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Phosphinic acid analogues of GABA are antagonists at the $GABA_B$ receptor in the rat anococcygeus

[1]Judith M. Hills, Andrew J. Sellers, Jayshree Mistry, Marianne Broekman & William Howson

SmithKline Beecham Pharmaceuticals Ltd., The Frythe, Welwyn, Herts. AL6 9AR

CGP 35348 (3-aminopropyl(diethoxymethyl)phosphinic acid) and 3-aminopropyl(n-hexyl)phosphinic acid (3-APHPA) were tested in the rat anococcygeus muscle against CGP 27492 (3-aminopropylphosphinic acid), a selective $GABA_B$ agonist, for their antagonist activity. Their antagonist potency was compared with that of 2-hydroxysaclofen. The pA_2 values for CGP 35348, 3-APHPA and 2-hydroxysaclofen were 5.38, 4.86, 4.45 respectively in the rat anococcygeus muscle. These results confirm the previous reports of $GABA_B$ antagonist activity for these compounds and show a marginal improvement in potency over 2-hydroxysaclofen.

Introduction A physiological role for $GABA_B$ receptors in the mammalian central nervous system has recently been suggested (Dutar & Nicoll, 1988) as a result of work with the $GABA_B$ antagonist, phaclofen (Kerr *et al.*, 1987). However phaclofen, and analogues derived from it such as saclofen and 2-hydroxysaclofen, bind relatively weakly to the $GABA_B$ receptor (Bowery, 1989) and are effective only at high concentrations in pharmacological assay systems (Kerr *et al.*, 1987; Curtis *et al.*, 1988; Hills *et al.*, 1989). A series of phosphinic acids have recently been reported to show $GABA_B$ receptor antagonist activity (Bittiger *et al.*, 1990). They are similar in structure to the potent $GABA_B$ receptor agonist CGP 27492 (see Figure 1) (Hills *et al.*, 1989), which is the phosphinic acid derivative of GABA. Here we have examined two of these compounds for $GABA_B$ antagonist activity in the field-stimulated rat anococcygeus muscle.

Methods Rat anococcygeus muscles were prepared as previously described (Hills *et al.*, 1989). Three cumulative agonist concentration-response curves were constructed on each preparation; control curve to agonist alone, curve in the presence of antagonist and wash out curve with a 30 min period between each. At least four concentrations of antagonists were tested on at least four preparations. pA_2 values were derived from Schild plots following statistical analysis carried out using a statistical package (fitline in RSE) and 95% confidence intervals calculated.

The following compounds were used; 3-amino-propylphosphinic acid (CGP 27492, for reference see Hills *et al.*, 1989; prepared by W. Howson at SmithKline Beecham); 2-hydroxysaclofen (Tocris Neuramin); 3-aminopropyl(diethoxymethyl)phosphinic acid (CGP 35348); 3-aminopropyl(n-hexyl)phosphinic acid (3-APHPA, Ciba Geigy). The Ciba-Geigy compounds were prepared as described in their recent patent (Bayliss *et al.*, 1989). All compounds were dissolved in distilled water, dilutions being made in distilled water, and compounds were added to the organ bath in volumes no greater than 1% total volume.

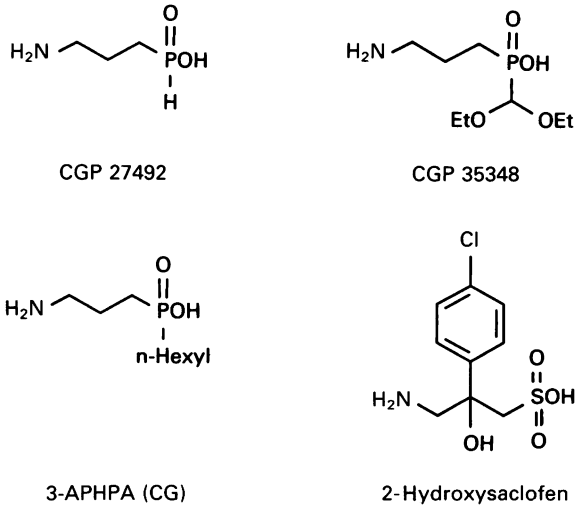

Figure 1 Chemical structures of the $GABA_B$ agonist 3-aminopropylphosphinic acid (CGP 27492) and the $GABA_B$ antagonists, 3-aminopropyl(diethoxymethyl)phosphinic acid (CGP 35348), 3-aminopropyl(n-hexyl)phosphinic acid (3-APHPA) and 2-hydroxysaclofen.

Results 3-Aminopropylphosphinic acid caused a concentration-dependent inhibition of the electrical stimulation induced contractions in the rat anococcygeus muscle as previously reported (Hills *et al.*, 1989). The IC_{50} value obtained was $0.09 \pm 0.01\,\mu M$ ($n = 12$) and 2-hydroxysaclofen caused a concentration-dependent reversible antagonism. The pA_2 value obtained from the Schild plot was 4.45 (4.23, 4.79 95% CL) with a slope of 1.03 (0.72, 1.31 95% CL) indicating competitive antagonism. Similarly, CGP 35348 and 3-APHPA, caused a concentration-dependent, reversible antagonism of concentration-response curves to the $GABA_B$ agonist. The pA_2 value obtained from straight line Schild plots for CGP 35348 was 5.38 (4.4, infinity 95% CL) with a calculated slope of 0.67 (−0.10, 1.44 95% CL), and for 3-APHPA, 4.86 (4.68, 5.10 95% CL) with a slope of 0.95 (0.76, 1.13 95% CL). Although CGP 35348 appears to be the most potent antagonist, despite repeated experimentation, the confidence limits obtained are wide. All three compounds tested caused a parallel shift in the concentration-response curve to 3-aminopropylphosphinic acid.

Discussion These results provide the first demonstration of functional $GABA_B$ receptor antagonism by phosphinic acids in the peripheral tissue studied here. CGP 35348 has previously been shown to antagonize baclofen-mediated hyperpolarizations in hippocampal cells at $100\,\mu M$ and to be active *in vivo* against baclofen at $30\,mg\,kg^{-1}$ in the rotarod test (Bittiger *et al.*, 1990). No published data are available which specifically relate to 3-APHPA (see Bayliss *et al.*, 1989) and we are therefore able to confirm reported data with CGP 35348 and extend the information available by demonstrating functional $GABA_B$ antagonism in the rat anococcygeus with CGP 35348 and 3-APHPA.

[1] Author for correspondence.

Previous work with 2-hydroxysaclofen, the sulphonic acid analogue of baclofen, has reported antagonism of $GABA_B$-mediated inhibition in the guinea-pig ileum (Kerr *et al.*, 1988) and other tissues (Kerr *et al.*, 1988; Curtis *et al.*, 1988) and in our experiments, clear antagonism was obtained in the rat anococcygeus.

Although these results confirm that CGP 35348 and 3-APHPA are functional $GABA_B$ antagonists *in vitro*, they fail to show any significant improvement in terms of potency over antagonists such as 2-hydroxysaclofen either studied here or elsewhere. They are however active *in vivo* (Bayliss *et al.*, 1989; Bittiger *et al.*, 1990) and this together with their CNS penetrating properties (Olpe *et al.*, 1989), may well turn out to be the feature of this class of compounds which makes them superior to those already available.

The authors wish to thank Mr Brian Bond for carrying out the statistical analysis and Dr Mike Parsons for his helpful guidance.

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Antipyretic actions of human recombinant lipocortin-1

[1]J. Davidson, *R.J. Flower, A.S. Milton, *S.H. Peers & [2]D. Rotondo

Division of Pharmacology, Marischal College, University of Aberdeen, Aberdeen AB9 1AS and *Department of Biochemical Pharmacology, The Medical College of Saint Bartholomew's Hospital, Charterhouse Square, London EC1M 6BQ

The effect of human recombinant lipocortin-1 (hrLC-1) on the pyrogenic actions of the synthetic polyribonucleotide polyinosinic:polycytidylic acid (poly I : C) has been studied in conscious rabbits. Poly I : C (2.5 $\mu g\,kg^{-1}$) given i.v. produced a biphasic fever with a first peak after 90–105 min and a second peak between 225–240 min. hrLC-1 (50 $\mu g\,kg^{-1}$) given i.v. simultaneously with the poly I : C produced a significant reduction in the febrile response but without complete suppression. The thermal response index over 5 h (TRI_5) was 4.69 ± 0.51 for poly I : C given with saline and the TRI_5 for poly I : C given with hrLC-1 was 2.66 ± 0.45 (values are for $n = 5$ ± s.e.mean, $P < 0.05$). hrLC-1 administered alone had no effect on body temperature and its antipyretic activity was lost on heating. In a separate series of experiments 1 h pretreatment with dexamethasone (1 $mg\,kg^{-1}$) given i.v. reduced the pyrogenic response (TRI_5) to poly I : C (2.5 $\mu g\,kg^{-1}$) from 4.87 ± 0.54 without dexamethasone to 2.00 ± 0.25 ($n = 5$, $P < 0.05$) and dexamethasone given alone had no effect on body temperature. These data demonstrate that LC-1 possesses antipyretic actions and raises the possibility that the antipyretic actions of dexamethasone are mediated through the induction of LC-1.

Introduction Lipocortin-1 (LC-1, also called annexin-1 or calpactin-2; Crumpton & Dedman, 1990) is a member of a diverse group of proteins which exhibit calcium and phospholipid binding properties. A common structural feature of this group of proteins is a 70 amino acid repeat unit containing a highly conserved consensus region of 17 amino acids which is thought to be important for calcium/phospholipid binding (Pepinsky *et al.*, 1988). Apart from a core sequence, members of this family of proteins have structurally different N-termini which possibly confer different biological properties on each member of the family.

LC-1 applied externally to cells can inhibit eicosanoid generation (Cirino *et al.*, 1989) and has potent anti-inflammatory actions when injected locally (Cirino *et al.*, 1989) or intravenously (Browning *et al.*, 1990) into the rat. The mechanism of action by which LC-1 inhibits eicosanoid biosynthesis could occur by inhibition of phospholipase A_2 but this is not clear and has recently been disputed.

Systemic treatment of man with glucocorticoids results in an induction of LC-1 synthesis and the appearance of LC-1 on the cell-surface of monocytes/macrophages (Goulding *et al.*, 1990). This evidence suggests that the reduction of eicosanoid release in response to glucocorticoids may be mediated through the induction of LC-1. Recently it has been shown that pretreatment of rabbits with dexamethasone attenuates the febrile response to exogenous immunomodulatory agents such as polyinosinic : polycytidylic acid (poly I : C) and lipopolysaccharide (Milton *et al.*, 1989). Both these pyrogenic agents appear to produce a febrile response by increasing blood levels of prostaglandin E_2(PGE_2), dexamethasone attenuates both the fever and the increase in circulating PGE_2 in response to these pyrogens (Milton *et al.*, 1989). The attenuation of both the fever and increase in blood levels of PGE_2 by dexamethasone is only observed if animals are pretreated for between 30 and 60 min, indicating that this action of dexamethasone may require the induction of a mediator, possibly lipocortin. In the present investigation, therefore, the effect of human recombinant LC-1 (hrLC-1) on poly I : C-induced fever has been studied.

Methods Male Dutch rabbits, weighing 1.7–2.0 kg were lightly restrained in conventional stocks throughout each experiment. To minimize any error in body temperature measurements due to restraint stress, all rabbits were accustomed to the stocks over a period of 5 days before beginning this series of experiments. All experiments were conducted at the same time of day (10 h 00 min–16 h 00 min) and carried out at an ambient temperature of 22 ± 1°C. Body temperature was measured continuously with rectal thermistor probes (Yellow Springs Instruments-401 series), inserted to a depth of 9 cm, which were connected to a Jacquet chart recorder. Rabbits were left unhandled after insertion of the probes until body temperature was stable for at least 1 h before any drugs were administered.

hrLC-1 produced by transfected CHO cells was obtained from Biogen, Boston, MA, U.S.A. The protein was stored as a sterile solution at a concentration of 1 $mg\,ml^{-1}$ and repetitive freeze-thawing was avoided as this leads to denaturation of the protein. The purity of the preparation was greater than 97% as ascertained by SDS/polyacrylamide gel electrophoresis and reverse phase high performance liquid chromatography (h.p.l.c.). hrLC-1, polyinosinic: polycytidylic acid obtained from Sigma Chemicals (Dorset) and dexamethasone sodium-21-phosphate provided as a gift from Merck Sharp and Dohme (Herts) were prepared using aseptic techniques, and were dissolved in sterile saline (Travenol Laboratories, Norfolk). In experiments where heated hrLC-1 was administered, the required volume of hrLC-1 was transferred to sterile Eppendorf tubes and placed in a water bath at 90°C for 30 min. All agents were administered i.v. via the marginal ear vein. Poly I : C was administered first and hrLC-1 was given several seconds later. Poly I : C and dexamethasone solutions were passed through Millex-GS 0.22 μm filters immediately prior to injection. The amount of agent in each dose was adjusted to give an injection volume of 0.5–1.0 ml. The results are expressed as either the change in temperature from basal (ΔT) in °C or as thermal response indexes where the magnitude of the febrile response is determined by integrating the change in temperature (°C) against time (h) to give a thermal response index (TRI). Data, expressed as the mean of n experiments ± the s.e.mean, were analysed by a paired Student's t test. Treatments were randomised, each rabbit acting as its own control and the differences were considered significant when $P < 0.05$.

[1] Author for correspondence.
[2] Present address: Division of Biochemical Sciences, Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB.

Results The dose of poly I : C used ($2.5\,\mu g\,kg^{-1}$) has previously been shown to give a reproducible fever in rabbits (Rotondo *et al.*, 1987). Poly I : C given i.v. alone produced a biphasic increase in body temperature with a first peak occurring after 90–105 min and a second peak between 225–240 min. The ΔT_{max} for the first peak was 1.14 ± 0.13 and 1.29 ± 0.16 for the second peak ($n = 5$). hrLC-1 ($50\,\mu g\,kg^{-1}$) given i.v. simultaneously with the poly I : C produced a significant reduction in the febrile response but without complete suppression (Figure 1). The ΔT_{max} for poly I : C with hrLC-1 were 0.76 ± 0.09°C for the first peak and 0.78 ± 0.16°C for the second peak ($n = 5$). The TRI_5 was 4.69 ± 0.51 for poly I : C given with saline and the TRI_5 for poly I : C given with hrLC-1 was 2.66 ± 0.45 ($n = 5$, $P < 0.05$). A lower dose of hrLC-1 ($25\,\mu g\,kg^{-1}$) did not produce a significant attenuation of the febrile response to poly I : C (TRI_5 4.01 ± 0.29, $n = 3$, range 3.44–4.38). hrLC-1 ($50\,\mu g\,kg^{-1}$) administered alone had no effect on body temperature, a response similar to that obtained with saline. The antipyretic activity of hrLC-1 was lost if it was heated at 90°C for 30 min and allowed to cool before being administered. The pyrogenicity of poly I : C when given with heated hrLC-1 was similar to its pyrogenicity when given alone. In a separate series of experiments 1 h pretreatment with dexamethasone ($1\,mg\,kg^{-1}$) given i.v. reduced the pyrogenic response (TRI_5) to $2.5\,\mu g\,kg^{-1}$ poly I : C (Figure 1) from 4.87 ± 0.54 without dexamethasone to 2.00 ± 0.25 ($n = 5$, $P < 0.05$). Dexamethasone given alone had no effect on body temperature. The dose of dexamethasone given ($1\,mg\,kg^{-1}$) and the pretreatment time have previously been shown to produce a reproducible attenuation of poly I : C fever (Abul *et al.*, 1987). In addition it was shown that if dexamethasone is given with poly I : C then no reduction in the pyrogenic response is observed and 1 h pretreatment appears to be optimal (Abul *et al.*, 1987).

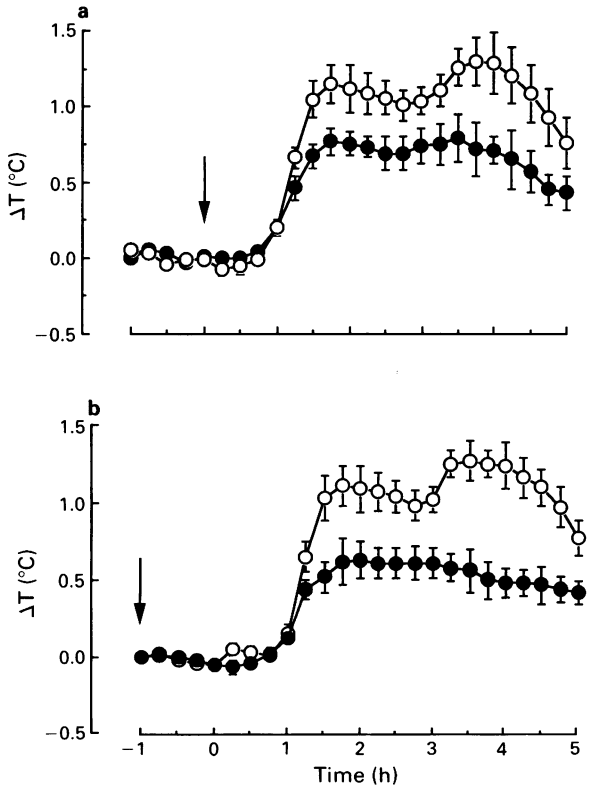

Figure 1 The effect of lipocortin-1 and dexamethasone on the pyrogenic action of polyinosinic: polycytidylic acid (poly I : C). Rabbits were injected i.v. with (as indicated by the arrows) either saline (○) or (a) human recombinant lipocortin-1 ($50\,\mu g\,kg^{-1}$, ●) and (b) dexamethasone ($1\,mg\,kg^{-1}$, ●). Poly I : C ($2.5\,\mu g\,kg^{-1}$) was given i.v. at time zero in both cases. Values are means of $n = 5$, s.e.means shown by vertical bars.

Discussion This report clearly demonstrates that hrLC-1 attenuates the pyrogenic response of rabbits to poly I : C. A similar magnitude of attenuation of poly I : C pyrogenicity has been observed with dexamethasone (Figure 1). The dose of dexamethasone used was approximately $1.75\,\mu mol\,kg^{-1}$ ($1\,mg\,kg^{-1}$) and the dose of hrCL-1 used in the present study was approximately $1.39\,nmol\,kg^{-1}$ ($50\,\mu g\,kg^{-1}$) therefore, on this molar basis the potency of hrLC-1 in attenuating the pyrogenicity of poly I : C is in the order of 1000 fold greater than dexamethasone. It is most unlikely that this difference in potency could be accounted for by differences in the pharmacokinetic properties of the two agents. LC-1 is a protein and would have a lower ability to penetrate into various compartments than the steroid dexamethasone. The higher potency of LC-1 in comparison to dexamethasone is in agreement with the observations of Cirino *et al.* (1989) who observed that $20\,\mu g$ hrLC-1 injected into the rat paw produced a slightly lower response than 1 mg dexamethasone in reducing oedema induced by various inflammatory stimuli. This effect was also observed in adrenalectomized rats indicating that adrenal hormones are not involved (Cirino *et al.*, 1989).

LC-1 is found in many tissues (Pepinsky *et al.*, 1988) and is particularly abundant in cells of the monocyte/macrophage lineage and the protein appears to be well conserved in a wide variety of species including man, rat, mouse, hamster and monkey (Pepinsky *et al.*, 1986). Goulding *et al.* (1990) observed that monocytes contain the largest amount of LC-1 of any cell type in human blood. Furthermore, only monocytes were able to respond to intravenously administered hydrocortisone by producing greater amounts of LC-1 both intracellularly and pericellularly within 2 h. Investigations into LC-1 induction by Browning *et al.* (1990) also demonstrate that the monocyte/macrophage is a sensitive target for the inducing action of glucocorticoids. Monocytes have also been shown to respond to pyrogenic agents by releasing PGE_2, this is attenuated by dexamethasone and appears to occur via the induction of a protein mediator, possibly LC-1 (Milton *et al.*, 1989). Dexamethasone given intravenously may, therefore, induce the biosynthesis of LC-1 from monocytes and many other tissues and increase the level of circulating LC-1. As dexamethasone requires at least a 1 h pretreatment period before it can attenuate the pyrogenic action of poly I : C (Milton *et al.*, 1989) and has been shown in other systems to increase the synthesis of LC-1 this raises the possibility that the suppression of pyrogenic responses by dexamethasone may be mediated in part by a lipocortin, possibly LC-1.

The mechanism by which LC-1 attenuates poly I : C pyrogenicity is not clear. Poly I : C appears to exert its pyrogenic action by increasing circulating levels of PGE_2 (Rotondo *et al.*, 1988). In the same series of experiments Rotondo *et al.* (1988) showed that the non-steroidal anti-inflammatory agent, ketoprofen, given i.v. completely abolished the poly I : C-induced fever and also reduced the blood levels of PGE_2 to almost undetectable levels in several animals. In addition it was shown by Milton *et al.* (1989) that dexamethasone suppressed the increase in circulating levels of PGE_2 and the fever in response to poly I : C. The antipyretic action of LC-1 could possibly involve an attenuation of PGE_2 biosynthesis as a property which LC-1 possesses is the ability to inhibit eicosanoid generation both *in vivo* and *in vitro* (Cirino *et al.*, 1989; Browning *et al.*, 1990). Although it has been suggested that the mechanism of lipocortin action is to inhibit phospholipase A_2 therefore inactivating the enzyme, no direct experimental evidence has been cited in the literature. Recently it has been suggested that phospholipase A_2 inhibition could be due to lipocortin binding to substrate; however, the exact mechanism of eicosanoid inhibitory action remains to be resolved.

Apart from its actions on the arachidonate cascade, little is known of the exact role lipocortins play in immunological processes. This study shows that LC-1 can attenuate an immunological action, fever which is the most easily measured parameter of the acute phase response to infection, *in vivo*

when administered systematically. Although no direct evidence has been presented in the current study this nevertheless raises the possibility that the antipyretic action of dexamethasone is mediated by LC-1.

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SPECIAL REPORT

Influence of angiotensin II on the α-adrenoceptors involved in mediating the response to sympathetic nerve stimulation in the rabbit isolated distal saphenous artery

[1]W.R. Dunn, J.C. McGrath & [2]V.G. Wilson

Autonomic Physiology Unit, Institute of Physiology, University of Glasgow, Glasgow G12 8QQ

Under normal experimental conditions, sympathetic nerve-mediated responses to electrical field stimulation in the isolated distal saphenous artery of the rabbit are sensitive to prazosin (0.1 μM) and so, by definition, are mediated by α_1-adrenoceptors. In the presence of angiotensin II (A II, 0.05 μM) however, a component of the response to nerve stimulation became resistant to prazosin. This 'uncovered' response was virtually abolished by the selective α_2-adrenoceptor antagonist rauwolscine (1 μM), a concentration that in the absence of A II had enhanced nerve-mediated responses. Exposure to A II therefore, allows the clear demonstration of a role for postjunctional α_2-adrenoceptors in mediating the contractile response to sympathetic nerve stimulation in this arterial preparation.

Introduction From observations in whole animals, it was initially suggested that postjunctional α_1-adrenoceptors were 'innervated', responding to neuronally released noradrenaline, while postjunctional α_2-adrenoceptors were located extrajunctionally responding to circulating catecholamines (McGrath, 1982). Studying such a generality *in vitro* has proved very difficult. This is primarily the result of the difficulty associated with the demonstration of prazosin-resistant, rauwolscine-sensitive responses in isolated vascular smooth muscle, particularly so in arteries (McGrath *et al.*, 1989). This problem is compounded by the well-documented prejunctional actions of agents which interact with α_2-adrenoceptors (Starke, 1987). We have recently demonstrated that the expression of postjunctional α_2-adrenoceptor-mediated (prazosin-resistant) responses to the synthetic agonist UK-14304 in the isolated distal saphenous artery of the rabbit, is dependent upon prior exposure to angiotensin II (Dunn *et al.*, 1989). This observation led us to examine the influence of this peptide on the responses of this preparation to sympathetic nerve stimulation.

Methods Male albino rabbits (2.5–2.7 kg) were killed by stunning followed by exsanguination. A length of distal saphenous artery was removed and placed in modified Krebs-Henseleit solution (for composition: see Daly *et al.*, 1988), and subsequently divided into 'ring' segments, 3–4 mm in length. Each segment was suspended between two wire supports (0.2 mm thick) and prepared for measurement of isometric tension. A modified glass tissue holder encompassing platinum plate electrodes connected to an electrical stimulator was used to apply frequency-dependent electrical field stimulation to each preparation.

Following exposure to a sighting concentration (3 μM) of noradrenaline (NA), tissues were subjected to electrical field stimulation with the following parameters: 16 Hz for 1 s, pulse width 0.03 ms at a supramaximal voltage of 35 V. Repetitive stimulation was applied once every 5 min until constant responses were achieved. Subsequently a control frequency-response curve (FRC) was obtained for each preparation. The FRC was constructed by obtaining responses to the following frequencies of stimulation: 4, 8, 16, 32, 64 Hz at a stimulation duration of 1 s and 4 and 8 Hz at a stimulation duration of 10 s. Each preparation was initially exposed to α,β-methylene ATP (3 μM) to remove a small purinergic (<10% of total) component of responses to nerve stimulation (unpublished observation). The distal saphenous artery was used in the present study (cf. Burnstock & Warland, 1987).

In some experiments preparations were exposed to α-adrenoceptor antagonists 45 min prior to obtaining an FRC. In others, preparations were exposed to A II (0.05 μM) at least 20 min before the start of another FRC. Results are expressed as a % of the response to 64 Hz (1 s duration) obtained in the first FRC. Differences between means were considered statistically significant if $P < 0.05$ (Student's t test).

Results Electrical stimulation of ring segments of isolated distal saphenous artery of the rabbit resulted in frequency-dependent contractile responses, which were reproducible for three FRC's, and susceptible to tetrodotoxin (0.1 μM) (result not shown). For clarity, only the results obtained at 8 Hz and 64 Hz, 1 s duration (submaximal and maximal responses) are shown in Figure 1. These results are representative of the findings at all parameters examined. Responses to electrical field stimulation were virtually absolished by the selective α_1-adrenoceptor antagonist prazosin (0.1 μM) (Figure 1a). In contrast, nerve-mediated responses were enhanced in the presence of both the selective α_2-adrenoceptor antagonists rauwolscine (1 μM) (Figure 1b) and A II (0.05 μM) (Figure 1c). This concentration of A II produced a transient contraction, the peak of which was equivalent to 60.2 ± 7.9% ($n = 13$) of the response to 64 Hz for 1 s, which returned to baseline after 12–15 min.

A II (0.05 μM) was without effect on nerve-mediated responses in preparations previously exposed to rauwolscine (1 μM) (Figure 1b). In contrast, responses in preparations previously exposed to prazosin (0.1 μM) were markedly enhanced in the presence of A II (0.05 μM) (Figure 1a). These enhanced responses observed in the presence of prazosin and A II were subsequently susceptible to rauwolscine (1 μM) (Figure 1d).

Discussion The difficulty associated with demonstration of prazosin-resistant responses to α-adrenoceptor agonists in isolated vascular preparations has generally precluded an examination of the physiological role for postjunctional α_2-adrenoceptors (McGrath *et al.*, 1989). Furthermore, resolution of this problem is compounded by the mutually opposing actions of agents which interact with α_2-adrenoceptors at both pre- and postjunctional locations (Starke, 1987). These problems have limited the demonstration

[1] Author for correspondence at: Department of Pharmacology, College of Medicine, Given Building, University of Vermont, Burlington, Vermont, 05405, U.S.A.

[2] Present address: Department of Physiology and Pharmacology, Medical School, Queen's Medical Centre, Nottingham NG7 2UH.

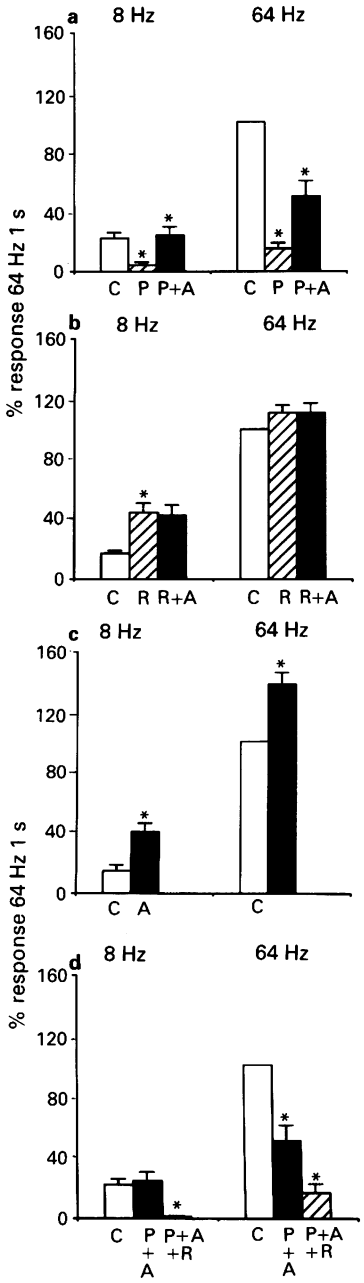

Figure 1 The effects of angiotensin II (0.05 μM) on the various components of sympathetic nerve-mediated contractile responses of rings of isolated distal saphenous artery of the rabbit. Stimulation was applied at 8 and 64 Hz for 1 s. Control responses (C) were abolished by tetrodotoxin 0.1 μM. (a) Elimination of the α_1-adrenoceptor- mediated component by prazosin 0.1 μM leaves a small residual response (P), which is subsequently potentiated by angiotensin II (P + A). (b) Blockade of α_2-adrenoceptors by rauwolscine 1 μM results in a potentiated response (R) (at 8 Hz only), presumably by interruption of prejunctional α_2-adrenoceptor-mediated negative feedback. However, this response is not blocked by angiotensin II (R + A), suggesting that potentiation by angiotensin II requires that α_2-adrenoceptors are available for activation. (c) The control response is potentiated by angiotensin II (A). (d) The 'potentiated response' revealed by angiotensin II after prazosin (P + A) is blocked by rauwolscine 1 μM (P + A + R) and is, therefore, mediated by postjunctional α_2-adrenoceptors. Responses are expressed as a % of the control response at 64 Hz. Each column represents the mean from 4–7 tissues; s.e.mean shown by vertical bars. * Significant difference from the mean value in the preceding column (Student's t test, $P < 0.05$).

of responses to sympathetic nerve stimulation, mediated via postjunctional α_2-adrenoceptors, to a very few isolated vascular (generally venous) preparations (e.g. Docherty & Hyland, 1985; Flavahan *et al.*, 1985). In the present study we have shown in an arterial preparation that, despite the sensitivity, of sympathetic nerve-mediated responses to prazosin, postjunctional α_2-adrenoceptors are present on the vascular smooth muscle and respond to neuronally-related NA but only after exposure of the tissue to the physiological peptide, A II.

Responses to sympathetic nerve stimulation in the isolated distal saphenous artery of the rabbit were virtually abolished by prazosin (0.1 μM), a concentration which has previously been shown to be selective for α_1-adrenoceptors in this preparation (Dunn *et al.*, 1989). This clearly indicates that under normal *in vitro* experimental conditions, nerve-released NA acts upon postjunctional α_1-adrenoceptors to produce the functional contractile response. Under these conditions rauwolscine (1 μM) enhanced nerve-mediated responses, an effect consistent with its well-documented action on prejunctional α_2-adrenoceptor-mediated autoregulation (Starke, 1987). A II also caused a potentiation of responses to nerve stimulation. While this may be due partly to an increase in the release of neuronal transmitter (Story & Ziogas, 1987), a postjunctional site of action is implicated by the observation that this agent introduced a prazosin-resistant component of responses to sympathetic nerve stimulation as it does to the adrenoceptor agonist UK-14304 (Dunn *et al.*, 1989). This prazosin-resistant response introduced by A II was abolished by rauwolscine at a concentration which has previously been shown to be selective for α_2-adrenoceptors in this preparation (Dunn *et al.*, 1989) and which, prior to exposure to A II, had enhanced responses to sympathetic nerve stimulation. The present study therefore clearly demonstrates a role for postjunctional α_2-adrenoceptors in mediating the end-organ response of the distal saphenous artery to electrical field stimulation, although this is dependent upon the presence of A II to simulate *in vivo* conditions more closely. Furthermore, it demonstrates that sensitivity to prazosin (or equivalent drug) cannot be taken as an unequivocal demonstration of the absence of postjunctional α_2-adrenoceptors in isolated vascular preparations (see also Dunn *et al.*, 1989; Templeton *et al.*, 1989).

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Antiarrhythmic properties of tedisamil (KC8857), a putative transient outward K^+ current blocker

G.N. Beatch, [1]S. Abraham, B.A. MacLeod, N.R. Yoshida & [2]M.J.A. Walker

Department of Pharmacology & Therapeutics, Faculty of Medicine, The University of British Columbia, Vancouver, B.C., V6T 1W5, Canada

1 Rats were used to evaluate the antiarrhythmic properties of tedisamil, a novel agent with the electrophysiological properties of a Class III antiarrhythmic drug. Tedisamil was tested against coronary artery occlusion-induced arrhythmias in conscious animals.

2 The actions of tedisamil on the ECG, as well as responses to electrical stimulation, were compared with those on the configuration of epicardial intracellular action potentials recorded *in vivo*.

3 Tedisamil (1–4 mg kg^{-1}, i.v.) caused bradycardia, elevated blood pressure and dose-dependently reduced ventricular fibrillation (VF) induced by occlusion of the left anterior descending coronary artery. Other ischaemia-associated arrhythmias were not so well suppressed. Antiarrhythmic activity was greatest when the tedisamil-induced bradycardia was prevented by electrically-pacing the left ventricle.

4 Tedisamil dose-dependently lengthened the effective refractory period and prevented electrically-induced VF. *In vivo*, tedisamil (0.5–4 mg kg^{-1}, i.v.) prolonged the duration of epicardial intracellular action potentials by up to 400%.

5 Results showed that tedisamil possessed antifibrillatory actions in rats that were related to Class III electrophysiological actions as revealed by electrical stimulation and electrophysiological analyses.

Introduction

Vaughan Williams (1984) classified antiarrhythmic drugs on the basis of effects of action potential morphology. The clinical and experimental usefulness of the classification is constantly under review, particularly for Class III antiarrhythmics which act mainly to prolong action potential duration (APD) presumably by inhibition of repolarizing cardiac K^+ currents. The testing of Class III drugs against arrhythmias is limited by the lack of adequate models and selective drugs. Clinically available Class III agents have mixed electrophysiological actions although newer drugs with greater Class III selectivity are claimed e.g. (+)-sotalol plus its imadizolium derivatives (Lis *et al.*, 1987), N-acetylprocainamide (Dangman & Hoffman, 1981) and its stable amide (methanesulphonamide) sematilide, (Lumma *et al.*, 1987), as well as radically new agents such as tedisamil, UK 68,798 and risotilide (Walker & Beatch, 1988; Gwilt *et al.*, 1989; Colatsky *et al.*, 1989).

Tedisamil appears to inhibit selectively the transient outward potassium current, i_{to}, in cardiac tissue (Dukes & Morad, 1989). As a result it is bradycardic in all species tested (Buschmann *et al.*, 1989a) but markedly increases the Q-T_c interval as well as APD in the rat (Walker & Beatch, 1988; Beatch *et al.*, 1990). It also widens the Q-T_c interval in primates (Buschmann *et al.*, 1989b).

In view of the above, the present study was undertaken to assess the electrophysiological and antiarrhythmic actions of tedisamil in the rat. We have previously used rat models to demonstrate the dose-related efficacy of Class I and Class IV agents against ischaemia-induced arrhythmias (Abraham *et al.*, 1989; Curtis *et al.*, 1986).

The following experiments were performed in rats: (i) Evaluation of antiarrhythmic effectiveness against occlusion-induced arrhythmia. (ii) Evaluation of effects on the ECG and responses to electrical stimulation. (iii) Evaluation of actions on intracellular potentials *in vivo*.

The occurrence and density of specific potassium channels are tissue- and species-dependent (Irisawa, 1987). Thus we considered it important to conduct all studies in a single species. In a systematic manner we hoped to establish that tedisamil increased action potential duration *in vivo*, resulting in Q-T_c widening and increasing ventricular refractoriness. An increase in refractoriness would be expected to inhibit the occurrence and severity of ischaemia-induced arrhythmias. Thus, tedisamil might be a useful drug in rats with which to investigate the antifibrillatory and antiarrhythmic effectiveness of a potent and efficacious Class III compound.

Methods

Male Sprague-Dawley rats (250–350 g) were used throughout. When required, pentobarbitone (45 mg kg^{-1}) was used for anaesthesia and animals artificially ventilated with O_2 at a stroke volume of 10 ml kg^{-1}, 60 min^{-1}; a regimen which has been found to keep blood gases at normal levels (Maclean & Hiley, 1988). Body temperature was maintained at 37.0 ± 0.5°C by means of a thermocouple linked to a heating lamp. Tedisamil (KC8857), i.e. N,N'dicyclopropylmethyl-9, 9-tetramethylene-3,7- diazabicyclo(3,3,1) nonane 2 HCl, was supplied by Kali-Chemie, FRG. It was dissolved in saline.

Doses and route of administration of tedisamil

In a separate study we determined the maximum tolerated dose of tedisamil. Tedisamil at 4 mg kg^{-1}, i.v. (given over 2 min) produced no symptoms of adverse effects, whereas 8 mg kg^{-1} produced respiratory symptoms of gasping and disturbed breathing. None of these doses produced arrhythmias. Adverse effects were attenuated when the drug was injected more slowly. The antiarrhythmic actions of tedisamil in conscious rats were therefore studied at 1, 2 and 4 mg kg^{-1} with doses administered as an infusion over 10 min. In electrical stimulation studies, tedisamil was administered in a cumulative manner with doses of 0.5, 0.5, 1, 2, and 4 mg kg^{-1} given 15 min apart; each dose was infused over 2 min. The same dosing schedule was used for *in vivo* intracellular studies. In order to allow for comparisons between the different dosing regimens, equivalent Q-T_c widenings in different preparations

[1] Present address: Department of Pharmacology, Israel Institute for Biological Research, Ness Ziona, Israel.
[2] Author for correspondence.

were assumed to indicate equivalence of plasma concentrations.

Evaluation of antiarrhythmic properties

Ischaemic arrhythmias were produced in conscious rats surgically implanted 7 days previously with a loosely-applied left anterior descending (LAD) coronary artery occluder, ECG electrodes (approximate lead V3) as well as arterial and venous cannulae as described previously (Johnston *et al.*, 1983). Experiments were performed one week after surgery. The measured variables included serum potassium, blood pressure (BP), heart rate (HR) and ECG changes as well as arrhythmias before and after occlusion of the LAD coronary artery. Arrhythmias were recorded 0–0.5 h, 0.5–4 h and 24 h after occlusion plus, in some cases, one week and one month later. As a summary of arrhythmic history, arrhythmia scores (AS) were calculated according to the occurrence of ventricular premature beats (VPBs) and the number and duration of ventricular tachycardia (VT), and VF episodes as justified elsewhere (Curtis & Walker, 1988). One day after occlusion, rats were killed and their occluded zone (OZ or zone-at-risk) estimated as described previously (Johnston *et al.*, 1983).

Doses of 1, 2, and 4 mg kg^{-1} of tedisamil were administered to groups of 9 rats as a 10 min infusion with a repeat dose 1 h after occlusion. Control rats ($n = 9$) received saline. The design of the experiments and the analysis of arrhythmias followed the Lambeth Conventions (Walker *et al.*, 1988). In keeping with the Conventions, the number of VPB in any time period were log transformed before statistical analysis in order to normalize statistically such data.

As a control for tedisamil-induced bradycardia, separate groups of rats had their ventricles electrically-stimulated so as to overcome the bradycardia. On the day of occlusion, chronically prepared rats were fitted with silver wire intraventricular electrodes, under pentobarbitone anaesthesia, in order to pace their hearts at 6.5 Hz, i.e. the rate found in control rats. Tedisamil (2 mg kg^{-1}, i.v.) was infused as above. Ventricles were stimulated from 4–15 min following occlusion, i.e., the vulnerable period for arrhythmias.

In separate experiments, animals with one day, one week, and one month old infarcts were found to have variable incidences of ventricular arrhythmias. The effects of tedisamil against these arrhythmias was tested using the cumulative dose regimen previously described, with 15 min between doses. Drug-effects were assessed in terms of an increase, or decrease (by 90%) in the control rate of appearance of spontaneous arrhythmias.

Electrical stimulation

The technique of ventricular electrical stimulation in intact rats was used as an index of the electrophysiological actions of tedisamil. To control for possible effects of anaesthesia and acutely-performed surgery, electrical stimulation was performed in acutely prepared pentobarbitone-anaesthetized rats, and chronically prepared halothane-anaesthetized rats.

In pentobarbitone-anaesthetized and artificially ventilated rats, the right jugular vein and left carotid artery were cannulated for drug administration and blood pressure recording, respectively. Teflon coated silver wire electrodes were implanted in the left ventricle as described previously (Curtis & Walker, 1986). The suitability of electrode location was confirmed by stable threshold values for stimulation, and verified by post mortem examination.

The following were measured: (a) Current threshold for single ventricular extra-systoles and for VF (VF_t) at 50 Hz and 2 times threshold pulse width as determined for the animal being investigated; (b) maximum following frequency (MFF) obtained by smoothly increasing pacing frequency (at twice threshold current and pulse width) from 6 Hz until the heart failed to follow, on a 1:1 basis, the increasing stimulation rate; (c) effective refractory period (ERP) obtained by pacing the heart at 7.5 Hz (at 2 times threshold pulse width and current) and randomly adding an extra stimulus at an increasing delay. The shortest interval required to produce a premature extrasystole was taken as the effective refractory period.

ECG and blood pressure changes were recorded throughout. Tedisamil was administered by a cumulative dose regimen and determinations made 10–15 min after each dose. Triplicate determinations were made at each dose level and six animals were tested per group.

In chronically-prepared animals, abdominal aortic and inferior vena caval cannulae were implanted together with a loose electrode carriage (Walker & Beatch, 1988) one week prior to testing. This carriage, fashioned out of PE 10 polyethylene tubing, was designed to reversibly position stainless steel electrodes (2.5 mm apart) against the left ventricular epicardium. On the day of experiment, animals were anaesthetized with halothane (1%) and blood pressure and ECG recorded. The electrode was positioned against the ventricle wall by gentle traction on the electrode assembly. Lead II was used to assess effects on the ECG, while lead III served to detect electrically-induced arrhythmias. The above protocol for acutely prepared rats was followed, except that only doses of 0.5, 0.5, 1 and 2 mg kg^{-1} tedisamil were studied.

