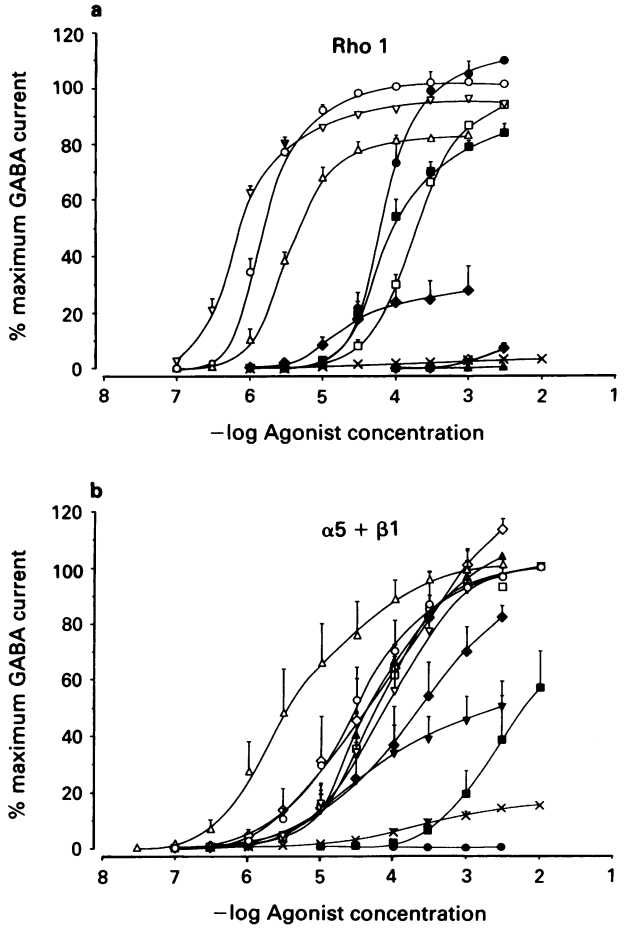


Figure 1 Log concentration-response curves for GABA ρ1 (a) and $\alpha_5\beta_1$ (b) as expressed in *Xenopus* oocytes. Agonists used were GABA (○), TACA (▽), CACA (■), muscimol (△), isoguvacine (□), CAMP (●), TAMP (◆), THIP (▲), P4S (▼), ZAPA (◇), and I4AA (X). Oocytes were voltage clamped at − 70 mV, and agonists were applied via the superfusing medium until the maximum current was reached. Several curves could be obtained from each oocyte. To compile data from several experiments, data from each oocyte were first fitted to Equation 1 by nonlinear regression, then the responses to each agonist tested at that oocyte were normalized to the GABA response, whose I_m was defined as 100%. Means and s.e.mean are shown. For abbreviations, see text.

Table 1 Comparison of various parameters of agonists at ρ1 and $\alpha_5\beta_1$ receptors expressed in *Xenopus oocytes*

Agonists	(n) (R, A)	K_d (μM)			*Relative* I_m		*Hill n*	
		ρ1	$\alpha_5\beta_1$	A/R	ρ1	$\alpha_5\beta_1$	ρ1	$\alpha_5\beta_1$
GABA	(13, 15)	1.7 ± 0.2	52 ± 10	30	1.00	1.00	2.1 ± 0.1	1.1 ± 0.1
Muscimol	(4, 5)	3.5 ± 0.4	20 ± 12	5.8	0.83 ± 0.01	1.01 ± 0.004	1.6 ± 0.5	1.1 ± 0.1
TACA	(6, 3)	0.62 ± 0.05	88 ± 34	140	0.90 ± 0.02	0.99 ± 0.03	1.6 ± 0.1	1.1 ± 0.2
TAMP	(4, 4)	20 ± 5	370 ± 170	18	0.25 ± 0.07	0.96 ± 0.04	1.7 ± 0.1	0.9 ± 0.1
CACA	(6, 3)	74 ± 17	9700 ± 5500	130	0.78 ± 0.04	0.97 ± 0.05	1.8 ± 0.2	1.1 ± 0.2
CAMP	(5, 3)	68 ± 8	> 10,000	> 150	1.07 ± 0.04	–	2.0 ± 0.2	–
I4AA	(3, 3)	16 ± 4	70 ± 19	4.5	0.033 ± 0.008	0.15 ± 0.08	1.0 ± 0.1	1.1 ± 0.1
Isoguva.	(4, 3)	140 ± 40	85 ± 35	0.62	0.72 ± 0.06	1.05 ± 0.06	1.5 ± 0.1	1.0 ± 0.1
ZAPA	(4, 4)	> 10,000	190 ± 90	< 0.02	–	1.22 ± 0.06	–	0.9 ± 0.1
P4S	(4, 3)	> 10,000	62 ± 40	< 0.006	–	0.47 ± 0.07	–	0.9 ± 0.01
THIP	(4, 4)	> 10,000	55 ± 19	< 0.005	–	0.97 ± 0.08	–	1.2 ± 0.1

Values are given as means ± s.e.mean for the number of oocytes given in columns 2 and 3 for ρ1 and $\alpha_5\beta_1$ receptors, respectively. The A/R ratio, given in column 6, was obtained by dividing the dissociation constant for the $\alpha_5\beta_1$ by that for the ρ1 receptor. This ratio shows that the cyclic agonists, P4S, and THIP as well as ZAPA, are specific for the $\alpha_5\beta_1$ receptor, whereas CAMP is highly specific for the ρ1 receptor. For abbreviations, see text.

γ_{2L} subunits, TACA and GABA were about equipotent, whereas CACA was less than 3% as potent (data not shown).

Hill coefficients differed between the ρ1 homooligomers and the $\alpha_5\beta_1$ heterooligomers (Table 1). The Hill coefficient for ρ1 was close to 2, indicating significant positive cooperativity, while coefficients for $\alpha_5\beta_1$ receptors, around unity, failed to provide evidence for such cooperativity. Maximal responses elicited at ρ1 receptors also displayed gradations in I_m values (Figures 1 and 2; Tables 1 and 2). Thus TACA was more potent than GABA, but its I_m value was around 10% lower. TAMP was about three times as potent as CAMP. However, TAMP's I_m was only 25% that of GABA while CAMP's I_m was at least as great as that of GABA itself (Table 1).

Several of these pharmacological features could also be demonstrated in an independent COS cell expression system. For GABA the apparent K_d was 1.7 ± 0.3 μM (s.e.mean), and the Hill coefficient was 1.9 ± 0.3 (s.e.mean) for 6 experiments. Figure 2a shows a representative TACA concentration-response curve with points closely spaced to optimize the precise estimation of the apparent K_d and n. Figure 2b represents experiments aimed at determining if differences in I_m for TACA and CACA were significant; data were sampled at high drug concentrations. All these results, indistinguishable from those obtained from oocytes (Tables 1 and 2), indicated that the unique pharmacology displayed by the ρ1 receptor was independent of constitutive proteins resident in one of these expression systems and unchanged by possible differences in post-translational modifications.

Most of the weak agonists studied could block the effects of more potent agonists, suggesting partial agonist properties. Imidazole-4-acetic acid (I4AA) displayed maximal agonist responses only 3% of those reached by GABA (Table 1), but it shifted the GABA dose-response curve to the right. For a simple competitive model the following function holds:

$$I(A,B) = \frac{I_m}{1 + \left(\frac{K_a}{[A]}\right)^n + \left(\frac{K_a}{[A]}\right)^n \left(\frac{[B]}{K_b}\right)^m} \quad \text{Equation 2}$$

in which [B] is the concentration of the antagonist, K_b is its apparent dissociation constant, and m the corresponding Hill coefficient; the rest of the symbols are as in Equation 1 (Methods).

In the experiment shown in Figure 3, $I_m = 212$ nA; $K_a = 1.86$ μM; $K_b = 0.58$ μM; n = 2.44; and m = 1.44. A sub-model, in which m was constrained to unity was rejected (*F*-test; $P << 0.001$). Plots predicted by this relationship, shown in Figure 3, are in excellent agreement with the observed data. The mean K_b values for three experiments, 0.64 ± 0.17 μM (s.e.mean), were thus about 25 times smaller than the apparent affinities of I4AA acting as an agonist (Table 1). In an analogous experiment using the $\alpha_5\beta_1$ recep-

Table 2 Comparison of agonist parameters of *trans*-4-aminoacrotonic acid (TACA) and *cis*-aminocrotonic acid (CACA) at voltage clamped COS cells

Agonist	K_d (μM)	*Hill n*	*Relative* I_m (CACA:TACA)
TACA	0.72 ± 0.13 (6)	1.9 ± 0.3 (7)	
			0.88 ± 0.03 (4)
CACA	77. ± 8. (6)	2.0 ± 0.2 (7)	

COS cells transfected with ρ1 were held at −60 mV by whole cell voltage clamp. Both agonists were applied in random order to each cell with a microsuperfuser attached to a U-tube. Parameters were found by fitting currents to Equation 1 by nonlinear regression. Means of different cells (number in parentheses) ± s.e.mean are shown.

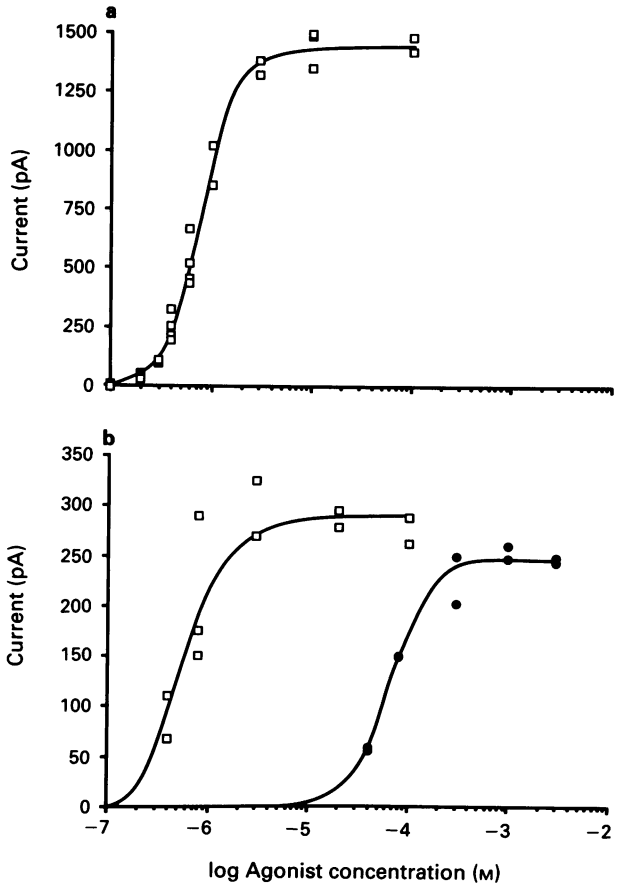


Figure 2 Concentration-response curves for TACA (□) and CACA (●) at whole cell voltage clamped COS cells transfected with ρ1 cDNA. The two panels exemplify two different protocols used. In (a) data points are sampled more heavily at the mid and lower range to maximize the precision of the estimates of the apparent K_d and the Hill coefficient, n. In (b) data points are sampled mostly at the high concentration ends of the curves to maximize the precision of the estimates of I_m, which differed between the two agonists. All data in each panel were collected from a single cell. Agonists were applied by microsuperfusion in random order. For abbreviations see text.

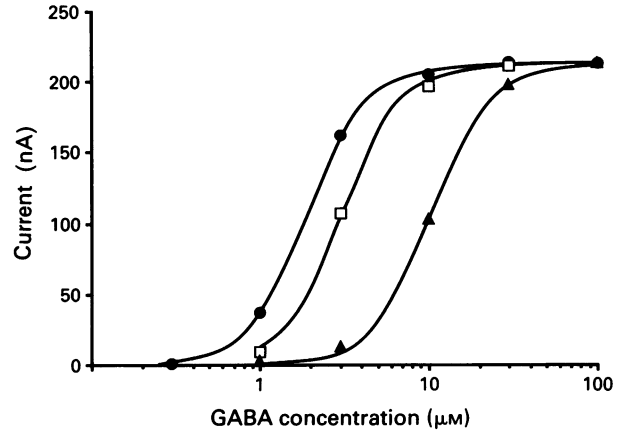


Figure 3 Concentration-response curves for GABA alone (●) and GABA plus 1 μM (□) or 10 μM (▲) imidazole-4-acetic acid at the ρ1 receptor expressed in a single oocyte. Imidazole-4-acetic acid had no observable activity alone at the concentrations used in this experiment. The curves are predicted values based on competitive antagonism, as expressed in Equation 2. All points were fitted simultaneously.

tor, $k_b = 90\,\mu M$, which was close to its apparent dissociation constant as an agonist here (Table 1). In one experiment, the partial agonist TAMP was about 100 times more potent as an antagonist than as an agonist at the $\rho 1$ receptor (data not shown).

Despite the observed partial agonism at $\rho 1$ receptors by agents active as full agonists at heterooligomeric $GABA_A$ receptors, several antagonists at these $GABA_A$ receptors were ineffective at $\rho 1$ receptors. Thus, $\rho 1$ was not only insensitive to blockade by bicuculline (Cutting *et al.*, 1991; Shimada *et al.*, 1992), but it also produced responses to $10\,\mu M$ GABA that were undimished by coapplication of $10\,\mu M$ SR-95531, $100\,\mu M$ securinine, and $100\,\mu M$ (+)-tubocurarine.

Discussions

The present results add to previous data defining the unique pharmacological profile of GABA $\rho 1$ receptors, expressed as homooligomers, and detail specific features of the recognition of $\rho 1$ agonists. This profile is quite distinct from that expressed by one defined model of the $GABA_A$ receptor, an $\alpha_5\beta_1$ heterooligomer. Agonists' affinities at $\rho 1$ displayed striking differences. Muscimol was the most potent agonist at the $\alpha_5\beta_1$ receptor, but TACA was the most potent at $\rho 1$. The cyclic agonists isoguvacine, THIP, and P4S, which displayed intermediate activity at the $\alpha_5\beta_1$ receptor, were among the weakest at $\rho 1$. ZAPA, potent at the $\alpha_5\beta_1$ receptor, was almost inert at $\rho 1$. Conversely, CAMP, a full agonist that was most specific for $\rho 1$, was inert at the $\alpha_5\beta_1$ receptor.

These different structure activity relationships of the $\rho 1$ and $\alpha_5\beta_1$ receptors can be rationalized if the recognition sites for agonists at the respective receptors differ. GABA's anionic (COO^-) and cationic (NH_3^+) groups are likely to bind at distinct subsites on both receptors. Subsite geometries demand specific distances between agonist charge centres and specific directions of the carboxylate group with respect to the cationic site. Other forces that can stabilize the receptor-ligand complex probably include short range, van der Waals interactions between the agonist's intermediate chain, which links its anionic and cationic sites, and the receptor's recognition site. The recognition site for agonists undergoes conformation changes that accompany changes in the functional state of the receptor. Binding of an agonist to any of these states will be disfavoured if the agonist intrudes on space already occupied by the receptor protein.

The high activity of TACA at both receptors and its constrained conformation make it a useful template for defining GABA pharmacophores. TACA's conformation is defined by the two dihedral angles that position its anionic and cationic ends, O = C-C = C, τ_1, and C = C-C-N, τ_2. τ_1 will be 180° (or 0°) at physiological pH, since ionization removes the distinction between the two oxygen atoms, and the conjugated O = C-C = C will be coplanar. The high $GABA_A$ potency of muscimol, in which the anionic center is contained within the planar isoxazole ring, gives strong support to the view that τ_1 must be 180° for this receptor (Andrews & Johnston, 1979). Conformational freedom thus resides in τ_2. This τ_2 angle is constrained to be small ($\approx 15°$) in the cyclic agonists, THIP, isoguvacine, and P4S. The high potency of these cyclic agonists, of substituted agonists, and of bicuculline at $GABA_A$ receptors argues that the optimal τ_2 for $GABA_A$ receptor recognition is also small (e.g. between 0° and ± 40°) corresponding to a 'partially folded' conformation (Figure 4a, left; see Krogsgaard-Larsen *et al.*, 1977; Nicholson *et al.*, 1979; Andrews & Johnston, 1979;

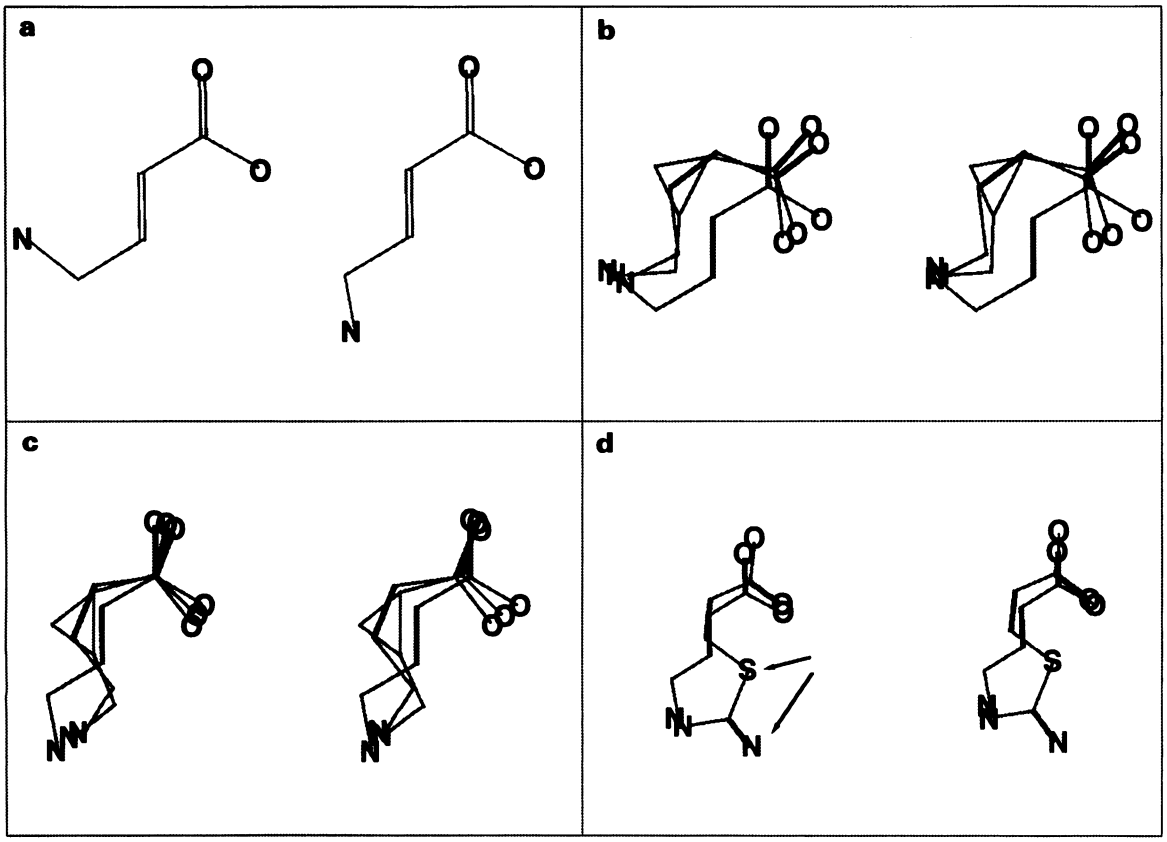


Figure 4 Conformations of some of the agonists. To improve clarity, hydrogen atoms are not shown. (a): TACA shown in the partially folded (left) and the extended (right) conformations. (b): Stereo view of CACA (top) and CAMP (middle) stacked on the partially folded conformation of TACA. While the ionic ends approach reasonable overlap, the direction of the COO^- of CACA and CAMP deviate from that of TACA by about 60°. (c): Stereo view of CACA (top) and CAMP (middle) stacked on the extended conformation of TACA. (d): Stereo view of ZAPA stacked on the extended conformation of TACA. Arrows point to a region of possible steric hindrance to binding at the $\rho 1$ receptor. For abbreviations, see text.

Krogsgaard-Laarsen, 1988). The feeble $GABA_A$ activities of CACA and CAMP, which poorly fit the 'partially folded' conformation (Figure 4b), reinforce this notion of the $GABA_A$ pharmacophore.

The structure-activity data obtained here can be rationalized on the basis of a different pharmacophore for the ρ1 receptor exemplified by the 'extended' conformation of TACA, in which τ_1 remains 180° but $\tau_2 \approx 180°$ (Figure 4a, right). The 'partially folded', cyclic agonists such as THIP displayed high preference for $\alpha_5\beta_1$ compared to ρ1 receptors (Figure 1; Table 1), as expected for a $GABA_A$ receptor. Their extremely weak activity at the ρ1 receptor suggests that the 'partially folded' conformation may be inappropriate. Conversely, ρ1-preferring CACA and CAMP (Table 1), which superimpose poorly on the 'partially folded' conformation (Figure 4b), fit fairly well on the 'extended conformation' (Figure 4c). Adopting the hypothesis that the 'extended' conformation is appropriate for the ρ1 receptor qualitatively explains the activities of all the agonists of Table 1 except for I4AA and ZAPA.

Imidazole-4-acetic acid (I4AA), in its planar conformation, can superimpose almost perfectly on TACA in its extended conformation. Its affinity was fairly high, 16 μM, but its intrinsic activity (I_m value) was only 3% that of GABA (Table 1). Conceivably, this loss of intrinsic activity could be attributable to dispersion of the charge of this weak base ($pK_a = 7.46$; Bowery & Jones, 1976) between its two nitrogens. Weakening of charge density at the *anionic* end of agonists through greater dispersion of charge has been linked to the reduced $GABA_A$ potency of isomuscimol and to the antagonism of 4,5,6,7-tetrahydroisoxazolo[3,4-c]pyridin-5-ol (iso-THAZ; Boulanger *et al.*, 1987). However ZAPA, which has similar charge dispersion, was active at the $\alpha_5\beta_1$ receptor.

Steric hindrance may offer another explanation. ZAPA is an effective agonist at $GABA_A$ receptors (Figure 1; Table 1; Allan *et al.*, 1986) that can be superimposed on both the 'partially folded' (Allan *et al.*, 1986) and, better, on the 'extended' conformations of TACA (Figure 4d). It displays cationic charge dispersion similar to that of I4AA, but it was virtually inert at ρ1 receptors. Conceivably, adverse steric interactions displayed by intermediate portions of the ZAPA molecule (Figure 4d) could explain its poor activity. Such steric hindrance could also contribute to the ρ1 inactivities of the cyclic agonists THIP, P4S, and isoguvacine, which, when overlaid on the 'extended' TACA configuration with ionic groups superimposed, project portions of their ring structures into the same region of space as does ZAPA (Figure 4d).

Could steric hindrance oppose binding, channel activation, or both? The apparent dissociation constant of an antagonist reflects its affinity for the resting state of the receptor. If a drug only blocked binding to the resting state, the apparent dissociation constants obtained from the drug's agonist and antagonist actions would be similar; if it inhibited activation, the apparent dissociation constant obtained from activation would be larger. The latter result was observed for I4AA and TAMP. The agonist to antagonist ratio was 25 and 100 to one, respectively. An agonist with unfavourable binding in the activated state would be expected to behave as a partial agonist with diminished intrinsic activity, as observed for these compounds.

Marked differences in ρ1 receptor intrinsic activity I_m values were displayed by different agonists; the relative rank order of intrinsic activity did not necessarily reflect the compound's relative potency (Tables 1 and 2). TAMP was more potent (displayed a lower apparent K_d value) at ρ1 receptors than its geometric isomer CAMP, but lacked almost all of CAMP's intrinsic activity in inducing GABA-like responses. The recognition site of the ρ1 'activated state' could be more sterically confined than the corresponding site mediating activation of the $\alpha_5\beta_1$ receptor. TAMP's cyclopropyl ring protrudes from the plane defined by the COO^- group. This protruding ring could form a steric obstacle slowing activation through reduced affinity for the ρ1 receptor's activated state but still activating the $\alpha_5\beta_1$ receptor evoking substantial intrinsic activity (Table 1). These ideas could be tested by single channel (patch clamp) recordings. The differences in intrinsic activity were much more apparent with the ρ1 than the $\alpha_5\beta_1$ receptor (Table 1).

Of the conventional $GABA_A$ antagonists tested, bicuculline, SR-95531, securinine, and (+)-tubocurarine (which is also a potent competitive antagonist of nicotinic receptors) did not block ρ1 receptors. Each of these compounds displays bulky 'accessory' groups that could prevent ρ1 recognition despite carbonyl and amine regions that can be superimposed well on an 'extended' conformation of TACA.

Hill coefficients distinguish drug activities at ρ1 receptors from those at $GABA_A$ receptors. GABA agonists display Hill coefficients close to 2 at ρ1, but closer to unity at $GABA_A$ receptors. Persistence of these differences among all agonists tested suggests differences in subunit-binding or cooperativity among these receptor types.

These results confirm the baclofen- and bicuculline-resistant properties of ρ1 receptors that define the $GABA_C$ receptor (Johnston, 1986). However, the low activity of CACA in comparison to TACA contradicts this classification.

The ρ1 receptor thus represents a unique GABA-responsive homooligomeric, ligand-gated ion channel with apparently distinguishing requirements for agonist structure and conformation to confer both receptor fit and intrinsic activity. Similar pharmacological features have been identified in *Xenopus* oocytes expressing mRNA extracted from whole retina (Polenzani *et al.*, 1991), a tissue which also expressed the highest concentrations of ρ1 mRNA. Horizontal cells from perch retina also express a GABA receptor showing similar properties to ρ1 (Quian & Dowling, 1992). Conceivably, these features of the ρ1 GABA recognition site could have evolved to match the special features of retinal GABA-ergic neurotransmission.

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Counteraction of the rapid tolerance to 8-OH-DPAT-induced corticosterone secretion in rats by activation of the $GABA_A$ receptor-chloride channel complex

Diana Kelder & [1]Svante B. Ross

Department of Neuropharmacology, Astra Arcus AB, S-151 85 Södertälje, Sweden

1 The effect of various classes of compounds on the rapidly developed tolerance to 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT)-induced corticosterone secretion was examined.

2 Compounds activating the γ-aminobutyric $acid_A$ ($GABA_A$) receptor-chloride complex, i.e. muscimol (3 mg kg^{-1}), diazepam (5 mg kg^{-1}), flunitrazepam (1 mg kg^{-1}), sodium pentobarbitone (10–30 mg kg^{-1}) and chlormethiazole ethane disulphonate (50 mg kg^{-1}) counteracted the development of tolerance when injected before or simultaneously with, but not 15 min after 8-OH-DPAT.

3 At these doses the compounds produced an acute increase in serum corticosterone but had, with the exception of muscimol, no effect on the response to the challenge dose of 8-OH-DPAT 24 h later. Muscimol significantly decreased the response.

4 The $GABA_A$ chloride channel antagonist, picrotoxin (1 mg kg^{-1}, s.c.), but not bicuculline (1 mg kg^{-1}, i.p.) potentiated the development of tolerance to 8-OH-DPAT-induced corticosterone secretion.

5 A number of compounds with widely differing pharmacological actions were examined and found to have no effect on the development of tolerance to 8-OH-DPAT-induced corticosterone secretion.

Keywords: Benzodiazepines; chlormethiazole; corticosterone; $GABA_A$ receptor complex; $5\text{-}HT_{1A}$ receptor; muscimol; 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT); pentobarbitone; tolerance

Introduction

The 5-hydroxytryptamine$_{1A}$ ($5\text{-}HT_{1A}$) receptor agonist, 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) (Hjorth *et al.*, 1982; Tricklebank *et al.*, 1984) produces in rats pharmacological responses such as the $5\text{-}HT_{1A}$ behavioural syndrome (elongated body and forepaw treading), inhibition of the cage-leaving response, hypothermia and secretion of corticosterone, probably via activation of postsynaptic $5\text{-}HT_{1A}$ receptors (Hjorth, 1985; Hutson *et al.*, 1987; Larsson *et al.*, 1990; Przegalinski *et al.*, 1989; Kelder & Ross, 1992). A common characteristic of these responses is their rapid and long lasting attenuation (tolerance) produced by prior exposure to a single dose of 8-OH-DPAT (Larsson *et al.*, 1990; Kelder & Ross, 1992; Rényi *et al.*, 1992; Ross *et al.*, 1992).

The tolerance to the 8-OH-DPAT-induced corticosterone secretion can be counteracted by pretreatment of the rats with N-methyl-D-aspartate (NMDA) receptor antagonists acting at the ion channel, the neurotransmitter recognition site or the glycine modulatory site of the receptor complex (Ross *et al.*, 1992). These observations indicate that glutamatergic systems are mechanistically involved in the development of a rapid attenuation of the $5\text{-}HT_{1A}$ receptor-mediated response.

Because the excitatory glutamatergic systems are often balanced by the inhibitory γ-aminobutyric acid (GABA) systems in the brain, we have in the present study examined the effect of compounds activating GABA receptors on the development of tolerance to 8-OH-DPAT-induced corticosterone secretion. We also report the negative results obtained with various other classes of compounds tested.

Methods

Animals

Male Sprague-Dawley rats weighing 200–240 g were obtained from Alab Laboratorietjänst, Sollentuna, Sweden. They were housed 5 per cage and kept under constant temperature with lights on between 06 h 00 min and 18 h 00 min and were allowed free access to food and water. The compounds were injected subcutaneously (s.c.) in a volume of 1 and 2 ml kg^{-1} unless otherwise stated. The animals were killed by decapitation.

Treatment

Rats were given daily injections of saline subcutaneously (s.c.) for 5 to 7 days before the start of the experiment in order to habituate the animals to the injections and thereby avoid acute stress-induced increases in serum corticosterone. Eight groups of 5 animals, controls included, were treated with the challenge compounds or saline within a time period of 30 min and the rats were killed 60 min after the injection. The experiments were performed between 11 h 00 min and 15 h 00 min. The trunk blood was collected in plastic tubes, the serum was obtained by centrifugation of the clotted sample after 30 to 60 min at room temperature and was stored at − 70°C.

Serum corticosterone

Corticosterone was determined with a radioimmunoassay (RIA) technique using a RSL rat corticosterone [^{3}H] kit no. 07-120002 from ICN Biomedicals Inc., Carson, CA, U.S.A.

Statistical analysis

Statistical comparisons were made with one way ANOVA followed by Student-Newman-Keuls multiple comparison test.

Compounds

Baclofen hydrochloride, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 8-hydroxy-2-(di-n-propylamino)tetralin hydrobromide (8-OH-DPAT), ifenprodil hydrochloride, and (−)-

[1] Author for correspondence.

quinpirole hydrochloride were purchased from Research Biochemicals Inc. (RBI), Natick, MA, U.S.A. N-(6-aminohexyl)-5-chloro-1-naphthalene sulphonamide hydrochloride (W7), bicuculline, indomethacin, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine hydrochloride (H7), N^G-methyl-L-arginine, muscimol, nifedipine, nordihydroguaiaretic acid, 5-α-pregnane-3α-ol-20-one, progesterone, trifluoperazine dihydrochloride and verapamil hydrochloride were bought from Sigma Chemical Co, St. Louis, MO, U.S.A. Diazepam and flunitrazepam were gifts from Kabi-Pharmacia AB, Uppsala, Sweden and F. Hoffman-LaRoche AG, Basel, Switzerland respectively. Picrotoxin was obtained from Fluka AG, Buchs, Switzerland. Alaproclate hydrochloride, chlormethiazole ethane disulphonate, raclopride hydrogen tartrate and sodium pentobarbitone were from Astra Arcus AB, Södertälje, Sweden. Rimcazole dihydrochloride was a gift from Burroughs Wellcome Co, Research Triangle Park, N.C., U.S.A. Ritanserin and ketanserin tartrate were donated by Janssen Pharmaceutica N.V., Beerse, Belgium. Opipramol dihydrochloride was a gift from Ciba-Geigy A.G., Basel, Switzerland. 2,3-Dihydroxy-6-nitro-7-sulphamoyl-benzo(F)-quinoxaline (NBQX) was donated by Novo Nordisk A/S, Målov, Denmark. All other chemicals were of highest purity available.

Results

Muscimol

The $GABA_A$ receptor agonist, muscimol at 3 mg kg^{-1}, s.c., produced marked sedation and muscle relaxation and increased the serum concentration of corticosterone when determined 60 min after the injection to the same level as that seen after giving 0.1 mg kg^{-1}, s.c., of 8-OH-DPAT (Table 1). When muscimol was given 24 h beforehand, muscimol decreased significantly the effect of the challenge dose of 8-OH-DPAT, but not the effect of a second injection of muscimol. This decrease was, however, much less than that produced by pretreatment with 8-OH-DPAT, 0.3 mg kg^{-1}, s.c. Muscimol at 3 mg kg^{-1}, s.c., but not at 1 mg kg^{-1}, s.c., counteracted the attenuation of 8-OH-DPAT-induced corticosterone secretion when tested with a challenge dose of 0.1 mg kg^{-1}, s.c., 8-OH-DPAT 24 h later (Exp. 2, Table 1).

Benzodiazepines

Diazepam and flunitrazepam at 5 and 1 mg kg^{-1}, s.c., respectively, increased the serum corticosterone concentration significantly when determined 60 min after the injection (Exp. 1, Table 2), but neither flunitrazepam (Exp. 2, group 2, Table 2) nor diazepam (control: 31 ± 10 ng ml^{-1}; 10 mg kg^{-1}, s.c. diazepam: 61 ± 11 ng ml^{-1} serum corticosterone) had any significant effect 24 h after the injection. Both compounds antagonized the 8-OH-DPAT-induced attenuation of the corticosterone secretion when given 15 min before or simultaneously with 8-OH-DPAT, 0.3 mg kg^{-1}, s.c., 24 h before the challenge injection. Flunitrazepam was more potent than diazepam in this respect. When diazepam, 10 mg kg^{-1} was injected 15 min after 8-OH-DPAT (0.3 mg kg^{-1}) no counteraction of the attenuating effect of the latter compound was seen (Exp. 3, group 6, Table 2). A high dose of flunitrazepam (10 mg kg^{-1} s.c.) 24 h before the challenge dose of 8-OH-DPAT did not cause any change in the corticosterone secretion by the latter compound.

Pentobarbitone

Like muscimol and the benzodiazepines, pentobarbitone at the hypnotic dose of 30 mg kg^{-1}, i.p., produced an acute increase in the serum corticosterone concentration when measured 60 min after the injection (Table 3). The serum corticosterone level was still significantly elevated compared to saline controls 24 h after this dose of pentobarbitone when determined 60 min after a challenge injection of saline (138 ± 28 ng ml^{-1}, $n = 5$, vs 23 ± 4 ng ml^{-1}, $n = 5$; $P < 0.05$). This dose of pentobarbitone completely counteracted the 8-OH-DPAT-induced attenuation of the corticosterone secretion when given 15 min before or simultaneously with, but not 15 min (Exp. 3, group 8) after 8-OH-DPAT (0.3 mg kg^{-1}, s.c.) 24 h before the challenge dose of 8-OH-DPAT. Sodium pentobarbitone had a significant antagonizing effect at 10, but not at 5 mg kg^{-1}, i.p.

Chlormethiazole

Chlormethiazole at the hypnotic dose of 50 mg kg^{-1}, i.p., increased the serum corticosterone concentration (Table 4). This dose together with a dose of 75 mg kg^{-1} i.p. significantly antagonized the 8-OH-DPAT-induced attenuation of the corticosterone secretion when given before or simultaneously

Table 1 Effects of muscimol on rat serum corticosterone and on the tolerance to 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT)-induced corticosterone secretion

Injection 1	*Dose* (mg kg^{-1})	*Injection 2*	*Dose* (mg kg^{-1})	*Injection 3*	*Dose* (mg kg^{-1})	*Corticosterone* (ng ml^{-1})	P*
Exp. 1							
1 Saline				Saline		41 ± 9	
2 Saline				Muscimol	3	455 ± 18	2 vs 1
3 8-OH-DPAT	0.3			Muscimol	3	432 ± 19	
4 Muscimol	3			Saline		36 ± 11	
5 Muscimol	3			Muscimol	3	422 ± 19	
6 Muscimol	3			8-OH-DPAT	0.1	378 ± 37	6 vs 7
7 Saline				8-OH-DPAT	0.1	504 ± 15	
8 8-OH-DPAT	0.3			8-OH-DPAT	0.1	49 ± 17	8 vs 7
Exp. 2							
1 Saline		Saline		Saline		40 ± 21	
2 Saline		Saline		8-OH-DPAT	0.1	461 ± 36	2 vs 1
3 Saline		8-OH-DPAT	0.3	8-OH-DPAT	0.1	145 ± 80	3 vs 2
4 Muscimol	3	Saline		Saline		36 ± 11	
5 Muscimol	1	8-OH-DPAT	0.3	8-OH-DPAT	0.1	156 ± 36	
6 Muscimol	3	8-OH-DPAT	0.3	8-OH-DPAT	0.1	330 ± 28	6 vs 3

Injection 1 was made 15 min before injection 2 and 24 h before injection 3 (the challenge dose). Blood was collected 60 min after the challenge dose. All injections were subcutaneous. Serum corticosterone was determined with RIA technique. Each value is the mean ± s.e.mean from 5 animals.

*$P < 0.05$ (Student-Newman-Keul multiple comparison test following ANOVA).

Table 2 Effect of benzodiazepine derivatives, diazepam and flunitrazepam, on rat serum corticosterone and on the tolerance to 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT)-induced corticosterone secretion

Injection 1	*Dose* (mg kg^{-1})		*Injection 2*	*Dose* (mg kg^{-1})	*Injection 3*	*Dose* (mg kg^{-1})	*Corticosterone* (ng ml^{-1})	P*
Exp. 1								
1					Saline		7 ± 2	
2					Diazepam	5	370 ± 17	2 vs 1
3					Flunitrazepam	1	356 ± 11	3 vs 1
Exp. 2								
1 Saline			Saline		Saline		51 ± 12	
2 Flunitrazepam	1		Saline		Saline		131 ± 30	
3 Saline			Saline		8-OH-DPAT	0.1	561 ± 16	2 vs 1
4 Saline			8-OH-DPAT	0.3	8-OH-DPAT	0.1	90 ± 14	4 vs 3
5 Flunitrazepam	0.3		8-OH-DPAT	0.3	8-OH-DPAT	0.1	244 ± 51	
6 Flunitrazepam	1		8-OH-DPAT	0.3	8-OH-DPAT	0.1	373 ± 47	6 vs 4
7 Flunitrazepam	10		Saline		8-OH-DPAT	0.1	454 ± 45	7 vs 4
Exp. 3								
1 Saline			Saline		Saline		20 ± 7	
2 Saline			Saline		8-OH-DPAT	0.1	426 ± 16	2 vs 1
3 Saline			8-OH-DPAT	0.3	8-OH-DPAT	0.1	102 ± 19	3 vs 2
4 Diazepam	5		8-OH-DPAT	0.3	8-OH-DPAT	0.1	272 ± 71	4 vs 3
5 Diazepam	10	+[a]	8-OH-DPAT	0.3	8-OH-DPAT	0.1	294 ± 42	5 vs 3
6 8-OH-DPAT	0.3		Diazepam	10	8-OH-DPAT	0.1	113 ± 25	
7 Flunitrazepam	1		8-OH-DPAT	0.3	8-OH-DPAT	0.1	297 ± 23	7 vs 3
8 Flunitrazepam	1	+[a]	8-OH-DPAT	0.3	8-OH-DPAT	0.1	337 ± 49	8 vs 3

Injection 1 was performed 15 min before or [a]simultaneously with injection 2 and 24 h before injection 3 (the challenge dose). All injections were subcutaneous. Blood was collected 60 min after the challenge injection and corticosterone in serum was determined with RIA technique. Each value is the mean ± s.e.mean from 5 rats.
*$P < 0.05$ (Student-Newman-Keul multiple comparison test following ANOVA).

Table 3 Effect of sodium pentobarbitone on rat serum corticosterone and on the tolerance to 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT)-induced corticosterone secretion

Injection 1	*Dose* (mg kg^{-1})		*Injection 2*	*Dose* (mg kg^{-1})	*Injection 3*	*Dose* (mg kg^{-1})	*Corticosterone* (ng ml^{-1})	P*
Exp. 1								
1					Saline		58 ± 15	
2					Pentobarbitone	30	290 ± 30	2 vs 1
Exp. 2								
1 Saline			Saline		Saline		57 ± 18	
2 Saline			Saline		8-OH-DPAT	0.1	624 ± 24	2 vs 1
3 Saline			8-OH-DPAT	0.3	8-OH-DPAT	0.1	49 ± 11	3 vs 1
4 Pentobarbitone	30		8-OH-DPAT	0.3	8-OH-DPAT	0.1	601 ± 38	4 vs 3
Exp. 3								
1 Saline			Saline		Saline		27 ± 18	
2 Saline			Saline		8-OH-DPAT	0.1	600 ± 50	2 vs 1
3 Saline			8-OH-DPAT		8-OH-DPAT	0.1	29 ± 9	3 vs 2
4 Pentobarbitone	5		8-OH-DPAT		8-OH-DPAT	0.1	153 ± 78	
5 Pentobarbitone	10		8-OH-DPAT		8-OH-DPAT	0.1	363 ± 88	5 vs 3
6 Pentobarbitone	20		8-OH-DPAT		8-OH-DPAT	0.1	466 ± 124	6 vs 3
7 Pentobarbitone	30	+[a]	8-OH-DPAT		8-OH-DPAT	0.1	688 ± 50	7 vs 3
8 8-OH-DPAT	0.3		Pentobarbitone	30	8-OH-DPAT	0.1	147 ± 55	

Injection 1 was performed 15 min before or [a]simultaneously with injection 2 and 24 h before injection 3 (the challenge dose). Sodium barbitone was injected intraperitoneally and 8-OH-DPAT subcutaneously. Blood was collected 60 min after the last injection and serum corticosterone was determined with RIA technique. Each value is the mean ± s.e.mean from 5 rats.
*$P < 0.05$ (Student-Newman-Keul multiple comparison test following ANOVA).

with, but not 15 min after (Exp. 3, group 8) 8-OH-DPAT. Chlormethiazole, 75 mg kg^{-1}, did not significantly elevate the serum corticosterone concentration when measured 24 h after the injection (83 ± 22 ng ml^{-1}, $n = 5$, vs saline controls 23 ± 4 ng ml^{-1}, $n = 5$; $P > 0.05$).

Picrotoxin and bicuculline

The convulsant agent, picrotoxin hydrochloride, 1 mg kg^{-1}, s.c., injected 15 min before 8-OH-DPAT potentiated significantly the 8-OH-DPAT-induced attenuation of the corticosterone secretion so that a dose as low as 0.01 mg kg^{-1}, s.c., of 8-OH-DPAT produced a marked attenuation of the effect of the challenge dose 24 h later (Table 5).

In contrast to picrotoxin, the $GABA_A$ receptor antagonist, bicuculline, at a subconvulsant dose (1 mg kg^{-1}, i.p.) did not potentiate the tolerance to 8-OH-DPAT (0.03 mg kg^{-1}, s.c.) as determined in two separate experiments (data not shown).

Compounds without effect

The following compounds did not produce any significant antagonism to the 8-OH-DPAT-induced attenuation of the cortiscosterone secretion at the dose examined (dose in mg kg^{-1}), indicating that these compounds did not produce any interaction with the development of the tolerance to 8-OH-DPAT: The 5-HT uptake inhibitor, alaproclate (Lindberg *et al.*, 1978) (50, i.p.); the $GABA_B$ receptor agonist,

Table 4 Effect of chlormethiazole ethane disulphonate on rat serum corticosterone and on the tolerance to 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT)-induced corticosterone secretion

Injection 1	*Dose* (mg kg^{-1})	*Injection 2*	*Dose* (mg kg^{-1})	*Injection 3*	*Dose* (mg kg^{-1})	*Corticosterone* (ng ml^{-1})	P*
Exp. 1							
1				Saline		58 ± 15	
2				Chlormethiazole	50	370 ± 32	2 vs 1
Exp. 2							
1 Saline		Saline		Saline		42 ± 15	
2 Saline		Saline		8-OH-DPAT	0.1	456 ± 60	2 vs 1
3 Saline		8-OH-DPAT	0.3	8-OH-DPAT	0.1	37 ± 5	3 vs 2
4 Chlormethiazole	50	8-OH-DPAT	0.3	8-OH-DPAT	0.1	240 ± 34	4 vs 3
Exp. 3							
1 Saline		Saline		Saline		20 ± 7	
2 Saline		Saline		8-OH-DPAT	0.1	426 ± 109	2 vs 1
3 Saline		8-OH-DPAT	0.3	8-OH-DPAT	0.1	102 ± 19	3 vs 2
4 Chlormethiazole	75 +[a]	8-OH-DPAT	0–3	8-OH-DPAT	0.1	345 ± 26	4 vs 3
5 8-OH-DPAT	0.3	Chlormethiazole	75	8-OH-DPAT	0.1	157 ± 23	

Injection 1 was made 15 min before or [a]simultaneously with injection 2 and 24 h before injection 3 (the challenge dose). Chlormethiazole was injected intraperitoneally and 8-OH-DPAT subcutaneously. Blood was collected 60 min after the last injection and serum corticosterone was determined with RIA technique. Each value is the mean ± s.e.mean from 5 rats.
*$P < 0.05$ (Student-Newman-Keul multiple comparison test following ANOVA).

Table 5 Potentiation by picrotoxin of the development of tolerance to 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT)-induced corticosterone secretion in the rat

Injection 1	*Dose* (mg kg^{-1})	*Injection 2*	*Dose* (mg kg^{-1})	*Injection 3*	*Dose* (mg kg^{-1})	*Corticosterone* (ng ml^{-1})	P*
Exp. 1							
1 Saline		Saline		Saline		10 ± 3	
2 Saline		Saline		8-OH-DPAT	0.1	506 ± 11	2 vs 1
3 Saline		8-OH-DPAT	0.03	8-OH-DPAT	0.1	475 ± 37	
4 Picrotoxin	1	8-OH-DPAT	0.03	8-OH-DPAT	0.1	194 ± 57	4 vs 3
Exp. 2							
1 Saline		Saline		Saline		55 ± 17	
2 Saline		Saline		8-OH-DPAT	0.1	491 ± 30	2 vs 1
3 Saline		8-OH-DPAT	0.01	8-OH-DPAT	0.1	384 ± 88	
4 Picrotoxin	1	8-OH-DPAT	0.01	8-OH-DPAT	0.1	172 ± 67	4 vs 3
5 Saline		8-OH-DPAT	0.03	8-OH-DPAT	0.1	427 ± 38	
6 Picrotoxin	1	8-OH-DPAT	0.03	8-OH-DPAT	0.1	82 ± 23	6 vs 5
Exp. 3							
1 Saline		Saline		Saline		26 ± 3	
2 Saline		Saline		8-OH-DPAT	0.1	385 ± 50	2 vs 1
3 Picrotoxin	1	Saline		Saline		116 ± 29	
4 Picrotoxin	1	Saline		8-OH-DPAT	0.1	343 ± 55	

Injection 1 was performed 15 min before injection 2 and 24 h before injection 3 (the challenge dose). All injections were subcutaneous. Blood was collected 60 min after the last injection and serum corticosterone was determined with RIA technique. Each value is the mean ± s.e.mean from 5 rats.
*$P < 0.05$ (Student-Newman-Keul multiple comparison test following ANOVA).

baclofen (Hill & Bowery, 1981) (25, s.c.); ethanol (750–2200, i.p.), ifenprodil (25, s.c.), which blocks the polyamine binding site associated with the NMDA receptor (Carter *et al.*, 1989); the prostaglandin cyclo-oxygenase inhibitor, indomethacin (Vane, 1971) (5, s.c.); the calcium channel blockers nifedipine (5, s.c.) and verapamil (10, s.c.) (Godfraind *et al.*, 1986); progesterone (10, s.c.) and 5α-pregnane-3α-ol-20-one (5, s.c.), which modulate the $GABA_A$ receptor complex at the steroid binding site (Turner *et al.*, 1989); the sigma ligands, rimcazole (50, i.p.) and opipramol (25, i.p.) (Walker *et al.*, 1990); the 5-HT_2 receptor antagonists, ritanserin (0.5, s.c.) and ketanserin (1, s.c.) (Leysen *et al.*, 1985); the dopamine D_2 receptor antagonist, raclopride (Köhler *et al.* 1985) (2, s.c.); the dopamine D_2 receptor agonist, (–)-quinpirole hydrochloride (Ruffolo & Shaar, 1983) (2, s.c.); the calmodulin inhibitor, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine hydrochloride (H7) (Weiss, 1983) (100, s.c.); the protein kinase C inhibitor, N-(6-aminohexyl)-5-chloro-1-naphtalene sulphonamide hydrochloride (W7) (Hidaka & Hagiwara, 1987) (100, s.c.); the nitric oxide synthase inhibitor, N^G-methyl-L-arginine (Palmer & Moncada, 1989) (200, s.c.); nordihydroguaiaretic acid (200, i.p.), an inhibitor of arachidonic acid production (Lanni & Becker, 1985); the non-NMDA receptor antagonists, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (50, s.c.) and 2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo(F)-quinoxaline (NBQX) (25, s.c.) (Watkins *et al.*, 1990). The calmodulin inhibitor, trifluoperazine dihydrochloride (Weiss, 1983) (10, s.c.) caused a pronounced increase in serum corticosterone, when determined 24 h after the injection (647 ± 127 ng ml^{-1}, $n = 5$), and therefore gave a false impression of counteraction of the tolerance to 8-OH-DPAT-induced corticosterone secretion.

Discussion

The results obtained in the present study show that compounds which open the chloride channels in the $GABA_A$ receptor complex antagonize the 8-OH-DPAT-induced attenuation of the corticosterone secretion. Thus, muscimol

opens the channel by activation of the $GABA_A$ receptor (Curtis *et al.*, 1971), the benzodiazepines bind to a specific site of the receptor complex and increase the frequency of the opening of the chloride channel (Study & Barker, 1981), barbiturates bind to another site and prolong the length of opening of the channel (Study & Barker, 1981) and chlormethiazole acts both by potentiating the effects of GABA and by modulating channel opening directly (Harrison & Simmonds, 1983; Moody & Skolnick, 1989; Cross *et al.*, 1989). Elevation of the serum corticosterone concentration produced acutely by these compounds does not appear *per se* to be the cause of the counteraction of the tolerance to 8-OH-DPAT-induced corticosterone secretion, since picrotoxin also acutely increased the corticosterone level but potentiated the tolerance to 8-OH-DPAT. It can be argued that the sedative and hypnotic effects of these compounds produce a nonspecific counteraction of the development of the attenuation. However, these pharmacological effects show that the interaction with the $GABA_A$ receptor complex is strong at the doses employed. Moreover, picrotoxin, a selective antagonist of the $GABA_A$ receptor-gated chloride channel (Olsen, 1981), markedly potentiated the 8-OH-DPAT-induced attenuation which is in accordance with the view that the chloride channels of the $GABA_A$ receptor complex are specifically involved in the modulation of the 8-OH-DPAT response. However, the $GABA_A$ receptor antagonist, bicuculline, at a non-convulsive dose (1 mg kg^{-1}) did not potentiate the tolerance to 8-OH-DPAT. The reason for this discrepancy is unclear but may be simply a reflection of the pharmacokinetic and pharmacodynamic properties of the compound. Further studies with other compounds, *e.g.* inverse agonists of the benzodiazepine receptors have to be performed in order to elucidate this problem.

The lack of effect of the compounds when given 15 min after 8-OH-DPAT indicates that tolerance to a second dose of 8-OH-DPAT was triggered very rapidly. This may contribute to the short lasting behavioural actions of 8-OH-DPAT.

It is possible that activation of NMDA receptors is a general mechanism involved in the development of rapid functional tolerance to various drugs. Thus, Khanna *et al.* (1991, 1992) reported that the noncompetitive NMDA receptor antagonists, dizolcipine and ketamine block the development of tolerance to ethanol. Dizolcipine also blocks development of tolerance to morphine (Ben-Eliyahu *et al.*, 1992). Whether activation of the $GABA_A$ receptor chloride channel complex also antagonizes the development of ethanol and morphine tolerance has not, as far as we know, been studied. The lack of effect of a large number of compounds from a wide range of pharmacological classes may well indicate the importance of these inhibitory and excitatory systems to the development of tolerance to the effects of a wide range of centrally acting drugs.

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Whole cell and single channel analysis of the kinetics of glycine-sensitive N-methyl-D-aspartate receptor desensitization

[1]Chris G. Parsons, Xiangang Zong & Hans D.Lux

Department of Neurophysiology, Max-Planck Institute for Psychiatry, am Klopferspitz 18a, 8033 Planegg-Martinsried, Germany

1 The kinetics of glycine-sensitive, N-methyl-D-aspartate (NMDA) receptor desensitization were investigated in cultured neurones with the patch clamp technique.

2 The degree of fast NMDA-receptor desensitization was inversely related to glycine concentration. Thus, increasing concentrations of glycine from 30 nM to 2.5 μM potentiated desensitized NMDA responses (873% ± 101%) to a greater degree than peak responses (260% ± 27%).

3 The desensitization was due to a decrease in the affinity of glycine for the strychnine-insensitive, glycine modulatory site ($glycine_B$ site) following activation of the NMDA-receptor complex. Thus, the A_{50} for glycine in potentiating peak responses (77 nM, 95% confidence limited 58–104 nM) was five fold lower than that for plateau responses (399 nM, 340–468 nM).

4 The rate of desensitization was related to glycine concentration such that a reciprocal plot of desensitization rate ($1/\tau$ S^{-1}) against glycine concentration had a slope of $9.5*10^6$ M^{-1} S^{-1}.

5 Recovery from desensitization following step increases in glycine or L-alanine concentration in the continuous presence of NMDA (200 μM) reflected the association kinetics of the $glycine_B$ agonist used.

6 The rate and degree of NMDA receptor desensitization was independent of holding potential.

7 NMDA receptor desensitization was also evident at the single channel level.

8 The $glycine_B$ antagonist 7-chlorokynurenic acid (7-Chl-Kyn 3 and 10 μM) concentration-dependently induced an identical form of desensitization in the presence of 1 μM glycine.

9 In contrast, the competitive NMDA antagonist (±)-amino-phosphonovaleric acid (APV 30 to 300 μM) concentration-dependently antagonized and slowed the onset kinetics of NMDA responses.