Epicardial intracellular action potential recording in vivo

Under pentobarbitone anaesthesia and artificial ventilation, the carotid artery and jugular vein were cannulated, and ECG electrodes placed subcutaneously. The heart was exposed through an incision at the level of 4th–5th intercostal space and a portion of the left ventricular epicardial surface immobilized by suturing it to a looped silver/silver chloride reference electrode. Epicardial action potentials were recorded with 3 M KCl fibre-filled microelectrodes and a floating-tip technique. This technique has been used in the evaluation of the effects of tetrodotoxin on action potential configuration (Abraham *et al.*, 1989). Doses of tedisamil (0.5, 0.5, 1, 2 and 4 mg kg^{-1}, i.v.) were administered consecutively every 15 min. A multiple impalement technique was used to assess drug effects upon action potential height, maximum value of dV/dt for the rising phase of the action potential (dV/dt_{max}) and APD at 10, 25, 50 and 75% of repolarization (APD_{10}, etc).

Data analysis

Most studies were conducted according to blind and random protocols. Statistical analysis was performed by ANOVA. Differences between means was determined by Duncan's multiple range test. Contingency tables were used to determine significance for nonparametric data.

Results

Occlusion study

The time course of tedisamil and occlusion effects on blood pressure and heart rate were summarized in Figure 1 which illustrates that tedisamil produced a dose-dependent bradycardia and an increase in mean arterial blood pressure. At 30 min post-occlusion the bradycardic effect of tedisamil was still apparent, whereas the pressor response was lost. At 1 h and 4 h post-infusion, heart rate and blood pressure were restored to control values. Serum potassium levels were not different in any of the groups with average values of 3.4–3.6 mM.

The effects of tedisamil on P–R, QRS and Q–T_c intervals of the ECG prior to occlusion are shown in Table 1. Tedisamil significantly widened the Q–T_c interval, and prolonged P–R duration, but had no effect on QRS interval. Tedisamil also influenced the ECG after occlusion by increasing the degree of 'S–T' segment elevation induced by occlusion (Figure 2). Similar effects on R-waves were not noted.

The effects of tedisamil on arrhythmias as measured by the incidence of VT, VF, as well as $\log_{10}$ of total VPB in any time

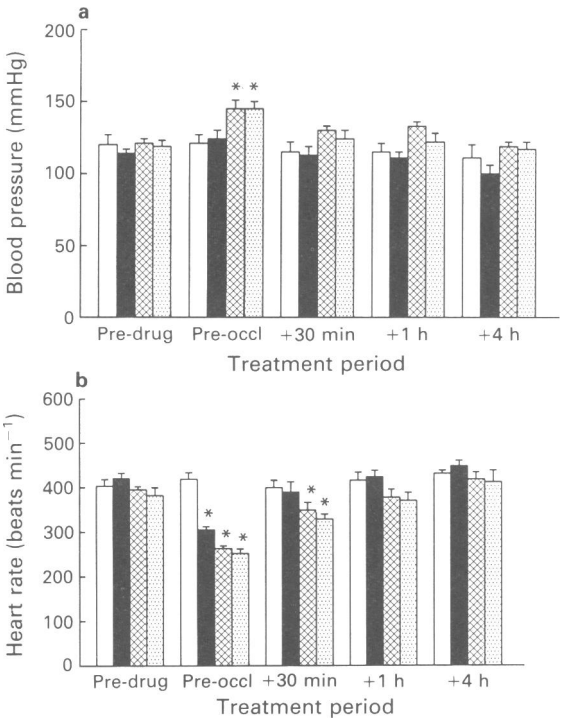

Figure 1 Effects of treatment and coronary artery occlusion on mean arterial blood pressure (a) and heart rate (b) in conscious chronically prepared rats. Tedisamil was administered as a 10 min infusion and occlusion performed 5 min after infusion. Open columns = saline; closed columns = 1 mg kg^{-1}; cross-hatched = 2 mg kg^{-1} and stippled = 4 mg kg^{-1} tedisamil. Values were determined before drug (pre-drug), before occlusion (pre-occl) and 30 min, 1 and 4 h after occlusion of the LAD coronary artery. Each column is the mean of $n = 5$–9 rats/group; s.e.mean shown by vertical bars. * $P < 0.05$ vs. control.

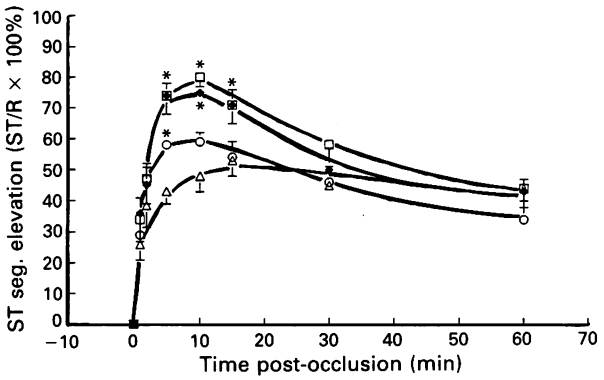

Figure 2 Effects of tedisamil on 'S–T' segment changes induced by LAD coronary occlusion in conscious chronically prepared rats. Drug was administered as a 10 min i.v. infusion and occlusion performed 15 min after beginning infusion. Symbol (△) indicates saline; (○) 1 mg kg^{-1}; (□) 2 mg kg^{-1} and (◆) 4 mg kg^{-1} tedisamil. 'S–T' segment amplitude is presented as percentage of R-wave amplitude. Each point is a mean of $n = 9$ rats/group; s.e.mean shown by vertical bars. For the sake of clarity only the first hour of occlusion is shown. * $P < 0.05$ vs. control.

period are summarized in Table 2. Tedisamil caused a dose-dependent reduction in the occurrence of VF, completely preventing this arrhythmia at 4 mg kg^{-1}. However, despite suppressing VF, the less serious arrhythmias (VT and VPB) were not obtunded. While tedisamil did not decrease the incidence of VT, it did modify its morphology. In control animals, and in those given 1 mg kg^{-1} tedisamil, VT patterns were of short cycle length and wide complex and were accompanied by precipitous falls in blood pressure (data not shown). In contrast, with 2 and 4 mg kg^{-1} tedisamil, VT was of a narrow-complex type with a long cycle length (e.g., 140 ms = 430 b min^{-1}) and was accompanied by minimal reductions in BP. Thus, although the incidence of VT in all groups was similar, the episodes were not equivalent. AS values, calculated according to the occurrence of VPBs and number and duration of VT and VF (Johnston *et al.*, 1983), were dose-dependently reduced by tedisamil: in the 0–30 min post occlusion period, 2 mg kg^{-1} tedisamil reduced AS to 2.4 ± 0.4, and 4 mg kg^{-1} tedisamil to 1.2 ± 0.5 ($P < 0.05$ for both effects), from the control value of 4.1 ± 0.8. For the 0.5–4 h time period, 2 mg kg^{-1} tedisamil reduced AS to 1.3 ± 0.4, from a control value of 2.6 ± 0.7 ($P < 0.05$).

Table 1 Effects of tedisamil on ECG variables in conscious rats

Dose (mg kg^{-1})	*P–R* (ms) *Pre*	*Post*	*QRS* (ms) *Pre*	*Post*	*Q–T$_c$* (ms) *Pre*	*Post*
Saline	41 ± 1	42 ± 1	22 ± 1	22 ± 1	215 ± 10	210 ± 10
1	38 ± 1	44 ± 1	22 ± 1	23 ± 1	220 ± 5	280 ± 9*
2	38 ± 2	47 ± 1*	23 ± 1	23 ± 1	205 ± 5	320 ± 7**
4	44 ± 2	53 ± 2*	22 ± 1	23 ± 1	195 ± 5	330 ± 9*

Tedisamil was administered to conscious rats as a 10 min i.v. infusion (see Methods). ECG values were recorded before (pre) and 4 min after (post) the end of infusion. Values are mean ± s.e.mean for $n = 9$ rats per group.
* $P < 0.05$; ** $P < 0.01$ compared with saline.

Table 2 Effects of tedisamil on the occurrence of occlusion-induced arrhythmias in conscious rats

	Arrhythmias in 0–0.5 h and 0.5–4 h post-occlusion periods						
	Incidence						
	VT		*VF*			*log$_{10}$ VPB*	
Dose	*Time post occlusion* (h)						
(mg kg^{-1})	*0–0.5*	*0.5–4*	*0–0.5*	*0.5–4*	*Mort*	*0–0.5*	*0.5–4*
Saline	8/9	3/5	7/9	3/5	4	1.5 ± 0.1	2.0 ± 0.1
1	7/9	5/7	6/9	3/7	2	1.8 ± 0.2	2.5 ± 0.2
2	8/9	2/8	4/9	1/8	1	1.6 ± 0.2	2.1 ± 0.4
4	4/9	4/9	1/9*	0/9	0*	1.4 ± 0.2	2.1 ± 0.3

Tedisamil was administered to conscious chronically prepared rats as a 10 min i.v. infusion as indicated in Methods. Occlusion was performed 5 min after the end of infusion. A repeat dose was given 1 h after occlusion. Arrhythmias occurring in the 0–0.5 h and 0.5–4 h periods are expressed as incidence (number of rats per group having one or more events) of VT or VF and the mean ± s.e.mean of $\log_{10}$ VPB. Initial group size was 9, but animals dying (Mort) in the first post-occlusion period (0–0.5 h) reduced the number of survivors in the second period (0–4 h).
* $P < 0.05$ compared with saline.

Variations in occluded zone size may account for variations in the incidence and severity of arrhythmias and so these were measured in all animals and were not found to be altered by tedisamil treatment. Values ranged from a mean of 34 to 37% and were not substantially different from each other. The similarity in occluded zone size might have been expected to result in similar 'S–T' segment changes being induced by occlusion but this was not found to be the case (see above).

To compensate for tedisamil-induced bradycardia, a separate group of paced rats was administered 2 mg kg^{-1} tedisamil. Ventricular pacing at 6.5 Hz appeared to increase the efficacy of tedisamil such that VT incidence (2/11), VF incidence (3/11) and AS (1.0 ± 0.4) were significantly lower ($P < 0.05$) in the electrically-paced rats than in controls (8/9, 9/9 and 3.8 ± 0.4, respectively). When comparison was made between paced and unpaced rats treated with 2 mg kg^{-1}, the VT incidence was reduced significantly (see Table 2 and above) in paced rats whereas VF incidence was unaffected. In the pacing study, the total duration of the ECG complex almost equalled the cycle length, i.e., Q–T/R–R = 0.95.

Effects in infarcted rats

Administration of tedisamil to rats with one-day, one-week, or one-month old infarcts did not affect blood pressure but produced bradycardias similar in magnitude to those seen in other rats. For example, control values for heart rate were 440 ± 12, 410 ± 15 and 330 ± 30 beats min^{-1} in one-day, one-week and one-month infarcted rats, respectively. At 4 mg kg^{-1} of tedisamil, heart rate in the three groups fell to 259 ± 9, 245 ± 16 and 225 ± 18 beats min^{-1}, respectively. Tedisamil exhibited antiarrhythmic activity in one-day infarcted animals suppressing VPBs in 8/14 rats. The effective dose ranged from 1–4 mg kg^{-1}. In one-week and one-month infarcted rats the incidence of arrhythmias was too low to test for antiarrhythmic effects. Proarrhythmic actions, consisting of an increase in VPB, bigeminy, or alternating bradytachycardia, were only encountered in 8/14 one-day infarcted rats. This occurred at a median cumulative dose of 7 mg kg^{-1}. Proarrhythmic effects of increased VPB and short episodes of VT were seen in 1/5 rats that had been infarcted for one-week. These occurred after cumulative doses of 1 and 4 mg kg^{-1} tedisamil. A similar finding occurred in 1/5 rats infarcted for 1 month after it had received a cumulative dose of 8 mg kg^{-1}. Tedisamil also elevated the 'S–T' segment in infarcted rats as is illustrated in Figure 3.

Effects of tedisamil on electrical stimulation

The effects of tedisamil on Q–T intervals, blood pressure, heart rate and electrical stimulation variables in chronically-prepared halothane anaesthetized rats are summarized in Table 3. Tedisamil lacked marked effects on blood pressure, but decreased heart rate as seen in the previous experiment. At doses of 4 mg kg^{-1} or greater, tedisamil completely inhibited electrical-induction of VF despite only moderately increasing VF_t threshold. At 4 mg kg^{-1}, tedisamil reduced MFF to 7 Hz and increased ERP to twice control values. The above responses were accompanied by marked $Q–T_c$ prolongation and insignificant P–R and QRS prolongation of the ECG. The effects of tedisamil on electrically-induced arrhythmias were quantitatively and qualitatively similar in acutely-prepared pentobarbitone anaesthetized rats although control ERP and VF_t values tended to be lower.

Effects on intracellular action potentials recorded in vivo

Representative epicardial transmembrane potentials before and after tedisamil are illustrated in Figure 4. As shown in the figure, tedisamil caused a marked prolongation of the epicardial intracellular potential. Table 4 shows that tedisamil prolonged APD by up to 500% at the higher dose levels. In addition, at the highest doses, tedisamil depressed the maximum rise rate (dV/dt_{max}) of phase 0 of the action poten-

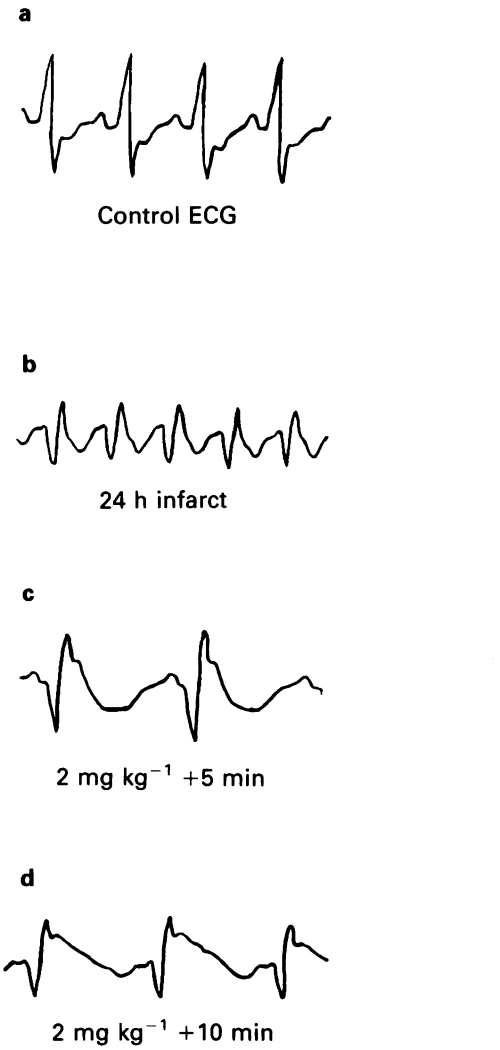

Figure 3 Effects of tedisamil on S–T segment elevation in 1-day infarcted rats. Representative ECG traces were sampled (a) before occlusion (control), (b) 24 h post occlusion and (c) 5 and (d) 10 min after administration of 2 mg kg^{-1} of tedisamil. Chart speed 100 mm s^{-1}.

Table 3 Effects of tedisamil on electrical stimulation characteristics of halothane-anaesthetized rats

Dose (mg kg^{-1})	*MFF* (Hz)	*ERP* (ms)	*VF_t* (μA)
0	13 ± 1	64 ± 7	230 ± 40
0.5	12 ± 2	68 ± 10	230 ± 50
1	11 ± 2	76 ± 12	250 ± 50
2	9 ± 2*	94 ± 26*	270 ± 60
4	7 ± 2**	107 ± 39*	VF not inducible

Dose (mg kg^{-1})	*Q–T* (ms)	*BP* (mmHg)	*HR* (beats min^{-1})
0	55 ± 3	105 ± 6	410 ± 15
0.5	100 ± 3**	106 ± 7	360 ± 20*
1	115 ± 5**	110 ± 6	335 ± 13**
2	130 ± 6**	104 ± 6	290 ± 25**
4	155 ± 8**	109 ± 10	245 ± 16**

Tedisamil was administered cumulatively every 15 min with each dose infused over 2 min. Doses are expressed cumulatively. Measurements were made 10 min after injections. All values represent mean ± s.e.mean for 6 rats per group. Effective refractory period (ERP), maximum following frequency (MFF) and current threshold for ventricular fibrillation (VF_t) were measured as detailed in Methods.
* $P < 0.05$; ** $P < 0.01$ from pre-drug.

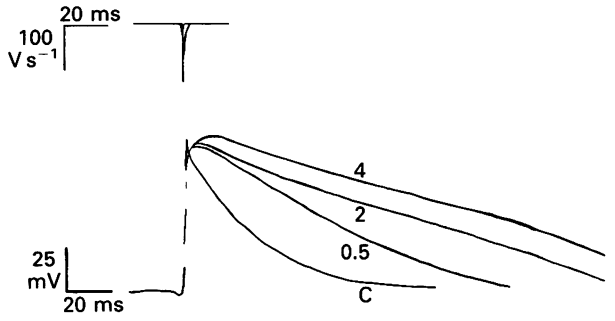

Figure 4 Representative epicardial action potential recorded *in vivo*, before (C) and 5 min after administration of tedisamil 0.5, 2 and 4 mg kg^{-1}, i.v. Redrawn from original records.

tial. The depression of dV/dt_{max} was not associated with a decrease in action potential height, instead (as shown in Figure 4) tedisamil tended to round-off the action potential spike without reducing its ultimate height.

Discussion

In rats, antifibrillatory doses of tedisamil widened the Q–T_c interval of the ECC and prolonged APD which justifies its classification as a Class III antiarrhythmic drug in this species. Effects on Q–T_c intervals, intracellular potentials, and responses to electrical stimulation were probably due to potassium channel blockade (see later). Prolongation of APD widened the Q–T_c interval and prolonged ventricular refractoriness. An increased refractoriness accounted for the observed decrease in MFF. Presumably, tedisamil prevented both ischaemia and electrically-induced VF by reducing the ability of the heart to follow high rates of stimulation.

Studies in rat isolated myocytes indicate that tedisamil speeds inactivation of the transient outward current (i_{to}) (Dukes & Morad, 1989), a major repolarizing current in rat ventricle (Irisawa, 1987). Blockade of this current would therefore widen the action potential in the rat. Blockade of i_{to} also explains why tedisamil is bradycardic in various species (Buschmann *et al.*, 1989a). Tedisamil prolongs sinoatrial node potentials (Oexle *et al.*, 1987) since i_{to} contributes to repolarization in these cells (Irisawa, 1987). In our study, Q–T_c prolongation paralleled bradycardia. Bradycardia alone does not widen rat (unlike guinea-pig) ventricular epicardial action potentials recorded *in vivo* (unpublished observations from our laboratory). Thus, the antifibrillatory activity of tedisamil is most easily explained by its Class III action.

Tedisamil widens Q–T_c interval in rats and baboons (Buschmann *et al.*, 1989b) but there is debate as to whether drugs which prolong Q–T interval prevent, or precipitate, arrhythmias. Association of Q–T prolongation with bradycardia and hypokalaemia has been clinically associated with arrhythmias, particularly Torsade de Pointes (Bacaner *et al.*, 1986; Singh, 1987; Surawicz, 1987; 1989).

The importance of K^+ channels in maintaining and terminating cardiac action potentials is recognized (Noma, 1987; Cook, 1988). The antiarrhythmic efficacy, or arrhythmogenic potential, of selective K^+ blockade merits investigation. Ideal antiarrhythmic drugs have yet to be found (Brugada, 1987), particularly those for ischaemia-induced arrhythmias. Tedisamil, with its marked Class III effects and lack of action on blood pressure, is thus useful for antiarrhythmic studies in rats subjected to myocardial ischaemia.

The *in vivo* electrophysiological actions of tedisamil were consistent with the findings of Dukes & Morad (1989) regarding action potential widening plus Class I actions at the higher doses or concentrations. The slight reduction of dV/dt and prolongation of P–R interval suggested ventricular sodium channel blockade (Buchanan *et al.*, 1985) at higher doses. However, any blockade must have been slight since neither elevations in thresholds for stimulation-induced VF, nor QRS prolongation, occurred. A fall in dV/dt normally reduces action potential height, but this did not occur in this study, presumably because the reductions in repolarizing potassium currents allowed the action potential to approach closer to the sodium equilibrium potential. In conclusion, the limited Class I actions of tedisamil did not contribute to its antiarrhythmic profile. The antiarrhythmic profile of tedisamil was markedly different from that for Class I drugs. The latter do not preferentially suppress VF but do increase VF_t (Walker & Beatch, 1988). Furthermore, the antiarrhythmic actions of Class I drugs do not convert VF to the particular form of VT seen with tedisamil. Another mechanism unlikely to have contributed to the antiarrhythmic actions of tedisamil was bradycardia. Previous studies have failed to show a relationship between bradycardia and antiarrhythmic actions (Curtis *et al.*, 1987; Abraham *et al.*, 1989).

Re-entry and abnormal automaticity are arrhythmogenic mechanisms which differ in their response to different drugs (Pogwizd & Corr, 1987; Brugada, 1987; Gitant & Cohen, 1988). An increase in refractoriness may be antiarrhythmic by selectively abolishing re-entry or, alternatively, by reducing the time available for arrhythmias. Action potentials occupying the whole cycle would leave no 'free-time' for arrhythmias. In our 'unpaced' study the T–Q interval (normally 70 ms), a measure of 'free-time', was not reduced by tedisamil suggesting that a simple increase in refractoriness did not account for the antiarrhythmic actions observed. However, in rats subjected to pacing such that T–Q was less than 10 ms, both VT and VF were reduced.

Re-entry circuits can be abolished by prolonging refractoriness within the circuit. Increased refractoriness can be expressed as an increase in minimal cycle time and this results either in termination of the re-entry, or a circuit of longer path-length. With VT, an increased path-length results in a slower VT providing conduction velocity remains unchanged. Such a mechanism would explain the preferential abolition of VF and occurrence of slow VT seen in our study. Tedisamil, 4 mg kg^{-1}, specifically abolished VF (stimulation or ischaemia-induced), slowed VT, reduced MFF to 7 Hz and prolonged effective refractory period to 107 ms. If, under these conditions, the re-entrant circuit had a conduction velocity of 0.6 m s^{-1}, the minimal path-length would be 6.42 cm. In the absence of tedisamil, a refractory period of 64 ms and the

Table 4 Effects of tedisamil on epicardial action potential variables recorded *in vivo*

	APD				*Action potential*	
Dose (mg kg^{-1})	10%	25%	50% (ms)	75%	dV/dt (V s^{-1})	*AP height* (mV)
Control	4.8 ± 0.4	10 ± 0.4	19 ± 0.7	45 ± 3	183 ± 6	97 ± 3
0.5	8 ± 1	19 ± 2	41 ± 3	81 ± 5	173 ± 8	103 ± 3
1	11 ± 1	26 ± 2	58 ± 6	95 ± 4	175 ± 7	105 ± 2*
2	17 ± 1	37 ± 1	69 ± 2	114 ± 3	170 ± 15	110 ± 2*
4	24 ± 4	53 ± 8	100 ± 14	162 ± 22	115 ± 18*	102 ± 4
8	29	67	125	195	165	107

After pre-drug recording, tedisamil was injected every 15 min according to a cumulative dose regimen. Each point is a mean ± s.e.mean of 6 rats with values averaged 14–15 min after dosing. At the 8 mg kg^{-1} dose level error is not given since n was less than 6. APD is shown at 10, 25, 50 and 75% of repolarization. The trend for tedisamil to increase APD at all levels was statistically highly significant ($P < 0.001$); * indicates $P < 0.05$ for difference from control in all other cases.

above conduction velocity, the minimal path-length would be 3.84 cm. This path-length would allow for multiple re-entry (i.e., VF) in the rat heart.

It was notable that tedisamil (0–4 mg kg^{-1}) was not arrhythmogenic in non-occluded rats but was arrhythmogenic in animals subjected to myocardial infarction. In a previously reported series of experiments, proarrhythmic effects of tedisamil in non-infarcted rats were seen at doses above 15 mg kg^{-1} and these arrhythmias depended upon the presence of an intact autonomic nervous system and signs of gNa^+ blockade (Howard *et al.*, 1989). Thus bradycardia and Q–T prolongation alone were not sufficient to induce arrhythmias. A 'substrate' of pathology (i.e. infarction) had to be present to reveal the arrhythmogenic actions of high doses of tedisamil.

The 'S–T' segment elevation induced by tedisamil was probably due to alterations in myocardial repolarization patterns since all animals had the same size OZs. If coronary vasoconstriction was the cause of the elevated 'S–T' segment (Wergia *et al.*, 1949) elevation should have been present in non-occluded rats, but this was not the case. The 'S–T' elevating actions of tedisamil may render it useful in the diagnosis of ischaemia/infarction.

In conclusion, tedisamil had Class III antiarrhythmic actions in the rat and was antifibrillatory. These actions were associated with increased APD, Q–T interval and refractoriness. Antifibrillatory actions were only seen at doses giving a four fold increase in APD. Previously available Class III antiarrhythmics produced only limited AP widening and thus may have limited antifibrillatory activity in the setting of myocardial ischaemia.

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Bicuculline blocks nicotinic acetylcholine response in isolated intermediate lobe cells of the pig

[1]Zhong-Wei Zhang & Paul Feltz

Institut de Physiologie (UA309 CNRS), Université Louis Pasteur, 21 Rue René Descartes, 67084 Strasbourg Cedex, France

1 The effect of bicuculline on nicotinic acetylcholine (ACh) responses in isolated intermediate lobe (IL) cells of the pig was investigated by use of patch-clamp techniques. Bicuculline was found to reduce ACh-evoked whole-cell currents (I_{ACh}) in all cells tested ($n = 40$).

2 The blocking effect of bicuculline on I_{ACh} was dose-dependent, the concentration producing half-maximal blockade being 43.8 μM.

3 The blockade of I_{ACh} by bicuculline was not voltage-dependent at membrane potentials above −60 mV, but a slight voltage-dependence was observed at holding potentials (HP) of −80 and −100 mV.

4 The inhibitory effect of bicuculline on I_{ACh} was partially competitive at a HP of −60 mV.

5 Neither SR 95531, a pyridazinyl γ-aminobutyric acid derivative, nor *t*-butylbicyclophosphorothionate (TBPS) blocked I_{ACh} in IL cells.

6 It is concluded that bicuculline interacts directly with the ACh receptor-ionophore complex on porcine IL cells.

Introduction

The selective antagonism of bicuculline to the γ-aminobutyric acid (GABA)-mediated responses in vertebrate systems has been studied, *in vivo* and *in vitro*, by the measurement of several parameters such as cell firing, GABA-gated Cl^- currents and specific binding of $GABA_A$ ligands (Reviews by Nistri & Constanti, 1979; Bormann, 1988). Stable radiolabelled derivatives of bicuculline have also been used to assay biochemically the kinetics of GABA receptors (Olsen & Snowman, 1983). Thus, bicuculline is applied as an important tool in tracking the physiologically relevant function of GABA-ergic synaptic activities in various experimental situations ranging from electrophysiological studies of post and pre-synaptic inhibition in neural networks, to integrated studies of behavioural regulation (for review see, Krogsgaard-Larsen *et al.*, 1988).

Using the pars intermedia of the pituitary as a model to study neural control of endocrine systems, we and others have demonstrated the functional role of GABA postsynaptic actions in the IL cells, and, the presence on these cells of $GABA_A$ and $GABA_B$ receptors with different sensitivity to bicuculline (Taraskevich & Douglas, 1982; Demeneix *et al.*, 1984; 1986a). *In vitro* electrophysiological studies (Davis *et al.*, 1985; Williams *et al.*, 1989) showed that stimulation of pituitary stalk produced in the pars intermedia, a fast depolarization (excitatory postsynaptic potential e.p.s.p.), followed by a slow hyperpolarization (inhibitory postsynaptic potential, i.p.s.p.). The latter was concluded to be a dopaminergic inhibitory effect. The fast e.p.s.p. was interpreted as mediated by $GABA_A$ receptors, as it was suppressed by bicuculline. We raised another possibility in our recent study, where we reported a neuronal-type nicotinic acetylcholine (ACh) receptor in the IL cells maintained in primary culture (Zhang & Feltz, 1990). ACh, as well as nicotine induced depolarization and action potentials in the cells by opening a cation selective conductance. This effect was sensitive to (+)-tubocurarine, hexamethonium and mecamylamine, but not to α-bungarotoxin (Zhang & Feltz, 1990).

In the present study, we investigated the effect of the $GABA_A$ antagonist, bicuculline on these nicotinic ACh receptors in isolated lobe cells of the pig.

[1] Author for correspondence.

Methods

Preparation

The primary culture of porcine IL cells was prepared as described previously (Demeneix *et al.*, 1986b). In brief, porcine pituitary glands were collected from the local abattoir, the anterior lobes were removed by careful dissection and the IL cells were dissociated with collagenase (1 mg ml^{-1}) and trypsin (0.5 mg ml^{-1}). The cell suspension was purified on a bovine serum albumin gradient. Cells were collected and resuspended in culture medium containing 65% Dulbecco's modified Eagle's medium (DMEM), 25% HAM's nutrient mixture F12 and 10% foetal calf serum. The suspension was plated in Costar 35 mm dishes and was kept in a humidified atmosphere of 95% air and 5% CO_2 at 37°C for up to 10 days.

Experiments were carried out between 2 to 8 days after plating. Only isolated cells were chosen for recording.

Recording and analysis

Experiments were performed at room temperature (15–18°C). Cells were recorded in the whole-cell configuration (Hamill *et al.*, 1981) with a List patch-clamp amplifier (EPC-7). Recording pipettes were pulled from soft capillary glass (WV-Mainz) and coated with Sylgard (Rhodorsil RTV-141, Rhone Poulenc). When filled with recording solution, electrodes had a resistance of 3 to 5 MΩ. Current and voltage signals were monitored on a digitizing oscilloscope (Tektronix, 5223) and stored simultaneously on videotape (Sony, PCM-701: bandwidth: d.c. to 10 kHz). Data were usually filtered at 250 Hz (8-pole Bessel-type: −3 dB), and digitized at 500 Hz with a data acquisition board (scientific solutions, 40 kHz) operated by an IBM compatible computer (Olivetti M28).

Solutions

The extracellular solution contained (in mM): NaCl 127, KCl 3, $MgCl_2$ 2, $CaCl_2$ 2, HEPES/NaOH 5 (pH 7.4). The intracellular solution contained (in mM): CsCl 130, $MgCl_2$ 2, $CaCl_2$ 0.9, EGTA 10, HEPES/CsOH 5 (pH 7.2). Acetylcholine chloride and bicuculline methiodide were obtained from Sigma. *t*-Butylbicyclophosphorothionate (TBPS) was obtained from New England Nuclear Corp. The synthetic antagonist, 2-(carboxy - 3' - propyl) - 3 - amino - 6 - paro - methoxyphenyl -

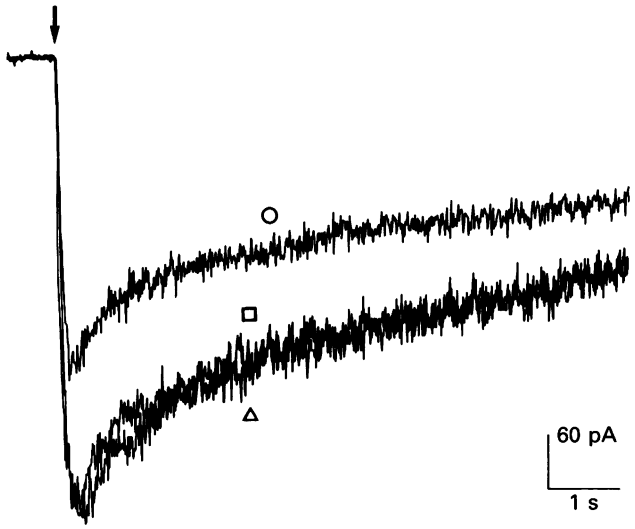

Figure 1 The effect of bicuculline methiodide on acetylcholine (ACh)-induced currents in an isolated IL cell voltage-clamped at −60 mV. Control response to ACh 100 μM (□), response to ACh 100 μM in the presence of bicuculline methiodide 20 μM (○) and recovery (△). Recordings were filtered at 100 Hz and digitized at 200 Hz.

pyridazinium bromide (SR 95531) was generously provided by SANOFI Recherche (Montpellier). Drugs were applied with a microperfusion system (Fenwick *et al.*, 1982).

Results

Experiments were carried out on 40 cells from 9 cell cultures. The blocking effect of bicuculline was observed in all cells tested. Figure 1 illustrates an example of the effect of bicuculline on nicotinic ACh responses in IL cells. Bicuculline reversibly reduced ACh-induced peak current by 39% on the cell voltage-clamped at −60 mV.

The dose-dependence of the effect of bicuculline was examined on 5 cells. Figure 2 shows the dose-responses of bicuculline for the inhibition of ACh-evoked currents. The 50% inhibition (K_D) was observed at 43.8 μM.

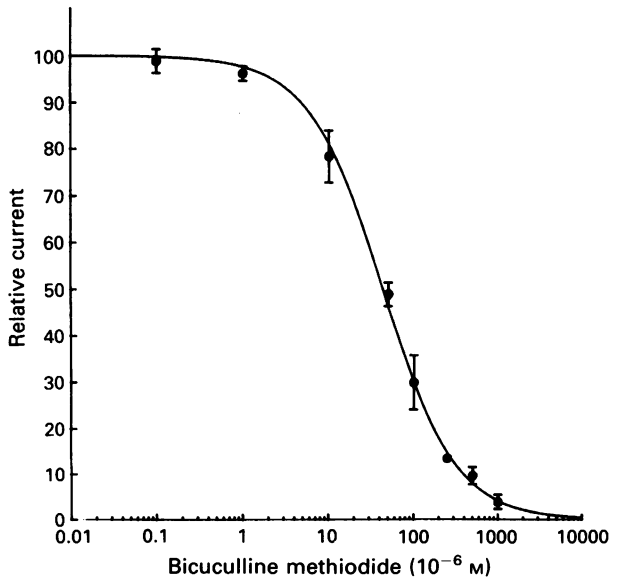

Figure 2 The dose-dependence of the effect of bicuculline methiodide on the peak current evoked by acetylcholine 100 μM. Data obtained from 5 cells held at −60 mV. Vertical bars indicate the s.e.mean.

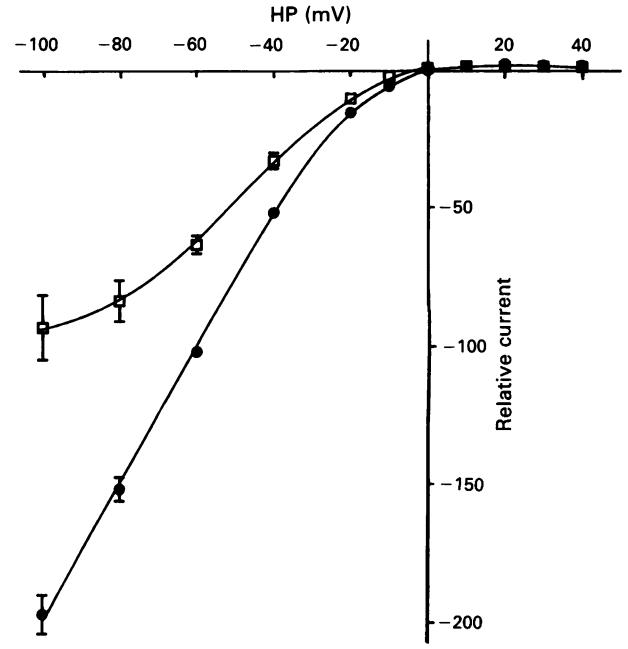

Figure 3 Current-voltage relation of responses induced by acetylcholine 100 μM in the absence (●) and presence (□) of bicuculline methiodide 20 μM. Results obtained from 4 cells; vertical bars show s.e.mean.

Figure 3 shows the voltage-dependence of the effect of bicuculline. Cells were held at different membrane potentials. Bicuculline methiodide was applied simultaneously with ACh by microperfusion. The action of bicuculline was not voltage-sensitive at membrane potentials above −60 mV. However, from −80 to −100 mV, a slight voltage-dependence was observed, the blocking effect being more important at these holding potentials.

Dose-response curves to ACh were established on cells voltage-clamped at −60 mV. The dose-response curve in the absence of bicuculline (Figure 4) was sigmoid with a half maximal value (EC_{50}) of 112 μM. Bicuculline (10 μM, Figure 4) shifted the curve to the right by increasing the EC_{50} to 352 μM. The maximal response was slightly decreased by 8% in the presence of bicuculline (10 μM).

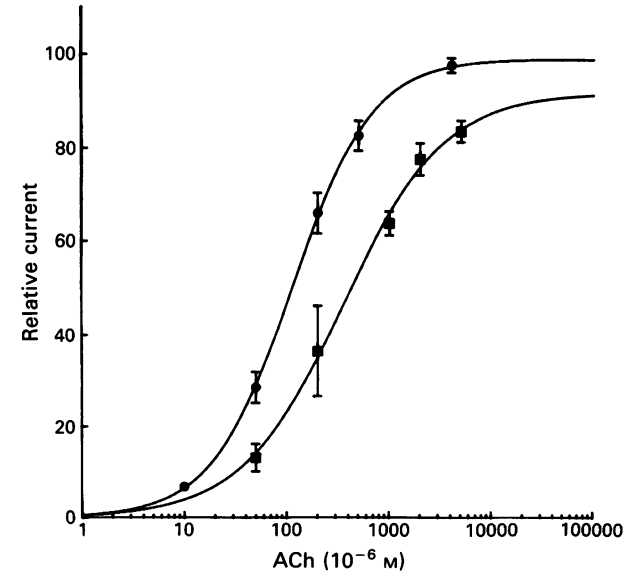

Figure 4 Acetylcholine (ACh) dose-response curves. Control (●); with bicuculline methiodide 10 μM (■). Cells were held at −60 mV. Data were mean values obtained in 4 experiments; vertical bars show s.e.mean.

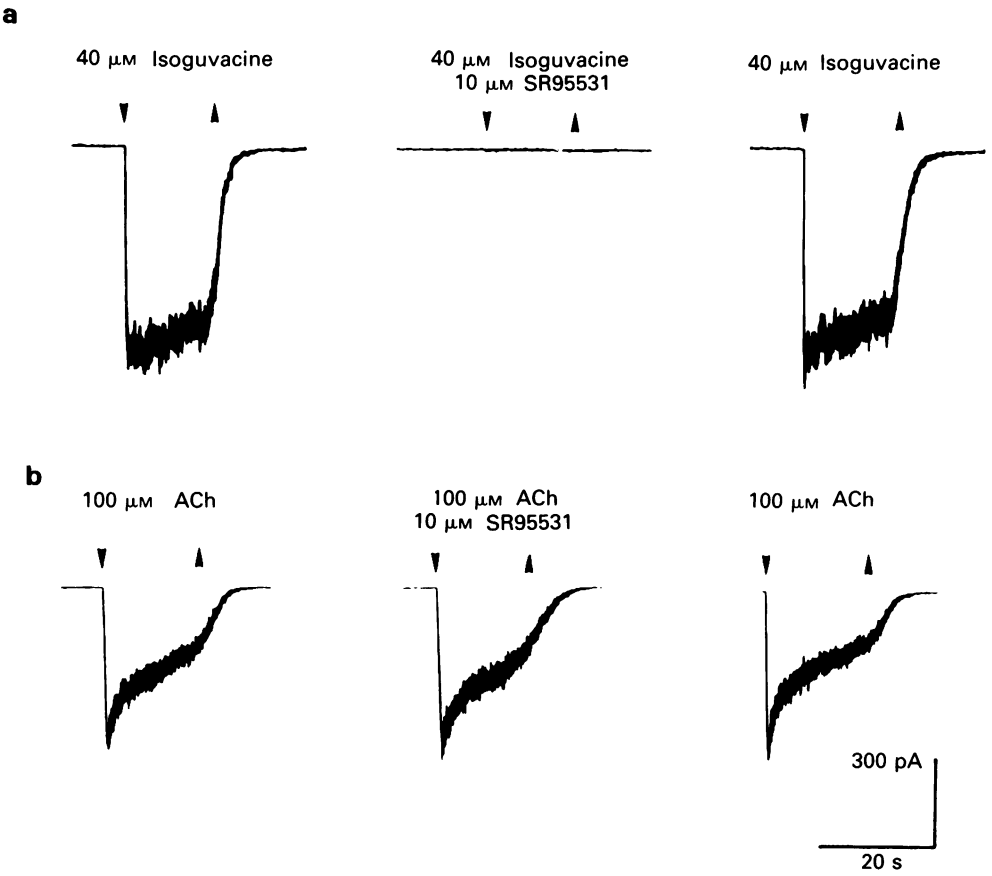

Figure 5 The effect of SR 95531 on responses to stimulation of GABA receptors (a) and acetylcholine receptors (b) (chart display). Results obtained from the same cell with a holding potential of −60 mV.

It is important to know whether other GABA antagonists are able to block the responses of ACh as well as bicuculline. SR 95531 has been demonstrated to be a potent competitive antagonist of $GABA_A$ receptors (Wermuth & Biziere, 1986; Mienville & Vicini, 1987; Hamann *et al.*, 1988). Figure 5a illustrates its efficiency on GABA responses in IL cells. However, SR 95531 failed to reduce ACh-induced responses (Figure 5b and in 7 other cells). TBPS, a non-competitive antagonist of $GABA_A$ receptors (Squires *et al.*, 1983), was not able to block the cholinoceptor responses (3 cells).