Keywords: Desensitization; N-methyl-D-aspartate (NMDA); glycine; patch clamp; electrophysiology; superior colliculus cultured neurones

Introduction

Although the phenomenon of N-methyl-D-aspartate (NMDA) receptor desensitization has been extensively documented, the nature of the processes that underlie it remain a matter of controversy. At least five types of desensitization have been reported to date. Glycine-sensitive desensitization was first reported by Mayer *et al.* (1989) in cultured mouse hippocampal neurones and subsequently confirmed for NMDA receptors expressed in *Xenopus* oocytes (Lerma *et al.*, 1990). Three forms of Ca^{2+}-sensitive desensitization have also been reported; one shows similarities to a pH-sensitive Na^+ current (Grantyn & Lux, 1988), another seems to be dependent on activation of a Ca^{2+}-sensitive Cl^- conductance (Leonard & Kelso, 1990) and another may be related to a direct influence of Ca^{2+} on NMDA receptors (Clark *et al.*, 1990; Zilberter *et al.*, 1991). In addition, calcium-and glycine-independent desensitization has been observed in outside-out patches of mouse cortical neurones (Sather *et al.*, 1992). Ca^{2+}-sensitive desensitization has a relatively slow time course of decay ($\tau \approx 2$ s), the magnitude of which is dependent on extracellular Ca^{2+} concentration. Glycine-sensitive desensitization has a much faster time course ($\tau \approx 100$–500 ms) and is most readily observed in the absence of Ca^{2+}-sensitive desensitization i.e. in the presence of low concentrations of extracellular Ca^{2+} (≤ 0.2 mM). Glycine is a coagonist at the NMDA receptor complex and its binding to a specific, strychnine-insensitive recognition site (hereafter termed the $glycine_B$ site) is an absolute prerequisite for channel activation by agonists at the NMDA recognition site (Johnson & Ascher, 1987; Kleckner & Dingledine, 1988; Kushner *et al.*, 1988). The degree of fast NMDA receptor desensitization is inversely related to glycine concentration. Thus, NMDA responses desensitize to a plateau of around 10 to 20% of the peak response in the presence of low nM concentrations of glycine whereas little desensitization occurs at glycine concentrations of around 10 μM. Furthermore, $glycine_B$ antagonists such as 7-chlorokynurenic acid (7-Chl-Kyn) concentration-dependently induce an identical form of desensitization in the presence of μM concentrations of glycine (Benveniste *et al.*, 1990b; Henderson *et al.*, 1990; Kemp & Priestley, 1991). It has been suggested that the desensitization is due to a decrease in the affinity of the $glycine_B$ site following binding of agonists at the NMDA recognition site as the potency of glycine in potentiating the plateau response was 5 fold less than its potency in potentiating the peak response (Lerma *et al.*, 1990). In this respect it is important to note that the rate of desensitization seen at low nM concentrations of glycine is similar to the dissociation rate of glycine from $glycine_B$ sites. As such, it has been suggested that the true rate of desensitization may in fact be much faster but masked by the slow dissociation kinetics of glycine (Benveniste *et al.*, 1990a). The purpose of this study was to characterize further the kinetics of glycine-sensitive desensitization to test this hypothesis.

Methods

Patch clamp recordings were made from cultured superior colliculus neurones (10 to 21 days *in vitro*) in whole cell and single channel outside-out modes with the aid of an EPC-7 amplifier (List). Electrodes were pulled with a horizontal

[1] Author for correspondence at present address: Merz + Co. GmbH & Co., Eckenheimer Landstraße 100-104, 6000 Frankfurt am Main 1, Germany.

puller (DMZ) and had an internal tip diameter between 1.2 and 1.6 μM and a tip resistance of 4 to 10 MOhms. Cells were continuously superfused via one of eight channels of a fast superfusion system (Konnerth *et al.*, 1987). Test substances were then applied by rapidly switching channels–complete exchange of the superfused solution was achieved within <10 ms (confirmed by the speed of block of voltage-activated calcium channels when switching to a solution containing cadmium). The application of solutions and the synchronized online electronic acquisition of data (sample rate 8 kHz) were controlled by the PDP 11 programme INTESV (Zucker, MPI Munich). Subsequently, AUTESP for IBM (Zucker, MPI Munich) was utilized to filter digitally the samples at 2.5 kHz and semi-automatically analyse the data offline. Kinetics fits to the data were made by a least squares routine in AUTESP. Only results from stable cells were accepted for inclusion in the final analysis.

Superior colliculi were obtained from rat embryos (E20 to E21) and were then transferred to calcium- and magnesium-free Hank's buffered salt solution (Gibco) on ice. Cells were mechanically dissociated in 0.05% DNAase/0.3% ovomucoid (Sigma) following a 15 min preincubation with 0.66% trypsin/0.1% DNAase (Sigma). The dissociated cells were then centrifuged at 18 *g* for 10 min, resuspended in minimum essential medium (MEM, Gibco) and plated at a density of 200,000 cells cm^{-2} onto poly-L-lysine (Sigma)-precoated plastic petri dishes (NUNC). The cells were nourished with $NaHCO_3$/HEPES-buffered MEM supplemented with 5% foetal calf serum and 5% horse serum (Gibco) and incubated at 37°C with 5%CO_2. The MEM was completely exchanged with MEM containing a low concentration of ketamine (20 μM, Sigma) to block the toxic effects of glutamate present in serum, following inhibition of further glial mitosis with cytosine-β-D-arabinofuranoside (20 μM Sigma) after about 7 days *in vitro*. Thereafter the MEM was partially exchanged twice weekly.

The contents of the intracellular (electrode) solution were as follows (mM): CsCl 120, TEACl 20, EGTA 10, $MgCl_2$ 1, $CaCl_2$ 0.2, glucose 10, ATP 2, adenosine 3′:5′-cyclic monophosphate (cyclic AMP) 0.25. The extracellular solutions had the following basic salt composition (mM): NaCl 140, KCl 3, glucose 10, HEPES 10, $CaCl_2$ 0.2, sucrose 4.5. In addition, neurones were pharmacologically isolated from one another by the inclusion of 0.5 to 1.0 μM tetrodotoxin to block voltage-activated sodium currents and bicuculline methiodide (25 μM) to block spontaneous γ-aminobutyric acid-mediated miniature inhibitory postsynaptic potentials. Test substances were added to this basic solution in concentrations detailed in results. In addition, the bath solution and one superfusion solution contained 1.5 mM $CaCl_2$ to aid formation of a 'giga Ohm seal'.

The following drugs (with sources) were used: N-methyl-D-aspartate (Sigma), glycine (Sigma), L-alanine (Sigma), 7-chlorokynurenic acid (Tocris), (±)-amino-phosphonovaleric acid (APV, Tocris) and bicuculline methiodide (Sigma).

Results

The first series of experiments were undertaken to investigate the effects of glycine on the absolute magnitude and degree of desensitization of responses to NMDA. Cells were exposed

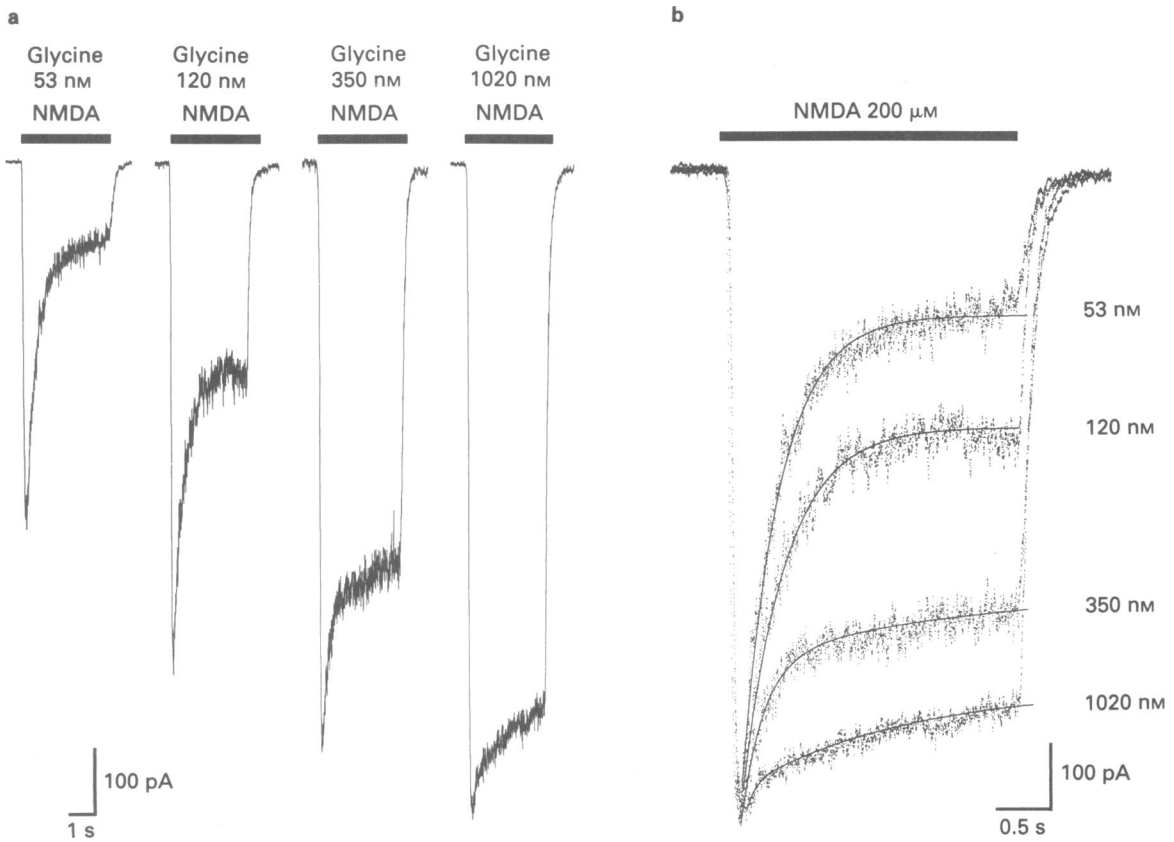


Figure 1 Desensitization of responses to N-methyl-D-aspartate (NMDA)-dependence of the magnitude and kinetics on glycine concentration. NMDA (200 μM) was applied for 2.5 s every 95 s in the continuous presence of various, corrected concentrations of glycine (see Results). (a) NMDA responses of a single cell in the presence of glycine 53 nM, 120 nM, 350 nM and 1020 nM are shown from left to right. (b) Superimposed responses following normalization of the peak response. Note the decrease in magnitude but faster kinetics of desensitization in the presence of increasing concentrations of glycine. The rate of desensitization seen with higher concentrations of glycine 350 nM and 1020 nM was best described by a double exponential fit (350 nM τ 201 and 1546 ms, 1020 nM τ 80 and 1616 ms) whereas the desensitization seen with lower concentrations was best described by a single exponential fit (53 nm τ 414 ms, 120 nM τ 395 ms).

to a stable concentration of glycine before, during and after application of NMDA 200 μM. Responses to NMDA in the continuous presence of glycine 53 nM showed profound desensitization (τ417 ± 12 ms, $n = 42$) such that the plateau response at the end of a 2.5 s application of NMDA was around 35% of the peak response. Increasing the concentration of glycine from 53 nM to 1020 nM caused a progressive potentiation of current responses to NMDA which was more evident for the plateau response than for the peak response i.e. the degree of desensitization was inversely related to glycine concentration (Figure 1a). The change in the relative magnitude of desensitization was even more apparent when the responses were normalized such that the magnitude of the peak response was the same (Figure 1b). Plateau responses recorded with 2.5 μM glycine were 873% ± 101% greater than those recorded with glycine 30 nM whereas this potentiation was only 260% ± 27% for peak responses. Analysis of pooled data illustrated in Figure 2a indicates that this desensitization is due to a five fold decrease in the affinity of $glycine_B$ sites after activation of the NMDA receptor-channel complex by NMDA with a resultant rightward shift in the glycine concentration-response curve of plateau compared to peak responses. Thus, glycine potentiated peak responses with an A_{50} of 77 nM (95% confidence limits, 58–104 nM) whereas the A_{50} for plateau responses was 399 nM (340–468 nM). The potency of glycine in preventing desensitization was between these two values, i.e. the A_{50} was 139 nM (121–160 nM) when the ratio of plateau over peak current was plotted against glycine concentration (Figure 2b).

It should be noted that all glycine concentration-response curves were corrected for contamination of solutions with a background glycine concentration of around 20 nM. This value was estimated by extrapolation of the linear region of the glycine concentration-response curve (10 to 100 nM) and probably accounts for the small response seen with NMDA in the absence of added glycine (data not shown). In contrast, glycine 1 μM evoked no response in the absence of added NMDA indicating the absence of contaminating concentrations of glutamate or aspartate.

The rate of desensitization seen with glycine 30 nM was similar to the τ_{off} following step changes in glycine concentration from 1 μM to nominally zero (approx 20 nM) in the continuous presence of NMDA 200 μM (τ_{off} 524 ± 16 ms, $n = 25$) indicating the possibility that the true rate of change in the affinity of the $glycine_B$ site is much faster but is masked by the slow dissociation kinetics of glycine from its recognition site. If this were exclusively the case, then the rate of desensitization should be independent of glycine concentration. However, a reciprocal plot of desensitization rate ($1/\tau s^{-1}$) against corrected glycine concentration had a slope of $9.5*10^6$ M^{-1} S^{-1} indicative of a influence of glycine concentration on the rate of desensitization (Figure 3) similar to that observed by Benveniste *et al.* (1990a). They attributed this effect to a glycine-dependent increase in the rate constant for recovery from desensitization with little direct effect on the rate constant for onset of desensitization.

As such, further experiments with step increases in the concentration of glycine in the continuous presence of NMDA were performed to test this hypothesis. Responses to glycine 30 nM showed profound desensitization whereas responses of the same cells to glycine 350 nM exhibited much less desensitization (Figure 4a). Following desensitization of responses in glycine 30 nM a step change was made to glycine 350 nM in the continued presence of NMDA. The evoked current increased to a new plateau level with a time course faster than the desensitization (τ_{on} 205 ± 9 ms, $n = 5$) and similar to the association rate of glycine 350 nM (Benveniste *et al.*, 1990a) suggesting that the association kinetics of glycine are the rate limiting step in this process. This assumption was supported by the results of similar experiments with L-alanine which associates more quickly than glycine at the $glycine_B$ site at the higher concentrations needed. Thus, following step changes in L-alanine concentration from 3.3 to

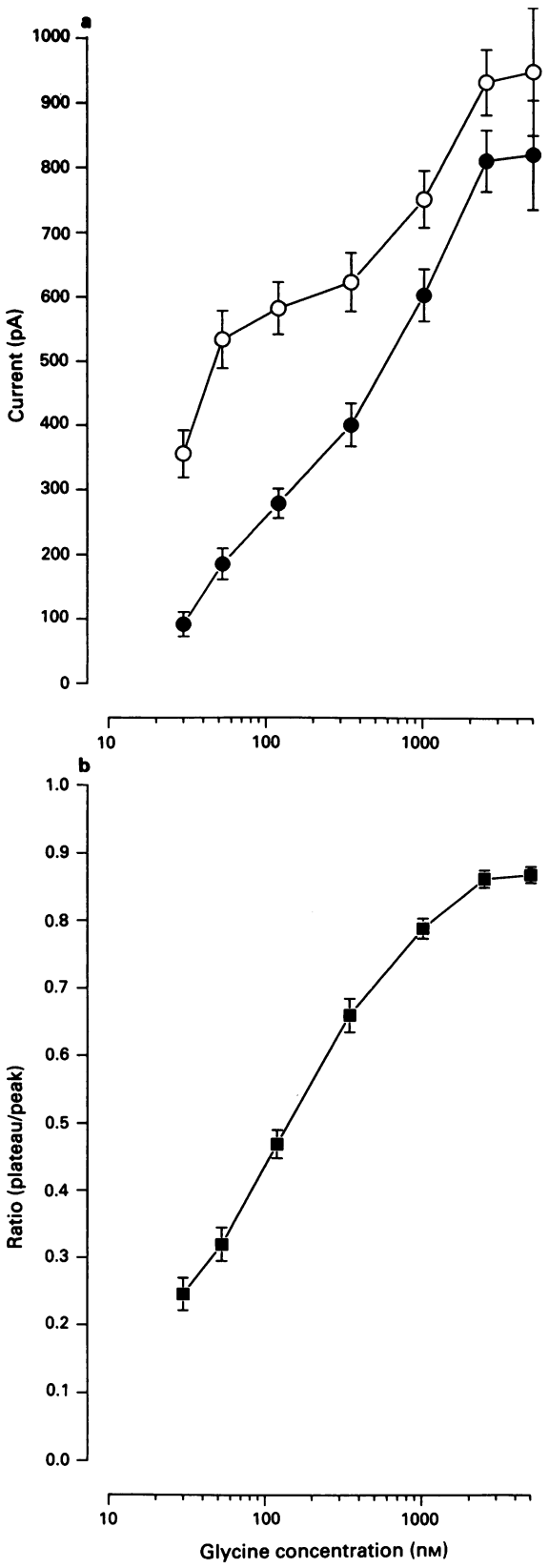


Figure 2 Concentration-dependent facilitation of peak and plateau responses to N-methyl-D-aspartate (NMDA) by glycine. NMDA (200 μM) was applied as in Figure 1. Pooled data are plotted as means (± s.e.mean) against corrected glycine concentration. (a) Desensitization of NMDA responses was accompanied by a rightward shift in the glycine concentration-response curve for plateau responses (●) compared to peak responses (○). (b) Responses to NMDA showed less desensitization in the presence of increasing concentrations of glycine. See Table 1 for the number of cells tested at each concentration.

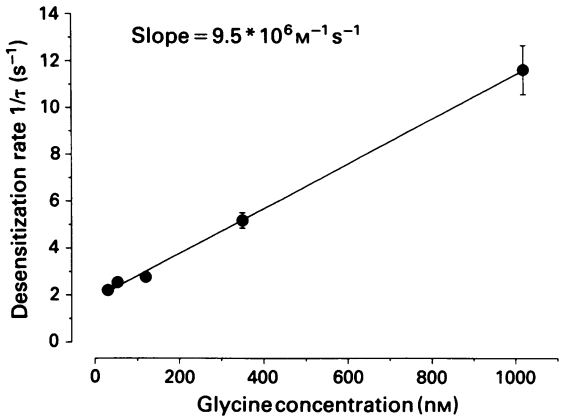


Figure 3 Glycine concentration-dependent change in the rate of N-methyl-D-aspartate (NMDA) receptor desensitization. NMDA (200 μM) was applied as in Figure 1. The rate of desensitization seen with glycine 350 nM and 1020 nM was best described by a double exponential fit, the faster component of which is illustrated (see discussion). Pooled data are plotted as mean reciprocal desensitization rate (± s.e.mean) against corrected glycine concentration. Linear regression analysis was used to determine the slope of $9.51*10^6\ M^{-1}\ S^{-1}$.

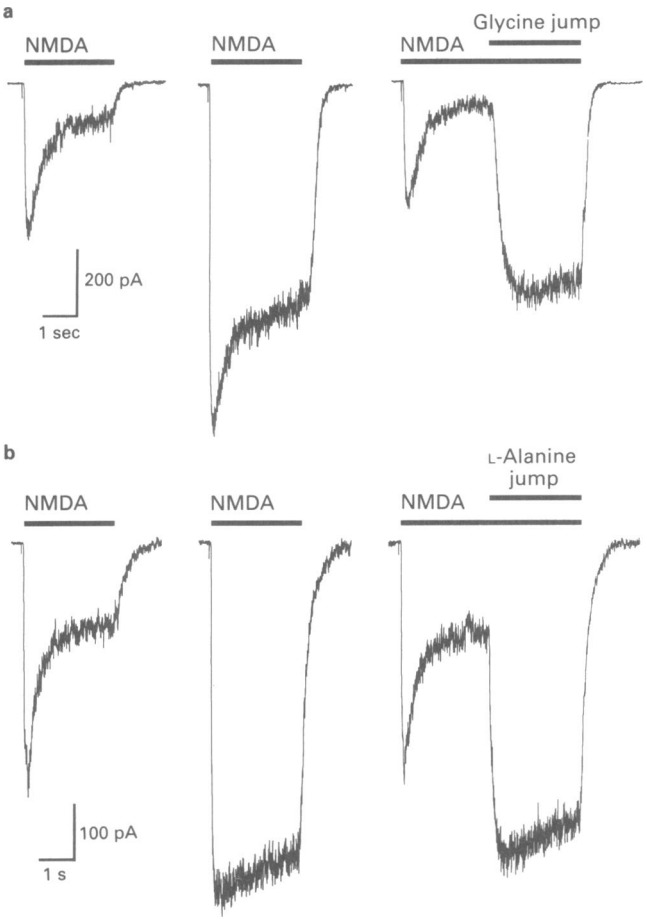


Figure 4 Recovery of N-methyl-D-aspartate (NMDA) receptor desensitization following step increases in glycine or L-alanine concentration. NMDA (200 μM) was applied for 2.5 s (left and middle) or 5 s (right) every 95 s in the continuous presence of glycine (a) or L-alanine (b) NB: two different cells. Traces on the left show desensitization of NMDA responses in the presence of low concentrations of glycine (30 nM) and L-alanine (3.3 μM). Traces in the middle show the facilitation of NMDA responses and decreased degree of desensitization in higher concentrations of glycine (350 nM) and L-alanine (33 μM). Traces on the right show the recovery of desensitized responses following step increases in glycine (30 to 350 nM) or L-alanine (3.3 to 33 μM) concentration 2.5 s after starting the application of NMDA. Note that this recovery was faster for L-alanine than for glycine.

33 μM desensitized responses recovered to a new plateau level at a faster rate than that seen with glycine (Figure 4b, τ_{on} 48.1 ± 9.4 ms, $n = 8$). However, in similar experiments with step changes in glycine concentration from 53 to 350 nM the rate of recovery of responses was faster than the association rate of glycine (τ_{on} 105 ± 11 ms, $n = 6$). This finding lends support to the hypothesis that glycine concentration-dependently increases the rate of recovery from desensitization. These results suggest that the rate of observed glycine-sensitive desensitization is dependent on two opposed mechanisms: (1) the slow dissociation of glycine from the $glycine_B$ site following a rapid decrease in affinity subsequent to activation of the receptor complex by NMDA and (2) a glycine concentration-dependent increase in the rate of recovery from desensitization.

As desensitization only occurs subsequent to activation of the receptor complex by NMDA and glycine it seemed pertinent to examine if the rate and degree of desensitization is dependent on biophysical parameters influencing the microenvironment of, and ion flow through, the NMDA receptor channel i.e. holding potential. This was not the case. Thus, both peak and plateau components of desensitizing responses in the presence of glycine 53 nM showed a linear current-voltage relationship with a reversal potential around 0 mV (Figure 5). Furthermore, the rates of desensitization and the onset and offset rates of NMDA responses were similar over the full voltage range (− 100 to + 50 mV) tested to those measured at − 70 mV (τ desensitization 377 ± 32 ms, τ_{on} 16.7 ± 3.4 ms, τ_{off} 225 ± 37 ms, $n = 7$). These data also provide evidence that glycine-sensitive NMDA receptor desensitization is a property of a homogeneous population of NMDA receptors and is not dependent on the recruitment of secondary mechanisms such as Ca^{2+}-sensitive Cl^- channels.

This conclusion was supported by experiments performed to investigate if the effects of glycine on NMDA desensitization are manifested at the single channel level. Initially, the desensitization of NMDA responses in the continuous presence of glycine 53 nM were recorded in the whole cell mode (Figure 6b). Subsequently, an outside out patch was pulled from the cell to record responses of a single NMDA channel under the same conditions. Eight representative responses to NMDA from such a patch are illustrated in Figure 6a. Single channel analysis was then performed on responses after equal division into transient and plateau components i.e. the first and last 1.25 s of the response. Although averaging of single channel data from several samples indicates that desensitization is indeed exhibited at the single channel level (Figure 6c) it seems unlikely that the small decrease in mean open time and increase in mean close time without a change in the mean amplitude (see Table 2) can solely account for this effect. This probably reflects the inability of conventional single channel analysis used to take account of the long periods of channel silence observed after an initial burst of varying duration. The degree of desensitization seen at the single channel level was less when recordings were made in the continuous presence of glycine 350 nM (Figure 7).

Glycine-sensitive desensitization may represent an endogenous mechanism to differentiate between transient synaptic activation of NMDA receptors and sustained activation through increased basal levels of excitatory amino acids (EAAs). As such, the $glycine_B$ site could be a promising target for pharmaceutical agents aimed at reducing the pathological consequences of sustained increases in basal EAAs and glycine known to occur following e.g. ischaemia. Indeed, the $glycine_B$ antagonist 7-Chl-Kyn concentration-dependently induced desensitization of responses to NMDA in the continuous presence of glycine 1 μM (Figure 7). The effect of increasing 7-Chl-Kyn was very reminiscent of that of decreasing glycine concentration i.e. the degree of desensitization was greater whereas the rate of desensitization was slower (Figure 8). This implies that the kinetics and magnitude of desensitization seen with 7-Chl-Kyn is dependent on

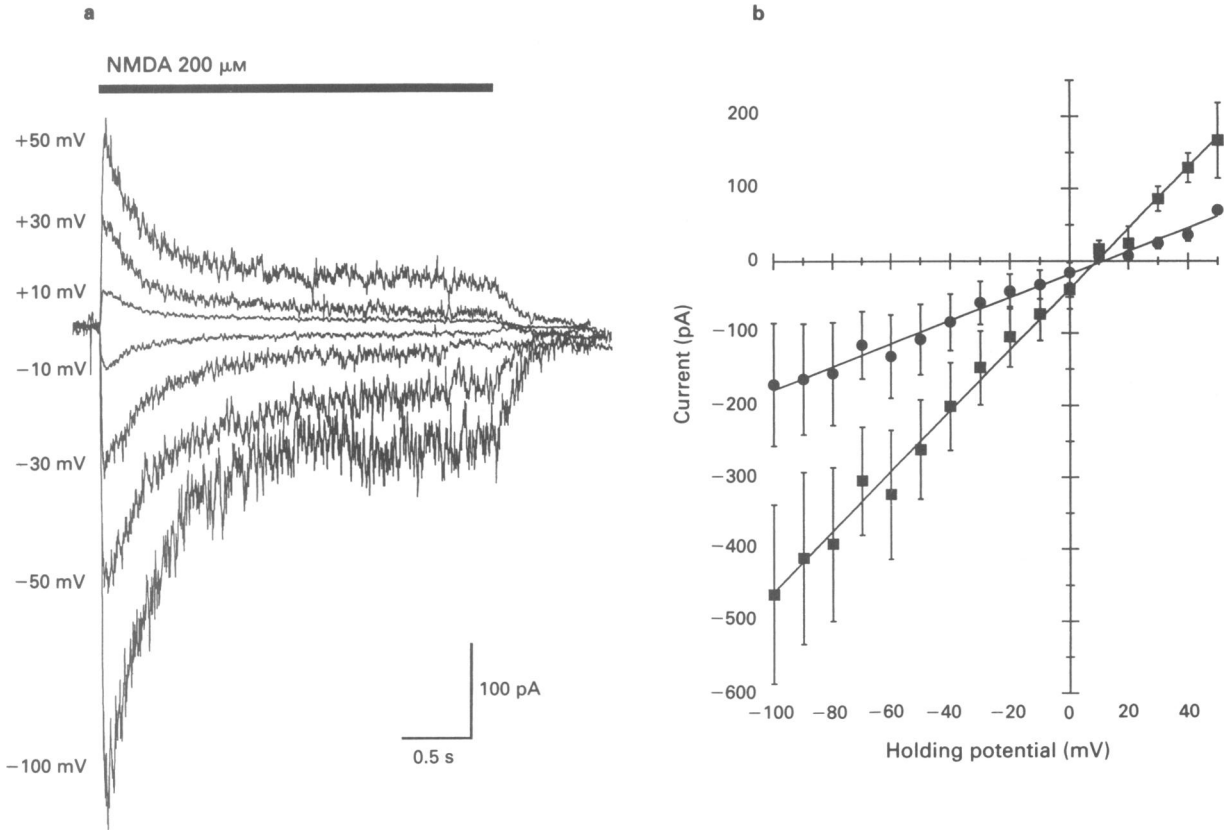


Figure 5 Voltage-independence of the rate and degree of desensitization of N-methyl-D-aspartate (NMDA) responses. (a) NMDA (200 μM) was applied for 2.5 s every 95 s in the continuous presence of glycine 53 nM and at various holding potentials (− 100 mV to + 50 mV). NB: step changes in holding potential were made at least 15 s before and after NMDA applications to avoid distortion of responses by voltage activated currents. (b) Pooled data showing linear current-voltage relationships for both peak (■) and plateau (●) components of responses to NMDA applied as in (a). Data show means ± s.e.mean ($n = 6$).

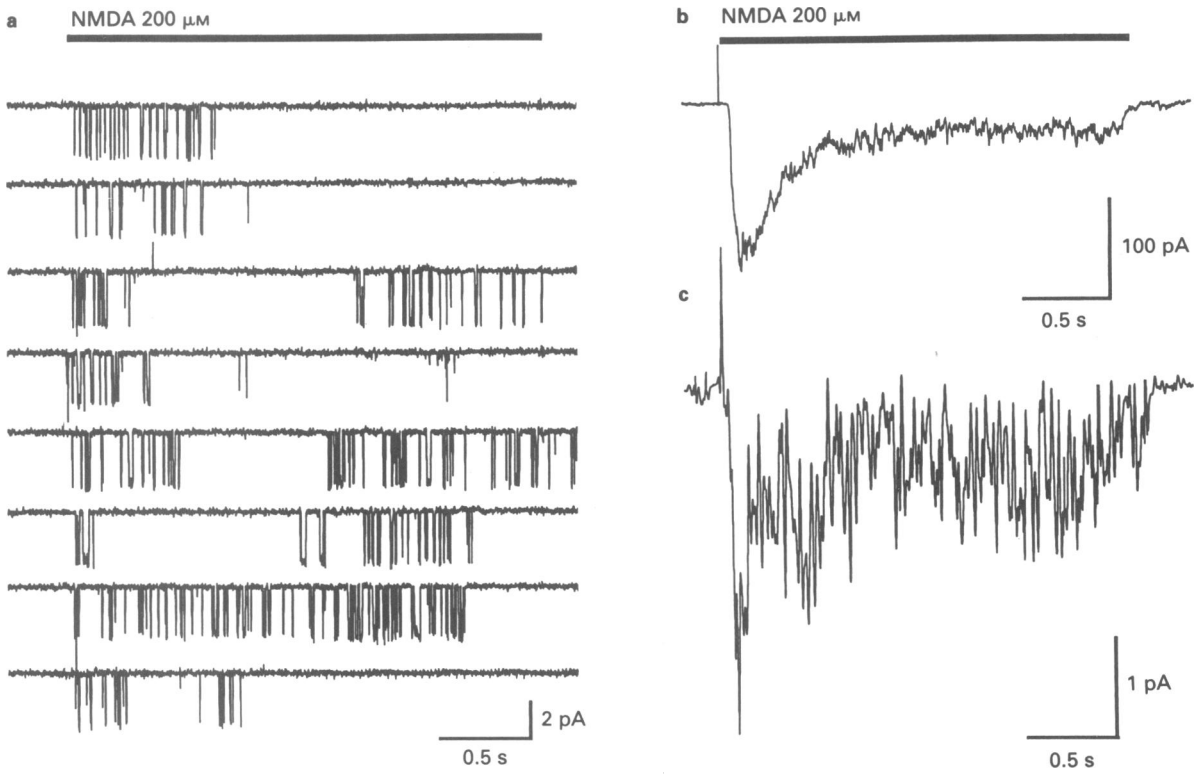


Figure 6 Glycine-sensitive desensitization of single N-methyl-D-aspartate (NMDA) receptor channels. Responses to NMDA 200 μM in the continuous presence of glycine 53 nM were first recorded in whole cell mode (b). Subsequently a patch with a single NMDA channel was pulled from the same cell. Representative single responses to eight applications to NMDA are illustrated. (a). Averaging of single channel responses to 33 NMDA applications (c) revealed a similar degree and kinetics of desensitization to that seen in the whole cell mode.

Table 1 Effects of glycine concentration on the degree and kinetics of NMDA receptor desensitization

Conc (nM)	n	*Peak* (pA)	*s.e.mean*	*Plateau* (pA)	*s.e.mean*	τ_{on} (ms)	*s.e.mean*	*Desen 1* (ms)	*s.e.mean*	*Desen 2* (ms)	*s.e.mean*	τ_{off} (ms)	*s.e.mean*
2520	45	934	51	813	47	24.4	0.9	n/c	n/c	n/c	n/c	103	10
1020	52	753	44	604	41	30.2	2.5	111	8	1715	212	153	13
350	46	624	45	401	34	25.6	2.5	216	10	1689	266	208	16
120	47	583	41	279	23	36.4	3.1	383	15	n/c	n/c	180	20
53	42	534	45	186	24	35.4	4.1	417	12	n/c	n/c	143	12
30	19	356	37	92	19	53.7	9.1	479	24	n/c	n/c	131	47

NMDA was applied as in Figure 1. The rate of desensitization seen with glycine 350 nM and 1020 nM was best described by a double exponential fit, both components of which are presented (Desen 1 and Desen 2). The rise and decay times of responses to NMDA are respectively given under τ_{on} and τ_{off}. n/c = not calculated.

Table 2 Glycine-sensitive desensitization of single NMDA receptor channels recorded from 6 outside out patches

	Peak			*Plateau*		
Glycine (nM)	τ_{open} (ms)	τ_{closed} (ms)	*Conductance* (pS)	τ_{open} (ms)	τ_{closed} (ms)	*Conductance* (pS)
53	3.7	24.4	54.7	3.3	34.4	53.6
53	3.2	18.7	59.2	3.1	24.8	59.5
53	5.4	40.4	53.3	4.3	44.1	53.9
53	4.2	16.8	55.2	4.2	21.5	55.6
350	3.7	28.9	56.7	2.9	28.2	54.8
350	3.5	22.6	57.5	3.6	25.4	58.1

NMDA (200 μM) was applied for 2.5 s every 95 s in the continuous presence of glycine 53 nM or 350 nM and at a holding potential of − 70 mV. The data from 30 to 40 NMDA applications per patch were used for conventional analysis of changes in channel kinetics after division of responses into peak and plateau components.

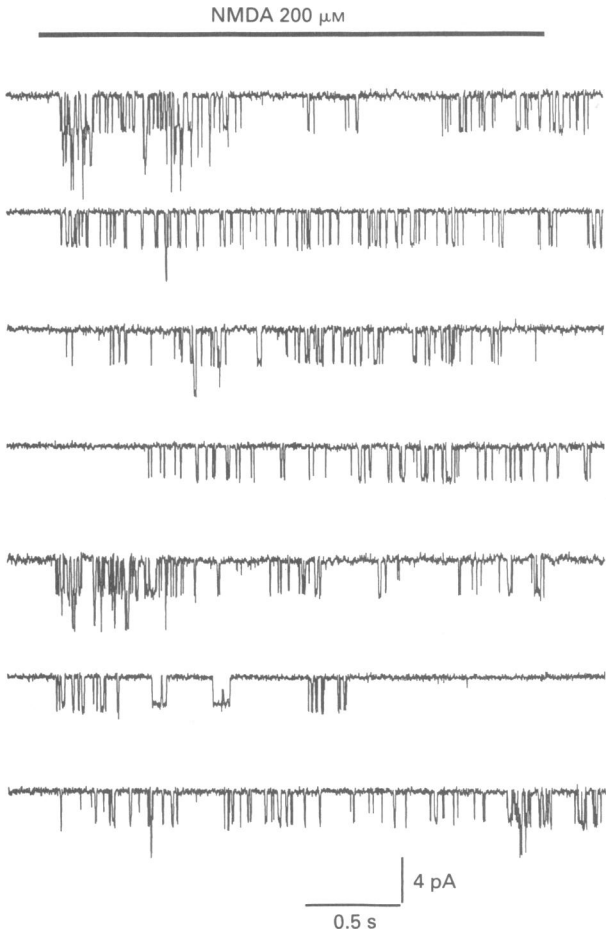


Figure 7 Desensitization of N-methyl-D-aspartate (NMDA) receptor channels is less evident at higher concentrations of glycine. Responses to NMDA 200 μM in the continuous presence of glycine 350 nM were recorded from an outside-out patch. Representative responses to seven applications of NMDA are illustrated. Note the simultaneous activity of 2 or 3 single channels in this patch at the beginning of some responses to NMDA.

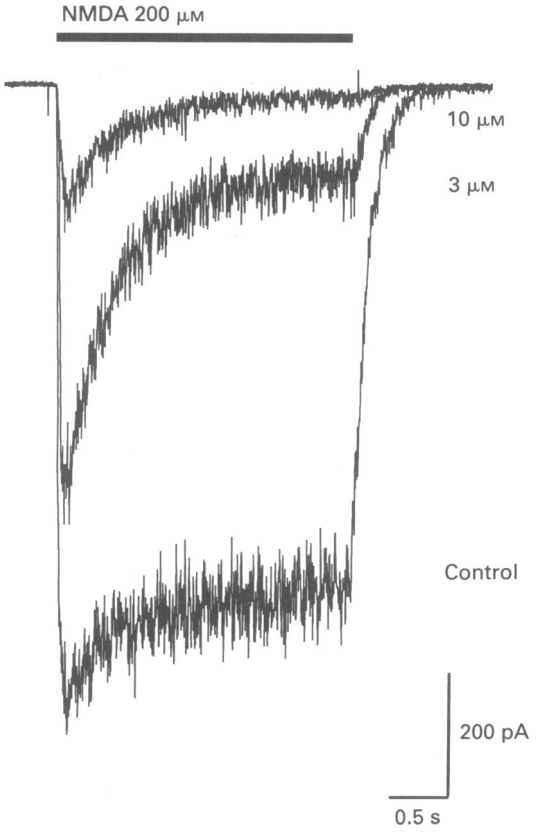


Figure 8 Desensitization of responses to N-methyl-D-aspartate (NMDA) induced by 7-chlorokynurenic acid (7-Chl-Kyn): dependence of the magnitude and kinetics on 7-Chl-Kyn concentration. Responses of a single cell to NMDA (200 μM) in the continuous presence of glycine 1 μM and various concentrations of 7-Chl-Kyn (0 μM, 3 μM and 10 μM) have been superimposed.

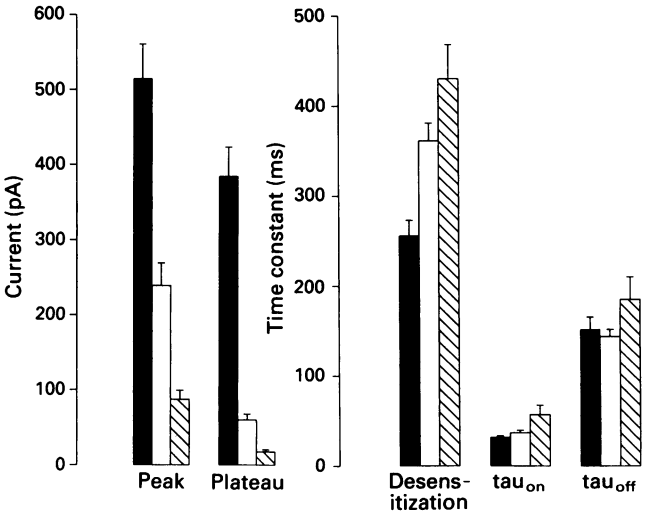


Figure 9 (a) Pooled peak and plateau responses to N-methyl-D-aspartate (NMDA, 200 μM) in the continuous presence of glycine 1 μM and various concentrations of 7-chlorokynurenic acid (7-Chl-Kyn). (b) Pooled kinetic analysis of responses to NMDA (200 μM) in the continuous presence of glycine 1 μM and various concentrations of 7-Chl-Kyn. Pooled data are presented as means ± s.e.mean ($n = 9$). Filled columns = control, open columns = 7-Chl-Kyn 3 μM; hatched columns = 7-Chl-Kyn 10 μM.

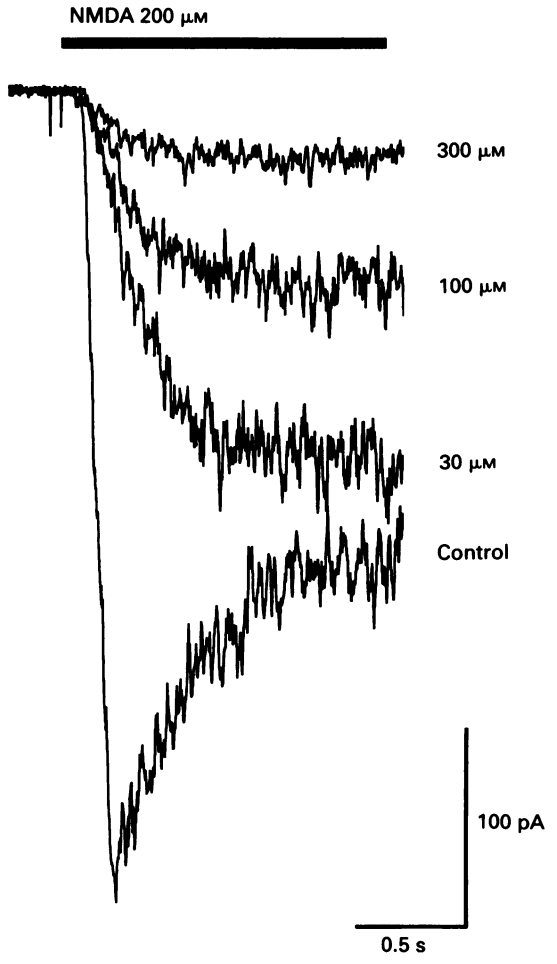


Figure 10 Concentration-dependent antagonism of and slowing in the onset kinetics of N-methyl-D-aspartate (NMDA) responses by (±)-amino-phosphonovaleric acid (APV). Responses of a single cell to NMDA (200 μM) in the continuous presence of glycine 120 nM and various concentrations of APV (control, 30 μM, 100 μM and 300 μM) have been superimposed.

the residual interaction of glycine with the glycine$_B$ site. Moreover, τ_{on} and τ_{off} for NMDA responses were not obviously dependent on the concentration of glycine or 7-Chl-Kyn (Table 1 and Figure 9).Thus it seems improbable that glycine concentration affects the affinity of NMDA for its recognition site as this would be reflected in a consistent, concentration-dependent change in the association/dissociation kinetics of NMDA.

In contrast, the competitive NMDA antagonist APV concentration-dependently antagonized peak responses to NMDA (glycine 120 nM) more than plateau responses (Figure 10) and was associated with a concentration-dependent slowing in τ_{on} for NMDA responses which probably reflects the slow dissociation of APV from the NMDA recognition site. Thus control NMDA responses had a τ_{on} of 51 ± 2.3 ms ($n = 12$) which was slowed to 151 ± 14 ms ($n = 12$), 185 ± 14 ms ($n = 8$) and 286 ± 19 ms ($n = 5$) in the presence of APV 30 μM, 100 μM and 300 μM respectively.

Discussion

The effect of glycine concentration on NMDA receptor desensitization

The magnitude, kinetics and glycine-sensitivity of the desensitization seen in this study with cultured rat superior colliculus neurones was very similar to that previously reported for cultured mouse hippocampal neurones (Mayer *et al.*, 1989; Benveniste *et al.*, 1990a) and for NMDA receptors expressed in *Xenopus* oocytes (Lerma *et al.*, 1990) and indicates receptor homology between the species and brain regions studied. Furthermore, the potency of glycine in potentiating plateau responses is in good agreement with electrophysiological studies at equilibrium (Kleckner & Dingledine, 1988; Kushner *et al.*, 1988) and functional binding studies on the facilitation of MK-801 [(+)-5-methyl-10,11-dihydro-5H-dibenzo-[a,d]cyclohepten-5,10-imine maleate] binding by glycine (Reynolds *et al.*, 1987; Bakker *et al.*, 1991; Pullan *et al.*, 1991).

Taken together, the data provide strong supportive evidence that a 5 fold change in the affinity of glycine$_B$ sites after activation of the NMDA receptor-channel complex underlies the desensitization. This change was reflected by a rightward shift of the glycine concentration-response curve for plateau responses compared to peak responses. The rate of desensitization became faster with increasing glycine concentration with a slope of $9.5*10^6$ M^{-1} s^{-1}. At higher concentrations of glycine the desensitization was best described by a double exponential fit, presumably due to the appearance of slower, calcium-dependent desensitization. This effect of glycine is in good agreement with the data from Benveniste *et al.* (1990a) and has been attributed to a glycine-dependent increase in the rate constant for recovery from desensitization with little effect on the rate constant for onset of desensitization.

The similarity in the rate of desensitization in the presence of glycine 30 nM with the dissociation kinetics of glycine (see also Benveniste *et al.*, 1990a) is indicative that slow dissociation of glycine from the glycine$_B$ site is the rate limiting step in the desensitization process at low nM concentrations of glycine. As such, one would expect the rate of desensitization with L-alanine to be much faster than with glycine due to the faster dissociation kinetics of L-alanine from the glycine$_B$ site (Benveniste *et al.*, 1990b). At first site this did not seem to be the case, single exponential fits of the rate of desensitization seen with L-alanine were very similar to those obtained in the presence of low nM concentrations of glycine (most data not shown but see Figure 4b). However, it is likely that this reflects contamination of all solutions with around 20 nM glycine, a concentration similar to that estimated by Lerma *et al.* (1990) and Benveniste *et al.* (1990a). These problems confounded similar quantitative analysis of peak and plateau concentration-response curves for L-alanine.

Step increases in glycine or L-alanine concentration

Further evidence that the desensitization is due to a change in the affinity of glycine$_B$ sites was provided by experiments where step increases in glycine or L-alanine concentration were made in the continuous presence of NMDA. The rates of recovery of desensitized responses following step changes in glycine and L-alanine concentration were similar to their respective τ_{on}s (see Benveniste *et al.*, 1990b) i.e. responses recovered more quickly following a step change in L-alanine than following a step change in glycine. However, it seems unlikely that the recovery rate of responses was exclusively-dependent on the association rate of the glycine agonists used as the rate of recovery was slower following step changes in glycine from 30 nM to 350 nM than following changes from 53 nM to 350 nM. Thus, it is probable that the forward rate constant with which glycine binds to its recognition site (Benveniste *et al.*, 1990a) also influences the rate of desensitization.

Activation of the NMDA receptor complex by agonists at both the NMDA and glycine recognition sites is necessary for desensitization

It has previously been claimed that binding of aspartate to the NMDA recognition site in the absence of channel activation i.e. in the absence of glycine, is, *per se*, sufficient to initiate a change in the affinity of glycine$_B$ sites (Chizhmakov *et al.*, 1990). However, contamination of solutions with low nM concentrations of glycine is unavoidable and thus invalidates the use of preincubations with an NMDA agonist in the absence of added glycine to test this hypothesis. Thus the small response to NMDA without added glycine seen in our study was almost certainly attributable to the estimated contamination of all solutions with 20 nM glycine. Although the authors claimed that such preincubations evoked no responses, the contrary was evident from the data presented and they themselves provided evidence for some degree of glycine contamination as preincubation with aspartate plus the glycine$_B$ antagonist 7-Chl-kyn was unable to influence subsequent desensitization.

It therefore seems likely that activation of the NMDA receptor complex is necessary for desensitization. However, the desensitization seen at low nM concentrations of glycine does not seem due to changes in the conductance of NMDA channels or recruitment of secondary mechanisms such as Ca^{2+}-activated Cl^- channels. Thus the rate and relative magnitude of desensitization seen in the presence of 53 nM glycine was not dependent on holding potential. Both peak and plateau responses showed a linear current voltage relationship with a reversal potential of around 0 mV. This is as would be predicted when both components of the current are directly attributable to ion flow through NMDA channels under the conditions used and in the absence of Mg^{2+} and provides strong evidence against a role of Ca^{2+}-activated Cl^- channels (see Leonard & Kelso, 1990) or pH-sensitive Na^+ channels (see Grantyn & Lux, 1988) in mediating the desensitization.

Single channel analysis

Glycine-sensitive desensitization was also observed at the single channel level. This effect was partially manifested as a small decrease in the mean open time and a small increase in the mean close time without an effect on channel conductance. However, the observed changes in these conventional parameters were too small to account exclusively for the desensitization. More pronounced changes were apparent in the pattern of firing. Single channel responses to NMDA in the presence of glycine 53 nM were characterized by an initial burst of channel activity of variable duration followed by long periods of channel inactivity. This was much less evident for single channel responses recorded in the continuous presence of glycine 350 nM. It is tempting to speculate that these bursts occur whilst glycine remains bound to the glycine$_B$ site for hundreds of milliseconds and that the periods of inactivity occur following dissociation of glycine.

Ca^{2+}-sensitive NMDA receptor desensitization

Although these data indicate that the desensitization seen at low nM concentrations of glycine can be attributed to changes in the kinetics of a homologous species of NMDA receptors it should be noted that the second slower component of desensitization which became apparent at concentrations of glycine greater than 350 nM had a time course which was similar to that seen in the presence of 1.5 mM Ca^{2+} (data not shown). This probably reflects the appearance of Ca^{2+}-sensitive desensitization following near maximal activation of NMDA receptors and probably accounts for the inability of even the highest concentrations of glycine to eliminate desensitization completely. Further studies in the complete absence of extracellular calcium and/or blockers of Ca^{2+}-activated Cl^- channels such as flufenamic acid are required to perform quantitative concentration-response analysis at higher concentrations of glycine and to assess the nature of this Ca^{2+}-sensitive desensitization.

7-Chlorokynurenic acid

This study confirmed the finding that the glycine$_B$ antagonist, 7-Chl-Kyn, induces desensitization of NMDA responses in a manner reminiscent to that of decreasing glycine concentration i.e. the concentration-dependent increase in the magnitude of desensitization was accompanied by a slowing in kinetics. As the association/dissociation kinetic of 7-Chl-Kyn are much faster than those of glycine (Benveniste & Mayer, 1991a) it seems likely that the kinetics of the desensitization seen in the presence of 7-Chl-Kyn is dependent on the residual interaction of glycine with the glycine$_B$ site. Moreover, the affinity of NMDA for its recognition site was not influenced by changes in glycine or 7-Chl-Kyn concentration as this would have been reflected in changes in the τ_{on} and/or τ_{off} of NMDA responses.

Buffered diffusion

It should be noted that the on and off rates of NMDA responses were slower than those reported in mouse hippocampal neurones (Patneau & Mayer, 1990) but faster than those reported for *Xenopus* oocytes (Lerma *et al.*, 1990). It has been pointed out that such differences can be attributed to slow solution exchange times. This seems unlikely as the exchange time was estimated to be under 10 ms and responses to AMPA under the same conditions were very fast (τ_{on} around 6 to 8 ms, τ desensitization around 30 ms, data not shown). It seems more likely that the phenomenon of 'buffered diffusion' in the restricted extracellular space between neurones and glial cells could partially account for this difference and highlights the necessity of using young cultures with few glia when studying the on and off rates of agonists and antagonists. However, this phenomenon should not affect glycine-sensitive desensitization if this process is indeed dependent on a change in the affinity of glycine$_B$ sites in a constant concentration of agonist.

Therapeutic consequences

Glycine-dependent desensitization could have very important consequences for patho-physiological processes such as those occurring following ischaemia or in cerebrovascular dementia as regional parallel increases in the extracellular levels of glycine and glutamate have been reported following an ischaemic insult (Baker *et al.*, 1991; Globus *et al.*, 1991a,b). This might be expected to worsen the damage caused by excessive NMDA receptor activation by preventing desen-

sitization. Systemically active glycine$_B$ antagonists could therefore prove to be useful therapeutic tools as they might be expected to facilitate desensitization of NMDA receptors following sustained activation by pathologically raised levels of EAA. Moreover, although the NMDA component of excitatory postsynaptic currents mediated at glutamatergic synapses has a duration of several hunded milliseconds (Lester *et al.*, 1990) its time course is still within the window of glycine-sensitive desensitization and, as such, would be expected to be relatively less affected by glycine antagonists. It is interesting to note that the same does not appear to be true for + HA-966 which is a partial agonist at the glycine$_B$ site but suppresses peak responses to a greater degree than plateau responses and reduces the affinity of glutamate for the NMDA recognition site (Kemp & Priestley, 1991). This effect is similar to that seen with competitive NMDA antagonists (see also Benveniste & Mayer 1991b) and both classes of antagonist might therefore be expected to influence synaptic transmission more than pathological processes and will probably be burdened with a variety of side effects similar to those seen with uncompetitive NMDA channel blockers such as phencyclidine and MK-801.

In conclusion, glycine-sensitive, NMDA receptor desensitization is due to a rapid change in the affinity of glycine for glycine$_B$ receptors following activation of the receptor-channel complex by NMDA. The rate of desensitization seen at low concentrations of glycine is largely dependent on the dissociation kinetics of glycine and is likely to be slower than the NMDA-induced change in the properties of the glycine$_B$ site. At higher concentrations, the forward rate at which glycine binds to its recognition site also plays a role in determining the observed rate of desensitization.

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Effects of chronic treatment with nitric oxide synthase inhibitors on regional haemodynamic responses to vasodilators in conscious Brattleboro rats

[1]Sheila M. Gardiner, Philip A. Kemp & Terence Bennett

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

1 The effects of acute inhibition of nitric oxide (NO) synthase on cardiovascular responses to vasodilator challenges have already been described. We now report the responses to vasodilators during and after chronic NO synthase inhibition.

2 In conscious Brattleboro rats, the regional haemodynamic effects of 3 min infusions of acetylcholine (4 $\mu g\ min^{-1}$), sodium nitroprusside (15 $\mu g\ min^{-1}$) or adrenaline (0.2 $\mu g\ min^{-1}$) were assessed (from areas under or over curves (AUC, AOC)) under control conditions, 6 and 72 h after the addition of the NO synthase inhibitor, N^G-monomethyl-L-arginine (L-NMMA) to the drinking water (1 $mg\ ml^{-1}$), and 6, 24 and 48 h after the withdrawal of L-NMMA. In a separate group of Brattleboro rats, responses to acetylcholine, sodium nitroprusside and adrenaline were assessed before and 6 h after the onset of oral ingestion of the more potent nitric oxide synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME; 0.05 $mg\ ml^{-1}$).

3 Acetylcholine caused renal vasodilatation (87 ± 11 units) and mesenteric vasoconstriction (−31 ± 5 units), sodium nitroprusside caused vasodilatation in renal (96 ± 12 units), mesenteric (222 ± 13 units) and hindquarters (49 ± 15 units) vascular beds, whereas adrenaline caused hindquarters vasodilatation (92 ± 8 units). Seventy two h after the onset of oral ingestion of L-NMMA, acetylcholine had a decreased renal vasodilator (59 ± 9 units) effect, sodium nitroprusside had an increased renal vasodilator (142 ± 23 units) action, while adrenaline had a decreased hindquarters vasodilator (55 ± 6 units) influence. Twenty four h after withdrawal of L-NMMA, the renal vasodilator effect of acetylcholine was greater than the control response (106 ± 14 units), but the regional haemodynamic effects of sodium nitroprusside and adrenaline were not different from those under control conditions. Hence, the increased renal vasodilator response to acetylcholine was probably due to changes in muscarinic receptor-mediated mechanisms rather than to any increase in guanylyl cyclase or its sensitivity to NO.

Keywords: Nitric oxide; vasodilators; haemodynamics

Introduction

The discovery that nitric oxide (NO) is a major endothelium-derived relaxing factor (see Moncada *et al.*, 1991a), the synthesis of which can be inhibited by analogues of L-arginine, such as N^G-monomethyl-L-arginine (L-NMMA) and N^G-nitro-L-arginine methyl ester (L-NAME) (Moore *et al.*, 1990; Rees *et al.*, 1990), has made it possible to assess the involvement of NO in the haemodynamic responses to vasodilator agents *in vivo*; such studies have produced some surprising results. Thus, acute, *in vivo* treatment with L-NAME inhibits, but does not abolish, the renal vasodilator effects of acetylcholine (Gardiner *et al.*, 1990d; 1991a,b), and the hindquarters vasodilator effects of adrenaline (Gardiner *et al.*, 1991c). In contrast, acute treatment with L-NAME enhances the mesenteric vasodilator effects of sodium nitroprusside (Gardiner *et al.*, 1991b). The questions left unanswered by those earlier studies are (1) what effect does more long-term inhibition of NO synthase have, i.e., does the system in any way adapt, and (2) what happens when treatment with the NO synthase inhibitor is withdrawn i.e., is the system reset?

Chronic inhibition of NO synthase can be achieved by addition of L-NMMA or L-NAME to the drinking water of polydipsic, vasopressin-deficient, Brattleboro rats (Gardiner *et al.*, 1990b; 1992). We did not use the congenic control (i.e., Long Evans) rats in those studies because, like other normal strains, Long Evans rats only drink when they eat (i.e., during the dark cycle) and hence addition of L-NMMA or L-NAME to their drinking water would not have resulted in sustained inhibition of NO synthase. Therefore, the objectives of the present work were to determine, in conscious Brattleboro rats, the regional haemodynamic responses to 3 min infusions of acetylcholine, sodium nitroprusside and adrenaline (at the doses used previously in Long Evans rats) before, during and after chronic oral ingestion of L-NMMA. We intended also to assess the effects of L-NAME in the same model. However, we found that instrumented Brattleboro rats showed signs of respiratory distress even when, as here (see also Gardiner *et al.*, 1992), they were given L-NAME to drink at a concentration of 0.05 $mg\ ml^{-1}$ for periods in excess of 24 h. Therefore, the experiments with L-NAME were confined to assessing the effects of the vasodilators before and 6 h after the onset of L-NAME ingestion.

Methods

All experiments were carried out on male, homozygous Brattleboro rats (350–450 g). Under sodium methohexitone anaesthesia (60 $mg\ kg^{-1}$, i.p., supplemented as required), pulsed Doppler probes (Haywood *et al.*, 1981) were sutured around the left renal and superior mesenteric arteries, and the distal abdominal aorta (to monitor hindquarters blood flow). Probe wires were led subcutaneously to emerge at the back of the neck where they were anchored to the skin by a suture. Animals were given ampicillin (Penbritin, Beecham,

[1] Author for correspondence.

7 mg kg^{-1}, i.m.) and returned to individual home cages with free access to water and food. At least 7 days later, animals were anaesthetized (sodium methohexitone 40 mg kg^{-1}, i.p.) and had an intra-arterial catheter (distal abdominal aorta via the ventral caudal artery) and 3 intravenous catheters (right jugular vein) implanted. The catheters ran subcutaneously to emerge at the same point as the probe wires. The latter were soldered into a microconnector (Microtech Inc., Boothwyn, USA) that was clamped into a harness fitted to the rat. A flexible spring was connected to the harness, and the catheters ran through this for protection. The connector to the pulsed Doppler probes was taped to the spring and the latter was supported by a counter-balanced lever system that allowed the rat free movement in its home cage to which it was returned with free access to food and water. Experiments did not begin until 48 h later.

On the first experimental day, baseline recording of phasic and mean arterial blood pressures, instantaneous heart rate, and phasic and mean Doppler shift signals [using a modified (Gardiner *et al.*, 1990a) Crystal Biotech VF1 mainframe] were made over 30 min (between 06 h 00 min and 06 h 30 min), during which time rats were drinking water. While still drinking water, animals were given randomized 3 min infusions of acetylcholine (4 μg min^{-1}), sodium nitroprusside (15 μg min^{-1}) and adrenaline (0.2 μg min^{-1}) over the next 30 min. These doses were chosen on the basis of our previous studies in Long Evans rats (Gardiner *et al.*, 1991b,c), showing that they evoked reproducible and readily quantifiable responses. (Since the animals studied had intact baroreflexes it was not feasible to obtain full dose-response curves to the vasodilators, and we considered it unethical to subject the animals to ganglion blockade in these chronic experiments).

At 07 h 00 min the drinking water was exchanged for an aqueous solution of L-NMMA (1 mg ml^{-1}) or L-NAME (0.05 mg ml^{-1}), and cardiovascular recordings were made every h for the next 6 h. At that juncture, animals were re-challenged with infusions of acetylcholine, sodium nitroprusside and adrenaline, as before. The experiment involving oral ingestion of L-NAME was stopped at this stage because the majority of animals showed signs of respiratory distress (they recovered rapidly when given normal tap water to drink). However, since all animals drinking the solution of L-NMMA showed no adverse signs, this experiment was continued for another 66 h (i.e. 72 h in total) with the animals ingesting L-NMMA. At the end of 72 h, these animals were re-challenged with acetylcholine, sodium nitroprusside and adrenaline before they were switched back to drinking tap water. Six, 24 and 48 h after withdrawal of L-NMMA from the drinking water the rats were again given 3 min infusions of acetylcholine, sodium nitroprusside and adrenaline.

Under resting conditions heart rate, mean arterial blood pressure and mean Doppler shift signals were averaged (by eye) over 20 s epochs. During infusion of acetylcholine, sodium nitroprusside or adrenaline, values straddling the 1, 2 and 3 min time points during the infusion were measured. Since all animals acted as their own controls throughout the experiment, absolute values (kHz) for Doppler shift signals were taken as indices of flow. Vascular conductances were calculated by dividing mean arterial blood pressure into mean Doppler shift.

Data analysis

Within group analysis was carried out by Friedman's test (Theodorsson-Norheim, 1987), applied to resting values. Comparison of responses to acetylcholine, or to sodium nitroprusside, or to adrenaline, under different experimental conditions in the same animals, were made by Friedman's test applied to areas under or over curves representing measurements made during the 3 min infusions. Comparison of the effects of L-NMMA and L-NAME on responses to the infusions of acetylcholine, sodium nitroprusside or adrenaline were made with the Mann-Whitney U test applied to the difference between the two respective control response and the response in the presence of L-NMMA or L-NAME. A P value <0.05 was taken as significant.

Drugs

L-NMMA acetate was synthesized by Dr H. Hodson (Wellcome Research Laboratories). L-NAME hydrochloride, acetylcholine chloride, adrenaline bitartrate and sodium nitroprusside were obtained from Sigma (UK). All drugs except L-NMMA and L-NAME were dissolved in sterile saline (154 mmol l^{-1} NaCl); L-NMMA and L-NAME were dissolved in tap water. The stock solution of adrenaline contained 0.1 M ascorbic acid, and this was diluted 1:100 before use. Drug infusions were given at the rate of 0.15 ml min^{-1}; infusion of saline at this rate had no cardiovascular effects. Throughout the experiment, when cardiovascular variables were not being recorded, the arterial catheter was connected to a fluid-filled swivel (Brown *et al.*, 1976) through which saline was infused (10 u ml^{-1} heparin at 0.3 ml h^{-1}) to keep the catheter patent.

Results

Experiment 1

Effects of L-NMMA on resting cardiovascular variables Under control conditions, cardiovascular variables in the animals that were going to ingest L-NMMA were similar in all phases of the experiment (Table 1). As noted previously (Gardiner *et al.*, 1990b; 1992), oral ingestion of L-NMMA caused progressive hypertension, a variable bradycardia and initial vasoconstriction in renal, mesenteric and hindquarters vascular beds (data not shown). In the present experiment, 6 h after the start of L-NMMA ingestion, mean arterial blood pressure was increased in association with mesenteric and hindquarters vasoconstriction (Table 1). By 72 h after the start of L-NMMA ingestion, although the increase in mean arterial blood pressure was generally well-maintained, mesenteric vascular conductance was no longer reduced, and the hindquarters vasoconstriction was tending to diminish (Table 1). Six h after withdrawal of L-NMMA, all variables were not different from baseline, with the exception of mesenteric vascular conductance, which was elevated. This latter effect was even more marked 24 h after withdrawal of L-NMMA, and at this time there was a slight, but significant, reduction in mean arterial blood pressure relative to the pre-L-NMMA baseline (Table 1). Thereafter, mean arterial blood pressure returned to control levels, although mesenteric vascular conductance remained elevated (Table 1).

Responses to acetylcholine in the absence and presence of L-NMMA When animals were drinking water, infusion of acetylcholine caused a slight hypotension, tachycardia, a clear increase in renal flow, a reduction in mesenteric flow, but no change in hindquarters flow; there was renal vasodilatation, mesenteric vasoconstriction, but no change in hindquarters vascular conductance (Table 2).

Six h after the start of L-NMMA ingestion, the pattern of response to acetylcholine was as before, although the hypotensive effect was increased and the renal vasodilator, and the mesenteric vasoconstrictor effects were attenuated (Table 2). After the animals had been ingesting L-NMMA for 72 h, the hypotensive response to acetylcholine was still augmented and the renal vasodilator effect was still attenuated, but the mesenteric vasoconstrictor response was not different from control (Table 2). Six h after withdrawal of L-NMMA, the effects of acetylcholine were not different from the control responses (Table 2). However, 24 h after withdrawal of L-NMMA, there was an increase in the hypotensive and renal vasodilator effects of acetylcholine, and the latter effect per-

Table 1 Resting cardiovascular variables (i.e. prior to challenge with acetylcholine, sodium nitroprusside or adrenaline) in the same, conscious Brattleboro rats ($n = 9$) under control conditions, 6 h and 72 h after the start of oral ingestion of N^G-monomethyl-L-arginine (L-NMMA) in the drinking water, and 6 h, 24 h and 48 h after withdrawal of L-NMMA

				On L-NMMA			*Off L-NMMA*	
			Control (a)	6 h (b)	72 h (c)	6 h (d)	24 h (e)	48 h (f)
Heart rate (beats min^{-1})		Pre-acetylcholine	305 ± 4	294 ± 9de	275 ± 10^{adef}	316 ± 11	311 ± 3	307 ± 4
		Pre-nitroprusside	298 ± 5	303 ± 6	293 ± 14	313 ± 6	323 ± 8	306 ± 7
		Pre-adrenaline	309 ± 6	298 ± 10	292 ± 14	314 ± 8	305 ± 6	303 ± 5
Mean arterial blood pressure (mmHg)		Pre-acetylcholine	110 ± 2	132 ± 3^{adef}	128 ± 5^{adef}	110 ± 4	103 ± 2^{af}	108 ± 3
		Pre-nitroprusside	109 ± 2	128 ± 3^{adel}	123 ± 4^{adef}	107 ± 2	106 ± 2^{a}	106 ± 3
		Pre-adrenaline	113 ± 4	136 ± 4^{acdef}	121 ± 3^{adef}	114 ± 3^{ef}	105 ± 3^{a}	106 ± 3^{a}
Vascular conductance ([kHz $mmHg^{-1}$]10^3)	Renal	Pre-acetylcholine	72 ± 7	59 ± 6	66 ± 6	72 ± 5	72 ± 8	71 ± 8
		Pre-nitroprusside	72 ± 7	60 ± 6	71 ± 7	76 ± 7	68 ± 7	67 ± 8
		Pre-adrenaline	72 ± 7	58 ± 6	73 ± 10	77 ± 6	67 ± 7	71 ± 7
	Mesenteric	Pre-acetylcholine	84 ± 9	58 ± 7^{acdef}	74 ± 6^{def}	90 ± 9^{ef}	109 ± 10^{a}	101 ± 8^{a}
		Pre-nitroprusside	77 ± 7	61 ± 5^{acdef}	80 ± 5^{def}	90 ± 8^{ae}	111 ± 10^{af}	98 ± 8^{a}
		Pre-adrenaline	80 ± 9	57 ± 6^{acdef}	78 ± 4^{def}	94 ± 7^{aef}	105 ± 9^{a}	106 ± 9^{a}
	Hindquarters	Pre-acetylcholine	39 ± 4	24 ± 1^{acdef}	34 ± 4^{def}	40 ± 4	42 ± 3	41 ± 2
		Pre-nitroprusside	39 ± 4	26 ± 1^{acdef}	32 ± 3^{adef}	39 ± 4^{f}	41 ± 4	41 ± 2
		Pre-adrenaline	36 ± 4	23 ± 2^{acdef}	33 ± 4^{ef}	34 ± 3^{ef}	41 ± 2	43 ± 3

Values are mean ± s.e.mean.
Superscript letters indicate significant ($P < 0.05$) differences from the values in the columns labelled with those letters (Friedman's test).

Table 2 Cardiovascular changes (areas under or over curves) during 3 min infusions of acetylcholine (4 μg min^{-1}) in the same, conscious Brattleboro rats ($n = 9$) under control conditions, 6 h and 72 h after the start of oral ingestion of N^G-monomethyl-L-arginine (L-NMMA) in the drinking water, and 6 h, 24 h and 48 h after withdrawal of L-NMMA

	On L-NMMA			*Off L-NMMA*		
	Control (a)	6 h (b)	72 h (c)	6 h (d)	24 h (e)	48 h (f)
ΔHeart rate (beats)	92 ± 9*	94 ± 21	118 ± 10	103 ± 13	121 ± 14	105 ± 14
ΔMean arterial blood pressure (mmHg min)	−18 ± 3*	−36 ± 6^{adf}	−35 ± 8^{ad}	−18 ± 4^{e}	−31 ± 5^{a}	−26 ± 4
ΔRenal conductance (([kHz $mmHg^{-1}$]10^3) min)	87 ± 11*	59 ± 8^{adef}	59 ± 9^{adef}	79 ± 11^{ef}	106 ± 14^{a}	109 ± 11^{a}
ΔMesenteric conductance (([kHz $mmHg^{-1}$]10^3) min)	−31 ± 5*	−11 ± 4^{acdef}	−33 ± 6	−38 ± 13	−37 ± 9	−34 ± 7
ΔHindquarters conductance (([kHz $mmHg^{-1}$]10^3) min)	5 ± 1	6 ± 1	7 ± 2	−6 ± 2	6 ± 3	6 ± 2

Values are mean ± s.e.mean.
*$P < 0.05$ for change versus baseline; superscript letters indicate significant ($P < 0.05$) differences from the values in the columns labelled with those letters (Friedman's test).

sisted at 48 h, although at that time the hypotensive response to acetylcholine was not significantly enhanced (Table 2).

Responses to sodium nitroprusside in the absence and presence of L-NMMA Under control conditions, sodium nitroprusside caused hypotension, tachycardia, a marked increase in mesenteric blood flow, a slight fall in renal blood flow, but no significant change in hindquarters flow (Table 3); all three vascular beds showed vasodilatation, with the rise in mesenteric vascular conductance being the greatest (Table 3).

Six h after L-NMMA was added to the drinking water, there was an enhancement of the hypotensive response to sodium nitroprusside (i.e., it fell from a higher level to the same nadir), but no significant change in any of its other effects (Table 3). However, by 72 h after the start of L-NMMA ingestion, the augmented hypotensive effect of sodium nitroprusside was accompanied by an increased renal vasodilator response (Table 3). Six h after withdrawal of L-NMMA, all responses to sodium nitroprusside were not different from those under control conditions, and this was also the case 24 h and 48 h after withdrawl of L-NMMA (Table 3).

Responses to adrenaline in the absence and presence of L-NMMA Under control conditions, adrenaline caused no significant change in mean arterial blood pressure, but there was a tachycardia and a marked increase in hindquarters blood flow, while mesenteric flow was reduced slightly and renal flow was unchanged; there was marked hindquarters vasodilatation, but no significant change in renal or mesenteric vascular conductance (Table 4).

Six h after L-NMMA had been added to the drinking water, adrenaline still had no significant effect on mean arterial blood pressure, but the hindquarters vasodilator effect was reduced, and its tachycardic effect was no longer significant (although the change in heart rate was not significantly different from that under control conditions (Table 4)). Seventy two h after the start of L-NMMA ingestion, adrenaline infusion caused a significant pressor effect that was different from the response under control conditions (Table 4), and it was accompanied by a further reduction in the hindquarters vasodilator effect (Table 4). Although there was no significant tachycardia overall, the variability in this effect led to there being no significant difference from the control response (Table 4). Likewise, while there was a significant mesenteric vasoconstrictor response to adrenaline 72 h after the start of L-NMMA ingestion, this effect was not different from the pre-L-NMMA response (Table 4). However, there was a significant renal vasoconstriction that was different from the control response (Table 4). Six h after withdrawal of L-NMMA all responses to adrenaline were not different from those seen under control conditions, and similar results were obtained 24 and 48 h after withdrawal of L-NMMA (Table 4).

Table 3 Cardiovascular changes (areas under or over curves) during 3 min infusions of sodium nitroprusside (15 μg min^{-1}) in the same, conscious Brattleboro rats ($n = 9$) under control conditions, 6 h and 72 h after the start of oral ingestion of N^G-monomethyl-L-arginine (L-NMMA) in the drinking water, and 6 h, 24 h and 48 h after withdrawal of L-NMMA

		On L-NMMA		*Off L-NMMA*		
	Control (a)	6 h (b)	72 h (c)	6 h (d)	24 h (e)	48 h (f)
ΔHeart rate (beats)	307 ± 38*	230 ± 50	203 ± 31	218 ± 24	197 ± 36	261 ± 18
ΔMean arterial blood pressure (mmHg min)	−124 ± 9*	−188 ± 10^{adef}	−206 ± 17^{adef}	−141 ± 4	−137 ± 10	−131 ± 6
ΔRenal conductance (([kHz mmHg^{-1}]10^3) min)	96 ± 12*	111 ± 9^{e}	142 ± 23^{ade}	103 ± 6	91 ± 13^{f}	109 ± 9
ΔMesenteric conductance (([kHz mmHg^{-1}]10^3) min)	222 ± 13*	232 ± 29	259 ± 36	204 ± 23	204 ± 24	249 ± 33
ΔHindquarters conductance (([kHz mmHg^{-1}]10^3) min)	49 ± 15*	63 ± 15	96 ± 23	67 ± 15	57 ± 14	49 ± 11

Values are mean ± s.e.mean.
*$P < 0.05$ for change versus baseline; superscript letters indicate significant ($P < 0.05$) differences from the values in the columns labelled with those letters (Friedman's test).

Table 4 Cardiovascular changes (areas under or over curves) during 3 min infusions of adrenaline (0.2 μg min^{-1}) in the same, conscious Brattleboro rats ($n = 9$) under control conditions, 6 h and 72 h after the start of oral ingestion of N^G-monomethyl-L-arginine (L-NMMA) in the drinking water, and 6 h, 24 h and 48 h after withdrawal of L-NMMA

		On L-NMMA		*Off L-NMMA*		
	Control (a)	6 h (b)	72 h (c)	6 h (d)	24 h (e)	48 h (f)
ΔHeart rate (beats)	98 ± 30*	64 ± 24	25 ± 8	70 ± 28	46 ± 14	35 ± 9
ΔMean arterial blood pressure (mmHg min)	−12 ± 5	9 ± 3^{c}	18 ± 6^{adef}	−9 ± 5	−7 ± 3	−9 ± 3
ΔRenal conductance (([kHz mmHg^{-1}]10^3) min)	11 ± 3	−13 ± 4^{cdf}	−20 ± 5^{adf}	13 ± 3^{e}	−9 ± 4	−8 ± 2
ΔMesenteric conductance (([kHz mmHg^{-1}]10^3) min)	−20 ± 7	−22 ± 6	−38 ± 5	−30 ± 6	−24 ± 4	−26 ± 6
ΔHindquarters conductance (([kHz mmHg^{-1}]10^3) min)	92 ± 8*	69 ± 7^{acdef}	55 ± 6^{adef}	92 ± 9	98 ± 10	88 ± 8

Values are mean ± s.e.mean.
*$P < 0.05$ for change versus baseline; superscript letters indicate significant ($P < 0.05$) differences from the values in the columns labelled with those letters (Friedman's test).

Experiment 2

Effects of L-NAME on resting cardiovascular variables Six h after the start of oral ingestion of L-NAME there was an increase in mean arterial blood pressure accompanied by reductions in heart rate, mesenteric and hindquarters blood flow, and renal, mesenteric, and hindquarters vascular conductances (Table 5). The generally more marked effects of L-NAME than L-NMMA on resting cardiovascular variables are consistent with previous observations (Gardiner *et al.*, 1990c; 1991a), and were not attributable to any differences between pretreatment values in the two groups of rats (Tables 1 and 5).

Responses to acetylcholine in the absence and presence of L-NAME Acetylcholine caused hypotension, tachycardia, an increase in renal and a decrease in mesenteric flows and vascular conductances, while hindquarters haemodynamics were unchanged (Table 6). In the presence of L-NAME (i.e. 6 h after the start of ingestion) the hypotensive effect of acetylcholine was enhanced while its mesenteric vasoconstrictor and renal vasodilator effects were inhibited. In the presence of L-NAME there was a greater inhibition of the renal haemodynamic effects of acetylcholine than there was in the presence of L-NMMA (Tables 2 and 6).

Responses to sodium nitroprusside in the absence and presence of L-NAME Sodium nitroprusside caused hypotension, tachycardia, and an increase in mesenteric, and a decrease in hindquarters blood flow; there were increases in renal, hindquarters and, particularly, mesenteric vascular conductance (Table 7). Six h after the start of L-NAME ingestion, the hypotensive and renal vasodilator effects of sodium nitroprusside were enhanced (Table 7); however, these effects were not significantly different from those in the presence of L-NMMA (Table 3).

Responses to adrenaline in the absence and presence of L-NAME In this group of animals, adrenaline caused a slight hypotension, tachycardia, a reduction in mesenteric flow, and a marked increase in hindquarters flow, together with a slight increase in renal vascular conductance, and a pronounced increase in hindquarters vascular conductance (Table 8). Six h after the start of L-NAME ingestion, adrenaline caused a slight, but significant, pressor effect, there was a slight bradycardia and there was a significant attenuation of the hindquarters vasodilatation; all effects were different from those seen in the absence of L-NAME (Table 8), but only the latter effect was significantly greater than that seen 6 h after the start of ingestion of L-NMMA (Table 4).

Discussion

Effects of L-NMMA or L-NAME on resting cardiovascular variables

The present results, showing sustained hypertension with hindquarters vasoconstriction in conscious, Brattleboro rats drinking solutions of L-NMMA or L-NAME, are consistent with our previous observations (Gardiner *et al.*, 1990b; 1992). As pointed out elsewhere (Gardiner *et al.*, 1992), the finding that none of the vascular beds monitored showed a sustained reduction in flow, and only the hindquarters showed a persistent reduction in vascular conductance with chronic inhibition of NO synthesis, indicates that elevation in cardiac output, and/or reduced aortic compliance might have contributed to the hypertension under these conditions.

Table 5 Resting cardiovascular variables (i.e. prior to challenge with acetylcholine, sodium nitroprusside or adrenaline) in the same, conscious Brattleboro rats ($n = 8$) under control conditions, and 6 h after the start of oral ingestion of N^G-nitro-L-arginine methyl ester (L-NAME) in the drinking water

			Control	*L-NAME*
Heart rate (beats min^{-1})		Pre-acetylcholine	328 ± 7	229 ± 9*
		Pre-nitroprusside	331 ± 8	302 ± 12*
		Pre-adrenaline	333 ± 7	315 ± 10
Mean arterial blood pressure (mmHg)		Pre-acetylcholine	114 ± 3	145 ± 6*
		Pre-nitroprusside	117 ± 3	147 ± 5*
Vascular conductance		Pre-adrenaline	116 ± 3	140 ± 8*
(([kHz $mmHg^{-1}$]10^3)	Renal	Pre-acetylcholine	72 ± 9	50 ± 2*
		Pre-nitroprusside	73 ± 9	49 ± 5*
		Pre-adrenaline	68 ± 7	55 ± 5*
	Mesenteric	Pre-acetylcholine	73 ± 8	46 ± 8*
		Pre-nitroprusside	72 ± 10	44 ± 6*
		Pre-adrenaline	69 ± 7	48 ± 7*
	Hindquarters	Pre-acetylcholine	39 ± 5	21 ± 3*
		Pre-nitroprusside	39 ± 4	19 ± 2*
		Pre-adrenaline	37 ± 4	21 ± 2*

Values are mean ± s.e.mean.
*$P < 0.05$ versus control (Friedman's test).

Table 6 Cardiovascular changes (areas under or over curves) during 3 min infusions of acetylcholine (4 μg min^{-1}) in the same, conscious Brattleboro rats ($n = 8$) under control conditions, and 6 h after the start of oral ingestion of N^G-nitro-L-arginine methyl ester (L-NAME) in the drinking water

	Control	*L-NAME*
ΔHeart rate (beats)	133 ± 26*	124 ± 24
ΔMean arterial blood pressure (mmHg min)	−30 ± 9*	−50 ± 11†
ΔRenal conductance (([kHz $mmHg^{-1}$]10^3) min)	99 ± 8*	29 ± 6†
ΔMesenteric conductance (([kHz $mmHg^{-1}$]10^3) min)	−30 ± 8*	−11 ± 5†
ΔHindquarters conductance (([kHz $mmHg^{-1}$]10^3) min)	7 ± 4	10 ± 2

Values are mean ± s.e.mean.
*$P < 0.05$ for change versus baseline; †$P < 0.05$ versus control (Friedman's test).

Table 7 Cardiovascular changes (areas under or over curves) during 3 min infusions of sodium nitroprusside (15 μg min^{-1}) in the same, conscious Brattleboro rats ($n = 8$) under control conditions, and 6 h after the start of oral ingestion of N^G-nitro-L-arginine methyl ester (L-NAME) in the drinking water

	Control	*L-NAME*
ΔHeart rate (beats)	282 ± 30*	302 ± 40
ΔMean arterial blood pressure (mmHg min)	−151 ± 15*	−236 ± 29†
ΔRenal conductance (([kHz $mmHg^{-1}$]10^3) min)	98 ± 12*	155 ± 31†
ΔMesenteric conductance (([kHz $mmHg^{-1}$]10^3) min)	198 ± 23*	210 ± 36
ΔHindquarters conductance (([kHz $mmHg^{-1}$]10^3) min)	44 ± 22*	74 ± 23

Values are mean ± s.e.mean.
*$P < 0.05$ for change versus baseline; †$P < 0.05$ versus control (Friedman's test).

Table 8 Cardiovascular changes (areas under or over curves) during 3 min infusions of adrenaline (0.2 μg min^{-1}) in the same, conscious Brattleboro rats ($n = 8$) under control conditions, and 6 h after the start of oral ingestion of N^G-nitro-L-arginine methyl ester (L-NAME) in the drinking water

	Control	*L-NAME*
ΔHeart rate (beats)	103 ± 24*	−35 ± 13†
ΔMean arterial blood pressure (mmHg min)	−21 ± 8*	22 ± 6†
ΔRenal conductance (([kHz $mmHg^{-1}$]10^3) min)	22 ± 7*	−10 ± 4
ΔMesenteric conductance (([kHz $mmHg^{-1}$]10^3) min)	−20 ± 7	−27 ± 7
ΔHindquarters conductance (([kHz $mmHg^{-1}$]10^3) min)	105 ± 18*	52 ± 10†

Values are mean ± s.e.mean.
*$P < 0.05$ for change versus baseline; †$P < 0.05$ versus control (Friedman's test).

Responses to acetylcholine in the absence and presence of L-NMMA or L-NAME

As noted previously (Gardiner *et al.*, 1991b) at the dose used, infusion of acetylcholine caused hyperaemic vasodilatation only in the renal vascular bed. This effect was significantly inhibited 6 h after the start of L-NMMA ingestion, but was much more suppressed 6 h after the start of L-NAME ingestion. Moreover, this effect of L-NAME was greater than that seen 60 min after the onset of i.v. infusion of L-NAME (at 1 mg kg^{-1} h^{-1}) (Gardiner *et al.*, 1991b). These findings indicate that almost all the renal hyperaemic vasodilator effect of acetylcholine in conscious, Brattleboro rats can be prevented by adequate inhibition of NO synthesis.

The inhibitory effect of L-NMMA on the renal vasodilator response to acetylcholine was maintained, but 6 h after withdrawal of L-NMMA, responses to acetylcholine were not different from control responses. Moreover, when increased responses to acetylcholine were present, 24 h after withdrawal of L-NMMA, resting renal vascular conductance was normal and mean arterial blood pressure was decreased, rather than increased. One explanation for this could be increased responsiveness of guanylyl cyclase to NO, following chronic inhibition of NO synthesis. However, if this were the case, then increased responses to sodium nitroprusside would have been expected, but these did not occur (see below). Thus, it may be that with chronic inhibition of NO synthase there was increased production of this enzyme and, hence, on withdrawal of L-NMMA, there was enhanced stimulation of NO production by acetylcholine. However, whether or not the effects observed were due to increased amounts of NO synthase, and/or changes at the level of muscarinic receptors remains to be determined (see below).

Responses to sodium nitroprusside in the absence and presence of L-NMMA or L-NAME

Sodium nitroprusside caused hypotension and renal, mesenteric and hindquarters vasodilatation, although only the mesenteric vascular bed showed an increase in flow, and hence the renal and hindquarters vasodilatation could have been autoregulatory, as noted elsewhere (Gardiner *et al.*, 1990d; 1991a,b).

Six h after the start of L-NMMA or L-NAME ingestion there was a significant increase in the hypotensive effect of sodium nitroprusside, and 72 h after the start of L-NMMA ingestion the hypotensive effect of sodium nitroprusside was still enhanced. Since none of the vascular beds studied showed an increased flow response the increased renal vasodilator effect of sodium nitroprusside in the presence of L-NMMA (Table 3) or L-NAME (Table 7) might still have been due to renal autoregulation.

One possible explanation for the lack of any signs of an increased mesenteric vasodilator action of sodium nitroprusside, in the presence of L-NMMA or L-NAME, is that the enhanced hypotensive response under these conditions caused more marked activation of the renin-angiotensin system, and the mesenteric vasoconstrictor effects of angiotensin II acted to offset the vasodilatation.

The absence of signs of increased responses to sodium nitroprusside, following withdrawal of L-NMMA, indicates that the changes in the presence of NO synthesis inhibition were probably due to increased responsiveness of guanylyl cyclase to exogenous NO when endogenous NO production was inhibited (Moncada *et al.*, 1991b; Gardiner *et al.*, 1991b), rather than to the presence of increased amounts of the enzyme.

Responses to adrenaline in the absence and presence of L-NMMA or L-NAME

Six h after the start of oral ingestion of L-NMMA or L-NAME, there was significant inhibition of the hindquarters vasodilator effect of adrenaline; as in other instances, the effect of L-NAME was greater than that of L-NMMA (Gardiner *et al.*, 1990c; 1991a,b). However, the effects of L-NMMA were progressive, and by 72 h after the start of its ingestion the inhibition of the hindquarters vasodilator effect of adrenaline was similar to that seen 6 h after the start of L-NAME ingestion. This observation is consistent with cumulative inhibition of NO synthase during the 72 h exposure to L-NMMA, but, the fact that the inhibitory effects of L-NMMA on the hypotensive and renal hyperaemic vasodilator effects of acetylcholine were not incremental (Table 2), indicates other factors might have been involved. Nevertheless, there was internal consistency in the profile of effect of adrenaline during the 3 day exposure to L-NMMA, since the incremental reduction in the hindquarters vasodilator response was accompanied by a progressive enhancement of the renal vasoconstrictor effect of adrenaline, such that, after 72 h exposure to L-NMMA, adrenaline had a pressor action, although this and the renal vasoconstriction were only slight. While the mesenteric vasoconstrictor effect of adrenaline became significant in the presence of L-NMMA, this response was not significantly different from that seen under control conditions. However, the overall picture was similar to that observed following i.v. L-NAME (Gardiner *et al.*, 1991c), and is consistent with an inhibition of β-adrenoceptor-mediated hindquarters vasodilatation and an unmasking of α-adrenoceptor-mediated renal and mesenteric vasoconstrictor effects of adrenaline in the presence of L-NMMA, as we suggested occurred in Long Evans rats given i.v. L-NAME (Gardiner *et al.*, 1991c).

It is notable that, following withdrawal of L-NMMA, there were no signs of enhanced responses to the effects of adrenaline. Thus, if at this stage there were increased amounts of NO synthase present (which was one explanation offered above for our findings with acetylcholine), then it seems that β-adrenoceptor stimulation did not activate this system. Whilst this is feasible, it is more likely that the enhanced responses to acetylcholine were due to changes at the level of muscarinic receptors (or their post-receptor coupling) rather than to increased amounts of NO synthase.

In conclusion, chronic treatment of Brattleboro rats with L-NMMA or L-NAME indicates that the major component of the renal vasodilator response to acetylcholine is mediated by NO, and that this response and the hypotensive effect of acetylcholine are enhanced following withdrawal of L-NMMA, at a time when there are no changes in responses to sodium nitroprusside. This suggests that the increased responses to acetylcholine are probably not due to increased amounts, and/or responsiveness, of guanylyl cyclase. During chronic exposure to L-NMMA the progressive reduction in the hindquarters hyperaemic effect of adrenaline is consistent with an important component of this response being dependent upon NO. However, the fact that, following withdrawl of L-NMMA, there was no increased response to adrenaline when there was to acetylcholine, indicates that the increased effects of the latter are probably due to changes in muscarinic receptor-mediated mechanisms, rather to an increase in activity of NO synthase.

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Contractile and relaxant effects of phorbol ester in the intestinal smooth muscle of guinea-pig taenia caeci

[1]Minori Mitsui & Hideaki Karaki

Department of Veterinary Pharmacology, Faculty of Agriculture, The University of Tokyo, Bunkyo-ku, Yayoi 1-1-1, Tokyo 113, Japan

1 Effects of phorbol esters on the cytosolic Ca^{2+} level ($[Ca^{2+}]_i$) and muscle tension in the intestinal smooth muscle of guinea-pig taenia caeci were examined.

2 12-Deoxyphorbol 13-isobutyrate (DPB, 1 μM) did not change the $[Ca^{2+}]_i$ and tension in resting muscle.

3 In high K^+-stimulated muscle, 1 μM DPB transiently augmented the contraction and decreased $[Ca^{2+}]_i$. 12-Deoxyphorbol 13-isobutyrate 20-acetate (1 μM) and phorbol 12, 13-dibutyrate (1 μM) showed similar effects to DPB whereas phorbol 12-myristate 13-acetate (1 μM) and phorbol 12, 13-didecanoate (1 μM) were ineffective.

4 DPB (1 μM) inhibited both $[Ca^{2+}]_i$ and tension stimulated by 300 nM carbachol or 3 μM histamine. In the presence of a higher concentration of carbachol (1 μM), DPB decreased $[Ca^{2+}]_i$ and transiently increased muscle tension.

5 In the muscle strips permeabilized with bacterial α-toxin, 1 μM DPB shifted the Ca^{2+}-tension curve to the left. An inhibitor of protein kinase C, H-7 (30 μM), inhibited the effect of DPB.

6 DPB did not change the high K^+-induced contraction in the muscle strips pretreated with 3 μM phorbol 12-myristate 13-acetate for 24 h.

7 These results suggest that activation of protein kinase C has dual effects; it augments contraction by increasing the Ca^{2+} sensitivity of the contractile elements and it inhibits contraction by decreasing $[Ca^{2+}]_i$.

Keywords: Smooth muscle (intestinal); phorbol ester; cytosolic Ca^{2+} levels; protein kinase C; permeabilized smooth muscle

Introduction

Since Nishizuka (1984) reported the biological effect of tumour promoting phorbol esters, these esters have been widely used to examine the role of protein kinase C in various biological systems. In the intestinal smooth muscle, conflicting results have been reported on the effects of phorbol esters on contractility. Phorbol esters potentiated the contractions induced by high K^+ (Menkes *et al.*, 1986; Sasaguri & Watson, 1989; Xu *et al.*, 1991) and histamine (Holzer & Lippe, 1989) possibly by activating Ca^{2+} channel (Menkes *et al.*, 1986; Xu *et al.*, 1991). In contrast, phorbol esters inhibited the contractions induced by histamine (Baraban *et al.*, 1985; Holzer & Lippe, 1989), carbachol (Menkes *et al.*, 1986; Sasaguri & Watson, 1989) and substance P (Holzer & Lippe, 1989) possibly by the activation of Na^+-K^+-ATPase (Sasaguri & Watson, 1990) or a negative feedback mechanism (Baraban *et al.*, 1985; Menkes *et al.*, 1986).

In order to understand the role of protein kinase C in the intestinal smooth muscle, we examined the effects of phorbol esters on muscle tension and cytosolic Ca^{2+} level ($[Ca^{2+}]_i$) in the guinea-pig taenia caeci.

Methods

Measurement of contractions in isolated muscle strips

Male guinea-pigs, weighing 250–300 g, were killed by a blow on the neck and bled. A section of taenia (5–10 mm in length) was dissected from the caecum. The normal physiological salt solution contained (mM): NaCl 136.9, KCl 5.4, glucose 5.5, $NaHCO_3$ 23.8, $CaCl_2$ 1.5, $MgCl_2$ 1.0 and EDTA 0.01. High K^+ (45 mM) solution was made by adding KCl to the PSS. In some experiments, all the NaCl was substituted with KCl, and pyruvate (5 mM) and indomethacin (10 μM) were added because Na^+ deficiency may interfere with glucose uptake (Karaki *et al.*, 1982) and release endogenous prostaglandins (Satoh & Karaki, 1988). These solutions were aerated with a 95% O_2 and 5% CO_2 mixture at 37°C (pH 7.4). Muscle tension was recorded isometrically with a force-displacement transducer. Passive tension of 2 mN was initially applied and tissues were allowed to equilibrate for 60–90 min until the contractile response to 45 mM K^+ solution became stable.

Measurement of cytosolic Ca^{2+} concentration

Cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured simultaneously with muscle contraction as described previously (Ozaki *et al.*, 1987; Sato *et al.*, 1988; Mitsui & Karaki, 1990). Muscle strips were treated with 10 μM acetoxymethyl ester of fura-2 (fura-2/AM) for approximately 12 h at 4°C. The non-cytotoxic detergent, cremophor EL (0.02%), was added to increase the solubility of fura-2/AM. The muscle strip was illuminated alternately (48 Hz) with two excitation wave lengths (340 nm and 380 nm), and the intensity of the 500 nm fluorescence (F340 and F380) was measured with a fluorimeter (CAF-100, JASCO, Tokyo, Japan). High K^+ (45 mM) solution for this experiment was made by substituting appropriate concentration of NaCl with equimolar KCl in PSS, because hypertonicity interferes with the fura-2-Ca^{2+} fluorescence (Himpens & Somlyo, 1988; Williams & Fay, 1990). Absolute Ca^{2+} concentration was not calculated because the dissociation constant of fura-2 for Ca^{2+} may be different from that obtained *in vitro* (Karaki, 1989). In the present study, the ratio of F340 to F380 (R340/380) was used as an indicator of $[Ca^{2+}]_i$, and the quantitative comparison of

[1] Author for correspondence.

the $[Ca^{2+}]_i$ was made by taking the resting and high K^+ stimulated $[Ca^{2+}]_i$ as 0% and 100%, respectively (Ozaki *et al.*, 1987; Sato *et al.*, 1988). It was not possible to measure $[Ca^{2+}]_i$ for a period longer than 90 min, because of the leakage of fura-2 from the cell.

Measurement of contractions in permeabilized smooth muscle

Permeabilized muscle was made by treating the isolated tissue with *Staphylococcus aureus* α-toxin as described by Nishimura *et al.* (1988) and Kitazawa *et al.* (1989). A thin bundle of muscle strip (0.1 mm wide and 2 mm long) was treated for 30 min with 100 μg ml^{-1} α-toxin in a relaxing solution containing (mM): K^+-propionate 130.0, $MgCl_2$ 4.0, Na_2ATP 5.0, Tris-maleate 20.0, creatine phosphate 2.0, creatine phosphokinase 10 u ml^{-1} and EGTA 2.0 (pH 6.8). Ca^{2+} concentrations were changed by adding an appropriate amount of $CaCl_2$. The apparent binding constant of EGTA for Ca^{2+} was considered to be 1×10^6 M^{-1} at pH 6.8 (Harafuji & Ogawa, 1980). The contractile tension of the permeabilized muscle was recorded isometrically.

Down-regulation of protein kinase C

Long-term exposure to phorbol esters has been shown to reduce the protein kinase C activity and also the amount of protein kinase C molecules in the cell (Jaken *et al.*, 1981; Collins *et al.*, 1982). In order to down-regulate the protein kinase C activity, taenia strips were incubated in Dulbecco's modified Eagle medium with 10% foetal calf serum for 24 h at 37°C in a CO_2 incubator in the presence of phorbol ester (3 μM phorbol 12-myristate 13-acetate or 3 μM DPB).

Chemicals

Chemicals used were 12-deoxyphorbol 13-isobutyrate (DPB), 12-deoxyphorbol 13-isobutyrate 20-acetate, phorbol 12, 13-dibutyrate, phorbol 12, 13-didecanoate, phorbol 12-myristate 13-acetate (Funakoshi, Tokyo, Japan), carbachol, indomethacin (Sigma Chemicals, St. Louis, MO, U.S.A.), histamine (Wako Pure Chemicals, Tokyo, Japan), clemophor EL (Nacalai Tesque, Kyoto, Japan), EGTA, fura-2/AM (Dojindo Laboratories, Kumamoto, Japan), H-7 (1-(5-isoquinolinesulphonyl)-2-methylpiperidine, Seikagaku, Corp., Tokyo, Japan). *Staphylococcus aureus* α-toxin was donated by Dr Iwao Kato.

Statistics

Results of the experiments are expressed as the mean ± s.e.mean. Student's *t* test was used for statistical analysis of the results and $P<0.05$ was considered to indicate a significant difference.

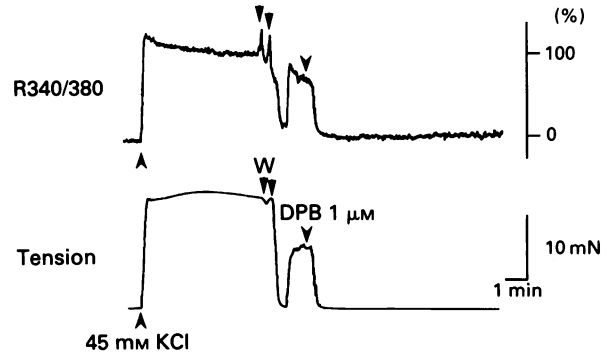


Figure 1 Effect of 45 mM KCl and 1 μM 12-deoxyphorbol 13-isobutyrate (DPB) on $[Ca^{2+}]_i$ and muscle tension in fura-2 loaded taenia caeci. Traced from a typical result of 4 experiments.

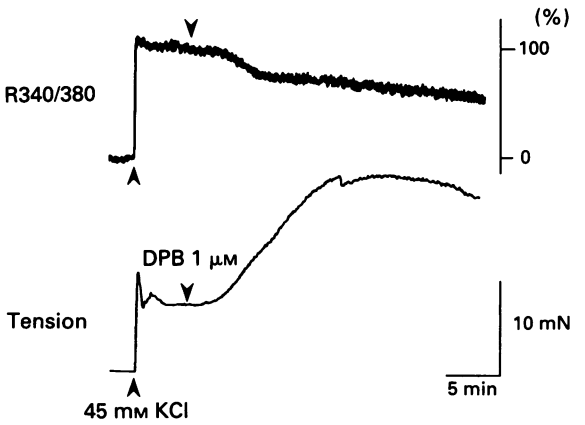


Figure 2 Effect of 1 μM 12-deoxyphorbol 13-isobutyrate (DPB) on $[Ca^{2+}]_i$ and muscle tension in the presence of 45 mM KCl. Traced from a typical result of 4 experiments.

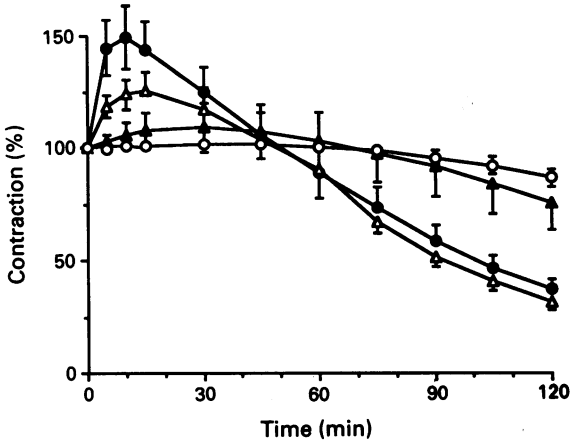


Figure 3 Time course of the effects of the various concentrations of 12-deoxyphorbol 13-isobutyrate (DPB) on 45 mM KCl-induced contractions. A single dose of DPB (10 nM, ▲; 100 nM, △; 1 μM, ●) was added after the contraction reached a steady level. (○) In the absence of DPB. Each point represents mean ± s.e.mean of 4 experiments.

Results

Effects of phorbol esters on $[Ca^{2+}]_i$ and muscle tension

As shown in Figure 1, addition of high K^+ induced sustained increase of $[Ca^{2+}]_i$ and muscle tension in the taenia. After removal of high K^+, spontaneous rhythmic changes of $[Ca^{2+}]_i$ and muscle tension were observed. In the presence of 1 μM DPB, both muscle tension and $[Ca^{2+}]_i$ stayed at their respective resting levels.

The effects of DPB on high K^+-induced increase of $[Ca^{2+}]_i$ and contraction are shown in Figures 2 and 3. DPB (1 μM) decreased high K^+-stimulated $[Ca^{2+}]_i$, although it gradually increased muscle tension reaching the maximum within 10–15 min followed by a decrease. Because of the leakage of fura-2 from the cells, it was possible to examine the effects of DPB for only 30 min. 12-Deoxyphorbol 13-isobutyrate 20-acetate (1 μM) and 1 μM phorbol 12, 13-dibutyrate showed similar effects to DPB, although 1 μM phorbol 12-myristate 13-acetate and 1 μM phorbol 12, 13-didecanoate were ineffective ($n = 4$ each).

Figure 3 shows the concentration-response relationships for the effects of DPB. DPB at a concentration of 100 nM and 1 μM induced augmentation followed by inhibition of the

high K^+-induced contraction whereas 10 nM DPB was ineffective. DPB, 1 μM, also transiently augmented and then inhibited the contraction induced by Na^+-free, 166.1 mM K^+-solution to 16.5 ± 6.7% at 120 min ($n = 4$).

We also examined the effects of DPB on the contraction and $[Ca^{2+}]_i$ stimulated by carbachol and histamine. DPB (1 μM) rapidly inhibited $[Ca^{2+}]_i$ whereas it augmented the contraction for only a short period of time in the muscle stimulated by 300 nM carbachol (Figure 4a) or 3 μM histamine (Figure 4b). In the presence of a higher concentration of carbachol (1 μM), 1 μM DPB gradually decreased $[Ca^{2+}]_i$ and transiently increased and then decreased muscle tension (Figure 4c).

Effect of 12-deoxyphorbol 13-isobutyrate on permeabilized muscles

As shown in Figure 5a, 1 μM Ca^{2+} induced transient increase followed by a sustained increase of muscle tension in permeablilized taenia. DPB (1 μM) potentiated the Ca^{2+}-induced contraction to 391.0 ± 76.7% ($n = 4$). An inhibitor of protein kinase C, 30 μM H-7 (Hidaka *et al.*, 1984), inhibited the contraction to 173.5 ± 31.2% ($n = 3$, $P < 0.01$). In contrast, 30 μM H-7 did not inhibit the contraction induced by 1 μM Ca^{2+} in the absence of DPB ($n = 3$, Figure 5b). Figure 5c shows that DPB (1 μM) shifted the Ca^{2+}-tension relationships to the left in permeabilized muscle strips.

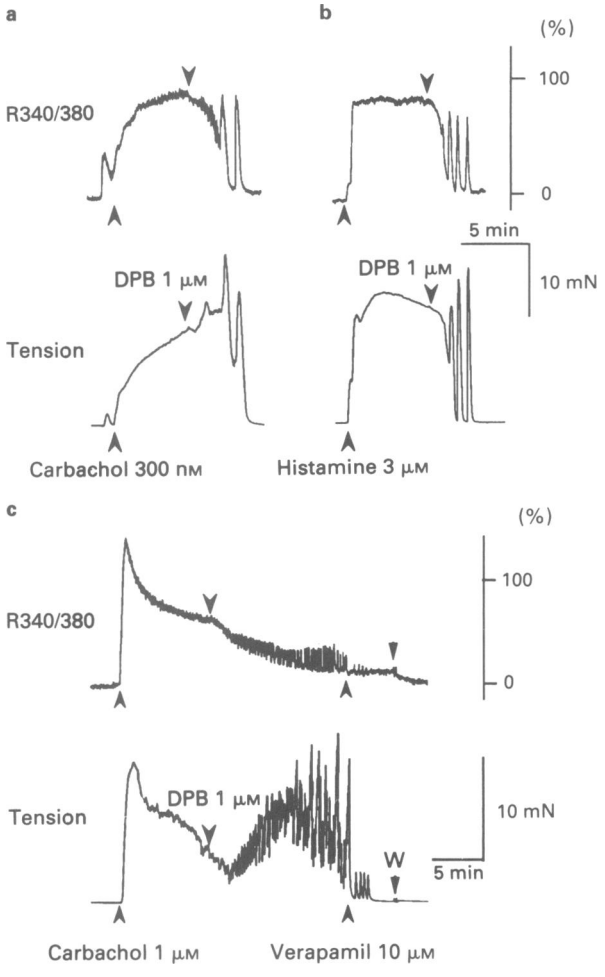


Figure 4 Effects of 1 μM 12-deoxyphorbol 13-isobutyrate (DPB) on $[Ca^{2+}]_i$ and muscle tension in the presence of carbachol (a, 300 nM; c, 1 μM) and histamine (b, 3 μM). Traced from typical results of 4 experiments.

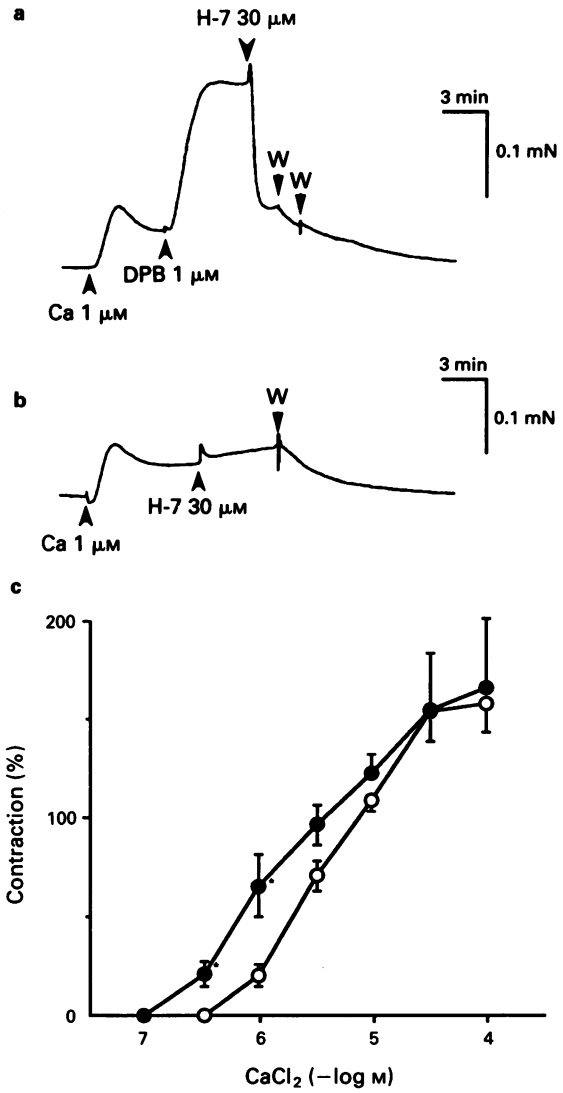


Figure 5 Effects of 1 μM 12-deoxyphorbol 13-isobutyrate (DPB) (a) and 30 μM H-7 (b) on the Ca^{2+}-induced contraction in α-toxin permeabilized taenia strips. Traced from typical results of 3–4 experiments. (c) Ca^{2+}-tension relationships in the absence (○) or presence of 1 μM DPB (●). Ca^{2+} was added cumulatively in the absence or presence of 1 μM DPB. Each point represents mean ± s.e.mean of 6–11 experiments; 100% represents the magnitude of contraction induced by a single application of 10 μM Ca^{2+} performed before the experimental procedure.

Effects of pretreatment with phorbol esters

In muscle strips incubated for 24 h at 37°C, 1 μM DPB increased the high K^+-induced contraction to 186.9 ± 12.0% ($n = 6$, Figure 6a). In muscle pretreated with 3 μM phorbol 12-myristate 13-acetate for 24 h at 37°C, 1 μM DPB did not augment the high K^+-induced contraction ($n = 9$, Figure 6b). Pretreatment with 3 μM DPB for 24 h at 37°C partially inhibited the ability of 1 μM DPB to augment the high K^+-induced contraction (120.7 ± 3.0%, $n = 8$, $P < 0.01$ vs the value without phorbol ester-pretreatment, Figure 6c).

Discussion

Phorbol ester-induced augmentation of contraction

In the present experiments, we found that the effects of DPB on muscle tension and $[Ca^{2+}]_i$ are different. DPB transiently augmented the contractions induced by high K^+ or a high

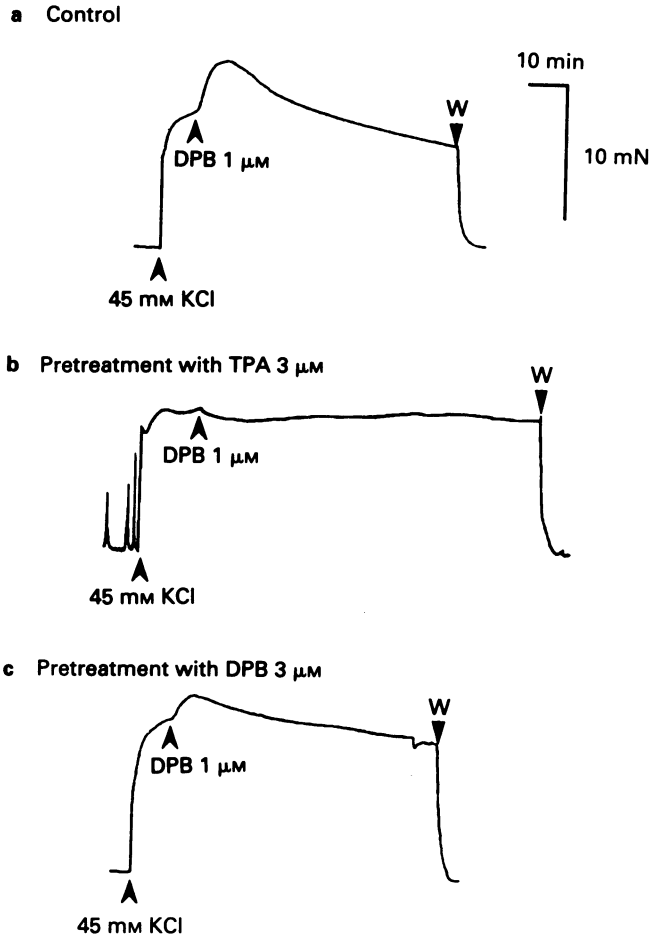


Figure 6 Effect of 12-deoxyphorbol 13-isobutyrate (DPB) on 45 mM KCl-induced contraction in the taenia strip without (a, control) or with preincubation with phorbol ester (b, phorbol 12-myristate 13-acetate (TPA) 3 μM and c, DPB 3 μM) for 24 h. DPB 1 μM was added when the muscle tension reached a steady level. Traced from typical results of 6–9 experiments.

concentration of carbachol (1 μM) whereas it decreased $[Ca^{2+}]_i$ (Figures 2 and 4c). The augmentation of contraction may be explained by an increase in Ca^{2+} sensitivity, as it has been shown that phorbol esters increase Ca^{2+} sensitivity of the contractile elements in vascular and intestinal smooth muscles (Itoh *et al.*, 1988; Nishimura & Van Breemen, 1989; Sato *et al.*, 1992; Ozaki *et al.*, 1993). In order to confirm this possibility, we examined the effect of DPB on the Ca^{2+}-induced contraction in permeabilized muscle strips. It was found that 1 μM DPB shifted the Ca^{2+}-tension relationship to the left (Figure 6c), supporting the suggestion that DPB augmented contraction by increasing the Ca^{2+} sensitivity of the contractile elements. In the resting muscle, DPB did not induce contraction (Figure 1) possibly because resting $[Ca^{2+}]_i$ was lower than threshold level even when the Ca^{2+} sensitivity was increased. Although we did not examine the mechanism of Ca^{2+} sensitization, it has been shown that DPB increases Ca^{2+} sensitivity of myosin light chain phosphorylation in vascular smooth muscle (Sato *et al.*, 1992; Hori *et al.*, 1992).