Discussion

Following its description as an antagonist of GABA in vertebrates, bicuculline has been widely studied in a variety of invertebrate and vertebrate preparations in the context of its effect on $GABA_A$ responses. The studies in invertebrates have given contradictory results (for review see, Nistri & Constanti, 1979). The effect of bicuculline appeared to be species and tissue-dependent and was much less potent than in vertebrate systems. At high concentrations ($>10^{-4}$ M) bicuculline was shown to antagonize GABA responses in invertebrate muscles (Takeuchi & Onodera, 1972; Earl & Large, 1974; Shank *et al.*, 1974). However, binding studies failed to demonstrate it as a competitive antagonist of GABA (Olsen *et al.*, 1976; Mann & Enna, 1980). Furthermore, with voltage-clamp techniques, Benson (1988) showed that bicuculline was inactive in blocking GABA-induced Cl^- conductance on locust ganglion neurones, whereas it did inhibit ACh-evoked cation channels in these cells. This is, to our knowledge, the only example of blockade of a cation conducting channel by bicuculline, via a competition with a ligand other than GABA.

Despite its role as a potent competitive antagonist of the $GABA_A$ receptor in the mammalian nervous system, a number of studies have questioned the selectivity of bicuculline for GABA (Godfraind *et al.*, 1970; Straughan *et al.*, 1971). Experiments *in vivo* have shown bicuculline to be a weak antagonist of GABA-ergic synaptic inhibition on rat cortical neurones (Straughan *et al.*, 1971; Hill *et al.*, 1973). These investigations prompted biochemical studies *in vitro* revealing bicuculline to be a competitive inhibitor of acetylcholinesterase (AChE) in mammalian brain (Svenneby & Roberts, 1973; Breuker & Johnston, 1975; Olsen *et al.*, 1976), results which were confirmed by electrophysiological recordings in rat central neurones (Miller & Mclennan, 1974). However, neuronal-type nicotinic receptors in the central nervous system (CNS) were neglected in these early studies, and the effect of bicuculline was attributed to an indirect potentiation of muscarinic response via the inhibition of AChE. Our studies in IL cells demonstrate a direct interaction of bicuculline with the ACh receptor-channel complex on vertebrate cells. This finding corroborates the previous findings and suggests a possible blocking effect of bicuculline on ACh receptors, in particular the neuronal-type ACh receptors in mammalian nervous systems. Furthermore, our results emphasize the case for using synthetic GABA antagonists, such as the pyridazinyl GABA derivatives, in the physiological studies of synaptic transmissions in mammalian CNS.

In locust ganglion neurones, the blocking effect of bicuculline was independent of membrane potential (Benson, 1988). The I-V curves obtained from porcine IL cells showed a different profile. A slight voltage-dependence could be observed at holding potentials over the range of −60 to −100 mV. This suggests a possible channel blocking effect of bicuculline.

The conventional dose-response data of our experiments indicate that at −60 mV, the effect of bicuculline (10 μM) on the I_{ACh} is at least partially competitive. The dose-response curve was shifted to the right in the presence of bicuculline.

The slight decrease of maximal response might be due to a weak non-competitive channel blocking effect of bicuculline.

It is of interest to mention that several other AChE inhibitors (e.g. neostigmine and physostigmine) can also antagonize nicotinic ACh responses (Slater *et al.*, 1986; Sadoshima *et al.*, 1988). This effect is supposed to be related to their structural similarity with ACh. ACh, bicuculline methiodide and other quaternary derivatives of bicuculline bear some similarity in structure, including the positive charged quaternary ammonium group. This analogy could be the basis of the interaction of bicuculline with ACh receptors.

In summary, our present study shows a direct inhibition of bicuculline on ACh-induced responses in isolated IL cells. This blocking effect of bicuculline is reversible and partially competitive.

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Sympathetic neurotransmission in the rabbit isolated central ear artery is affected as early as one week following a single dose of X-irradiation

A.L. Stewart-Lee, K.I. Maynard, J. Lincoln & [1]G. Burnstock

Department of Anatomy and Developmental Biology and Centre for Neuroscience, University College London, Gower Street, London WC1E 6BT

1 The short-term effect of a single dose of 4500 rad X-irradiation on sympathetic neurotransmission (involving both noradrenergic and purinergic components) was assessed in the rabbit central ear artery, 1, 4 and 6 weeks post-irradiation.

2 Neurally mediated contractions were reduced as early as 1 week post-irradiation, with responses to lower frequency stimulation being initially most affected. This suggests that the purinergic component of the contractile response is affected earlier than the adrenergic component.

3 There was no change in the amplitude or sensitivity of treated preparations to the cumulative application of noradrenaline when compared with untreated preparations. In contrast, contractions to α,β-methylene ATP (1 μM), a P_2-purinoceptor agonist, were significantly increased at 4 and 6 weeks post-irradiation, although not at 1 week post-irradiation.

4 There were no apparent changes in the pattern of catecholamine fluorescence as a result of irradiation. However, the tissue content of noradrenaline was significantly reduced 6 weeks post-irradiation when compared with control preparations.

5 It is concluded that damage to sympathetic cotransmission is one of the early effects of irradiation, with initial impairment predominantly of the purinergic component.

Introduction

It is well established that blood vessels are sensitive to X-irradiation (see Fajardo & Berthrong, 1988). However, studies to date have concentrated almost entirely on morphological damage to the various parts of the vessel wall, without considering the functioning of the vessel.

In this study, we have investigated changes in the nervous control of the rabbit central ear artery at intervals of 1, 4 and 6 weeks following exposure to a single dose of X-irradiation. Since it is now generally accepted that sympathetic transmission involves noradrenaline (NA) and adenosine 5'-triphosphate (ATP) as cotransmitters in many tissues (see Burnstock, 1988), including the rabbit ear artery (Kennedy *et al.*, 1986; Saville & Burnstock, 1988), the effects of irradiation on both components are considered.

Methods

X-irradiation procedure

Both ears of male New Zealand white rabbits (2–3.5 kg) were locally irradiated with a single dose of X-rays (4500 rad), whilst animals were anaesthetized with fentanyl citrate and fluanisone (collectively known as 'hypnorm') (0.3 mg kg^{-1}, i.p.) and midazolam hydrochloride ('hypnovel') (up to 2 mg kg^{-1}, i.p.). Control animals were also anaesthetized by this procedure, but not irradiated. Irradiation was administered at 383 rad min^{-1} from a General Electric Maximar 250 kV X-ray apparatus. The type of X-rays given were 215 kV at 12.5 mA with no added filtration, 30 cm from the source with a field size diameter of 17.75 cm.

At intervals of 1, 4 and 6 weeks post-irradiation, animals were killed by stunning followed by exsanguination, and the entire ear artery was dissected from the ear and divided into three segments. The proximal segment was used for the pharmacological investigations since this region responds comparatively better than distal regions for this purpose (de la Lande & Waterson, 1968; Griffith *et al.*, 1982; Owen *et al.*, 1983). The middle segment was used for the noradrenaline assay and sections from the distal segment of the artery were used for the histochemistry. The investigations were performed on identical segments from both control and irradiated animals, since regional differences have been reported along the length of the vessel (Griffith *et al.*, 1982).

Pharmacology

Rings (5 mm) obtained from the proximal segment of the rabbit ear artery were mounted horizontally under isometric conditions in 10 ml organ baths so that changes in tension could be measured according to the method of Bevan & Osher (1972). The tissues were bathed in Krebs solution of the following composition (mM): NaCl 133, KCl 4.7, $NaHPO_4$ 1.35, $NaHCO_3$ 16.3, $MgSO_4$ 0.61, glucose 7.8 and $CaCl_2$ 2.52 (Bülbring, 1953). The solution was gassed with 95% O_2 and 5% CO_2 and maintained at a temperature of 37°C. Preparations were allowed to equilibrate for at least 1 h under a resting tension of 1.0 g. Responses of the circular smooth muscle were recorded with a Grass polygraph (model 79).

Electrical field stimulation was provided by a Grass S11 stimulator and carried out with two platinum wire electrodes placed parallel to and on either side of the vessel segment and supplied at supramaximal voltage, 0.1 ms pulse duration for 1 s at 4, 8, 16, 32 and 64 Hz. Contractions elicited by electrical field stimulation were abolished in the presence of tetrodotoxin (1 μg ml^{-1}) which confirms that the contractions were entirely due to neural stimulation with no direct muscle stimulation. Responses to electrical field stimulation were recorded every minute. NA concentration-response curves were constructed by adding cumulative doses of NA to the baths (0.1–100 μM). α,β-methylene ATP (α,β-MeATP) was

[1] Author for correspondence.

added as a single dose (1 μM) and was washed out once a maximum response was established.

Histochemistry

The catecholamine-containing nerves were demonstrated by the glyoxylic acid technique of Lindvall & Bjorklund (1974) and Furness & Costa (1975). Vessel segments were immersed in a freshly prepared 2% w/v glyoxylic acid solution (pH 7.2, adjusted with 5 M NaOH) at room temperature for 1.5 h. After incubation, the segments were stretched onto slides adventitial side uppermost and air-dried until they assumed a transparent appearance. They were then incubated at 100°C for 4 min after which they were mounted in paraffin, viewed and photographed under a Zeiss photomicroscope equipped with a 3RS epi-illumination system. The catecholamine fluorescence was examined with the following filters: exciter-interference BP436/8, barrier-LP 470 and a FT 460 dichromatic beam splitter.

Noradrenaline assay

Segments of rabbit ear artery from control and irradiated animals 6 weeks post-irradiation were cleaned and frozen in liquid nitrogen until assay. After measurements of length and weight were taken, arteries were homogenized in 500 μl of 0.1 M perchloric acid containing 0.4 mM sodium bisulphite and 12.5 ng dihydroxybenzylamine (DHBA) in a motor-driven glass-glass homogenizer. Following low-speed centrifugation, the supernatants were subjected to alumina extraction (Keller *et al.*, 1976). NA and DBHA levels were measured by high performance liquid chromatography with electrochemical detection. Separation was achieved on a radial pak 10 μ Bondapak C18 reverse-phase column (Waters Assoc.) using a mobile phase of 0.1 M sodium dihydrogen phosphate (pH 5.0) containing 5 mM heptane sulphonate, 0.1 mM EDTA and 10% (v/v) methanol at a flow rate of 2 ml min^{-1}. Quantitation was performed with a glassy carbon electrode set at a potential of +0.72 V. NA levels were corrected for recovery by use of the DHBA internal standard.

Treatment of results and statistical methods

Pharmacological and NA assay data are expressed as mean ± s.e.mean. Responses to electrical field stimulation and NA are expressed as grams contractions. Responses to α,β-MeATP are expressed as a percentage of the maximum NA contraction for each preparation. NA content is expressed as ng cm^{-1} length artery. Mean values were calculated from a number of preparations under each of the test conditions (i.e. control, 1, 4 and 6 weeks post-irradiation) denoted as 'n' in the figure legends. pD_2 values were calculated from the mean −log (concentration of noradrenaline) ± s.e.mean which produced 50% of the maximal response. Results were analysed by Student's unpaired t test. A probability of $P < 0.05$ was considered significant.

Drugs used

Noradrenaline bitartrate (NA), α,β-methylene ATP (α,β-MeATP), tetrodotoxin (TTX) and glyoxylic acid monohydrate were all obtained from Sigma Chemical company. A fresh solution of NA in 100 μM ascorbic acid was made up each day. α,β-MeATP was dissolved in distilled water and stored at −20°C at a concentration of 1000 μM. DHBA and heptane sulphonate were obtained from Aldrich Chemicals. 'Hypnorm' (fentanyl citrate (0.315 mg ml^{-1}) and fluanisone (10 mg ml^{-1})) was obtained from Janssen Pharmaceutical Ltd. and 'hypnovel' (midazolam hydrochloride, 5 mg ml^{-1}) was obtained from Roche.

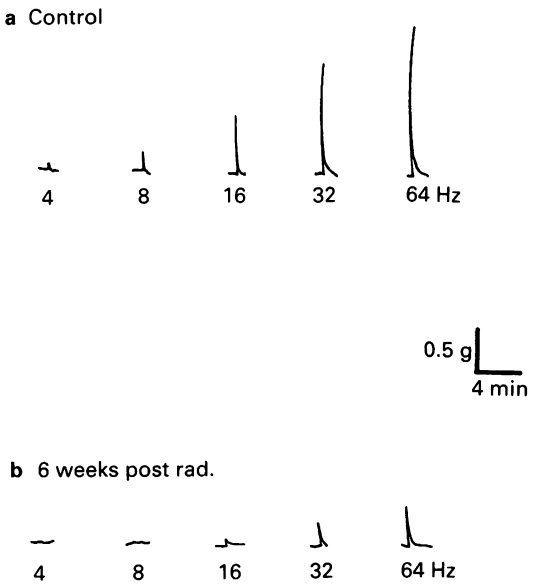

Figure 1 Contractions of the rabbit isolated ear artery to 1 s periods of field stimulation (0.1 ms duration, supramaximal voltage of 60 V; 4, 8, 16, 32 and 64 Hz). Contractions produced in (a) control preparations and (b) preparations 6 weeks following irradiation.

Results

Pharmacology

Field stimulation resulted in rapid, frequency-dependent contractions in control preparations (Figure 1a). However, the effect of radiation was to reduce significantly the contractile response as early as 1 week post X-irradiation with a further reduction at 4 and 6 weeks post-irradiation (Figures 1 and 2). At 4 Hz, only the control vessels produced contractile responses to field stimulation (0.07 ± 0.03 g) and at 8 Hz there were minimal contractile responses in some of the vessels which had been irradiated 1 week previously (0.01 ± 0.003 g), but no response at this frequency in the 4 and 6 weeks post-irradiated vessels. At 16, 32 and 64 Hz, all the irradiated vessels showed a contractile response to field stimulation, although the responses were significantly reduced when compared with the control vessels. Maximum reduction in the contractile response at 16, 32 and 64 Hz appeared to be established by 4 weeks post-irradiation with the responses remaining at a similar level of significance at 6 weeks

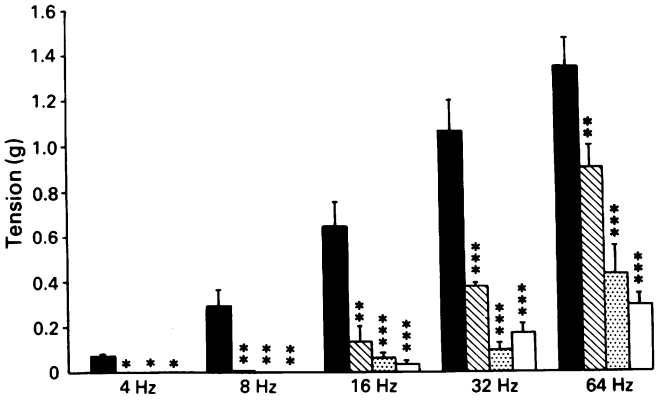

Figure 2 Contractions of the rabbit isolated ear artery to 1 s periods of field stimulation (0.1 ms duration, supramaximal voltage of 60 V; 4, 8, 16, 32 and 64 Hz). Contractions produced in control preparations (solid columns, $n = 7$), are compared with those 1 (hatched columns, $n = 7$), 4 (stippled columns, $n = 7$) and 6 weeks (open columns, $n = 6$) post-irradiation. Vertical bars denote s.e.mean. Significant differences between control and post-irradiation contractions are shown as: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

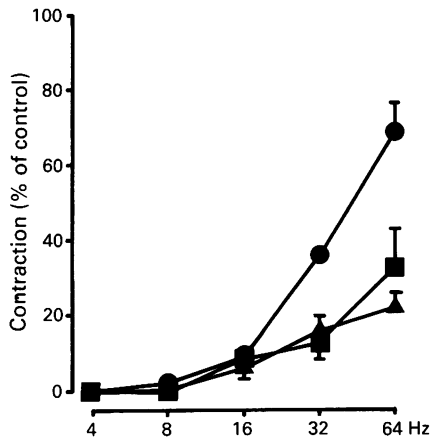

Figure 3 Contractions of the rabbit isolated ear artery to 1 s periods of field stimulation (0.1 ms duration; supramaximal voltage of 60 V; 4, 8, 16, 32, and 64 Hz). Contractions are expressed as a percentage of the control contractions at each frequency, 1 ($n = 7$) (●), 4 ($n = 7$) (■) and 6 weeks ($n = 6$) (▲) post-irradiation treatment. Vertical bars denote s.e.mean. No s.e. bars are shown when they are smaller than the symbols used.

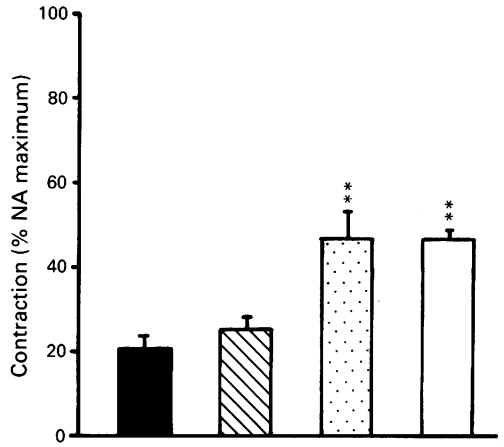

Figure 5 Contractions of the isolated rabbit ear artery to a single dose of α,β-methylene ATP (1 μM) expressed as a percentage of the maximum response to noradrenaline (NA). Contractions produced in control preparations (solid column, $n = 7$) are compared with those 1 (hatched column, $n = 7$), 4 (stippled column, $n = 6$) and 6 weeks (open column, $n = 4$) post-irradiation treatment. Vertical bars denote s.e.mean. ** shows a significant difference at $P < 0.01$.

post-irradiation. However, when results are expressed as mean percentage variation from control values, the percent reduction in contractions are greatest at the lowest frequencies (Figure 3).

The cumulative application of NA (0.1–100 μM) produced a concentration-dependent contraction in all preparations tested. In control preparations, NA produced a maximum contraction of 2.95 ± 0.05 g, giving a pD_2 value of 6.00 ± 0.09. There was no significant difference in either the maximum contractions or pD_2 values between control and irradiated preparations 1, 4 and 6 weeks after irradiation (Figure 4).

Application of α,β-MeATP (1 μM) to preparations at resting tone produced a rapid contraction of 20.83 ± 3.06% NA maximum response. There was no significant variation in this response 1 week post-irradiation (25.43 ± 3.38% NA max). However, preparations 4 and 6 weeks post-irradiation produced a significantly increased response (46.85 ± 6.49 and 47.00 ± 2.14% NA max respectively) (Figure 5).

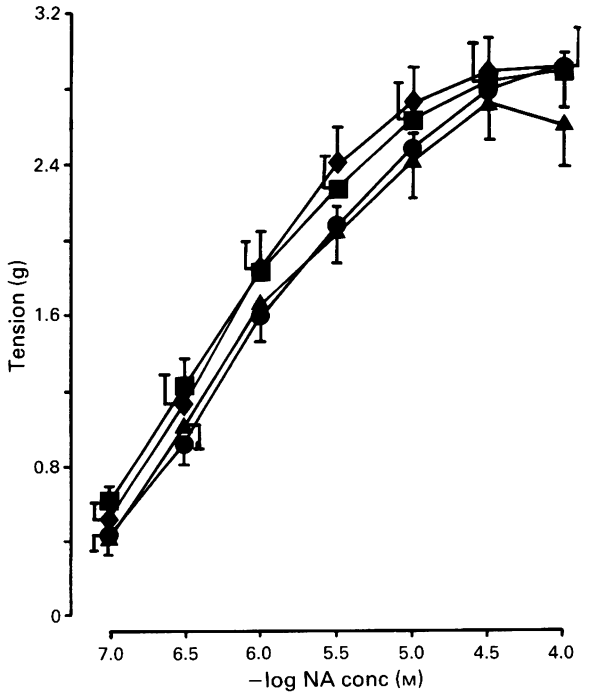

Figure 4 Cumulative dose-response curves to noradrenaline (0.1–100 μM) in isolated ring preparations of the rabbit ear artery. Control preparations (●) ($n = 7$) are compared with preparations 1 (■) ($n = 8$), 4 (▲) ($n = 7$) and 6 weeks (◆) ($n = 9$) post-irradiation. Vertical bars denote s.e.mean.

Fluorescence histochemistry

Histochemical localization of catecholamine-containing nerves revealed clear noradrenergic innervation in both control and irradiated preparations, with no apparent changes in the pattern of fluorescence 1, 4 or 6 weeks after irradiation. Although the intensity of fluorescence could not be quantitatively assessed, there appeared to be no obvious difference amongst the irradiated vessels.

Noradrenaline content

The mean content of NA in control tissue was 3.87 ± 0.3 ng cm^{-1}. However, tissues examined from irradiated sections taken 6 weeks post-irradiation showed a significant reduction ($P < 0.01$) in NA content (2.56 ± 0.2 ng cm^{-1}).

Discussion

This functional study gives us a clear indication that X-irradiation induces changes in sympathetic neurotransmission in the rabbit isolated central ear artery which occur as early as 1 week post-irradiation and shows that endothelial cells are not necessarily the component of blood vessels most sensitive to irradiation as previously reported (Fischer-Dzoga *et al.*, 1984).

The alteration in sympathetic neurotransmission after irradiation treatment could be attributed to pre- and/or postjunctional factors involving noradrenergic and/or purinergic systems. However, since there was no alteration in the contractile response to NA following treatment, damage to the smooth muscle contractile mechanism or to postjunctional α-adrenoceptors can be effectively ruled out.

It is difficult to judge absolute tissue levels of catecholamines based purely on the intensity of histofluorescence (Furness & Costa, 1975). Had catecholamine levels been reduced dramatically, this would probably have been obvious histochemically. Nevertheless, fluorescence histochemistry did enable us to observe that sympathetic nerves were still present and had not been destroyed by the irradiation treatment.

The biochemical assay results, however, indicate a reduction in the NA tissue content when measured 6 weeks following irradiation. This suggests that there was a depletion in the prejunctional levels of NA being stored in perivascular nerves, which could be a contributory factor in the reduction in nerve-mediated constrictor responses.

ATP has been shown to be a cotransmitter with NA in sympathetic nerves in the rabbit central ear artery, acting postjunctionally via P_{2X}-purinoceptors situated on the muscle to elicit a constrictor response (Kennedy & Burnstock, 1985; Kennedy *et al.*, 1986; Saville & Burnstock, 1988). Furthermore, Kennedy *et al.* (1986) found that ATP was the primary transmitter responsible for neurogenic contractions at lower stimulation frequencies. In our study, at 1 week post-irradiation, the percentage reduction in neurally mediated contractions is greater at lower frequencies than at higher frequencies. By 4 weeks post-irradiation, this effect is not so evident because the higher frequencies are also greatly affected. This would suggest that the predominantly purinergic component of the nerve-mediated response at low frequencies is damaged earlier in comparison with the predominantly adrenergic component seen at higher frequencies.

When α,β-MeATP (1 μM) was applied exogenously to preparations 1 week post-irradiation, there was no significant difference in the contractions. This indicates that the postjunctional P_{2X}-purinoceptors are functioning normally, thus suggesting that any purinergic contribution to the initial reduction in nerve-mediated contractions is likely to be located prejunctionally.

The responses to exogenous application of α,β-MeATP at 4 and 6 weeks post-irradiation, showed a significant increase in contrast to the responses to exogenous application of NA, which remained unchanged. This suggests that there may be a selective increase in the sensitivity of the postjunctional P_{2X}-purinoceptors as a result of the initial changes.

In summary, our study has shown that sympathetic neurotransmission is sensitive to X-irradiation, a previously unrecognized consequence which would certainly contribute to early alterations in vasomotor control.

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Temporal changes in the calcium-force relation during histamine-induced contractions of strips of the coronary artery of the pig

Katsuya Hirano, [1]Hideo Kanaide, Shimako Abe & Motoomi Nakamura

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

1 We examined temporal changes in the relationship between cytosolic calcium concentrations ($[Ca^{2+}]_i$) and developed tension during histamine-induced contractions of strips of the coronary artery of the pig, by making use of simultaneous measurements of fura-2 fluorescence and force.

2 The relationship between $[Ca^{2+}]_i$ and developed tension observed with cumulative applications of extracellular Ca^{2+} ($[Ca^{2+}]_o$), ranging from 0 mM to 10 mM, during 118 mM K^+-depolarization was similar to that observed in chemically skinned strips of the porcine coronary artery, as noted by other investigators. $[Ca^{2+}]_i$ at 0 mM $[Ca^{2+}]_o$, at 50% of maximum, and at maximum tension development were 76 nM, 424 nM, and 3050 nM, respectively.

3 Cumulative applications of histamine induced dose-dependent increases in $[Ca^{2+}]_i$ and tension and the extent of tension for a given change in $[Ca^{2+}]_i$ increased, i.e. greater effectiveness of $[Ca^{2+}]_i$-tension relationship, than seen with K^+-depolarization.

4 When histamine 10^{-5} M was applied, $[Ca^{2+}]_i$ abruptly rose and reached the first peak within several seconds. After a slight dip at 30 s, $[Ca^{2+}]_i$ reached a second peak at 3 min, and then gradually declined. On the other hand, tension developed rapidly reached a maximum at 4 min, then gradually declined. The relation between $[Ca^{2+}]_i$ and tension in the early, rising phase of contraction was similar to that obtained during depolarization. At the time of maximum tension development, the relation was greater than that observed during depolarization, which persisted in the phase of declining tension.

5 To examine the role of protein kinase C in the increased effectiveness of $[Ca^{2+}]_i$-tension relation of histamine-induced contractions, we used 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H-7), as an inhibitor. Exposure of strips to H-7, 10^{-5} M, reduced the effectiveness of $[Ca^{2+}]_i$-tension relation toward the level observed during depolarization, while it had no effect on the $[Ca^{2+}]_i$-tension relation in the early phase of contraction either in the presence or absence of extracellular Ca^{2+}.

6 In intact smooth muscle of the coronary artery of the pig, the relationship between $[Ca^{2+}]_i$ and developed tension varies during contraction. Histamine, a receptor-mediated stimulus, induced a greater tension for a given change in $[Ca^{2+}]_i$ during the steady state of contraction than did depolarization. The greater effectiveness of contraction, particularly in the later phase, seems to be mediated by activation of an H-7-sensitive mechanisms.

Introduction

Changes in cytosolic concentrations of Ca^{2+} ($[Ca^{2+}]_i$) play a primary role in the regulation of contraction of vascular smooth muscle cells (Sommerville & Hartshorne, 1986). The initiation of contraction is associated with Ca^{2+} binding to calmodulin with the subsequent activation of myosin light chain kinase (Kamm & Stull, 1985). Phosphorylation of the 20,000 dalton myosin light chain by Ca^{2+}-calmodulin-activated myosin light chain kinase results in an increase in actin-activated myosin ATPase activity which is closely linked with the initiation of contraction. Thus, alterations in $[Ca^{2+}]_i$ may be the primary determinant of the initiation and the extent of contraction. However, it has been reported that receptor-mediated stimulation produces a proportionately greater tension for a given change in $[Ca^{2+}]_i$ than does depolarization (Morgan & Morgan, 1984; Bruschi *et al.*, 1988; Himpens & Somlyo, 1988; Rembold & Murphy, 1988; Sato *et al.*, 1988). It was also noted that activation of G-protein enhanced the effectiveness of $[Ca^{2+}]_i$ during agonist stimulation (Nishimura *et al.*, 1988; Kitazawa *et al.*, 1989) and that regulatory mechanisms of tension maintenance differ from those related to tension development (Rasmussen *et al.*, 1987; Murphy, 1989). In the late phase of contractions, the developed tension is maintained by a reduction in ATP consumption and by other Ca^{2+}-dependent regulatory mechanisms (Murphy, 1989). Thus, the $[Ca^{2+}]_i$-tension relationship may vary during a contraction.

Some agonist-receptor interactions stimulate phospholipase C-catalyzed hydrolysis of phosphoinositides, via G-protein, which yield two intracellular messengers, inositol 1,4,5-trisphosphate and diacylglycerol (Nishizuka, 1984). While the formation of the former is transient during stimulation, the formation of the latter is sustained and activates protein kinase C (PKC) (Nishizuka, 1984; Takuwa *et al.*, 1986; Griendling *et al.*, 1986). Although the role of PKC in the regulation of vascular smooth muscle contraction remains controversial, there is evidence suggesting that activation of PKC may increase the effectiveness of the $[Ca^{2+}]_i$-tension relation (Itoh *et al.*, 1988; Nishimura & van Breemen, 1989a). Despite the growing interest in these events, the temporal relation between $[Ca^{2+}]_i$ and developed tension during the receptor-mediated contraction is not fully understood, possibly because of difficulty in recording the former, in intact tissue.

We made use of front-surface fluorometry of fura-2 and examined the temporal changes in the $[Ca^{2+}]_i$-tension relation during histamine-induced contractions in intact smooth muscle strips of the porcine coronary artery. The effect of a relatively specific inhibitor of PKC, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H-7) (Hidaka *et al.*, 1984), on the $[Ca^{2+}]_i$-force relation was also determined.

[1] Author for correspondence.

Methods

Tissue preparation

Hearts from adult pigs of either sex were obtained from a local slaughterhouse immediately after the animals had been killed. These hearts were placed in ice-cold saline solution and transferred to our laboratory. Left circumflex arteries were isolated and segments 2–3 cm from the origin were excised. A cotton swab was passed over the luminal surface to remove the endothelium. Following removal of the adventitia, medial preparations were cut into approximately 1 × 5 mm circular strips, 0.1 mm thick.

Fura-2 loading

The vascular strips thus prepared were loaded with the Ca^{2+} indicator dye, fura-2 by incubating in medium containing 2.5×10^{-5} M fura-2/AM (an acetoxymethyl ester form of fura-2) and 5% foetal bovine serum for 3–4 h at 37°C. The strips were then washed with physiological salt solution (PSS) to remove dye in the extracellular space and then were further incubated in PSS for 1 h before initiation of the measurements. Fura-2-loaded strips showed a specific peak (500 nm) of emission spectrum and specific peak (340 nm) and valley (380 nm) of the excitation spectra, as determined with a fluorescence spectrophotometer (model 650-40, Hitachi, Tokyo, Japan). Loading the strips with fura-2 did not alter the time courses and the maximum levels of tension development during 118 mM K^+-depolarization. Assuming the maximum developed tension during 118 mM K^+-depolarization before fura-2 loading to be 100%, it was 96.0 ± 10.7% ($n = 3$) after fura-2 loading. Thus, in the present study, there was neither Ca^{2+}-buffering by fura-2 nor tissue damage by possible acidification of the cells due to formaldehyde released on acetoxymethyl-ester hydrolysis (Tsien *et al.*, 1982; Rink & Pozzan, 1985).

Measurement of tension

Strips were mounted vertically in a quartz organ bath, and contractile force was monitored with a strain gauge (TB-612T, Nihon Koden, Japan). During a 1 h fura-2 equilibration period, the strips were stimulated with 60 mM K^+-depolarization every 15 min, and the resting tension was increased stepwise. After equilibration, the resting tension was adjusted to 250 mg wt. The response of each strip to 118 mM K^+-depolarization was recorded before starting the protocol. The developed tension was expressed as a percentage, assuming the values in PSS (5.9 mM K^+) and 118 mM K^+ PSS to be 0% and 100%, respectively.

Front-surface fluorometry

Changes in fluorescence intensity of the fura-2-Ca^{2+} complex were monitored with a fluorometer, specifically designed for front-surface fura-2 fluorometry (model CAM-OF-1), with the collaboration of Japan Spectroscopic Co. (Tokyo, Japan). In brief, strips were illuminated by guiding the alternating (400 Hz) 340 and 380 nm excitation light from a Xenon light source through quartz optic fibres arranged in a concentric inner circle (diameter = 3 mm). Surface fluorescence of strips was collected by glass optic fibres arranged in an outer circle (diameter = 7 mm) and introduced through a 500 nm band-pass filter into a photomultiplier. Special care was taken to keep the distance between a strip and the end of the optic fibres as short as possible, and constant during each measurement.

The ratio of the fluorescence intensities at 340 nm excitation to those at 380 nm excitation was monitored and expressed as a percentage assuming the values in PSS (5.9 mM K^+) and 118 mM K^+ PSS to be 0% and 100%, respectively. The values of $[Ca^{2+}]_i$ were calculated from the % ratio (R), using the following equation (Grynkiewicz *et al.*, 1985):

$$[Ca^{2+}]_i = K_d(R - R_{min})/(R_{max} - R)$$

where K_d is a dissociation constant and assumed to be 224 nM (Grynkiewicz *et al.*, 1985). R_{max} was determined by the addition of 2.5×10^{-5} M ionomycin in the presence of extracellular Ca^{2+} (1.25 mM Ca^{2+}) and then R_{min} was determined in the absence of extracellular Ca^{2+} (0 mM Ca^{2+}; 2 mM ethylene-glycol-*bis*(β-amino-ethylether)-N,N,N′,N′-tetraacetic acid (EGTA)). Mean values ($n = 10$) of R_{max} and R_{min} were 156.3 ± 11.8% and −76.1 ± 22.9%, respectively. The mean values of $[Ca^{2+}]_i$ at rest (0%) and during 118 mM K^+-depolarization (100%) were 108 nM and 715 nM, respectively. Since $[Ca^{2+}]_i$ was determined on the assumption that the K_d value of fura-2 in the cytosol of vascular smooth muscle in the present experimental condition was 224 nM (a K_d for fura-2 in the buffer with normal ionic strength at pH 7.05 and 37°C (Grynkiewicz *et al.*, 1985)), the $[Ca^{2+}]_i$ value obtained is an approximation to the true $[Ca^{2+}]_i$.

Drugs and solutions

The normal physiological salt solution (normal PSS) we used was of the following composition (in mM): NaCl 123, KCl 4.7, $NaHCO_3$ 15.5, KH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 1.25, and D-glucose 11.5. The Ca^{2+}-free version of PSS (Ca^{2+}-free PSS) contained 2 mM EGTA instead of 1.25 mM $CaCl_2$. The only difference between the high potassium PSS and normal PSS was equimolar substitution of KCl for NaCl. PSS was gassed with a mixture of 5% CO_2 and 95% O_2 (pH 7.4; 37°C). Histamine dihydrochloride (Wako, Osaka, Japan), mepyramine maleate (Sigma, St. Louis, MO, U.S.A.), H-7 (Seikagaku Kogyo, Tokyo, Japan), and fura-2/AM (Molecular Probes, Eugene, OR, U.S.A.) were purchased. Cimetidine was kindly donated by Fujisawa Pharmaceutical Co., Ltd. (Tokyo, Japan).

Statistical analysis

Values are expressed as mean ± standard error. Student's t test was used to determine the statistical significance and a P value of less than 0.05 was considered to have a statistical significance. The $[Ca^{2+}]_i$-tension curve of contractions induced by cumulative applications of Ca^{2+} during 118 mM K^+-depolarization was obtained by fitting the data to Hill's equation (Segel, 1976):

$$\log(T/(T_{max} - T)) = n_H(\log[Ca^{2+}]_i - \log K_d)$$

where T represents the measured value of developed tension, expressed in percent, and $[Ca^{2+}]_i$ represents the value of cytosolic Ca^{2+} concentration calculated as described for front-surface fluorometry. n_H and K_d are constant values. n_H is a Hill's coefficient. T_{max} represents the estimated maximum tension development and was calculated from the following equation and least squares method (Scatchard, 1949).

$$T/[Ca^{2+}]_o = (T_{max} - T)/K$$

where $[Ca^{2+}]_o$ represents the extracellular Ca^{2+} concentration and K is a constant.

Results

Changes in $[Ca^{2+}]_i$ and tension development induced by K^+-depolarization and by histamine

A representative recording of the changes in fura-2 fluorescence (fluorescence intensities at 340 nm and 380 nm excitations and their ratio) and the development of tension induced by 118 mM K^+-depolarization in normal PSS and by cumula-

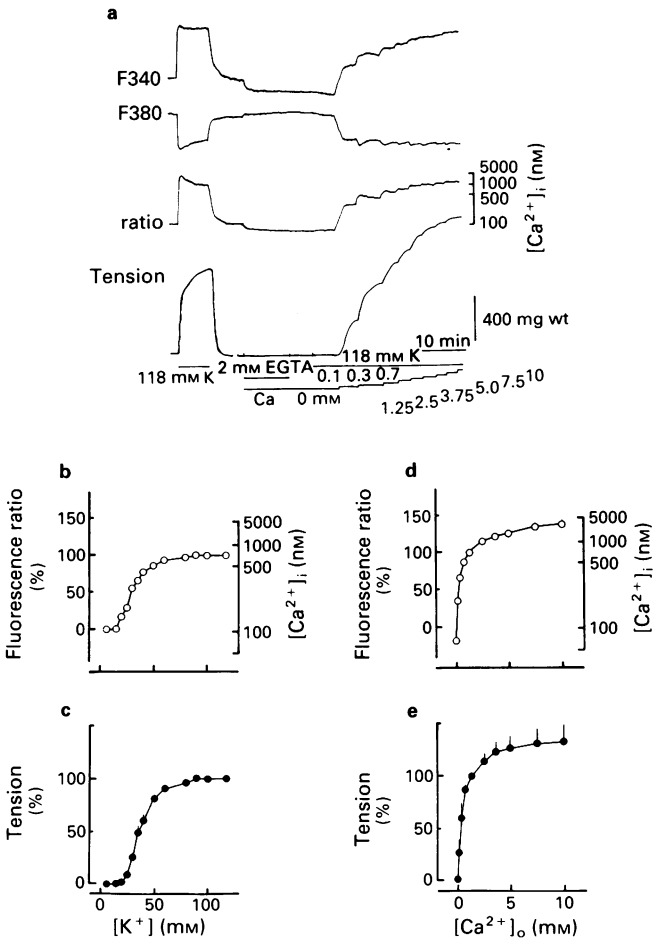

Figure 1 Changes in fluorescence intensities and tension development induced by K^+-depolarization. (a) Representative time courses of changes in 500 nm fluorescence intensities at 340 nm excitation (F_{340}) and at 380 nm excitation (F_{380}), their ratio (F_{340}/F_{380}), and of tension development induced by 118 mM K^+ in normal PSS and cumulative applications of Ca^{2+} in 118 mM K^+-depolarization. Concentration-related responses in fluorescence ratio (b) and tension development (c) induced by single dose applications of K^+-depolarization in normal PSS containing 1.25 mM Ca^{2+}. Data were obtained at the time of maximum tension development. Concentration-related responses in fluorescence ratio (d) and tension development (c) induced by cumulative applications of Ca^{2+} (from 0 mM to 10 mM) in 118 mM K^+-depolarization. Data were obtained at the time of maximum tension development. Data are the mean with s.e. shown by vertical lines in (e) ($n = 4$ for (b) and (c), $n = 6$ for (d) and (e)).

tive applications of Ca^{2+} in 118 mM K^+-depolarization is shown in Figure 1a. When vascular strips were exposed to high K^+-PSS, fluorescence ratio and tension reached maximum levels within 30 s and 3 min, respectively. These levels were either sustained or slightly reduced during depolarization. The level of $[Ca^{2+}]_i$ and the extent of tension development were concentration-dependent (Figure 1b and c). After incubation in Ca^{2+}-free PSS containing 2 mM EGTA followed by 5 min exposure to Ca^{2+}-free PSS without EGTA, contractions were initiated by cumulative applications of extracellular Ca^{2+} during 118 mM K^+-depolarization (Godfraind & Kaba, 1969). Fluorescence ratio and tension increased stepwise with elevations in extracellular Ca^{2+}. Data from six different measurements are summarized in Figure 1d and e. The fluorescence ratio increased from $-19.4 \pm 5.2\%$ (76 nM) to $139.0 \pm 5.9\%$ (3050 nM) and tension increased from 0% to $133.1 \pm 16.4\%$. Cumulative application of extracellular Ca^{2+} indicated that $[Ca^{2+}]_i$ at 0 mM $[Ca^{2+}]_o$, at 50% of maximum, and at maximum tension development were 76 nM, 424 nM, and 3050 nM, respectively.

Figure 2 shows a representative recording of changes in fura-2 fluorescence (fluorescence intensities at 340 and 380 nm excitation and their ratio) and tension development induced by histamine 10^{-5} M in normal PSS (a) and Ca^{2+}-free PSS (b). Figure 3 also shows time courses of fura-2 fluorescence ratio (and $[Ca^{2+}]_i$) and tension obtained from 4 different measurements. When histamine 10^{-5} M was added to the normal PSS, tension developed rapidly, reached a maximum after 4.3 ± 0.1 min, and then declined gradually. The extent of tension developed at the maximum and after 30 min was $121.2 \pm 2.6\%$ and $33.9 \pm 8.5\%$ of that observed during 118 mM K^+-depolarization, respectively ($n = 4$). $[Ca^{2+}]_i$ rose abruptly and reached the first peak within several seconds. After a slight dip at 30 s, $[Ca^{2+}]_i$ reached a second peak at 3.0 ± 0.3 min ($n = 4$) and then gradually declined but remained higher than the pre-stimulation level (resting level). The levels of $[Ca^{2+}]_i$ at the first peak, at the second peak and at 30 min were $70.9 \pm 21.4\%$ (421 nM), $75.8 \pm 4.9\%$ (424 nM), and $38.7 \pm 2.2\%$ (218 nM), respectively ($n = 4$). Elevation of $[Ca^{2+}]_i$ even at 30 min was statistically significant ($P < 0.01$) compared with findings in the case of the resting level (108 ± 27 nM, $n = 10$). There was no statistically significant difference of $[Ca^{2+}]_i$ between the first peak, at the dip, and at

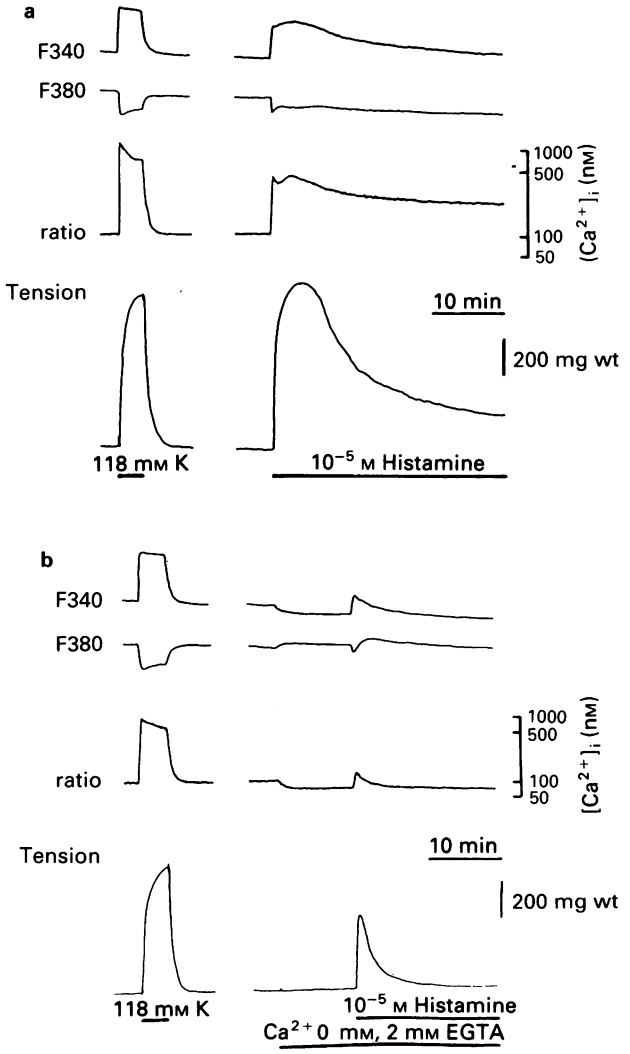

Figure 2 Changes in fluorescence intensities and tension development induced by histamine. Representative time courses of changes in 500 nm fluorescence intensities at 340 nm excitation (F_{340}) and at 380 nm excitation (F_{380}), their ratio (F_{340}/F_{380}), and of tension development induced by 118 mM K^+-depolarization and 10^{-5} M histamine in normal PSS (a) and Ca^{2+}-free PSS (b). In (b), vascular strips were exposed to Ca^{2+}-free PSS 10 min before the application of histamine.