DPB induced similar but much faster changes in $[Ca^{2+}]_i$ and muscle tension in the taenia stimulated by a lower concentration of carbachol (300 mM) or histamine (3 μM) (Figure 5a,b). This result may be due to the ability of DPB to decrease $[Ca^{2+}]_i$ more rapidly under these conditions than in the presence of higher concentrations of stimulants.

Inhibitory effect of phorbol esters on $[Ca^{2+}]_i$

On the mechanism of DPB-induced decrease in $[Ca^{2+}]_i$, it has been reported that phorbol ester activates Na^+-K^+-ATPase in tracheal (Schramm & Grunstein, 1989; Souhrada & Souhrada, 1989) and intestinal smooth muscles (Sasaguri & Watson, 1990) which results in a membrane hyperpolarization and inhibition of the voltage-dependent Ca^{2+} channels. The second possibility is an inhibition of Ca^{2+} channels mediated more directly by the activation of protein kinase C (Shearman *et al.*, 1989). We have reported that a Ca^{2+} channel blocker, verapamil, inhibited the contraction induced by a higher concentration of carbachol more strongly than that induced by a lower concentration in the guinea-pig taenia caeci (Karaki & Mitsui, 1988). In the present experiments, we found that DPB inhibited the contractions induced by lower concentrations of carbachol and histamine more strongly than those induced by higher concentrations. Since the lower concentrations of agonists are expected to induce smaller membrane depolarization than those induced by higher concentrations, membrane hyperpolarization resulting from activation of Na^+-K^+-ATPase may be responsible for the inhibitory effect of phorbol ester on the contractions induced by carbachol and histamine. However, we also found that 1 μM DPB inhibited the contraction induced by Na^+-free, 166.1 mM K^+-solution in which stimulation of Na^+-K^+ATPase activity would not be possible.

Detailed analysis is necessary to determine whether there is an inhibitory effect of phorbol ester on Ca^{2+} channels.

Role of protein kinase C

It has been reported that an inhibitor of protein kinase C, H-7, non-selectively inhibits smooth muscle contraction in intact vascular smooth muscle (Ratz *et al.*, 1990). In a preliminary experiment, we confirmed this in the taenia. In the permeabilized taenia caeci, however, H-7 inhibited the DPB-induced augmentation of the Ca^{2+}-induced contraction without changing the contraction in the absence of DPB (Figure 5). Furthermore, pretreatment of the muscle with phorbol 12-myristate 13-acetate or DPB, which may cause down-regulation of protein kinase C, inhibited the effects of DPB (Figure 6b,c). These results suggest that effects of DPB are due to activation of protein kinase C.

Spedding (1987) showed that phorbol 12-myristate 13-acetate had no effects on the Ca^{2+}-induced contraction in the high K^+depolarized taenia caeci. In the present experiments, we also found that phorbol 12-myristate 13-acetate had no effect on the high K^+-induced contraction. However, pretreatment of the muscle with phorbol 12-myristate 13-acetate seemed to down-regulate the protein kinase C activity more strongly than DPB (Figure 6b,c). Mattingly *et al.* (1987) also reported that down-regulation of protein kinase C by phorbol 12-myristate 13-acetate was stronger than phorbol 12,13-dibutyrate. The reason for this difference has not been examined in the present experiments.

In conclusion, it is suggested that phorbol esters decrease $[Ca^{2+}]_i$ and increase Ca^{2+} sensitivity of the contractile element (shifting the Ca^{2+}-tension relationship to the left) in the taenia.

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Role of platelet-activating factor (PAF) in platelet accumulation in rabbit skin: effect of the novel long-acting PAF antagonist, UK-74,505

F. Pons, A.G. Rossi, K.E. Norman, T.J. Williams & [1]S. Nourshargh

Department of Applied Pharmacology, National Heart and Lung Institute, Dovehouse Street, London SW3 6LY

1 The contribution of platelet-activating factor (PAF) to platelet deposition and oedema formation induced by exogenous soluble mediators, zymosan particles and associated with a reversed passive Arthus (RPA) reaction in rabbit skin was investigated by use of a novel long-acting PAF receptor antagonist, UK-74,505.

2 Oedema formation and platelet accumulation were simultaneously measured by i.v. injection of [^{125}I]-albumin and ^{111}In-labelled rabbit platelets. UK-74,505 was either administered i.v. or used to pretreat radiolabelled platelets *in vitro* before their injection into recipient animals. Platelets pretreated with UK-74,505 were also labelled with the fluorescent calcium indicator, Fura-2, to assess their *ex vivo* reactivity to PAF at the end of the *in vivo* experiment.

3 UK-74,505 (0.5 mg kg^{-1}), administered i.v., inhibited PAF-induced oedema formation, but did not affect oedema induced by zymosan particles, bradykinin (BK), histamine, formyl-methionyl-leucyl-phenylalanine (FMLP), zymosan-activated plasma (ZAP, as a source of C5a des Arg), leukotriene B_4 (LTB_4) or interleukin-8 (IL-8).

4 UK-74,505, administered i.v. also suppressed the small platelet accumulation induced by exogenous PAF, but had no effect on accumulation induced by IL-8 or ZAP. Although oedema induced by zymosan was not affected by i.v. UK-74,505, zymosan-induced platelet accumulation was significantly attenuated by the antagonist.

5 The RPA reaction in rabbit skin was associated with marked oedema formation and platelet accumulation which were both inhibited by i.v. UK-74,505.

6 *In vitro*, UK-74,505 inhibited aggregation and the increase in intracellular calcium concentration induced by PAF in rabbit washed platelets in a concentration-dependent manner ($IC_{50} = 1.6 \times 10^{-8}$ M and 1.1×10^{-8} M, respectively). Platelets pretreated with 10^{-6} M UK-74,505, and maintained at 37°C, were unresponsive to PAF, whilst responding normally to thrombin, for up to 4 h.

7 In a second series of *in vivo* experiments, platelets were labelled with ^{111}In and loaded with Fura-2. The platelets were then pretreated with 10^{-6} M UK-74,505, washed, and injected into recipient rabbits. These platelets, prepared from blood samples taken at the end of the *in vivo* experiments, exhibited an 80% reduction in their response to PAF as measured *ex vivo* with Fura-2. However, in contrast to the effects of i.v. UK-74,505, platelets pretreated with the antagonist did accumulate effectively in the RPA reaction, a significant reduction only being observed in responses at the lowest antibody dose. In addition, pretreatment of platelets had no effect on the small platelet accumulation induced by PAF.

8 These results suggest that PAF is an important mediator of oedema formation and platelet accumulation in the RPA reaction in rabbit skin. However, they question the role of PAF receptors on platelets in this model. The results also indicate that PAF may be involved in platelet accumulation induced by zymosan in rabbit skin.

Keywords: Reversed passive Arthus (RPA) reaction; oedema formation; platelet accumulation; platelet-activating factor (PAF); PAF antagonist; inflammation

Introduction

Platelet accumulation has been observed in different animal models of acute inflammation including carrageenin-induced paw oedema (Vincent *et al.*, 1978), the dermal reversed passive Arthus (RPA) reaction (Henson & Cochrane, 1969) and the local Shwartzman reaction (Movat & Burrowes, 1985). Platelet accumulation has also been associated with certain pathological conditions in man such as the Adult Respiratory Distress Syndrome (Heffner *et al.*, 1987). Platelets can potentially contribute to the development of inflammation as they contain a wide variety of proinflammatory substances, including vasoactive amines and peptides, proteases, chemotactic factors for leucocytes and components of the coagulation pathway. Several studies have suggested that platelets are involved in the early stages of inflammation, often prior to the development of tissue damage, haemorrhage and thrombosis (Issekutz *et al.*, 1983), but the sequence of events leading to platelet accumulation and its relationship to other components of the inflammatory process are unknown.

Together with oedema formation, neutrophil infiltration and haemorrhage, platelet accumulation is a characteristic feature of the Arthus reaction (Stetson, 1951; Humphrey, 1955a; Cochrane & Janoff, 1974; Kravis & Henson, 1977). This model of vascular injury is initiated by the local deposition of immune complexes within the vessel wall and previous reports have demonstrated the importance of neutrophils (Humphrey, 1955a; Cochrane & Janoff, 1974; Warren *et al.*, 1989) and complement fragments such as C5a (Cochrane *et al.*, 1970; Cochrane & Janoff, 1974; Lewis & Turk, 1975; Jose *et al.*, 1983; Yeh *et al.*, 1991; Rossi *et al.*, 1992). The role of platelets and their contribution to the development of the reaction remain unclear. Some studies reported an inhibition of the Arthus reaction in platelet-depleted animals (Henson & Cochrane, 1969), whereas other

[1] Author for correspondence.

investigators did not observe any modification of the reaction in the absence of platelets (Humphrey, 1955b; Cochrane & Janoff, 1974).

The phospholipid platelet-activating factor (PAF) appears to play an important role in the development of the Arthus reaction as PAF antagonists, administered locally or i.v., have been shown to reduce oedema formation associated with RPA reactions in rabbit and rat skin (Deacon *et al.*, 1986; Hellewell & Williams, 1986; Issekutz & Szejda, 1986; Williams *et al.*, 1986; Warren *et al.*, 1989; Hellewell, 1990; Rossi *et al.*, 1992). Neutrophil depletion has been shown to suppress oedema formation in RPA reactions in rat skin, an inhibition that is reported to be partially reversed by local injection of neutrophils (Warren *et al.*, 1989). Preincubation of the neutrophils with a PAF antagonist restored this inhibition (Warren *et al.*, 1989). Furthermore, it has been shown that i.v. injection of PAF antagonists partially suppresses platelet accumulation (Issekutz & Szejda, 1986). Kravis & Henson (1977) have suggested that PAF released from basophils or mast cells by an IgE-dependent mechanism is responsible for the early phase of platelet accumulation observed in the Arthus reaction. However, endothelial cells, neutrophils undergoing phagocytosis, and platelets themselves are also possible sources of PAF (Chignard *et al.*, 1980; Camussi *et al.*, 1983; Hellewell & Williams, 1986). It is not known whether PAF mediates platelet accumulation by acting directly on the platelet *in vivo*, or by an indirect mechanism that involves PAF interacting with receptors on other inflammatory cells such as the neutrophil.

The objectives of this study were to investigate the accumulation of platelets and its relationship to oedema formation in defined inflammatory reactions. For this purpose we monitored the accumulation of ^{111}In-labelled platelets and [^{125}I]-albumin in rabbit skin in response to soluble inflammatory mediators and particulate stimuli. Two types of the latter were employed: zymosan, and immune complexes deposited in an Arthus reaction. We used a novel PAF antagonist, UK-74,505 (Cooper *et al.*, 1990; Parry *et al.*, 1990; Alabaster *et al.*, 1991), to investigate the contribution of PAF in platelet deposition and oedema formation *in vivo*. UK-74,505 has the advantage of potency and a long duration of action which enabled measurements to be made *in vivo* over either short (30 min) or long (4 h) periods required for development of responses to exogenous mediators or particulate stimuli respectively. Dissociation of the compound from PAF receptors is very slow (Cooper *et al.*, 1990; Parry *et al.*, 1990; Alabaster *et al.*, 1991), which permitted PAF receptors to be blocked on ^{111}In-platelets by pretreatment *in vitro* before i.v. injection. This enabled us to investigate the role of platelet PAF receptors in platelet deposition *in vivo*.

Methods

Animals

New Zealand White rabbits (2.5–3.5 kg) were purchased from Froxfield Farm, Hampshire.

Generation of antiserum for the Arthus reaction

Arthus antiserum, anti-bovine-γ-globulin (anti-BGG) antibody, was raised in rabbits as previously described (Hellewell & Williams, 1986). Briefly, 4 × 0.25 ml of BGG (2 mg ml^{-1} in saline) emulsified with an equal volume of Freund's complete adjuvant was administered subcutaneously. This was followed 14 days later by booster s.c. injections (4 × 0.25 ml) of the same concentration of BGG in Freund's incomplete adjuvant. At day 28, a s.c. injection of alum-precipitated BGG (300 μg/rabbit) was given. Blood was collected by carotid cannulation at day 38. The serum from five rabbits was pooled, heat-inactivated at 56°C for 30 min and stored in aliquots at − 20°C. Heat-inactivated rabbit serum from normal rabbits was used as the control.

Preparation of zymosan-activated plasma (ZAP)

ZAP as a source of rabbit C5a des Arg was prepared by incubating heparinized (10 u ml^{-1}) rabbit plasma with zymosan (5 mg ml^{-1}) for 30 min at 37°C. Zymosan was removed by centrifugation (2 × 10 min, 2500 *g*) and ZAP was stored in aliquots at − 20°C. The C5a des Arg content of ZAP was approximately 5×10^{-7} M as measured by radioimmunoassay (Jose *et al.*, 1983).

Isolation and preparation of rabbit washed platelets

Washed platelets were prepared from rabbit blood as previously described (Radomski & Moncada, 1983). Briefly, blood was collected from a carotid artery or marginal ear vein into tri-sodium citrate (0.38%, w/v) and centrifuged at 250 *g* for 20 min to obtain platelet-rich plasma (PRP). The isolated PRP was then used for aggregation assays or further centrifuged at 750 *g* for 10 min in the presence of prostacyclin (PGI_2, 300 ng ml^{-1}) for the preparation of washed platelets. The resultant platelet pellet was resuspended in 10 ml Tyrode solution containing 300 ng ml^{-1} PGI_2 (PGI_2/Tyrode). The washed platelets were then centrifuged at 720 *g* for 10 min and finally resuspended in PGI_2-free Tyrode. The platelet concentration in PRP or Tyrode solution was determined with a Coulter counter (model ZM, Coulter Electronics Ltd, Luton, Bedfordshire).

Measurement of platelet aggregation

Aggregation of rabbit platelets either in suspension in plasma (PRP) or after washing with Tyrode solution was assessed photometrically in a dual channel aggregometer (Chronolog 440 VS) linked to a dual pen recorder (Chronolog 707). Aliquots of platelet suspension (250 μl) were incubated for 5 min at 37°C with continuous stirring at 700 r.p.m. and then stimulated with PAF or thrombin. Responses were allowed to develop for 3 min and expressed as a percentage of maximum light transmission. Platelet-poor-plasma and Tyrode solution were used as references (100% light transmission) for PRP and washed platelet suspension respectively. Platelet concentrations ranged from 190,000 to 560,000 platelets per μl in PRP and 200,000 to 250,000 platelets per μl in washed platelet suspensions. In some experiments, the effect of UK-74,505 on PAF or thrombin-induced washed platelet aggregation was investigated either by adding the antagonist to the platelet suspension 2 min before the aggregation assay or by pretreating the platelets during their preparation (see below).

Measurement of platelet intracellular calcium concentration ($[Ca^{2+}]_i$) with Fura-2

Platelet $[Ca^{2+}]_i$ was measured by monitoring the fluorescence of platelets loaded with the calcium indicator Fura-2 (Grynkiewicz *et al.*, 1985). Briefly, Fura-2 acetoxymethyl ester was added to PRP at a final concentration of 2 μM and the cell suspension was incubated for 45 min at 37°C in the presence of PGI_2 (300 ng ml^{-1}). The PRP was then centrifuged at 750 *g* for 10 min and washed platelets were prepared as described above. For the assay, aliquots of platelet suspension (200,000 to 300,000 platelets per μl in 200 μl) were dispensed into quartz cuvettes and the external $[Ca^{2+}]$ adjusted to 1 mM with $CaCl_2$. Changes in fluorescence induced by PAF or thrombin were monitored by a Perkin Elmer LS50 fluorescence spectrometer fitted with a thermostatically controlled cell holder incorporating an electronic stirrer and maintained at 37°C by a water circulator. Fluorescence readings were taken at excitation wavelengths 340 nm and 380 nm and emission wavelength 510 nm. The $[Ca^{2+}]_i$ was calculated from the ratio of the two fluorescence

readings using a K_d value of 224 nM. Responses were allowed to develop for 3 min and data were expressed as maximal increase in $[Ca^{2+}]_i$ over the basal levels.

^{111}In-labelling of washed rabbit platelets

Rabbit platelets ($8 \times 10^9 - 2 \times 10^{10}$), in some experiments loaded with Fura-2 as described above, were resuspended in PGI_2/Tyrode and incubated with $^{111}InCl_3$ (100 to 200 μCi in 10 to 20 μl) chelated with 2-mercaptopyridine-*N*-oxide (40 μg in 0.1 ml 50 mM phosphate buffered saline (PBS), pH 7.4). After a 10 min incubation at room temperature, the suspension was diluted to 10 ml with PGI_2/Tyrode and centrifuged for 10 min at 720 *g*. The platelets were then washed twice by repeating the same procedures of centrifugation and resuspension in PGI_2/Tyrode. In some experiments, platelets were treated with UK-74,505 before their last wash, as described below. The cells were finally resuspended in PGI_2-free Tyrode before injection into the animals. Supernatant aliquots were collected during washes and after the final suspension in order to assess labelling efficiency. In these experiments, labelling efficiency values varied between 80 and 95% and were not statistically different between control and UK-74,505-treated platelets.

Pretreatment of rabbit platelets with UK-74,505

Labelled or unlabelled platelets were pretreated with UK-74,505 before their last wash. At this time, the platelet pellet was resuspended in 2 ml of PGI_2/Tyrode and divided into 2 aliquots of 1 ml. UK-74,505 was added to one aliquot at a final concentration of 10^{-6} M and the other aliquot was used as control. After a 15 min incubation at room temperature, both suspensions were diluted to 10 ml with PGI_2/Tyrode, centrifuged and finally resuspended in PGI_2-free Tyrode.

Measurement of platelet accumulation and plasma protein leakage in rabbit skin

Platelet accumulation and oedema formation in rabbit dorsal skin were simultaneously measured by i.v. injection of ^{111}In-labelled platelets, in some experiments also loaded with Fura-2, and ^{125}I-labelled human serum albumin ([^{125}I]-HSA). Rabbits were anaesthetized with i.v. Sagatal and their dorsal skin shaved. [^{125}I]-HSA (5 μCi kg^{-1}) mixed with Evans blue dye (10 mg kg^{-1}) in saline was injected i.v., followed 5 min later by platelets in 3 ml volumes. Platelets were labelled with ^{111}In only for experiments investigating the effects of i.v. UK-74,505, and double labelled with Fura-2 and ^{111}In in experiments where platelets were pretreated with UK-74,505. The number of platelets injected into pairs of animals varied between 3.5 and 8×10^9 but was the same within each pair of animals. When necessary, UK-74,505 or its vehicle, was injected i.v. 10 min after the platelets. After a further 10 min, the agents under investigation, all prepared in saline containing 0.1% bovine serum albumin (BSA) (saline/BSA) were injected i.d. in 0.1 ml volumes, each agent having six replicates per animal according to a balanced site plan. Where indicated, agonists were co-injected with prostaglandin E_2 (PGE_2) (3×10^{-10} mol/site) as a potentiating agent of plasma protein leakage. The RPA reaction was elicited by injecting anti-BGG antiserum i.d. undiluted (100%), diluted with saline/BSA 1/2 (50%) and 1/4 (25%) at the same time as the other agents tested, followed 5 min later by an i.v. injection of BGG (5 mg kg^{-1}). In this model, no significant reaction was observed when saline was given i.v. instead of BGG. In experiments where the platelets were double labelled with ^{111}In and Fura-2, the animals were bled after 4 h via the carotid artery and platelets isolated for measurement of $[Ca^{2+}]_i$, as described above. In other experiments, at the end of the *in vivo* test period, 30 min or 4 h, a 10 ml blood sample was collected into heparin by cardiac puncture. The animals were then killed by an overdose of anaesthetic, the dorsal skin removed and the skin sites excised with a 17-mm diameter punch. Radioactivity in skin sites and plasma samples was counted in a gamma counter with automatic spill-over and cross talk correction (Packard Cobra, Meridien, CT, U.S.A.). Oedema formation was expressed as μl of plasma by dividing skin sample ^{125}I-counts by ^{125}I-counts in 1 μl of plasma. The ^{111}In-count per platelet was determined and used to express platelet accumulation in terms of number of platelets per site per 10^9 cells injected. Blood samples were also used to determine the percentage of circulating labelled platelets and the level of free ^{111}In. In all experiments, the level of free ^{111}In was less than 3% and the percentage of circulating labelled platelets varied between 75 and 100% of those injected. These values were the same in all groups of animals.

Materials

BSA (low endotoxin and fatty acid free), BGG, bradykinin (BK), histamine, zymosan, formyl-methionyl-leucyl-phenylalanine (FMLP), PGE_2, and PGI_2 were purchased from Sigma Chemical Co, Poole, Dorset. [^{125}I]-HSA (20 mg ml^{-1} albumin in sterile isotonic saline, 50 μCi ml^{-1}) and $^{111}InCl_3$ (2 mCi in 0.2 ml sterile pyrogen free 0.04 N HCl) were from Amersham International, Amersham, Buckinghamshire. Sagatal (pentobarbitone sodium, 60 mg ml^{-1}) was from May and Baker, Dagenham, Essex. Fura-2 acetoxymethyl ester was from Cambridge Bioscience, Cambridge. Freund's complete and incomplete adjuvant were from Difco Laboratories, West Molesey, Surrey. Evans blue was from British Drug House, Poole, Dorset. Viaflex (sterile, pyrogen-free isotonic saline solution) was from Baxter Healthcare Ltd., Thetford, Norfolk. PAF was from Bachem, Saffron Walden, Essex. Leukotriene B_4 (LTB_4) was from Cascade Biochem Ltd., Reading, Berkshire. Recombinant human interleukin-8 (IL-8) was from British Bio-technology Ltd., Cowley, Oxford. Sterile Tyrode solution (composition, mM: NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 0.5, NaH_2PO_4,H_2O 0.36, $NaHCO_3$ 12, glucose 5.6) was from Gibco BRL, Paisley. UK-74,505 [4-(2-chlorophenyl)-1,4-dihydro-3-ethoxycarbonyl-6-methyl-2-[4-(2-methylimidazo[4,5-c]pyrid-1-yl)phenyl]-5-[N-(2-pyridyl)carbamoyl)pyridine], initially dissolved in 0.1 N HCl and diluted in saline, was a gift from Dr M.J. Parry, Pfizer Central Research, Sandwich, Kent.

Statistical analysis

All data are presented as the mean ± s.e.mean of the indicated number of experiments. Statistical significance was assessed by use of two way analysis of variance for *in vivo* experiments and paired and unpaired Student's *t* tests for *in vitro* and *ex vivo* experiments, respectively. *P* values less than 0.05 were considered to be significant.

Results

Effect of systemic UK-74,505 on agonist-induced oedema formation over 30 min in rabbit skin

Initially, we determined the effective dose of UK-74,505 required to inhibit the PAF-induced inflammatory response in rabbit skin. Figure 1 (a) shows that PAF ($10^{-11} - 10^{-9}$ mol/site) administered i.d. in the presence of the vasodilator PGE_2 (3×10^{-10} mol/site) induced a dose-dependent oedema formation over a 30 min test period. In this system, as previously reported (Wedmore & Williams, 1981), PAF and PGE_2 act synergistically to induce oedema formation. Only small responses were produced by PAF alone at the top dose employed. In animals pretreated i.v. with 0.1 mg kg^{-1} UK-74,505, the oedema responses evoked by the different doses of PAF were inhibited by greater than 80%. Increasing the

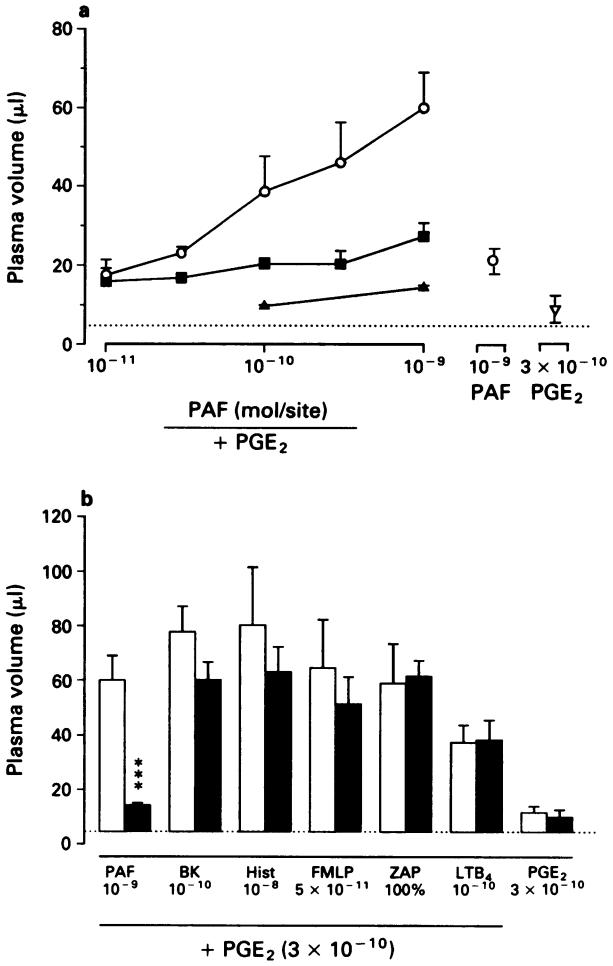


Figure 1 Effect of UK-74,505 on oedema formation induced by PAF (a) and by different soluble inflammatory stimuli (b) in rabbit skin. (a) Rabbits received an i.v. injection of either vehicle (open symbols) or UK-74,505 (0.1 mg kg^{-1}, ■ or 0.5 mg kg^{-1}, ▲) followed 10 min later by i.d. injections of increasing doses (mol/site) of PAF in the presence of prostaglandin E_2 (PGE_2, 3×10^{-10} mol/site). (b) Rabbits received an i.v. injection of either vehicle (open columns) or 0.5 mg kg^{-1} UK-74,505 (solid columns) followed 10 min later by i.d. injections of the indicated doses (mol/site) of PAF, bradykinin (BK), histamine (Hist), formyl-methionyl-leucyl-phenylalanine (FMLP), zymosan-activated plasma (ZAP) and leukotriene B_4 (LTB_4), all tested in the presence of PGE_2 (3×10^{-10} mol/site). After 30 min, the animals were killed and plasma protein exudation was measured as indicated in the Methods section. Results are expressed as μl plasma and presented as mean ± s.e.mean of $n = 3-6$ experiments. The dotted line represents control responses obtained after i.d. injection of saline/BSA. In (a) the open circle and triangle represent the responses obtained in PAF (10^{-9} mol/site) and PGE_2 (3×10^{-10} mol/site) injected sites respectively. Significant differences from control are indicated by $^{***}P < 0.001$.

dose of UK-74,505 to 0.5 mg kg^{-1} resulted in almost total suppression of PAF-induced oedema formation.

The specificity of UK-74,505 was then evaluated by examining its effect on plasma exudation induced by other inflammatory mediators over a 30 min test period. Figure 1 (b) shows that the optimum dose of 0.5 mg kg^{-1} UK-74,505, virtually abolished the response induced by PAF (10^{-9} mol/site), but had no effect on plasma leakage induced by BK, histamine, FMLP, ZAP (as a source of C5a des Arg) or LTB_4, all tested in the presence of PGE_2 (3×10^{-10} mol/site).

Effect of systemic UK-74,505 on oedema formation and platelet accumulation induced by inflammatory stimuli over 4 h in rabbit skin

Having determined an effective and specific dose for the i.v. injection of UK-74,505 (i.e. 0.5 mg kg^{-1}), we investigated the effect of this PAF antagonist on oedema formation (Figure 2a) and platelet accumulation (Figure 2b) induced by exogenous soluble inflammatory mediators and zymosan particles measured over a 4 h period. As found with the 30 min experimental protocol, oedema formation induced by PAF + PGE_2 over 4 h was effectively inhibited in rabbits pretreated with 0.5 mg kg^{-1} i.v. UK-74,505 (Figure 2a). In control animals, zymosan (which induces endogenous prostaglandin production (Williams & Jose, 1981)), IL-8 + PGE_2 and ZAP + PGE_2 all induced marked oedema formation. These responses were unchanged in animals pretreated with i.v. UK-74,505 (Figure 2a).

In these experiments, PAF, PAF + PGE_2, IL-8, IL-8 + PGE_2, or ZAP induced a small, but measurable, accumulation of ^{111}In-labelled platelets (5,000–15,000 platelets/site) in the skin, whereas platelet deposition evoked by zymosan or ZAP + PGE_2 was much more pronounced (25,000–30,000

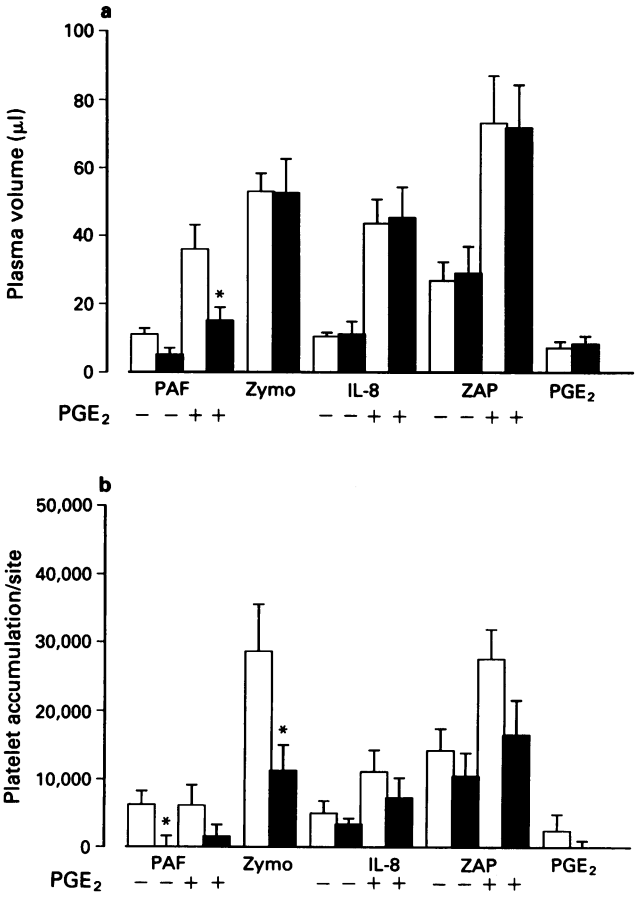


Figure 2 Effect of UK-74,505 on oedema formation (a) and platelet accumulation (b) induced by different soluble inflammatory stimuli in rabbit skin. Rabbits received an i.v. injection of either vehicle (open columns) or 0.5 mg kg^{-1} UK-74,505 (solid columns) followed 10 min later by i.d. injections of PAF (10^{-9} mol/site), zymosan (Zymo, 300 μg/site), interleukin-8 (IL-8, 3×10^{-11} mol/site) and undiluted zymosan activated plasma (ZAP), in the presence or the absence of prostaglandin E_2 (PGE_2, 3×10^{-10} mol/site). After 4 h, the animals were killed and plasma protein exudation and platelet accumulation were measured as indicated in the Methods section. Results are expressed as μl plasma (oedema formation) and number of platelets per site per 10^9 platelets injected (platelet accumulation) and presented as mean ± s.e.mean of responses obtained after subtraction of saline/BSA values in $n = 5-6$ rabbits. In control animals, oedema formation and platelet accumulation in response to i.d. injection of bradykinin (BK) (10^{-10} mol/site) + PGE_2 (3×10^{-10} mol/site) were 62.5 ± 6.2 μl ($n = 7$) and 6074 ± 527 platelets/site ($n = 7$) respectively. Oedema formation and platelet accumulation in saline/BSA injected sites were 8.9 ± 1.7 μl and 8866 ± 1773 platelets/site in control animals and 9.7 ± 1.8 μl and 8684 ± 1373 platelets/site in animals pretreated with UK-74,505. Significant differences from control are indicated by $^{*}P < 0.05$.

platelets/site) (Figure 2b). PGE_2 had a marked potentiating effect on platelet accumulation when in combination with IL-8 and ZAP. BK + PGE_2 induced large oedema responses, but only small numbers of platelets accumulated in this case (see Figure 2 legend). The platelet deposition induced by PAF + PGE_2, IL-8, IL-8 + PGE_2, ZAP or ZAP + PGE_2 was not significantly affected in animals given 0.5 mg kg^{-1} i.v. UK-74,505, whereas platelet accumulation provoked by PAF alone or zymosan was significantly suppressed, the latter being inhibited by greater than 50% (Figure 2b). Interestingly, the inhibition of zymosan-induced platelet accumulation occurred without any effect on the plasma leakage response (Figure 2).

Effect of UK-74,505 on rabbit platelet aggregation and changes in $[Ca^{2+}]_i$ induced by PAF in vitro

The potency of UK-74,505 in inhibiting PAF-induced responses of rabbit washed platelets was assessed *in vitro*. Figure 3a shows the concentration-dependent aggregation of washed platelets evoked by PAF (10^{-11} to 3×10^{-8} M). Maximal aggregation of $48.3 \pm 4.2\%$ ($n = 4$ preparations) was observed at 10^{-8} M PAF and the calculated ED_{50} value for the autacoid was $3.86 \pm 0.41 \times 10^{-10}$ M ($n = 4$ preparations). PAF was one hundred times less potent in inducing aggregation of platelets in PRP ($ED_{50} = 5.03 \pm 0.80 \times 10^{-8}$ M, $n = 3$ preparations) and its maximal effect on this preparation ($32.0 \pm 3.3\%$) was smaller than on washed platelets (Figure 3a). The effect of UK-74,505 was investigated against washed platelet aggregation induced by the submaximal dose of 3×10^{-9} M PAF (Figure 3b). The antagonist incubated for 2 min prior to platelet stimulation inhibited the response induced by PAF in a concentration-dependent manner (Figure 3b), with a calculated IC_{50} value of $1.63 \pm 0.13 \times 10^{-8}$ M ($n = 3$ preparations).

As found for aggregation, PAF induced a concentration-dependent increase in platelet $[Ca^{2+}]_i$ (Figure 3c). The range of PAF concentrations used was similar to those necessary to trigger an aggregatory response and the calculated ED_{50} value was $3.24 \pm 0.85 \times 10^{-10}$ M ($n = 5$ preparations). UK-74,505 incubated for 2 min prior to stimulation inhibited the increase in $[Ca^{2+}]_i$ evoked by 3×10^{-9} M PAF in a concentration-dependent manner (Figure 3d), with a calculated IC_{50} value of $1.13 \pm 0.28 \times 10^{-8}$ M ($n = 4$ preparations). These results demonstrate that UK-74,505 exhibits similar inhibitory activity towards rabbit platelet aggregation and increases in $[Ca^{2+}]_i$ induced by PAF. With both *in vitro* test systems maximal inhibition of the effects of PAF was achieved with 10^{-6} M of the antagonist.

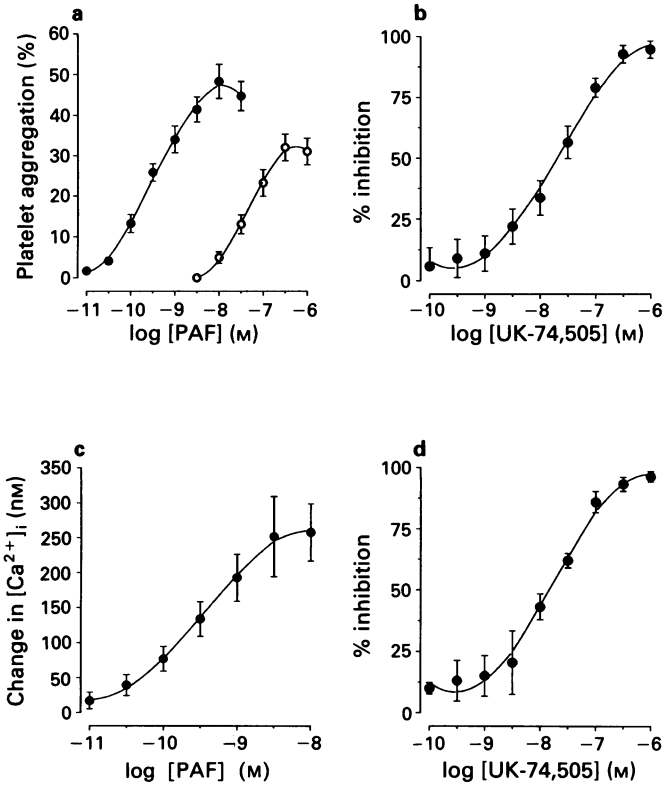


Figure 3 Effect of UK-74,505 on platelet aggregation and changes in $[Ca^{2+}]_i$ induced by PAF in rabbit washed platelets. (a) Concentration-dependent PAF-induced aggregation of platelets in PRP (○) or in Tyrode solution (●). Results are expressed as a percentage of light transmission and presented as mean ± s.e.mean of $n = 3$ preparations for PRP and $n = 4$ preparations for washed platelets. (b) Inhibition by UK-74,505 of washed platelet aggregation induced by 3×10^{-9} M PAF. UK-74,505 was added to the platelet suspension 2 min prior to challenge. Results are expressed as percentage inhibition of aggregation induced by 3×10^{-9} M PAF and presented as mean ± s.e.mean of $n = 3$ preparations. (c) Concentration-dependent PAF-induced changes in $[Ca^{2+}]_i$ in washed platelets. Results are expressed as $[Ca^{2+}]_i$ after subtraction of basal values and presented as mean ± s.e.mean of $n = 5$ preparations. Basal $[Ca^{2+}]_i$ was 191 ± 36 nM ($n = 5$). (d) Inhibition by UK-74,505 of changes in $[Ca^{2+}]_i$ induced by 3×10^{-9} M PAF in washed platelets. UK-74,505 was added to the platelet suspension 2 min prior to challenge. Results are expressed as percentage inhibition of the increase in $[Ca^{2+}]_i$ induced by PAF and presented as mean ± s.e.mean of $n = 4$ preparations. Basal $[Ca^{2+}]_i$ was not significantly different before and after addition of UK-74,505.

Pretreatment of rabbit platelets with UK-74,505

Based on the above experiments, a concentration of 10^{-6} M UK-74,505 was used to pretreat labelled rabbit platelets. However, before initiating the *in vivo* experiments, the time-course of the inhibitory activity of this dose was assessed *in vitro* on PAF-induced platelet aggregation. These experiments were carried out in order to determine whether pretreatment with UK-74,505 antagonized platelet PAF receptors for the 4 h *in vivo* period necessary to develop an RPA reaction in rabbit skin. Washed platelets were pretreated with UK-74,505, washed again to remove unbound compound and maintained for 4 h at 37°C. At the end of the incubation period, aggregation in response to 3×10^{-9} M PAF and, for comparison, 3.2 u ml^{-1} thrombin was assessed. As indicated in Figure 4, untreated washed platelets maintained for 4 h at 37°C retained their responsiveness to both PAF and thrombin throughout this incubation period. In contrast, platelets

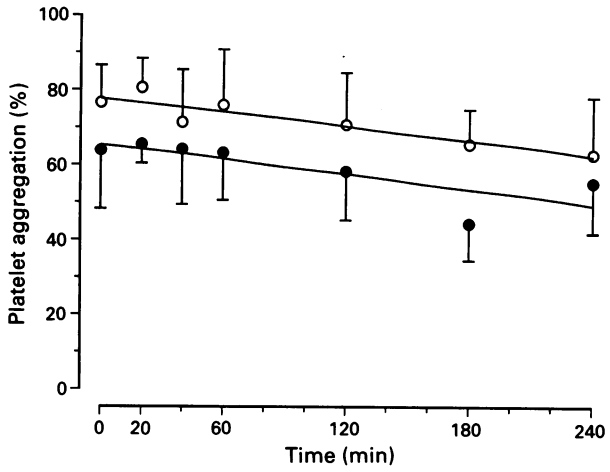


Figure 4 Time-course of rabbit washed platelet aggregation induced by 3×10^{-9} M PAF (●) or 3.2 u ml^{-1} of thrombin (○). After their final suspension, washed platelets were kept at 37°C in a water bath. Platelet aliquots were taken at different times during the 4 h incubation that followed and their aggregation to PAF or thrombin was assessed. Results are expressed as a percentage of maximum light transmission and presented as mean ± s.e.mean of $n = 4$ preparations.

pretreated with UK-74,505 exhibited a significantly inhibited response when tested with PAF after the 4 h period (Figure 5a). This inhibition was specific since the responses to thrombin were unaffected by pretreatment with UK-74,505 (Figure 5a). UK-74,505 remained bound to platelet PAF receptors during the 4 h incubation at 37°C, since pretreated platelets centrifuged for 10 min and resuspended in fresh Tyrode solution at the end of the 4 h period still exhibited an inhibited aggregatory response to PAF (Figure 5a). Furthermore, the

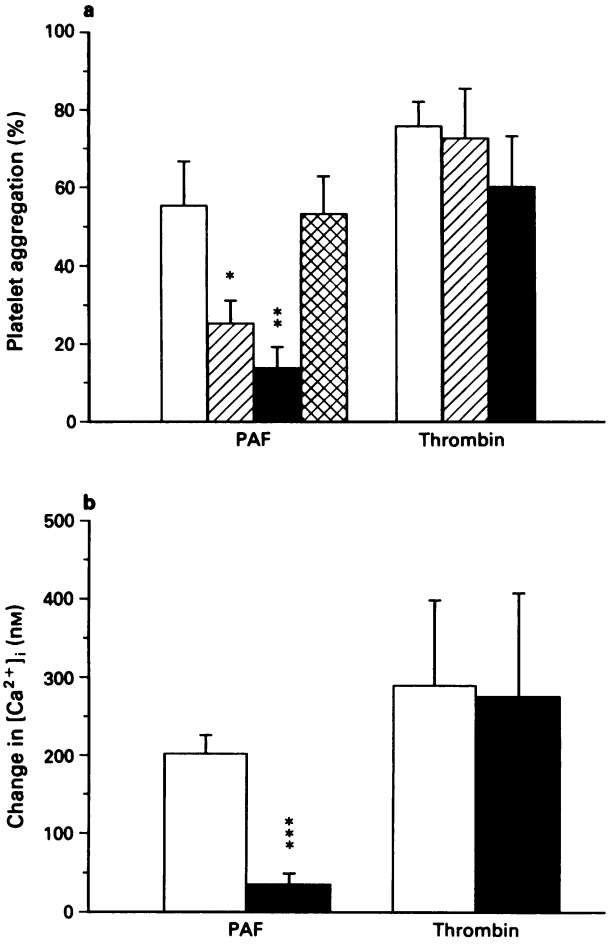


Figure 5 *In vitro* (a) and *ex vivo* (b) responsiveness to PAF and thrombin of control and UK-74,505 pretreated platelets after a 4 h period. (a) PAF- or thrombin-induced aggregation of control and UK-74,505 pretreated washed rabbit platelets incubated at 37°C for 4 h. Washed platelets were prepared and pretreated with UK-74,505 (10^{-6} M for 15 min) as indicated in the Methods section. The platelet preparations were then kept at 37°C for 4 h and at the end of the incubation period their reactivity to PAF (3×10^{-9} M) or thrombin (3.2 u ml^{-1}) was assessed. Results are expressed as a percentage of maximum light transmission and presented as mean ± s.e.mean of $n = 4$ preparations (open columns, control platelets; hatched columns, UK-74,505-pretreated platelets; solid columns, UK-74,505-pretreated platelets centrifuged and resuspended in fresh Tyrode solution; cross-hatched columns, control platelets incubated for 2 min with the supernatant of UK-74,505-pretreated platelets at a final dilution of 1/10). $*P<0.05$; $**P<0.01$, paired Student's t test. (b) *Ex vivo* measurement of changes in $[Ca^{2+}]_i$ induced by PAF (10^{-7} M) or thrombin (1 u ml^{-1}) in labelled platelets isolated from recipient rabbits. Washed platelets were loaded with Fura-2 and labelled with ^{111}In and pretreated (solid columns) or not (open columns) with UK-74,505, as indicated in the Methods section. Platelets were then injected into different recipient rabbits in order to measure their accumulation in response to various stimuli. After 4 h, rabbits were bled and their platelets isolated. Results are expressed as $[Ca^{2+}]_i$ after subtraction of basal values and presented as mean ± s.e.mean of $n = 6$ preparations. Basal values of $[Ca^{2+}]_i$ were 120 ± 19 nM and 123 ± 8 nM for control and UK-74,505-pretreated platelets, respectively ($n = 6$ preparations). Significant differences from control are indicated by $***P<0.001$.

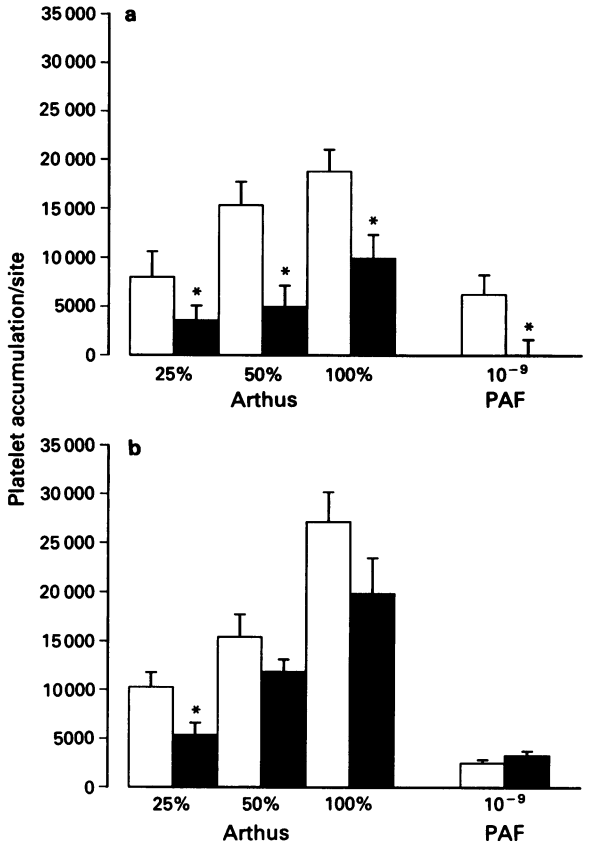


Figure 6 Platelet accumulation induced by PAF (10^{-9} mol/site) and increasing concentrations of Arthus antiserum in rabbit skin: effect of i.v. injection of 0.5 mg kg^{-1} UK-74,505 (a, solid columns) or pretreatment of ^{111}In-labelled platelets with UK-74,505 (b, solid columns). Platelet accumulation was expressed as number of platelets per site per 10^9 platelets injected and presented as mean ± s.e.mean of the responses obtained after subtraction of normal rabbit serum or saline/BSA values in $n = 6-7$ rabbits. Significant differences from control are indicated by $*P<0.05$. Oedema formation in response to 50% Arthus antiserum was significantly reduced in animals pretreated with i.v. UK-74,505 compared to control animals (14.9 ± 4.2 µl ($n = 6$) versus 30.5 ± 3.9 µl ($n = 6$), $P<0.05$). Platelet accumulation and oedema formation in response to normal rabbit serum were not significantly different from saline/BSA controls.

4 h supernatant of UK-74,505-pretreated platelets obtained after centrifugation, did not inhibit aggregation of control platelets in response to PAF (Figure 5a). However, centrifugation of UK-74,505-pretreated platelets and resuspension in fresh Tyrode solution at the end of the 4 h incubation period did not affect their normal responsiveness to thrombin (Figure 5a).

In order to determine if PAF receptors on platelets pretreated with the antagonist remained blocked after 4 h *in vivo*, platelets were ^{111}In-labelled and loaded with Fura-2. The platelets were then treated with UK-74,505 and washed. Treated and untreated platelets for comparison were then injected i.v. into rabbits for the experiments described in the next section. At the end of a 4 h period, blood samples were taken and platelets prepared. Responses to PAF were then determined in terms of changes in $[Ca^{2+}]_i$ measured with Fura-2. Platelets pretreated with the antagonist clearly remained unresponsive to PAF after the 4 h period *in vivo* whereas responses to thrombin were unaffected (Figure 5b).

Effect of systemic UK-74,505 or in vitro *pretreatment of rabbit platelets with UK-74,505 on oedema formation and platelet accumulation in an RPA reaction in rabbit skin*

Intradermal administration of increasing amounts of Arthus antiserum (25%, 50% and 100%) in rabbits, followed by i.v.

injection of antigen, provoked a significant and dose-dependent platelet accumulation over the 4 h test period (Figure 6, open columns). A similar dose-response curve for oedema formation was obtained (data not shown). Platelet deposition triggered by the different dilutions of Arthus antiserum was significantly inhibited when rabbits were pretreated with 0.5 mg kg^{-1} i.v. UK-74,505 (Figure 6a). The small platelet deposition induced by i.d. PAF was also inhibited in these animals (Figure 6a). Intravenous injection of the PAF antagonist also suppressed oedema responses in the RPA reaction (see legend to Figure 6), as shown previously with other antagonists (Hellewell & Williams, 1986; Issekutz & Szejda, 1986).

Thus, i.v. UK-74,505 effectively inhibited platelet accumulation in RPA reactions; however, radiolabelled platelets with their PAF receptors blocked by pre-incubation with the antagonist still accumulated in high numbers in RPA sites (Figure 6b). A significant reduction in accumulation was only seen in sites receiving 25% antiserum. Further, the small platelet accumulation induced by i.d. PAF was also unaffected by preincubating platelets with the antagonist (Figure 6b). In these experiments, oedema responses in Arthus sites were not significantly different in the two groups of rabbits (data not shown).

Discussion

In this study a novel PAF antagonist, UK-74,505, was employed to investigate the possible contribution of endogenous PAF to oedema formation and platelet accumulation induced in rabbit skin in response to soluble mediators and particulate stimuli. A unique property of the compound is avid binding to PAF receptors which enabled the effect of selectively blocking platelet receptors to be studied.

Intravenous UK-74,505 blocked oedema formation induced by intradermally-injected PAF, but had no effect on oedema formation induced by BK and histamine, or on neutrophil-dependent oedema formation induced by ZAP (as a source of C5a des Arg), FMLP, LTB_4 or IL-8 (Figures 1 and 2). Mediators were tested in the presence of the vasodilator, PGE_2, in order to induce substantial oedema responses by synergism. These experiments demonstrated the potency and selectivity of the antagonist. This selectivity is not seen with certain other PAF antagonists such as CV-3988 (Hellewell & Williams, 1989) which makes interpretation of some previous results difficult (Issekutz & Szejda, 1986). Oedema induced by i.d. injection of zymosan particles, which stimulates the generation of C5a and a vasodilator prostaglandin locally, was also unaffected by i.v. UK-74,505.

PAF, ZAP, IL-8 and zymosan were tested i.d. for their ability to induce the accumulation of ^{111}In-labelled platelets in rabbit skin (Figure 2). Interestingly, PAF alone, or PAF + PGE_2 which induced substantial oedema responses, induced only small, but significant, platelet accumulation. Some platelet accumulation was induced by IL-8 and this was significantly potentiated by PGE_2. ZAP-induced platelet accumulation and large accumulation was seen with the addition of PGE_2 (to our knowledge this is the first demonstration of potentiation of platelet accumulation by a prostaglandin). Zymosan particles also induced substantial platelet accumulation. Only the platelet accumulation induced by PAF and zymosan was significantly suppressed by i.v. UK-74,505. A striking observation was the effective inhibition of zymosan-induced platelet accumulation by the antagonist, with no effect on the oedema response. These results suggest that PAF is generated locally in response to zymosan and that it is important in platelet accumulation. These results are in agreement with data published by Dewar *et al.* (1984), showing that PAF causes platelet accumulation in guinea-pig skin. However, in our model, endogenous PAF does not appear to be important for zymosan-induced oedema formation, perhaps because this is masked by the powerful oedema-inducing effect of endogenous C5a by a neutrophil-dependent process. The observation that neutrophil chemoattractants induced platelet accumulation that is not significantly inhibited by i.v. UK-74,505 may suggest that this platelet accumulation is secondary to neutrophil adherence to the endothelium, e.g. platelet adherence to stimulated neutrophils (Issekutz *et al.*, 1983) or, alternatively, release of platelet stimulatory activity from neutrophils (Chignard *et al.*, 1986). ZAP appeared to be very active in this respect, which may reflect its more protracted action in the skin. Exogenous PAF induced substantial oedema when in combination with PGE_2, but small platelet accumulation. PAF is unstable in tissue fluid and these results may indicate that a sustained production of PAF is important for platelet accumulation, whereas an acute effect on endothelial cells can induce plasma protein leakage. These points require further investigation, but it is clear that platelet accumulation is not merely a consequence of increased microvascular permeability as BK + PGE_2 induced large oedema responses with very little platelet accumulation (Figure 2 legend).

These observations and the special binding properties of the antagonist led us to address the importance of PAF receptors on platelets in the process of platelet accumulation *in vivo*. PAF induced a concentration-related aggregation of rabbit platelets *in vitro* correlating with changes in $[Ca^{2+}]_i$ (Figure 3). Both these responses were inhibited by UK-74,505 (Figure 3). Platelets retained their responsiveness to PAF and thrombin when tested for aggregation after a 4 h incubation period at 37°C (Figure 4). Responses to PAF remained selectively blocked when platelets were incubated with UK-74,505, washed and then aggregation responses were tested after the 4 h incubation period (Figure 5a).

^{111}In-labelled platelets, loaded with Fura-2 and with their PAF receptors blocked by pre-incubation with UK-74,505, were then administered i.v. to rabbits. RPA reactions were induced in the skin, and platelet accumulation and plasma protein leakage measured after 4 h. Platelets prepared from blood samples taken at 4 h remained selectively unresponsive to PAF, as evidenced by changes in $[Ca^{2+}]_i$ (Figure 5b). However, these blocked platelets were still able to accumulate in Arthus sites (Figure 6b), whereas i.v. UK-74,505 (with access to PAF receptors on other cells as well as platelets) was far more effective in suppressing platelet accumulation (Figure 6a). Similarly, platelet accumulation induced by exogenous PAF was inhibited by i.v. antagonist and not modified in animals injected with UK-74,505-pretreated platelets. Intravenous UK-74,505 also suppressed oedema formation in the Arthus sites, (Figure 6 legend), as shown previously with other selective antagonists (Hellewell & Williams, 1986; Issekutz & Szejda, 1986).

These results suggest that endogenous PAF is not an important secondary mediator for oedema formation and platelet accumulation induced by the soluble mediators tested in this study. However, endogenous PAF is implicated in oedema formation in the RPA reaction, although not in response to zymosan. This difference may be explained by the different sites of PAF generation in these two responses. Both immune complexes and zymosan can activate complement locally and generate C5a that will cause neutrophil accumulation and neutrophil-dependent oedema formation. In the RPA reaction, PAF is likely to be generated by phagocytosing neutrophils very close to the microvascular endothelium and can increase permeability by a direct effect on endothelial cells. When zymosan is injected i.d. the particles will be relatively remote from vessels and PAF liberated during phagocytosis may be metabolized before reaching an effective concentration near the endothelium.

The situation with respect to platelet accumulation appears to be different. In this case i.v. PAF antagonist inhibited the response induced by both zymosan (Figure 2b) and immune complexes (Figure 6a). To account for this, we propose that released PAF acts over a short range and stimulates the secondary release of an unidentified, relatively-stable med-

iator that induces platelet accumulation. The source of endogenous PAF may be the neutrophil and it is possible that this PAF may have an autocrine function in acting on neutrophil PAF receptors to release the secondary mediator. This hypothesis is supported by the results of the final experiments (Figure 6b) demonstrating that platelets in which PAF receptors are blocked by preincubation with the antagonist still accumulate effectively in Arthus sites. The results with i.d. PAF also support this idea although platelet accumulation was small presumably because of the instability of PAF *in vivo*. Platelet accumulation induced by intradermally-injected PAF was clearly blocked by i.v. antagonist, which we propose would prevent PAF stimulating release of the secondary mediator. However, preincubation of platelets with the antagonist had no effect on platelet accumulation induced by i.d. PAF. An alternative explanation for the results obtained in Figure 6 (b) is that PAF, added exogenously or generated by neutrophils during the RPA reaction, activates the recipient animal's own platelets which then in turn cause recruitment of the antagonist-treated radiolabelled platelets by releasing a mediator other than PAF. However, our results in Figure 5 (b) render this latter hypothesis unlikely since platelets isolated from recipients receiving UK-74,505-treated platelets (this platelet preparation containing both Fura-2 loaded PAF-receptor blocked platelets and the recipient animal's own untreated platelets) exhibited only a minimal increase in $[Ca^{2+}]_i$ in response to PAF.