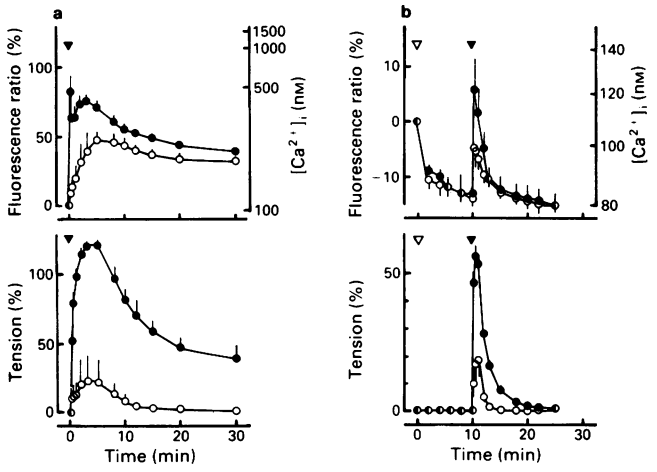

Figure 3 Effects of 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H-7) on time courses of changes in fluorescence and tension development induced by histamine, 10^{-5} M. The left (a) and right (b) panels show time courses of contractions induced by histamine, 10^{-5} M, in the presence and absence of extracellular Ca^{2+}, respectively: data obtained in (○) 10^{-5} M H-7-treated strips and in (●) non-treated strips. H-7 was applied 10 min before and during histamine-induced contractions. Histamine 10^{-5} M was applied at the time indicated by closed triangles (▼). Open triangles (▽) in (b) indicate the time at which strips were exposed to Ca^{2+}-free media. Data are expressed as mean of 4 different measurements; s.e. shown by vertical lines.

the second peak ($P > 0.05$). Mepyramine maleate (10^{-5} M), a selective H_1-receptor antagonist, completely inhibited elevations of both $[Ca^{2+}]_i$ and tension, but cimetidine (10^{-5} M), a selective H_2-receptor antagonist, did not (data not shown). The dose-response relationships of maximum tension development and corresponding $[Ca^{2+}]_i$ induced by histamine in normal PSS were obtained from contractions induced by cumulative applications of histamine (Figure 4). Both the extent of maximum tension and the level of corresponding $[Ca^{2+}]_i$ increased dose-dependently with concentrations between 10^{-7} M and 10^{-4} M. Such a high level of fluorescence ratio as that seen with a high concentration ($> 10^{-5}$ M) of histamine seems to reach the saturation of fluorescence signals, and there should be no further increases in fluorescence signals even when $[Ca^{2+}]_i$ increases. However, such was not the case, because the fluorescence ratio exceeded 150% when histamine (10^{-5} M) was applied during 118 mM K^+-depolarization (data not shown).

In Ca^{2+}-free PSS, histamine induced transient increases of both $[Ca^{2+}]_i$ and tension (Figure 2b and 3b). When strips were exposed to Ca^{2+}-free PSS, $[Ca^{2+}]_i$ gradually decreased to reach the steady state level ($-13.6 \pm 1.7\%$ (83 nM); $n = 4$), while the tension remained unchanged. Applictaion of 10^{-5} M histamine after 10 min incubation in Ca^{2+}-free PSS led to abrupt elevations of $[Ca^{2+}]_i$ and tension, to produce a peak within 1 min, followed by a rapid decline to pre-stimulation levels, within 10 min. The extent of transient elevations of $[Ca^{2+}]_i$ and tension were concentration-dependent within the range of 10^{-6} M to 10^{-4} M (Figure 5).

Effects of H-7 on histamine-induced changes in $[Ca^{2+}]_i$ and tension

The effects of H-7, a relatively specific inhibitor of PKC, on the time courses of histamine-induced changes in $[Ca^{2+}]_i$ and tension development are shown in Figure 3. In normal PSS (Figure 3a), exposure of vascular strips to H-7 10^{-5} M for 10 min before and during the application of histamine (10^{-5} M) inhibited increases in both $[Ca^{2+}]_i$ and tension development. Resting levels of $[Ca^{2+}]_i$ and tension were not affected by treatment with H-7. The histamine-induced abrupt elevation of $[Ca^{2+}]_i$ (the first peak) disappeared in the H-7-treated strips. $[Ca^{2+}]_i$ gradually reached the peak at 4.8 ± 0.6 min ($n = 4$), and then declined to a fairly steady level which was slightly and significantly ($P < 0.05$) lower than that observed in non-treated strips. On the other hand, development of tension was markedly inhibited in the H-7-treated strips: tension gradually increased to a maximum at 4.4 ± 1.3 min and then declined to the pre-stimulation level in 24.8 ± 5.5 min ($n = 4$). H-7 dose-dependently inhibited the development of maximum tension and corresponding $[Ca^{2+}]_i$ elevation induced by 10^{-5} M histamine. As shown in Figure 4, H-7 inhibited the development of maximum tension and corresponding $[Ca^{2+}]_i$ induced by cumulative applications of histamine, in a different manner; increases in tension were inhibited non-competitively while increases in $[Ca^{2+}]_i$ were inhibited competitively.

In Ca-free PSS, exposure of strips to H-7 inhibited transient elevations of $[Ca^{2+}]_i$ and tension induced by 10^{-5} M histamine (Figure 3b). Decreases in $[Ca^{2+}]_i$ during exposure to Ca^{2+}-free media and resting tension were not affected by H-7. The inhibitory effects of H-7 on the peak levels of $[Ca^{2+}]_i$ transient and tension developed were concentration-dependent. Figure 5 shows the effects of H-7 on the dose-related response to histamine in Ca^{2+}-free PSS. The mode of inhibition could not be determined because we were unable to obtain evidence of any saturation of responses.

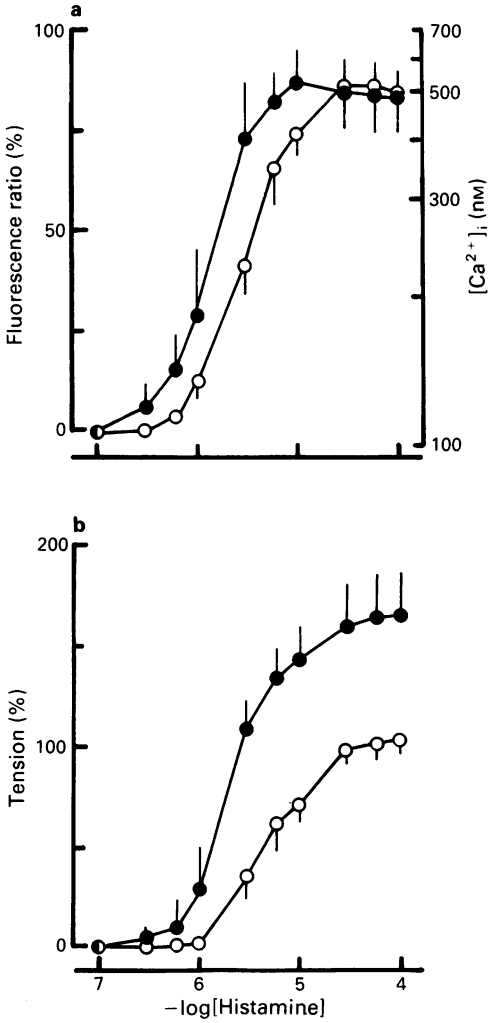

Figure 4 Effects of 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H-7) on concentration-related responses in (a) fluorescence ratio and (b) tension development induced by histamine in normal PSS: (○) H-7-treated strips and (●) non-treated strips. Strips were stimulated by the cumulative applications of histamine. H-7 (10^{-5} M) was applied 10 min before the histamine. Data are mean of 4 different measurements with s.e. shown by vertical lines.

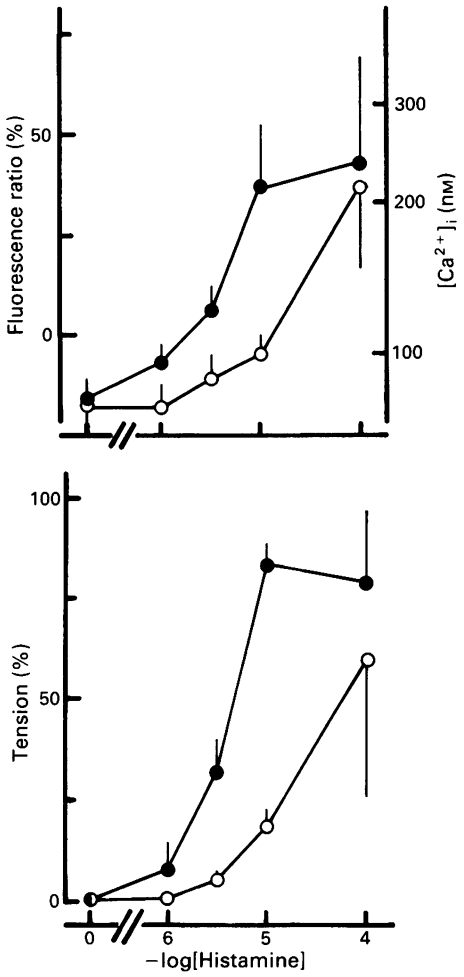

Figure 5 Effects of 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H-7) on concentration-related responses in fluorescence ratio and tension development induced by histamine in Ca^{2+}-free PSS: (○) H-7-treated strips and (●) non-treated strips. Each strip was stimulated by a certain concentration of histamine after 10 min incubation in Ca^{2+}-free PSS. H-7 (10^{-5} M) was applied at the time of exposure to Ca^{2+}-free PSS. Data are mean of 4 different measurements; s.e. shown by vertical lines.

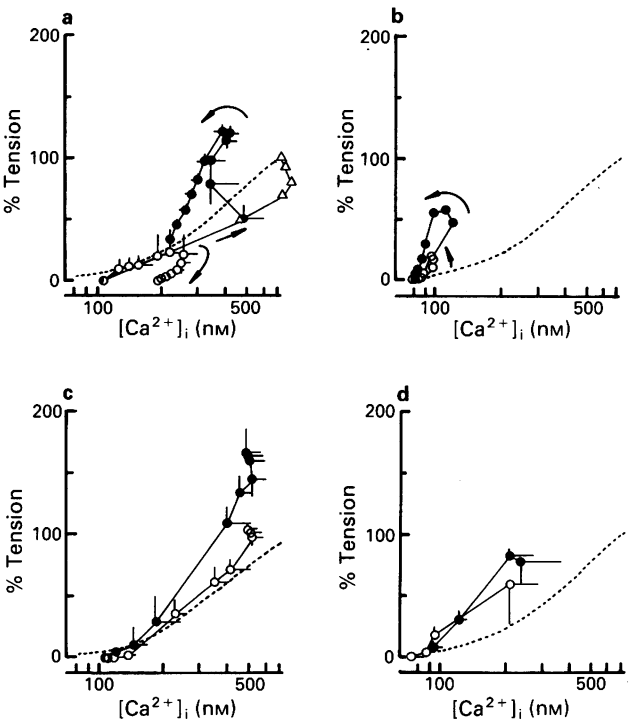

Figure 6 Effects of 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H-7) on $[Ca^{2+}]_i$-tension relationship of histamine-induced contractions. In (a) and (b) are shown the changes in $[Ca^{2+}]_i$-tension relation with time after application of histamine (10^{-5} M) in normal PSS (a) and in Ca^{2+}-free PSS (b). (c) and (d) Show the $[Ca^{2+}]_i$-tension relation at various concentrations of histamine, obtained by cumulative applications of histamine in normal PSS (c) and by a single application of histamine in Ca^{2+}-free PSS (d). In (a) and (b), curved arrows indicate the direction of time. Values in (c) and (d) were obtained at the time of maximum development at each concentration of histamine: (○) H-7-treated strips and (●) non-treated strips, respectively. The $[Ca^{2+}]_i$-tension curves in (a), (b), (c) and (d) were constructed from data shown in Figures 3a, 3b, 4, and 5, respectively. The $[Ca^{2+}]_i$-tension curve of contractions induced by cumulative applications of extracellular Ca^{2+} during 118 mM K^+-depolarization is depicted as a dashed curve in all panels, for comparison and as constructed from data shown in Figure 1d and e. In (a) is also shown the $[Ca^{2+}]_i$-tension relation obtained at 15 s, 30 s, 1 min, 2 min, and 3 min during 118 mM K^+-depolarization (△).

$[Ca^{2+}]_i$-tension relationship of histamine-induced contractions: effects of H-7

The relationships between $[Ca^{2+}]_i$ and tension of histamine-induced contractions under various conditions were examined by making use of $[Ca^{2+}]_i$-tension curves (Figure 6). In this figure, the $[Ca^{2+}]_i$-tension curve of contractions induced by cumulative applications of extracellular Ca^{2+} during 118 mM K^+-depolarization is also presented. K_d values ($[Ca^{2+}]_i$ at 50% of maximum tension) and Hill's coefficient of the $[Ca^{2+}]_i$-tension relationship of the depolarization were 424 ± 70 nM and 1.9 ± 0.5 ($n = 6$), respectively, thereby suggesting that this relationship was similar to the Ca^{2+}-tension relationship obtained in saponin-treated coronary arterial strips of the pig (Itoh *et al.*, 1982). During 118 mM K^+-depolarization, the Ca^{2+}-tension relationship showed a counter-clockwise rotation, with time (Figure 6a; △). Figure 6a also shows the time course of $[Ca^{2+}]_i$-tension relationship of histamine (10^{-5} M)-induced contractions in H-7-treated and non-treated strips in normal PSS. In non-treated strips, the relationship shows a counter-clockwise rotation with time, as indicated by an arrow. On the other hand, the $[Ca^{2+}]_i$-tension relation in H-7-treated strips shows a curve similar to that observed during depolarization at the time of maximum tension development, and a clockwise rotation at the late phase of contraction. In contrast, the $[Ca^{2+}]_i$-tension relation in Ca^{2+}-free PSS shows a counter-clockwise rotation with time, both in the treated and non-treated strips (Figure 6b). Figure 6 shows the $[Ca^{2+}]_i$-tension relation at the time of maximum tension development induced by various concentrations of histamine, in normal PSS. In non-treated strips, histamine produced a greater tension for a given change in $[Ca^{2+}]_i$ than did K^+-depolarization. Treatment with H-7 reduced the extent of tension developed for a given change in $[Ca^{2+}]_i$ and shifted the $[Ca^{2+}]_i$-tension curve toward that obtained during K^+-depolarization. In contrast, in Ca^{2+}-free PSS, $[Ca^{2+}]_i$-tension relation curves showed no difference between the treated and non-treated strips (Figure 6d).

Discussion

The present study shows that in the presence of histamine, there was a greater extent of tension development for a given change in $[Ca^{2+}]_i$, a greater effectiveness of $[Ca^{2+}]_i$ than seen with depolarization and that there was a greater effectiveness in the late than in the early phase of histamine-induced contractions in normal PSS. These events lead

to a counter-clockwise rotation of the instantaneous $[Ca^{2+}]_i$-tension relationship. The $[Ca^{2+}]_i$-tension relationship of the contractions induced by cumulative applications of Ca^{2+} during K^+-depolarization in intact smooth muscle of porcine coronary artery is similar to that obtained with chemically skinned preparations (Itoh *et al.*, 1982). The difference between histamine-induced contractions and contractions induced by increasing extracellular Ca^{2+} during depolarization in intact muscle seems to be related to differences in the activation of membrane-bound proteins. The activation of G-protein was reported to enhance the Ca^{2+}-sensitivity of myofilaments in α-toxin permeabilized smooth muscles (Nishimura *et al.*, 1988; Kitazawa *et al.*, 1989). In the present study, we obtained evidence that the extent of maximum tension development obtained with histamine required a lesser degree of $[Ca^{2+}]_i$ elevation and was greater than that obtained with Ca^{2+}-reapplication during K^+-depolarization. Thus, histamine seems to enhance the Ca^{2+}-sensitivity of the contractile elements, and as a result, increases the extent of tension development for a given change in $[Ca^{2+}]_i$ in intact smooth muscle of the coronary artery of the pig. In normal PSS, the finding that $[Ca^{2+}]_i$ remained at levels markedly higher than levels at rest in the presence of histamine has to be given due attention. Controversy exists regarding $[Ca^{2+}]_i$ levels at the late phase of contraction induced by agonist application. There are reports that $[Ca^{2+}]_i$ returned to almost resting or slightly higher than resting levels at the late phase of contraction (Morgan & Morgan, 1984; Rasmussen *et al.*, 1987; Rembold & Murphy, 1988). The findings in the present study are consistent with reports of a sustained elevation of $[Ca^{2+}]_i$ during the contraction of vascular strips of various species and organs induced by application of several agonists (Sato *et al.*, 1988; Bruschi *et al.*, 1988; Himpens & Somlyo, 1988).

Effects of H-7 on the $[Ca^{2+}]_i$-tension relationship in normal PSS differed from those obtained in Ca^{2+}-free PSS. H-7 markedly decreased effectiveness of the $[Ca^{2+}]_i$-tension relation of histamine-induced contractions in normal PSS. In normal PSS, the time course of the $[Ca^{2+}]_i$-tension relationship of the vascular strip treated with histamine showed a counter-clockwise rotation curve, which became a clockwise rotation curve with H-7 treatment (Figure 6a). On the contrary, in Ca^{2+}-free PSS, the curve retained a counter-clockwise rotation in the H-7-treated strips, as in the non-treated ones (Figure 6b). The $[Ca^{2+}]_i$-tension relationship between maximum tension and corresponding $[Ca^{2+}]_i$ induced by various concentrations of histamine in normal PSS shifted to the right in the H-7-treated strips, to overlap closely with the relation observed during depolarization (Figure 6c). Such was not the case in Ca^{2+}-free PSS (Figure 6d). The $[Ca^{2+}]_i$-tension curves obtained in the present study indicate that contractions induced by histamine both in Ca^{2+}-free PSS and in the very early phase in normal PSS can be mostly attributed directly to $[Ca^{2+}]_i$-dependent contractile mechanisms which produce a similar $[Ca^{2+}]_i$-tension relationship to that obtained during K^+-depolarization. In the late phase of contractions, sustained tension development with increased effectiveness of $[Ca^{2+}]_i$-tension relation is attributed mainly to activation of H-7-sensitive mechanisms. It has to be noted that tension maintenance in the late phase of the histamine-induced contraction in normal PSS was associated with an elevation of $[Ca^{2+}]_i$. Hence this late phase of contraction may also be maintained by Ca^{2+}-dependent mechanisms.

We found that [^{3}H]-mepyramine bound specifically to histamine H_1-receptors in the sarcolemma from the coronary artery of the pig (Nishimura *et al.*, 1985). In the present study, elevations of both $[Ca^{2+}]_i$ and tension induced by histamine were completely inhibited by mepyramine, but not by cimetidine, as reported (Hagen & Paegelow, 1979; Matsumoto *et al.*, 1986). It was also noted that H_1-receptor stimulation induces phosphoinositide hydrolysis in intestinal smooth muscle, vascular endothelial cells, atrial myocytes, and the brain (Daum *et al.*, 1984; Donaldson & Hill, 1986; Lo & Fan, 1987; Sakuma *et al.*, 1988). Inositol 1,4,5-trisphosphate, a product of phosphoinositide hydrolysis, acts as a second messenger to induce mobilization of Ca^{2+} from intracellular storage sites (Suematsu *et al.*, 1984; Yamamoto & van Breemen, 1985; Somlyo *et al.*, 1985) and diacylglycerol acts as a second messenger to activate PKC (Nishizuka, 1984). While the formation of inositol 1,4,5-trisphosphate is transient, diacylglycerol formation is sustained for 10 min after stimulation (Takuwa *et al.*, 1986; Griendling *et al.*, 1986). In this study, we used H-7 as an inhibitor of PKC (Hidaka *et al.*, 1984). Although this compound also inhibits myosin light chain kinase with a 16 fold lesser potency than in the case of inhibition of PKC (Hidaka *et al.*, 1984), our finding that H-7 inhibited contractions in the late phase with little decrease in $[Ca^{2+}]_i$ but with a decrease in the effectiveness of $[Ca^{2+}]_i$-tension relation suggests that histamine-induced contractions, especially in the late phase, are maintained Ca^{2+}-dependently with increased effectiveness of $[Ca^{2+}]_i$. These events appear to be linked to the activation of an H-7-sensitive mechanism, probably PKC.

The role of PKC in the regulation of smooth muscle is controversial. Phorbol ester, an exogenous activator of PKC, was shown to induce slowly progressive contractions of vascular smooth muscle (Rasmussen *et al.*, 1984; Danthuluri & Deth, 1984; Chatterjee & Tejada, 1986) with little increase in $[Ca^{2+}]_i$ (Jiang & Morgan, 1987; Takuwa *et al.*, 1988). In saponin or α-toxin permeabilized smooth muscle, phorbol ester increased the amplitude of contraction evoked by Ca^{2+} (Itoh *et al.*, 1988; Nishimura & van Breemen, 1989a). The sites of phosphorylation in the 20,000 dalton myosin light chain by PKC differed from those phosphorylated by myosin light chain kinase (Endo *et al.*, 1982; Nishikawa *et al.*, 1983; Ikebe *et al.*, 1987; Bengur *et al.*, 1987) and such an ectopic phosphorylation by PKC inhibits conformational changes of myosin molecules (Umekawa *et al.*, 1985) and actin-activated myosin ATPase activity (Nishikawa *et al.*, 1983; Nishikawa *et al.*, 1984; Ikebe *et al.*, 1987). Furthermore, myosin light chain kinase activity decreases in the case of phosphorylation by PKC *in vitro* (Ikebe *et al.*, 1985; Nishikawa *et al.*, 1985). Thus, the contracting action of PKC, if any, may not be mediated by Ca^{2+}-calmodulin-dependent activation of myosin light chain kinase or by phosphorylation of myosin light chain. It was postulated that several proteins which may serve as substrates for PKC and differ from myosin light chain, might be involved in the late, sustained phase of the contraction of vascular smooth muscle (Rasmussen *et al.*, 1987; Nishimura & van Breemen, 1989b). PKC is a Ca^{2+}-, and phospholipid-dependent protein kinase and diacylglycerol or phorbol esters increase the affinity of PKC for Ca^{2+} ten fold (Nishizuka, 1986). This Ca^{2+}-dependency of PKC activity supports our finding that the late phase of histamine-induced contraction was maintained Ca^{2+}-dependently.

In the present study, H-7 inhibited initial increases in $[Ca^{2+}]_i$ both in normal and Ca^{2+}-free PSS. Thus, this compound may inhibit release of Ca^{2+} from intracellular stores, by a yet to be determined mechanism.

H-7, a relatively specific inhibitor of PKC, inhibited the late phase of histamine-induced contractions in the presence of extracellular Ca^{2+} and this inhibitory effect was accompanied by decreases in the effectiveness of the $[Ca^{2+}]_i$-tension relationship but with little decreases in $[Ca^{2+}]_i$. Histamine-induced contractions in the absence of extracellular Ca^{2+}, or at the early phase in the presence of extracellular Ca^{2+}, were also inhibited by H-7. This inhibition was accompanied by decreases in $[Ca^{2+}]_i$ but with no alteration of $[Ca^{2+}]_i$-tension relation. These decreases in $[Ca^{2+}]_i$ may be attributed to inhibitory effects of H-7 on Ca^{2+}-release mechanisms. Thus, the early phase of histamine-induced contractions may correlate with changes in $[Ca^{2+}]_i$. During the contraction, especially in the late phase, the histamine-induced contraction may be enhanced by the increasing effectiveness of $[Ca^{2+}]_i$, probably due to activation of PKC, under conditions of elevated $[Ca^{2+}]_i$. Regulatory proteins which can serve as sub-

strates for PKC may play some role in this increase in effectiveness of the $[Ca^{2+}]_i$-tension relationship.

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Bradykinin induces elevations of cytosolic calcium through mobilisation of intracellular and extracellular pools in bovine aortic endothelial cells

Kevin W. Buchan & [1]William Martin

Department of Pharmacology, University of Glasgow, Glasgow G12 8QQ

1 In the presence of 1.8 mM extracellular calcium, bradykinin (0.3 nM–100 nM) induced a biphasic elevation of intracellular calcium ($[Ca^{2+}]_i$) in bovine aortic endothelial cells, consisting of an initial, large transient component followed by a lower sustained component.

2 When endothelial cells were bathed in nominally calcium-free solution containing 0.5 mM EGTA, bradykinin induced only a transient elevation of $[Ca^{2+}]_i$: the magnitude of this was significantly smaller than that obtained in the presence of extracellular calcium and the sustained phase was abolished. In the continued presence of bradykinin, re-addition of extracellular calcium to achieve a level of around 1.8 mM resulted in the induction of a biphasic elevation of $[Ca^{2+}]_i$ consisting of a large initial component followed by a lower sustained component.

3 In the presence of 1.8 mM extracellular calcium, caffeine (5 mM) induced a small elevation of $[Ca^{2+}]_i$. When endothelial cells were bathed in nominally calcium-free solution containing 0.5 mM EGTA, the caffeine-induced elevation of $[Ca^{2+}]_i$ was almost completely abolished.

4 In the presence of 1.8 mM extracellular calcium, treatment of endothelial cells with the calcium influx blocker, nickel chloride (4 mM), had no effect on resting $[Ca^{2+}]_i$ or on the magnitude of the bradykinin-induced initial transient elevation of $[Ca^{2+}]_i$ but abolished the sustained component.

5 In the presence of 1 mM extracellular calcium, treatment with the calcium chelator EGTA (2 mM; 1 min) had no effect on resting $[Ca^{2+}]_i$ but the magnitude of the bradykinin-induced initial transient elevation of $[Ca^{2+}]_i$ was significantly reduced. Increasing the exposure time or concentration of EGTA resulted in no further reduction in the magnitude of the bradykinin-induced transient component.

6 Treatment of endothelial cells with the putative inhibitor of intracellular calcium release, 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester (TMB-8, 0.1 mM) increased resting $[Ca^{2+}]_i$ slightly but had no effect on the magnitude of the bradykinin-stimulated elevation of $[Ca^{2+}]_i$.

7 These findings suggest that, in bovine aortic endothelial cells, the bradykinin-induced initial transient elevation of $[Ca^{2+}]_i$ is completely dependent upon release of calcium from intracellular stores and the sustained component is due to calcium influx. They further suggest the possible existence of two intracellular calcium pools, one which is rapidly depleted in the absence of extracellular calcium and a second which is resistant to such depletion.

Introduction

The vascular endothelium plays an important role in the regulation of blood vessel tone through the release of potent vasodilators such as prostacyclin (Moncada *et al.*, 1976) and endothelium-derived relaxing factor (EDRF; Furchgott & Zawadzki, 1980). Release of these endothelium-derived vasodilators appears to be calcium-dependent (Weksler *et al.*, 1978; Singer & Peach, 1982; Furchgott, 1984; Griffith *et al.*, 1986).

Recent studies with fluorescent calcium indicators, such as quin-2, fura-2 and indo-1, have demonstrated that the endothelium-dependent vasodilators, bradykinin, adenosine triphosphate, thrombin and histamine elevate intracellular levels of calcium ($[Ca^{2+}]_i$) in endothelial cells obtained from a variety of vascular sites (Rotrosen & Gallin, 1986; Hallam & Pearson, 1986; Colden-Stanfield *et al.*, 1987; Hallam *et al.*, 1988a). These agonist-induced elevations of $[Ca^{2+}]_i$ are biphasic in nature, consisting of a large initial transient, followed by a lower but more sustained elevation of $[Ca^{2+}]_i$. There is general agreement that the sustained component is completely dependent upon influx of extracellular calcium as it is abolished upon removal of extracellular calcium following pretreatment with calcium chelators such as EGTA or with inhibitors of calcium influx such as nickel or lanthanum (Hallam & Pearson, 1986; Rotrosen & Gallin, 1986; Colden-Stanfield *et al.*, 1987; Hallam *et al.*, 1988a,b). In contrast, the precise nature of the initial transient elevation of $[Ca^{2+}]_i$ is less clear. In human umbilical vein endothelial cells (Hallam *et al.*, 1988a) and in bovine aortic endothelial cells (Lückhoff *et al.*, 1988a) removal of external calcium had no effect on the magnitude of the initial agonist-induced transient elevation of $[Ca^{2+}]_i$. These results suggest that the initial transient elevation of $[Ca^{2+}]_i$ is entirely due to release of calcium from an intracellular store. In contrast, other studies have demonstrated a reduction in the magnitude of the initial agonist-induced transient elevation of $[Ca^{2+}]_i$ in the absence of extracellular calcium (Colden-Stanfield *et al.*, 1987; Schilling *et al.*, 1988). These latter studies could be explained by either the presence of a calcium influx component in the initial transient elevation of $[Ca^{2+}]_i$ or, alternatively, depletion of intracellular calcium. The purpose of this study was to determine the contribution of intracellular and extracellular calcium pools in the biphasic elevation of $[Ca^{2+}]_i$ induced by bradykinin in bovine aortic endothelial cells.

Methods

Culture of endothelial cells

Bovine aortae were obtained from a local abattoir. Immediately after removal from the animal, the aorta was flushed with sterile saline containing benzyl penicillin (100 u ml^{-1}) and streptomycin (100 $\mu g\,ml^{-1}$). The vessel was subsequently

[1] Author for correspondence.

ligated at one end and cannulated at the other with a 60 ml syringe containing the same sterile saline. This saline was infused into the lumen and the aorta transported to the laboratory. After ligating the intercostal arteries, collagenase (type II, Sigma, 0.1% in Dulbecco's modification of Eagle's medium (DMEM)) was infused into the lumen and the vessel incubated for 25 min at 37°C. The endothelial cells were then harvested and washed twice by repeatedly spinning the cells and re-suspending them in DMEM. Cells were finally re-suspended in DMEM containing foetal bovine serum (10%), newborn bovine serum (10%), glutamine (4 mM), benzyl penicillin (100 u ml^{-1}) and streptomycin (100 $\mu g\,ml^{-1}$). The cells harvested from each aorta were seeded into 2 tissue culture flasks (80 cm^2) and grown in an incubator at 37°C under an atmosphere of 5% CO_2 in air. The culture medium was replaced the day after isolation and every 2 days subsequently. All tissue culture reagents were obtained from Gibco (Paisley, UK) except for HEPES-DMEM which was obtained from Northumbria Biologicals (Cramlington, UK). Upon reaching confluence (3–5 days), the cells were detached by 2–3 min exposure to trypsin (0.05%) and EDTA (0.02%) (Flow laboratories, Irvine, UK), washed twice, seeded onto sterile glass coverslips (11 × 42 mm) and grown to confluence, reached after 2–3 days.

Measurement of $[Ca^{2+}]_i$

Upon reaching confluence, endothelial cell monolayers were incubated for 45 min with the penta-acetoxymethyl ester form of fura-2 (2 μM, Novabiochem) at 37°C in HEPES (20 mM)-buffered DMEM containing 1% bovine serum albumin (Fraction V, Sigma). The loaded cells were transferred to HEPES (10 mM)-buffered Krebs solution (pH 7.4) containing (mM): NaCl 118, KCl 4.8, $MgSO_4$ 1, $NaHCO_3$ 2.4, glucose 11, HEPES 10 and $CaCl_2$ 1.8, and left at room temperature for 20 min, to allow for hydrolysis of fura-2 to the acid form. Most experiments were carried out in this HEPES (10 mM)-buffered Krebs solution, but in some the $CaCl_2$ content was varied as indicated in the Results. In the experiments involving the use of $NiCl_2$ (4 mM), the same solution was used except that $MgSO_4$ was replaced with 1 mM $MgCl_2$ and $NaHCO_3$ was omitted. Coverslips with fura-2 loaded cells were placed in HEPES (10 mM)-buffered Krebs solution in a quartz cuvette, in a Perkin Elmer LS3B fluorimeter, at an angle of 30° to the incident light. The beam irradiated the monolayer without passing through the coverslip. The cuvette was maintained at 37°C and stirred continuously. The excitation monochromator was computer driven (IBM-PC AT) alternating between 340 nm and 380 nm every 3.8 s and fluorescence emission collected at 509 nm. At the end of each experimental run, background auto-fluorescence (the inherent fluorescence emitted from cells, coverslip and cuvette at 340 nm and 380 nm) was obtained by the method of Hallam *et al.* (1988a). Ionomycin (1 μM) was added to permeabilise the cells to divalent cations and $MnCl_2$ (2 mM) was added to quench intracellular fura-2 fluorescence. Following subtraction of auto-fluorescence, the corrected fluorescence values obtained following excitation at 340 nm were divided by those obtained at 380 nm, giving a corrected ratio (R). $[Ca^{2+}]_i$ was then calculated by the computer using the equation of Grynkiewicz *et al.* (1985):

$$[Ca^{2+}]_i = K_d \times \frac{(R - R_{min})}{(R_{max} - R)} \times \frac{S_{f2}}{S_{b2}}$$

and a resultant experimental trace of $[Ca^{2+}]_i$ versus time obtained. R_{max} and R_{min} are the maximal and minimal fluorescence ratios of fura-2 obtained in medium containing saturating concentrations of calcium and in calcium-free medium (with 40 mM EGTA), respectively. S_{f2} and S_{b2} are the fluorescence values obtained at 380 nm in the absence of calcium and in presence of saturating levels of calcium, respectively. The K_d for the fura-2-Ca^{2+} complex was assumed to be 225 nM at 37°C. The following calibration values were obtained experimentally and used throughout to calculate $[Ca^{2+}]_i$: R_{max} 16.3, R_{min} 0.8 and S_{f2}/S_{b2} 7.3.

Levels of calcium in the Krebs solution following removal of calcium or addition of EGTA were measured with fura-2 in the acid form.

Drugs

Bradykinin triacetate, caffeine, ethylene glycol *bis* (β-aminoethyl ethane)N,N,N',N'- tetraacetic acid (EGTA) and 3,4,5-trimethoxybenzoic acid 8-(diethylamino) octyl ester (TMB-8) were obtained from Sigma. Fura-2 penta-acetoxymethyl ester (fura-2 AM), fura-2 acid, and ionomycin were obtained from Novabiochem. All drugs were dissolved in distilled water except for fura-2 AM and ionomycin which were dissolved in dimethyl sulphoxide (DMSO), TMB-8 which was dissolved in 100% ethanol and caffeine which was dissolved in HEPES-buffered Krebs.

Statistical analysis

Results are expressed as the mean ± s.e.mean and comparisons were made by Student's *t* test and by the Mann-Whitney test when there was unequal variance in samples. A probability of 0.05 or less was considered significant.

Results

Effects of bradykinin on $[Ca^{2+}]_i$

In the presence of 1.8 mM extracellular calcium, the resting level of $[Ca^{2+}]_i$ in the first passage bovine aortic endothelial cells was 102 ± 3 nM (n = 89). Bradykinin induced a biphasic elevation of $[Ca^{2+}]_i$ consisting of a large initial transient component, which peaked within 30 s, followed by a second component, which was relatively well maintained (Figure 1). Both components were concentration-dependent between 0.3 nM and 10 nM (EC_{50} = 2 nM) (Figure 1).

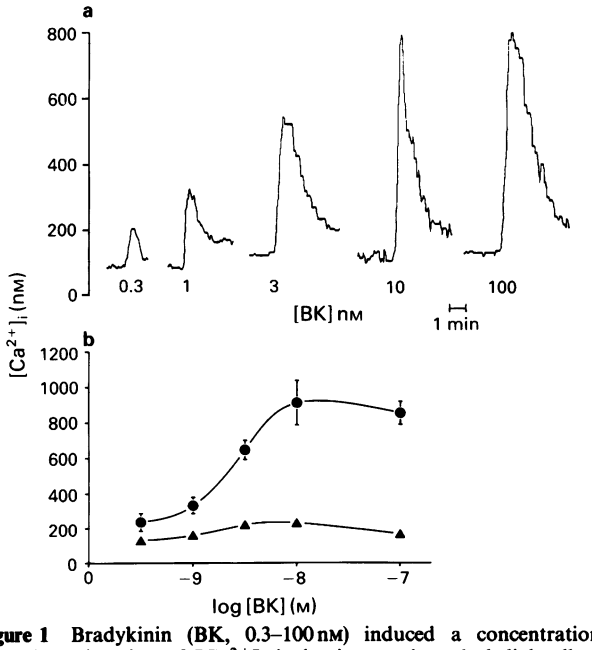

Figure 1 Bradykinin (BK, 0.3–100 nM) induced a concentration-dependent elevation of $[Ca^{2+}]_i$ in bovine aortic endothelial cells in the presence of 1.8 mM extracellular calcium. (a) Individual traces showing the biphasic nature of the response to bradykinin: a large initial transient elevation followed by a lower, more sustained component. (b) Concentration-effect curves showing the magnitude of the initial transient (●) increase in $[Ca^{2+}]_i$ and the sustained phase (▲) measured 5 min after addition of bradykinin. Individual points represent the mean of 8–29 observations and vertical bars indicate the s.e.mean. Where error bars are not seen, they are encompassed within the size of the symbols.

Effects of lowering extracellular calcium

Incubation in nominally calcium-free HEPES (10 mM)-buffered Krebs for 25 min, followed by addition of EGTA (0.5 mM) for 5 min reduced extracellular calcium to 12 ± 1 nM ($n = 4$) and reduced resting $[Ca^{2+}]_i$ from 88 ± 6 nM ($n = 11$) to 57 ± 8 nM ($n = 15$). Re-addition of Ca^{2+} (2.3 mM) restored $[Ca^{2+}]_i$ to 98 ± 5 nM ($n = 5$). In calcium-free Krebs in the presence of EGTA (0.5 mM) the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by bradykinin (10 nM) was reduced and the sustained component (Figure 2a and b) was abolished. In the continued presence of bradykinin (10 nM), re-addition of extracellular calcium (2.3 mM) induced a biphasic elevation of $[Ca^{2+}]_i$, consisting of a large transient component followed by a lower sustained component (Figure 2a and b).

The elevation of $[Ca^{2+}]_i$, induced by bradykinin (1 nM) was almost completely abolished following incubation in nominally calcium-free Krebs containing EGTA (0.5 mM; Figure 2c and d). Subsequent re-addition of extracellular calcium (2.3 mM) induced a biphasic elevation of $[Ca^{2+}]_i$, the magnitude of both components being similar to those induced by bradykinin (1 nM) in the presence of 1.8 mM extracellular calcium (Figure 2c and d).

In an attempt to determine the kinetics of the loss of the bradykinin-induced transient elevation of $[Ca^{2+}]_i$, we examined the effects of acute addition of EGTA to cells in calcium-containing Krebs. In the presence of 1 mM extracellular calcium, addition of EGTA (2 mM) for 1 min lowered extracellular calcium to 560 ± 30 nM ($n = 4$), had no effect on resting $[Ca^{2+}]_i$, but reduced the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by bradykinin (10 nM) and abolished the sustained component (Figure 3). Prolonging exposure to EGTA (2 mM) for 10 min had no effect on resting $[Ca^{2+}]_i$ but exposure to EGTA (5 mM) for 1 min, which lowered extracellular calcium to 101 ± 2 nM ($n = 4$), induced a small but significant fall in resting $[Ca^{2+}]_i$ (Figure 3). However, neither treatment led to a further reduction in the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by bradykinin (10 nM; Figure 3). The effects of higher concentrations of EGTA could not be examined since this led to significant detachment of cells.

Effects of nickel

In the presence of 1.8 mM extracellular Ca^{2+}, treatment with nickel chloride (4 mM) for 2 min had no effect on resting $[Ca^{2+}]_i$ and had no effect on the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by bradykinin (10 nM and 0.3 nM; Figure 4). This treatment with nickel (4 mM) did, however, abolish the sustained component of $[Ca^{2+}]_i$ induced by bradykinin (10 nM and 0.3 nM; Figure 4).

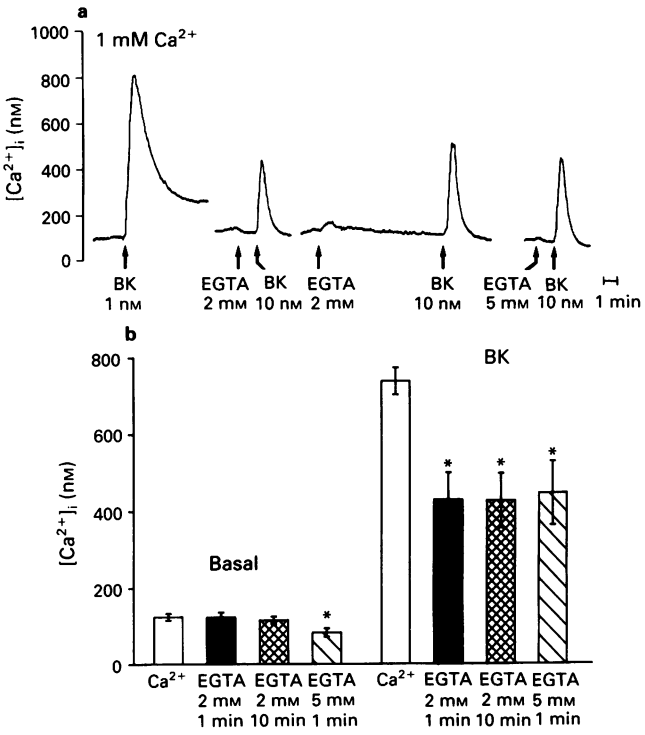

Figure 3 Individual traces (a) and a histogram (b) showing the effects of pretreatment with EGTA (2 mM and 5 mM) for 1 min or 10 min on resting (Basal) and bradykinin (BK, 10 nM)-stimulated levels of $[Ca^{2+}]_i$ in bovine aortic endothelial cells in the presence of 1 mM extracellular calcium. In the histogram, resting levels and the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ are given as the mean of 4–16 observations and vertical bars indicate the s.e.mean. * $P < 0.05$, indicates a significant difference from values obtained in the presence of 1 mM extracellular calcium.

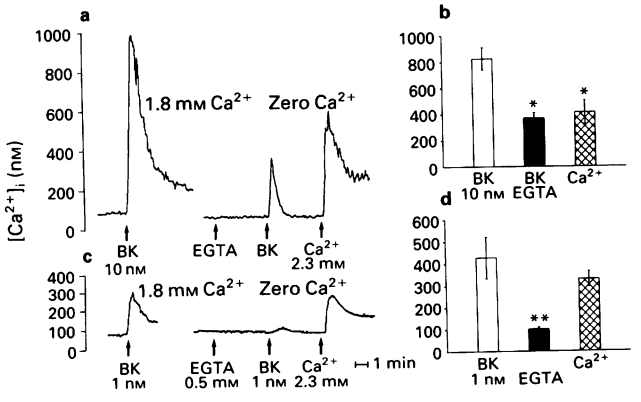

Figure 2 Individual traces (a and c) and histograms (b and d) showing the effects of bradykinin (BK, 10 nM and 1 nM) on $[Ca^{2+}]_i$ in bovine aortic endothelial cells in the presence of 1.8 mM calcium (open columns) and following the removal of extracellular calcium and addition of EGTA (0.5 mM, 5 min, solid columns). The effects of re-addition of calcium (Ca^{2+}, 2.3 mM) in the continued presence of bradykinin are shown in the cross-hatched columns. In the histograms, the magnitude of the initial transient elevations of $[Ca^{2+}]_i$ is given as the mean of 5–7 observations and vertical bars indicate the s.e.mean. * $P < 0.05$; ** $P < 0.01$, indicates a significant difference from the response to bradykinin in the presence of calcium.

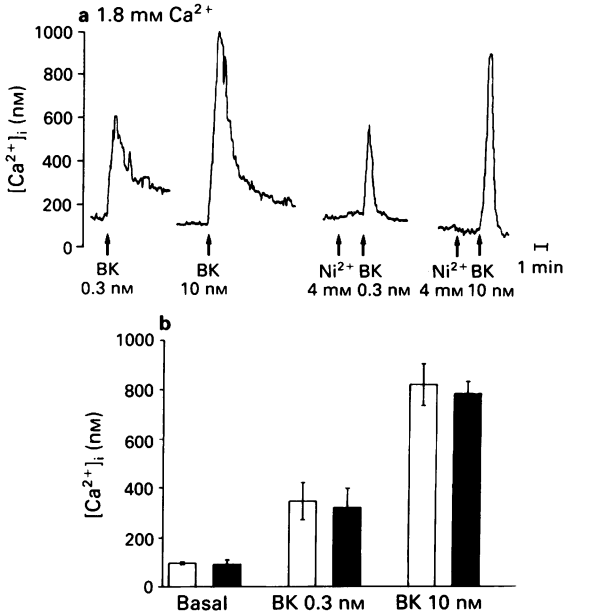

Figure 4 Individual traces (a) and a histogram (b) showing resting (Basal) and bradykinin (BK, 0.3 nM and 10 nM)-stimulated levels of $[Ca^{2+}]_i$ in bovine aortic endothelial cells in the presence of 1.8 mM extracellular calcium (open columns) and following pretreatment with nickel (Ni^{2+}, 4 mM, 2 min, solid columns). In the histogram, resting levels and the magnitude of the initial transient elevations of $[Ca^{2+}]_i$ are given as the mean of 4–19 observations and vertical bars indicate the s.e.mean.

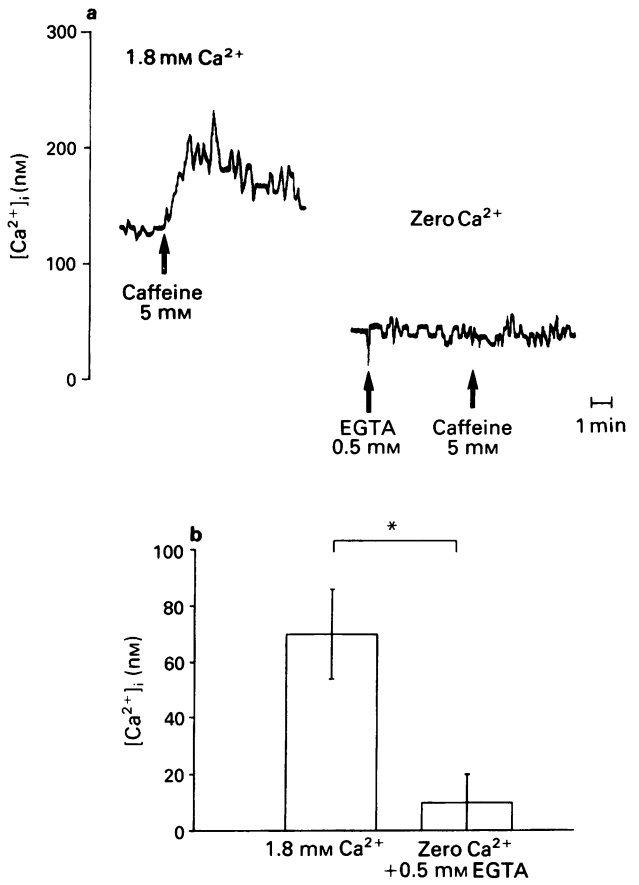

Figure 5 Individual traces (a) and a histogram (b) showing the effects of removal of extracellular calcium and addition of EGTA (0.5 mM, 5 min) on caffeine (5 mM)-stimulated elevations of $[Ca^{2+}]_i$ in bovine aortic endothelial cells. In the histogram, the magnitude of the elevations of $[Ca^{2+}]_i$ induced by caffeine in the absence and presence of 1.8 mM extracellular calcium are given as the mean of 5–7 observations and vertical bars indicate the s.e.mean. * $P < 0.05$, indicates a significant difference between groups joined by a bracket.

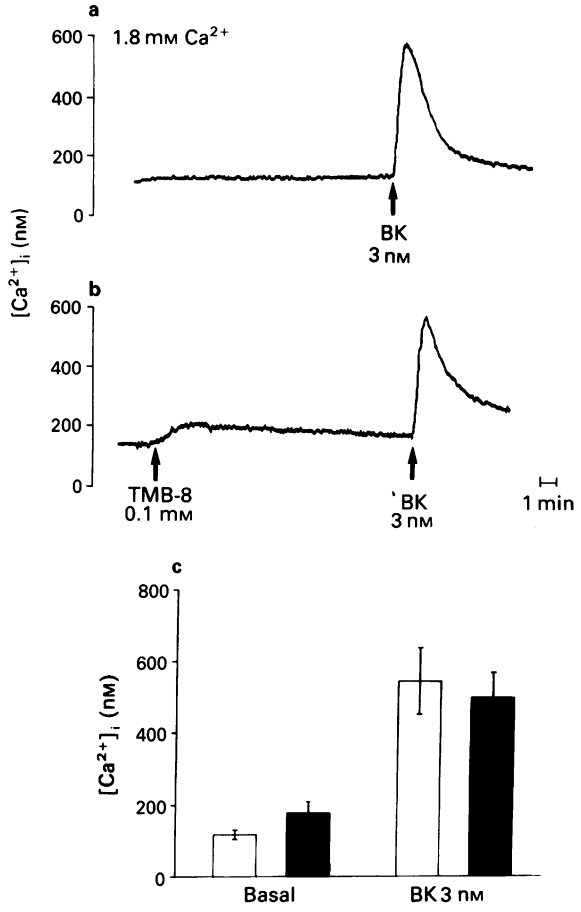

Figure 6 Individual traces (a and b) and a histogram (c) showing resting (Basal) and bradykinin (BK, 3 nM)-stimulated levels of $[Ca^{2+}]_i$ in bovine aortic endothelial cells in the presence of 1.8 mM extracellular calcium (open columns) and following treatment with 3,4,5-trimethoxybenzoic acid 8-(diethylamino) octyl ester (TMB-8, 0.1 mM, 15 min, solid columns). In the histogram, resting levels and the magnitude of the initial transient elevations of $[Ca^{2+}]_i$ are given as the mean of 4–8 observations and vertical bars indicate the s.e.mean.

Effects of caffeine

In the presence of 1.8 mM extracellular calcium, caffeine (5 mM), which is known to promote release of calcium from intracellular stores (Weber & Herz, 1968), induced a small elevation of $[Ca^{2+}]_i$ (Figure 5). Following incubation in nominally calcium-free Krebs for 25 min followed by addition of EGTA (0.5 mM) for 5 min, the resting $[Ca^{2+}]_i$ was reduced significantly and the ability of caffeine (5 mM) to elevate $[Ca^{2+}]_i$ was abolished (Figure 5).

Effects of TMB-8

In the presence of 1.8 mM extracellular calcium, treatment with TMB-8 (0.1 mM), a putative inhibitor of intracellular calcium release (Malagodi & Chiou, 1974), induced a small elevation of $[Ca^{2+}]_i$ (58 ± 6 nM; $n = 4$), but had no effect on the magnitude of the elevation of $[Ca^{2+}]_i$ induced by bradykinin (3 nM; Figure 6).

Discussion

In this study, bradykinin was found to induce a biphasic elevation of intracellular calcium ($[Ca^{2+}]_i$) in bovine aortic endothelial cells, consisting of a large, initial transient component followed by a lower, relatively well-sustained component. These findings are in agreement with previous observations made in endothelial cells from bovine aorta (Colden-Stanfield *et al.*, 1987; Lückhoff *et al.*, 1988a), human umbilical vein (Rotrosen & Gallin, 1986; Hallam *et al.*, 1988a,b; Brock & Capasso, 1988), bovine pulmonary artery (Ryan *et al.*, 1988) and pig aorta (Hallam & Pearson, 1986).

We found that the sustained component of the bradykinin-induced elevation of $[Ca^{2+}]_i$ was abolished when bovine aortic endothelial cells were placed in nominally calcium-free solution containing 0.5 mM EGTA. This observation has been made consistently in all endothelial cell types studied (Hallam & Pearson, 1986; Rotrosen & Gallin, 1986; Colden-Stanfield *et al.*, 1987). We also found that this component was abolished by treatment with nickel, which has been previously shown to block calcium influx into endothelial cells (Hallam *et al.*, 1988b). Both these observations suggest that the maintained elevation of $[Ca^{2+}]_i$ induced by agonists is due to influx of extracellular calcium. This conclusion is further supported by patch clamp analysis (Johns *et al.*, 1987), by $^{45}Ca^{2+}$ flux studies (Bussolino *et al.*, 1985), and by the finding that agonists can promote entry of another divalent cation, manganese, into cells which subsequently quenches fura-2 fluorescence (Hallam *et al.*, 1988b). Calcium influx is unlikely to occur via voltage-operated channels: electrophysiological studies have failed to show the existence of these channels in endothelial cells (Colden-Stanfield *et al.*, 1987), and depolarization with potassium chloride or treatment with calcium channel antagonists has no effect on $[Ca^{2+}]_i$ in endothelial cells (Hallam & Pearson, 1986; Colden-Stanfield *et al.*, 1987). It is therefore likely that bradykinin-stimulated calcium entry occurs via a receptor-operated channel. When bovine aortic endothelial cells were bathed in nominally calcium-free solution containing 0.5 mM EGTA, the magnitude of the bradykinin-induced

initial transient elevation of $[Ca^{2+}]_i$ was significantly reduced. This observation is consistent with the findings of Colden-Stanfield *et al.* (1987) and Schilling *et al.* (1988). In a separate study in which statistical validation data were not presented (Lückhoff *et al.*, 1988a), no reduction in the magnitude of this component was observed in bovine aortic endothelial cells. Under similar, nominally calcium-free conditions, variable reductions in the magnitude of the initial transient component have been observed in human umbilical vein endothelial cells in response to histamine or thrombin (Hallam *et al.*, 1988a; Brock & Capasso, 1988). In contrast, in a study in which the older, less sensitive probe, quin-2, was used, no reduction in the magnitude of the transient elevation of $[Ca^{2+}]_i$ induced by histamine was reported (Rotrosen & Gallin, 1986). In pig aortic endothelial cells the initial transient induced by ATP was also reduced slightly in calcium-free conditions (Hallam & Pearson, 1986). In conclusion, in statistically-validated studies using the more sensitive probes, fura-2 and indo-1, there appears general agreement that the magnitude of the initial agonist-induced elevation of $[Ca^{2+}]_i$ is reduced in calcium-free conditions.

From our studies performed in nominally calcium-free solution, it was not possible to determine whether the reduced magnitude of the bradykinin-induced initial transient elevation of $[Ca^{2+}]_i$ was due to an impaired ability to release intracellular calcium, to depletion of an intracellular store, or to loss of a transient calcium influx component. Bradykinin-induced hydrolysis of phosphoinositides is not affected when bovine aortic endothelial cells are placed in calcium-free solution (Derian & Moskowitz, 1986). It is unlikely, therefore, that reduced production of inositol-1,4,5-trisphosphate accounts for the reduction in the initial transient elevation of $[Ca^{2+}]_i$. Loss of a calcium influx component is also unlikely since in the presence of extracellular calcium, treatment with nickel, which blocks calcium influx (Hallam *et al.*, 1988b), had no effect on the magnitude of bradykinin-induced initial transient elevation of $[Ca^{2+}]_i$, although it completely blocked the sustained component. Lanthanum, another inhibitor of calcium influx, was similarly found to have little effect on the magnitude of the initial transient elevation of $[Ca^{2+}]_i$, despite blocking the sustained component (Colden-Stanfield *et al.*, 1987). Our novel observation that the ability of caffeine to release intracellular calcium (Weber & Herz, 1968) and so elevate $[Ca^{2+}]_i$, was blocked in the absence of extracellular calcium is consistent with the depletion of intracellular stores. The small magnitude of the elevation of $[Ca^{2+}]_i$ induced by caffeine shows, however, that the caffeine-sensitive calcium pool is a small proportion of the total intracellular content. It is therefore likely that the bradykinin-induced initial transient elevation of $[Ca^{2+}]_i$ is completely dependent upon release of calcium from an intracellular store and that exposure to calcium-free solution leads to depletion of this store.

In a separate series of experiments we attempted to examine the kinetics of the loss of the bradykinin-induced initial transient elevation of $[Ca^{2+}]_i$ in calcium-free solution. In these experiments in which bovine aortic endothelial cells were bathed in 1 mM extracellular calcium and then exposed to the calcium chelator, EGTA (2 mM) for only 1 min, the magnitude of the bradykinin-induced initial transient elevation of $[Ca^{2+}]_i$ was significantly reduced. Increasing of either the concentration of EGTA or the time of exposure resulted in no further reduction in the bradykinin-induced initial transient elevation of $[Ca^{2+}]_i$. These novel observations suggest the possible existence of two intracellular calcium pools; one which is rapidly depleted in the presence of low extracellular calcium, and a second which is resistant to such depletion. The rapidly depleted store appears to contribute more to responses obtained to sub-maximal stimuli since, in the presence of EGTA, the initial transient elevation of $[Ca^{2+}]_i$ is blocked to a greater degree when the concentration of bradykinin is low. Whether these two pools suggested by our results are equivalent to the recently described separate guanosine triphosphate (GTP)-sensitive and inositol trisphosphate-sensitive pools (Ghosh *et al.*, 1989) remains to be determined.

When bovine aortic endothelial cells were bathed in nominally calcium-free solution in the presence of 0.5 mM EGTA, the bradykinin-induced transient elevation of $[Ca^{2+}]_i$ was complete within 90 s. In a novel series of experiments, we found that subsequent addition of extracellular calcium, to achieve a free concentration of around 1.8 mM, resulted in the induction of a biphasic elevation of $[Ca^{2+}]_i$, consisting of a large, initial transient component followed by a smaller, well-maintained component. It is likely that the latter is analogous to the sustained elevation fo $[Ca^{2+}]_i$ induced by bradykinin in the presence of extracellular calcium. However, the nature of the initial transient component is less clear. One possible explanation is that the re-addition of extracellular calcium results in calcium-induced calcium release. Procaine has been shown to inhibit calcium-induced calcium release (Weber & Hertz, 1968), but we saw no reduction in the magnitude of the initial component in the presence of procaine (1 mM, unpublished observations). Alternatively, the rapid, transient component obtained upon the re-addition of calcium may result from the rapid refilling of intracellular stores from the extracellular space followed by discharge of calcium from these stores into the cytosol.

TMB-8, a putative inhibitor of intracellular calcium release, inhibits prostacyclin production by endothelial cells (Seid *et al.*, 1983; Lückhoff *et al.*, 1988b; White & Martin, 1989). This observation appears consistent with the belief that prostacyclin production is more dependent upon release of calcium from intracellular stores than on calcium influx (Seid *et al.*, 1983; Hallam *et al.*, 1988a; White & Martin, 1989). Surprisingly, however, in this study, treatment with TMB-8 for up to 15 min had no effect on the bradykinin-induced initial transient elevation of $[Ca^{2+}]_i$. TMB-8 did induce a small elevation of $[Ca^{2+}]_i$ by itself but the mechanism of this is not known. We therefore obtained no evidence that TMB-8 inhibits release of calcium from intracellular stores. It would appear unlikely that TMB-8 inhibits endothelial production of prostacyclin by inhibiting release of intracellular calcium.

In conclusion, bradykinin induced a biphasic elevation of $[Ca^{2+}]_i$ in bovine aortic endothelial cells consisting of a large, initial transient component followed by a lower, sustained component. The initial transient component was not reduced following treatment with the calcium entry blocker, nickel, suggesting that it is completely dependent upon release of calcium from intracellular stores. Our observations suggest that bradykinin may release calcium from two intracellular pools; one which is rapidly depleted in the absence of extracellular calcium and a second which is resistant to such depletion. The lower, sustained elevation of $[Ca^{2+}]_i$ induced by bradykinin was dependent upon the presence of extracellular calcium and was abolished by pretreatment with nickel, suggesting that it is dependent upon influx of extracellular calcium.

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Presynaptic inhibition by neuropeptide Y in rat hippocampal slice *in vitro* is mediated by a Y_2 receptor

[1]*William F. Colmers, *Gloria J. Klapstein, †Alain Fournier, †Serge St-Pierre & *Kerri A. Treherne

*Department of Pharmacology, University of Alberta, Edmonton, Alberta, Canada, T6G 2H7, and †INRS Santé, Pointe-Claire, Québec, Canada, H9R 1G6

1 The action of analogues and C-terminal fragments of neuropeptide Y (NPY) was examined on excitatory synaptic transmission in area CA1 of the rat hippocampal slice *in vitro*, by use of intracellular and extracellular recordings, to determine by agonist profile the NPY receptor subtype mediating presynaptic inhibition.

2 Neither NPY, analogues nor fragments of NPY affected the passive or active properties of the postsynaptic CA1 pyramidal neurones, indicating their action is at a presynaptic site.

3 The full-sequence analogues, peptide YY (PYY) and human NPY (hNPY), were equipotent with NPY at the presynaptic receptor, while desamido hNPY was without activity.

4 NPY2–36 was equipotent with NPY. Fragments as short as NPY13–36 were active, but gradually lost activity with decreasing length. NPY16–36 had no effect on extracellular field potentials, but still significantly inhibited excitatory postsynaptic potential amplitudes. Fragments shorter than NPY16–36 had no measurable effect on synaptic transmission.

5 The presynaptic NPY receptor in hippocampal CA1 therefore shares an identical agonist profile with the presynaptic Y_2 receptor at the peripheral sympathetic neuroeffector junction.

Introduction

Neuropeptide Y (NPY) is an abundant, 36 amino acid peptide expressed and released by many types of nerve cells in the central (CNS) and peripheral (PNS) nervous systems of mammals (DeQuidt & Emson, 1986; Sundler *et al.*, 1986). In peripheral smooth muscle tissues, NPY has two sites of action: (1) on the muscle cell itself, relatively high concentrations of NPY induce contractions, and subthreshold concentrations of the peptide markedly enhance the contractile response to other agents, such as noradrenaline and histamine (Wahlestedt *et al.*, 1986; Wahlestedt, 1987); (2) on the presynaptic sympathetic nerve terminal, NPY inhibits its own release, as well as the release of noradrenaline (Wahlestedt *et al.*, 1986; Wahlestedt, 1987). In rat hippocampal slice *in vitro*, NPY has been shown to inhibit excitatory synaptic transmission at stratum radiatum-CA1 (glutamatergic) synapses (Colmers *et al.*, 1985; 1987; 1988; Haas *et al.*, 1987) by an action at the presynaptic terminals, probably the inhibition of voltage-dependent calcium influx (Colmers *et al.*, 1988).

To date, no antagonists at NPY receptors have been identified. However, studies which tested agonist fragments and analogues of NPY indicate that at least two subtypes of NPY receptor can be distinguished pharmacologically in peripheral sympathetic neuroeffector junctions (Wahlestedt *et al.*, 1986; Wahlestedt, 1987). The Y_1 subtype is located on the postsynaptic cell in blood vessels and vas deferens, and requires the intact NPY, or the very closely related peptide YY (PYY) molecule for its activation (Wahlestedt *et al.*, 1986). The Y_2 subtype is found on the presynaptic terminal, and can be activated by analogues and C-terminal fragments of NPY as short as NPY 13–36 (Wahlstedt *et al.*, 1986). The C-terminal desamido form of NPY has very little activity at both receptor subtypes (Wahlestedt *et al.*, 1986). The two receptor subtypes in peripheral tissues are also distinguished by the second messenger systems to which they are coupled; Y_1 receptor activation causes phosphatidylinositol hydrolysis, while Y_2 receptor activation inhibits adenylate cyclase (Wahlestedt, 1987; Westlind-Danielsson *et al.*, 1987; Hinson *et al.*, 1988; Perney & Miller, 1989).

To ascertain which NPY receptor subtype (if either) is responsible for the presynaptic inhibition observed in hippocampus, we compared the response of stratum radiatum-CA1 excitatory synaptic transmission to single concentrations of agonist analogues and fragments of NPY with that to the intact peptide. The results indicate that, in rat hippocampus *in vitro*, NPY inhibits excitatory synaptic transmission via a receptor which is pharmacologically similar in its agonist profile to the peripheral Y_2 subtype.

Methods

Peptide synthesis

Fragments of NPY were synthesized by the solid phase method with a manual home-made multireactor synthesizer. The syntheses were carried out with a benzhydrylamine resin (Pietta *et al.*, 1974) since the peptides bear an amide C-terminal function. All amino acids were coupled via the BOP/DMF method (Fournier *et al.*, 1988), according to a recently-described protocol (Forest & Fournier, 1989). The Boc amino acids with appropriate side-chain protection were obtained from commercial sources. Completed peptides were cleaved from the resin support and deprotected by a 90 min treatment at 0°C with liquid hydrofluoric acid containing *m*-cresol and dimethyl sulphide as scavengers (10:1:1 v/v).

After extraction from the resin and lyophilization, the peptides were purified by reverse-phase chromatography on a Waters Deltapak column, using an eluent of (A) H_2O (0.06% trifluoroacetic acid, TFA) and (B) acetonitrile-H_2O (0.06% TFA). Peptides were eluted with successive linear gradients of solvent B. Analytical high performance liquid chromatography (h.p.l.c.) of the individual fractions was carried out and the fractions corresponding to the purified peptide were lyophilized. The purified material was characterized by analytical h.p.l.c., capillary electrophoresis and amino acid analysis.

Electrophysiological studies

Intra- and extracellular recordings were made from area CA1 of rat hippocampal slices as described previously by Colmers

[1] Author for correspondence.

et al. (1987, 1988). Briefly, transverse slices (400–450 μm) of hippocampus were submerged in a recording chamber continuously perfused (2.5 ml min^{-1}) with saline (Colmers et al., 1985; 1987; 1988) saturated with 95% O_2/5% CO_2 and heated to 34 ± 0.2°C. Orthodromic stimuli (monophasic, square wave, 100–300 μs, 3–35 V) were applied through bipolar electrodes, placed on stratum radiatum of area CA1. Extracellular recordings were made from the stratum pyramidale of area CA1 with glass microelectrodes (3–15 MΩ; 2 M NaCl); intracellular recordings were made from CA1 pyramidal neurones in area CA1 with glass microelectrodes (85–150 MΩ; 2 M K^+ acetate). An Axoclamp 2A amplifier, used in the bridge current clamp mode was used both for extracellular and intracellular recordings. Current and voltage were displayed continuously on a d.c. coupled chart recorder (Gould 2200, frequency response d.c. 60 Hz), and selected parts of experiments were stored either on FM (Racal) or PCM coded videotape (Vetter). A Nicolet 4094 digital oscilloscope was used to average and store data on-line and analyse data off-line.

Analysis of electrophysiological data was performed as published previously (Colmers et al. 1985; 1987; 1988). Stimulus amplitudes were chosen to evoke responses between 70 and 85% of maximum, which is on the steepest, and therefore most sensitive, portion of the stimulus-response relationship (Colmers et al., 1985; 1987). Population spike (PS) amplitude was determined from the peak of the negativity following the stimulus artifact to the peak of the following positivity. Resting membrane potential was determined from chart records; excitatory postsynaptic potentials (e.p.s.ps) were measured as peak amplitudes. E.p.s.ps were evoked 40 ms after beginning of a hyperpolarizing current pulse applied to the electrode via the bridge circuit to prevent the neurone from achieving action potential threshold. Input resistance was determined by the slope of a least-squares linear regression line fitted to data obtained from families of hyperpolarizing and depolarizing constant current pulses (125 ms) applied to neurones under each condition.

Peptides and drugs were applied via the superfusate (Colmers et al., 1987; 1988). Native porcine NPY (Richelieu Biotechnologies, Québec, Canada or a generous gift from Dr T.O. Neild, Monash University, Melbourne, Australia) and analogues and fragments (synthesized as above) were prepared just prior to use at a final concentration of 1 μM. All analogues and fragments, except as noted, were porcine NPY sequence, C-terminally amidated peptides. Native, intact porcine NPY is referred to here simply as NPY. Fragments are referred to by the number of C-terminal residues.

Data were taken only from preparations where a significant recovery from drug effects occurred upon washout. Preparations were used as their own controls for statistical purposes, and peptide effects relative to control were assessed by Student's paired t test. Although we attempted to apply NPY itself to each preparation, it was not always possible; pooled data from fragments and analogues were therefore compared statistically with one another by use of Student's t test for 2 means.

Results

Results are based on recordings from 96 separate preparations: extracellular recordings were made in 83 different preparations; intracellular recordings were made of 62 different neurones in some of the preparations where field potentials were simultaneously recorded, and in 10 preparations without simultaneous extracellular recordings.

As we and others have observed previously (Colmers et al., 1985; 1987; 1988; Haas et al., 1987), NPY reversibly inhibited excitatory synaptic transmission from stratum radiatum to CA1 pyramidal cells, as measured by a reduction of the amplitude of the extracellular population spike (PS). A concentration-response relationship to NPY indicated the threshold for inhibition of the PS to be about 30 nM; 1 μM inhibited the PS by 89.9 ± 2.8% (n = 16). The EC_{50} for NPY on the PS was about 250 nM, similar to that seen in earlier studies (Colmers et al., 1985; 1987).

In addition, as reported earlier (Colmers et al., 1987; 1988; Haas et al., 1987), NPY also reversibly inhibited the intracellularly-recorded e.p.s.p. evoked in CA1 pyramidal cells by stratum radiatum stimulation. NPY, 1 μM, reduced the e.p.s.p. by 59.8 ± 2.3% (n = 8); the EC_{50} for NPY effects on e.p.s.p. was about 200 nM, in agreement with earlier findings (Colmers et al., 1987). As reported earlier, NPY had no effects on passive or active membrane properties in CA1 neurones (Colmers et al., 1987; 1988).

Peptide analogues

All peptide analogues and fragments tested in this study were without effects on membrane resting potential, input resistance or action potential amplitude or duration, consistent with earlier observations on NPY itself (Colmers et al., 1987; 1988).

Application of the full-sequence native analogue, PYY, at 1 μM also caused reduction of both PS and e.p.s.p. amplitudes, which reversed upon washout (Figure 1). The inhibition of PS was statistically indistinguishable from that seen with NPY (Figure 2) although it appeared greater and persisted longer. However, the effect of PYY on the simultaneously-recorded intracellular e.p.s.p. was indistinguishable from that of NPY (Figure 3) although the effect also appeared to persist longer than with NPY.

Human sequence NPY (hNPY), the amino acid sequence of which is identical with rat NPY and differs from that of porcine NPY only at position 17 (Corder et al., 1984; Minth et al., 1984; Allen et al., 1987), was as effective as NPY in inhibiting synaptic transmission measured both as PS (Figures 1 and 2) and e.p.s.p. (Figures 1 and 3). However, desamido hNPY (hNPYa) had no effects on synaptic transmission (Figure 2).

Peptide fragments

Amidated C-terminal fragments of (porcine sequence) NPY, including NPY2–36, 5–36, 11–36, 16–36, and 25–36, were also studied. Data are summarized in Figures 2, 3 and 4.

NPY2–36 was at least as potent as NPY in inhibiting excitatory synaptic transmission in CA1. No significant difference

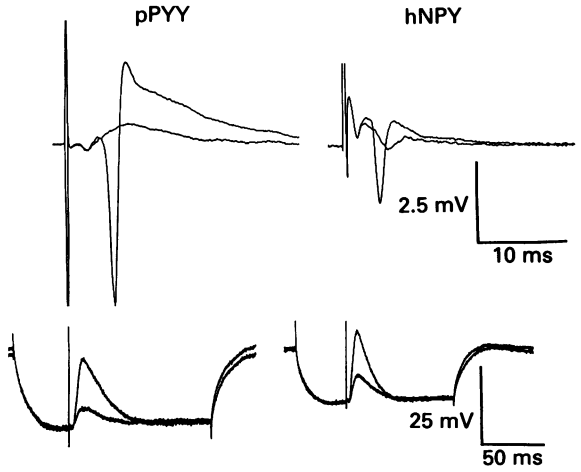

Figure 1 Effect of the full-sequence neuropeptide Y (NPY) analogues, peptide YY (PYY, left traces) and human NPY (hNPY, right traces), on amplitude of the population spike (PS, upper traces) and excitatory postsynaptic potential (e.p.s.p., lower traces) recorded simultaneously in CA1. Records for PYY and hNPY are from different preparations. Peptides were applied at a concentration of 1 μM. Control and peptide traces are shown superimposed for comparison. The responses recovered after prolonged washout (not illustrated).

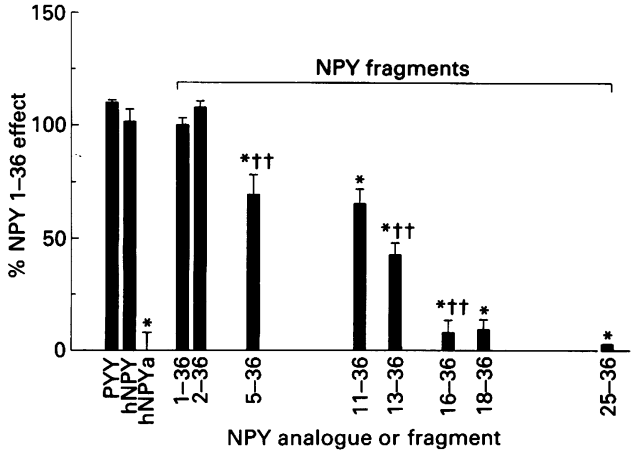

Figure 2 Relative effects of analogues and C-terminal fragments of neuropeptide Y (NPY) on population spike (PS) evoked in CA1 by stimulation of stratum radiatum. Values shown are means of effects (s.e.mean shown by vertical bars), expressed as a percentage of mean NPY (=100%) effect. Analogues are shown on the left of the figure, while pNPY and its fragments are shown to the right (under bar), listed in order of decreasing length on the X-axis, with spacing along this axis indicating the relative length of the fragments. * $P < 0.001$ difference from NPY; † $P < 0.02$ difference from previous fragment; †† $P < 0.001$ difference from previous fragment. All fragments were tested ≥5 times in different preparations. Fragments NPY16–36 and shorter were without significant effect at inhibiting the PS.

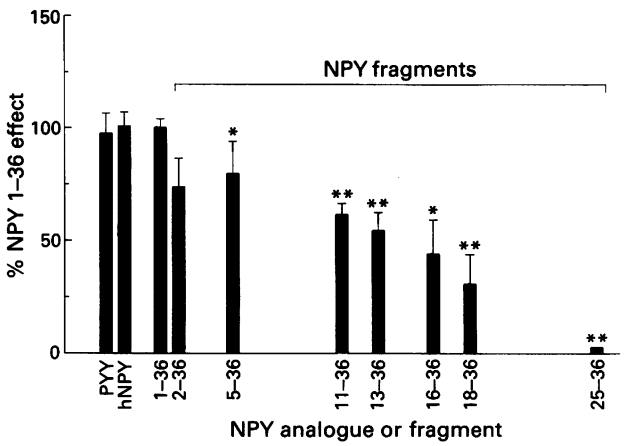

Figure 3 Relative effects of analogues and C-terminal fragments of neuropeptide Y (NPY) on excitatory postsynaptic potential (e.p.s.p.) evoked in CA1 pyramidal neurones by stimulation of stratum radiatum. Values shown are means of effects (s.e.mean shown by vertical bars), expressed as a percentage of mean NPY (=100%) effect. Analogues and fragments are distributed as in Figure 2. * $P < 0.005$ difference from NPY; ** $P < 0.001$ difference from NPY. Immediately neighbouring fragments were not statistically different from one another. All fragments were tested ≥5 times on different preparations. Fragments NPY18–36 and shorter were without effect on the e.p.s.p.

was seen between this fragment's actions on both PS and e.p.s.p. and those of NPY. While NPY5–36 also inhibited excitatory synaptic transmission, it was less active than NPY. At a concentration of 1 μM, this fragment was about 70% as active in inhibiting the PS and about 80% as active in inhibiting the e.p.s.p. as was NPY. NPY5–36 was also significantly less active than NPY2–36, NPY11–36 was slightly, but not significantly, less active at the presynaptic NPY receptor than was NPY5–36. However, NPY13–36 was significantly less active than NPY11–36, and was less than half as active as the intact peptide in inhibiting the PS, while its inhibition of the e.p.s.p. was somewhat more than 50% of that caused by NPY itself (Figure 2). By contrast, the slightly shorter NPY16–36 fragment did not significantly inhibit PS in CA1, although it had a small, but significant, inhibitory effect on the e.p.s.p. recorded intracellularly. NPY16–32 was, on average, 80% as active as NPY13–36 in inhibiting e.p.s.p. amplitudes. NPY18–36 was without any significant action on either the PS or the e.p.s.p., and NPY25–36 was without significant effects at 1 μM on PS or e.p.s.p. (Figure 3).

Discussion

In this study, there were no effects of NPY, its analogues or fragments on the passive or active membrane properties of the CA1 pyramidal neurones. This is in agreement with other work from this laboratory (Colmers *et al.*, 1987; 1988) and others (Haas *et al.*, 1987). Thus, all active peptides tested on stratum radiatum-CA1 synaptic transmission *in vitro* appear to act only at a presynaptic site.

The presynaptic receptor for NPY in the rat hippocampus appears to share an identical agonist profile with the Y_2 receptor first characterized at sympathetic neuroeffector junctions (Wahlestedt *et al.*, 1986; Wahlestedt, 1987). Thus, the full-sequence analogues PYY and hNPY are equipotent with NPY, while desamido hNPY was without measurable activity, confirming previous reports that the C-terminal amide is necessary for any activity of the peptide at either Y_1 or Y_2 receptors (Wahlestedt *et al.*, 1986). The 2–36 fragment of NPY was at least equipotent with NPY, although further reductions in fragment length caused a gradual decline in activity. There was a relatively sharp drop in activity between NPY13–36, which had roughly 50% of the activity of NPY, and NPY16–

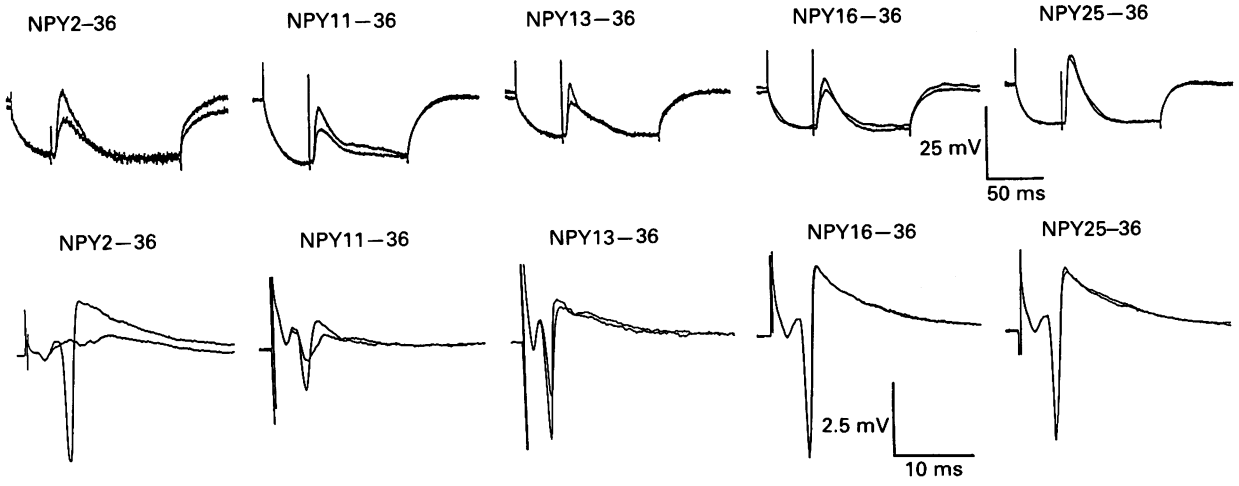

Figure 4 Representative effects of different C-terminal fragments of neuropeptide Y (NPY) on excitatory postsynaptic potential (e.p.s.p., upper traces) and population spike (PS, lower traces) applied at 1 μM in CA1. Fragment length is indicated above each trace. Records are all from different preparations. All responses showed recovery upon washout (not illustrated).

36, which had no measurable effect on the PS, and a small, but significant effect on the e.p.s.p. Further truncation to NPY18–36 eliminated all activity at the presynaptic receptor in hippocampus.

While NPY16–36 had a small but significant effect on e.p.s.ps, it did not affect PS in this study. Y_2 receptors in peripheral tissue have been shown to be sensitive to fragments as short as NPY23–36 (Grundemar & Håkanson, 1990); NPY18–36 inhibits calcium influx in dorsal root ganglion cells in culture (Colmers, Bleakman & Miller, unpublished). It therefore seems likely that, at the concentrations tested here, the actions of NPY16–36 were subthreshold for an effect on PS. It is therefore essential that both pre- and postsynaptic responses be examined in similar studies.

The Y_2 receptor has been shown in peripheral tissues and in homogenates of whole brain to inhibit the activity of adenylate cyclase (Wahlestedt, 1987; Westlind-Danielsson *et al.*, 1987). However, although the mechanism coupling the presynaptic NPY receptor in hippocampus to its effectors (probably calcium channels in presynaptic terminals; Colmers *et al.*, 1988) is not known, preliminary results indicate that the inhibition of adenylate cyclase is not responsible, as elevation of intracellular adenosine 3':5'-cyclic monophosphate levels with a membrane-soluble analogue did not affect the inhibition of synaptic transmission brought about by NPY (Klapstein *et al.*, 1990). The actual mechanism by which NPY exerts its presynaptic action is currently under investigation.

As the results of this and other studies indicate, some presynaptic nerve terminals in both the peripheral and central nervous systems bear NPY receptors which inhibit release of transmitter. Thus far, they all appear to be of the Y_2 subtype. The notable difference is that, in peripheral nerve terminals, the NPY receptor is an inhibitory autoreceptor, while in hippocampus, it is an inhibitory heteroreceptor. While the inhibition of synaptic transmission by action at a presynaptic terminal appears a common property of the Y_2 receptors studied until now, there seems to be a difference in the role they play in different neuronal systems.

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Differential effects of dopamine agonists upon stimulated limbic and striatal dopamine release: *in vivo* voltammetric data

[1]Jonathan A. Stamford, Zygmunt L. Kruk & Julian Millar

Department of Pharmacology (Physiology: JM), The London Hospital Medical College, Turner St, London E1 2AD

1 Fast cyclic voltammetry at carbon fibre microelectrodes was used in rats anaesthetized with chloral hydrate to monitor dopamine release in the caudate and nucleus accumbens evoked by electrical stimulation of the median forebrain bundle. Stimulation trains (50 Hz sinusoidal current, 100 ± 10 μA r.m.s., 2 s duration) were repeated every 5 min throughout the experiment.

2 The actions of the dopamine agonists quinpirole, pergolide, SKF 38393, bromocriptine, (+)-3-(3-hydroxyphenyl)-N-n-propylpiperidine ((+)-3PPP) and (−)-3PPP were compared in the two nuclei.

3 Bromocriptine (10 mg kg^{-1}, i.p.) did not affect release in either nucleus while SKF 38393 caused a fleeting decrease in limbic but not striatal dopamine release at a high dose (20 mg kg^{-1}, i.p.).

4 Quinpirole and pergolide (both 1 mg kg^{-1}, i.p.) decreased stimulated dopamine release in the nucleus accumbens while in the caudate the drugs each caused a transient, though not quite significant, elevation of stimulated dopamine release followed by decrease in release of the same magnitude as that seen in the nucleus accumbens.

5 The (−)-enantiomer of 3PPP (20 mg kg^{-1}, i.p.), a partial agonist at the dopamine autoreceptor, increased stimulated dopamine release in both nuclei although the action in the caudate was larger and more prolonged. (+)-3PPP (20 mg kg^{-1}, i.p.), a full agonist, decreased release in the nucleus accumbens. A small, transient and not significant increase in the caudate was followed by decreased release.