In conclusion, we have demonstrated a role for PAF in platelet accumulation induced by particulate, but not soluble inflammatory stimuli. Interestingly, the results suggest that platelet PAF receptors may not play a significant role in mediating platelet accumulation and the activation of PAF receptors on neutrophils rather than on platelets may be more important. In this respect, UK-74,505 is a valuable tool for assessing the role of PAF receptors on leucocytes in inflammatory reactions and such experiments are currently in progress. With long-acting, selective and potent PAF antagonists such as UK-74,505 a clearer understanding of the mechanisms involved in inflammatory reactions such as the RPA reaction may be forthcoming.

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Effects of vasoactive intestinal polypeptide on antigen-induced bronchoconstriction and thromboxane release in guinea-pig lung

[1]Giovanni Ciabattoni, Paolo Montuschi, Diego Currò, Giuseppina Togna & Paolo Preziosi

Department of Pharmacology, Catholic University School of Medicine, Largo F. Vito 1, 00168 Rome, Italy

1 Exogenous vasoactive intestinal polypeptide (VIP) infused into the pulmonary artery of isolated and ventilated lungs of guinea-pigs decreased, in a dose-dependent fashion (1.0–10.0 nmol), airway resistance and thromboxane B_2 (TXB_2, the stable hydrolysis product of TXA_2) release in the perfusion medium. Prostacyclin (PGI_2) synthesis, as reflected by the release of its stable hydrolysis product 6-oxo-$PGF_{1\alpha}$, was unaffected. Pretreatment with the 5-lipoxygenase inhibitor BWA4c (3.5×10^{-5} M) did not modify the bronchodilatory effect of VIP or its inhibitory action on TXB_2 release.

2 Basal release of immunoreactive VIP from perfused lungs decreased from an initial value of 0.96 ± 0.10 ng min^{-1} (mean ± s.e.mean) in the first 2 min to an average of 0.58 ± 0.10 ng min^{-1} in the following 15–20 min.

3 Antigen challenge with ovalbumin (0.1%) in sensitized lungs caused an anaphylactic reaction in 45% of tested lungs, concomitant with a 5 fold increase in both VIP and TXB_2 release. Tetrodotoxin pretreatment (10^{-6} M) reduced basal VIP release by >80% and abolished the VIP increase observed during anaphylaxis, without modifying TXB_2 release or the bronchoconstrictor response.

4 Indomethacin (10^{-6} M) inhibited TXB_2 synthesis and release by >90%, delayed the bronchoconstrictor response and blunted the increased VIP release during lung anaphylaxis, without influencing basal VIP release.

5 The 5-lipoxygenase inhibitor BWA4c (3.5×10^{-5} M) blunted the increase of TXB_2 and VIP release from guinea-pig lung and attenuated the bronchoconstrictor response following ovalbumin challenge.

6 The administration of exogenous VIP as a continuous infusion (10^{-8} M) attenuated the bronchoconstriction and the release of cyclo-oxygenase metabolites following antigen challenge.

7 Acetylcholine (10^{-6}–10^{-5} M) infused into the pulmonary artery induced a dose-dependent bronchoconstriction not associated with enhanced VIP or TXB_2 release.

8 The TXA_2 mimetic U-46619 (0.1–1.0 nmol) caused dose-dependent increases in airway resistance, concomitant with an up to 10 fold increase in VIP release. VIP inhibited arachidonate-induced *in vitro* aggregation of washed rabbit platelets in a dose-dependent manner over a dose range 10^{-8}–10^{-6} M. Despite the antiaggregatory effect of VIP, TXB_2 and PGE_2 synthesis was reduced only to a minor extent, and there was no redirection of arachidonate metabolism from TXA_2 to PGE_2, indicating that VIP does not act as a TX synthase inhibitor *in vitro*.

9 We conclude that VIP may play a role in regulating bronchial smooth muscle reactivity in lung anaphylaxis by inhibiting the synthesis and release of TXA_2, a potent vasoactive and bronchoconstrictor agent. TXA_2, on the other hand, strongly enhances neuronal VIP release.

Keywords: Vasoactive intestinal polypeptide; thromboxane A_2; lung anaphylaxis; guinea-pig isolated lung; BWA4c

Introduction

Vasoactive intestinal polypeptide (VIP), a 28-amino acid peptide, is the neuropeptide found in the highest concentrations in the lung of several mammalian species, including man (see Said, 1991a and Barnes, 1991 for reviews). Immunohistochemical studies have localized immunoreactive VIP in nerve fibres within airway smooth muscle, surrounding tracheobronchial mucous and serous glands, in the walls of airway blood vessels and in microganglia within the lungs (Dey *et al.*, 1981; Laitinen *et al.*, 1985; Lundberg *et al.*, 1984; Polak *et al.*, 1986; Said, 1988). Moreover, specific, high affinity receptors for VIP have been identified in membrane preparations of rat, mouse, guinea-pig and human lungs (Said, 1987) and on airway glands, epithelium and vascular smooth muscle of human and guinea-pig lung (Carstairs & Barnes, 1986; Uddman & Sundler, 1987).

VIP is the most favoured candidate for the role of neurotransmitter in non-adrenergic non-cholinergic (NANC) inhibitory nerves because, in animal airways, it mimics the electrophysiological changes produced by NANC nerve stimulation (Cameron *et al.*, 1983). It is released when these nerves are stimulated electrically (Matsuzaki *et al.*, 1980), and, when tolerance is induced by exposure to high concentrations of VIP, the NANC inhibitory nerve effects are selectively reduced (Ito & Takeda, 1982).

VIP is known to inhibit the bronchoconstrictor effects of histamine, prostaglandin $F_{2\alpha}$, ($PGF_{2\alpha}$) the sulphidopeptide leukotrienes (LT) C_4 and D_4, neurokinins A and B, and endothelin (see Said, 1991a for review). It has been reported that VIP can inhibit the release of LTC_4 from rat and guinea-pig lungs incubated in the presence of platelet-activating factor (PAF) and calcium ionophore (Beaubien *et al.*, 1984; Di Marzo *et al.*, 1989). Moreover, VIP selectively inhibits the contraction induced by LTD_4 in the small airways of guinea-pig lung (Samhoun *et al.*, 1987) and considerably reduces the release of cyclo-oxygenase products induced by LTD_4 in strips of guinea-pig lung parenchyma (Samhoun *et al.*, 1987; Conroy *et al.*, 1988a,b) and from guinea-pig perfused lung (Conroy *et al.*, 1988a,b; 1992).

Previous studies have characterized the release of thromboxane and leukotrienes from anaphylactic guinea-pig lungs

[1] Author for correspondence.

and found that TXB_2 (the stable hydrolysis product of TXA_2) is the most abundant cyclo-oxygenase product of arachidonate metabolism in anaphylactic lung perfusates (Robinson *et al.*, 1984). We have previously reported that exposure to toxicants that irritate the respiratory tract selectively enhances TXB_2 release from isolated, perfused and ventilated lung of guinea-pigs (Preziosi & Ciabattoni, 1986; 1987). The aim of this study was to characterize the release of VIP from guinea-pig sensitized isolated lungs undergoing anaphylactic reactions after an antigen challenge and to explore the hypothesis that VIP and arachidonate metabolites interact in regulating bronchial smooth muscle reactivity.

A preliminary report of this study was presented at the Fifth International Symposium on VIP and Related Peptides, held on November 12–15, 1991, at Shizuoka, Japan, and published in abstract form (*Biomed. Res.*, **12**, S1:34, 1991).

Methods

Isolated lungs from male Dunkin-Hartley guinea-pigs (400–600 g) were perfused according to the procedure originally described by Delaunois *et al.* (1956) and Preziosi *et al.* (1959). Each lung was removed from the chest of the animal, the pulmonary artery was cannulated and most of the heart cut away to allow perfusion. The lungs were perfused at 5 ml min^{-1} in a thermostatic Perspex chamber with Krebs Ringer bicarbonate solution at 37°C, gassed with 5% CO_2/95% O_2 and containing bacitracin (30 mg l^{-1}) and aprotinin (100,000 iu l^{-1}). A pressure transducer connected to the trachea allowed measurement of pressure-volume variations in the lung system on a Beckman Dynograph. A pump, which aspirated air from the closed chamber during one half of a cycle and pushed the same air back during the other half, enabled lung ventilation in sequence. The lung perfusion solution was removed from the bottom of the chamber by a peristaltic pump connected to a fraction collector. The solution was chilled on ice, centrifuged at 4°C and stored at − 25°C until assayed.

Sensitization of guinea-pigs

Guinea-pigs were sensitized with a 1% ovalbumin solution; 20 mg being given intraperitoneally and 20 mg subcutaneously after 10 min. Animals were used after 15 days.

VIP infusion

Exogenous VIP was given as an infusion over 1 min through the pulmonary artery in control non-sensitized lungs or after pretreatment with the lipoxygenase inhibitor BWA4c (N-(3-phenoxycinnamyl)-acetohydroxamic acid; Payne *et al.*, 1988). The latter compound was dissolved in the perfusion medium at a concentration 3.5×10^{-5} M and the lungs were perfused with this solution throughout the experiment. Pressure-volume variations in the lung were recorded under basal conditions and during and after exposure to three different doses of VIP (1, 5 and 10 nmol) given in a sequence at intervals of 6 min. For this purpose 5 ml of a VIP solution at concentrations ranging between 2×10^{-7} and 2×10^{-6} M was injected into the pulmonary artery within 1 min, during which the flow of the perfusion medium from the reservoir was stopped. The perfusate was recovered at intervals of 2 min for measurement of TXB_2 and 6-oxo-$PGF_{1\alpha}$, the stable hydrolysis product of prostacyclin (PGI_2; see below).

Release experiments

Ovalbumin-sensitized guinea-pig lungs were perfused, allowed to equilibrate for at least 10 min and challenged with a 0.1% ovalbumin solution given through the pulmonary artery. In 25 of all the lungs tested in this study ($n = 59$) a bronchoconstriction followed antigen challenge within a few minutes. Pressure-volume variations in the lung were monitored throughout the experiment and the perfusate was recovered at intervals of 2 min. Some of the sensitized lungs were pretreated with tetrodotoxin (TTX) 10^{-6} M, or indomethacin 10^{-6} M, or BWA4c (3.5×10^{-5} M) for at least 20 min before exposure to ovalbumin. These compounds were dissolved in the perfusing medium and the lungs were perfused with the appropriate solution throughout the experiment.

A total of 12 sensitized lungs were challenged with ovalbumin during administration of exogenous VIP. For this purpose, the lungs were perfused with a medium containing VIP 10^{-8} M, i.e. a concentration that has been shown to inhibit the release of cyclo-oxygenase products from guinea-pig isolated perfused lung (Conroy *et al.*, 1988a,b; 1992). After an equilibration period of 20 min they were exposed to ovalbumin; 5 of these underwent bronchoconstriction. Pressure-volume variations in the lung were recorded and the perfusate was collected at 2 min intervals.

Acetylcholine infusion

Acetylcholine was infused over 1 min through the pulmonary artery of non-sensitized lungs at concentrations ranging from 10^{-6} to 10^{-5} M (flow rate: 5 ml min^{-1}) to decrease the tracing recording the pressure-volume variations in the lung to an extent comparable to that observed in the lungs undergoing anaphylactic reaction after antigen challenge. The perfusate from these lungs was recovered at 2 min intervals.

Thromboxane-mimetic infusion

The stable thromboxane A_2 analogue U-46619 (15 S-hydroxy-11α, 9α-epoxymethanoprosta-5 *Z*, 13 *E*-dienoic acid) was infused over 1 min through the pulmonary artery of non-sensitized lungs. Three different doses (0.1, 0.5 and 1.0 nmol) were given in sequence at 6 min intervals. For this purpose 5 ml of a U-46619 solution at concentrations ranging between 2×10^{-8} and 2×10^{-7} M was injected into the pulmonary artery within 1 min, during which the flow of the perfusion medium from the reservoir was stopped. Pressure-volume variations in the lung were recorded, and the perfusate was recovered as above.

Washed-platelet experiments

Washed rabbit platelets were prepared according to the method originally described by Ardlie *et al.* (1970) with some modifications. Blood was drawn from New Zealand rabbits by cardiac puncture in plastic tubes with sodium citrate 3.8% in the ratio 9:1 (blood:anticoagulant), and centrifuged at 120 *g* for 15 min. The resulting platelet-rich plasma was aspirated and centrifuged at 1,100 *g* for 15 min at room temperature. The pellet was dissolved in 10 ml of washing solution (modified Tyrode solution, containing (mM): NaCl 129, KCl 2.6, Na_2PO_4 0.39, $NaHCO_3$ 12, glucose 5.5, $MgCl_2$ 2.0 and 0.35% bovine serum albumin) and kept for 15 min before centrifugation. The procedure was repeated twice and finally platelets were suspended in a HEPES-buffered Tyrode solution (the same solution described above, containing 1.8 mM $CaCl_2$, 5 mM HEPES, 30 mg l^{-1} bacitracin and 100,000 iu l^{-1} aprotinin). Platelets were stirred at 1,000 r.p.m. at 37°C in an automated aggregometer (Elvi-Logos) and incubated for 60 s in the presence of VIP; thereafter, sodium arachidonate was added at final concentrations ranging from $5–15 \times 10^{-6}$ M. Dose-dependent aggregation curves were repeated in the absence and in the presence of VIP at final concentrations ranging from 10^{-8} to 10^{-6} M.

Analytical methods

TXB_2, 6-oxo-$PGF_{1\alpha}$ and PGE_2 were measured by specific radioimmunoassay (RIA) systems developed in our laboratory. Details of these RIA techniques and validation of

the measurements by comparison with other independent assay methods are described elsewhere (Ciabattoni *et al.*, 1979; Patrono *et al.*, 1982; 1985).

LTC_4-like immunoreactivity (LTC_4-LI) was measured by RIA with anti-LTC_4 serum AS1 (Preziosi & Ciabattoni, 1986). This antiserum was a gift from the Merck Frosst Canada. It showed a 43% cross-reactivity with LTD_4, 6% with LTE_4 and less than 1% with other prostanoids (Hayes *et al.*, 1983).

VIP-like immunoreactivity (VIP-LI) was measured with a recently developed RIA system, employing a specific polyclonal rabbit antiserum and ^{125}I-labelled VIP. The entire procedure has been described in detail by D'Amato *et al.* (1992).

Materials

The following compounds were used: VIP (porcine, Sigma), aprotinin (Kir, Richter), bacitracin (Lusofarmaco), ovalbumin (Merck), TTX (Sigma), indomethacin (Merck Sharp & Dohme), BWA4c (Wellcome), acetylcholine (Merck), U-46619 (The Upjohn Company), arachidonic acid (Fluka), [^{3}H]-TXB_2 and [^{3}H]-PGE_2 (New England Nuclear), [^{3}H]-6-oxo-$PGF_{1\alpha}$, [^{3}H]-LTC_4 and [^{125}I]-VIP (Amersham).

Statistical analysis

The results were evaluated by means of a parametric analysis of variance for multiple comparisons and Student's *t* test for single comparisons. Chi-squared test was used to compare means calculated on values expressed as a percentage of the basal. All values are reported as mean ± s.e.mean. $P \leqslant 0.05$ was considered statistically significant.

Results

Effects of VIP infusion

When infused into non-sensitized guinea-pig lungs, exogenous VIP caused a statistically significant, dose-dependent increase in the amplitude of the tracing of the pressure-volume variations recorded through the trachea. This bronchodilator response was associated with a dose-related decrease in thromboxane release (Figure 1). Inhibition of TXB_2 release seemed to be selective, since PGI_2 release, as reflected by its hydrolysis product 6-oxo-$PGF_{1\alpha}$, was unaffected by VIP infusion (Figure 1). The lipoxygenase inhibitor, BWA4c, did not modify the bronchodilator effects of VIP nor its inhibitory effect on TXB_2 release (Figure 1).

Antigen challenge and VIP and TXB_2 release from guinea-pig lung

VIP-like immunoreactivity was identified in perfusates of guinea-pig isolated and ventilated lungs under basal conditions as well as after antigen challenge (Table 1). Basal release averaged 0.96 ± 0.10 ng min^{-1} during the first 2 min and decreased to 0.58 ± 0.10 ng min^{-1} within the following 6 min, thereafter remaining stable up to 15–20 min in lungs that were refractory to ovalbumin challenge, i.e. those that did not undergo an anaphylactic reaction (Table 1). In these lungs TXB_2 release averaged 3.36 ± 0.63 ng min^{-1} ($n = 6$), and no change was observed after ovalbumin injection into the pulmonary artery (3.29 ± 0.78 ng min^{-1}). In descending order of magnitude, ovalbumin refractory lungs released $TXB_2 > 6\text{-oxo-}PGF_{1\alpha} > PGE_2$. No LTC_4-LI was detected under these conditions. The corresponding values are shown in Table 2.

Ovalbumin challenge induced a bronchoconstrictor res-

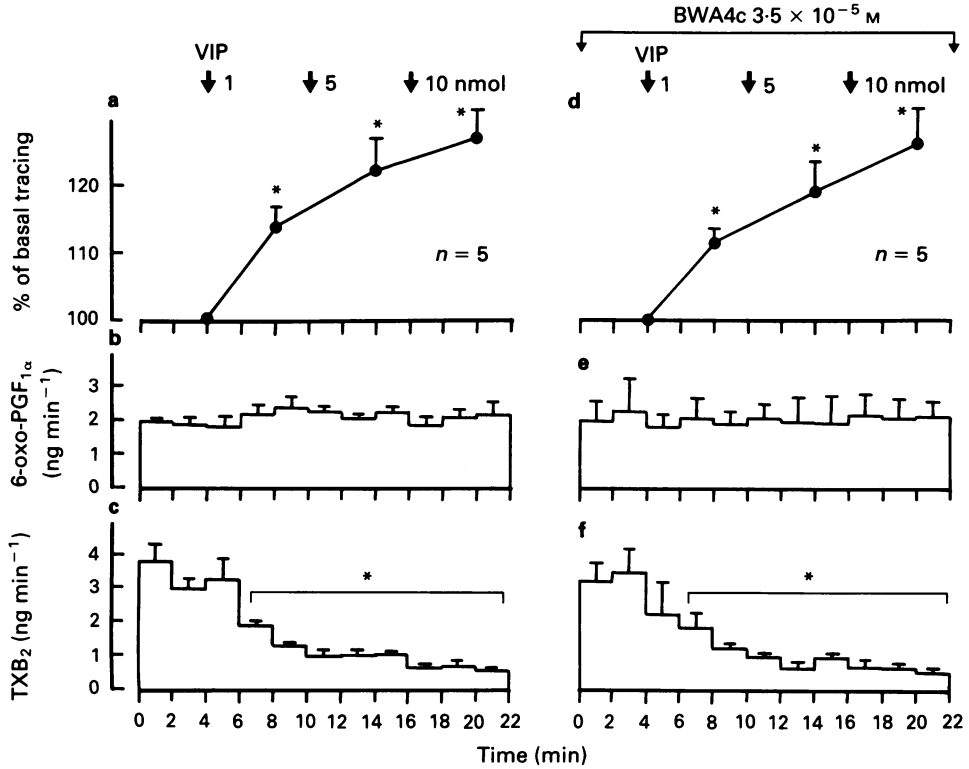


Figure 1 (a,b,c) Effects of vasoactive intestinal polypeptide (VIP) infusion into the pulmonary artery of guinea-pig isolated perfused and ventilated lung: (a) percentage of the basal tracing recording the pressure-volume variations in the lung; (b) and (c) 6-oxo-prostaglandin (PG) $F_{1\alpha}$ and thromboxane (TX) B_2 release in the perfusing medium. (d,e,f) The effects of VIP infusion on the same parameters after pretreatment with the lipoxygenase inhibitor BWA4c. *$P < 0.01$ vs basal.

Table 1 Vasoactive intestinal polypeptide (VIP) release from isolated, perfused and ventilated lung of the guinea-pig after antigen challenge

	VIP (ng min^{-1})	
	Before	*After*
Bronchoconstriction absent ($n = 6$)	0.58 ± 0.10	0.59 ± 0.11
Bronchoconstriction present ($n = 5$)	0.43 ± 0.07	2.10 ± 0.50*

*$P < 0.001$ vs basal release of 0.96 ± 0.10 ng min^{-1}.

ponse which did not resolve within the time course studied in 5 of the 11 sensitized lungs, as reflected by an 88.6 ± 1.4% reduction in the tracing of the pressure-volume variations measured through the trachea. In these lungs the bronchoconstriction was temporally accompanied by enhanced release of VIP (Table 1 and Figure 2) and the measured cyclo-oxygenase products, i.e. TXB_2 (3 fold increase), 6-oxo-$PGF_{1\alpha}$ (2 fold increase) and PGE_2 (2.6 fold increase: Figure 2 and Table 2). LTC_4-LI, which was unmeasurable under basal conditions, appeared after ovalbumin challenge (Table 2). VIP release as well as the release of all the measured eicosanoids was maximal in the collections performed during the reduction of the tracing recording the pressure-volume variations in the lung.

Effects of tetrodotoxin and indomethacin on VIP and TXB_2 release

Figure 2 shows the effects of TTX treatment on ovalbumin-sensitized lungs that underwent anaphylactic reaction after antigen challenge. Those lungs which were refractory to ovalbumin exposure were not considered. Basal VIP release was reduced by >80%, and the increase observed during the bronchoconstrictor response was completely abolished. However, the increase of TXB_2 release was unaffected by TTX treatment (Figure 2), and the decrease of the tracing of the pressure-volume variations in the lung observed after ovalbumin exposure was nearly identical to that observed in control experiments, as indicated by the slope and the plateau of the curve obtained by plotting the amplitude of this tracing as a function of time (Figure 3). In this graphical analysis the area under the continuous pen tracing was divided into segments of 12 s duration, and each segment was expressed as a percentage of the area covered in the same time under basal conditions, i.e. after equilibrations and before ovalbumin injection.

Indomethacin treatment almost abolished thromboxane synthesis and release under control conditions and prevented the increase observed after antigen challenge in lungs undergoing anaphylactic reaction (Figure 2). Moreover, although indomethacin did not modify basal release, it did prevent the increase in VIP observed during the bronchoconstrictor response (Figure 2). Indomethacin did not modify the maximal reduction of the pressure-volume variations induced in the lung as compared with controls (83.3 ± 5.6 vs 88.6 ± 1.4%), but it significantly ($P < 0.01$) modified the slope of the curve (Figure 3) by increasing the time required to achieve maximal bronchoconstriction from an average of 1.2 min in controls to 3.6 min after indomethacin treatment.

Effects of the 5-lipoxygenase inhibitor BWA4c on thromboxane and VIP release

Five of 12 sensitized lungs challenged with ovalbumin after BWA4c treatment underwent bronchoconstriction. In these

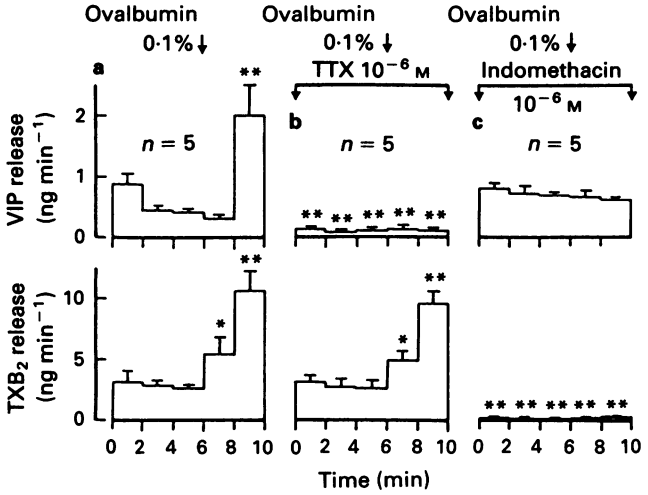


Figure 2 Vasoactive intestinal polypeptide (VIP) and thromboxane (TX) B_2 release in ovalbumin sensitized guinea-pig lungs undergoing anaphylactic bronchoconstriction after antigen challenge without any treatment (a) or after tetrodotoxin (TTX; b) or indomethacin (c) treatment. *$P < 0.01$ and **$P < 0.001$ vs basal of control experiments.

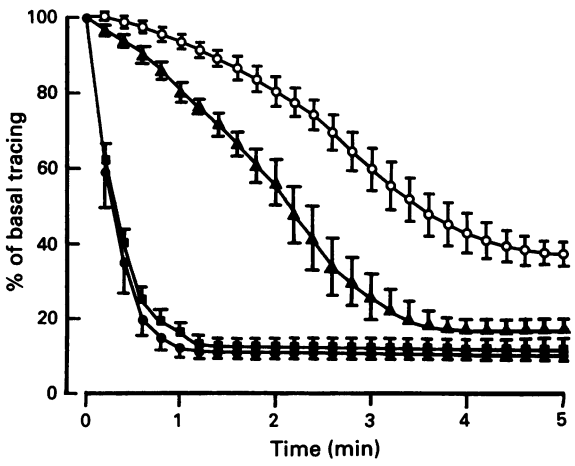


Figure 3 Pressure-volume variations in the perfused and ventilated guinea-pig lungs undergoing bronchoconstriction after antigen challenge: (●) control experiments ($n = 5$); (■) tetrodotoxin (10^{-6} M)-treated lungs ($n = 5$); (▲) indomethacin-treated lungs (10^{-6} M; $n = 5$); (○) VIP-treated lungs (10^{-8} M; $n = 5$).

Table 2 Eicosanoid release from isolated perfused and ventilated lung of guinea-pig after antigen challenge

	TXB_2 (ng min^{-1})	6-oxo-$PGF_{1\alpha}$ (ng min^{-1})	PGE_2 (ng min^{-1})	LTC_4-LI (ng min^{-1})
Bronchoconstriction absent ($n = 6$)	3.29 ± 0.78	1.95 ± 0.49	0.68 ± 0.12	<0.3
Bronchoconstriction present ($n = 5$)	10.68 ± 1.33*	3.96 ± 0.85*	1.81 ± 0.31*	5.06 ± 0.45*

*$P < 0.01$ vs basal

Table 3 Effects of acetylcholine (ACh) infusion into the pulmonary artery of perfused and ventilated lung of the guinea-pig

	VIP (ng min^{-1})	*TXB_2* (ng min^{-1})	*% reduction of the basal tracing*
Basal	0.61 ± 0.10	3.30 ± 0.13	0
ACh 10^{-6} M	0.66 ± 0.05	2.88 ± 0.10	21.2 ± 3.5*
ACh 5×10^{-6} M	0.57 ± 0.02	2.76 ± 0.16	52.1 ± 4.4**
ACh 10^{-5} M	0.59 ± 0.04	2.89 ± 0.20	85.3 ± 5.4**

The amplitude of the pen tracing recording pressure-volume variations in the lung was measured after equilibration and at the maximal effects produced by each dose of ACh. Numbers are means ± s.e.mean of 4 experiments.
*$P<0.01$ vs basal; **$P<0.001$ vs basal.

lungs the 5-lipoxygenase inhibitor did not modify the basal release of TXB_2 or VIP (2.90 ± 0.43 and 0.55 ± 0.09 ng min^{-1}, respectively), but significantly ($P<0.01$) blunted the increase of both compounds observed during the bronchoconstrictor response (4.42 ± 0.85 and 0.82 ± 0.16 ng min^{-1}, respectively). Moreover, BWA4c significantly attenuated the maximal reduction of the pressure-volume variations induced in the lung as compared with controls (72.9 ± 4.1 vs 88.6 ± 1.4%, $P<0.05$) and increased the time required to achieve the maximal bronchoconstriction from an average of 1.2 min in controls to 3.8 min after BWA4c.

Effects of exogenous VIP on the bronchoconstriction and the release of cyclo-oxygenase metabolites following ovalbumin challenge

Perfusion with exogenous VIP (10^{-8} M) attenuated the maximal reduction of the tracing recording the pressure-volume variations in the lung as compared with controls (63.9 ± 7.0 vs 88.6 ± 1.4%; $P<0.01$) and reduced the slope of the curve (Figure 3) by increasing the time required to achieve maximal bronchoconstriction from an average of 1.2 min in controls to 4.5 min after VIP pretreatment. This effect was even more evident than after indomethacin or BWA4c treatment. TXB_2 release was 1.78 ± 0.27 ng min^{-1} immediately before ovalbumin challenge and increased to 3.15 ± 0.68 ng min^{-1} in those lungs which underwent bronchoconstriction. Both basal release and the increase after antigen challenge were significantly lower than in controls ($P<0.05$). 6-oxo-$PGF_{1\alpha}$ and PGE_2 release was 1.82 ± 0.30 and 0.53 ± 0.05 ng ml^{-1}, respectively, before ovalbumin challenge. They increased to 2.56 ± 0.42 and to 0.92 ± 0.07 ng min^{-1}, respectively, during the bronchoconstrictor response. However, the percentage increase, although statistically significant ($P<0.05$), resulted significantly lower than in untreated lungs ($P<0.05$).

Effects of acetylcholine

When infused into the pulmonary artery of non-sensitized lungs in the concentration range 10^{-6}–10^{-5} M, acetylcholine induced a dose-dependent, reversible decrease of the tracing of the pressure-volume variations in the lung (Table 3). However, despite a degree of bronchoconstriction at the highest dose, substantially identical to that observed after anaphylactic reaction, no statistically significant changes in VIP or TXB_2 release were observed (Table 3).

Effects of thromboxane analogue U-46619

The TXA_2-mimetic, U-46619, in the dose range 0.1–1.0 nmol, infused over 1 min into the pulmonary artery, caused a dose-dependent decrease of the tracing of the pressure-volume variations in the lung, together with a significant increase in VIP release. The results are detailed in Figure 4. TXB_2 levels were not determined because of the high cross reactivity of U-46619 with anti-TXB_2 serum in the RIA system.

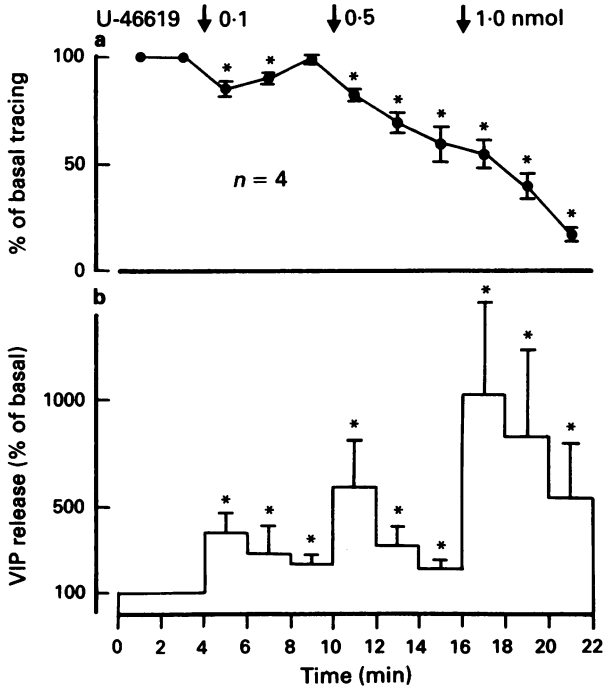


Figure 4 Percentage of the basal tracing recording the pressure-volume variations in the lung (a), and vasoactive intestinal polypeptide (VIP) release; (b) after infusion into the pulmonary artery of the endoperoxide PGH_2 analogue U-46619 in the dose-range 0.1–1.0 nmol. *$P<0.01$ vs basal.

Effects of VIP on platelet aggregation and thromboxane synthesis

VIP inhibited arachidonic acid (AA)-induced platelet aggregation in a dose-dependent manner over a dose-range 10^{-8}–10^{-6} M. A 28.3 ± 2.0% inhibition of maximal aggregation was achieved at a final concentration of 10^{-8} M VIP and 68.3 ± 5.7% at 10^{-6} M. Despite the antiaggregatory effect of VIP, TXB_2 levels in the supernatant collected after platelet aggregation were only marginally reduced by 12–20% ($P<0.01$; $n=12$). A nearly identical reduction of PGE_2 was observed (12.0 ± 4.0%–20.6 ± 1.9% at the same VIP concentrations). However, within the range of VIP concentrations used, no clear dose-response could be established between the reduction in TXB_2 or PGE_2 levels and VIP concentrations. Moreover, no redirection of arachidonate cyclo-oxygenation from TXB_2 to PGE_2 synthesis was observed.

Discussion

VIP relaxes airway smooth muscle *in vitro* in several species, including man, and represents the most potent endogenous

bronchodilator thus far discovered (Said, 1984; Palmer *et al.*, 1986). Previous studies have reported that VIP inhibits the release of TXA_2 induced by LTD_4 and bradykinin from guinea-pig isolated perfused lung (Conroy *et al.*, 1988a,b; 1992). In addition, these studies reported that VIP also inhibited the release of PGE_2 and PGI_2. Similarly Berisha *et al.* (1990) have shown that the release of several cyclo-oxygenase metabolites induced by oxidant injury was inhibited by VIP. Our study confirms, at least in part, these findings, because we have shown that exogenous VIP infusion into the pulmonary artery of isolated, perfused and ventilated guinea-pig lung inhibits the antigen-induced release of TXB_2, 6-oxo-$PGF_{1\alpha}$ and PGE_2. However, our findings are not in complete agreement with those of Conroy *et al.* (1992) who showed that the basal release of the measured cyclo-oxygenase metabolites was unaffected by VIP. In contrast, we found that when infused in non-sensitized lungs VIP causes a progressive, dose-related decrease of TXB_2 release into the perfusion medium. This effect is concomitant with a dose-related relaxation of bronchial smooth muscle and seems to be selective for thromboxane, since the relase of 6-oxo-$PGF_{1\alpha}$ was unmodified. Similarly, pre-treatment with VIP before ovalbumin challenge of sensitized lungs decreased the basal release of TXB_2, but not that of 6-oxo-$PGF_{1\alpha}$ or PGE_2. Discrepancies with the data of Conroy *et al.* (1992) might be explained on the basis of differences between the two experimental models. In our study, lung ventilation represents a mechanical stimulus which could increase to some extent the basal release of TXB_2.

Sulphidopeptide leukotrienes are known to stimulate TXA_2 synthesis in guinea-pig lung and it has recently been shown that VIP inhibits leukotriene synthesis and release from guinea-pig lung (Beaubien *et al.*, 1984; Samhoun *et al.*, 1987; Di Marzo *et al.*, 1989). Therefore, we have tested the effect of VIP infusion in the presence of the lipoxygenase inhibitor BWA4c at a dose that produces a nearly maximal inhibition of this enzyme *in vitro* (Payne *et al.*, 1988; Higgs *et al.*, 1988). Lipoxygenase inhibition did not modify the bronchodilator response nor the reduction in thromboxane release induced by VIP, suggesting that VIP directly affects TXA_2 synthesis in guinea-pig lung. This is not surprising, since the levels of LTC_4-LI found in the perfusate of lungs not exposed to ovalbumin or not undergoing anaphylactic reaction after antigen challenge were below the picogram detection limit of the assays used (Ciabattoni & Preziosi, 1985; Preziosi & Ciabattoni, 1986; 1987).

VIP has been proposed as a modulator of lung inflammation and airway constriction because of its ability to inhibit inflammatory cell function, antagonize major humoral mediators of inflammation and attenuate acute oedematous lung injury in several experimental models (Berisha *et al.*, 1990). We identified immunoreactive VIP in the perfusate of isolated, perfused and ventilated guinea-pig lung under basal conditions and found that antigen challenge causes an approximate five fold increase in VIP release in those lungs undergoing persistent bronchoconstriction after exposure to ovalbumin. Previous studies have characterized the pattern of arachidonate metabolite release by anaphylactic guinea-pig lung and identified TXB_2 as the most abundant product followed, in descending order of magnitude, by 6-oxo-$PGF_{1\alpha}$ and PGE_2 (Robinson *et al.*, 1984). A platelet source of TXA_2 was excluded by pretreating animals with aspirin 24 h before they were killed (Preziosi & Ciabattoni, 1986). Moreover, we have previously reported the presence of LTC_4-LI in the perfusates of anaphylactic guinea-pig lungs by means of two anti-LTC_4 sera showing a different cross-reactivity with LTD_4 and LTE_4 (Ciabattoni & Preziosi, 1985; Preziosi & Ciabattoni, 1986). The data presented in this study confirm these previous observations. However, because the cross-reactivity of our antisera with LTD_4 and LTE_4 is high, we cannot discriminate among the mixture of sulphidopeptide leukotrienes which may be present in lung perfusate.

TXA_2 plays an essential role in the bronchospasm that accompanies the anaphylactic reaction, as demonstrated by studies with selective TX-synthase inhibitors (Preziosi & Ciabattoni, 1986) and by the effect of indomethacin pretreatment which delays the development of bronchoconstriction. However, because indomethacin does not block the 5-lipoxygenase pathway of arachidonic acid metabolism, leukotriene synthesis is unaffected and a final bronchoconstriction results as an effect of these substances, once termed 'slow reacting substance of anaphylaxis'. In the present study we found increased VIP release that was concomitant with a massive increase in TXA_2 synthesis and release. This enhanced VIP release appears to be quite specific for an inflammatory reaction such as anaphylaxis, since acetylcholine infusion in non-sensitized lungs failed to increase either VIP or thromboxane release, despite a bronchoconstriction similar to (although reversible) that observed in anaphylactic lungs.

The neuronal origin of the VIP in lung perfusates was confirmed by TTX treatment, which inhibited by $>80\%$ the basal VIP release. TTX also abolished the increased VIP release observed during the anaphylactic reaction, without affecting thromboxane release or the bronchoconstrictor response. On the other hand, cyclo-oxygenase inhibition achieved by pretreating the lungs with indomethacin, reduced TXB_2 release by $>90\%$, delayed the bronchoconstrictor response and blunted the increase in VIP release during anaphylaxis, without modifying basal VIP release. Failure of TTX treatment to modify the bronchoconstrictor response seems feasible, since it inhibits the release of a bronchodilator substance. The observed effects should be an increased bronchoconstriction, but because this response after ovalbumin challenge is maximal, it is hard to suppose, although theoretically possible, a shift of the curve of Figure 3 toward the left.

To explore the hypothesis that thromboxane acts on NANC neurones by releasing VIP, we tested the effects of the TXA_2 mimetic U-46619 (Coleman *et al.*, 1981). This compound is a stable analogue of prostaglandin H_2, the precursor of TXA_2, and an agonist at TXA_2/PGH_2 receptor(s), which exists in many tissues (see Halushka *et al.*, 1989 for a review). As expected, U-46619 caused a dose-dependent bronchoconstrictor response. At the highest dose (1.0 nmol) the bronchoconstriction did not resolve within the time course studied and was comparable to that induced by lung anaphylaxis. Similarly, in a dose-related fashion, U-46619 increased VIP release up to ten fold. As no change in the release of this peptide was observed with comparable increases in airway resistance induced by acetylcholine, we might conclude that the enhanced VIP release is a consequence of the interaction of U-46619 with the TXA_2/PGH_2 receptor rather than a non-specific response to bronchoconstriction.

The selective inhibition of thromboxane synthesis and release induced by VIP infusion could be interpreted on the basis of an inhibition of the TX synthase, i.e. the enzyme converting PGH_2 into TXA_2. An inhibition of cyclo-oxygenase can be excluded because the synthesis and release of other prostaglandins, such as PGI_2, was unaffected. To answer this question, we investigated the effects of exogenous VIP on *in vitro* platelet TXA_2 synthesis. This model represents the most abundant source of TX-synthase, and redirection of arachidonate synthesis toward other prostanoids may be achieved with selective TX-synthase inhibitors (Patrignani *et al.*, 1984). The effects of VIP on *in vitro* platelet aggregation induced by AA are consistent with the reported ability of VIP to inhibit PAF-induced platelet aggregation and secretion (Cox *et al.*, 1984). However, despite a substantial reduction in the platelet aggregating capacity, the inhibition of TXB_2 release occurred only to a minor extent, not closely dose-related and without any evidence of a redirection of arachidonate metabolism toward other cyclo-oxygenase products. This finding can exclude an inhibitory effect of VIP on TX synthase.

Pretreatment with the 5-lipoxygenase inhibitor, BWA4c, was effective in reducing the release of TXB_2 and VIP from guinea-pig lung following ovalbumin challenge. It also delayed the development of bronchoconstriction and attenuated the extent of the final response. These data suggest a primary release of sulphidopeptide leukotrienes in lung anaphylaxis. Leukotrienes, on the other hand, enhance TXA_2 synthesis and release, which, in turn, might stimulate the release of VIP. Such a view could explain not only the partial protection of BWA4c against bronchoconstriction because of reduction in leukotriene release, but also the observed inhibition of both TXB_2 and VIP release after antigen challenge.

In recent studies on isolated, perfused lungs of rat and guinea-pigs, VIP has been shown to reduce all signs of acute lung injury caused by a series of experimental inflammatory injuries (Berisha *et al.*, 1988; 1990; Pakbaz *et al.*, 1988; 1990). Possible mechanisms by which VIP may reduce or prevent acute lung injury include the inhibition of inflammatory cell function and inhibition of the generation of inflammatory mediators, including eicosanoids (see Said, 1991a,b for reviews). In the present study the administration of exogenous VIP attenuated the bronchoconstriction following ovalbumin challenge to an extent even more evident than indomethacin or BWA4c. This effect was comcomitant with the reduction in the release of the measured cyclo-oxygenase metabolites. Recently, Trotz & Said (1991) have shown that VIP can inhibit phospholipase A_2 activity, i.e. the enzyme which releases arachidonic acid from membrane phospholipids. Also from the data of Conroy *et al.* (1992) it appears that VIP acts at a step preceding cyclo-oxygenase activation. Therefore, it is reasonable to suppose that the observed inhibitory effect of VIP on cyclo-oxygenase metabolite release as well as on antigen-induced bronchoconstriction might be the consequence of a reduced availability of arachidonic acid.

A possible explanation of the selective inhibition of thromboxane release by VIP could be represented by the cellular origin of the former. If we exclude platelets, an important source is represented by mononuclear cells, mast cells and leukocytes, i.e. cells that mediate and modulate inflammation. VIP binds to specific receptor sites on these cells and prevents the release of several inflammatory mediators (see Said, 1991a for a review). TXA_2 in guinea-pig lung might be included among these mediators.

In conclusion, these data suggest that VIP plays a complex role in regulating bronchial reactivity in lung anaphylaxis. A protective role against the activity of bronchoconstrictor mediators released by the immunological reaction is strongly suggested both by the increase in VIP release during lung anaphylaxis and by its ability to inhibit the synthesis and release of TXA_2. The latter, on the other hand, seems to enhance neuronal release of VIP. Thus, a balance might exist between these two opposite mediators in regulating bronchial smooth muscle reactivity in guinea-pig lung. In this balance, VIP might have a role as an endogenous modulator of both bronchoconstriction and pulmonary inflammation.

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Effects of the κ-opioid agonist U50,488 on parturition in rats

A.J. Douglas, *G. Clarke, *S.J.A. MacMillan, P.M. Bull, ‡I. Neumann, †S.A. Way, **D.M. Wright, B.G. McGrory & [1]J.A. Russell

Department of Physiology, University of Edinburgh, Teviot Place, Edinburgh EH8 9AG; *Department of Anatomy, University of Bristol, BS8 1TD; †Department of Anatomy, Downing Street, Cambridge CB2 3DY; ‡Department of Neurobiology, University of Leipzig, Leipzig, Germany and **Brighton Upjohn Clinical Research Unit, Brighton General Hospital, Elm Grove, Brighton BN2 3EW

1 The effects of the κ-opioid agonist U50,488 on parturition were studied in the rat.

2 Given directly after the birth of the second pup U50,488 (5 mg or 10 mg kg^{-1}, i.p.) delayed the birth of the subsequent 4 pups by ca. 100 min, acting like morphine (10 mg kg^{-1}, i.p.). In controls given the vehicle i.p., the birth of the 4 pups after treatment took 45.4 ± 4.6 min. The effects of U50,488 could be prevented by simultaneous naloxone injection (10 mg kg^{-1}). Injection of either U50,488 or morphine at 1 mg kg^{-1}, i.v. also significantly delayed parturition. The effects of U50,488 but not of morphine were fully prevented by preinjection with nor-binaltorphimine (0.5 mg kg^{-1}, i.v.) showing selective κ-opioid receptor-mediated inhibition by U50,488 of established parturition.

3 In rats with an indwelling jugular venous cannula, i.v. injection of U50,488 (5 mg kg^{-1}) after the birth of the second pup slowed parturition in a similar way to i.p. injection and significantly reduced blood plasma oxytocin concentration measured by radioimmunoassay compared with vehicle-injected controls.

4 Bolus i.v. injections of oxytocin (4 mu once per 5 min) significantly reduced the delay in parturition caused by i.v. U50,488, but continuous i.v. infusion of oxytocin (4 mu 5 min^{-1}) was less effective.

5 Since i.v. oxytocin did not immediately reverse the effects of U50,488 on parturition, direct effects of U50,488 on isometric uterine contractions *in vitro* were sought. U50,488 inhibited spontaneous or oxytocin-stimulated contractions of uteri from rats within 24 h after parturition in a dose-related manner; the inhibitory effect was not naloxone-reversible.

6 Thus U50,488 inhibited established parturition in the rat in a κ-opioid selective manner by reducing oxytocin secretion. The inhibitory effect may well have been potentiated by a direct non-opioid depressant action on contractile activity of the uterus.

Keywords: Morphine; naloxone; opiates; oxytocin; uterine contractility

Introduction

There are κ-opioid receptors (Herkenham *et al.*, 1986) on oxytocin-containing nerve terminals, and the κ-opioid agonist, U50,488 inhibits oxytocin secretion from the posterior pituitary *in vivo* and *in vitro* (Coombes & Russell, 1988; Zhao *et al.*, 1988) by a naloxone-reversible action (Coombes & Russell, 1988). In addition there are κ-opioid receptors in the supraoptic and magnocellular paraventricular nuclei where the cell-bodies of oxytocin neurones projecting to the posterior pituitary gland are located (Mansour *et al.*, 1988), and U50,488 inhibits their firing-rate both *in vivo* and *in vitro* (Russell *et al.*, 1989b; Leng *et al.*, 1990). Since the firing-rate of these neurones is the major determinant of oxytocin secretion, U50,488 would be expected to inhibit oxytocin secretion by a combination of actions on the terminals and cell-bodies of oxytocin neurones.

Oxytocin is secreted during suckling and U50,488 is known to inhibit the milk-ejection reflex, a process dependent on oxytocin (Clarke & Wright, 1989). Another major function of oxytocin is to promote the progress of parturition by stimulating uterine contractions (Higuchi *et al.*, 1986). The secretion of oxytocin during parturition is under tonic opioid inhibitory restraint since naloxone increases oxytocin secretion and accelerates parturition (Leng *et al.*, 1988). Similarly injection of the μ-opioid agonist, morphine, interrupts parturition and inhibits oxytocin secretion (Russell *et al.*, 1989a), acting centrally on the firing-rate of oxytocin neurones but not on the posterior pituitary (Wakerley *et al.*, 1983; Coombes & Russell, 1988; Pumford *et al.*, 1991). We have now studied the effects of the κ-agonist U50,488 on the time-course of parturition, and on oxytocin secretion during parturition in rats. In addition we have compared the effects of U50,488 with morphine and have investigated reversing the effects of U50,488 by the opioid antagonist naloxone, by the selective κ-opioid antagonist, norbinaltorphimine (nor-BNI) (Portoghese *et al.*, 1989) or by boosting the plasma levels of oxytocin. We have also studied the effects of U50,488 on spontaneous and oxytocin-stimulated uterine contractions *in vitro*.

Some of the data in the present paper have been briefly reported elsewhere (Clarke *et al.*, 1990; Douglas *et al.*, 1990).

Methods

Wistar (for i.p. injection experiments) or Sprague-Dawley rats were caged either individually with a proven fertile male to identify day zero of pregnancy by the appearance of a vaginal plug, or were caged in groups with a proven male and day zero identified by the appearance of spermatozoa in vaginal smears. Pregnant females were then caged individually in our standard conditions (Russell *et al.*, 1989a). The rats were observed continually during the lights-on phase from day 21 of pregnancy, and the time of birth of the first and each subsequent pup was noted. The number of live pups in each litter was counted approximately 1 h after completion of delivery of the litter.

Effects of U50,488 on parturition and reversibility by naloxone or norbinaltorphimine (norBNI)

Immediately after the birth of pup 2, the dams were given a mid-line intraperitoneal injection of either U50,488 (5 mg

[1] Author for correspondence.

kg^{-1} or 10 mg kg^{-1}), or vehicle (0.9% saline), or naloxone (10 mg kg^{-1}) or U50,488 (10 mg kg^{-1}) plus naloxone (10 mg kg^{-1}) or morphine (10 mg kg^{-1}); some rats were untreated. Drugs were diluted so that the injection volume was ca. 1 ml.

In a second experiment, to test lower doses of opioids and reversibility by the κ-selective antagonist, norbinaltorphimine (norBNI), early on day 20 of pregnancy rats were anaesthetized with ether and a cannula was inserted into a jugular vein and led subcutaneously to the back of the neck and exteriorized (see Russell *et al.*, 1991); the cannula was occluded with a stainless steel wire, withdrawn the next day when the cannula was connected via a length of tubing to a syringe for later drug injection without disturbance during parturition. Intravenous injections were given 1 and 6 min after the birth of the second pup as follows: either vehicle (0.9% saline 0.5 ml kg^{-1}), then vehicle again, or vehicle then U50,488 (1 mg kg^{-1}), or norBNI (0.5 mg kg^{-1}) then U50,488 (1 mg kg^{-1}) or vehicle then morphine (1 mg kg^{-1}), or norBNI (0.5 mg kg^{-1}) then morphine (1 mg kg^{-1}).

Effects of U50,488 on oxytocin secretion during parturition

Early on day 21 of pregnancy the rats were anaesthetized with ether and a cannula was inserted into a jugular vein and led subcutaneously to the back of the neck, exteriorized and connected via tubing to a heparinized syringe (see Russell *et al.*, 1991) thereby allowing intermittent blood-sampling with minimal further disturbance to the conscious animal. A first blood sample of 0.3 ml was taken into a heparinized syringe 2 h after completion of surgery, chilled on ice, centrifuged and the plasma separated, frozen and stored at −20°C prior to subsequent radioimmunoassay for oxytocin. The blood cells were re-suspended in 150 μl of 0.9% saline and re-infused. Further blood samples were taken and replaced in the same way on the birth of pup 2 and, at 5, 10, 20, 30, 60, 90 and 120 min later. Immediately following the blood-sample taken 5 min after the birth of pup 2, either U50,488 (5 mg kg^{-1}) or vehicle (0.5 ml kg^{-1}) was injected intravenously.

Effects of intravenous injections or infusion of oxytocin after U50,488 injection during parturition

A further group of 21 day pregnant rats were cannulated as described above for injections only. At the birth of pup 2, an i.v. injection of U50,488 (5 mg kg^{-1}) was given to all the rats. Then either one of the three following treatments was given intravenously: (1) a series of bolus injections of oxytocin (4 mu in 30 μl 0.9% saline) was given every 5 min for 90 min; (2) vehicle only was injected on the same regime; (3) a continuous infusion of oxytocin was started, by use of a slow-infusion pump (Braun, Perfusor VI, see Russell *et al.*, 1989a) modified to drive a 1 ml plastic syringe and infusing 4 mu oxytocin in 26.5 μl 0.9% saline per 5 min for 90 min.

Effects of U50,488 on parturient rat uterine contractile activity in vitro

Rats which had completed delivery within the preceding 12 h, but had not been given any treatment were killed by stunning and cervical dislocation and the uterine horns were removed. Each horn was halved transversely and a piece was suspended by one end from a force transducer with the other end fixed, the tissue being totally immersed in a 50 ml bath of de Jalon's solution gassed with 95% O_2/5% CO_2, kept at 37°C by a thermostatically-regulated water-jacket (see Russell *et al.*, 1989a, for full details). Each piece of uterus was tensioned to 10 g and contractile activity recorded isometrically on a flat-bed chart-recorder (see Russell *et al.*, 1989a). Three experiments were performed on each uterine horn with washes in between. The uterine horns were allowed to establish a stable pattern of contractile activity for 1 h and the activity recorded in the 10 min prior to each experiment was taken as the control. In the first experiment U50,488 was added to the medium in increasing doses at 10 min intervals (5.16, 10.3 and 15.5 × 10^{-6} M), followed by oxytocin (1.4 and 2.8 u l^{-1} 10 min apart, equivalent to 2.8 and 5.6 × 10^{-9} M).

For the second experiment oxytocin alone was added as above (1.4 then 2.8 u l^{-1}). In the third experiment U50,488 was added again (for each horn at the dose almost fully suppressing activity in the first experiment, 1.03 or 1.55 × 10^{-5} M), then after 10 min naloxone was added in three equal doses, 10 min apart (3.3 × 10^{-5} M each). Finally, 10 min after the last naloxone dose oxytocin was added for 10 min (5.6 × 10^{-9} M).

Drugs

U50,488 (*trans*-(±)-3,4-dichloro-N-methyl-N-(2-[1-pyrrolidinyl]cyclo-hexyl) benzene-acetamide methane sulphonate) was either purchased from Sigma or was a gift from Upjohn Laboratories; naloxone HCl was purchased from Sigma; norBNI, norbinaltorphimine (17,17′-*bis*(cyclopropylmethyl)-6,6′, 7,7′tetradehydro 4,5,4′5′-diepoxy-6,6′(imino)[7,7′-bimorphinan] -3,3′,14,14′tetroldihydrochloride) was purchased from Semat; the oxytocin used was Syntocinon (Sandoz Pharmaceuticals), and morphine sulphate, B.P. was used. For i.p. or i.v. injection the opioids were diluted in 0.9% sterile saline so that the injection volume was 0.5 ml kg^{-1}. Oxytocin for i.v. injection and i.v. infusion was diluted to 133.3 mu ml^{-1} and 151 mu ml^{-1} 0.9% sterile saline, respectively. For the *in vitro* experiments drugs were diluted in de Jalon's solution so that the volume added to the bath was 100 μl for each dose.

Oxytocin radioimmunoassay

The assay described by Higuchi *et al.* (1986) was used. Duplicate 50 μl plasma aliquots were assayed; all samples from the experiment were measured in the same assay. Sensitivity was 1 pg/tube, and intra-assay variation was 11.2%.

Statistical analysis

Data were analysed parametrically by analysis of variance (ANOVA), followed by Duncan's multiple range test to compare individual groups, or by Student's *t* test. Paired comparisons were made with paired *t* tests.

Results

U50,488 and parturition

After i.p. injection of U50,488 (5 or 10 mg kg^{-1}) or of morphine (10 mg kg^{-1}) following the birth of pup 2, the birth of the next three pups was significantly delayed (one-way ANOVA, $P<0.01$, Figure 1) with no difference between U50,488 at 5 and 10 mg kg^{-1}. The time from the birth of pup 6 to pup 8 was not significantly different between the vehicle and U50,488-treated groups (12.3 ± 1.8 min, $n=8$ vs. 19.65 ± 4.0 min, $n=20$), indicating recovery from the effects of U50,488 after ca. 100 min, and consequently the slopes of curves relating time to birth number were similar after pup 6 (Figure 1). Recovery from the delaying effect of morphine was similar (Figure 1).