6 The results are interpreted as being evidence for differences in the dopamine autoreceptor in the two nuclei, possibly in the affinity state of the receptor in each nucleus.

Introduction

Since its first demonstration *in vitro* (Farnebo & Hamberger, 1971) and *in vivo* (Kehr *et al.*, 1972) various groups have confirmed that dopamine agonists can decrease dopamine release or synthesis by an action at the nerve terminal level. These effects are receptor-mediated since they can be blocked by known dopamine antagonists. Since the receptors also respond to the endogenous transmitter they are termed 'autoreceptors' and are a potent means by which dopamine function is self-regulated. The nerve terminal autoreceptor is of the D_2 subtype (Stoof & Kebabian, 1984).

In recent experiments *in vivo* we were however surprised to observe that apomorphine, a commonly used agonist, appeared not to act as such in the caudate nucleus (CPu). Using voltammetry to measure the dopamine released by stimulation of the median forebrain bundle (MFB), we found that apomorphine reduced release in the nucleus accumbens (Acb) but was without any similar action in CPu. Indeed, the net effect was a slight, though not significant increase in release (Stamford *et al.*, 1987).

The purpose of the present study was to establish whether this effect of apomorphine was idiosyncratic or extended to other dopamine agonists. A series of dopamine agonists was therefore examined of differing D_1/D_2 selectivity, pre- and postsynaptic specificity and degree of partial to full agonist activity.

Methods

Animals

All experiments were performed on male Sprague-Dawley rats (250–350 g body weight) anaesthetized with chloral hydrate (400 mg kg^{-1}, i.p.). Anaesthesia was maintained throughout at a depth sufficient to abolish corneal and hind limb withdrawal reflexes. Core temperature was monitored with a rectal probe and kept at 36.5 ± 0.5°C by means of a homeothermic blanket under the rat.

Rats were positioned in a stereotaxic frame according to the cranial orientation of Pellegrino *et al.* (1979). Small holes were made with a dental drill and rose-headed burr, over the Acb, CPu and MFB regions to allow implantation of the electrodes.

Carbon fibre microelectrodes (see below) were implanted, with a dual electrode carrier, into the Acb (AP: +3.3, L: +1.3, V: −6.5 mm: coordinates relative to bregma and the cortical surface) and CPu (AP: +1.8, L: +2.8, V: −4.5 mm). Histology revealed that electrodes implanted in Acb were located just medial to the anterior commissure and at the same dorsoventral level. Striatal electrodes were positioned centrally within the nucleus. The reference (Ag/AgCl) and auxiliary (stainless steel) were implanted at a convenient location in the neck muscle.

A small concentric bipolar stimulating electrode (Rhodes SNE 100) was located in the region of the MFB, as previously described (Ewing *et al.*, 1983). Initially, the electrode tip was inserted 6 mm below the cortical surface (AP: −2.2, L: +1.2 mm). Stimulation current was switched on and the electrode was lowered (1 mm min^{-1}) until dopamine release was detected at the carbon fibre working electrodes in the forebrain. Current was disconnected for a rest period of 10 min before starting the experiment.

Electrodes and electrochemistry

Glass-insulated carbon fibre microelectrodes (8 × 20 μm tip size) were prepared as previously described (Armstrong James & Millar, 1979). Electrodes were immersed in phosphate-buffered saline for 1 h before implantation *in vivo* to allow full equilibration of the surface. No electrochemical or chemical surface treatments of the electrodes were performed at any stage. Any electrode giving unstable charging current in buffer was discarded.

All electrochemical measurements were made by fast cyclic voltammetry (Millar *et al.*, 1985). The input voltage profile to

[1] Author for correspondence at present address: Department of Pharmacology, Faculty of Basic Medical Sciences, Queen Mary and Westfield College, Mile End Road, London E1 4NS.

the potentiostat consisted of 1.5 cycles of a 75 Hz triangular waveform (−1.0 to +1.0 V vs Ag/AgCl, 300 V s^{-1} scan rate) sweeping initially in the cathodic direction. With these scan parameters, dopamine oxidized at a peak potential of +600 mV vs Ag/AgCl. Pharmacological, anatomical and electrophysiological studies have shown that, following electrical stimulation of the MFB, dopamine is the sole compound detectable in the striatal and limbic forebrain at this potential (Millar *et al.*, 1985; Stamford *et al.*, 1988c). Current at +600 mV was monitored with sample-and-hold circuits for each working electrode. The outputs from the circuits were displayed on separate chart recorders and an oscilloscope. Voltammetric scans were applied alternately to the two carbon fibre working electrodes at a rate of 20 scans per electrode per second. At the end of each experiment the electrodes were calibrated in solutions of dopamine in phosphate-buffered saline.

Electrical stimulations

Electrical stimulations (50 Hz sinusoidal current, 100 ± 10 μA r.m.s., 2 s train duration) were applied to the MFB at intervals of 5 min throughout the experiment. The first 6 stimulations acted as a control period. Peak dopamine release on these trains was averaged and expressed as 100%. Drugs or vehicle were administered immediately after stimulation 6 and their effects monitored for between 1 and 3 h.

Data presentation and statistics

Drug-induced changes in stimulated dopamine release were expressed as percentage changes (positive or negative) relative to the vehicle-treated controls. In each case, the changes in dopamine release in the controls were subtracted from those in the test animals to give the net effect (Stamford *et al.*, 1988a). Statistical comparisons of drug- and vehicle-treated animals were made by Mann Whitney U test. Peak dopamine release in CPu and Acb were compared by a paired *t* test.

Chemicals

Na_2HPO_4, KH_2PO_4 and NaCl were obtained from BDH chemicals. Phosphate-buffered saline consisted of 0.15 M solutions of each phosphate salt in 0.85% w/v aqueous NaCl. The solutions were mixed in an approximately 4:1 ratio (Na:K salt) and then adjusted to pH 7.4 with smaller additions as necessary. Dopamine hydrochloride, tartaric acid and chloral hydrate were obtained from Sigma.

Drugs and dosing

The following drugs were used at the doses stated: Quinpirole (LY 171555) hydrochloride (1 mg kg^{-}); bromocriptine mesylate (10 mg kg^{-1}); 7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1H-3- benzazepine (SKF 38393) hydrochloride (20 mg kg^{-1}); pergolide mesylate (1 mg kg^{-1}); (+)- and (−)-enantiomers of 3-(3-hydroxyphenyl)-N-n-propylpiperidine (3PPP, 20 mg kg^{-1} each). The drugs were dissolved in 1% w/v tartaric acid and administered intraperitoneally in a dose volume of 0.33 ml per 100 g body weight. All drug doses refer to the base. Control animals received vehicle alone.

Results

Electrical stimulation of the MFB evoked dopamine release in both CPu and Acb. With the parameters used in the present study (50 Hz, 100 ± 10 μA, 2 s train), the stimulation gave a peak dopamine release in CPu of 3.11 ± 0.31 μM (mean ± s.e.mean, $n = 43$). This was significantly ($P < 0.05$, paired *t* test) greater than in Acb (2.48 ± 0.21 μM). Dopamine release could be evoked repetitively with stimulations applied every 5 min for 4 h or more. Dopamine release on successive stimulations were reproducible and allowed drug effects to be quantified.

Figure 1 shows the effect of pergolide (1 mg kg^{-1}, i.p.) upon stimulated dopamine release over a 3 h period. Pergolide decreased limbic dopamine release (filled circles) by 30–40% over the course of the experiment. In CPu however (squares), the initial response was very variable although the group trend was a sharp elevation of release (+50%) reaching significance ($P < 0.05$) at its maximum (20 min post-dosing). This response subsequently diminished and was superseded by a consistent inhibitory effect on dopamine release from about 80 min onward. The inhibition of dopamine release was of similar magnitude (50%) to that seen in Acb.

Bromocriptine (10 mg kg^{-1}, i.p.) had no effect on dopamine release over the 3 h test period (Figure 2). Stimulated dopamine release in both nuclei showed a high degree of variability with increasing time post dosing.

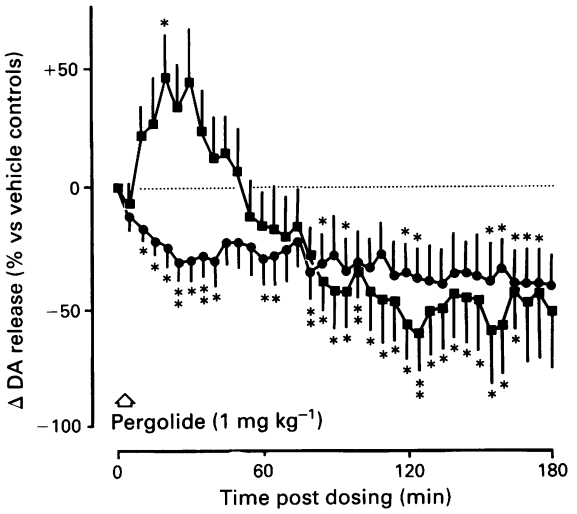

Figure 1 The effect of pergolide (1 mg kg^{-1}, i.p.) upon stimulated dopamine (DA) release in the caudate nucleus (■) and nucleus accumbens (●), expressed as a percentage change relative to the vehicle-treated controls (means are shown with s.e.mean indicated by vertical lines, $n = 7$). * $P < 0.05$; ** $P < 0.01$ vs vehicle controls (Mann Whitney U test).

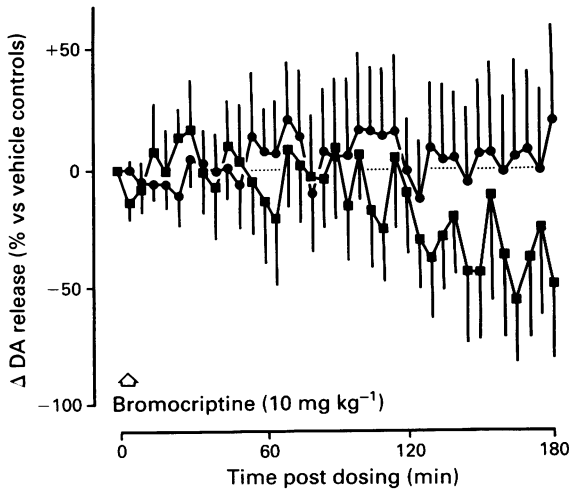

Figure 2 The effect of bromocriptine (10 mg kg^{-1}, i.p.) upon stimulated dopamine (DA) release in the caudate nucleus (■), and nucleus accumbens (●) expressed as a percentage change relative to the vehicle-treated controls (means with s.e.mean shown by vertical lines, $n = 5$).

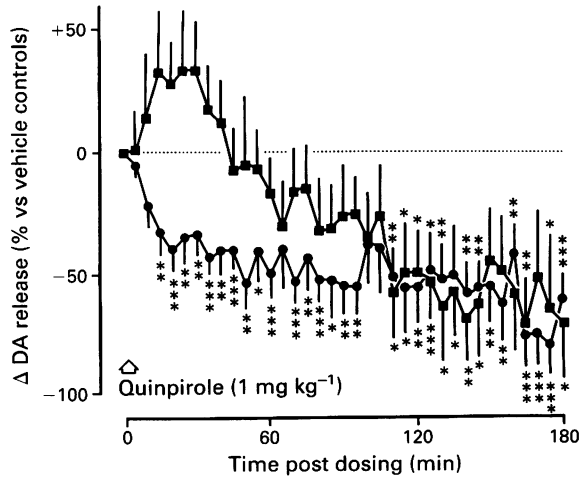

Figure 3 The effect of quinpirole (1 mg kg^{-1}, i.p.) upon stimulated dopamine (DA) release in the caudate nucleus (■) and nucleus accumbens (●) expressed as the percentage change relative to the vehicle-treated controls (means with s.e.mean shown by vertical lines, $n = 6$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs vehicle controls (Mann Whitney U test).

The action of quinpirole (1 mg kg^{-1}, i.p.) was similar to that of pergolide (Figure 3). In Acb, quinpirole caused a rapid (onset 15 min) and sustained decrease (50%) in stimulated dopamine release. However, in CPu there was generally an initial sharp rise (+30%) in dopamine release although a few animals showed a decrease. This heterogeneity was reflected in the group data such that the increase narrowly failed to reach significance. As with pergolide, this effect was followed by inhibition of dopamine release from about 100 min onwards.

SKF 38393 (20 mg kg^{-1}, i.p.) caused a fleeting reduction in stimulated limbic dopamine release (Figure 4). The effect was only observed for 20 min and was not matched by any action on dopamine release in CPu.

The effects of the enantiomers of 3PPP were studied over a period of 1 h. 3PPP is electroactive and could thus be detected *in vivo* with a broad oxidation current peaking at greater than +1000 mV vs Ag/AgCl. Nevertheless simultaneous detection of 3PPP did not affect the ability to detect changes in stimulated dopamine release above this elevated background signal. Following intraperitoneal administration, peak brain levels of

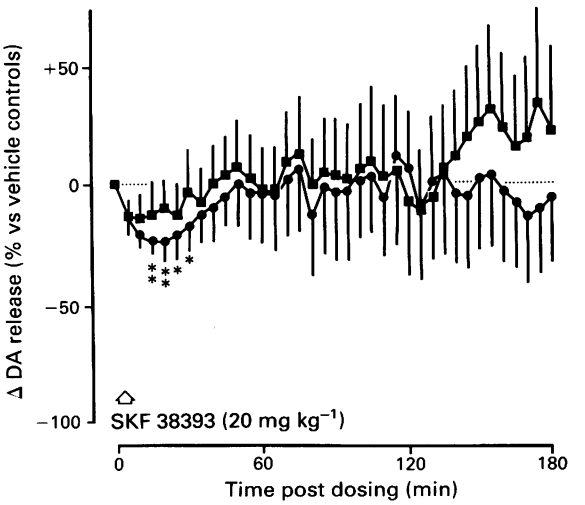

Figure 4 The effect of SKF 38393 (20 mg kg^{-1}, i.p.) upon stimulated dopamine (DA) release in the caudate nucleus (■) and nucleus accumbens (●) expressed as the percentage change relative to the vehicle-treated controls (means with s.e.mean shown by vertical lines, $n = 6$). * $P < 0.05$; ** $P < 0.01$ vs vehicle controls (Mann Whitney U test).

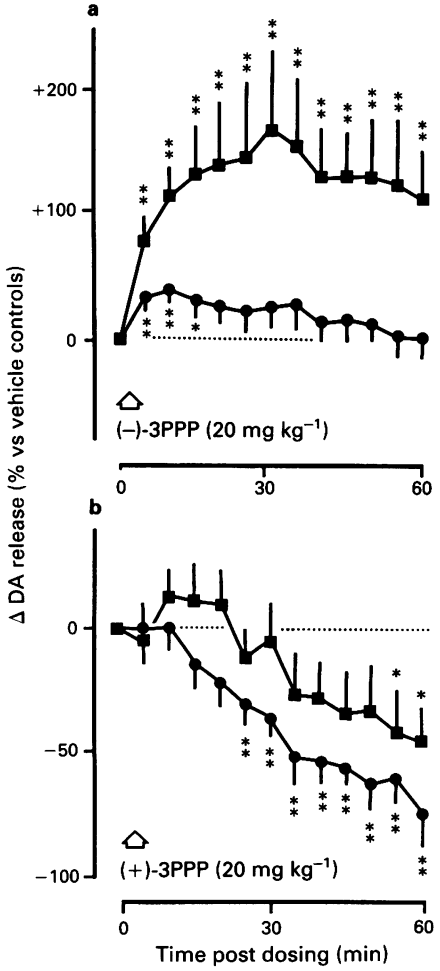

Figure 5 Effects of (−)-3PPP (a) and (+)-3PPP (b), both at 20 mg kg^{-1}, i.p., upon stimulated dopamine (DA) release in the caudate nucleus (■) and nucleus accumbens (●) expressed as the percentage change relative to the vehicle-treated controls (means with s.e.mean shown by vertical lines, $n = 5$). * $P < 0.05$; ** $P < 0.01$ vs vehicle controls (Mann Whitney U test).

3PPP occurred after about 20–30 min. The enantiomers of 3PPP had very different effects on dopamine release (Figure 5). (−)-3PPP elevated dopamine release (Figure 5a) in both CPu and Acb although the action in CPu was larger and of longer duration. (+)-3PPP decreased dopamine release in ACb (Figure 5b) while the action in CPu was qualitatively similar to pergolide and quinpirole: a small elevation (not significant) followed by a decrease in release.

Discussion

The experimental procedure used in the present study functionally isolates the nerve terminal from the cell body. Actions of drugs at the cell body autoreceptor alter the firing rate of the neurones. However, the present methodology prevents drug effects in the substantia nigra or ventral tegmental area from influencing those in the terminals since the protocol measures stimulated, not basal, dopamine release. Since the stimulating electrode is positioned in the MFB, impulse traffic to the terminals is controlled at this point rather than in the cell bodies. Drugs such as SCH 23390 that have no effect on the nerve terminal autoreceptor, but which increase cell firing (Mereu *et al.*, 1985) and hence dopamine metabolism (Saller & Salama, 1986) have no observable action on stimulated dopamine release in the present protocol (Stamford *et al.*, 1988a). Thus the methodology, in effect, measures the actions of drugs at the level of the nerve terminal. Although we cannot firmly

exclude actions upon local microcircuitry, preliminary experiments in our laboratory have failed, for example, to show any effect of cholinoceptor agonists or antagonists upon stimulated striatal dopamine release (Stamford unpublished) although this action is readily demonstrable *in vitro* (Lehmann & Langer, 1982) and might be expected to exert some local non-autoreceptor mediated control of dopamine release *in vivo*. Thus we cautiously propose that the present protocol measures solely effects at terminal dopamine autoreceptors.

The purpose of the study was to establish whether the previously seen effect of apomorphine (Stamford *et al.*, 1987) was observable with other agonists and which properties of the agonist might be responsible. Apomorphine is equipotent at D_1 and D_2 receptors (Andersen *et al.*, 1987; Seeman *et al.*, 1986). Thus, drugs were chosen which ranged from the selective D_1 agonist SKF 38393 (Setler *et al.*, 1978) to the D_2 agonist quinpirole (Stoof & Kebabian, 1984). Apomorphine also does not discriminate between pre- and postsynaptic dopamine receptors (Helmreich *et al.*, 1982) and thus drugs with mainly postsynaptic or presynaptic actions were used (bromocriptine and 3PPP respectively – Seeman, 1981; Watling, 1982). The doses chosen for each drug were selected to be in the middle to upper half of the dose-range described in the literature.

SKF 38393, the D_1 agonist (Setler *et al.*, 1978), had no effect on stimulated dopamine release in CPu over the 3 h post administration (Figure 4). In Acb the drug caused a transient decrease in dopamine release. The brevity of this effect and the absence of parallel action in CPu make it likely that the result is artefactual. SKF 38393 exerts D_1 receptor-mediated effects at 1.25–4.0 mg kg^{-1} (Andersen *et al.*, 1987; Braun & Chase, 1986) and has a D_1 type cooperative action on postsynaptic D_2 receptor-related behaviours at doses as low as 0.25 mg kg^{-1} (Sonsalla *et al.*, 1988). It is likely that the effect seen in the present study is a reflection of the high dose used. For instance, Imperato & Di Chiara (1988) showed that SKF 38393 applied locally at high concentration could decrease dopamine release in CPu in a manner unrelated to D_1 or D_2 receptor activation.

Bromocriptine (10 mg kg^{-1}, i.p.) had no effect on dopamine release in either nucleus (Figure 2). This is consistent with the reported postsynaptic selectivity of this drug (Jenner *et al.*, 1979). Bromocriptine has been shown to induce clear circling behaviour in rats at doses much lower than used here (Costall *et al.*, 1975). Bromocriptine is also found to be substantially less potent than apomorphine in several models of dopamine autoreceptor activity (Westfall *et al.*, 1983; Yarbrough *et al.*, 1984).

Pergolide (1 mg kg^{-1}, i.p.) and quinpirole (1 mg kg^{-1}, i.p.) evoked similar responses (Figures 1 and 3 respectively). In both cases the drugs caused a substantial reduction of stimulated dopamine release in Acb with a rapid onset of action (10–15 min). In CPu the drugs usually caused an initial elevation of stimulated dopamine release (with pergolide, significantly so). This was followed, after about an hour, by a reduction in release of similar magnitude to that in Acb. In both cases the actions of the drugs in each nucleus were qualitatively the same as those obtained for apomorphine (Stamford *et al.*, 1987).

Both pergolide and quinpirole have higher potency at D_2 than D_1 receptors (Andersen *et al.*, 1985). This and the absence of effect with SKF 38393 mean that the unusual caudate response of apomorphine is likely to be the result of D_2 receptor activity. This is supported by the fact that it is blocked by haloperidol (Stamford *et al.*, 1987). The lack of effect of bromocriptine also indicates that the response is mediated by presynaptic D_2 receptors.

Although the data suggest that the unusual effect seen in CPu is due to presynaptic D_2 receptor activation, the same conclusion applies to Acb where a classical agonist response was observed. The question of why the CPu response differs so markedly from the expected effect remains unanswered. This was therefore further investigated with the 3PPP enantiomers. (+)-3PPP is an agonist at both pre- and postsynaptic D_2 receptors (Clark *et al.*, 1985) while (−)-3PPP is a partial agonist at the autoreceptor whose effect is determined by the ongoing level of endogenous transmitter activity (Hjorth *et al.*, 1987). In the present study (+)-3PPP behaved as an autoreceptor agonist in CPu although the effect was slower in onset and less pronounced than in Acb (Figure 5). (−)-3PPP however showed strong autoreceptor 'antagonist' behaviour in CPu as previously reported (Arbilla & Langer, 1984). A very small potentiation of dopamine release was transiently observed in Acb.

The partial agonist action of (−)-3PPP provides a clue to possible interpretation of the data. The action of an autoreceptor agonist is determined, in part, by the existing 'tone' at the receptor (see Kenakin, 1987). In a circumstance where there is little endogenous transmitter activity, the effect of an agonist will be at least additive and thus inhibit transmitter release. It is reported, for example, that the autoreceptor activity of the dopamine agonists is most pronounced at low stimulation frequencies (Hoffmann *et al.*, 1986). However, at receptors that are maximally activated already, any agonist with less than full intrinsic activity will compete with the transmitter and behave functionally as an antagonist, as seen here with (−)-3PPP.

A qualitatively similar effect would be expected with different affinity states of the same receptor. A high affinity receptor would be maximally stimulated at a low transmitter level and thus agonists would compete with the transmitter and behave essentially as antagonists. Conversely, a low affinity receptor might not be fully activated and thus have room for further agonist stimulation.

The dopamine D_2 receptor can exist in two states: the high affinity state has a dissociation constant (K_d) for dopamine of 5 nM while the low affinity state has a K_d of 4 μM (Seeman *et al.*, 1986). These two levels are interconvertible and the state of the receptor expressed can be modified by, amongst others, guanine nucleotides (Mackenzie & Zigmond, 1984), temperature (Watanabe *et al.*, 1985), sodium and calcium ions (Hamblin *et al.*, 1984; Urwyler, 1987).

An explanation for the data of the present study (and previous report on apomorphine; Stamford *et al.*, 1987) can be made by postulating different autoreceptor states in CPu and Acb. In Acb, for instance, the agonists' action would be consistent with the autoreceptor existing in a low affinity state ($K_d = 4\,\mu$M). Thus peak dopamine release on stimulation (2.48 μM) would not cause maximal activation and agonists would be able to stimulate the receptor and thus inhibit release. Conversely, agonist action in CPu can be explained by a high affinity state. At this K_d (5 nM) the receptor would be saturated by the dopamine levels attained on stimulation (3.11 μM). Agonists with lower intrinsic activity would thus compete with the dopamine, and, to some extent, antagonize its effect.

Support for this hypothesis is obtained from the later effects of pergolide and quinpirole, where the CPu response shifts from functional 'antagonism' to the same effect as in Acb. It is established that prolonged activation can desensitize the autoreceptor (Arbilla *et al.*, 1985). One might thus expect persistent agonist exposure to cause high affinity sites to convert to the lower affinity state of the autoreceptor. Such interconversion has been shown to occur over a period of 15–30 min (Levesque & Di Paolo, 1988) and thus could explain the shift in response to pergolide and quinpirole seen over a similar time frame. No change occurred in Acb since the receptors were already (according to the hypothesis) in the low affinity state.

The hypothesis is further supported by two related studies from our laboratory. In the first we compared the diffusion and uptake of dopamine in CPu and Acb and observed a lower density of dopamine uptake sites in Acb (Stamford *et al.*, 1988b). The fewer uptake sites allow dopamine to exist longer in the extracellular space and thus may mean that autoreceptors are more persistently stimulated in Acb. This, in

turn, would be expected to lead to desensitization. In CPu, which has more uptake sites, the autoreceptors may be less exposed to dopamine and consequently exist in the high affinity state, as previously postulated (Seeman & Grigoriadis, 1987). The fact that stimulated dopamine release in the CPu is greater than in Acb may reflect other differences in the dopamine innervation of the two nuclei such as the relative size of the releasable and storage dopamine pools or a higher release rate in response to externally imposed impulse traffic.

In a second related set of experiments we compared the regional effects of dopamine antagonists (Stamford *et al.*, 1988a). Overall, the antagonists showed a greater potentiation of dopamine release in CPu than Acb, as would be predicted by the hypothesis of a different autoreceptor affinity state in the two nuclei. The proposed high affinity striatal receptor, being maximally activated by ongoing dopamine release, would be expected to show a greater response to subsequent blockade than the partially activated low affinity limbic autoreceptor.

In conclusion, although one cannot exclude the possibility that the drugs interact with other receptors or that differences in local circuitry underlie the drug effects, it is possible to say that the previously reported unusual CPu response to apomorphine is matched only by those drugs that are agonists at presynaptic D_2 receptors. Taking the various strands of evidence together, we interpret the results of the present study as indicating that dopamine agonists exert actions on stimulated striatal and limbic dopamine release that are the result of their own intrinsic activity at D_2 receptors and the affinity state of the autoreceptor.

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Actions of dipyridamole on endogenous and exogenous noradrenaline in the dog mesenteric vein

[1]Yajun Li, Guoliang Zhang, [2]Hikaru Suzuki & Hirosi Kuriyama

Department of Pharmacology, Faculty of Medicine, Kyushu University, Fukuoka 812, Japan

1 In the isolated mesenteric vein of the dog, dipyridamole inhibited both the excitatory junction potential (e.j.p.) and the slow depolarization evoked by perivascular nerve stimulation, to 60–70% of control, with no change in the postjunctional membrane potential. These inhibitory actions of dipyridamole were not modified by 8-phenyltheophylline or phentolamine, suggesting that the inhibition did not involve either the actions of endogenous adenosine or the prejunctional α-autoregulation mechanism.

2 Dipyridamole did not produce any detectable effects on either the facilitation process of the e.j.ps or the postjunctional membrane depolarization produced by exogenously applied noradrenaline (NA).

3 Dipyridamole reduced the outflow of both the NA and the 3,4-dihydroxyphenylglycol (DOPEG) evoked by perivascular nerve stimulation to below 10% of control, the effect being much greater than that of exogenously applied adenosine (to about 90% of the control).

4 Exogenously-added NA was degraded by incubation with a segment of the vein. Dipyridamole itself produced degradation of NA and accelerated the NA-induced degradation. By contrast, pyrogallol, but not pargyline or imipramine, prevented the NA-induced degradation.

5 It is suggested that dipyridamole degrades NA directly, and also indirectly through activation of catechol-*O*-methyl transferase, with no alteration of the activity of monoamine oxidase or of the uptake mechanisms of NA into nerve terminals.

Introduction

Dipyridamole decreases vascular resistance and increases both coronary blood flow and the oxygen tension in coronary sinus blood. Thus, this drug is used clinically in ischaemic heart disease (Berne *et al.*, 1983). The cellular mechanisms by which dipyridamole causes vasodilatation are thought to be mainly indirect, via inhibition of the uptake of adenosine into both blood cells and the vascular wall (Bunag *et al.*, 1964; Berne *et al.*, 1983). This agent also accelerates the release of prostacyclin (PGI_2) from the vascular wall (Moncada & Korbut, 1978), reduces the production of thromboxane A_2 (TXA_2), prevents thrombus formation, possibly by inhibiting adenosine 3′:5′-cyclic monophosphate (cyclic AMP) phosphodiesterase activity in platelets (Emmons *et al.*, 1965) and inhibits platelet aggregation (Gresele *et al.*, 1983). These actions are also important in mediating the dipyridamole-induced increase in blood flow.

Central regulation via vasomotor adrenergic nerves is one of the main components in the maintenance of vascular tone (Su, 1977). Adenosine inhibits release of transmitter substances from perivascular adrenergic nerves, as estimated from the measurement either of incorporated [^{3}H]-noradrenaline ([^{3}H]-NA) during perivascular nerve stimulation (Su, 1977) or of excitatory junction potentials (e.j.ps) recorded from vascular smooth muscle cells (Kuriyama & Makita, 1984). Therefore, it is to be expected that, in the presence of dipyridamole, an increased concentration of adenosine at the vessel wall might inhibit release of NA from adrenergic nerve endings.

We investigated the effects of dipyridamole on adrenergic transmission in the dog mesenteric vein using recordings of junction potentials from smooth muscle cells and by measuring outflows of NA. Adrenergic transmission in this vein has been investigated electrophysiologically: electrical stimulation of perivascular nerves elicits two types of membrane response (the e.j.p. and slow depolarization) in smooth muscle cells, and the reactions of these electrical responses to pharmacological agents have been well documented (Suzuki, 1984; Kou *et al.*, 1984; Komori *et al.*, 1989). Although the e.j.p. seems to be generated by substances other than NA (Burnstock & Kennedy, 1986), possible relationships between the electrical responses elicited by nerve stimulation and the outflow of NA have also been demonstrated in this vein (Seki & Suzuki, 1989). The outflow of a metabolite of NA, 3,4-dihydroxyphenylethylglycol (DOPEG) elicited by perivascular nerve stimulation was also measured to estimate the effects of dipyridamole on the uptake mechanisms of the released NA (Graefe & Henseling, 1983).

Methods

Male mongrel dogs, weighing 10–15 kg, were anaesthetized with sodium pentobarbitone (40 mg kg^{-1}, i.v.), and exsanguinated. The mesenteric vascular beds were isolated and kept in Krebs solution at room temperature.

For the electrophysiological experiments, the mesenteric vein (external diameter, 0.2–0.5 mm, 1–2 cm long) was dissected free, together with mesenteric membranes, mesenteric arteries, lympatic vessels and nerve bundles. To facilitate microelectrode penetration into venous smooth muscle cells, the vessels free from overlying fatty tissues were chosen. The isolated vessels were mounted in a recording organ bath made from lucite plate, with a capacity of about 2 ml. A silicon rubber plate (KE-66, Shin-Etsu Kagaku, Tokyo) was fixed at the bottom of the chamber, and the isolated vessel was firmly fixed to it with tiny pins. The tissue was superfused with warmed (35°C) Krebs solution at a constant flow rate of 2.5–3 ml min^{-1}.

Glass capillary microelectrodes were made from borosilicate glass tube (1.2 mm outer diameter with a fine filament inside, Hilgenberg, West Germany). The electrode was filled with 3 M KCl, and its resistance was 30–60 mΩ. The electrode was inserted into the venous smooth muscle from the adventitial side, through the overlying mesenteric membrane. Perivascular nerves were stimulated electrically by the point stimulation method of Suzuki (1984). Briefly, a silver wire coated with enamel was cut across and the square cut end was

[1] Author for correspondence.
[2] Present address: Department of Physiology, Nagoya City University Medical School, Mizuho-ku, Nagoya 467, Japan.

gently attached to the vessel. Brief electric currents (0.1–0.5 ms duration, 20–50 V intensity) were applied via this stimulating electrode, using an electric stimulator (SEN-7103, Nihon-Kohden, Tokyo). In preliminary experiments, the electrical responses of venous smooth muscle cells elicited by these stimuli were confirmed to be blocked all but completely by tetrodotoxin (3×10^{-7} M) (showing that the responses were generated via excitation of perivascular nerves). The recorded responses were displayed on a pen-recorder (Recticorder, Nihon-Kohden RJG-4014, Tokyo).

To enable measurement of catecholamine outflow, veins (6–7 cm long) were isolated from the mesenteric vascular bed and the surrounding tissue removed. The vessels were cleared of branches, opened by cutting longitudinally, and tied at both ends with fine threads. The spontaneous and evoked outflows of noradrenaline (NA) and 3,4-dihydroxyphenylethylglycol (DOPEG) from the isolated mesenteric vein were measured by methods described previously (Mishima *et al.*, 1984). Briefly, two Ag–AgCl wires (diameter, 0.5 mm; 10 cm long) were fixed parallel and vertically with a gap of about 1 mm, and the isolated mesenteric vein was fixed between these two wires using fine threads. Krebs solution (35°C) was dripped onto the tissue at a constant flow rate of 1 ml min^{-1}, so that the tissue was superfused evenly. Electric current pulses (1 ms duration and 100 V intensity) were applied for 1 min at 10 Hz frequency through these two silver wires. Preliminary experiments confirmed that in the presence of tetrodotoxin (3×10^{-7} M), the outflows of NA and DOPEG were not increased by these stimuli, indicating that the evoked outflows were entirely due to the excitation of perivascular nerves (Miyahara & Suzuki, 1985). The solutions were collected into a conical test tube (10 ml capacity) at the bottom of the tissue, for 5 min before and after application of nerve stimulation, since this period of time has been confirmed to be sufficient to collect nearly all catecholamines coming out into the perfusate during nerve stimulation (Mishima *et al.*, 1984).

The collected solutions were added to 50 μl of percholoric acid (60%), and assayed for their content of NA and DOPEG by the modified alumina adsorption method (Oishi *et al.*, 1983). The extracted samples were analysed by high performance liquid chromatography (h.p.l.c., Yanagimoto MFG, L-200L, Tokyo, Japan). After the experiments, the tissue was blotted with filter paper and weighed. The outflows of NA and DOPEG were expressed as ng g^{-1} wet weight of tissue. The ionic content of the Krebs solution was as follows (mM): Na^+ 137.4, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.5, HCO_3^- 15.5, $H_2PO_4^-$ 1.2, Cl^- 134 and glucose 11.5. The solution was bubbled with O_2 containing 3% CO_2, and its pH maintained at 7.3–7.4.

Drugs used were: dipyridamole (Boehringer Ingelheim Japan, Osaka, Japan), adenosine hemisulphate salt, noradrenaline hydrochloride, 8-phenyltheophylline, 1,2,3-trihydroxybenzene (pyrogallol), N-methyl-*N*-2-propynylbenzylamine (pargyline), and 10,11-dihydro-*N*,*N*-dimethyl-5H-dibenz-[b,f]azepine-5-propanamine (imipramine) (Sigma, St. Louis, MO, U.S.A.), phentolamine mesylate (CIBA Geigy, Basel, Switzerland) and tetrodotoxin (Sankyo, Tokyo, Japan).

Experimental values were expressed as mean ± standard deviation (s.d.), and statistical significance was tested by Student's *t* test. Probabilities of less than 5% ($P < 0.05$) were considered significant.

Results

Effects of dipyridamole on electrical responses evoked by nerve stimulation

The resting membrane potential of individual smooth muscle cells of the dog mesenteric vein was between −60 and −70 mV (Table 1). Electrical stimulation of perivascular nerves by the point stimulation method elicited an excitatory junction potential (e.j.p.) and a slow depolarization in these cells. Stimulation of perivascular nerves repetitively by a train of stimuli at 0.01–1 Hz frequency elicited e.j.ps with a facilitation of the slow depolarization. These electrical properties are similar to those reported previously (Suzuki, 1984).

Table 1 Membrane potentials of smooth muscle cells of the dog mesenteric vein in the presence of the drugs used in the experiments

Control (resting membrane potential)	−67.8 ± 2.8 mV (n = 36)
Dipyridamole 10^{-6} M	−68.5 ± 2.5 mV (n = 21)
8-Phenyltheophylline 10^{-5} M	−67.1 ± 2.6 mV (n = 15)
Adenosine 10^{-4} M	−68.6 ± 3.1 mV (n = 18)
Phentolamine 3×10^{-6} M	−68.0 ± 2.7 mV (n = 10)

Mean ± s.d. (n = number of observations).

Figure 1 shows the effects of dipyridamole on the e.j.ps and slow depolarization generated by a train of 10 stimuli at 0.33 Hz frequency. Application of dipyridamole (10^{-6} M) inhibited both the e.j.p. and the slow depolarization, with no alteration of the membrane potential (Table 1). The amplitude of the slow depolarization recovered to 80–90% of those generated before application of dipyridamole, after it had been washed out of the tissue for over 60 min (Figure 1a(iii)). Application of 8-phenyltheophylline (8-PT, 10^{-5} M), a blocker of the adenosine receptor (Griffith *et al.*, 1981), inhibited the amplitude both of the e.j.p. and of the slow depolarization to 60–80% of control (Figure 1b(ii)), with no significant change in the membrane potential (Table 1). In the presence of 8-PT, dipyridamole again inhibited both the e.j.p. and the slow depolarization (Figure 1b(iii)).

When the amplitudes of the e.j.p. and slow depolarization were expressed relative to those recorded before application of dipyridamole, the actions of dipyridamole on these potentials could be summarized as shown in Figure 2. Dipyridamole inhibited both the e.j.p. and the slow depolarization in concentrations over 10^{-7} M and, at 10^{-6} M these potentials were inhibited to 60–70% of control. These inhibitory actions of dipyridamole remained unchanged in the presence of 8-PT (10^{-5} M).

Adenosine inhibits adrenergic transmission by inhibiting the facilitation process of transmitter release, as estimated

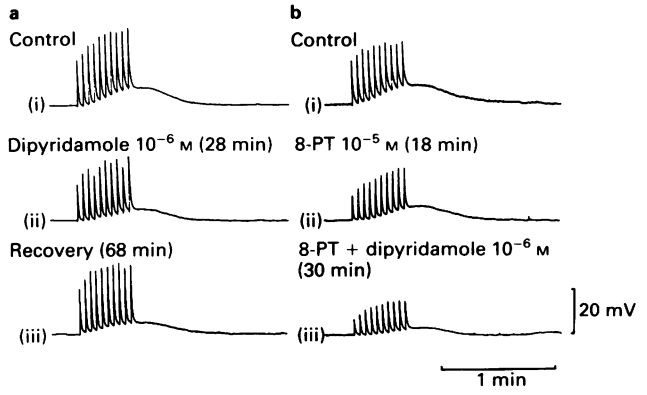

Figure 1 Electrical responses of smooth muscle cells elicited by nerve stimulation and their modulation by dipyridamole and 8-phenyltheophylline (8-PT). Dog mesenteric vein. (a) Perivascular nerve stimulation (0.03 ms duration, 25 V, 10 times at 3 s intervals) was applied before (i) control and during (ii) application of 10^{-6} M dipyridamole for 28 min; (iii) after washing out dipyridamole for 68 min. Membrane potential, −70 mV. (b) Electrical responses evoked before (i) control and after (ii) application of 8-PT (10^{-5} M) for 18 min. (iii) Responses elicited by nerve stimulation in the presence of 8-PT and dipyridamole 10^{-6} M for 30 min. Membrane potential, −66 mV. (a) and (b) were recorded from different single cells.

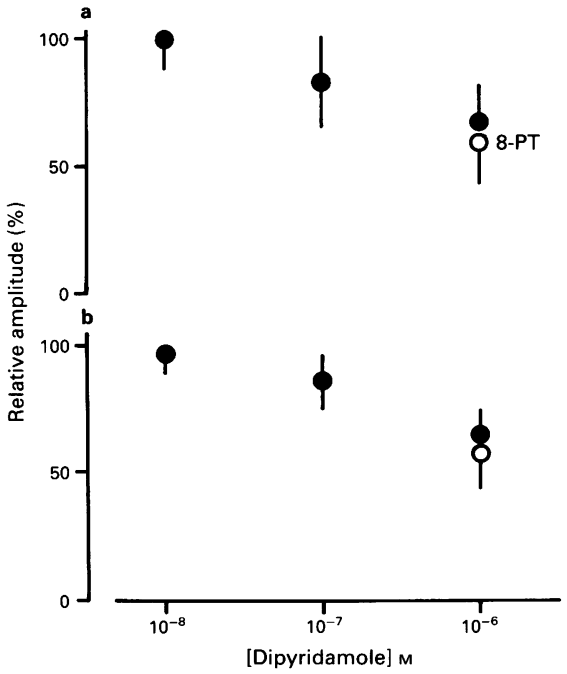

Figure 2 Effects of dipyridamole on (a) the amplitude of e.j.p. and (b) slow depolarization in the dog mesenteric vein. Perivascular nerves were stimulated 10 times at 0.33 Hz frequency, and the amplitude of the first e.j.p. of a train and the maximum amplitude of the slow depolarization during the stimulation period were expressed relative to those before application of dipyridamole (●); (○) responses observed in the presence of 8-phenyltheophylline (10^{-5} M). Mean of $n = 5$–12; s.d. shown by vertical lines.

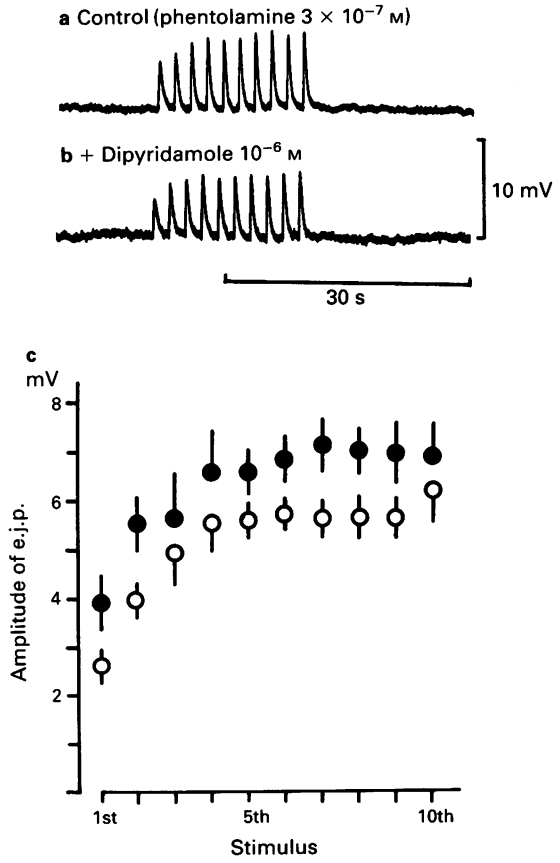

Figure 3 Effects of dipyridamole on the facilitation process of e.j.ps elicited by nerve stimulation in the dog mesenteric vein. Nerve stimulation (0.05 ms duration, 30 V intensity, 0.5 Hz frequency) was applied in the presence of phentolamine 3 × 10^{-7} M (a, control) and phentolamine plus dipyridamole 10^{-6} M (b). The mean amplitude of the e.j.ps evoked by each stimulus is summarized in (c); $n = 5$ for control (●) and 7 for dipyridamole 10^{-7} M (○); s.d. shown by vertical lines.

from the e.j.ps evoked by repetitive stimulation of perivascular nerves in the rabbit mesenteric artery (Kuriyama & Makita, 1984). Therefore, attempts were made to observe the effects of dipyridamole on the facilitation of repetitively evoked e.j.ps in the dog mesenteric vein. Experiments were carried out in the presence of phentolamine (3 × 10^{-7} M) to inhibit both the slow depolarization (Suzuki, 1984) and also the prejunctional α-autoregulation mechanisms which are reported to be involved in the facilitation of the e.j.ps (Starke, 1987). In the presence of phentolamine, therefore, stimulation of perivascular nerves evoked the e.j.ps which were facilitated, but there was now no associated slow depolarization (Figure 3a). Application of dipyridamole in the presence of phentolamine again inhibited the e.j.ps to an extent similar to that seen in the absence of phentolamine (i.e. to 60–70% of control, $n = 5$), with no alteration of the facilitation process of the e.j.p. (Figure 3b).

Effects of dipyridamole on membrane depolarization produced by noradrenaline

In the dog mesenteric vein, NA (10^{-8}–10^{-5} M) depolarizes the smooth muscle membrane in a concentration-dependent manner (Suzuki, 1984). Experiments were carried out to observe the effects of dipyridamole on the membrane depolarization produced by exogenously applied NA.

As shown in Figure 4, application of NA (5 × 10^{-7} M) produced a sustained depolarization by 15–18 mV from the resting potential, for up to 10 min. This NA-induced depolarization was unchanged by the addition of dipyridamole (10^{-6} M). By contrast, in similar experiments, application of adenosine (10^{-4} M) in the presence of NA inhibited the NA-induced depolarization by 2–5 mV (mean, 3.5 ± 1.5 mV, $n = 4$; Figure 4b), although in the absence of NA, this concentration of adenosine only marginally changed the membrane potential of the venous smooth muscles (Table 1).

Modulation of release of noradrenaline by dipyridamole

Even in the absence of nerve stimulation, there were detectable amounts of NA and DOPEG coming out into the perfusate (Table 2, shown as 'spontaneous outflow'). These spontaneous outflows of NA and DOPEG were both significantly decreased by phentolamine (10^{-7} M), while 8-PT (10^{-5} M) inhibited the spontaneous outflow of NA but had no

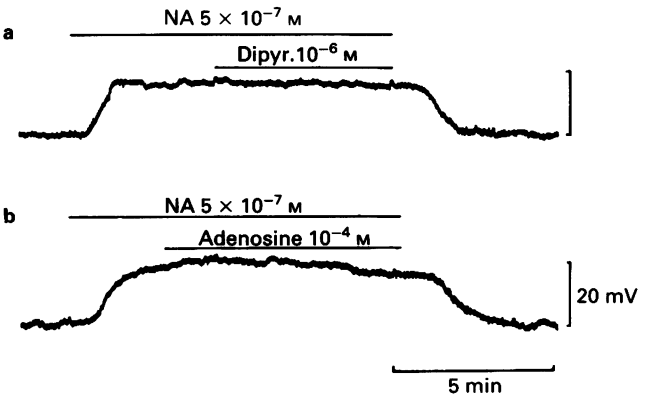

Figure 4 Effects of dipyridamole (a) and adenosine (b) on noradrenaline (NA)-induced depolarization in smooth muscle cells of the dog mesenteric vein. NA 5 × 10^{-7} M; dipyridamole (Dipym) 10^{-6} M; adenosine 10^{-4} M. (a) and (b) were recorded from different tissues. Membrane potential: (a) −69 mV; (b) −66 mV.

Table 2 Effects of phentolamine and 8-phenyltheophylline (8-PT) on spontaneous (A) and evoked (B) outflows of noradrenaline (NA) and 3,4-dihydroxyphenylethylglycol (DOPEG) from the dog mesenteric vein

	NA	*DOPEG*
(A) *Spontaneous outflow*		
Control	10.2 ± 1.4 (n = 18)	10.2 ± 1.1 (n = 18)
Phentolamine 10^{-7} M	8.1 ± 1.7 (n = 6)*	8.3 ± 1.1 (n = 6)*
8-PT 10^{-5} M	8.1 ± 1.2 (n = 8)*	10.4 ± 1.2 (n = 8)
Dipyridamole 10^{-6} M	0 (n = 16)*	0 (n = 16)*
(B) *Nerve stimulation* (S_1)		
Control	38.6 ± 17.7 (n = 18)	17.6 ± 6.5 (n = 18)
Phentolamine 10^{-7} M	28.9 ± 13.0 (n = 6)*	13.3 ± 4.7 (n = 6)*
8-PT 10^{-5} M	38.2 ± 13.9 (n = 8)	22.0 ± 5.2 (n = 8)

In (B) outflows evoked by the first nerve stimulation (S_1) are shown. Mean ± s.d. (ng g^{-1} wet weight of tissue). n = number of observations.
* Significant difference from control ($P < 0.05$). 0 in the table means below detectable level.

effect on the outflows of DOPEG. Dipyridamole (10^{-6} M) reduced the spontaneous outflow of both catecholamines to below the level of detection.

Application of perivascular nerve stimulation increased the outflow of both NA and DOPEG by 2–4 times the spontaneous levels (Table 2). The evoked outflows of NA and DOPEG were decreased successively by 5–10% when the nerve stimulation was applied repetitively at 30 min intervals, and therefore the amounts of NA and DOPEG coming out into the perfusates during each stimulation period were expressed relative to those of the first stimulation period (S_1). In the control condition, application of nerve stimulation 6 times at 30 min intervals resulted in outflows of NA and DOPEG at the 6th stimulation period which were 60% or 70% of those at S_1, respectively. When dipyridamole (10^{-6} M) was applied at the 3rd and 4th stimulation periods, outflows of NA and DOPEG were decreased to below 10% of control. The outflows were restored to the control level within 30 min by removal of dipyridamole from the perfusate (Figure 5a and b).

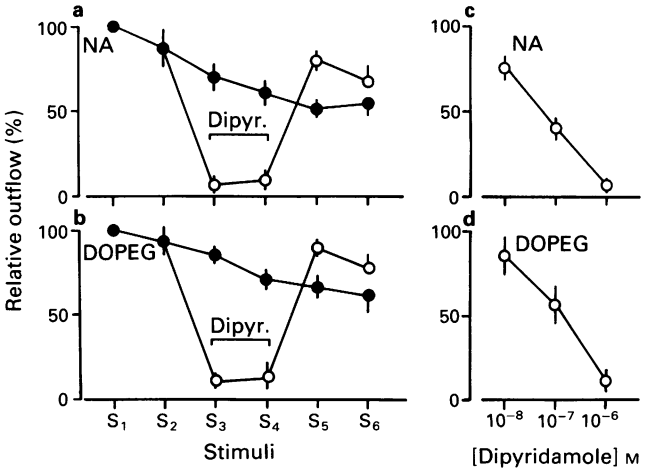

Figure 5 Effects of dipyridamole on the outflows of noradrenaline (NA) (a) and 3,4-dihydroxyphenylethylglycol (DOPEG) (b) evoked by nerve stimulation in the dog mesenteric vein. The nerve stimulation (600 stimuli at 10 Hz frequency) was applied 6 times (S_1–S_6) at 30 min intervals. Dipyridamole (Dipyr, 10^{-6} M) was applied for S_3 and S_4 periods. Outflows of NA and DOPEG are expressed relative to those evoked by S_1; actual values of the outflows of NA and DOPEG are given in Table 2B. (●) Control outflows in the absence of dipyridamole; (○) outflow during, and following application of dipyridamole. The concentration-response relationships of the actions of dipyridamole on the outflows of NA and DOPEG are shown in (c) and (d), respectively. Mean ± s.d. (n = 4–6).

The concentration-response relationship of the effects of dipyridamole on the evoked outflows of NA and DOPEG is shown in Figure 5c and d, in which the inhibitory actions of dipyridamole can be detected in concentrations over 10^{-8} M.

Application of adenosine (10^{-5} M) at the 3rd and 4th stimulation periods inhibited the evoked outflows of NA and DOPEG to 18.2 ± 3.4 ng g^{-1} (n = 6) and 18.3 ± 2.0 ng g^{-1} wet weight of tissue (n = 6), the values being about 88% and 89% of control, respectively ($P < 0.05$).

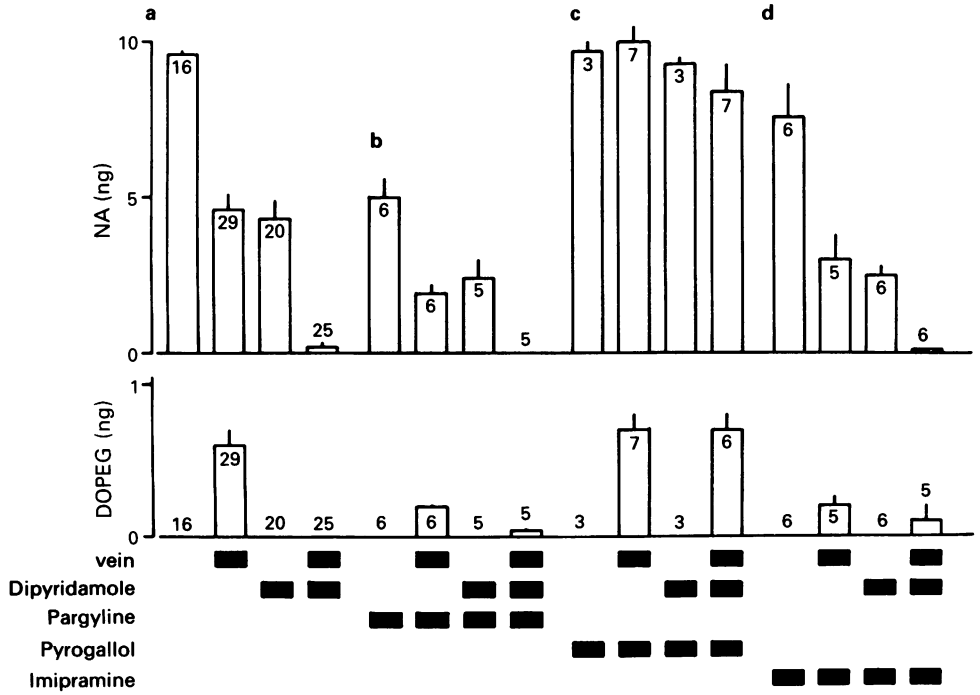

Figure 6 Digestion of exogenous noradrenaline (NA) by segments of mesenteric vein and dipyridamole in the absence (a, control) and presence of: pargyline 10^{-6} (b), pyrogallol 10^{-6} M (c) and imipramine 10^{-6} M (d). NA, 10 ng. Vein, 20.1 ± 1.6 mg, wet weight (n = 77). After incubation in Krebs solution (37°C) for 10 min, the content of NA (upper) and 3,4-dihydroxyphenylethylglycol (DOPEG, lower) in the solution was measured. Mean with s.d. indicated by vertical lines is shown. Number of experiments is shown in each column by small numbers. Experimental conditions are indicated at the bottom of each column: filled squares indicate the presence of vein and/or drugs in the NA-containing solution.

Degradation of noradrenaline

Attempts were made to observe the effects of dipyridamole on the degradation of exogenously applied NA. Experiments were carried out to measure the NA and DOPEG content of solutions in which 10 ng NA had been incubated with venous tissues, dipyridamole, pargyline (inhibitor of monoamine oxidase), pyrogallol (inhibitor of catechol-*O*-methyltransferase) or imipramine (inhibitor of the uptake of NA into nerves). After incubation of NA with the tissue and with various combinations of these drugs (in 5 ml Krebs solution at 37°C for 10 min) the amounts of NA and DOPEG in the solutions were measured by the alumina adsorption method.

In Krebs solution, over 90% of the exogenously-applied NA remained unmetabolized, and no detectable amount of DOPEG was found in the solution. Incubation of a segment of the dog mesenteric vein (wet weight, 19.6 ± 1.6 mg, $n = 29$) with NA decreased the content of NA to about half and produced DOPEG in the solution. Dipyridamole also decreased the added NA to about half, but with no production of DOPEG. Incubation of NA with venous tissues and dipyridamole together degraded over 97% of the NA in the solution, with no associated production of DOPEG (Figure 6a). Pargyline accelerated the degradation of NA in the absence of venous tissues. Degradation of NA by the venous tissue or dipyridamole was not altered by pargyline (Figure 6b).

Degradation of the added NA was prevented almost completely by pyrogallol and partially (to about 75%) by imipramine. In the presence of pyrogallol, the venous tissue slightly increased NA with an associated production of DOPEG, while the degradative actions of dipyridamole on NA were inhibited. By contrast, imipramine did not prevent the degradation of NA by the venous tissue or by dipyridamole (Figure 6c and d).

Discussion

The present experiments demonstrate that in the dog mesenteric vein, dipyridamole inhibits the amplitude of junction potentials (the e.j.p. and slow depolarization) and the outflows of NA and DOPEG, i.e., this drug inhibits the evoked release of transmitter substances from perivascular adrenergic nerves. Although multiple actions of dipyridamole on the cardiovascular system have been reported, the main actions of this drug on coronary vasodilatation are considered to be inhibition of the uptake of adenosine into blood cells and vascular walls, thus causing an increase in the concentration of endogenous adenosine in the serum (Bunag *et al.*, 1964). Adenosine is a potent vasodilator, and its cellular actions are mainly due to an increased production of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) through activation of adenylate cyclase (Haslam & Lynham, 1972; Berne *et al.*, 1983). Adenosine also inhibits the release of NA from perivascular adrenergic nerves, possibly by activating the autoinhibition systems (Hedqvist & Fredholm, 1976; Enero & Saidman, 1977; Katsuragi & Su, 1982; Su, 1985). Inhibition by adenosine of the e.j.p. has also been reported in some arteries (Kuriyama & Makita, 1984; Zhang *et al.*, 1989). Thus, the most reasonable interpretation of the inhibition by dipyridamole of adrenergic transmission in the dog mesenteric vein might seem to be an involvement of endogenous adenosine, a hydrolyzed substrate of released ATP.

However, there are some problems in accepting this idea, and the following findings did not accord with the adenosine hypothesis: (1) The inhibitory actions of dipyridamole on the electrical responses elicited by nerve stimulation and on the outflows of NA and DOPEG are resistant to 8-PT which antagonizes the actions of adenosine (Griffith *et al.*, 1981). (2) Adenosine inhibits the e.j.p. by inhibiting the facilitation process (Kuriyama & Makita, 1984); however, inhibition by dipyridamole of the e.j.p. does not alter the facilitation of the e.j.ps. (3) The NA-induced depolarization of the smooth muscle membrane is inhibited by adenosine but not by dipyridamole. (4) The inhibitory actions of dipyridamole on the outflows of NA and DOPEG are more potent than those of adenosine. Differences in the degree of inhibition by dipyridamole of the electrical responses and of the outflows of NA and DOPEG also do not favour an involvement of endogenous adenosine in the actions of dipyridamole.

The finding that accelerated degradation of exogenously-added NA by dipyridamole was prevented by pyrogallol, but not by pargyline, suggests that pyrogallol has actions which stimulate catechol-*O*-methyl transferase (COMT), with no alteration of the action of monoamine oxidase (MAO), because pyrogallol and pargyline are potent inhibitors of COMT and MAO, respectively (Weiner, 1985). The uptake of NA into perivascular adrenergic nerves may not be accelerated by dipyridamole, because imipramine, an inhibitor of the uptake of NA (Weiner, 1985), did not alter the actions of dipyridamole. DOPEG is an important indicator enabling estimation of the amount of NA taken up into nerves (Graefe & Henseling, 1983), and absence of DOPEG outflow in the presence of dipyridamole also suggests that dipyridamole does not activate the uptake mechanism for NA. Dipyridamole also acted directly to degrade exogenously-added NA and the potency was comparable to that of a segment of the dog isolated mesenteric vein. Therefore, such direct and indirect degradatory actions of dipyridamole on released NA would be expected to cause differences in the degree of inhibition of the junction potentials and of the outflows of NA. In the present experiments, we did not investigate the action of dipyridamole on ATP, a co-transmitter of NA. Further experiments would be required to understand the action of dipyridamole on the neuromuscular transmission process in the mesenteric vein including the role of ATP.

The present experiments also demonstrated that pargyline and imipramine act potently to degrade directly exogenously-added NA. Pyrogallol did not seem to inhibit uptake of NA into nerves, and, as a consequence, the production of DOPEG by the tissue segment would not be changed. This drug also inhibited the degradatory actions of dipyridamole on NA and DOPEG. Thus, each of these drugs was found to have actions on the metabolism of catecholamines, in addition to their specific actions on individual enzymes. Therefore, the multiple actions of dipyridamole have to be accounted for by the interpretation of the underlying mechanism of actions of dipyridamole found in visceral smooth muscle tissues (Maguire & Satchell, 1981).

It is concluded that in the dog mesenteric vein, dipyridamole directly accelerates the degradation of NA released from perivascular adrenergic nerves and probably indirectly accelerates it by activating COMT in the tissue, with no causal relation to endogenous adenosine. Such actions of dipyridamole would facilitate a decrease in concentration of released NA at the vessel wall, thus reducing or preventing the NA-induced vasoconstrictions.

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Ion transport in cultured epithelia from human sweat glands: comparison of normal and cystic fibrosis tissues

D.J. Brayden, R.J. Pickles & [1]A.W. Cuthbert

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

1 Cultured epithelia derived from whole human sweat glands, isolated secretory coils, isolated reabsorptive ducts and whole glands from cystic fibrosis (CF) subjects have been used to examine drug sensitivity by use of short circuit current recording.

2 Short circuit current increases were observed with lysylbradykinin, carbachol and histamine in epithelia of different origins. All responses were due to stimulation of electrogenic sodium absorption, evidenced by the inhibition of these responses by amiloride. The latter also abolished the basal current. The terpenes, thapsigargin and forskolin had no effect on transport.

3 The stimulation of a sodium current by agonists was dependent upon calcium, responses being inhibited by lanthanum ions and EGTA. Further A23187 induced a sodium current.

4 Pronounced oscillations in the sodium currents were a feature of the responses, implying synchronous, regulated cell activity.

5 Forskolin produced a ten fold increase in adenylate cyclase activity. All agonists listed in 2 except forskolin caused an increase in intracellular calcium $[Ca]_i$, $[Ca]_i$ responses in CF cells were not different from those of normal cells, except with thapsigargin where the responses were smaller.

6 It is concluded that in culture, cells develop ductal characteristics, whether derived from normal or CF glands, coils or ducts. An increase in $[Ca]_i$ followed by activation of calcium-sensitive potassium channels and apical membrane hyperpolarization may be the major mechanism for increasing sodium influx.

Introduction

The geometry of the human sweat gland makes investigation of transepithelial ion transport difficult. However, human sweat glands can be grown in primary culture, then disaggregated and plated upon pervious supports to form small epithelial sheets. These sheets can be short circuited and the nature of the transported species obtained from ion substitution experiments and use of blocking drugs. Recently data have been obtained with whole human sweat glands (Brayden *et al.*, 1988) and human sweat gland ducts (Pedersen *et al.*, 1987). In a few instances (Brayden *et al.*, 1988) experiments were done with cultures derived from separated sweat gland coils. These coil-derived epithelia demonstrated electrogenic sodium absorption under short circuit current (SCC) conditions *in vitro*, rather than chloride secretion. Indeed they behaved similarly to cultures from whole glands. Furthermore, cultures with duct-like characteristics responded to cholinoceptor agonists and isoprenaline, although it is not generally considered that salt reabsorption in the duct is under autonomic control. Cultures derived from whole cystic fibrosis (CF) glands also demonstrated electrogenic sodium absorption, but they showed a reduced sensitivity to amiloride compared to controls (Cuthbert *et al.*, 1990). This effect is considered to result from the reduced apical chloride conductance, characteristic of the disease (Welsh & Leidtke, 1986; Frizzell *et al.*, 1986).

This present study explores further features of cultured sweat gland epithelia. The spectrum of agents that promote sodium transport is expanded and additionally the effects of agents on cultures derived from separated coils and ducts is given. Particular attention has been paid to the role of the intracellular messengers $[Ca]_i$ and adenosine 3':5'-cyclic monophosphate (cyclic AMP) in the stimulation of sodium transport. Recently, the gene responsible for CF has been cloned (Rommens *et al.*, 1989). The normal gene apparently codes for a membrane protein of 1480 amino acids with two nucleotide binding folds called CFTR (cystic fibrosis transmembrane regulator). In CF a phenylalanine at position 508 is missing. The protein has a close similarity to mdr 1 (p-glycoprotein), the membrane protein responsible for multiple drug resistance although the precise function of CFTR is unknown. It is apparent that more than one cellular function is perturbed in CF. An understanding of the pharmacological responsiveness of CF epithelia may provide clues for possible therapeutic strategies for the disease. In this connection the improved lung function in CF patients receiving nebulised amiloride (Knowles *et al.*, 1990) is of interest.

[1] Author for correspondence.

Methods

Isolation and culture of sweat glands

Non-cauterized skin samples were obtained at surgery from normal and CF patients, and occasionally from punch biopsies. Permission was obtained from Huntingdon Health Authority Ethics Committe. The skin was placed in 10 ml of sterile buffer and sweat glands isolated by the procedure described by Lee *et al.* (1984).

Primary cultures of whole human sweat glands were grown as described previously (Brayden *et al.*, 1988). In some instances primary cultures were grown from secretory coil or from reabsorptive duct. Glands were microdissected as described by Lee *et al.* (1986), briefly by collagenase digestion and recovery in a medium containing bovine serum albumin after which the glands were gently uncoiled. The coil was distinguished on the basis of appearance and a wider diameter and both coil and duct were separated at a point distal from the transitional junction. The transitional junction region was placed in dilute neutral red buffer to check that a diffuse red area (i.e. coil) merged with a thin dark red line (duct) thus validating the integrity of the separated coil.

After two to three weeks the primary cultures were dispersed with trypsin-versene (as described by Brayden *et al.*, 1988) and used to seed small wells closed by Millipore filters ($0.2\,cm^2$) coated in Matrigel, using 0.2 to 0.5×10^6 cells per

well (Cuthbert et al., 1987). Cultured epithelia were ready for use in five to eight days. Measurements of transepithelial potential, transepithelial resistance and short circuit current (SCC) were made as described previously (Cuthbert et al., 1987). Briefly, a WP dual voltage clamp with a facility for fluid resistance compensation was used. After a period for stabilization (circa 20 min) the transepithelial potential was noted after which the monolayers were short-circuited throughout the rest of the experiment. Intermittently the voltage was clamped at ± 1 mV and the current required to do this used to calculate the resistance.

Solutions

The bathing solution used for the electrophysiological work was Krebs-Henseleit which had pH 7.5 at 37°C when bubbled with 95% O_2 and 5% CO_2. The composition of this solution was (mM): NaCl 117, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 11.1. This solution was modified in some experiments. When barium ions were used $MgSO_4$ was replaced with $MgCl_2$ and bicarbonate was replaced with HEPES-Tris (10 mM) and NaCl increased to 142 mM. The same was true when lanthanum was used and additionally KH_2PO_4 was omitted and KCl increased to 5.7 mM. Both of these modified solutions were gassed with O_2. To remove ionized calcium from the bathing solution the appropriate amount of EGTA solution, 36 mM was added which had been previously neutralized to pH 7.4 with NaOH. This solution did not alter the tonicity of the medium or its sodium concentration; the final EGTA concentration was 2.5 mM. Readdition of $CaCl_2$ solution was able to restore the original ionized calcium concentration in the presence of the EGTA. To make low chloride Krebs solution (5.0 mEq l^{-1}) NaCl and KCl were replaced with sodium gluconate and potassium gluconate respectively.

Measurement of $[Ca]_i$

Intracellular calcium ion concentrations $[Ca]_i$ were measured by Fura 2 fluorescence (Grynkiewicz et al., 1985). After 2–3 weeks in culture, cells were dispersed in trypsin-versene as above, separated by gentle centrifugation and resuspended in the following medium (mM): NaCl 137, KCl 5.4, $CaCl_2$ 1.0, KH_2PO_4 0.4, $MgSO_4$ 0.3, glucose 1.1, HEPES 10, bovine serum albumen 1 mg ml^{-1} and Fura 2-AM, 2 μM at pH 7.4. The cells were incubated, with gentle shaking, at room temperature for 45 min. Aliquots (2 ml) of the suspension, containing 2–3 $\times 10^6$ cells, were rapidly centrifuged and washed in buffer without Fura 2-AM, and the cells transferred to a cuvette maintained at 37°C and stirred. Fluorescence was measured in a Perkin-Elmer LS5B luminescence spectrometer at 510 nm with excitation at 340 nm and 380 nm. After subtraction of the autofluorescence the ratio of the intensities at 340 nm and 380 nm were computed every 5 s. $[Ca]_i$ was computed from the formula

$$[Ca]_i = K_d \times \frac{R - R_{min}}{R_{max} - R} \times S$$

where $K_d = 224$ nM, R is the ratio of intensity at 340 nm and 380 nm. R_{max} is the same ratio in the presence of excess calcium (12 mM) and R_{min} the ratio in the absence of calcium (10 mM EGTA). S is the ratio of the fluorescence at 380 nm in calcium-free and excess calcium conditions. When $[Ca]_i$ was measured continuously without drug addition there was a slow increase in basal level. This was due to Fura-2 leakage from the cells since after centrifugation the supernatant gave a fluorescence insensitive to digitonin but abolished by Mn. Consequently increases caused by agonists were calculated from the immediately preceding basal value.

Adenylate cyclase assay

Whole sweat glands were cultured as given above and scraped from the plastic surface after 2–3 weeks. To measure adenylate cyclase activity, sweat gland cells were homogenized in a buffer containing (mM): Tris-HCl 25, $MgCl_2$ 5 and creatine phosphate 20. Aliquots of this suspension were added to the reaction mixture containing (mM): Tris HCl 25, $MgCl_2$ 5, creatine phosphate 20, cyclic AMP 1, GTP 0.5, ATP 1, isobutylmethylxanthine (IBMX) 0.2, creatine phosphokinase 100 μg ml^{-1} and α^{32}P-ATP (5×10^6 c.p.m. per assay). The reaction was started by adding the homogenate and stopped by adding sodium dodecylsulphate. The reaction was carried out at 30°C and pH 7.5 for 15 min. To separate cyclic AMP from ATP the reaction mixtures were run over Dowex 50 columns onto alumina columns and the cyclic AMP eluted with imidazole buffer (0.1 M). Appropriate blanks (no reaction mixture or no homogenate) and controls (e.g. linearity between volume of homogenate and activity) were carried out. Protein concentrations of the homogenates were measured by the method of Lowry et al. (1951). Results were expressed as pmol cyclic AMP mg^{-1} protein h^{-1}.

Student's t test (unpaired) was used to test for significance, with $P < 0.05$ considered significant.

Drugs and chemicals

Forskolin, Fura 2-AM and ionomycin were obtained from Calbiochem, benzimidazole guanidine from Pfaltz & Bauer Inc., cimetidine from Aldrich Chemical Co Ltd. and the following drugs from Sigma Chemical Co. Ltd.: A23187, piroxicam, atropine, carbachol, dibutyryl cyclic AMP, dibutyryl cyclic GMP, histamine, mepyramine, isoprenaline, lysylbradykinin, neomycin sulphate. Peptides were from Peninsula Laboratories Inc. All other chemicals were of reagent grade. Thapsigargin was a gift from O. Thastrup.

In general, drugs were dissolved in distilled water at concentrations such that only minute volumes were added to preparations. An exception was neomycin which was dissolved in KHS because of the large volume of solution required to achieve the desired concentration.

Results

Spectrum of agonist sensitivity in normal and CF epithelia

Epithelial cultures derived from whole glands, subcultured for 5–8 days on Matrigel-coated filters had the following basal characteristics. For normal tissues prepared from skins of 12 subjects values were SCC, 16.8 ± 1.8 μA cm^{-2} ($n = 49$), transepithelial resistance 99.2 ± 10.7 Ω cm^2 ($n = 49$) and for transepithelial potential (apical side negative), 1.7 ± 0.3 mV ($n = 49$). Note that n gives the total number of epithelia examined. The comparable values derived from tissues from four CF patients were SCC = 28.5 ± 3.2 μA cm^{-2} ($n = 18$), R = 92.2 ± 19.4 Ω cm^2 ($n = 18$) and PD = 2.0 ± 0.4 mV ($n = 18$).

In a few experiments epithelial sheets were prepared from microdissected secretory coils or reabsorptive ducts from normal glands. The basal transporting characteristics of these tissues fell within a similar range to those above and were as follows. For secretory coil epithelia values were SCC = 24.3 ± 3.2 μA cm^{-2} ($n = 7$), PD = 2.3 ± 0.6 mV ($n = 7$) and R = 70.0 ± 11.8 Ω cm^2 ($n = 7$). Many reabsorptive ducts were required to produce enough cells to seed filters and only two confluent monolayers prepared exclusively from ducts were obtained. Mean values were 30 μA cm^{-2}, 0.7 mV and 28 Ω cm^2 respectively for SCC, PD and R.

One aim of this investigation was to explore the spectrum of agonists that could affect SCC in these epithelia. While the actions of some agonists have been described by us before (Brayden et al., 1988) their actions have not been described on cultures derived from separated coils and ducts. Supramaximal concentrations of agonists have been used as it was not possible to determine concentration-response relationships in these delicate structures, except by cumulative addi-

tion. However, marked desensitization precluded this option. It is also of interest to record agonists that were ineffective in increasing SCC, especially if they produce secondary changes in intracellular messengers. The bulk of this information is given in Table 1 which should be studied alongside Figures 1–4 and the statements below. Figure 1 shows results for epithelia cultured from whole sweat glands. The oscillations in SCC following histamine are characteristic of all agonists investigated. However oscillations were not seen invariably and the frequency of occurrence is given in Table 1. Oscillations were not exclusive to normal epithelia, occurring with a similar frequency in epithelia cultured from whole CF sweat glands. Figure 2 shows oscillations in CF epithelia following lysylbradykinin (LBK) and carbachol. Note both the agonist-induced current and oscillations, but not the basal current, were immediately curtailed by atropine following carbachol. The effect of atropine was different from that of amiloride (Figure 1) which abolished both the oscillations and basal current. Figure 3 is a tracing produced with an epithelium derived from secretory coils only. This was chosen to illustrate responses almost free of oscillations, although coil cultures could also show these. Cultured epithelia derived from reabsorptive ducts also showed sensitivity to LBK and carbachol and are also capable of oscillatory behaviour (Figure 4). The effects of other agonists not illustrated are given in Table 1. Some clues as to mechanisms were obtained by use of inhibitors. For example, piroxicam, 5 μM, failed to block the effects of LBK, suggesting the latter's effects were not due to prostaglandin formation. Atropine, 10 nM blocked the effects of carbachol or curtailed its effect if given after the agonist (Figures 2, 3) indicating an action at muscarinic receptors. Mepyramine, 100 nM, but not cimetidine, 1 μM, blocked the effects of histamine indicating the presence of H_1-receptors.

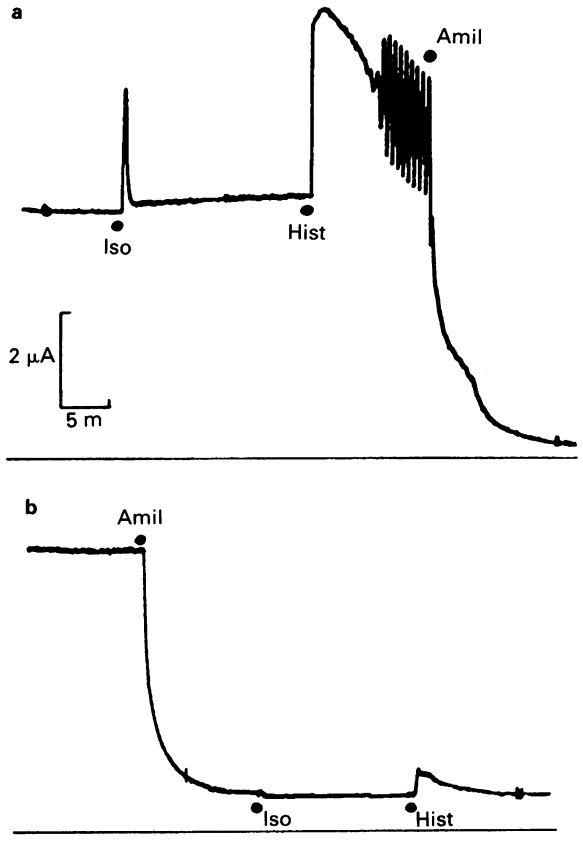

Figure 1 Illustrates the effects of histamine (Hist, 10 μM) on SCC in epithelia cultured from normal whole glands. With histamine the SCC response is sustained compared to that for isoprenaline (Iso, 10 μM). In (b) amiloride (10 μM) was added before either agonist, while the reverse was true for (a). Both preparations were from the same batch. Horizontal lines indicate zero SCC and calibrations are the same for both traces.

Table 1 Short circuit current responses (μA cm^{-2}) in cultures of whole sweat gland epithelia: comparison of normal and cystic fibrosis (CF) tissues

Agonist	*Normal*	*CF*
Lysylbradykinin (0.1 μM)	15.7 ± 1.7 (14/53)	11.2 ± 1.3 (4/10)
Carbachol (10 μM)	15.1 ± 1.8 (10/32)	14.3 ± 1.1 (4/5)
Histamine (10 μM)	13.0 ± 2.2 (7/18)	13.3 ± 3.7 (3/3)
Isoprenaline (10 μM)	6.2 ± 1.3 (2/11)	6.6 ± 1.6 (0/3)
A23187 (1 μM)	5.3 ± 0.9 (2/6)	4.2 (2/2)
Thapsigargin (170 nM)	−0.3 ± 0.4 (0/5)	1.6 ± 0.5 (0/4)
Ionomycin (1 μM)	11.3 ± 2.3 (0/3)	—
Adenosine triphosphate (1 μM)	—	33.8 (0/1)

Numbers in parentheses indicate number of separate preparations examined, the first of two figures indicating the number showing oscillations in response to particular agonists.

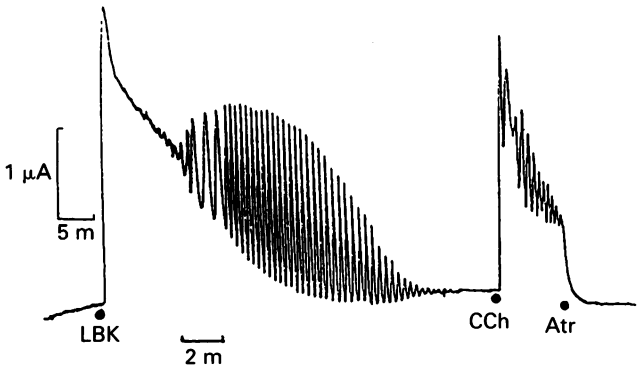

Figure 2 SCC responses of a whole gland culture made from cystic fibrosis glands. Notice that time scale was temporarily expanded to show the contour of the oscillations. Lysylbradykinin (LBK) 0.1 μM, carbachol (CCh) 10 μM, and atropine (Atr) 0.1 μM were added as indicated. Horizontal line indicates zero SCC.

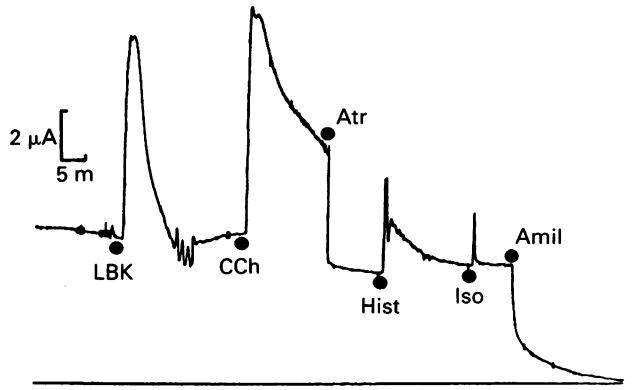

Figure 3 SCC responses in a culture derived from secretory coils from normal glands. Responses to lysylbradykinin (LBK) (0.1 μM), carbachol (CCh) (10 μM), histamine (Hist) (10 μM) and isoprenaline (Iso) (10 μM) are shown. Atropine (Atr) (10 nM) abolished the response to CCh. Note only minor oscillations are seen following LBK. Horizontal line indicates zero SCC.

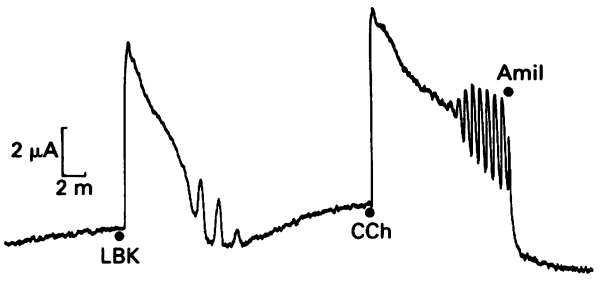

Figure 4 SCC responses in a culture produced from reabsorptive ducts from normal glands. Concentrations of drugs were lysylbradykinin (LBK, 0.1 μM), carbachol (CCh, 10 μM) and amiloride (Amil, 10 μM). Horizontal line indicates zero SCC.

A number of agents had no effect on SCC. For example, benzimidazole guanidine, which promotes sodium movement through amiloride-sensitive channels, caused minor SCC inhibition like amiloride. This is surprising as amiloride-sensitive sodium channels are clearly present (see later). It is shown later that an intact adenylate-cyclase system is present in sweat gland epithelia, yet no SCC responses were obtained in response to cyclic AMP, cyclic GMP (both as dibutyryl derivative at 0.1 μM) or forskolin, 10 μM. No effect of the neuropeptides vasoactive intestinal polypeptide, neuropeptide Y, arginine vasopressin or atrial natriuretic peptide were seen. There are reasons to expect the sweat gland epithelium would be sensitive to these agents (see Discussion). We did not find any agents which had exclusive actions on normal or CF epithelia. Finally the effects of the terpene thapsigargin are of particular interest since it is shown later that this agent has a profound effect on $[Ca]_i$ but is almost without effect on SCC (Table 1).

Nature of the transported ion

It is already apparent from Figures 1, 3 and 4 that the basal SCC or the additional current induced by agonists is removed by amiloride, 10 μM. At this concentration amiloride is specific for apical epithelial sodium channels. It may therefore be concluded that all the SCC is due to electrogenic sodium absorption, both in normal and CF tissues, and in secretory coil and duct derived epithelia. Electrogenic anion secretion might have been expected in the epithelia derived from secretory coils. Conditions were manipulated to increase the possibility of seeing a minor secretory response. Amiloride was added to block sodium absorption and to cause apical membrane hyperpolarization, a condition favouring chloride exit through the apical face. Secondly, the apical chloride concentration was reduced to 5.0 mEq l^{-1}, replacing with gluconate, to impose a favourable chloride gradient for secretion (Willumsen & Boucher, 1989). Small responses to several agonists were seen, no bigger than the residual ones seen in normal solution in the presence of amiloride.

To examine further if the responses to agonists were due to sodium absorption experiments were carried out in which agonists were added either before or after amiloride, 10 μM, with statistical comparison of the responses obtained. This was done with a batch of CF cultures and the agonists LBK and carbachol, in order to compare with similar data obtained in normal tissues previously (Brayden *et al.*, 1988). In the second experiment a batch of normal cultures were used to probe the effect of amiloride on the responses to isoprenaline and histamine. The data are summarized in Table 2. While there is a highly significant reduction in the size of the responses there were small residual agonist effects. These were too small to investigate systematically, but it is possible they may have a different ionic basis to those inhibited by amiloride.

Table 2 Effects of amiloride on responses to various agonists in cultures of whole normal glands and cystic fibrosis glands

	Before amiloride	*After amiloride*	P
Normal			
Isoprenaline	7.9 ± 2.5 (5)	0.8 ± 0.5 (5)	<0.025
Histamine	10.8 ± 3.8 (4)	1.1 ± 0.6 (4)	<0.025
Cystic fibrosis			
LBK	18.0 ± 3.2 (4)	2.4 ± 3.3 (5)	<0.001
Carbachol	17.9 ± 3.3 (5)	3.3 ± 0.7 (5)	<0.001

All values are of SCC ($\mu A\,cm^{-2}$). Amiloride (apical application) was used at 10 μM. Agonists are applied basolaterally at the following concentrations; isoprenaline 10 μM, histamine 10 μM, lysylbradykinin (LBK), 0.1 μM and carbachol 10 μM. An unpaired Student's *t* test was used to test for significance of the effect of amiloride.

Second messenger systems involved in the SCC responses

Adenylate cyclase activity in homogenates of normal cultured sweat gland cells showed a ten fold increase in response to forskolin, indicating the presence of the enzyme (Table 3). LBK can stimulate adenylate cyclase activity indirectly through the intermediary of prostaglandins. However, no stimulation of activity was found with LBK, as expected since responses to LBK were not modified by piroxicam. While isoprenaline caused a doubling of activity this increase was not significant. The presence of β-adrenoceptors coupled to adenylate cyclase in these cells is therefore unproven.

In contrast to the unlikely role for cyclic AMP in transport in these *in vitro* epithelia, a strong case can be made for the involvement of intracellular calcium ($[Ca]_i$). First, lanthanum ions added during the plateau of an agonist response immediately removes the added SCC without severely affecting the basal SCC (Figure 5a). Added before an agonist lanthanum ions had no immediate effect on basal SCC. This might suggest that there is a requirement for continued calcium influx during the plateau response, while the basal SCC is not so dependent. Secondly, A23187, a calcium ionophore was able to stimulate SCC and oscillations in cultured sweat gland epithelia. Further, as with lanthanum, removal of calcium influx by chelation with EGTA removed the stimulated component of the current while leaving the basal largely intact. The effect of EGTA was reversible by adding calcium ions (Figure 5b), and had no immediate effect on basal SCC when added alone.