When naloxone (10 mg kg^{-1}) was injected 1 min prior to the U50,488 it completely prevented the effects of U50,488 (Figure 1). When given alone, naloxone appeared to increase the rate of births compared with i.p. vehicle injection (NS, Figure 1), and the time to deliver pups 3 to 5 (25.4 ± 3.8 min, $n=15$) was even shorter in rats that were undisturbed (Figure 1).

When U50,488 was given intravenously at the lower dose of 1 mg kg^{-1} it significantly delayed parturition compared to vehicle-injected controls (Figure 2a, ANOVA, $P<0.05$ at pup 5), an effect clearly smaller than that seen with U50,488 at 5 or 10 mg kg^{-1} given i.p. or at 5 mg kg^{-1}, i.v. (Figures 1,

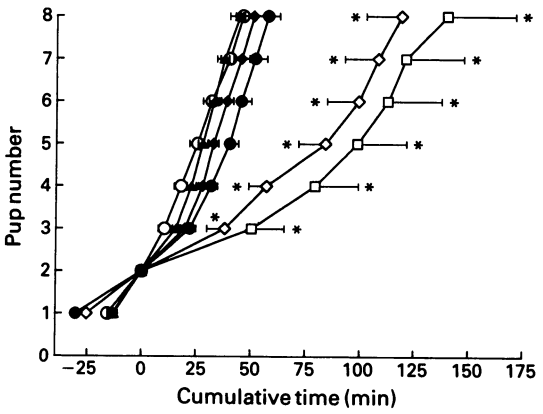


Figure 1 Effects of U50,488 on parturition, reversal by naloxone and comparison with morphine. Data are mean ± s.e.mean cumulative times to the births of successive pups after pup 2 when either no injection was given (○, $n = 15$), or vehicle (●, $n = 8$), or naloxone (10 mg kg^{-1}, ▲, $n = 8$), or U50,488 (at either 5, $n = 9$; or 10, $n = 11$, mg kg^{-1}; data combined, ◇, $n = 20$), or naloxone (10 mg kg^{-1}) plus U50,488 (10 mg kg^{-1}, ◆, $n = 10$), or morphine (10 mg kg^{-1}, □, $n = 9$) was administered by intraperitoneal injection. Statistical comparisons: *$P < 0.05$ vs. uninjected controls, and after pup 4 vs. vehicle-injected controls, Duncan's multiple range test.

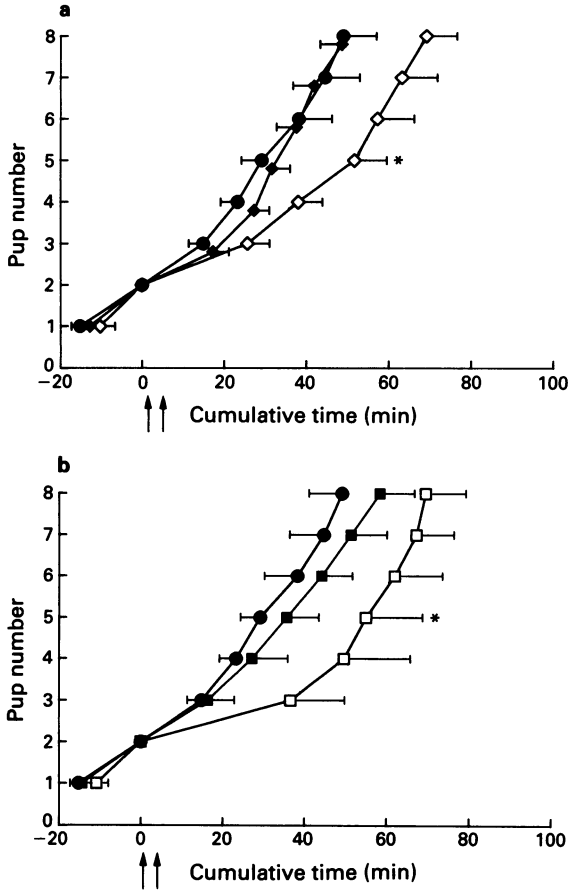


Figure 2 Effects of U50,488 or morphine on parturition and reversal by norbinaltorphimine (norBNI). Data are mean ± s.e.mean cumulative times to the births of successive pups after pup 2 when one of 5 regimens was administered i.v. with 5 min between drugs (arrows). (a) U50,488 alone or with norBNI. Treatments: (1) vehicle + vehicle (●, $n = 9$), (2) vehicle + U50,488 (1 mg kg^{-1}, ◇, $n = 9$), (3) norBNI (0.5 mg kg^{-1}) + U50,488 (1 mg kg^{-1}, ◆, $n = 6$). Statistical comparisons: *$P < 0.05$, vs. vehicle + vehicle and norBNI + U50,488, Duncan's multiple range test. (b) Morphine alone or with norBNI. Treatments: (1) vehicle + vehicle (●, $n = 9$, same as in (a)), (2) vehicle + morphine (1 mg kg^{-1}, □, $n = 5$), (3) norBNI (0.5 mg kg^{-1}) + morphine (1 mg kg^{-1}, ■, $n = 6$). Statistical comparisons: *$P < 0.05$, vs. vehicle + vehicle, Duncan's multiple range test.

2 and 3). The time for the birth of the three pups following administration of U50,488 at 1 mg kg^{-1} (51.7 ± 7.9 min, $n = 9$) was significantly greater than in vehicle injected controls (29.2 ± 4.9 min, $n = 9$), $P < 0.05$, Duncans) but the time for birth of the next three pups was not different compared to vehicle indicating rapid recovery from this dose (17.3 ± 3.6 min and 19.9 ± 3.6 min, respectively), so the delay due to U50,488 was about 22 min. Administration of norBNI (0.5 mg kg^{-1}) 5 min before U50,488 completely prevented the delaying effect of U50,488 (Figure 2a, at pup 5, ANOVA, $P < 0.05$).

Morphine at 1 mg kg^{-1} had a similar delaying effect on births to U50,488 at 1 mg kg^{-1} (Figure 2b, at pup 5, ANOVA $P < 0.05$), the main effect occurring within 40 min following treatment. NorBNI (0.5 mg kg^{-1}) appeared partially to antagonize the morphine (Figure 2b) but there was no significant difference at any pup between the vehicle + morphine and the norBNI + morphine groups.

U50,488 and oxytocin secretion during parturition

U50,488 at 5 mg kg^{-1} given by i.v. injection following the birth of pup 2, delayed parturition compared to i.v. vehicle (Figure 3, $P < 0.01$, t test), an effect similar to that of the high dose of U50,488 given i.p. previously (Figure 1). The time to birth of the first 3 pups after treatment was greater in the U50,488 group (140.3 ± 29.4 min, $n = 10$ vs. 52.3 ± 6.4 min, $n = 9$, $P < 0.05$, Student's t test), but the time for the birth of the next three pups (34.7 ± 8.9 min, $n = 10$) was not different compared to vehicle (30.2 ± 5.7 min, $n = 9$). Thus, U50,488 delayed the completion of parturition in this experiment by ca. 90 min.

Plasma oxytocin concentration was increased at the time of the birth of pup 2 compared with the post-surgery, pre-parturition sample (Figure 4, $P < 0.01$, all values, paired t test), but had fallen to the pre-parturition value 5 min later just before U50,488 or vehicle was injected ($P < 0.05$). In the control group, plasma oxytocin increased after i.v. vehicle and remained elevated compared with the pre-parturition value throughout the 2 h sampling period, which coincided with the duration of parturition (Figures 3 and 4). After i.v. injection of U50,488 plasma oxytocin concentration fell significantly by 5 min after injection (= time 10 min in Figure 4) and remained significantly less than in the control group ($P < 0.05$) throughout the 2 h sampling period, and was even significantly less than the pre-parturition value at 30 and 90 min.

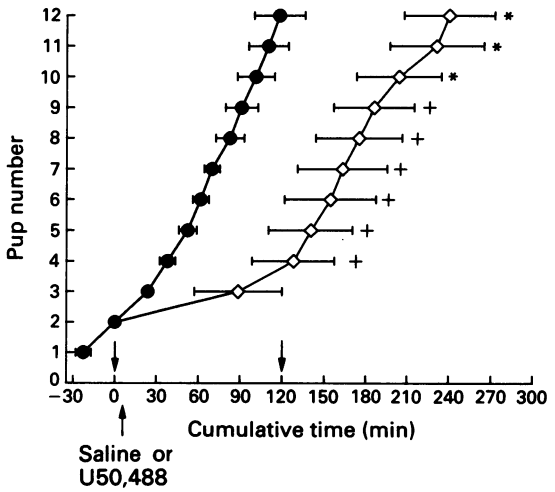


Figure 3 Effects of intravenous (i.v.) U50,488 on parturition in conscious rats with a jugular venous cannula for blood-sampling. The mean ± s.e.mean cumulative times to the births of successive pups after i.v. treatment on the birth of pup 2 are shown; either vehicle (●, $n = 9$) or U50,488 (5 mg kg^{-1}, ◇, $n = 10$) was injected (upward arrow). The downward arrows indicate the start and finish of blood-sampling (see Figure 4). Statistical comparisons: †$P < 0.05$; *$P < 0.01$ vs. control (Student's t test).

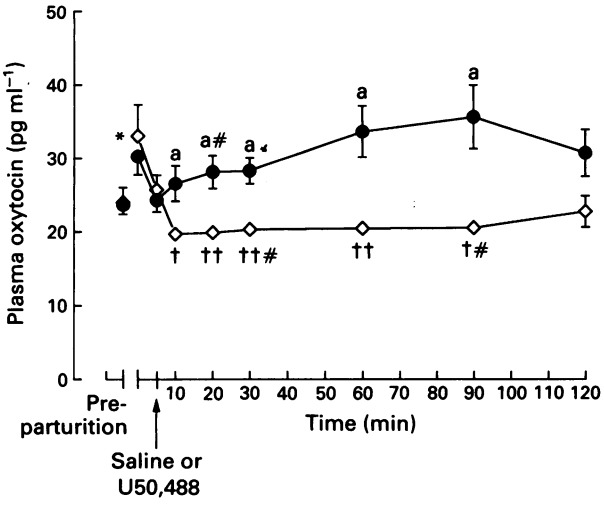


Figure 4 Effects of intravenous (i.v.) U50,488 on plasma oxytocin concentration during parturition. Values are mean ± s.e.mean. Either vehicle (●, $n = 8$) or U50,488 (5 mg kg^{-1}, ◇, $n = 9$) was injected 5 min following the birth of pup 2 (see Figure 3) arrow. Statistical comparisons: $^{*}P < 0.05$ (combined data, paired t test) vs. pre-parturition and pre-injection values; $\#P < 0.05$ vs. value on injection (paired t test); $^{a}P < 0.05$ vs. pre-parturition value (paired t test); $\dagger P < 0.05$, $\dagger\dagger P < 0.01$ vs. control value at same time (Student's t test).

U50,488 and oxytocin replacement during parturition

The time-course of parturition in the rats given i.v. U50,488 on the birth of pup 2 and then bolus i.v. vehicle injections for 90 min was similar to that in the U50,488-treated rats in the above experiments (Figures 1, 3 and 5). Oxytocin given by bolus injections to rats treated with U50,488 significantly hastened parturition compared with U50,488 and bolus saline injections but the effect was slow in onset and was only apparent 30 to 50 min following treatment (Figure 5). During the 90 min period of i.v. injections or infusion the number of pups born within the first 60 min was not significantly different between the treatment groups but by 90 min the number of pups born was greater in the group given bolus oxytocin injections (7.1 ± 1.3 pups) than in the bolus saline group (1.1 ± 0.9 pups, one-way ANOVA, $P < 0.01$; Duncan's test, $P < 0.05$). Although oxytocin was effective whether infused or given as a series of bolus injections, the continuous infusion of oxytocin reversed the effect more slowly (Figure 5).

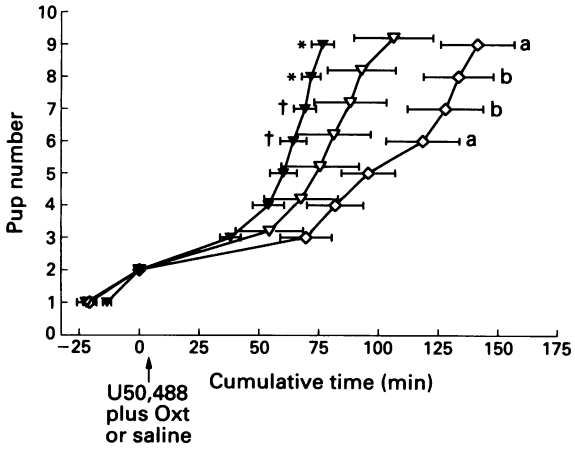


Figure 5 Effects of intravenous (i.v.) oxytocin on parturition retarded by U50,488. Mean ± s.e.mean cumulative times to the births of successive pups after i.v. treatment following the birth of pup 2 are shown; all rats were given, i.v. U50,488 (5 mg kg^{-1}) followed by either bolus i.v. vehicle injections (30 µl) at 5 min intervals for 90 min (arrow), (◇, $n = 8$, to pup 9) or bolus i.v. injections of oxytocin (Oxt 4 mu) at 5 min intervals for 90 min (▼, $n = 8$, to pup 10), or continuous i.v. infusion of oxytocin (4 mu 5 min^{-1}) for 90 min (▽, $n = 7$, to pup 9). Statistical comparison: $^{*}P < 0.01$; $\dagger P < 0.05$ (ANOVA); $^{a}P < 0.05$ (Duncan's multiple range test) compared to U50,488 + Oxt pulses; $^{b}P < 0.05$ vs. both U50,488 + Oxt pulses and U50,488 + Oxt infusion (Duncan's).

Pup viability

Assessed ca. 1 h after completion of delivery, pup viability (mean ± s.e.mean) in the i.v.-treated groups given saline or U50,488 and blood-sampled was 97.1 ± 2.3% ($n = 11$) and 80.1 ± 7.0% ($n = 11$) respectively ($P < 0.05$, U-test), and in the groups given i.v. U50,488 and bolus saline injections or bolus oxytocin injections or continuous oxytocin infusion was 73.7 ± 7.6% ($n = 9$), 83.7 ± 6.9% ($n = 8$) and 78.7 ± 11.3% ($n = 7$), respectively (NS).

Uterine activity in vitro

Contractile activity was decreased in a dose-related manner by U50,488 with almost total suppression of activity at a cumulative dose of 15.5 to 31 × 10^{-6} M (Figures 6 and 7), arising mostly from decreased mean contraction amplitude (reduced dose-dependently from 0.028 ± 0.005 to 0.016 ± 0.003, 0.004 ± 0.001 and 0.002 ± 0.001 N, $n = 6$, $P < 0.01$,

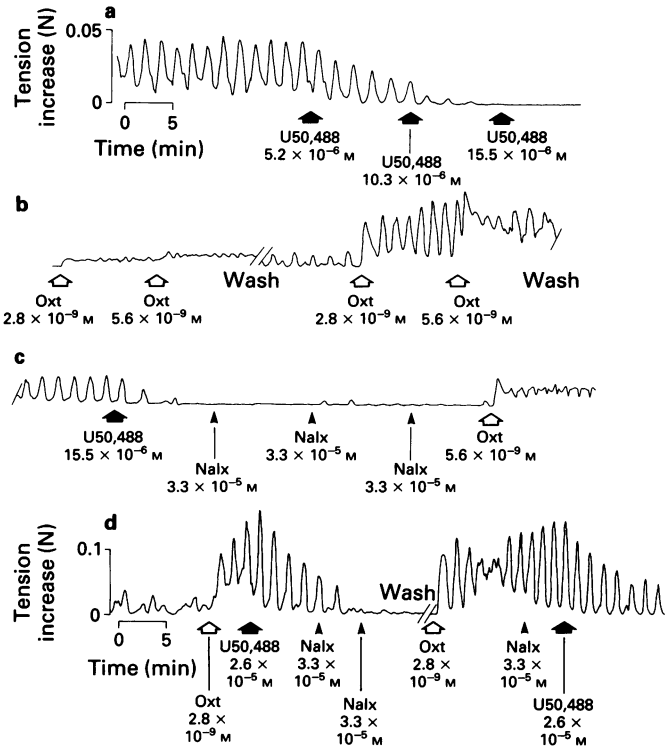


Figure 6 Effects of U50,488 on the contractile activity of parturient rat uterus *in vitro*. (a), (b), (c) Typical continuous recordings of tension developed by half uterine horn from a rat <12 h post partum; group results are shown in Figure 7. (a) Stable basal activity was established over 1 h. Increasing doses of U50,488 (large solid arrows) progressively inhibited contractile activity. (b) Addition of oxytocin (Oxt, open arrows) stimulated activity marginally; washout of the drugs (Wash: 10 min gap in record), restored spontaneous contractions; further oxytocin strongly stimulated activity. (c) After washout a single dose of U50,488 completely suppressed activity; addition of naloxone (Nalx arrow heads) had no effect on the inhibition by U50,488, and subsequent oxytocin was less effective than a lower dose in the absence of U50,488 (b). (d) Uterine contractile activity from another parturient rat (typical of four similar experiments). Oxytocin was added first, but U50,488 still suppressed contractile activity, and was not reversed by naloxone; after washout, contractile activity was again stimulated by oxytocin, and U50,488 still inhibited activity even if naloxone was added first.

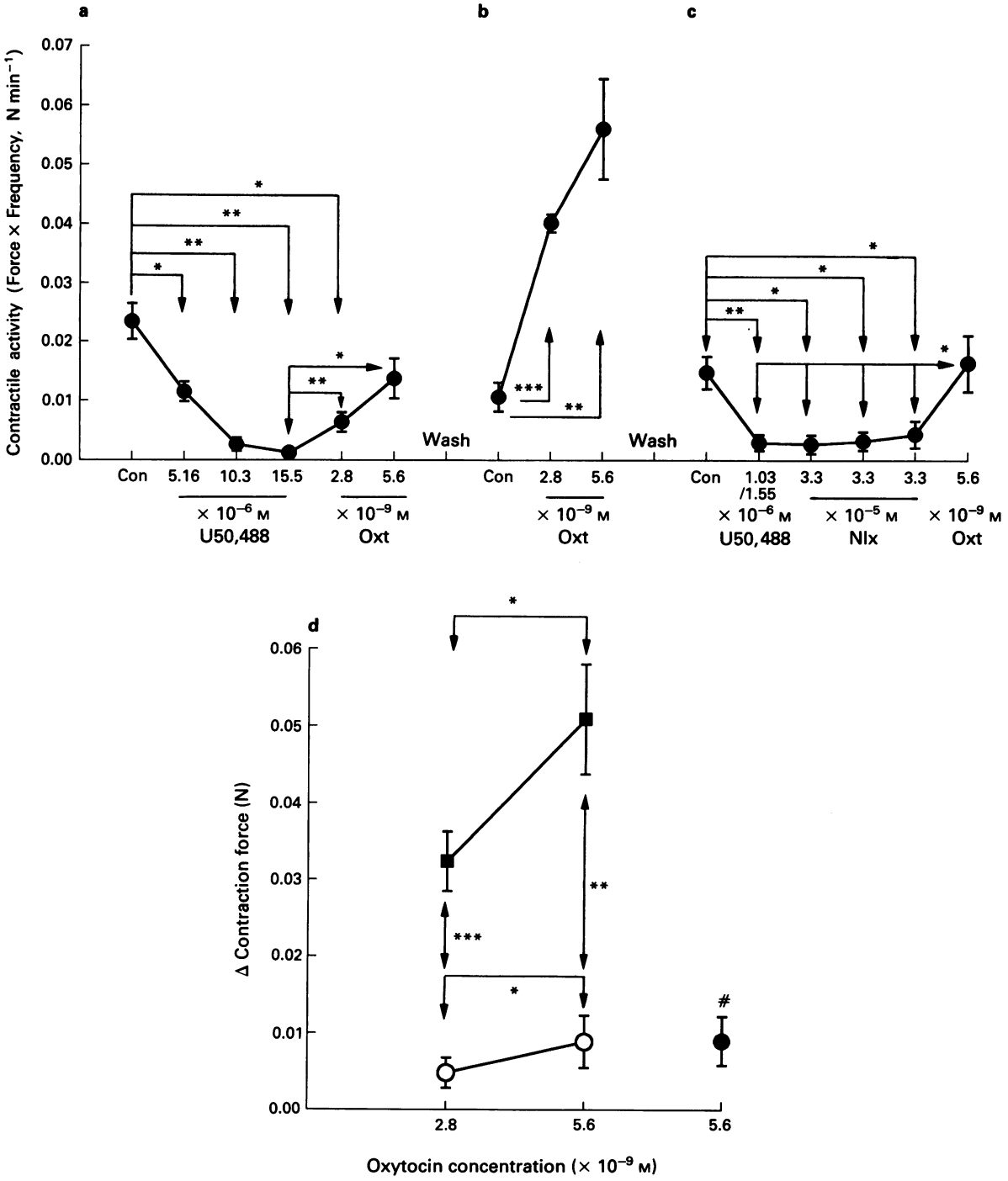


Figure 7 Quantitation of effects of U50,488 on the contractile activity of parturient rat uterus *in vitro*. (a,b,c) Contractile activity was calculated from records of experiments illustrated in Figure 6 (a,b,c) as the product of the force of each contraction (using force during maximal inhibition by U50,488 as baseline) and frequency of contractions (per min) in either the last 10 min of the basal period (Con) or in the last 7 of 10 min after each drug addition. Oxt: oxytocin; Nlx: naloxone. Values are mean ± s.e.mean, $n = 6$ rats, same uteri for each treatment. Statistical comparisons (paired t tests): $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$. (d) Increases in force of contractions after oxytocin. Values are mean ± s.e.mean within-animal differences between mean force of contractions after vs. before oxytocin, measured over the same times as above; $n = 6$ rats, same uteri for each treatment. (○) Oxytocin added after three doses of U50,488 (total 3.1×10^{-5} M, see Figure 6a and (a) above); (■) oxytocin added after washout of previous drugs (see Figure 6b, and (b) above); (●) oxytocin added after U50,488 ($1.03/1.55 \times 10^{-5}$ M) and naloxone (total 9.9×10^{-5} M). Statistical comparisons (paired t test): $^{*}P<0.05$; $^{**}P<0.005$; $^{***}P<0.001$; $\#P<0.01$ vs. low-dose oxytocin without drugs.

paired t test), the frequency of contractions only being significantly decreased after the highest dose of U50,488 (from 0.76 ± 0.071 to 0.32 ± 0.14 contractions per min, $P<0.05$, $n = 6$). Subsequent doses of oxytocin increased contractile activity during maximal inhibition by U50,488 (Figures 6 and 7), however, none of the variables was greater after U50,488 plus oxytocin than during the initial control period without oxytocin (Figures 6b and 7b). After washout of the drugs, oxytocin strongly stimulated contractile activity (Figures 6 and 7) due to a dose-related increase in amplitude of individual contractions (to 0.040 ± 0.003 N and 0.059 ± 0.006 N, respectively, $P<0.001$), with a reduction in frequency (to 0.93 ± 0.061 contractions per min at the highest dose, $P<0.05$). The dose-response relations for oxytocin in the absence or presence of U50,488 were non-parallel (Figure 7d).

Up to a 6.4 fold molar excess of naloxone had no significant effect on the inhibition of contractile activity by U50,488 (Figures 6c and 7c), and subsequent addition of oxytocin did not increase contractile activity above pre-drug control activity. In other experiments neither oxytocin nor naloxone in any combination were able to prevent inhibition of contractile activity by U50,488 (Figure 6).

Discussion

U50,488 is generally considered to be a relatively selective κ-agonist (Clarke & Pasternak, 1988), acting on the postulated κ_1 sub-type receptor (Clarke *et al.*, 1989), and morphine though less selective preferentially acts as a μ-opioid agonist (Kosterlitz, 1985). The results clearly show a transient, inhibitory action of U50,488 on the progress of established parturition that was dose-related (from 1 to 10 mg kg^{-1}) and similar to the delaying effect of morphine on parturition shown previously and confirmed in this study (Figures 1, 2a, b, Russell *et al.*, 1989a). While selectivity of U50,488 and morphine for κ- and μ-type receptors respectively may be less complete at the high doses, selectivity is expected at the low dose (1 mg kg^{-1}) used in this study (Figure 2). Since both U50,488 and morphine still delayed parturition similarly at this dose, this indicates that U50,488 was acting by κ-receptors and morphine by μ-receptors, and further that both κ- and μ-opioid receptors can mediate interruption of parturition.

Prevention by naloxone of the delaying action of U50,488 on parturition indicates that U50,488 acted via opioid receptors. The delaying action of morphine on parturition has similarly been previously shown to be prevented by naloxone (Russell *et al.*, 1989a). Naloxone is a very effective opioid antagonist but though it shows greater affinity for μ-type opioid receptors, it is also effective at κ-receptors (Kosterlitz, 1985) and is thus of limited usefulness in discriminating actions at the types of opioid receptor. NorBNI is a selective κ-antagonist (Portoghese *et al.*, 1988) and this was used to examine further whether U50,488 was acting at κ-receptors. Consequently, the complete prevention by norBNI of the delaying action of U50,488 on parturition does strongly indicate that U50,488 acted via κ-receptors. Conversely, the lack of a significant effect of norBNI on the delay of parturition by a low dose of morphine further indicates that μ-receptors mediate at least part of the inhibitory action of morphine; nonetheless the delay of parturition by the low dose of morphine appeared to be less after norBNI. This could be because morphine was partly acting via κ-receptors, or because norBNI was acting indirectly, antagonizing endogenous opioids. The former is unlikely, since at these doses norBNI does not antagonize the inhibition by morphine of the electrical activity of oxytocin neurones (unpubl.), and morphine alone does not inhibit oxytocin secretion from the posterior pituitary where there are only κ-receptors (Herkenham *et al.*, 1986; Coombes & Russell, 1988).

Although opioid antagonists prevented the delaying actions of U50,488 on parturition, naloxone alone has previously been shown to antagonize endogenous opioids, promoting parturition by increasing oxytocin secretion and antagonizing inhibitory effects of environmental disturbance (Leng *et al.*, 1988; Summy Long, 1989; Neumann *et al.*, 1991). In view of the previously reported actions of naloxone during parturition the reversal by naloxone of U50,488-induced inhibition of pup births (Figure 1) may have been due not only to antagonism of the opiate actions of U50,488 but may have included the effects of increased oxytocin secretion arising from antagonism of endogenous opioids (Bicknell *et al.*, 1988; Summy Long, 1989). The apparent (though non-significant) partial reversal of the effects of morphine by norBNI (Figure 2b) may also be attributed to its actions against endogenous opioids, especially at oxytocin nerve terminals in the posterior pituitary outside the blood brain barrier. Antagonism by norBNI of the delaying effects of U50,488 on parturition could similarly involve antagonism of endogenous opioid actions.

Plasma oxytocin concentration was increased early in parturition and it remained significantly elevated for 90 min as previously reported (Russell *et al.*, 1991), supporting the view that circulating oxytocin has an important role in propelling parturition (Higuchi *et al.*, 1986). Plasma oxytocin concentration fell quickly after U50,488 administration, and remained low (even less than pre-parturition values) for 90 min. There are two obvious mechanisms by which oxytocin secretion could be reduced by U50,488 during parturition. The first is direct inhibition of posterior pituitary oxytocin secretion; it is now well-established that κ-opioid mechanisms mediate opioid inhibition of oxytocin secretion at the level of oxytocin nerve terminals in the posterior pituitary (Bicknell & Zhao, 1989), and U50,488 acts at this site both *in vivo* and *in vitro* (Coombes & Russell, 1988; Zhao *et al.*, 1988). In addition, κ-type opioid receptors predominate in the supraoptic and paraventricular nuclei (Mansour *et al.*, 1988), although μ-receptors are also present (Sumner *et al.*, 1992), and κ-opioids inhibit the firing of oxytocin cell-bodies both directly *in vitro* or after intravenous administration (Russell *et al.*, 1989; Leng *et al.*, 1990; Pumford *et al.*, 1992) probably by actions within or close to the magnocellular supraoptic nuclei, acting both pre- and post-synaptically on oxytocin neurones themselves (McFadzean *et al.*, 1987; Inenaga *et al.*, 1990).

The second way in which oxytocin secretion could be reduced would be by inhibition of the excitatory neural input arising in the uterine cervix, and ascending via the spinal cord by incompletely described pathways to the magnocellular oxytocin neurones, establishing a positive feedback loop to promote oxytocin secretion as foetuses are propelled through the birth canal (Ferguson, 1941; Summerlee, 1981; Higuchi *et al.*, 1986). U50,488 acting on the spinal cord for example, could inhibit excitatory input to oxytocin neurones, as has been described for part of the inhibitory actions of opiates on the milk-ejection reflex (Wright, 1985). A direct inhibitory action of U50,488 on the contractile activity of the uterus would have the same result of removing a stimulus (e.g. the Ferguson reflex) to oxytocin secretion, but could also render oxytocin less effective (see below). In addition, since oxytocin has been shown to be synthesized in the uterus during pregnancy (Lefebvre *et al.*, 1992) the inhibitory action of U50,488 on uterine contractile activity may interfere with local mechanisms involving possible paracrine actions of the endogenous uterine oxytocin. To test the primary importance of inhibition of oxytocin secretion into the blood, the experiments involving oxytocin infusion were carried out. During suckling there is substantial evidence that oxytocin is secreted in discrete pulses every few minutes, and this leads to transient high circulating concentrations of oxytocin and to sharp increases in intramammary pressure, which are efficient for expelling milk (Higuchi *et al.*, 1986). There is evidence that oxytocin is also secreted into the blood in pulses every few minutes during parturition (Summerlee, 1981; Higuchi *et al.*, 1986; Summerlee *et al.*, 1989); in the present study, oxytocin concentration was higher in the sample which was taken on the birth of the second pup than in the sample taken 5 min later, indicating that the birth of a foetus coincides with, and may trigger secretion of a pulse of oxytocin (Higuchi *et al.*, 1986; Summerlee, 1989). In addition, there is a steady increase in oxytocin secretion during parturition (Higuchi *et al.*, 1986), also measured in the present study. To try to distinguish the effectiveness of the pulsatile vs. gradual increase patterns, oxytocin was given at the same average rate after U50,488 in the present study either as boluses (in a pattern expected to result from the synchronized bursting firing pattern of oxytocin neurones, Summerlee, 1989) or continuous infusion. The results showed first, that the depression of oxytocin secretion by U50,488 can account for a major part of the effects of U50,488 on parturition, and this

indicates that the uterus can respond *in vivo* to oxytocin after U50,488 treatment. Secondly, the greater effectiveness of bolus oxytocin injection (Figure 5) confirms that pulsatile secretion of oxytocin into the blood is important during normal parturition (Higuchi *et al.*, 1986; Summerlee, 1989).

However, oxytocin reversal of the U50,488 inhibition of parturition was not as rapid as expected, compared with vehicle-injected controls not given U50,488, even though the resulting plasma oxytocin concentrations can be estimated to be at least as great as those measured during parturition (Russell *et al.*, 1989a). Contractile activity of uterus from rats which had recently delivered their litters was strongly inhibited *in vitro* by U50,488, tested in doses intended to be similar to those in extracellular fluid after systemic administration and the inhibition was reversible by washing. U50,488 inhibited not only spontaneous contractile activity but also strongly attenuated, in a non-competitive way, the stimulatory action of oxytocin. Since this inhibitory action of U50,488 was not reversed by naloxone applied in excess, to take account of the relatively high and low affinities of the agonist and antagonist at κ-receptors respectively (Kosterlitz, 1985), then U50,488 did not inhibit uterine contractions via opioid receptors. U50,488 has been described as having non-opioid, inhibitory actions on smooth muscle (Hayes *et al.*, 1988) and this could explain the effects on uterine contractile activity that we found. In previous studies we have found that pethidine, a μ-selective opiate with anticholinergic properties, inhibits parturition and inhibits oxytocine secretion, but it also inhibits uterine contractile activity in a naloxone non-reversible way (Russell *et al.*, 1991). Morphine, also a μ-selective opiate, likewise inhibits parturition and oxytocin secretion in a naloxone reversible way, but it has no actions on uterine contractile activity (Russell *et al.*, 1989a). Therefore, it is likely that the slow reversal of the inhibition of pup births by exogenously administered oxytocin is related to the initial high concentration of U50,488 inhibiting the uterus directly.

Perinatal pup viability was reduced by i.v. U50,488 regardless of whether i.v. oxytocin was given to reverse the delay of parturition; conversely, we have found previously that the interruption of parturition by intracerebroventricular morphine does not reduce immediate perinatal pup viability (Russell *et al.*, 1989a), so the delay *per se* seems not to be the explanation. The expression of maternal behaviour is strongly inhibited by morphine given during parturition and pup survival is reduced (Russell *et al.*, 1989a). Morphine also inhibits established maternal behaviour in lactating rats (Russell & Spears, 1984) by a central action on μ-receptors, but intracerebroventricular U50,488 does not disrupt maternal behaviour (Mann *et al.*, 1991). Direct depressant effects on the pups either of U50,488 after trans-placental transfer or of the reduced uterine contractility and thus prolonged labour are a possibility.

The present study thus shows that a selective κ-opioid agonist can interrupt established parturition in the rat, and this involves inhibition of oxytocin secretion into the blood with the possibility of direct non-opioid inhibition of uterine contractility. The results further support an important role for pulsatile oxytocin secretion in parturition and indicate that actions of opiates on oxytocin secretion and on uterine responsiveness to oxytocin should be considered in assessing the usefulness of opiates as obstetric analgesics.

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A non-peptide NK_1-receptor antagonist, RP 67580, inhibits neurogenic inflammation postsynaptically

[1]S.M. Moussaoui, F. Montier, A. Carruette, J.C. Blanchard, P.M. Laduron & C. Garret

Rhone-Poulenc Rorer, Centre de Recherche de Vitry-Alfortville, 13, Quai Jules Guesde, 94403 Vitry-sur-Seine cedex, France

1 The non-peptide neurokinin NK_1-receptor antagonist, RP 67580 (3a**R**, 7a**R**), a perhydroisoindolone derivative, powerfully reduced plasma extravasation in rat hind paw skin induced by local application of xylene ($ID_{50} = 0.03$ mg kg^{-1}, i.v.) or capsaicin ($ID_{50} = 0.06$ mg kg^{-1}, i.v.), or by i.v. injection of exogenous substance P (SP) or septide ([pGlu^6,Pro^9]SP(6–11)) ($ID_{50} = 0.04$–0.05 mg kg^{-1}, i.v.). RP 67580 (1 mg kg^{-1}, i.v.) also abolished capsaicin-induced nasal fluid hypersecretion (by 82 ± 5%). These effects were found to be stereospecific, the enantiomer, RP 68651 (3a**S**, 7a**S**), being inactive at 1 mg kg^{-1}, i.v.

2 In rats neonatally treated with capsaicin (50 mg kg^{-1}, s.c.), plasma extravasation induced by SP was significantly increased (by 43 ± 7%). RP 67580 (1 mg kg^{-1}, i.v.) completely inhibited the SP-induced plasma extravasation in capsaicin neonatally treated-animals, as it did in control animals. This result suggests that RP 67580 acts at the postsynaptic level for the inhibition of plasma extravasation.

3 Opioid receptor agonists, μ-(morphine) and κ-(PD-117302) at 10 mg kg^{-1}, s.c., in contrast to NK_1-receptor antagonists, did not inhibit plasma extravasation induced by exogenous SP. They were, however, partially effective against plasma extravasation induced by electrical nerve stimulation (74 ± 4% and 48 ± 9% inhibition at 10 mg kg^{-1}, s.c. of morphine and PD-117302, respectively, compared to 90 ± 3% inhibition obtained with RP 67580, 3 mg kg^{-1}, s.c.). These results indicate the presynaptic action of opioid receptor agonists, in contrast to the postsynaptic action of NK_1-receptor antagonists for the inhibition of plasma extravasation.

4 Ligature of the saphenous nerve distal to the point of electrical stimulation, local application of lignocaine to the saphenous nerve, neonatal capsaicin pretreatment, and colchicine at very low doses (120 μg kg^{-1} day^{-1} given for 3 days) were found to prevent plasma extravasation elicited by electrical nerve stimulation.

5 The foregoing results demonstrate that the non-peptide NK_1-receptor antagonist, RP67580, is a potent inhibitor of plasma extravasation induced in skin by NK_1-receptor agonists, by local application of chemical irritants (capsaicin or xylene) or by electrical nerve stimulation. Moreover, opioid receptor agonists and colchicine inhibit plasma extravasation induced by electrical nerve stimulation but not that elicited by exogenous SP. Therefore, it is possible to inhibit neurogenic inflammation either at the presynaptic level with opioid receptor agonists and colchicine, or at the postsynaptic level with NK_1-receptor antagonists, and that the new non-peptide NK_1-receptor antagonists may have a great potential for alleviation of inflammation in various pathological syndromes in man.

Keywords: Non-peptide NK_1-receptor antagonists; opioid receptor agonists; colchicine; neurogenic inflammation; NK_1-receptors; substance P

Introduction

Neurogenic inflammation is a complex process involving vasodilatation, plasma protein extravasation and oedema. It can be elicited by antidromic stimulation of the unmyelinated C-fibres of sensory nerves or by topical application of chemical irritants, such as capsaicin, mustard oil and xylene (Jancso *et al.*, 1967; Chahl & Ladd, 1976; Lembeck & Holzer, 1979; Lynn & Shakhanbeh, 1988). If rats were treated neonatally with high doses of capsaicin, a substance that selectively destroys C-fibres (Nagy *et al.*, 1983), neurogenic inflammation cannot subsequently be elicited by stimulation (Gamse *et al.*, 1980). Therefore, C-fibres are directly involved in the inflammatory process. In 1953, Lembeck suggested that substance P (SP) is the primary afferent neurotransmitter of inflammation. Subsequent studies demonstrated the accuracy of this proposal: SP along with neurokinin A (Dalsgaard *et al.*, 1985) was found in the C-fibres of sensory neurones (Hökfelt *et al.*, 1975), indicating that it may play a role in neurogenic inflammation. Moreover, the application of SP to the skin, both in rodents and man, elicits local oedema and plasma extravasation, thus mimicking the vascular responses to antidromic nerve stimulation (Hägermark *et al.*, 1978; Gamse *et al.*, 1980; Foreman & Jordan, 1984; Foreman, 1987; 1988; Pedersen-Bjergaard *et al.*, 1991). The afferent sensory neurones containing SP were found to control vascular permeability in numerous tissues other than the skin, such as the air passages, nasal mucosa, meninges, eyes, urinary bladder and joints (Moskowitz *et al.*, 1979; Bill *et al.*, 1979; Saria *et al.*, 1983; Lundberg *et al.*, 1983; 1984; Pernow, 1985; Helme *et al.*, 1985; Lam & Ferrell, 1991).

There is good evidence that SP induces neurogenic inflammation via NK_1-receptors, whereas NK_2- and NK_3-receptors, for which neurokinin A and neurokinin B, respectively, are considered to be the preferential agonists (Regoli *et al.*, 1987), do not seem to be involved (Andrews *et al.*, 1989; Abelli *et al.*, 1991). Therefore, if SP is the main mediator of neurogenic inflammation, selective and potent NK_1-receptor antagonists potentially represent a new therapeutic approach to various pathologies. Recently, RP 67580, a perhydroisoindolone derivative, and CP-96,345, a quinuclidine derivative, were reported to be non-peptide NK_1-receptor antagonists that both act competitively on NK_1-receptors (Garret *et al.*, 1991; Snider *et al.*, 1991). Moreover, RP 67580 (3a**R**, 7a**R**) and CP-96,345 were reported to be antinociceptive agents in rodents (Garret *et al.*, 1991; Yamamoto &

[1] Author for correspondence.

Yaksh, 1991; Moussaoui *et al.*, 1992a). We previously showed that RP 67580, but not its enantiomer RP 68651 (3a**S**, 7a**S**), inhibits plasma extravasation induced in rat skin by electrical stimulation of the saphenous nerve (Garret *et al.*, 1991), and induced by NK_1-receptor agonists in rat urinary bladder (Moussaoui *et al.*, 1992a). We thought these NK_1-receptor antagonists could be useful in extending our understanding of the role of SP and NK_1-receptors in physiological and pathological processes. In the present work, we examined the importance of NK_1-receptors in plasma extravasation in the rat paw and on nasal mucus production in the air ways.

Methods

Male Wistar rats (200–300 g, Charles River Laboratories, St Aubin lès Elbeuf, France) were used in all experiments except in the test of xylene-induced plasma extravasation. They were anaesthetized with sodium pentobarbitone (40 mg kg^{-1}, i.p.). In the experiments on xylene-induced plasma extravasation, male Sprague-Dawley hairless rats (220–260 g, Iffa Credo, L'Arbresle, France) were used and anaesthetized with urethane (1.10 g kg^{-1}, i.p.). The administration time for each drug was decided on the basis of preliminary results of pilot experiments (see below).

Plasma extravasation induced by electrical nerve stimulation (neurogenic inflammation)

Saphenous nerves were stimulated electrically (25 V, 2 ms, 10 Hz, duration 15 min) according to the procedure of Lembeck & Holzer (1979). Plasma extravasation was visualized with Evans blue as previously described (Garret *et al.*, 1991). The inhibitory effect of drugs was calculated by comparison with the effect of vehicle, and ID_{50} was determined by regression analysis (graphic method).

(±)-CP-96,345 (racemate) was given i.v. 5 min before the saphenous nerve was stimulated. RP 67580 (3a**R**, 7a**R**) and opioid receptor agonists were administered s.c. 30 min before electrical nerve stimulation. Lignocaine (20 µl of a 2% solution) was applied directly to the saphenous nerve 2 min before electrical stimulation. Colchicine (120 µg kg^{-1} day^{-1}) was administered i.p. and Evans blue extravasation, induced by electrical nerve stimulation, was evaluated in rats (treated for 1, 2 or 3 consecutive days, 4 h) after the last administration of colchicine. Capsaicin (50 mg kg^{-1}) or its vehicle (100 µl of saline containing 10% ethanol and 10% Tween 20) was given s.c. to 2-day-old rats, and 2 months later, the male rats (200–250 g) were used for neurogenic inflammation studies. In additional experiments, the saphenous nerve was ligated; its proximal part was stimulated and plasma extravasation was determined as previously described.

Plasma extravasation induced by intravenous injection of peptide agonists

Plasma extravasation was induced in hind paw skin by NK_1-receptor agonists, exogenous SP or septide (1 µg kg^{-1}, i.v.), and was determined by the method described by Saria *et al.* (1983). A simultaneous injection of NK_1-receptor agonist (1 µg kg^{-1}, i.v.) and Evans blue (30 mg kg^{-1}, i.v.) was given 5 min after i.v. injection of RP 67580 or RP68651; 30 min after s.c. injection of RP 67580, morphine or PD-117 302; or 4 h after the last administration of colchicine. Colchicine and capsaicin pretreatments were performed as described above.

Plasma extravasation induced by topical application of capsaicin or xylene to skin

Capsaicin was applied topically to the shaven skin of one hind paw, with a paper patch that had been soaked with 50 µl of a 7.5 mg ml^{-1} capsaicin solution in olive oil. The corresponding area of the other hind paw, used as a control, was covered with a patch soaked with olive oil alone. Test drug and Evans blue were administered i.v. 5 min before capsaicin was applied. Thirty minutes later, the animals were killed and the skin under the patches was removed and weighed and its dye concentration was assayed. In the test of xylene-induced plasma extravasation, a mixture of meta-xylene/ethanol (1:1) was applied directly to the skin of one hind paw, and vehicle of xylene (10 µl of a mixture of 0.9%

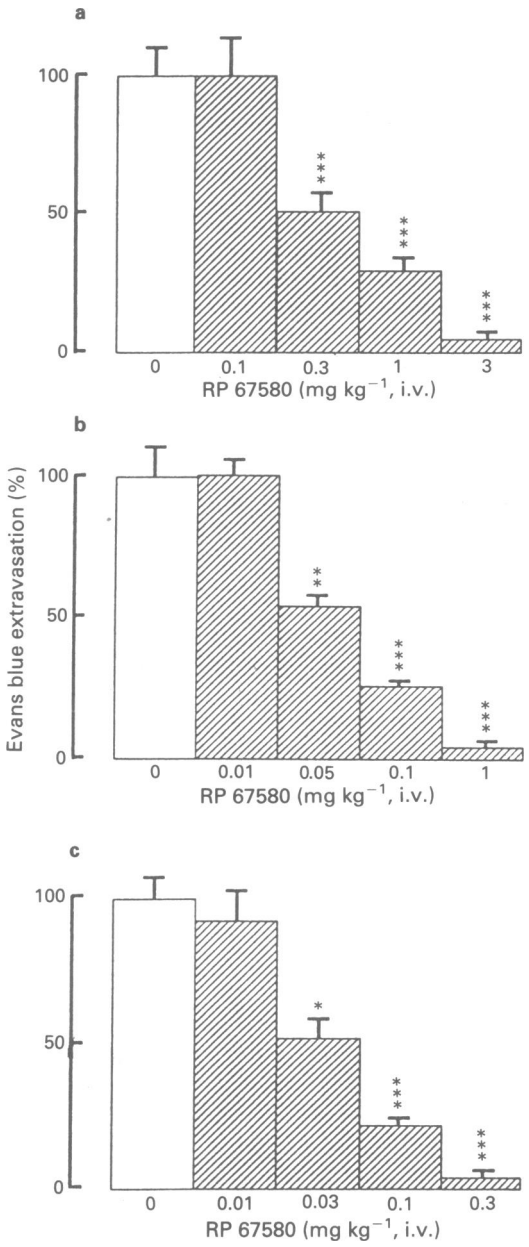


Figure 1 Effects of RP 67580 on plasma extravasation in hind paw skin of rat induced by electrical stimulation of the saphenous nerve (a), by intravenous injection of substance P (b), and by topical application of xylene (c). In (a) RP 67580 or vehicle (control) was administered s.c. 30 min before the saphenous nerve was stimulated, and Evans blue extravasation in the hind paw skin area innervated by this nerve was determined. In (b) Evans blue extravasation in hind paw skin of rat was determined 5 min after simultaneous i.v. injection of RP 67580 and substance P (1 µg kg^{-1}, i.v.). In (c) RP 67580 and Evans blue were administered i.v., 5 min before the topical application of xylene or vehicle. Xylene or vehicle was applied to a hind paw skin area for 30 min before the Evans blue extravasation in this same skin area was determined. Data (mean ± s.e.mean, $n = 6$) are expressed as a percentage of the value obtained with the control (mean value of animals treated with vehicle of RP 67580). $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ (Student's t test). Open column: controls; hatched columns: RP 67580-treated.

NaCl solution/ethanol, 1:1) was applied to the skin of the other hind paw of hairless rats. The dye concentration in the skin was assayed as described above.

Nasal fluid hypersecretion

Hypersecretion of nasal fluid was induced according to the method described by Petersson *et al.* (1989). Dry strips of filter paper were weighed and inserted into the nasal cavities for 5 min. Upon removal, the strips were reweighed and the weight gain (i.e. the basal nasal secretion) was calculated (see below). Another pair of paper strips was prepared (one strip loaded with 25 µl of a 0.2% capsaicin solution in ethanol and the other one, with ethanol alone) and introduced into the nasal cavities for 5 min Capsaicin-stimulated nasal secretion was considered to be that remaining after what was measured in the vehicle-treated contralateral nasal cavity had been subtracted. RP 67580 or its enantiomer, RP 68651, was administered i.v. after determination of basal nasal secretion and 5 min before capsaicin or vehicle application.

Materials

RP 67580 (3a**R**, 7a**R**) (7,7-diphenyl-2-(1-imino-2-(2-methoxy phenylethyl) perhydroisoindol-4-one), RP 68651 (3a**S**, 7a**S**) (7,7-diphenyl-2-(1-imino-2-(2-methoxyphenylethyl)perhydro-isoindol-4-one), (±)-CP-96,345 (2**RS**, 3**RS**) (*cis*-2-(diphenyl-methyl)-*N*-(2-methoxyphenyl)-methyl-1-azabicyclo(2.2.2)octan-3-amine) and PD-117302 (±)-(*trans*-*N*-methyl-*N*-(2-(1-pyro-lidinyl)-cyclohexyl-)benzo-(b)-thiopene-4-acetamide were obtained from the Department of Chemistry, Rhône-Poulenc Rorer (France). Morphine was purchased from Francopia (France). Naloxone, colchicine and capsaicin were purchased from Sigma (France). SP and septide ([*p* Glu^6,Pro^9]-SP(6–11)) which is a selective NK_1-receptor agonist (Laufer *et al.*, 1986) were purchased from Bachem (Switzerland). Lignocaine was from Laboratoires Roger Bellon (France).

Results

Plasma extravasation induced by electrical nerve stimulation (neurogenic inflammation)

Electrical stimulation of the rat saphenous nerve induced an intense Evans blue extravasation (2.49 ± 0.24 µg per 100 mg of tissue) in the hind paw skin innervated by this nerve (compared to the basal Evans blue extravasation which was equal to 0.07 ± 0.003 µg per 100 mg of tissue). Used by i.v. route, (±)-CP-96,345 potently reduced plasma extravasation (ID_{50} = 0.6 mg kg^{-1}, i.v.), with a similar potency to RP 67580 (ID_{50} = 0.15 mg kg^{-1}, i.v. as we previously found: Garret *et al.*, 1991). Given subcutaneously, RP 67580 was able to inhibit plasma extravasation in a dose-dependent manner (ID_{50} = 0.3 mg kg^{-1}, s.c.) (Figure 1a).

Both µ- (morphine) and κ-(PD-117302) opioid receptor agonists inhibited neurogenic inflammation (Table 1). However, in contrast to the NK_1-receptor antagonist, RP 67580, neither of these opioid receptor agonists reduced the neurogenic inflammation by more than 80%, even when they were used at high doses of 10 mg kg^{-1}, s.c. (Table 1). The opioid receptor antagonist naloxone (10 mg kg^{-1}, s.c.) was without effect on neurogenic inflammation. However, it reversed the effect of the opioid agonists used here, but not that of RP 67580 (not shown).

Plasma extravasation was totally blocked by a saphenous nerve ligation distal to the site of stimulation, but was not blocked by a ligation proximal to this site. Local lignocaine application to the nerve before its stimulation also blocked the extravasation (Table 1). Colchicine treatment and neonatal capsaicin treatment also strongly reduced neurogenic plasma extravasation (Table 1).

Plasma extravasation induced by exogenous peptides

The intravenous injection (1 µg kg^{-1}, i.v.) of SP or septide produced an intense plasma extravasation in hind paw skin (3.03 ± 0.15 and 3.10 ± 0.34 µg per 100 mg of tissue, respectively). The basal Evans blue extravasation was 0.08 ± 0.01 µg per 100 mg of tissue.

Table 1 Effects of RP 67580, opioid receptor agonists, nerve ligature, lignocaine local application, and capsaicin and colchicine pretreatments on plasma extravasation induced by electrical nerve stimulation or by exogenous substance P

		Plasma extravasation elicited by: *Electrical nerve stimulation* (% of control)	*Exogenous substance P* (% of control)
RP 67580	1 mg kg^{-1}, s.c.	41 ± 8***	9 ± 3***
Morphine	1 mg kg^{-1}, s.c.	69 ± 4**	–
	10 mg kg^{-1}, s.c.	26 ± 4***	105 ± 6
PD 117302	1 mg kg^{-1}, s.c.	64 ± 7**	–
	10 mg kg^{-1}, s.c.	52 ± 9***	97 ± 8
Ligature of saphenous nerve		8 ± 7***	–
Local application of lignocaine to saphenous nerve		7 ± 1***	–
Neonatal capsaicin treatment (50 mg kg^{-1}, s.c.)		5 ± 1***	143 ± 7***
Colchicine 120 µg kg^{-1} day^{-1}, i.p. for:	- 1 day	97 ± 26	–
	- 2 days	62 ± 14*	–
	- 3 days	24 ± 5***	89 ± 5

Data (mean ± s.e.mean of 6 values) are expressed as a percentage of the control value (mean value of non-treated animals). RP 67580 and opioid receptor agonists were administered s.c. 30 min before the saphenous nerve was stimulated or the intravenous injection of substance P. The saphenous nerve was ligated at the part distal to the stimulation. Lignocaine (20 µl of a 2% solution) was directly applied to the saphenous nerve before the electrical stimulation. Capsaicin (50 mg kg^{-1}) was administered s.c. to two-day-old rats, and 2 months later, the rats were used for plasma extravasation studies. Colchicine (120 µg kg^{-1} day^{-1}) was given i.p. for 1, 2 or 3 consecutive days. In all cases, Evans blue extravasation in hind paw skin innervated by the saphenous nerve was measured as described in the Methods section. Data (mean ± s.e.mean, $n = 6$) are expressed as a percentage of the control value (mean value of non-treated animals).
*$P<0.05$; **$P<0.01$; ***$P<0.001$ (Student's t test).

RP 67580 reduced, in a dose-dependent manner, septide- or SP-induced plasma extravasation in the skin (ID_{50} = 0.04 and 0.05 mg kg^{-1}, i.v., respectively) (see Figure 1b for results obtained with SP). Given by s.c. route, RP 67580 was also able to reduce vascular permeability (Table 1). The effect of RP 67580 was stereospecific since its enantiomer, RP 68651 (1 mg kg^{-1}, i.v.), did not affect septide- and SP-induced plasma extravasation.

Neither opioid agonists nor colchicine affected plasma extravasation when it had been induced by SP, but they did inhibit it when it had been evoked by electrical nerve stimulation (see above and Table 1).

Neonatal capsaicin treatment led to an increase of SP-induced plasma extravasation (by 43 ± 7.26%) (Table 1). RP 67580 at 1 mg kg^{-1}, i.v., blocked SP-induced plasma extravasation in capsaicin-treated animals (96 ± 1.5% inhibition).

Capsaicin- or xylene-induced plasma extravasation

Local application of capsaicin or xylene to a cutaneous area of hind paw elicited an intense Evans blue extravasation (5.64 ± 0.61 μg per 100 mg of wet skin and 8.85 ± 2.00 μg in the whole stained skin area, respectively), compared to the basal Evans blue extravasation induced by vehicle of capsaicin or xylene (0.10 ± 0.06 or 0.15 ± 0.12 μg, respectively). This chemically induced plasma extravasation was powerfully inhibited by RP 67580 (ID_{50} = 0.06 and 0.03 mg kg^{-1}, i.v. for capsaicin and xylene, respectively; see Figure 1c for xylene-induced plasma extravasation), while it was not affected by the enantiomer, RP 68651, used at 1 mg kg^{-1}, i.v.

Nasal fluid hypersecretion

The topical application of capsaicin to nasal cavities increased nasal fluid secretion from 4.51 ± 0.36 mg (vehicle-treated) to 7.81 ± 0.77 mg (capsaicin-treated). RP 67580 (1 mg kg^{-1}, i.v.) inhibited the nasal hypersecretion induced by capsaicin by 82% (Figure 2).

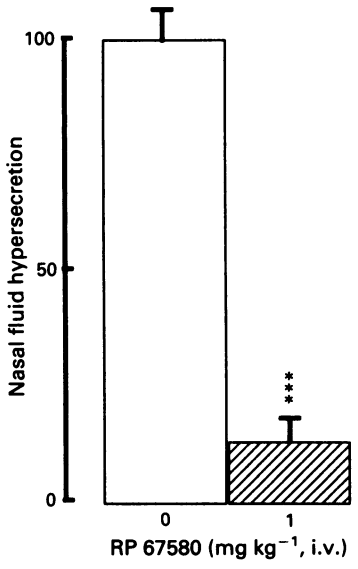


Figure 2 Effect of RP 67580 on capsaicin-induced nasal fluid hypersecretion. Nasal hypersecretion was induced by inserting into nasal cavities strips of filter paper soaked in a capsaicin solution (25 μl, 0.2% in ethanol). RP 67580 was given 5 min before capsaicin or vehicle application. Data (mean ± s.e.mean, n = 6) are expressed as a percentage of the control value (mean value of animals treated with vehicle of RP 67580). ***$P<0.001$ (Student's t test). Open column: controls; hatched column: RP 65780-treated.

Discussion

The major findings in the present study are that the NK_1-receptor antagonist, RP 67580 at low doses (0.3 ± 1 mg kg^{-1}), inhibits plasma extravasation induced in skin either by NK_1-receptor agonists (SP and septide) or by chemical irritants (xylene or capsaicin applied topically). In addition, RP 67580 blocks capsaicin-elicited nasal fluid hypersecretion in the rat. Opioid receptor agonists and colchicine inhibit plasma extravasation induced by electrical nerve stimulation, but not that elicited by exogenous SP. All these results clearly indicate that NK_1-receptor antagonists act at the post-synaptic level while opioid receptor agonists and colchicine act at the presynaptic site to inhibit neurogenic vascular permeability.

RP 67580, used in our experiments as an antagonist of NK_1-receptors, has been described as highly selective for this receptor type (Garret *et al.*, 1991). Indeed, RP 67580 was found to act potently and competitively at NK_1-receptors (K_i = 2.9–3.3 nM), without interaction with NK_2- and NK_3-receptors and had no affinity for a variety of peptide receptors, nor with numerous other receptors of classical neurotransmitters, as shown in binding assays (Fardin *et al.*, 1992) or in *in vitro* functional studies (Carruette *et al.*, 1992). In contrast, (±)-CP-96,345, a second non-peptide NK_1-receptor antagonist, was recently reported to interact with calcium channels (Guard & Watling, 1992) and to present cardiovascular effects (Donnerer *et al.*, 1992) unrelated to actions on NK_1-receptors. The fact that RP 67580 is highly selective for NK_1-receptors without interaction with other receptors indicates that the inhibition of plasma extravasation we obtained when using this compound is due to selective blockade of NK_1-receptors. Moreover, the effect of RP 67580 was found to be stereospecific, since its enantiomer (RP 68651) at a high dose of 1 mg kg^{-1}, i.v. was without effect on plasma extravasation induced by NK_1-receptor agonists, capsaicin and xylene.

(±)-CP-96,345 was slightly less active than RP 67580 in our experiments, because we used it as racemate (its inactive enantiomer was not available) while RP 67580 is the active (3a**R**, 7a**R**) enantiomer, RP 68651 (3a**S**, 7a**S**) being the inactive enantiomer. The difference in potency in inhibiting plasma extravasation between RP 67580 and (±)-CP-96,345 found in the present work could also be explained by the lower affinity of (±)-CP-96,345 than of RP 67580 for rat NK_1-receptors as compared to those of other species such as rabbit and guinea-pig (Beresford *et al.*, 1991; Fardin *et al.*, 1992). Indeed, CP-96,345 was found to be 15 times less potent than RP 67580 for the inhibition of [^{3}H]-SP binding in subcortical structures of brain and in submaxillary gland of rat (Fardin *et al.*, 1992).

Our results, in accordance with other reports (Jessel & Iversen, 1977; Morton & Chahl, 1980; Barthó & Szolcsányi, 1981; Brodin *et al.*, 1983), confirm that the μ-opioid receptor agonist, morphine, inhibits neurogenic plasma extravasation at presynaptic level. Recently, κ-agonists have received particular attention owing to their potentially analgesic properties. Indeed, systemic administration of non-peptide opioid receptor agonists, such as PD-117302, induce antinociception (Leighton *et al.*, 1987). We showed that this κ-agonist, like morphine, was effective in inhibiting plasma extravasation induced by electrical nerve stimulation, but not that induced by exogenous SP (Table 1), suggesting that κ-agonists act through receptors located presynaptically on afferent fibres to inhibit tachykinin release. In our experiments, the blockade of neurogenic inflammation by both μ- and κ-agonists was not complete even when these substances were used at high doses. The reason for this is not clear: other substances, such as histamine, may increase tachykinin release when nerves are electrically stimulated (see Tani *et al.*, 1990 for review).

Furthermore, we have shown that colchicine inhibited plasma extravasation induced by electrical stimulation, but not that induced by exogenous SP (Table 1). This is of

interest because colchicine is very effective in various pathological inflammatory states in man, such as acute gouty attacks and familial Mediterranean fever (Famey, 1988; Zemer *et al.*, 1991). It is possible that SP contributes to the pain and inflammation that accompany these pathologies and that colchicine, by inhibiting axonal transport at the presynaptic level, indirectly reduces tachykinin release. Previous studies have shown SP to be present in C-fibres of nerves supplying small dermal vessels and sweat glands as well as in free nerve endings in the epidermis (Hökfelt *et al.*, 1975; Hartschuh *et al.*, 1983; Moussaoui *et al.*, 1992b). We demonstrated that neonatal capsaicin treatment leads to an increase of SP-induced plasma extravasation (see above). This suggests the existence of an up-regulation of NK_1-receptors in capsaicin-treated animals due to the chronic lack of tachykinin release from nerve terminals. However, other explanations such as effects of capsaicin on a second messenger system or on blood flow cannot be excluded. Our results also suggest that NK_1-receptors are probably not located on terminals of C-fibres, since the destroying of these fibres by neonatal capsaicin treatment leads to an increase of SP-induced plasma extravasation.

Our results showing that RP 67580 is a potent inhibitor of plasma extravasation are in accordance with those of Lembeck *et al.* (1992) who demonstrated that CP-96,345, the other non-peptide NK_1-receptor antagonist, is also a potent blocker of plasma extravasation induced in rat by SP, mustard oil or electrical nerve stimulation.

In conclusion, non-peptide NK_1-receptor antagonists, such as RP 67580, inhibit vascular permeability in the skin and the nasal mucosa, and consequently, they could be of great potential for the treatment of dermatosis and rhinitis. They also could represent a new therapeutic approach to various other pathologies in which neurogenic inflammation often accompanies pain.

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Induction by endotoxin of nitric oxide synthase in the rat mesentery: lack of effect on action of vasoconstrictors

Jane A. Mitchell, [1]Kathy L. Kohlhaas, Raffaella Sorrentino, [2]Timothy D. Warner, [1]Ferid Murad & John R. Vane

The William Harvey Research Institute, The Medical College of Saint Bartholomews' Hospital, Charterhouse Square, London EC1M 6BQ

1 Male Sprague-Dawley or Wistar rats were injected with bacterial lipopolysacharride (LPS; 5 mg kg^{-1}, i.p.) and killed after 1, 3, 6, 15, and 24 h. The brains, mesenteries, spleens, lungs, livers, kidneys, hearts, aortae and diaphragms were removed and frozen immediately. Control rats were injected with sterile saline and killed after 6 h.

2 The organs were homogenized in a semi-frozen state and NO synthase (NOS) activity measured in tissues from both LPS-treated and saline-treated groups by the ability of homogenates to convert [^{3}H]-L-arginine to [^{3}H]-L-citrulline in a NADPH-dependent manner.

3 The NOS activity in all organs taken from control animals was found to be calcium-dependent, with the highest activity being in the brain. After LPS-treatment an induced calcium-independent NOS was detected in all tissues tested, with the exception of the brain. The spleen, lung, mesentery and liver had the highest amounts of LPS-induced NOS activity. No induction of calcium-dependent NOS was detected.

4 Induction of NOS was maximum 6 h after administration of LPS and had returned to control levels in 24 h.

5 The constitutive NOS in brain and mesentery and the LPS-induced activities in the spleen, lung, liver and mesentery were inhibited by N^G-monomethyl-L-arginine (L-NMMA) or N^G-nitro-L-arginine methyl ester (L-NAME) according to concentration. The IC_{50} for L-NAME was 2.5 μM against the constitutive NOS from brain, and 20–25 μM against the inducible NOS. For L-NMMA the IC_{50} was 20–25 μM against either NOS isoform.

7 The vascular responses to endothelin-1 (ET-1), the thromboxane A_2-mimetic 11$_\alpha$,9$_\alpha$-epoxymethano prostaglandin $F_{2\alpha}$ (U46619), phenylephrine (PE) or 5-hydroxytryptamine (5-HT) were measured in the simultaneously perfused arterial and venous mesenteric vascular beds from both control and LPS-treated (6 h) rats. Vasoconstrictor responses to all agonists tested were unaffected by LPS treatment. In the presence of L-NAME (100 μM) vasoconstrictor responses were potentiated in both the arterial and venous portion of the mesenteric beds from both control and LPS-treated rats. The potentiation of responses to U46619 was significantly greater in beds from LPS-treated rats.

8 Injection of LPS i.p. is associated with induction of NOS in all organs tested, except for the brain. In the mesentery this is not accompanied by a hyporesponsiveness to constrictor agents suggesting an increased sensitivity, particularly to U46619. This may explain the poor perfusion and tissue damage in the splanchnic circulation associated with sepsis.

Keywords: Nitric oxide; L-arginine; N^G-monomethyl-L-arginine; N^G-nitro-L-arginine methyl ester; hypotension; cytokine; thromboxane A_2; 5-hydroxytryptamine; endothelin-1; phenylephrine

Introduction

Many mammalian cells including endothelial cells and some neuronal cells generate nitric oxide (NO; Palmer *et al.*, 1987) from L-arginine (L-Arg) via a constitutive NO synthase (NOS). Several isoforms of NOS have now been isolated and purified (Förstermann *et al.*, 1991). The isoform present in endothelial cells is a 135 kD protein located in the membrane fraction (Pollock *et al.*, 1991) whereas neuronal or brain NOS is a 155 kD protein located in the soluble fraction (Bredt & Snyder, 1990; Mitchell *et al.*, 1991a; Schmidt *et al.*, 1991). As well as these two constitutive NOS enzymes, bacterial lipopolysaccharide (LPS) or cytokines induce various cells including vascular smooth muscle and macrophages to express a different NOS (Busse & Mulsch, 1990; Fleming *et al.*, 1990; Gross *et al.*, 1990; Julou-Schaeffer *et al.*, 1990; Knowles *et al.*, 1990; Rees *et al.*, 1990; Stuehr *et al.*, 1991; Mitchell *et al.*, 1992a). Although the constitutive and inducible NOS isoforms have the same cofactor requirements for NADPH and tetrahydrobiopterin (BH_4), and contain/require FAD and FMN (see Förstermann *et al.*, 1991) they differ in that the constitutive NOS enzymes are calcium-dependent, and the inducible NOS calcium-independent. In addition, the L-Arg analogues, N^G-monomethyl-L-arginine (L-NMMA) and N^G-nitro-L-arginine methyl ester (L-NAME) show different potencies as inhibitors of the constitutive and inducible isoforms of NOS. For instance, L-NAME more potently inhibits NO release from endothelial cells *in vitro* (Moore *et al.*, 1989) and from purified constitutive NOS from endothelial cells (Pollock *et al.*, 1991) or brain (Schmidt *et al.*, 1991), and more potently increases blood pressure *in vivo* (Hecker *et al.*, 1990). In contrast, L-NMMA more potently inhibits NO formed by cytokine-treated cells *in vitro* (Gross *et al.*, 1990). The use of such analogues to treat conditions resulting from over production of NO is currently being investigated, particularly for the hypotensive crisis associated with septic shock. NO production is responsible for the immediate and late phase hypotension observed following LPS administration (Thiemermann & Vane, 1990; Nava *et al.*, 1991; Petros *et al.*, 1991; Szabo *et al.*, 1993), the latter stage being associated with the induction of NOS in

[1]Present address: Department 47S, AP10/2, Abbott Laboratories, Abbott Park, Illinois 60064, U.S.A.
[2]Author for correspondence.

various organs (Salter et al., 1991) and a hyporesponsiveness of vessels to vasoconstrictor agents, as assessed ex vivo. Here we investigated whether NOS is induced in the mesenteric bed of the rat following LPS administration and whether this activity compromises the vascular responses ex vivo of the arterial and venous portions of the mesenteric bed (Warner, 1990). Some of these observations have been published in abstract form (Mitchell et al., 1992b; Sorrentino et al., 1992).

Methods

Measurement of NO synthase activity

Results obtained in two laboratories were combined. The time course and levels of induction of NOS were measured in both albino Wistar rats (250–300 g, UK) and Sprague-Dawley rats (250–300 g, USA) and found to be comparable. At the designated times 3–6 rats from each group were anaesthetized with sodium pentobarbitone and decapitated. The organs were then removed and frozen in liquid nitrogen and stored at − 70°C until NOS activity was measured. The organs were homogenized on ice in 5 volumes of the following buffer: Tris/HCl 50 mM; EDTA 0.1 mM; EGTA 0.1 mM; 2-mercaptoethanol 12 mM; leupeptin 2 μM; pepstatin 1 μM; and phenylmethylsulphonyl fluoride 1 mM at pH 7.4. NOS activity was measured by the ability of homogenates to convert [^{3}H]-L-Arg to [^{3}H]-L-citrulline ([^{3}H]-L-Cit), the co-product of NO formation, as described previously (Bredt & Snyder, 1990; Mitchell et al., 1991a). Briefly, 50 μl of tissue homogenate (approximately 200 μg of protein) was incubated in the presence of L-Arg/[^{3}H]-L-Arg (10 μM; 5000 Bq/tube), NADPH (1 mM), tetrahydrobiopterin (3 μM), calmodulin (30 nM) and calcium (2 mM). The reaction volume was made up to 100 μl by the addition of HEPES buffer (10 mM; pH 7.5) and incubated for 20 min. The reaction was stopped by the addition of 1 ml of 20 mM HEPES buffer (pH 5.5) containing EGTA (2 mM) and EDTA (2 mM). The reaction mixture was then applied to Dowex-50W (Na form) columns (1 ml) and the eluted [^{3}H]-L-Cit measured in a Beckman scintillation counter. As other enzymes, e.g. arginase and ornithine transcarbamoylase, convert L-Arg to L-Cit we increased the specificity of this assay by including in incubates L-valine (60 mM; Salter et al., 1991), which inhibits arginase activity and the specific NOS inhibitor L-NMMA, which inhibits the formation of L-Cit via the L-Arg/NO pathway.

To test the calcium-dependency of NOS in various organs the activity in the presence of calcium (2 mM) was compared to that in the absence of calcium and the presence of EGTA (1 mM). L-Cit production occurring in the absence of NADPH was taken as non-NOS specific conversion. The protein concentrations in the homogenates were measured using Bradford reagent (Bradford, 1976) with bovine serum albumin as a standard.

Double perfused mesentery

Male albino Wistar rats were killed with pentobarbitone sodium (100 mg kg^{-1}, i.p.). The abdomen was opened and the ileocolic and colic branches of the superior mesenteric artery ligated. A cannula (Portex, size 3FG, external diameter 1.02 mm, internal diameter 0.61 mm) was inserted into the portal vein and passed into the superior mesenteric vein. The superior mesenteric artery was cannulated and the superior mesenteric vascular bed perfused via the artery for 5 min (2 ml min^{-1}) with Krebs buffer containing heparin (100 u ml^{-1}). The intestine was then separated from the mesentery and the preparation supported on a petri-dish while the arteries and veins were perfused independently at 2 ml min^{-1} with warmed (37°C) and gassed (95% O_2:5% CO_2) Krebs buffer containing indomethacin (6 μM) to block cyclo-oxygenase activity. Changes in perfusion pressures were measured with transducers approximately 15 cm from the tips of the arterial and venous cannulae (Warner, 1990). Following an equilibration period of 45 min vasoconstrictor responses of the two sides of the preparation were tested by injection of bolus doses (1–3 μl) of agents into both parts of the circulation, with a time delay between the injections into each side of less than 5 s.

Materials

L-NMMA was a gift from Dr James Kerwin, Abbott Laboratories, U.S.A. and U46619 was a gift from Upjohn Co., U.S.A. [^{3}H]-L-Arg was purchased from Amersham International, U.K. All other compounds were obtained from Sigma Chemical Company, U.K.

Results

Distribution of calcium-dependent and calcium-independent NOS activity in the organs of rats following LPS-treatment

Generally, the ability of organ homogenates to convert [^{3}H]-L-Arg to [^{3}H]-L-Cit was dependent on NADPH (Table 1). Exceptions were the liver and kidney from control animals in which all the L-Cit producing activity (in the presence of L-valine) was independent of NADPH, and therefore not NOS.

In control animals, very low levels of constitutive, calcium-dependent, NOS activity were measured in homogenates of heart, kidney, liver, aorta, lung, diaphragm or spleen (all <2 pmol L-Cit mg^{-1} 20 min^{-1}). There was significant activity in the mesentery (9.4 ± 4 pmol L-Cit mg^{-1} 20 min^{-1}) and even more in the brain (100 ± 10 pmol L-Cit mg^{-1} 20 min^{-1}) (Table 1).

After LPS administration (5 mg kg^{-1} for 6 h) there was a

Table 1 Distribution of NO synthase (NOS) activity in the organs from control rats and those treated with bacterial lipopolysaccharide (LPS, 5 mg kg^{-1} for 6 h)

Organ	Control with Ca^{2+}	Control without Ca^{2+}	LPS with Ca^{2+}	LPS without Ca^{2+}
Brain	100 ± 10	3 ± 2	80 ± 4	2 ± 4
Mesentery	9.5 ± 4	2 ± 4	43.2 ± 4	33.7 ± 3
Spleen	6.3 ± 3	4.6 ± 4	99.9 ± 1.4	90.4 ± 1.2
Lung	5.7 ± 2	3.4 ± 2	100 ± 10	95 ± 2
Liver	<2	<2	50 ± 7	48 ± 2
Kidney	<2	<2	42 ± 5	45 ± 2
Heart	<2	<2	12.5 ± 5	10.4 ± 2
Aorta	<2	<2	17 ± 6	15 ± 2
Diaphragm	<2	<2	8.9 ± 3	9.5 ± 2

The table shows the amount of NOS activity determined by the production of L-citrulline from L-arginine (pmol mg^{-1} 20 min^{-1}) which was dependent upon the presence of NADPH. The calcium-dependent activity (with Ca^{2+}) was determined in the presence of 1 mM calcium and the calcium independent (without Ca^{2+}) activity was measured in the absence of calcium and in the presence of 1 mM EGTA. The results shown represent the mean ± s.e.mean from 3–12 determinations using the organs from 3–6 animals.

significant induction of calcium-independent NOS activity in the mesentery (43 ± 4 pmol L-Cit mg^{-1} 20 min^{-1}). The lung and spleen showed strong NOS induction (100 ± 10 and 99.9 ± 1.4 pmol L-Cit mg^{-1} 20 min^{-1} respectively) whereas the aorta, heart and diaphragm showed less induced activity (17 ± 6, 12.5 ± 5 and 8.9 ± 3 respectively). NOS induction in the liver (50 ± 5 pmol L-Cit mg^{-1} 20 min^{-1}) and kidney (45 ± 2 pmol L-Cit mg^{-1} 20 min^{-1}) was similar in magnitude to that in the mesentery, although 30–40% of the total L-Cit production remained independent of NADPH. There was no induction of NOS in the brain (Table 1).

Time-course for induction of NOS activity

The NOS activity in the mesentery of rats increased maximally 6 h after LPS administration and declined to control levels by 24 h (Figure 1). NOS activity in the other organs similarly reached a peak 6 h after LPS treatment (Table 1) and had returned to control levels by 24 h ($n = 3$ for each; data not shown).

Inhibitory effects of N^G-monomethyl-L-arginine and N^G-nitro-L-arginine methylester on NOS activity

Both L-NMMA and L-NAME inhibited NOS activity in a concentration-dependent manner with respective IC_{50} values of 20–25 μM and 2.5 μM against brain NOS (Figure 2c) and 20–25 μM against the induced NOS in homogenates of mesenteries (Figure 2a) or spleens (Figure 2b) from LPS-treated rats.

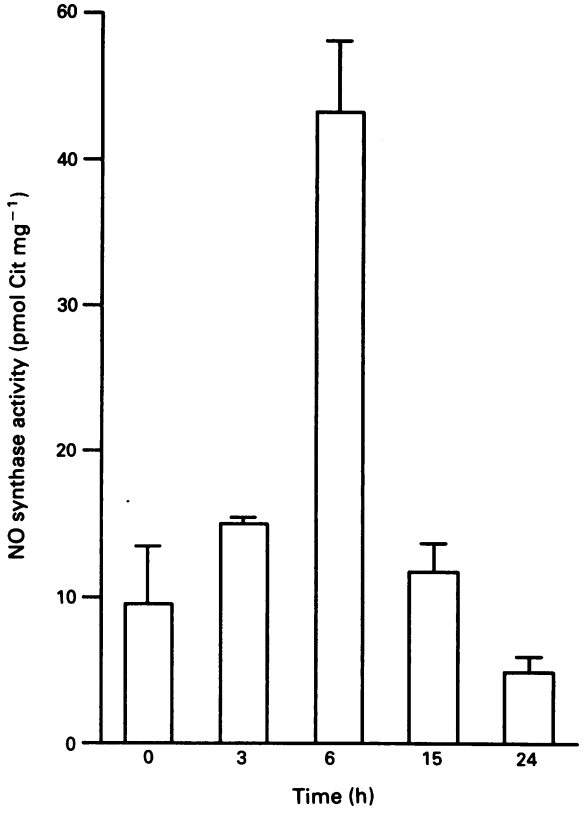


Figure 1 Nitric oxide synthase is induced in a time-dependent manner in the mesenteries of rats treated with bacterial lipopolysaccharide (LPS, 5 mg kg^{-1}). The figure shows the activity of NO synthase (NOS) (pmol L-citrulline mg^{-1} protein 20 min^{-1}) measured in homogenates from mesenteries removed from rats after treatment with LPS for 0, 3, 6, 15 and 24 h. Measurements of NOS activity were performed in the presence of excess cofactors, as described in Methods. Results are presented as the mean ± s.e.mean from 3 experiments. *Denotes a significant difference in activity from that at time 0.

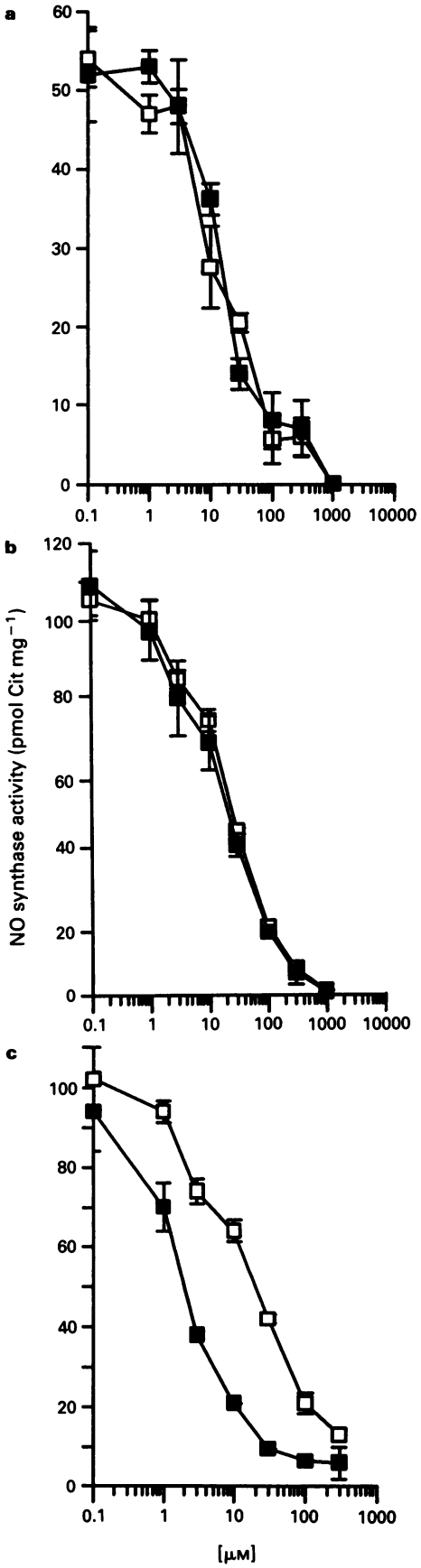


Figure 2 N^G-monomethyl-L-arginine (L-NMMA) and N^G-nitro-L-arginine methyl ester (L-NAME) inhibit NO synthase (NOS) activity. See Methods section for details of the assay. The figure shows the concentration-dependent inhibition of NOS activity by L-NAME (■) and L-NMMA (□) of NOS: (a) against NOS from spleens of bacterial lipopolysaccharide (LPS)-treated animals (5 mg kg^{-1}, i.p., 6 h); (b) against NOS from mesenteries of LPS-treated animals; (c) against activity in brain (from non-LPS treated rats). The results represent the mean ± s.e.mean from 3 experiments.

Vascular responses of the isolated double perfused mesenteric bed

The arterial and venous portions of mesenteric beds removed from control and LPS-treated rats constricted to bolus injections of endothelin-1 (ET-1; 3–30 pmol), 5-hydroxytryptamine (5-HT; 1–10 nmol), phenylephrine (PE; 1–10 nmol) or U46619 (30–300 pmol). There were no significant decreases in the vasoconstrictor responses to any of these agonists in either side of the circulation in beds removed from LPS-treated animals (Figure 3; Table 2). In separate experiments, L-NAME (100 μM) was included in the Krebs solution perfusing both portions of the mesenteric bed. Although this had no effect on the basal perfusion pressures the responses of the arterial vessels from control animals to all agonists used were significantly potentiated (Table 3 versus Figure 4), but there was no change in the venous vessels. In the presence of L-NAME the responses of the mesenteric arterial vessels from LPS-treated rats were potentiated to a similar degree to control rats for ET-1, 5-HT and PE. However, the responses to U46619 were potentiated further than in control rats (Figure 4; Table 3). In the venous vessels from LPS-treated animals L-NAME also potentiated vasoconstrictions in response to U46619, but not to ET-1, 5-HT or PE in comparison to control vessels treated with L-NAME (Figure 4; Table 3).

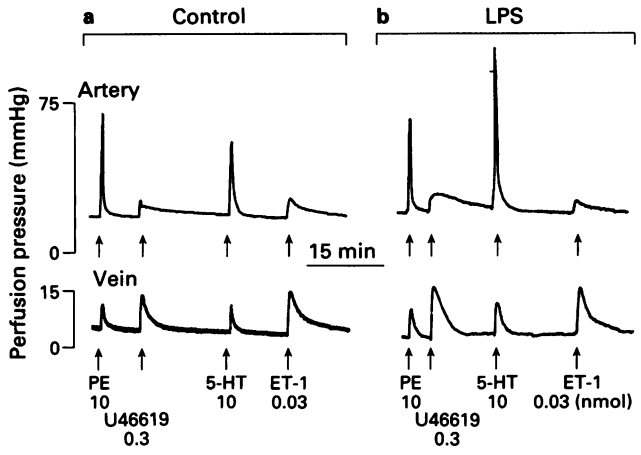


Figure 3 Treatment of rats with bacterial lipopolysaccharide (LPS, 5 mg kg^{-1}; 6 h) does not impair the vasoconstrictor responses to phenylephrine (PE; 10 nmol), U46619 (0.3 nmol), 5-hydroxytryptamine (5-HT; 10 nmol) or endothelin-1 (ET-1; 0.03 nmol). In (a) are shown responses from control preparations and in (b) those from LPS-treated animals. The vasoconstrictions of the arterial portions of the mesenteric beds are shown at the top and those of the venous portions at the bottom. Traces are representative of 4–6 experiments for each treatment.

Table 2 Effect of bacterial lipopolysaccharide (LPS) administration on the vasoconstrictor actions of various agonists

		Control		*LPS*	
		arterial	venous	arterial	venous
ET-1	3	0.5 ± 0.2	1.5 ± 0.3	0.6 ± 0.2	0.9 ± 0.3
	10	1.8 ± 0.3	3.0 ± 0.7	2.1 ± 0.4	2.6 ± 0.4
	30 (pmol)	4.5 ± 1.2	7.4 ± 0.7	5.2 ± 0.9	7.0 ± 1.7
U46619	30	1.4 ± 0.6	4.8 ± 0.9	2.0 ± 1.0	6.4 ± 1.4
	100	6.0 ± 2.5	8.2 ± 0.6	4.3 ± 1.6	9.8 ± 1.2
	300 (pmol)	10.5 ± 4.1	9.1 ± 1.0	5.8 ± 1.2	13.0 ± 1.7
5-HT	1	6.6 ± 2	2.9 ± 0.4	13.4 ± 4.9	2.7 ± 1.1
	3	13.8 ± 3.4	6.5 ± 0.6	23.6 ± 7.4	4.0 ± 1.1
	10 (nmol)	26.8 ± 5.4	6.2 ± 0.7	35.1 ± 8.2	4.5 ± 0.9
PE	1	2.0 ± 0.5	2.2 ± 0.5	3.2 ± 0.6	1.6 ± 0.4
	3	9.7 ± 2.2	4.4 ± 1.1	14.4 ± 5.4	3.2 ± 0.8
	10 (nmol)	19.7 ± 3.9	5.7 ± 0.7	29.9 ± 9.8	4.4 ± 0.8

The constrictor response to endothelin-1 (ET-1), 5-hydroxytryptamine (5-HT), phenylephrine (PE) and U46619 are given as increase in mmHg. There was no significant effect of LPS administration (5 mg kg^{-1}, i.p.; 6 h) on the activities of any of the agonists used measured by the responses of either the arterial or venous portions of the mesenteric bed. The table represents the mean ± s.e.mean from 3–5 separate experiments.

Table 3 Comparison of vasoconstrictor responses in the arterial and venous portion of the mesenteric bed from control and bacterial lipopolysaccharide (LPS)-treated rats (5 mg kg^{-1} for 6 h) in the presence of N^{G}-monomethyl-L-arginine (100 μM)

		Control		*LPS*	
		arterial	venous	arterial	venous
ET-1	3	1.4 ± 0.3	1.5 ± 0.2	1.3 ± 0.2	2.6 ± 0.7
	10	6.2 ± 1.8	5.4 ± 1.0	5.8 ± 1.6	7.3 ± 1.9
	30 (pmol)	12.8 ± 3.0	9.6 ± 1.3	18.6 ± 3.6	15.1 ± 3.0
U46619	30	4.3 ± 1.4	10.9 ± 1.4	8.5 ± 3.0	25 ± 2.9*
	100	9.9 ± 3.4	13.5 ± 1.2	20 ± 2.9*	46.6 ± 15
	300 (pmol)	21.9 ± 4.8	12.9 ± 1.5	30.5 ± 4.7	27.8 ± 4*
5-HT	1	25.5 ± 6.1	4.2 ± 0.6	19.7 ± 3.1	4.9 ± 0.5
	3	65.1 ± 3.8	7.5 ± 1.4	67.1 ± 13	5.7 ± 0.7
	10 (nmol)	85.6 ± 11	7.6 ± 1.0	81.9 ± 18	5.7 ± 1.0
PE	1	4.1 ± 6.1	3.0 ± 0.6	3.4 ± 0.7	5.8 ± 1.2
	3	25.3 ± 3.8	4.6 ± 1.4	18.1 ± 1.4	7.3 ± 1.4
	10 (nmol)	71.8 ± 8.5	5.6 ± 1.0	98.9 ± 10	7.1 ± 1.5

*Denotes significant ($P < 0.05$) differences between responses in the corresponding portion of the bed in mesenteries from control and LPS-treated rats ($n = 4–6$).

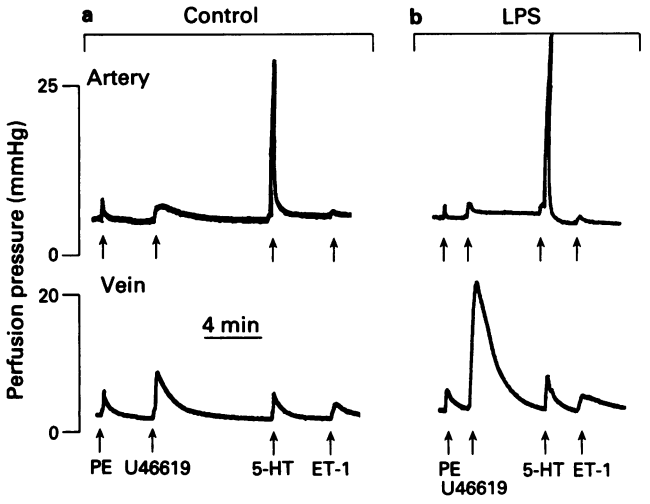


Figure 4 N^G-nitro-L-arginine methyl ester (L-NAME, 300 μM) potentiates the vasoconstrictor effects of U46619 more in mesenteric beds from rats treated with bacterial lipopolysaccharide (LPS, 5 mg kg^{-1}; 6 h) than in mesenteries from control rats. The details of the figure are the same as in Figure 3. The figure shows traces representative of results from 4–6 experiments for each treatment.

Discussion

Here we show that the vessels of mesenteries removed from rats treated with LPS (5 mg kg^{-1} for 6 h) are not hyporesponsive to vasoconstrictor agents, despite significant induction of NOS. Indeed, comparing responses in the absence of NO, i.e. in L-NAME-treated preparations, it can be seen that LPS treatment potentiated the vasoconstrictor actions of U46619 on both sides of the circulation. This finding differs from previous studies, employing similar protocols, which showed that *in vivo* induction of NOS depressed the *ex vivo* responsiveness of both the aorta (Fleming *et al.*, 1990) and the coronary circulation (Smith *et al.*, 1990) to vasoconstrictor agents, an effect which was reversed by L-NMMA. These contrasting actions are even more intriguing as we found that the induction of NOS in the aorta and the heart was significantly lower than that in the mesentery.

The phenomenon of enhanced vasoconstriction of the mesenteric bed to U46619 may be the result of an increased sensitivity of the receptors, or of a portion of the signal transduction pathway leading to constriction. The finding that the vasoconstrictions induced by ET-1, 5-HT, or PE were unaffected by LPS-treatment, either in the presence or in the absence of L-NAME, suggests that the increased NOS activity in the mesentery following LPS-treatment is balanced by an increased vascular responsiveness to these agents. The potentiation by L-NAME of vasoconstrictor responses in control preparations shows, however, that the activity of NOS can modulate the responses to vasoconstrictors in the mesenteric vessels. This confirms our earlier observations that removal of the endothelium or treatment with haemoglobin, which scavenges NO, or methylene blue, which inhibits guanylyl cyclase activation by NO, all potentiate contractile responses in this preparation (Warner *et al.*, 1989).

Thromboxane A_2 contributes to the gastro-intestinal damage induced by endotoxin (Wallace & Whittle, 1987; Whittle *et al.*, 1987; Boughton-Smith *et al.*, 1989). Our results suggest that this contribution may be due to an increase in the responsiveness of this vascular bed to thromboxane A_2 and prostaglandin endoperoxides. This change in sensitivity may also explain earlier observations that in various models of cardiogenic shock, blood flow to the stomach (Bulkley *et al.*, 1985), liver (Bulkley *et al.*, 1986) and intestine (Bailey *et al.*, 1986) is reduced by a disproportionately greater amount than the overall reduction in cardiac output. Thus, in these conditions, in which there are elevated levels of circulating vasoconstrictors, the mesenteric circulation would constrict more strongly, and blockade of NO production (Hutcheson *et al.*, 1990) would produce disproportionately greater damage in this vascular bed.

The differences in the degree of NOS induction measured in the various organs both here and by others (Salter *et al.*, 1991) may be a result of the relative populations of inducible cells, which will become identifiable with the advent of selective and sensitive antibodies. Interestingly, all tissues showed the same time course of induction of NOS with a maximum of 6 h, as in the liver (Salter *et al.*, 1991). The half-life of the induced enzyme is not known, but the waning of its activity over 24 h may be associated with damage by free radicals such as NO and O_2^- (Mitchell *et al.*, 1991b).

Crude homogenates from the brain and mesentery of the rat contained constitutive NOS activity which was calcium-dependent and more sensitive by about ten fold to inhibition by L-NAME than by L-NMMA. This is consistent with previous findings measuring NO release from endothelium *in vitro* (Moore *et al.*, 1990) and NO formation by isolated NOS derived from endothelial cells (Pollock *et al.*, 1991) or brain (Schmidt *et al.*, 1991). This activity differed from that induced in the mesentery, spleen or lung where L-NMMA and L-NAME were equipotent as inhibitors. We found the IC_{50} value for L-NMMA for constitutive and inducible NOS to be approximately the same. This differs from Gross and co-workers (1990) who found L-NMMA to be ten fold more potent against NO release from cytokine-activated macrophages. This may be related to the different incubation times used in that study and here, for prolonged exposure of inducible NOS to L-NMMA produces irreversible inhibition (Olken *et al.*, 1991).

It is clear that during sepsis NOS is induced throughout the body with the exception of the brain (this paper, Salter *et al.*, 1991). Thus inhibitors of NOS, particularly of the inducible form, could be of benefit in the treatment of the hypotension associated with sepsis (Thiemermann & Vane, 1990). However, as we show here, in the mesenteric circulation induction of NOS is not inevitably linked to a hyporesponsiveness to vasoconstrictors. Thus, treatment with a NOS inhibitor during endotoxic shock, even one selective for inducible NOS, may result in an increase in intestinal damage due to the strong elevation in responsiveness to circulating and locally produced vasoconstrictors.

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The diuretic action of 8-cyclopentyl-1,3-dipropylxanthine, a selective A_1 adenosine receptor antagonist

R.J. Knight, C.J. Bowmer & [1]M.S. Yates

Department of Pharmacology, Worsley Medical and Dental Building, The University of Leeds, Leeds LS2 9JT

1 The diuretic effect of the selective A_1 adenosine receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine (CPX), was investigated in anaesthetized rats.

2 CPX (0.1 mg kg^{-1}, i.v.) produced significant increases in urine flow, and the excretion rate and fractional excretion of both sodium and chloride. By contrast, CPX administration did not result in any significant change in the excretion of potassium.

3 The diuretic effect of CPX was accompanied by a transient increase in inulin clearance although *p*-amino-hippurate clearance was unaffected, indicating the CPX induced a temporary elevation of glomerular filtration rate but no change in renal blood flow.

4 The fractional excretion of lithium (a marker of delivery of fluid out of the proximal tubule) was also significantly increased by CPX. However, other measures of tubular function derived from lithium clearance indicated that there were no changes in the handling of sodium or water in the distal regions of the nephron.

5 CPX did not significantly alter the relationship between either free water reabsorption or free water clearance and the distal delivery of sodium, which suggests that CPX does not affect the renal concentration/dilution mechanism.

6 The results of this study show that the diuresis and increased excretion of sodium and chloride induced by CPX (0.1 mg kg^{-1}) in the rat, occurs with only transient elevation in glomerular filtration rate and no change in renal blood flow. The primary reason for the diuresis appears to be inhibition of sodium reabsorption in the proximal tubule. Furthermore, the results provide evidence that production and release of endogenous adenosine modifies renal excretory function via stimulation of the A_1 receptor subtype.

Keywords: 8-Cyclopentyl-1,3-dipropylxanthine; A_1 adenosine receptor antagonism; diuresis; lithium clearance; free water reabsorption; free water clearance; inulin clearance; *p*-amino-hippuric acid clearance

Introduction

The diuretic actions of alkylxanthines have been appreciated for over a century (Koschlakoff, 1864). However, they were of limited clinical value due to lack of potency, tolerance to repeated administration and numerous adverse effects, including cardiac stimulation and psychomotor effects (Meyers, 1924; Persson *et al.*, 1986). The diuretic effect of alkylxanthines has been attributed to a combination of haemodynamic and tubular actions (Jacobson *et al.*, 1991), on the basis that adenosine affects both these components to exert an antidiuresis. However, alkylxanthines can produce a diuresis and natriuresis in the absence of a rise in glomerular filtration rate (GFR) suggesting that an elevation in GFR is not an essential component of xanthine-induced diuresis (Churchill & Bidani, 1990). The pharmacological basis for the diuretic action is believed to be adenosine antagonism, since studies in conscious rats have shown that 8-phenyltheophylline and 8-cyclopentyl-1,3-dipropylxanthine (CPX) which have high affinities for adenosine receptors are diuretic whilst enprofylline, a phosphodiesterase inhibitor with low affinity for adenosine receptors, does not elicit a diuresis (Collis *et al.*, 1986; 1991). The receptor subtype responsible for the increase in urine output evoked by adenosine antagonism appears to be of the A_1 subtype as CPX has diuretic effects at doses that produce selective blockade of A_1-receptors *in vivo* (Collis *et al.*, 1991) and adenosine antagonists with A_2 selectivity do not show any diuretic or natriuretic properties (Suzuki *et al.*, 1992).

The studies of 8-phenyltheophylline and CPX by Collis *et al.* (1986; 1991) consisted solely of measuring urine output and Na^+, K^+ and Cl^- excretion in response to an oral saline load and consequently did not allow any determination of the mechanism underlying the diuresis and natriuresis. The aim of the present study was to establish the basis for CPX-induced diuresis by identifying its haemodynamic and/or tubular actions and thereby elucidating further the role of A_1-adenosine receptors in the renal excretion of electrolytes. CPX is a useful tool to investigate the diuretic effects of selective A_1-adenosine receptor antagonism since it has no marked effects on heart rate and blood pressure (Knight *et al.*, 1991) at doses that block adenosine-induced falls in renal blood flow (Kellett *et al.*, 1989). Furthermore, CPX has little effect on renal phosphodiesterases (Panjehshahin *et al.*, 1992) and at diuretic doses (Collis *et al.*, 1991) has no central stimulant activity (Griebel *et al.*, 1991). A study of the diuretic effect associated with blockade of A_1-adenosine receptors is of particular interest because this class of compound represents a novel approach to treatment and prevention of acute renal failure (Kellett *et al.*, 1989; Knight *et al.*, 1991; Suzuki *et al.*, 1992).

Methods

Surgical protocol

Male Wistar albino rats (250–350 g) or Long Evans hooded rats, Brattleboro strain (250–250 g) were anaesthetized with thiobutabarbitone (180 mg kg^{-1}, i.p.) and cannulae placed in: the trachea for artificial ventilation (80 strokes min^{-1}, 10 ml kg^{-1}); the left jugular vein for drug or isotonic saline (0.9% w/v NaCl) administration; the right carotid artery for measurement of systemic blood pressure and the bladder for collection of urine. In some experiments a cannula was also

[1] Author for correspondence.

placed in the left femoral vein for the delivery of hypertonic or hypotonic saline. Blood pressure was measured via a pressure transducer (Druck PDCR 75) with heart rate obtained electronically from the blood pressure signal. Body temperature was maintained at 37°C with a rectal thermometer and heating lamps. After completion of surgery the following experiments were performed in separate groups of animals.

Ion excretion

Two ml of a solution with the following composition: 0.9% w/v NaCl, 0.07% w/v LiCl (16 mM), 0.35 μCi ml^{-1} [^{3}H]-inulin and 0.055 μCi ml^{-1} *p*-[^{14}C]-aminohippurate (PAH) were administered via the jugular vein to Wistar rats; followed by an infusion (100 μl min^{-1}) of this solution for the remainder of the experiment. The loading dose and infusion rate of lithium was that employed by Johns (1988). A 60 min equilibration period followed the start of infusion to allow stabilization of urine flow. The experiment consisted of 8 clearance periods, each of 15 min duration, during which urine was collected into pre-weighed tubes and a blood sample (0.5 ml) was taken at the mid-point of each collection period. Blood samples were centrifuged (MSE Micro Centaur) and plasma separated for subsequent analysis. The erythrocytes were suspended in an equal volume of saline and transfused back into the animal. The first two clearance periods took place prior to drug administration and the mean measurements of renal function made during these two periods were taken as control values. A statistical comparison of the indices of renal function obtained in these two control clearance periods revealed no significant differences ($P > 0.05$), indicating that equilibration of renal function had been achieved during the 60 min following the start of the i.v. infusion. After completion of the second clearance period, either CPX (0.1 mg kg^{-1}) or its vehicle (1 ml kg^{-1} of 1% v/v dimethyl sulphoxide, 0.75% v/v 1 M NaOH in saline) were given as a bolus injection via the jugular vein. Plasma and urinary levels of [^{3}H]-inulin and [^{14}C]-PAH were determined by dual isotope liquid scintillation counting. Plasma and urinary concentrations of Na^+, K^+ and Li^+ were determined by flame photometry (Corning 480) and chloride levels were measured with a chloride meter (Corning 925). The urinary concentration of uric acid was also determined using a standard spectrophotometric assay.

Standard formulae were used to calculate clearances of sodium (C_{Na^+}), potassium (C_{K^+}), chloride (C_{Cl^-}), lithium (C_{Li^+}), inulin (C_{IN}) and PAH (C_{PAH}). Filtration fraction was determined from $C_{IN}/C_{PAH} \times 100$ and fractional excretion of the various ions was derived from: clearance values/$C_{IN} \times 100$. Plasma lithium concentrations recorded in vehicle and CPX-treated rats were 490 ± 30 μM, and 500 ± 30 μM, $n = 6$ respectively. These concentrations are the mean levels determined during the two control clearance periods. Lithium reabsorption is reported to take place largely in the proximal tubule and to the same extent as sodium and water, and therefore gives a marker of fluid delivery out of the proximal tubule (Thomsen, 1984). As a result the following assumptions can be made:

- C_{Li^+}/C_{IN} = fractional excretion of fluid from proximal tubule
- $C_{IN} - C_{Li^+}$ = absolute amount of fluid reabsorbed by proximal tubule
- $C_{Li^+} - V$ = absolute amount of water reabsorbed beyond proximal tubule
- $C_{Li^+} - C_{Na^+}$ = absolute amount of sodium reabsorbed beyond proximal tubule
- V/C_{Li^+} = fractional excretion of water beyond proximal tubule
- C_{Na^+}/C_{Li^+} = fractional excretion of sodium beyond proximal tubule

where V = urine flow (Johns, 1988).

Free water reabsorption ($T^c_{H_2O}$)

Both food and water were removed from Wistar rats 16 h before the start of each experiment. Following surgery, s.c. injections of [Arg^8] vasopressin (ADH, 0.1 units h^{-1}) were administered into the neck. A bolus injection (0.2 ml) of isotonic saline containing 3.5 μCi ml^{-1} [^{3}H]-inulin was then given via the jugular vein, followed by an infusion of this solution at a rate of 10 μl min^{-1}. Thirty minutes later, either CPX (0.1 mg kg^{-1}) or its vehicle (1 ml kg^{-1}) were given via the jugular vein. At the same time 2% w/v NaCl was infused via the femoral vein at a rate of 100 μl min^{-1}. After 10 min of hypertonic saline infusion, urine was collected for two successive 15 min clearance periods with a midpoint blood sample taken to calculate osmolar clearance (C_{OSM}). The infusion rate of 2% saline was progressively increased from 100 to 200, 300, 400, 500 and finally to 750 μl min^{-1} and after a change of infusion rate, 10 min was allowed for equilibration before the next two collection periods. Two 15 min urine collections and appropriate blood samples were taken for C_{OSM} determination during each saline infusion.

Plasma and urinary osmolality were measured by freezing point depression (Advanced Digimatic 3DII). Free water reabsorption ($T^c_{H_2O}$) was calculated from osmolar clearance – urine flow ($C_{OSM} - V$). A graph was then constructed depicting fractional free water reabsorption $T^c_{H_2O}/C_{IN}$ as a function of the fractional distal delivery of sodium. The fractional osmolar clearance (C_{OSM}/C_{IN}) is taken as an index of distal sodium delivery (Opava-Stitzer & Martinez-Maldonado, 1978).

Free water clearance (C_{H_2O})

Male Long Evans hooded rats (Brattleboro strain) were used to determine free water clearance because of their congenital deficiency of ADH. As soon as surgery was complete, an infusion was started via the femoral vein of hypotonic saline (0.225% w/v NaCl) at a rate of 100 μl min^{-1}. After which, a bolus injection and infusion of [^{3}H]-inulin in saline was given via the jugular vein, as described for the free water reabsorption experiments. An hour was allowed for stabilization of urine flow, then either CPX (0.1 mg kg^{-1}) or its vehicle (1.0 ml kg^{-1}) was injected into the jugular vein. Animals received successive infusions of 0.225% saline increasing from 100 to 200 and 300 μl min^{-1}, after which 0.45% w/v NaCl was infused at 200, 300 and 400 μl min^{-1} (Martinez-Maldonado & Opava-Stitzer, 1978). At each infusion rate a 10 min equilibration period preceded two 15 min urine collections with midpoint blood samples in order to measure both C_{Na^+} and C_{OSM}.

C_{H_2O} was calculated from $V - C_{OSM}$. In a simular manner to the free water reabsorption experiments, a graph was constructed of fractional free water clearance (C_{H_2O}/C_{IN}) as a function of fractional distal sodium delivery. In these experiments however, $(C_{Na^+} + C_{H_2O})/C_{IN}$ was used as the index of fractional distal sodium delivery (Martinez-Maldonado & Opava-Stitzer, 1978).

Analysis of data

Results are given as mean ± s.e.mean. Means were compared by either a paired or non-paired Student's *t* test. Paired comparisons were made before and after vehicle or CPX injection in the analysis of renal function data in the ion excretion experiments. The results of these comparisons are detailed in the Results section. In addition, the various indices of renal function following vehicle and CPX treatment were compared by a non-paired test. This analysis revealed that CPX induced the same pattern of statistically significant changes in renal function as was noted for the paired comparisons.

Graphs of fractional free water reabsorption ($T^c_{H_2O}/C_{IN}$) versus fractional distal sodium delivery (C_{OSM}/C_{IN}), and frac-

tional free water clearance (C_{H_2O}/C_{IN}) as a function of fractional distal sodium delivery ($C_{Na^+} + C_{H_2O})/C_{IN}$ were fitted by non-linear least squares regression analysis to the following equation $y = a.x/(x + b)$, where a and b are constants. Applying this equation to experimental plots; a is the maximum value of $T^c_{H_2O}/C_{IN}$ or C_{H_2O}/C_{IN} and b is the fractional distal delivery of sodium which gives half maximum $T^c_{H_2O}/C_{IN}$ or C_{H_2O}/C_{IN}.

Materials

[^{3}H(G)]-inulin (355.5 mCi g^{-1}) and *p*-[glycyl-1-^{14}C]-aminohippuric acid (60 mCi mmol^{-1}) were obtained from NEN Research Products. The stated radiochemical purity of each isotope was greater than 98%. CPX was synthesized by Dr R. James of ICI Pharmaceuticals. The reagent kit for the assay of uric acid was purchased from Sigma Chemical Co. and [Arg^8] vasopressin was obtained from Parke Davis Co.

Results

In a series of experiments designed to assess the effect of CPX (0.1 mg kg^{-1}) on ion excretion, free water reabsorption and free water clearance, administration of vehicle or CPX produced no statistically significant effect ($P > 0.05$) on either mean arterial blood pressure or heart rate. For example, in the ion excretion experiments mean arterial blood pressure and heart rate measured 5 min after CPX injection were: 105 ± 6 mmHg and 401 ± 8 beats min^{-1}, $n = 6$; compared to control values of 99 ± 8 mmHg and 392 ± 6 beats min^{-1}, $n = 6$.

Ion excretion

Intravenous administration of the vehicle for CPX (1 ml kg^{-1}) had no statistically significant effect ($P > 0.05$) on urine flow, renal haemodynamics or any of the excretory variables monitored during these experiments. However, injection of CPX (0.1 mg kg^{-1}) resulted in a marked diuresis with urine flow increasing 3 fold in the collection period (0–15 min) immediately following drug administration (Figure 1a). The rate of urine production remained elevated throughout the experimental period, but was only significantly ($P < 0.05$) elevated for the first two collection periods, 0–30 min (Figure 1a). The increase in urine flow was accompanied by an initial rise of 39% ($P < 0.05$) in C_{IN} which returned to control levels by the second clearance period (Figure 1b). By contrast to the changes in urine flow and C_{IN}, CPX administration produced no significant changes in C_{PAH} (Figure 1c). Figure 1d shows there was a small increase in filtration fraction from 43% to 57% following injection of CPX. However, this change was not statistically significant ($P > 0.05$) and subsequently, there were no marked changes in filtration fraction.

Table 1 shows that the excretion rate of Na^+ and Cl^- increased approximately 3.5 fold in the first collection period after CPX, before slowly declining during the course of the experiment. The rate of excretion of both ions was significantly elevated ($P < 0.05$) in the first two collection periods (Table 1). The fractional excretion of Na^+ and Cl^- was also significantly ($P < 0.05$) elevated during these periods, reflecting reduced tubular reabsorption of these ions. The excretion rate of K^+ also increased immediately following injection of CPX; but by contrast to Na^+ and Cl^-, this rise was not statistically significant ($P > 0.05$) and fell to

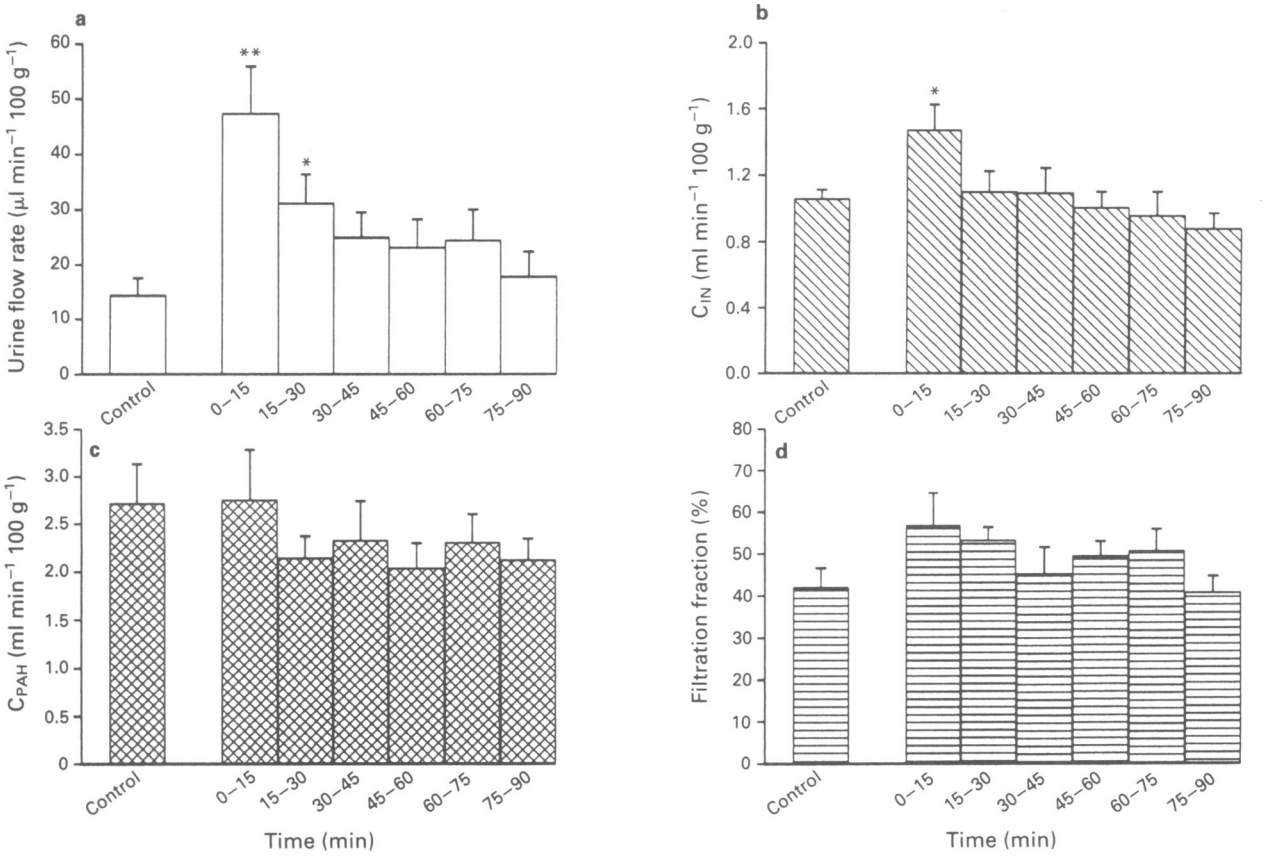


Figure 1 The effect of 8-cyclopentyl-1,3-dipropylxanthine (CPX, 0.1 mg kg^{-1}, i.v.) on (a) urine flow, (b) [^{3}H]-inulin clearance (C_{IN}), (c) [^{14}C]-*p*-aminohippuric acid clearance (C_{PAH}) and (d) filtration fraction in Wistar rats. The control values are the mean of two clearance periods prior to CPX administration. Columns represent the mean with s.e.mean ($n = 6$). *$P < 0.05$; **$P < 0.01$ relative to controls (paired t test).

Table 1 Effect of 8-cyclopentyl-1,3-dipropylxanthine (CPX, 0.1 mg kg^{-1}, i.v.) on sodium, potassium and chloride excretion in the rat

	Control	*Time after CPX injection* (min) (0–15)	(15–30)	(30–45)	(45–60)	(60–75)	(75–90)
Na^+ excr. (μmol min^{-1} 100 g^{-1})	2.0 ± 0.7	7.3 ± 1.7**	6.4 ± 1.4**	4.9 ± 1.2	4.2 ± 1.1	4.4 ± 0.9	3.3 ± 0.6
FE Na^+ (%)	1.4 ± 0.5	3.8 ± 1.1**	3.9 ± 0.8**	3.0 ± 0.5	2.9 ± 0.7	3.2 ± 0.9	2.6 ± 0.5
K^+excr. (μmol min^{-1} 100 g^{-1})	1.1 ± 0.2	2.9 ± 1.0	1.4 ± 0.2	1.2 ± 0.2	1.1 ± 0.2	1.3 ± 0.2	1.0 ± 0.2
FE K^+ (%)	35 ± 5.5	56 ± 13	37 ± 5.1	32 ± 5.8	32 ± 6.0	36 ± 6.5	31 ± 4.5
Cl^- excr. (μmol min^{-1} 100 g^{-1})	2.8 ± 0.7	10 ± 1.8**	7.1 ± 1.0*	5.9 ± 1.0	6.4 ± 1.9	5.3 ± 0.9	4.0 ± 0.9
FE Cl^- (%)	2.7 ± 0.8	7.0 ± 1.4*	5.8 ± 0.7*	5.3 ± 1.1	4.6 ± 2.0	5.3 ± 1.2	4.4 ± 1.2

Results are given as mean ± s.e.mean ($n = 6$). Control values are the mean of two collection periods prior to CPX administration. FE = fractional excretion i.e. clearance/C_{IN}.
*$P < 0.05$; **$P < 0.01$ (paired t test) relative to controls.

control levels by the second collection period (Table 1). In addition the fractional excretion of K^+ showed only a modest rise ($P > 0.05$) in the first period following CPX, returning to the control level by the second period. The urinary excretion of uric acid was not significantly ($P > 0.05$) affected by administration of CPX. The control excretion rate was 5.3 ± 1.9 nmol min^{-1} 100 g^{-1}($n = 6$) and in the collection periods 0–15, 30–45 and 75–90 min following CPX the excretion rates were: 4.5 ± 0.8, 7.3 ± 2.1 and 5.4 ± 0.2 nmol min^{-1} 100 g^{-1} ($n = 6$), respectively. These data indicate that CPX at a dose of 0.1 mg kg^{-1} has neither uricosuric properties nor impairs the urinary excretion of uric acid.

CPX induced a 2 fold increase in the fractional excretion of Li^+ in the first clearance period after drug administration. This suggests that a greater proportion of the fluid filtered at the glomerulus leaves the proximal tubule and enters the loop of Henlé (Table 2). Following this rise, the fractional excretion of Li^+ remained elevated throughout the experiment, but was only statistically different ($P < 0.05$) from control values during the first two periods (0–30 min). The increase in fractional excretion of Li^+ was accompanied by a fall in the absolute amount of fluid reabsorbed from the proximal tubules ($C_{IN} - C_{Li+}$). This was maintained throughout the experiment, but elevations of $C_{IN} - C_{Li+}$ were not significantly different from the control value. Table 2 also shows that in the first clearance period after CPX injection there were 3 fold rises in the reabsorption of both water ($C_{Li+} - V$) and sodium ($C_{Li+} - C_{Na+}$) in regions beyond the proximal tubule. Following the first clearance period, the distal reabsorption of water and sodium declined, although remaining higher than control levels throughout the experiment. However, in none of the clearance periods following CPX administration were there any statistically significant ($P < 0.05$) differences in comparison to control values. In addition, there were no significant differences ($P > 0.05$) between control estimates of fractional excretion of water (V/C_{Li+}) or sodium (C_{Na+}/C_{Li+}) from distal nephron regions and any of the respective estimates made after CPX injection (Table 2).

Free water reabsorption and clearance

The relationship between fractional free water reabsorption ($T^c_{H_2O}/C_{IN}$) and fractional osmolar clearance (C_{OSM}/C_{IN}) after administration of either vehicle or CPX is shown in Figure 2. It is clear from this figure that the relationship is non-linear and that the data can be fitted to a rectangular hyperbola, $y = a.x/(x + b)$. The values of a and b derived from these curves showed that there was no detectable effect of CPX on the reabsorption of free water. The mean value of a in the rats treated with vehicle was 0.14 ± 0.02, $n = 8$ compared to 0.23 ± 0.08, $n = 8$ in CPX-treated rats whilst the values of b were 0.17 ± 0.06 and 0.33 ± 0.17 for vehicle and CPX treatment respectively. For each parameter there were no

Table 2 Effect of 8-cyclopentyl-1,3-dipropylxanthine (CPX, 0.1 mg kg^{-1}, i.v.) on lithium clearance and related indices of tubular function in the rat

	Control	*Time after CPX injection* (min) (0–15)	(15–30)	(30–45)	(45–60)	(60–75)	(75–90)
FE_{Li+} (%)	29 ± 6.0	62 ± 19*	54 ± 12*	48 ± 12	54 ± 15	57 ± 18	45 ± 12
$C_{IN}-C_{Li+}$ (μl min^{-1} 100 g^{-1})	758 ± 84	573 ± 200	530 ± 147	611 ± 149	517 ± 171	562 ± 184	546 ± 132
$C_{Li+}-V$ (μl min^{-1} 100 g^{-1})	283 ± 30	870 ± 315	574 ± 140	504 ± 140	496 ± 125	493 ± 121	365 ± 72
V/C_{Li+} (%)	5.8 ± 2.0	9.9 ± 4.1	7.2 ± 1.8	5.9 ± 0.9	5.1 ± 0.5	6.5 ± 1.8	5.7 ± 1.2
$C_{Li+}-C_{Na+}$ (μl min^{-1} 100 g^{-1})	297 ± 26	869 ± 322	546 ± 148	496 ± 137	491 ± 125	490 ± 121	367 ± 92
C_{Na+}/C_{Li+} (%)	5.4 ± 2.1	6.0 ± 2.3	6.2 ± 1.2	7.3 ± 1.1	6.4 ± 1.0	6.8 ± 1.2	6.3 ± 1.1

Results are given as mean ± s.e.mean ($n = 6$). Control values are the mean of two collection periods prior to CPX administration. FE_{Li+} = fractional excretion of lithium, $C_{IN}-C_{Li+}$ = absolute reabsorption of fluid in the proximal tubule, $C_{Li+}-V$ = reabsorption of water from distal nephron regions, V/C_{Li+} = fractional excretion of water from distal nephron regions, $C_{Li+}-C_{Na+}$ = absolute reabsorption of sodium from distal nephron regions, C_{Na+}/C_{Li+} = fractional excretion of sodium from distal nephron regions.
*$P < 0.05$ (paired t test) relative to controls.

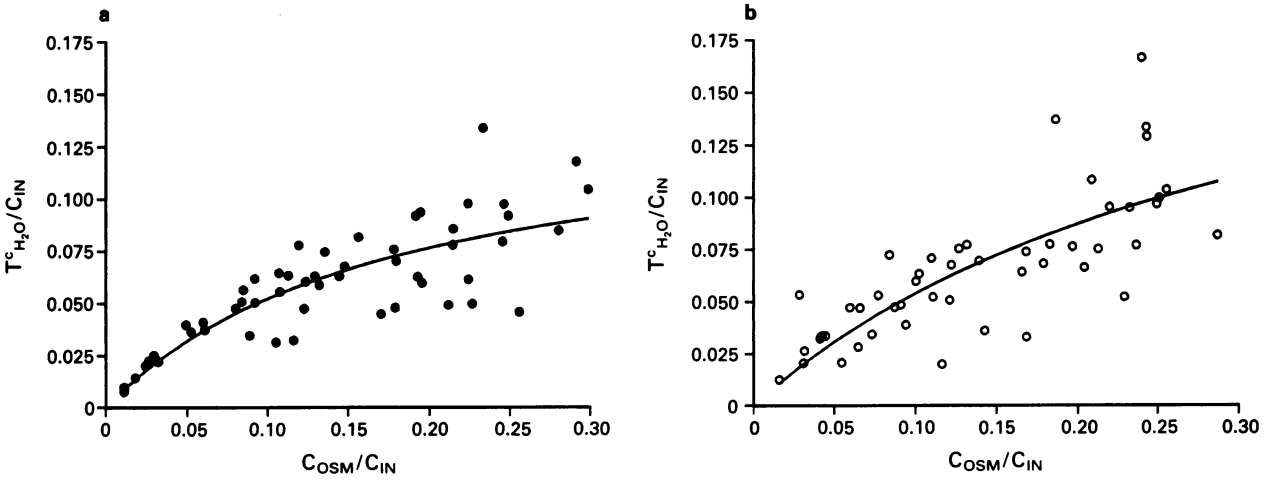


Figure 2 The relationship between fractional free water reabsorption ($T^c_{H_2O}/C_{IN}$) and fractional distal delivery of sodium which was derived from fractional osmolar clearance (C_{OSM}/C_{IN}) in Wistar rats which received [Arg^8] vasopressin (0.1 unit h^{-1}) and were treated with either (a) vehicle (1.0 ml kg^{-1}, i.v., $n = 8$) or (b) 8-cyclopentyl-1,3-dipropylxanthine (0.1 mg kg^{-1}, i.v., $n = 8$). The data were fitted to the equation $y = a.x/(x + b)$ and the line of best fit is shown.

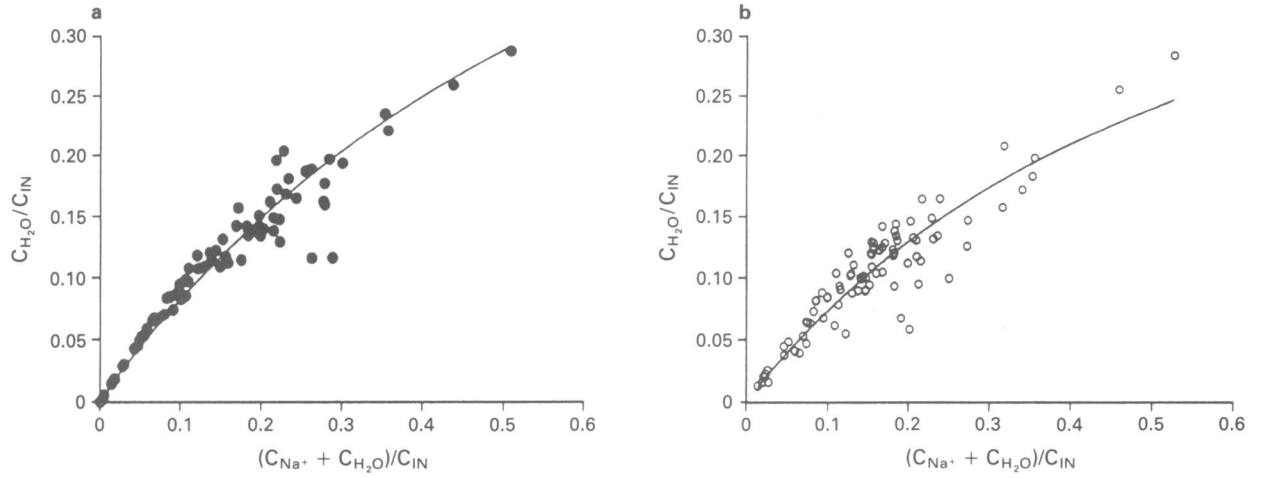


Figure 3 The relationship between fractional free water clearance (C_{H_2O}/C_{IN}) and fractional distal delivery of sodium which was derived from ($C_{Na^+} + C_{H_2O}$)/C_{IN} in Long Evans hooded rats (Brattleboro strain) treated with either (a) vehicle (1.0 ml kg^{-1}, i.v., $n = 8$) or (b) 8-cyclopentyl-1,3-dipropylxanthine (0.1 mg kg^{-1}, i.v., $n = 8$). The data were fitted to the equation $y = a.x/(x + b)$ and the line of best fit is shown.

significant differences ($P > 0.05$) between values derived from vehicle and CPX-treated rats.

The relationship between fractional free water clearance (C_{H_2O}/C_{IN}) and fractional distal delivery of sodium, ($C_{OSM} + C_{Na^+}$)/C_{IN}, was also fitted to the equation $y = a.x/(x + b)$ as shown in Figure 3. CPX was without effect on this relationship with no significant differences ($P > 0.05$) in values of a and b between CPX and vehicle-treated groups. The mean values of a were 0.78 ± 0.14 and 0.55 ± 0.09 and for b were 0.85 ± 0.19 and 0.65 ± 0.14 in vehicle ($n = 8$) and CPX ($n = 8$)-treated rats respectively.

Discussion

This study has shown that following i.v. administration, CPX produces a diuresis and increases in sodium and chloride excretion which are not accompanied by a commensurate increase in potassium excretion. We have demonstrated that despite a brief elevation of C_{IN}, the primary diuretic mechanism of CPX appears to be of tubular origin. Significant increases in the fractional excretion of sodium and chloride indicate that a greater proportion of these ions, once filtered are excreted from the nephron following CPX administration. Although rises in potassium excretion were noted immediately after CPX was given, at no time was there a significant elevation of either absolute or fractional potassium excretion above control levels. Several studies have shown that the natriuresis induced by alkylxanthines is not associated with a corresponding increase in potassium excretion (Persson *et al.*, 1982; Andersson *et al.*, 1984; Collis *et al.*, 1991). The mechanism behind this potassium sparing phenomenon is unknown.

The fractional excretion of lithium was also significantly increased implicating the proximal tubule as a principal location for the decrease in sodium reabsorption produced by CPX. The non-selective adenosine antagonist aminophylline has also been reported to reduce sodium reabsorption in the proximal tubule as part of its diuretic action (Brater *et al.*, 1983). Under most conditions lithium ions are reabsorbed by the proximal tubule to the same extent as sodium ions but undergo no further reabsorption by distal regions of the tubule (Thomsen, 1984). Distal reabsorption of lithium has been noted in animals fed a low sodium diet (Thomsen & Leyssac, 1986). However, in the current study rats were fed on a standard diet and consequently, lithium clearance is able to give a marker of the amount of sodium, and therefore fluid, leaving the proximal tubule. Using this technique it was

possible to show that CPX induced a significant rise in the volume of fluid leaving the proximal tubule and entering the loop of Henlé. The data also showed that there were no statistically significant changes in the fractional excretion of sodium or water through distal regions of the nephron. However, the data for fractional lithium clearance and other estimates of tubular function dependent on lithium clearance showed considerable variation which may have obscured any effect of CPX on the distal nephron.

We found the brief increase in C_{IN} induced by CPX was not accompanied by a rise in C_{PAH} indicating that the elevation in glomerular filtration rate (GFR) that occurs 0–15 min after injection of CPX is not associated with an increase in renal blood flow. This suggests that the rise in C_{IN} is a consequence of either a rise in glomerular capillary pressure resulting from an increase in post to pre glomerular arteriolar resistance, in which total renal resistance remains unchanged, or an increase in the glomerular ultrafiltration coefficient. One interesting feature of the diuretic effect of CPX is that despite the increased distal delivery of sodium there was no fall in C_{IN} or C_{PAH} triggered by the tubuloglomerular feedback mechanism. This mechanism, whereby an increase in sodium load at the macula densa results in a fall in single nephron GFR, is believed to be mediated by adenosine stimulating A_1 adenosine receptors present on the afferent arterioles (Schnermann *et al.*, 1990). CPX has been demonstrated to block this mechanism (Schnermann *et al.*, 1990) and this blockade may form a component of the diuretic effect of CPX in which a reduction of proximal sodium reabsorption is linked with an impaired ability of the tubuloglomerular feedback mechanism to adjust GFR to the increased delivery of sodium to the macula densa.

The findings of this investigation also suggest that CPX had no significant effect upon the concentration/dilution mechanism of the thick ascending limb of the loop of Henlé. Transport of sodium and chloride into the medullary interstitium by this water impermeable region of the tubule is responsible for the dilution of tubular fluid and the creation of solute 'free' water. This free water can later be reabsorbed by the distal tubules and collecting ducts under the influence of ADH and the medullary hypertonicity generated by the thick ascending limb. We have determined $T^c_{H_2O}$ and C_{H_2O} in order to assess both of these functions and found no modification of either process at varying rates of solute delivery (Figures 2 and 3). This contrasts with the findings of Brater *et al.* (1983), who reported a modest 15% fall in C_{H_2O} following theophylline administration to man, although in this study there were no measurements of C_{H_2O} in response to a range of solute loads.

Since CPX at the dose used in the present study (0.1 mg kg^{-1}) has been shown to be a potent and selective A_1 antagonist *in vivo* (Kellett *et al.*, 1989; Collis *et al.*, 1991), the current findings provide further support that a diuresis can be induced by A_1 adenosine antagonism. This diuretic effect may result from inhibition of sodium-dependent phosphate uptake and sodium-coupled glucose transport because these transport pathways have been shown to be stimulated via activation of A_1 adenosine receptors in cultured opossum kidney cells, a continuous cell line that resembles the proximal tubule (Coulson *et al.*, 1991). Further evidence for the diuretic effect of A_1 adenosine antagonism is provided by the report of a non-xanthine A_1 selective adenosine antagonist which also possesses natriuretic and diuretic activity (Terai *et al.*, 1990). In addition, alkylxanthines which are selective A_2 adenosine receptor antagonists appear to be devoid of diuretic effects (Suzuki *et al.*, 1992). The duration of the diuretic effect of CPX noted in the present study was short in that urine flow had almost returned to control levels by the last collection period (75–90 min). However, the duration of adenosine A_1 receptor blockade produced by 0.1 mg kg^{-1} of CPX, as determined by antagonism of adenosine-induced bradycardia, persists for longer than 5 h (Knight *et al.*, 1991). This difference in time course could be due to an increase in renal adenosine levels, as a consequence of increased distal tubule sodium delivery, which attenuates the adenosine antagonism afforded by CPX. Future studies using higher doses of CPX or multiple administrations may help to resolve this question.

In summary, the results of this study show that the diuresis and increased excretion of sodium and chloride induced by CPX in the rat, occurs with only transient changes in GFR and no change in renal blood flow. In addition, our findings suggest an inhibition of sodium reabsorption in the proximal tubule is the primary reason for the increased urine flow without any detectable change in the concentration/dilution mechanism. This study also provides further evidence that endogenous adenosine affects renal excretory function via stimulation of the A_1 receptor subtype. A_1 receptors generally have affinities for adenosine analogues in the nanomolar range; three orders of magnitude greater than A_2 receptor affinity (Olsson & Pearson, 1990). Such high affinity receptors have a slow dissociation rate, and are likely to be well suited to provision of tonic background modulation of electrolyte and water excretion by the kidney (Olsson & Pearson, 1990).

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Effect of tienoxolol, a new diuretic β-blocking agent, on urinary prostaglandin excretion in the rat

François Caussade & [1]Alix Cloarec

Laboratoires UPSA, 128, rue Danton, BP 325, 92506 Rueil-Malmaison Cedex, France

1 The effects of tienoxolol, (ethyl 2-[3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]-5-[(2-thienylcarbonyl) amino] benzoate, hydrochloride), a novel drug exhibiting both diuretic and β-adrenoceptor blocking properties, were investigated on urinary 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) and PGE_2 excretion in the rat and compared to those of reference diuretic (furosemide) and β-adrenoceptor antagonists (acebutolol, propranolol). Since tienoxolol was shown to bind to A_1 and A_2 adenosine receptors, the action of theophylline was also evaluated.

2 Tienoxolol (8–128 mg kg^{-1}, p.o.) induced a dose-related increase of 6-keto-$PGF_{1\alpha}$ excretion from 32 mg kg^{-1} but a significant elevation of urinary PGE_2 levels was only reached after administration of 128 mg kg^{-1}. However, renal prostaglandin concentrations were not modified by tienoxolol.

3 Furosemide (32 mg kg^{-1}) displayed a strong diuretic activity but did not enhance 6-keto-$PGF_{1\alpha}$ excretion. Likewise, the latter was unaffected by acebutolol and propranolol (128 mg kg^{-1}) and no significant diuresis was observed following administration of these two β-blocking agents. Theophylline (64 mg kg^{-1}), like tienoxolol, was able to induce both diuresis and urinary prostaglandin excretion. Furthermore, they bound with similar affinities to A_1 and A_2 adenosine receptors. This led to the suggestion that a relationship between P_1-purinoceptors, prostaglandin release, diuresis and natriuresis could exist.

4 Oral co-administration of NECA (0.2 mg kg^{-1}) with tienoxolol markedly reduced the urinary 6-keto-$PGF_{1\alpha}$ excretion observed when tienoxolol was administered alone. However, neither diuresis nor natriuresis were modified, demonstrating that the proposed relationship was untenable.

5 In conclusion, PGI_2 probably does not participate in the diuretic and natriuretic activity of tienoxolol. The increase of urinary 6-keto-$PGF_{1\alpha}$ excretion may result not only from the haemodynamic properties of the drug but also from the rise of the urinary flow induced by tienoxolol.

Keywords: Diuretic drug; diuresis; β-adrenoceptor antagonist; natriuresis; adenosine receptor antagonist; urine; PGE_2; kidney; 6-keto-$PGF_{1\alpha}$

Introduction

Prostaglandins are formed in both the renal medulla and cortex, and participate in several aspects of renal physiology. Prostacyclin (PGI_2) mainly produced in the glomerulus, modulates cortical functions (maintenance or increase of renal blood flow, regulation of glomerular filtration rate, renin secretion) (Anderson *et al.*, 1976; Weber *et al.*, 1976; Dunn & Hood, 1977; Whorton *et al.*, 1977; Terragno *et al.*, 1978). Prostaglandin E_2 (PGE_2), synthesized primarily in the papilla, regulates medullary functions (water, sodium and chloride excretion) (Anderson *et al.*, 1976; Fine & Trizna, 1977; Stokes, 1979; Sraer *et al.*, 1982).

Diuretic-induced hypotension and salt and water loss are considered to be, at least in part, mediated by prostaglandins (Gullner *et al.*, 1980; Tannenbaum *et al.*, 1975).

Tienoxolol (Figure 1) is a novel drug, combining in the same molecule diuretic, natriuretic and β-adrenoceptor blocking properties which have previously been demonstrated not only in normotensive conscious rats and monkeys after oral dosing (Cloarec *et al.*, 1984; Bouley *et al.*, 1986), but also in man (Berdeaux *et al.*, 1988). Furthermore, tienoxolol binds to A_1 and A_2 adenosine receptors (previous unpublished observations).

Since the mechanism(s) underlying the diuretic and natriuretic effects of this compound is (are) not yet known, the aim of the present study was to investigate whether urinary prostaglandin excretion and binding to adenosine receptors could be involved in tienoxolol-induced diuresis and natriuresis.

[1] Author for correspondence.

Methods

Collection of urine and kidneys

Male CD rats (Charles River France) weighing 130–140 g were fasted overnight with free access to water. Tienoxolol (8–128 mg kg^{-1}), acebutolol (128 mg kg^{-1}), propranolol (128 mg kg^{-1}), furosemide (32 mg kg^{-1}) and theophylline (64 mg kg^{-1}) were administered orally (0.5 ml 100 g^{-1} body weight).

All the animals received an intraperitoneal water load of 25 ml kg^{-1}, and were then housed in groups of two in metabolism cages. Six hours later, urine samples were collected with aspirin 11.5 mM and frozen until PGE_2 and 6-keto-$PGF_{1\alpha}$ determination. Urine volume was measured in each metabolism cage.

Rats were also treated by oral co-administration of 5'-(N-ethylcarboxamido) adenosine (NECA) (0.2 mg kg^{-1}) with tienoxolol (128 mg kg^{-1}) or theophylline (64 mg kg^{-1}). At the end of the 6 h period, urine was collected either in the presence of aspirin 11.5 mM to determine prostaglandin levels or in the absence of aspirin to measure natriuresis by flame

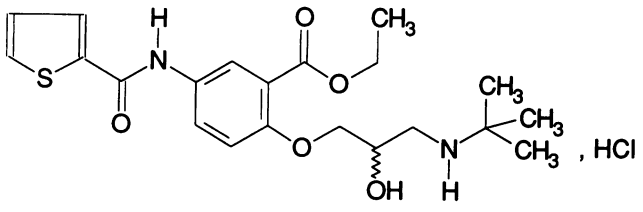


Figure 1 Chemical structure of tienoxolol.

photometry. Thirty min, 2 h, 4 h and 6 h after oral administration of tienoxolol (8, 32 and 128 mg kg^{-1}), rats were killed. Whole kidneys were quickly excised and frozen until PGE_2 and 6-keto-$PGF_{1\alpha}$ extraction.

Prostaglandin extraction

PGE_2 and 6-keto-$PGF_{1\alpha}$ extraction procedure was carried out as previously described (Powell, 1980) with slight modifications. Kidneys were thawed at room temperature and homogenized in 10 volumes (w/v) of ice-cold buffer (50 mM Tris HCl, 11.5 mM aspirin, pH 7.4). Homogenization was performed with a tissue disrupter Ultra-Turrax (Janke & Kunkel, Stauffen, Germany) for 30 s at 4°C (60–70% of the maximal speed), followed by a glass-teflon potter homogenizer (Prosciences, Paris, France) (setting 6). Aliquots (1 ml) of the homogenate were mixed with absolute ethanol (3 ml) and the mixtures were allowed to stand for 5 min. Distilled water (16 ml) was then added and the mixtures were centrifuged at 480 *g* for 20 min at 4°C (centrifuge J2-21 M/E, Beckman, Gagny, France). The supernatant was removed, transferred to another tube, and the pH was adjusted to 3–4 with 0.1 N HCl (250 µl).

An octadecylsilyl silica column (Amersham, Les Ulis, France) was used for PGE_2 and 6-keto-$PGF_{1\alpha}$ extraction. The column was first conditioned with 20 ml absolute ethanol followed by 20 ml distilled water. The acidified supernatant was then introduced to the column and eluted sequentially with 20 ml ethanol/water (15:85), 20 ml petroleum ether and 5 ml methyl formate. The methyl formate fraction was evaporated under a stream of nitrogen and the dried extract was dissolved in 500 µl absolute ethanol and 500 µl distilled water. The dissolved extract was stored at −20°C until prostaglandin determination.

Since the use of unextracted urine samples has been validated by many authors (Granstrom & Kindahl, 1978; Quilley *et al.*, 1986), urinary PGE_2 and 6-keto-$PGF_{1\alpha}$ concentrations were measured in unextracted samples.

Prostaglandin radioimmunoassay

PGI_2 was measured with a radioimmunoassay kit for 6-keto-$PGF_{1\alpha}$, the stable hydrolysis product of PGI_2. Briefly, 100 µl aliquots of urine or renal tissue extract, [^{125}I]-6-keto-$PGF_{1\alpha}$ ($\approx$33000 c.p.m.), 6-keto-$PGF_{1\alpha}$ antiserum (cross-reactivity of less than 1% with PGE_2, $PGF_{2\alpha}$, PGE_1, $PGF_{1\alpha}$. TXB_2) and buffer (0.05 M phosphate buffer, pH 6.8, with 0.05% bovine serum albumin) were incubated in a total volume of 500 µl overnight at 4°C. Standards covered the range 3.1 pg up to 400 pg of 6-keto-$PGF_{1\alpha}$. Bound and free antigen were separated by means of 500 µl of donkey anti-rabbit second antibody bound to magnetizable polymer particles. Then, separation was carried out by application of a magnetic field and the supernatant was removed. The radioactivity in the pellet was determined by solid scintillation spectroscopy using a Kontron Gamma Counter at a counting efficiency of 76%. Each assay was performed in duplicate.

PGE_2 was measured with a radioimmunoassay kit for methyl oximated PGE_2 obtained by conversion of PGE_2 by methoxamine hydrochloride. Following methyl oximation of PGE_2 contained in extracted or unextracted samples, the latter (100 µl) were incubated for 2 h at room temperature with [^{125}I]-PGE_2 (methyl oximate derivative) ($\approx$35000 c.p.m.) and PGE_2 antiserum specific for the methyl oximate derivative (cross-reactivity of less than 1% with PGD_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, TXB_2), in a total volume of 300 µl. Standards covered the range 1.25 pg up to 160 pg of methyl oximated PGE_2. Separation of bound and free antigen was then achieved by using 250 µl of donkey anti-rabbit serum coated onto magnetizable polymer particles. The remainder of the experiment was similar to that described for 6-keto-$PGF_{1\alpha}$ radioimmunoassay.

Preparation of brain membranes for adenosine binding assays

To study A_1 adenosine receptors, crude rat brain membranes were prepared according to a previously described method (Schwabe & Trost, 1980). Male CD rats (Charles River France), weighing 180 to 280 *g*, were killed. Whole brains were quickly removed and homogenized in 25 volumes of ice-cold 50 mM Tris HCl, pH 7.4, using an Ultra-Turrax (30 s at 70% maximal setting). The homogenate was centrifuged at 1000 *g* for 10 min at 4°C and the resulting supernatant was centrifuged at 48000 *g* for 20 min at 4°C. The pellet was resuspended in 4 volumes of ice-cold homogenization buffer, incubated with adenosine deaminase (1 µg ml^{-1}) for 30 min at room temperature and centrifuged as before. The final pellet was resuspended in 10 volumes of ice-cold 50 mM Tris-HCl, pH 7.4, and membranes were used either immediately or after storage at −20°C.

A_2-adenosine receptors were studied in rat striatal membranes prepared as previously described (Bruns *et al.*, 1986). Briefly, striata were disrupted in 10 volumes of ice-cold buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.7) for 30 s with an Ultra-Turrax (70% maximal setting). The homogenate was centrifuged at 50000 *g* for 10 min at 4°C. The pellet was resuspended in 10 volumes of buffer, incubated with adenosine deaminase (1 µg ml^{-1}) for 30 min at room temperature and finally centrifuged as before. The resulting pellet was resuspended in 5 volumes of the same buffer and frozen at −70°C.

Radioligand binding assays

The measurement of (−)-N6-**R**-[G-^{3}H]-phenylisopropyl-adenosine ([^{3}H]-PIA) binding to whole brain membranes was performed as described elsewhere (Schwabe & Trost, 1980; Lohse *et al.*, 1984) with slight modifications. Incubations (2 ml) were carried out at 20°C for 30 min in polystyrene tubes containing incubation buffer (50 mM Tris-HCl, pH 7.4), 400 µl of the membrane suspension, 2.5 nM [^{3}H]-PIA and various concentrations of the competing drugs. The reaction was terminated by the addition of 3 ml of cold incubation buffer, followed by rapid filtration through Whatman GF/B glass fibre filters. The filters were immediately washed twice with 3 ml of ice-cold Tris-HCl buffer, transferred into liquid scintillation vials and 10 ml of scintillation fluid were added. Radioactivity was counted by liquid scintillation spectroscopy (Kontron Beta Counter) at a counting efficiency of 56%. Non specific binding was defined as that remaining in the presence of 10 µM N6-(L-2-phenylisopropyl)-adenosine.

The binding of 5'-N-ethylcarboxamido [8(n)-^{3}H]-adenosine ([^{3}H]-NECA) to striatal membranes was achieved according to the method described by Bruns *et al.* (1986) with minor modifications. Membranes (100 µl) were incubated at 25°C during 1 h with 4 nM [^{3}H]-NECA, various concentrations of the test compounds and incubation buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 0.117 µM N6-cyclopentyladenosine (CPA), pH 7.7) in a total volume of 2 ml. CPA was included in the medium to eliminate A1 receptor binding. The reaction was terminated by the addition of 3 ml of ice-cold buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.7) and filtration through Whatman GF/B glass fibre filters. The subsequent washing and counting procedures were as described above. Non specific binding was determined by measurement of the amount of radioactivity retained on filters when incubations were performed in the presence of 5 µM unlabelled NECA.

Data analysis

Data are shown as mean ± s.e.mean.

The one-way analysis of variance was used to identify statistically significant differences between prostaglandin levels in control and treated groups. When a significant difference was reached, comparisons were carried out with

Student's t test for unpaired data. A P value of 0.05 or less was considered to be significant.

Radioligand binding data were analysed by the non linear regression programme LIGAND (Munson & Rodbard, 1980), adapted for an IBM-PC (McPherson, 1985) and obtained from Elsevier-Biosoft (Cambridge, England). The concentration of unlabelled drug causing 50% displacement of [^{3}H]-PIA or [^{3}-H]-NECA from binding sites (IC_{50} value) was calculated by log-logit linear regression analysis of data (EBDA). Then, the latter were analysed assuming models of one and two independent binding sites. A two-site model was accepted over a one-site fit only if it was preferred $P<0.05$ using the partial F-test of the programme. Inhibition constant value (K_i) was calculated according to the Cheng-Prusoff equation (1973). The average K_i values were determined from two to four values obtained from independent experiments carried out with different whole brain or striatal membrane preparations.

Materials

Propranolol, PIA, NECA, CPA and adenosine deaminase type VI were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). [^{3}H]-PIA (35–60 Ci mM^{-1}), [^{3}H]-NECA (20–40 Ci mM^{-1}), [^{125}I]-6-keto-prostaglandin $F_{1\alpha}$ and [^{125}I]-prostaglandin E_2 radioimmunoassay kits were purchased from Amersham International (Amersham, England).

In all *in vivo* studies, tienoxolol (hydrochloride salt, Carpibem, Rueil-Malmaison, France), furosemide (Hoechst, Paris-La Défense, France), acebutolol (Rhône-Poulenc Santé, Vitry, France), propranolol, theophylline (Coopération Pharmaceutique Française, Melun, France) and NECA were prepared as suspensions in aqueous solution (0.1% sodium chloride, 1% arabic gum and 0.01% Tween 80). In radioligand binding studies, drugs were dissolved in dimethylsulphoxide/ethanol (DMSO)/(EtOH) (80/20, v/v).

Aspirin (115 mM) was prepared in a 5% sodium bicarbonate solution then diluted in 50 mM Tris-HCl buffer, pH 7.4, in order to obtain 11.5 mM (final concentration).

Absolute ethanol, petroleum ether and methyl formate were supplied by Prolabo (Paris, France).

Results

Effects of tienoxolol on urinary PGE_2 and 6-keto-$PGF_{1\alpha}$ excretion

The effects of various single oral doses of tienoxolol relative to a vehicle control group on urinary PGE_2 and 6-keto-$PGF_{1\alpha}$ levels are shown in Figure 2. Tienoxolol induced a dose-related increase of urinary 6-keto-$PGF_{1\alpha}$ concentration which was statistically significant from 32 mg kg^{-1} (+100%, $P<0.001$). At 64 mg kg^{-1} and 128 mg kg^{-1}, urinary 6-keto-$PGF_{1\alpha}$ excretion increase was 200% and 284%, respectively. On the other hand, PGE_2 excretion was enhanced only at the highest dose (+115%, $P<0.01$).

Effects of tienoxolol on PGE_2 and 6-keto-$PGF_{1\alpha}$ concentrations in the kidney

The effects of various single oral doses of tienoxolol, at different times, on renal tissue PGE_2 and 6-keto-$PGF_{1\alpha}$ levels are shown in Figure 3. Tienoxolol did not affect prostaglandin concentrations in the kidney.

Comparative effects of tienoxolol, theophylline, furosemide and β-blocking agents on urinary PGE_2 and 6-keto-$PGF_{1\alpha}$ excretion

Urinary 6-keto-$PGF_{1\alpha}$ excretion was weakly enhanced by furosemide (+23%) and propranolol (+39%), while theophylline (+77%, $P<0.01$) produced significant effects on 6-keto-$PGF_{1\alpha}$ excretion (Figure 4). The most marked effect was observed with tienoxolol, whilst acebutolol was completely inactive.

Since tienoxolol and theophylline were able to stimulate 6-keto-$PGF_{1\alpha}$ excretion, the effects of both compounds were investigated on PGE_2 excretion. The latter was markedly increased by theophylline (+269%, $P<0.001$) as compared to tienoxolol (+115%, $P<0.01$) (Figure 4). Theophylline produced much more pronounced effects on urinary excretion of PGE_2 than of 6-keto-$PGF_{1\alpha}$, whereas the reverse was observed with tienoxolol.

Affinity of tienoxolol for A_1 and A_2 adenosine receptors in rat brain homogenates

Tienoxolol and theophylline bound to A_1 and A_2 adenosine receptors, with some selectivity for A_1 binding sites (Table 1). The two compounds exhibited similar affinities on both receptor subtypes although a slightly greater selectivity was observed with tienoxolol ($K_iA_2/K_iA_1 = 8$) than for theophylline ($K_iA_2/K_iA_1 = 5$). Acebutolol bound to A_1 binding sites with a very modest affinity ($K_i = 165 \pm 43$ μM), while propranolol and furosemide did not displace [^{3}H]-PIA and [^{3}H]-NECA binding.

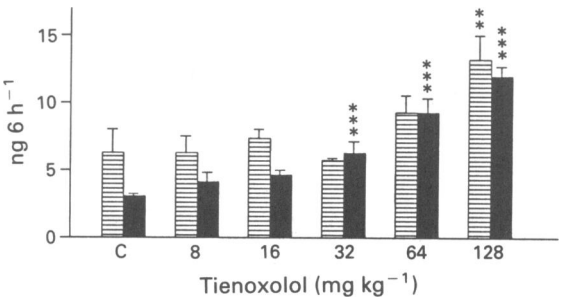


Figure 2 Effects of tienoxolol on urinary prostaglandin E_2 (PGE_2) and 6-keto-$PGF_{1\alpha}$ excretion. Tienoxolol was given by gavage at doses ranging from 8 to 128 mg kg^{-1}. Results represent the accumulation of PGE_2 (horizontally lined columns) and 6-keto-$PGF_{1\alpha}$ (solid columns) in urine at the end of a 6 h period. Values are expressed as mean ± s.e.mean from 6 metabolism cages for PGE_2; from 6 cages (16 and 64 mg kg^{-1}), 12 cages (8 and 32 mg kg^{-1}) and 18 cages (128 mg kg^{-1} and vehicle) for 6-keto-$PGF_{1\alpha}$. **$P<0.001$, ***$P<0.001$ as compared to control group, Student's t test using residual variance.

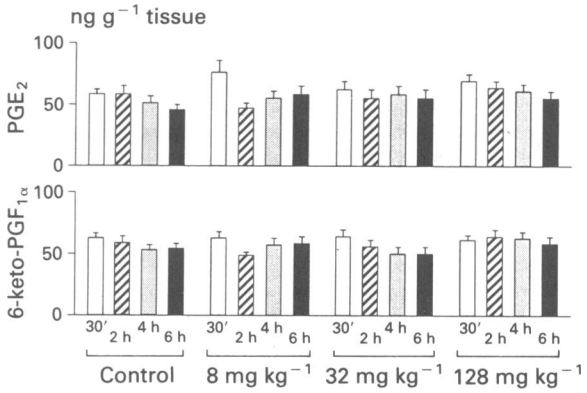


Figure 3 Effects of tienoxolol on prostaglandin E_2 (PGE_2) and 6-keto-$PGF_{1\alpha}$ concentrations in the kidney. Results represent the renal tissue levels of PGE_2 and 6-keto-$PGF_{1\alpha}$, 30 min, 2 h, 4 h or 6 h after oral administration of tienoxolol (8, 32 and 128 mg kg^{-1}) or vehicle. Values are expressed as mean ± s.e.mean from 6 rats (time 30 min) or 12 rats (other times).

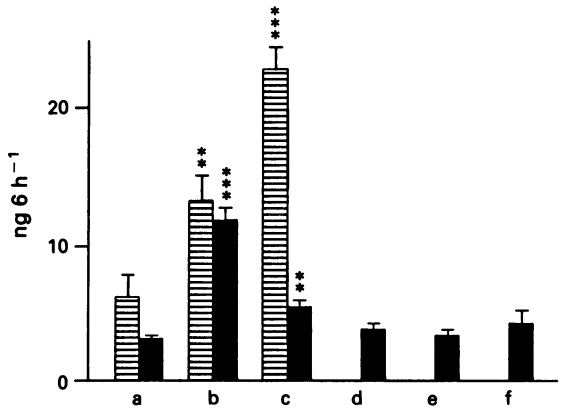


Figure 4 Comparative effects of oral (b) tienoxolol (128 mg kg^{-1}), (c) theophylline (64 mg kg^{-1}), (d) furosemide (32 mg kg^{-1}), (e) propranolol (128 mg kg^{-1}) and (f) acebutolol (128 mg kg^{-1}) on urinary prostaglandin E_2 (PGE_2) and 6-keto-$PGF_{1\alpha}$ excretion; (a) = control. Results represent the accumulation of PGE_2 (horizontally lined columns) and 6-keto-$PGF_{1\alpha}$ (solid columns) in urine at the end of a 6 h period. Values are expressed as mean ± s.e.mean (vertical bars) from 6 metabolism cages for PGE_2, from 5 cages (propranolol), 6 cages (acebutolol), 12 cages (furosemide and theophylline) and 18 cages (tienoxolol and control) for 6-keto-$PGF_{1\alpha}$ excretion. The effects of acebutolol, propranolol and furosemide were not investigated on urinary PGE_2 excretion. $^{**}P<0.01$, $^{***}P<0.001$ as compared to control group, Student's t test using residual variance.

Table 1 Affinity for A_1 and A_2 adenosine receptors of diuretics, β-blockers and theophylline in rat brain homogenate

	Inhibition of [^{3}H]-*PIA binding* A_1 receptor K_i (μM)	*Inhibition of* [^{3}H]-*NECA binding* A_2 receptor K_i (μM)
Tienoxolol	2.64 ± 0.07	20.49 ± 1.67
Theophylline	5.55 ± 0.34	30.50 ± 0.37
Acebutolol	165 ± 43	> 1000
Propranolol	> 1000	> 1000
Furosemide	> 1000	> 1000

The K_i values represent mean ± s.e.mean from 2 to 4 values obtained from independent experiments carried out on each drug with different brain membrane preparations. In a given experiment, K_i values were determined from at least 6 concentrations of each compound as triplicate incubations. Propranolol and furosemide were tested only at 1 mM on both receptor subtypes.

Effects of simultaneous administration of NECA with tienoxolol and theophylline on urinary PGE_2 and 6-keto-$PGF_{1\alpha}$ excretion

Co-administration of vehicle with tienoxolol led to a marked increase in 6-keto-$PGF_{1\alpha}$ levels (19.3 ± 1.9 ng 6h^{-1}, $P<0.001$) which was comparable with that measured when tienoxolol was administered alone (11.9 ± 0.8 ng 6h^{-1}, $P<0.001$). Likewise, theophylline given orally with vehicle induced similar effects to that observed when the drug was administered alone, although increase of urinary 6-keto-$PGF_{1\alpha}$ excretion was not statistically significant after co-treatment with theophylline and vehicle (Figure 5).

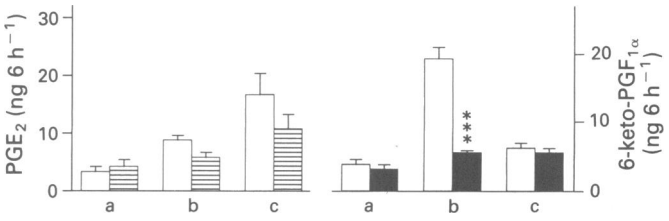


Figure 5 Effects of oral co-administration of 5'-(N-ethylcarboxamido)adenosine (NECA, 0.2 mg kg^{-1}) with tienoxolol (128 mg kg^{-1}) (b), theophylline (64 mg kg^{-1}) (c) or vehicle (control) (a) on urinary prostaglandin E_2 (PGE_2) and 6-keto-$PGF_{1\alpha}$ excretion. In the absence of NECA (open columns for both PGE_2 and 6-keto-$PGF_{1\alpha}$), rats received orally first the vehicle, then the drug (tienoxolol or theophylline) (vehicle-drug group), or two successive administrations of vehicle (control). In the presence of NECA (horizontally lined columns for PGE_2, solid columns for 6-keto-$PGF_{1\alpha}$), animals received orally first NECA, then the drug (tienoxolol or theophylline) (NECA-drug group), or NECA followed by the vehicle (control). Results represent the accumulation of PGE_2 and 6-keto-$PGF_{1\alpha}$ in urine at the end of a 6 h period. Values are expressed as mean ± s.e.mean from 6 metabolism cages. $^{***}P<0.001$ indicates a statistically significant difference between the NECA-drug group value and the corresponding vehicle-drug group value.

A very strong and highly significant PGE_2 urinary excretion was induced by simultaneous administration of vehicle with theophylline (+ 388%, $P<0.01$). Urinary PGE_2 levels were also enhanced by tienoxolol but to a lesser degree (+ 156%). This result was similar to that obtained when tienoxolol was given alone by mouth (+ 115%). In the presence of NECA, tienoxolol-induced urinary 6-keto-$PGF_{1\alpha}$ excretion was dramatically reduced (− 72%, $P<0.001$) while PGE_2 was only slightly reduced (− 31%). On the other hand, NECA impaired more significantly excretion of PGE_2 induced by theophylline (− 36%) than that of 6-keto-$PGF_{1\alpha}$ (− 11%) (Figure 5).

Effects of simultaneous administration of NECA with tienoxolol and theophylline on diuresis and natriuresis

Diuresis and natriuresis were both increased after simultaneous oral administration of tienoxolol and theophylline with vehicle. When the latter was replaced by NECA, tienoxolol and theophylline-induced diuresis and natriuresis remained unchanged (Table 2).

Discussion

It is generally recognized that urine PGE_2 is mostly of renal origin (renal medulla) (Frölich *et al.*, 1975) while urinary

Table 2 Effects of oral co-administration of 5'-(N-ethylcarboxamido)adenosine (NECA) with tienoxolol, theophylline and vehicle on diuresis and natriuresis

		Control	*Tienoxolol* (128 mg kg^{-1})	*Theophylline* (64 mg kg^{-1})
Diuresis	Vehicle	8.8 ± 0.4	14.5 ± 0.7	10.6 ± 0.7
ml 6 h^{-1}	NECA (0.2 mg kg^{-1})	7.7 ± 0.5	16.1 ± 0.8	10.2 ± 0.5
Natriuresis	Vehicle	0.10 ± 0.03	0.76 ± 0.10	0.83 ± 0.11
mEq 6 h^{-1}	NECA (0.2 mg kg^{-1})	0.02 ± 0.01	0.72 ± 0.07	0.86 ± 0.10

Drugs were given simultaneously with vehicle or NECA, and diuresis and natriuresis were determined at the end of a 6 h period. Values are expressed as mean ± s.e.mean from 9 metabolism cages (diuresis) or 3 cages (natriuresis).

excretion of 6-keto-$PGF_{1\alpha}$, the stable metabolite of prostacyclin, could reflect not only renal synthesis of PGI_2 (renal cortex) but also its own arterial blood levels or those of PGI_2 (Rosenkranz *et al.*, 1981; Zipser & Martin, 1982).

Tienoxolol stimulates urinary 6-keto-$PGF_{1\alpha}$ excretion at doses similar to those required for diuresis and natriuresis (Cloarec *et al.*, 1984), whereas PGE_2 levels are significantly enhanced only at the higher doses. Theophylline also causes diuresis and natriuresis accompanied by more pronounced effects on urinary excretion of PGE_2 than of 6-keto-$PGF_{1\alpha}$. Since PGI_2 and PGE_2 are diuretic and natriuretic (Anderson *et al.*, 1976; Bolger *et al.*, 1978), it is tempting to speculate that both prostaglandins are involved in tienoxolol and theophylline-induced diuresis and natriuresis. Furthermore, as the principal renal sites of PGI_2 and PGE_2 synthesis are not the same, the differing effects of the two compounds on prostaglandin excretion suggest that their renal distribution is different. Tienoxolol may be mainly distributed in the cortex and also at the vascular level, but poorly in the medulla, which could stimulate 6-keto-$PGF_{1\alpha}$ excretion. On the other hand, theophylline may be mainly distributed in the medulla and could activate PGE_2 excretion. The different physical molecular characteristics and pharmacokinetic properties of tienoxolol and theophylline may explain such a tissue distribution.

Surprisingly, tienoxolol does not change prostaglandin levels in the kidney which seems to be inconsistent with data obtained in urine. This apparent discrepancy could be explained by the fact that an accumulation of prostaglandins is measured in urine, while 6-keto-$PGF_{1\alpha}$ and PGE_2 levels are determined only at a given time in the renal tissue. Moreover, prostaglandin extraction is performed from whole kidney so that a slight, but significant, rise of 6-keto-$PGF_{1\alpha}$ and/or PGE_2 in a well defined renal area could be masked. Therefore, it would be of a great interest to evaluate the effects of tienoxolol on 6-keto-$PGF_{1\alpha}$ and PGE_2 concentrations in the cortical and medullary tissues.

Propranolol induces a weak diuresis (+23% at 128 mg kg^{-1}) as compared to tienoxolol (+90% at 128 mg kg^{-1}), but does not stimulate 6-keto-$PGF_{1\alpha}$ excretion in urine. Acebutolol (128 mg kg^{-1}) modifies neither diuresis nor urinary excretion of prostaglandins. Consequently, in this study, tienoxolol is the only β-adrenoceptor antagonist able to promote both diuresis and prostaglandin excretion.

Furosemide (32 mg kg^{-1}) produces a marked diuresis but does not change the urinary excretion of 6-keto-$PGF_{1\alpha}$. Although furosemide markedly enhances urinary prostaglandin excretion both in animals and man (Abe *et al.*, 1977; Oliw & Anggård, 1979; Patak *et al.*, 1979), results obtained in this study corroborate those of other authors (Halushka *et al.*, 1979; Tan & Mulrow, 1977) showing urinary prostaglandin excretion to remain unchanged when long collection periods are used. Prostaglandin excretion is maximal within 15 to 40 min returning to control excretion rates 1 to 2 h following either oral or intravenous doses of furosemide (Scherer & Weber, 1979; Wilson *et al.*, 1982), this gives an unchanged net excretion rate when urine is collected for more than 2 h. These data confirm that factors such as urinary volume are able to affect prostaglandin excretion (Kirschenbaum & Serros, 1980) and, on the other hand, suggest that the time course of urinary prostaglandin excretion induced by tienoxolol could be different from that of furosemide. Furthermore, the latter is a much more potent diuretic agent than tienoxolol so that urinary volume probably does not play a role as important as with furosemide.

Inasmuch as tienoxolol and theophylline bind to A_1 and A_2 adenosine receptors with similar affinities, and although the radioligand binding studies were not performed in the kidney but in the brain, it is proposed that P_1-purinoceptors could be involved in prostaglandin release which could then contribute to diuresis and natriuresis induced by both drugs. This assumption is supported by the fact that NECA, a potent adenosine receptor agonist which affects neither prostaglandin excretion nor diuresis and natriuresis at the studied dose (0.2 mg kg^{-1}), markedly reduces tienoxolol-stimulated urinary 6-keto-$PGF_{1\alpha}$ excretion while exhibiting only a moderate effect on urinary PGE_2. Likewise, a decrease, though not significant, of PGE_2 level in urine is observed after co-administration of NECA and theophylline. Considering that tienoxolol and theophylline may have a preferential renal tissue distribution, it is possible that NECA may impair tienoxolol-induced 6-keto-$PGF_{1\alpha}$ excretion in the cortex and theophylline-induced PGE_2 release in the medulla. In other words, NECA may be distributed in both renal areas and compete with each of these drugs for common binding sites, namely adenosine receptors.

However, the impairment of 6-keto-$PGF_{1\alpha}$ and PGE_2 excretion does not modify the diuretic and natriuretic activities of tienoxolol and theophylline. Therefore, prostacyclin and PGE_2 probably do not participate in the diuresis and natriuresis observed. Interestingly, enprofylline, a xanthine derivative with a low affinity for P_1-purinoceptors (Schwabe *et al.*, 1985; Bruns *et al.*, 1986), stimulates prostaglandin excretion at doses that have no effect on urine volume or electrolyte (Takeuchi *et al.*, 1981; Baer *et al.*, 1983). Taken together, these data suggest that adenosine receptor inhibition mediates the prostaglandin excretion observed with tienoxolol and xanthines, but this excretion appears to be unrelated to diuresis and natriuresis. Regarding the effects of tienoxolol on 6-keto-$PGF_{1\alpha}$ excretion, P_2-purinoceptors should also be considered in this pharmacological response since they were shown to modulate prostaglandin formation by Burnstock (1978) and Carter *et al.*, (1988). As suggested above, tienoxolol-induced urinary 6-keto-$PGF_{1\alpha}$ excretion may result from an action of the drug at the vascular level. Prostacyclin is the major prostaglandin produced by most blood vessels and the stimulation of its release from aortic endothelial cells of porcine (Pearson *et al.*, 1983) or bovine (Van Coevorden & Boeynaems, 1984) origin can be obtained by the activation of P_{2Y} receptors (Needham *et al.*, 1987). Furthermore, PGI_2 release may also result from an activation of nucleotide receptors located on smooth muscle cells (Demolle *et al.*, 1988; O'Connor, 1992) and recent studies have demonstrated the presence of P_{2Y}-purinoceptors in the rat renal cortex (Nanoff *et al.*, 1990).

Finally, the increase of PGI_2 biosynthesis induced by different antihypertensive agents could also result initially from the reduction of arterial blood pressure (Frölich, 1990). Such a reduction was previously described following tienoxolol administration in man (Coupez *et al.*, 1989). Moreover, the increase of urinary 6-keto-$PGF_{1\alpha}$ excretion observed after treatment with diuretics may be mainly related to the increased urinary flow (Frölich *et al.*, 1985). It is conceivable therefore that tienoxolol may stimulate 6-keto-$PGF_{1\alpha}$ excretion by these two mechanisms.

To summarize, the hypothesis of a relationship between P_1-purinoceptors, prostaglandin release, diuresis and natriuresis appears to be untenable. A causal role of prostaglandins in the mechanism underlying the diuretic and natriuretic effects of tienoxolol is unlikely, while adenosine receptor inhibition may play a role. In this respect, A_2 adenosine receptors may represent the site of action for the diuretic effects of methylxanthines such as theophylline and caffeine (Freissmuth *et al.*, 1987). The pharmacological significance of urinary 6-keto-$PGF_{1\alpha}$ and PGE_2 excretion evoked by tienoxolol remains to be clarified as does the tissue origin of PGI_2 and the mechanism of its production. However, data obtained in the renal tissue seem to indicate that this enhanced 6-keto-$PGF_{1\alpha}$ is of prerenal origin.

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