In contrast to the effects of lanthanum ions, barium ions affected the basal SCC. Barium acts as a weak blocker of calcium-sensitive potassium channels present in sweat gland cells (Henderson *et al.*, 1990). Barium (5 μM) applied basolaterally caused SCC to decrease by 54 ± 5% ($n = 6$) in epithelia from normal glands and by 35% (mean of 2) in CF epithelia. Responses to the agonists LBK, carbachol and histamine were not attenuated by barium (Figure 6). In 8 experiments made

Table 3 Adenylate cyclase activity in cultured normal sweat gland cells

	Cyclic AMP ($pmol\,mg^{-1}\,h^{-1}$)
Control (3)	98.6 ± 31.6
Forskolin 10 μM (3)	1024.3 ± 210.0*
LBK, 0.1 μM (3)	116.0 ± 48.1
Isoprenaline, 10 μM (2)	229.0 ± 63.6

* $P < 0.01$, unpaired *t* test. Numbers in parentheses indicate number of observations of cultures from separate subjects. LBK: lysylbradykinin.

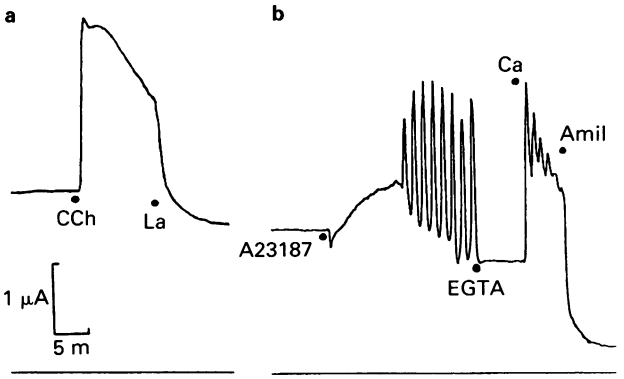

Figure 5 (a) Shows the effect of lanthanum ions (1 mM) applied basolaterally on the response to carbachol (CCh, 10 μM). (b) Effect of A23187 (1 μM) on SCC after which external calcium ions were removed then replaced by addition of EGTA and $CaCl_2$ respectively. Finally amiloride (10 μM) was added. Both preparations were cultured from whole cystic fibrosis glands. Horizontal lines indicate zero SCC.

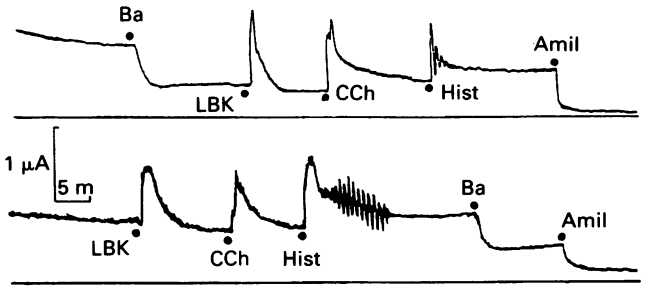

Figure 6 Responses of two normal epithelia from the same batch derived from whole glands. Lysylbradykinin (LBK, 0.1 μM), carbachol (CCh, 10 μM) and histamine (Hist, 10 μM) were added in one instance after and in the other before barium ions (5 mM) were added to the basolateral side. Amiloride, 10 μM, was added at the end of each experiment, apically. Horizontal lines indicate zero SCC and calibration applies to both traces.

with two batches of epithelia the effects of LBK, 0.1 μM given before or after barium were respectively $4.7 \pm 1.5\,\mu A\,cm^{-2}$ and $6.2 \pm 3.6\,\mu A\,cm^{-2}$, thus barium ions were not an effective blocker of kinin action. Interestingly apically applied barium ions did not affect the basal current indicating a probable effect on basolateral K-channels.

Agonist effects on intracellular calcium $[Ca]_i$

To confirm the presumed effects of agonists on $[Ca]_i$ direct measurements with fura-2 fluorescence were made with cell suspensions. The aims were two fold; first to discover if all agonists could increase $[Ca]_i$ and secondly to establish for given agonists, namely LBK and carbachol, whether the responses were different in extent in normal and CF tissues.

The experimental design posed some problems because of the paucity of experimental material. It was necessary to add more than one agonist to the cell suspension in the cuvette to conserve cells. However, it is known in some situations (Pickles, unpublished observations) that when agonists are given close upon one another the response to the second can be impaired, presumably because both release from the same intracellular store. Consequently agonists were added to cell suspensions at least 5 min apart. Figure 7 illustrates typical traces for single suspensions of sweat gland cells from normal and CF tissues. Reference to Table 4 shows the results of 17 experiments, 10 with CF cells and 7 with normal cells. In the experiments with CF cells, carbachol and LBK were given alternately either first or second. While, in general, when an agonist was given second the responses, both peak heights and plateau values, were smaller they were not significantly so compared with the first responses. This analysis was not carried out for the results with control cells as carbachol was

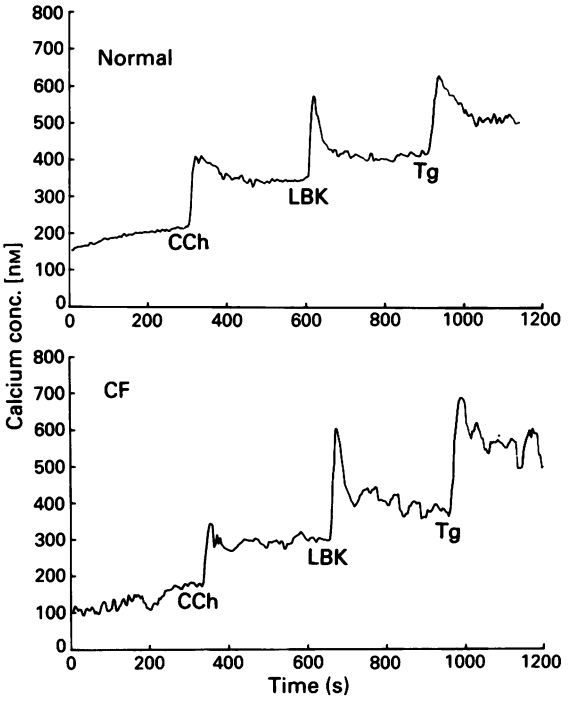

Figure 7 Illustrative effects of carbachol (CCh, 10 μM), lysylbradykinin (LBK, 0.1 μM) and thapsigargin (Tg, 170 nM) on $[Ca]_i$ measured in suspensions of normal (a) and cystic fibrosis (b) sweat gland cells. Peak heights and plateau values given in Table 4 were measured from the immediately pre-existing value before the agonist was added.

only given second in two of the six measurements, and yielded the largest responses. Table 4 reveals several interesting features as follows: (a) Basal $[Ca]_i$ values in normal and CF cells are identical; (b) there are no significant differences in the responses to LBK or carbachol in normal and CF cells when comparing either the peak responses or the maintained plateau levels, furthermore responses to LBK are less well sustained than those to carbachol, and (c) there appears to be an enhanced response to thapsigargin in normal cells compared to CF. Two other agonists, namely ATP and histamine, are shown to increase $[Ca]_i$ in both normal and CF cells. The data here are limited and no comment can be made about comparability, only capability.

A likely mechanism for the increase in $[Ca]_i$ following LBK is via activation of the phospholipase C system with the consequent production of inositol phosphates and diacylglycerol (Shayman & Morrison, 1985). To test this hypothesis the phospholipase inhibitor neomycin was used. In four normal whole gland cultures the response to LBK (0.1 μM) was 2.11 ± 0.19 nEq (in these experiments the response was mea-

Table 4 Intracellular calcium increases in response to lysylbradykinin (LBK), carbachol and thapsigargin

	Control cells			*CF cells*		
Agonist	Peak	Plateau	*n*	Peak	Plateau	*n*
Carbacol 10 μM				172 ± 39 (F)	147 ± 37 (F)	5
				114 ± 27 (S)	85 ± 18 (S)	4
	161 ± 37	101 ± 21	6	146 ± 26 (T)	119 ± 23 (T)	9
LBK 0.1 μM				232 ± 67 (F)	45 ± 14 (F)	5
				182 ± 18 (S)	70 ± 7 (S)	3
	184 ± 109	41 ± 18	6	214 ± 41 (T)	54 ± 10 (T)	8
Thapsigargin 160 nM	531 ± 110	252 ± 40	4	260 ± 25	156 ± 32	7
				$P < 0.02$	$0.05 < P < 0.1$	
ATP 1.0 mM	216	101	3	342	185	1
Histamine 10 μM	107	53	2	118	0	1
Basal	189 ± 13	—	7	195 ± 8	—	10

Note: F, S and T refer respectively to first responses, second responses and total first and second responses. Statistical comparisons are to the relevant control value. All values are given as nM, basal values are the values immediately after the cell suspensions are placed in the cuvette and have achieved 37°C.

sured as total charge transfer to account for both the size and duration of the responses). In four other preparations from the same batch, exposed to neomycin 15 mM, the response to LBK was reduced significantly to 0.70 ± 0.16 nEq ($P < 0.005$). Neomycin had a transient effect on SCC which returned to normal before the experiment commenced. This effect was not due to the vehicle which had no effect alone.

Discussion

Reported for the first time in this study is the effect of a variety of agonists on transepithelial ion transport in cultured epithelia from normal and CF whole sweat glands and from cultures derived from separated coils and ducts of whole sweat glands.

In general, the transepithelial potentials were lower than expected from measurements in whole glands (10 mV, lumen negative in normals and 70 mV in CF glands; Bijman & Quinton, 1984; Quinton, 1983; 1986; Bijman & Fromter, 1986) which is probably due to structural imperfections in the monolayers, allowing additional shunt pathways to reduce the potential. It was found (data not shown) that substitution of impermeant anions, such as gluconate, did not substantially alter the potentials indicating pathways permeant to these large anions must exist.

Cable analysis in microperfused ducts gave a value for transepithelial resistance of $10\,\Omega\,cm^2$ due it is claimed to high chloride conductance of the ductal cell membranes rather than to leaky tight junctions (Bijman & Fromter, 1986). Our resistance values are up to an order of magnitude greater, which may result from the multilayered structures which develop *in vitro* (Brayden *et al.*, 1988).

We have increased substantially the number of different receptor types now known to be located in the membranes of cultured human sweat glands, both normal and CF. These include histamine H_1 receptors in normal glands, receptors for LBK, histamine, carbachol (muscarinic) and isoprenaline in CF cells. Further, muscarinic receptors and those for LBK are found in cultures derived entirely from secretory coils or from absorptive ducts. Table 1 shows that the responses to LBK, carbachol, histamine and isoprenaline are remarkably similar in normal and CF tissues. Again all four agonists can produce SCC oscillations, but this is not invariably so. These oscillations must indicate that the transporting activity of individual cells can be synchronized, presumably by intercellular communication, although the signal oscillator generating this activity is unknown.

It has been shown here that the effects of histamine and isoprenaline in normal cells and LBK and carbachol in CF cells are significantly inhibited by amiloride, 10 μM. The unique specificity of amiloride used at this concentration (Cuthbert & Shum, 1974) is strong evidence that these agents stimulate electrogenic sodium absorption. As the basal SCC is also abolished by amiloride at this concentration it is likely that the only substantial activity shown by any of the cultures we have examined is sodium absorption. The failure to see chloride secretion in coil cultures is puzzling as this is the normal function of this section of the gland. Attempts to enhance the small residual effects seen after amiloride by imposing a favourable chloride gradient were unsuccessful. Unfortunately the residual responses were too small to investigate systematically.

Considering the epithelia are sodium absorbing, it is of interest that arginine vasopressin and atrial natriuretic peptide which can stimulate and inhibit respectively electrogenic sodium absorption in other tissues (Orloff & Handler, 1962; Light *et al.*, 1989) had no effect. Similarly benzimidazole guanidine which can open epithelial sodium channels (Zeiske & Lindemann, 1974) had no stimulatory effect in sweat glands. Although not included in the results, a number of attempts were made to modify the responses to agonists by preincubation with agents shown elsewhere to alter transporting characteristics. These included prolactin, oestrogens and progesterone (Robertson *et al.*, 1986; Zeitlin *et al.*, 1989). No changes were detected following these procedures.

Cyclic nucleotides and forskolin were without effect on SCC in sweat gland epithelia. Nevertheless an intact adenylate cyclase system was present which could be stimulated by forskolin (Table 3). Neither LBK nor isoprenaline caused an increase in cyclic AMP generation and there is evidence that isoprenaline produces an effect in salivary glands via a calcium signal (Cook *et al.*, 1988).

Considerable evidence was obtained to support the view that agonists promoting sodium absorption in the glands do so by mobilizing calcium. This includes (a) an increase in SCC following A23187 (including oscillations) and ionomycin, two calcium ionophores, (b) removal of agonist-induced currents by lanthanum ions, (c) presumed inhibition of phospholipase C with neomycin inhibits the response to LBK and (d) reversible inhibition of agonist-induced currents by EGTA. This latter implies, at least, that external calcium is required to maintain the response. More direct evidence comes from the measurement of $[Ca]_i$ in cell suspensions by fura-2 fluorescence. No major differences in the responses of normal and CF cells were found (Table 4). Although only few experiments were performed with some agonists there was but one exception to the rule that agonists increasing SCC also increased $[Ca]_i$. The exception was thapsigargin, an agent which apparently raises $[Ca]_i$ by inhibiting a calcium-ATPase which unbalances the pump-leak system in intracellular organelles (Thastrup *et al.*, 1990), allowing calcium to leak out thus raising cytosolic $[Ca]_i$. While this agent stimulates SCC in some epithelia (Brayden *et al.*, 1989) it has no effect on sweat gland epithelia (Table 1). Nevertheless $[Ca]_i$ is raised, apparently more so in controls than in CF cells. (Table 4). This result supports the notion that a temporally orchestrated and locationally correct increase in $[Ca]_i$ is required to trigger a physiological-type response (Putney, 1986). It remains a puzzle that A23187 can stimulate SCC and oscillations through a non-specific interaction, even though its effects are noticeably weaker than the receptor-operated increases.

Recently the presence of calcium-activated K-channels has been reported in sweat gland epithelia (Henderson *et al.*, 1990; Henderson & Cuthbert, 1990) which are considered to be located basolaterally. Opening of these K channels by increased $[Ca]_i$ effectively hyperpolarizes the apical membrane increasing the sodium influx. Similar mechanisms have been proposed before (Maruyama *et al.*, 1983; Mandel *et al.*, 1986) and it may well be that this is the major way in which sodium transport is enhanced by $[Ca]_i$. Indeed, in other situations membrane currents or potentials can be used to monitor intracellular $[Ca]_i$ with ion channels acting as an immediate reporter of $[Ca]_i$. For example, in smooth muscle cells, membrane potential and $[Ca]_i$ oscillate in unison (Yada *et al.*, 1986), while in pancreatic acinar cells, calcium-dependent chloride currents oscillate in phase with changes in $[Ca]_i$ (Opsichuk *et al.*, 1990). Thus there are strong precedents for our proposal. Alternatively, it is possible that apical sodium channels are directly regulated by $[Ca]_i$. However in another epithelium, the cortical collecting tubule, $[Ca]_i$ inhibits rather than promotes sodium entry (Palmer & Frindt, 1987). Barium ions proved to be a weak blocker of basal SCC and to be ineffective against maximally effective concentrations of LBK. With patch-clamping barium ions were found to have only a minor effect on K-channel open time and that only at depolarized potentials (Henderson & Cuthbert, 1990). It is not surprising, therefore, that in the relatively cruder experiments given here that effective block was not shown.

Finally it is necessary to consider if the receptors expressed *in vitro* are consistent with what is known about intact glands *in vivo*. Sweat gland coils receive both cholinergic and adrenergic innervation (Uno & Montagna, 1975; Sáto, 1977; Uno, 1977), while vasoactive intestinal peptide (VIP) immunoreactive nerve endings appear to innervate both the coil and duct (Heinz-Erian *et al.*, 1985). Others have postulated that VIP

co-exists with acetylcholine in cholinergic nerves supplying the sweat gland (Hökfelt *et al.*, 1980). It is expected and found that the secretory coil cultures respond to both cholinoceptor and adrenoceptor agonists. However, while they do respond to carbachol and isoprenaline they have the characteristics of ductal cells, showing sodium absorption rather than a secretory process. On the other hand whole gland cultures were insensitive to VIP, as they were to other agents shown to increase cyclic AMP.

It is possible using primary cultures of whole glands that when the cells are disaggregated before seeding the culture wells there is a selection process (e.g. selective killing of one cell type) which results in epithelia with ductal characteristics. This possibility is negated by the experiments in which transporting epithelia were derived from either coils or ducts. Both tissues gave similar epithelia from a functional standpoint, and although the studies were limited there was no indication that the properties of epithelia were different from those derived from whole glands.

By use of microelectrodes it was found that individual cells in cultures of secretory coils showed amiloride sensitivity (Jones *et al.*, 1988) suggesting that both secretory and absorptive processes can occur in this segment. A reasonable interpretation of the present data is that in our culture conditions, sweat gland cells revert to a less differentiated form where sodium absorption is the predominant transport process, yet able to generate receptors which may be more characteristic of the coil. In an embryonic sense the duct is more primitive and forms first (Hashimoto *et al.*, 1982) while in some species (e.g. cat) sweat glands of the foot pad contain non-functioning ducts (Brusilow & Munger, 1962). In regard to responses to the autacoids LBK and histamine, it may be that local production of these substances is able to modify sweat gland function.

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Effects of N^G-nitro-L-arginine methyl ester or indomethacin on differential regional and cardiac haemodynamic actions of arginine vasopressin and lysine vasopressin in conscious rats

[1]Sheila M. Gardiner, Alix M. Compton, Philip A. Kemp & Terence Bennett

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

1 Measurements of changes in renal, mesenteric and hindquarters haemodynamics or cardiac haemodynamics in response to i.v. bolus doses of arginine vasopressin (AVP) or lysine vasopressin (LVP, 0.7 and 7.0 pmol) were made in conscious, chronically-instrumented Long Evans rats.

2 In some experiments AVP and LVP were administered during an infusion of N^G-nitro-L-arginine methyl ester (L-NAME; 1.0 or 0.3 mg kg^{-1} h^{-1}) to determine whether or not inhibition of nitric oxide production influenced the cardiovascular effects of the peptides. In other experiments, indomethacin (bolus dose of 5 mg kg^{-1} followed by infusion at 5 mg kg^{-1} h^{-1}) was given to determine the possible involvement of cyclo-oxygenase products in the responses to AVP and LVP.

3 Under control conditions, the lower dose of LVP had significantly greater effects than AVP on heart rate, mean arterial blood pressure, renal, mesenteric and hindquarters conductances, total peripheral conductance, cardiac index, peak aortic flow and $+dF/dt_{max}$. The higher dose of LVP had significantly greater effects than AVP on all variables (i.e. including stroke index and central venous pressure).

4 In the presence of L-NAME (1 mg kg^{-1} h^{-1}) there was a sustained increase in mean arterial blood pressure ($+23 \pm 3$ mmHg) and reductions in mesenteric ($-38 \pm 4\%$) and hindquarters ($-30 \pm 6\%$) vascular conductances. Under these conditions the difference in the pressor effects of AVP and LVP was abolished, but their differential effects on regional and cardiac haemodynamics persisted. This dose of L-NAME did not change cardiac baroreflex sensitivity.

5 During infusion of L-NAME at a lower rate (0.3 mg kg^{-1} h^{-1}), baseline cardiovascular status was unchanged and regional haemodynamic effects of AVP and LVP were enhanced, but the differences in the regional vasoconstrictor responses to the two peptides persisted.

6 Indomethacin (5 mg kg^{-1} bolus, then 5 mg kg^{-1} h^{-1} infusion) augmented the renal vasoconstrictor responses to AVP and LVP, but abolished the difference in the hindquarters vasoconstrictor responses to the two peptides. However, the differences in the pressor and the renal and mesenteric vasoconstrictor effects of AVP and LVP still occurred in the presence of indomethacin.

7 The results indicate that AVP normally has lesser cardiovascular effects than LVP but this difference does not seem to be due to more effective stimulation of nitric oxide-mediated or cyclo-oxygenase-dependent vasodilator mechanisms by AVP than LVP.

Introduction

Substantial evidence now exists that nitric oxide is the major endothelium-derived relaxing factor (see Palmer *et al.*, 1987; Moncada *et al.*, 1989a,b; Moncada & Higgs, 1990). Moreover, since the original demonstration that nitric oxide is synthesized from L-arginine in endothelial cells (Palmer *et al.*, 1988), similar L-arginine-nitric oxide pathways have been shown to be of functional significance in a variety of other cell types and systems (see Moncada & Higgs, 1990).

Administration of L-arginine alone under normal, resting conditions does not exert cardiovascular effects, indicating that substrate availability is not rate-limiting in the formation of endothelium-derived nitric oxide in these circumstances (Rees *et al.*, 1989a,b; Aisaka *et al.*, 1989; Gardiner *et al.*, 1990a). However, following inhibition of nitric oxide biosynthesis with N^G-monomethyl-L-arginine (L-NMMA) or N^G-nitro-L-arginine methyl ester (L-NAME), administration of L-arginine has marked haemodynamic effects that oppose those of L-NMMA or L-NAME (Rees *et al.*, 1989b; Gardiner *et al.*, 1990c,f). In addition, it is known that many vasoconstrictors cause concurrent activation of endothelium-dependent vasodilator processes that oppose their vasoconstrictor effects. For example, in the perfused mesenteric vascular bed of the rat, the vasoconstrictor effects of arginine vasopressin (AVP) are enhanced by the removal of the endothelium (Randall *et al.*, 1988), but it is not known if similar modulation of the vasoconstrictor effects of lysine vasopressin (LVP) occurs. However, Altura (1973) demonstrated that AVP was more potent than LVP in causing mesenteric vasoconstriction *in vivo* in anaesthetized rats, although he did not address the possibility of differential involvement of endothelium-mediated mechanisms. Therefore, we measured regional and cardiac haemodynamic effects of AVP and LVP in conscious, Long Evans rats in the absence and in the presence of L-NAME. However, since the latter compound is not a selective inhibitor of endothelial cell nitric oxide biosynthesis, and since there is some evidence that afferent neuronal function may involve nitric oxide-mediated mechanisms (Duarte *et al.*, 1990), it seemed possible that treatment with L-NAME *in vivo* might influence baroreflex mechanisms in such a way as to obscure any differential changes in the haemodynamic effects of AVP and LVP caused by L-NAME. Therefore, in order to test the possibility that L-NAME might interfere with baroreflex function, we also made a formal assessment of cardiac baroreflex sensitivity in the absence and presence of L-NAME.

Although we found that AVP and LVP did have quantitatively different regional and cardiac haemodynamic effects, these differences persisted in the presence of L-NAME, in spite of there being no apparent changes in cardiac baroreflex sensitivities (see Results). One of the possible explanations of this observation was that the differences in the effects of AVP and

[1] Author for correspondence.

LVP were due to differential activation of vasodilator mechanisms, but mediated through cyclo-oxygenase products (Hofbauer *et al.*, 1983; Walker, 1985; Lote *et al.*, 1987; Walker *et al.*, 1988) rather than through nitric oxide. Therefore, in a separate experiment we investigated the regional haemodynamic effects of AVP and LVP in the absence and in the presence of indomethacin.

Methods

All experiments were carried out on male Long Evans rats (350 g–450 g) bred in the Animal Unit in Nottingham. Details of the techniques used have been published elsewhere (Gardiner *et al.*, 1990a,c). Briefly, animals were anaesthetized (sodium methohexitone, 60 mg kg^{-1} i.p., supplemented as required) and had miniaturized pulsed Doppler probes (Haywood *et al.*, 1981) implanted to monitor changes in renal, superior mesenteric and hindquarter blood flows (Gardiner *et al.*, 1990a,c), or an electromagnetic flow probe implanted to measure ascending thoracic aortic blood flow (Smith & Hutchins, 1979; Smits *et al.*, 1982; Gardiner *et al.*, 1990e). At least 7 days later animals were briefly re-anaesthetized (sodium methohexitone 40 mg kg^{-1}, i.p.) for the insertion of intravascular catheters. Experiments began the following day, when the animals were fully conscious and unrestrained, and the protocols extended over two days.

The animals bearing pulsed Doppler probes were connected to a pulsed Doppler flow meter (Crystal Biotech VF-1, Hollis-ton, MA, USA) modified to operate with a pulse repetition frequency of 125 kHz (Gardiner *et al.*, 1990b) and fitted with HVPD-20 modules to avoid problems with aliasing of the renal and mesenteric signals (Gardiner *et al.*, 1990b). Continuous recordings were made of renal, mesenteric and hindquarters Doppler shift signals, and percentage changes in flow were calculated from these (Haywood *et al.*, 1981). The Doppler shift and corresponding mean arterial blood pressure signals were used to calculate percentage changes in regional vascular conductances (Gardiner *et al.*, 1990c).

The animals with electromagnetic flow probes were connected to a flow meter (MDL 1401, Skalar, Delft, Netherlands) interfaced with an haemodynamics microprocessor (University of Limburg, Department of Instrument Services: Schoemaker, 1989; Gardiner *et al.*, 1990e) and a Tandon 386 microcomputer. This system digitized data (every 2 ms) and, off-line, provided averaged (every 2 s) data for heart rate, mean arterial blood pressure, cardiac index, stroke index, peak aortic flow, maximum rate of rise of aortic flow ($+dF/dt_{max}$), total peripheral conductance and mean central venous pressure (Gardiner *et al.*, 1990e). Peak aortic flow and $+dF/dt_{max}$ were taken as indirect indices of contractility (Schoemaker, 1989).

Regional haemodynamic effects of arginine vasopressin and lysine vasopressin in the absence and presence of L-NAME

Animals ($n = 8$) received AVP (0.7 and 7 pmol i.v.) or LVP (0.7 and 7 pmol i.v.) in random order on the first day, but the low doses were given before the high doses. The low doses were separated by at least 60 min and the high doses by 90 min. On the second day the animals were given AVP and LVP in the same order as on the first day but starting 90 min after the onset of L-NAME infusion (1 mg kg^{-1} h^{-1}). The latter caused significant hypertension and reductions in mesenteric and hindquarters vascular conductances (see Results; Gardiner *et al.*, 1990d,f); thus, while it was possible to compare the responses to AVP and LVP under these conditions, it was less straightforward to consider any differences in the responses to AVP or those to LVP in the absence and presence of L-NAME (because of the baseline shift). Therefore, in a second group of animals ($n = 7$) responses to AVP and LVP were assessed in the absence and presence of a lower dose of L-NAME (0.3 mg kg^{-1} h^{-1}) that had no significant effect on resting cardiovascular status.

Cardiac baroreflex sensitivities in the presence and absence of L-NAME

In order to cover the possibility of changes in baroreflex function in the presence of L-NAME (see Introduction), the animals in the first study mentioned above had their cardiac baroreflex sensitivities (Gardiner & Bennett, 1988) assessed in response to depressor (sodium nitroprusside, 100 μg ml^{-1} infused at 0.2 ml min^{-1}) and pressor (methoxamine, 400 μg ml^{-1} infused at 0.2 ml min^{-1}) stimuli in the absence and in the presence of L-NAME (1 mg kg^{-1} h^{-1}).

Cardiac haemodynamic effects of arginine vasopressin and lysine vasopressin in the absence and presence of L-NAME

The higher dose of L-NAME (1 mg kg^{-1} h^{-1}) abolished the difference between the pressor effects of the higher doses of AVP and LVP (see Results). Therefore, we assessed the cardiac haemodynamic effects of AVP and LVP under these conditions (following the protocol described above) in a group ($n = 8$) of Long Evans rats with thoracic aortic electromagnetic flow probes.

Regional haemodynamic effects of arginine vasopressin and lysine vasopressin in the absence and presence of indomethacin

Although L-NAME had effects on the responses to AVP and LVP (see Results), significant differences remained in the responses to these peptides in the presence of L-NAME. Therefore, in a separate group of animals ($n = 8$) regional haemodynamic responses to AVP and LVP were assessed in the absence and presence of indomethacin (5 mg kg^{-1} bolus, 5 mg kg^{-1} h^{-1} infusion; Gardiner *et al.*, 1990a). Indomethacin was given by primed infusion to ensure adequate cyclo-oxygenase inhibition throughout the experiment.

Data analysis

Measurements were made at time points selected to represent the full profile of the effects of AVP and LVP for 20 min following the i.v. injection. Within-group changes relative to baseline were assessed by Friedman's Test (Theodorsson-Norheim, 1987). Comparisons between the responses to AVP and LVP, or the responses to either peptide under different conditions, were made by applying Wilcoxon's ranks sums test to maximum responses and durations and areas under or over curves (Gardiner *et al.*, 1990f). $P < 0.05$ was taken to be significant.

Peptides and drugs

AVP and LVP (Bachem UK Ltd) were dissolved in isotonic (154 mM NaCl) saline containing 1% bovine serum albumin (Sigma Chemical Co.). Injections were given in 100 μl flushed in with 100 μl isotonic saline (catheter dead space). Administration of vehicle alone in these volumes had no consistent cardiovascular effects. L-NAME hydrochloride (Sigma) was dissolved in isotonic saline and infused at 0.3 ml h^{-1}. Indomethacin (Merck Sharp & Dohme Ltd) was dissolved in 10 mM sodium bicarbonate; the bolus dose was given in 0.34 ml over 10 min and the infusion at 0.3 ml h^{-1}. Methoxamine hydrochloride (Sigma) and sodium nitroprusside (Sigma) were dissolved in isotonic saline.

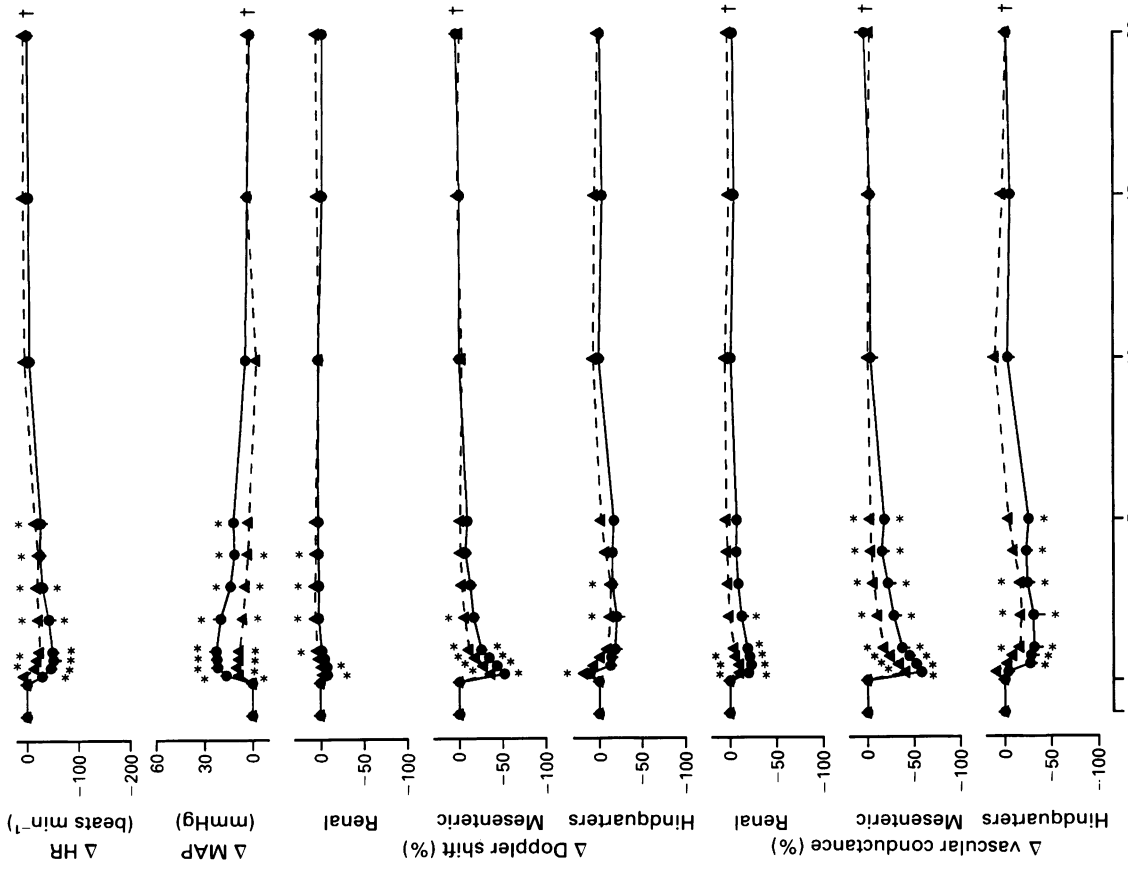

Figure 1 Cardiovascular changes (Δ) in conscious Long Evans rats ($n = 8$) following i.v. bolus injections of arginine vasopressin (AVP, ▲, 0.7 pmol) or lysine vasopressin (LVP, ●, 0.7 pmol). * $P < 0.05$ versus baseline (Friedman's test); † $P < 0.05$ AVP versus LVP (Wilcoxon's test applied to areas under or over curves). Values are mean with s.e.mean shown by vertical lines; where the latter do not appear they lie within the area covered by the symbol. HR = heart rate; MAP = mean systemic arterial blood pressure.

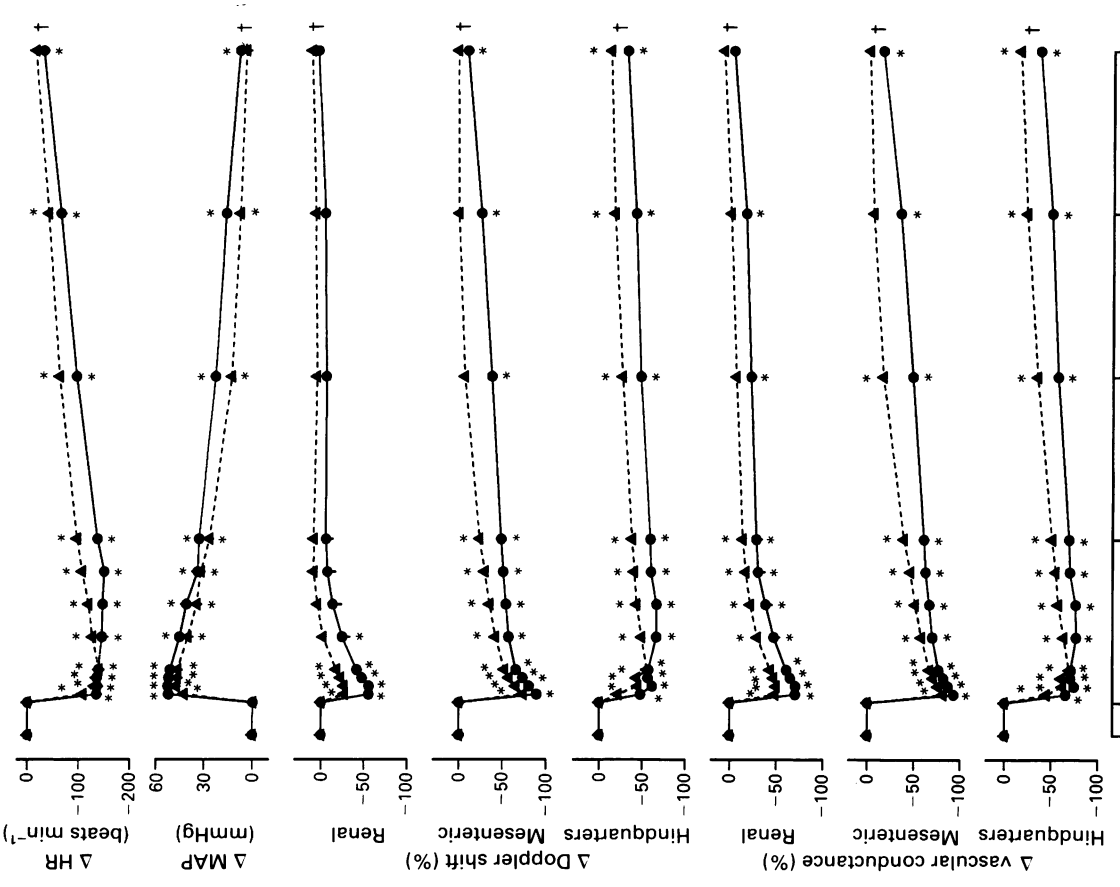

Figure 2 Cardiovascular changes (Δ) in the same conscious Long Evans rats ($n = 8$) as in Figure 1, following i.v. bolus injections of arginine vasopressin (AVP, ▲, 7.0 pmol) or lysine vasopressin (LVP, ●, 7.0 pmol). * $P < 0.05$ versus baseline (Friedman's test); † $P < 0.05$ AVP versus LVP (Wilcoxon's test applied to areas under or over curves). Values are mean with s.e.mean shown by vertical lines; where the latter do not appear they lie within the area covered by the symbol. HR = heart rate; MAP = mean systemic arterial blood pressure.

Table 1 Peak changes in regional haemodynamic variables in response to arginine vasopressin (AVP, 0.7 or 7.0 pmol) or lysine vasopressin (LVP 0.7 and 7.0 pmol) in the presence of N^G-nitro-L-arginine methyl ester (1 mg kg^{-1} h^{-1}) in conscious, Long Evans rats

	0.7 pmol		7.0 pmol	
	AVP	*LVP*	*AVP*	*LVP*
Heart rate (beats min^{-1})	−38 ± 10*	−66 ± 5*†	−122 ± 16*	−159 ± 23*
Mean arterial pressure (mmHg)	+18 ± 4*	+35 ± 1*†	+37 ± 2*	+39 ± 3*
Renal flow (%)	−7 ± 3*	−21 ± 5*†	−45 ± 4*	−86 ± 2*†
Mesenteric flow (%)	−24 ± 3*	−45 ± 2*†	−70 ± 3*	−89 ± 2*†
Hindquarters flow (%)	−13 ± 5*	−33 ± 4*†	−46 ± 7*	−63 ± 6*†
Renal conductance (%)	−18 ± 4*	−38 ± 5*†	−58 ± 3*	−90 ± 2*†
Mesenteric conductance (%)	−33 ± 3*	−56 ± 2*†	−77 ± 2*	−91 ± 1*†
Hindquarters conductance (%)	−20 ± 6*	−44 ± 3*†	−59 ± 5*	−72 ± 4*†

Values are mean ± s.e.mean, $n = 8$; * $P < 0.05$ versus baseline; † $P < 0.05$ LVP versus AVP.

Results

Regional haemodynamic effects of arginine vasopressin and lysine vasopressin in the absence and presence of L-NAME

The lower dose of AVP and LVP caused rises in mean arterial blood pressure and bradycardias. Renal and hindquarters flows changed little, but there were substantial reductions in mesenteric flow. However, all 3 vascular beds showed vasoconstrictions (Figure 1). The higher dose of AVP and LVP caused marked reductions in renal, mesenteric and hindquarters blood flows (Figure 2), in addition to pressor, bradycardic and vasoconstrictor effects.

In the absence of L-NAME, LVP (0.7 pmol) had significantly greater effects than AVP on heart rate, mean arterial blood pressure, mesenteric blood flow and renal, mesenteric and hindquarters vascular conductances (Figure 1). At the higher dose (7.0 pmol) LVP had significantly greater effects than AVP on all variables (Figure 2), although the difference in the pressor effects was less than at the lower dose.

In the presence of L-NAME (1 mg kg^{-1} h^{-1}) the differences between the effects of the low dose of AVP and LVP persisted (Table 1), but the pressor responses to the high dose of the peptides were not different under these conditions (Table 1). However, L-NAME itself caused both sustained hypertension (+23 ± 3 mmHg) and mesenteric and hindquarters vasoconstrictions (−38 ± 4 and −30 ± 6% change in vascular conductances, respectively) and thus we could not dismiss the possibility that this influenced the results. Nonetheless, LVP still had significantly greater effects than AVP on regional haemodynamics (Table 1).

In the group of rats that was to receive the lower dose of L-NAME (0.3 mg kg^{-1} h^{-1}) the differential responses to LVP and AVP in the absence of L-NAME were similar to those described above. Infusion of L-NAME caused no significant cardiovascular changes, but in its presence there was a significant prolongation in the reduction of hindquarters vascular conductance following the low dose of LVP (from 5 to 15 min) which is consistent with inhibition of nitric oxide release. However, there were no other significant changes in the responses to the low dose of LVP or AVP in the presence of L-NAME (0.3 mg kg^{-1} h^{-1}), and the differential effects of LVP and AVP persisted.

The higher dose of LVP and AVP had similar pressor effects (+43 ± 1 and 49 ± 5 mmHg, respectively) in the presence of L-NAME (0.3 mg kg^{-1} h^{-1}). Under these conditions the maximum falls in vascular conductance in the renal (−54 ± 3%) and hindquarters (−65 ± 3%) vascular beds in response to AVP were significantly greater than the corresponding changes seen in the absence of L-NAME (−42 ± 4% and −53 ± 2% respectively). In addition, the durations of the renal and mesenteric vasoconstrictor responses to AVP were increased (from 2 to 5 and from 5 to 10 min, respectively). Some responses to LVP were also affected by L-NAME (0.3 mg kg^{-1} h^{-1}). Thus, in the presence of L-NAME the maximum fall in renal vascular conductance (−81 ± 4%) following the high dose of LVP was significantly greater than in its absence (−68 ± 4%) and the durations of the renal and mesenteric vascular responses were increased (both from 15 to 20 min).

In the presence of L-NAME the magnitudes and durations of the regional vascular conductance changes evoked by LVP (renal −81 ± 4% maximum, 20 min duration; mesenteric −94 ± 4% maximum, 20 min duration; hindquarters −76 ± 4% maximum, 20 min duration) were greater than those to AVP (renal −54 ± 3%, 5 min duration; mesenteric −81 ± 2%, 10 min duration; hindquarters −65 ± 3%, 15 min duration).

Cardiac baroreflex sensitivities in the absence and presence of L-NAME

There were no significant differences between cardiac baroreflex sensitivities to pressor or to depressor stimuli in the absence and presence of L-NAME (1 mg kg^{-1} h^{-1}; Table 2).

Table 2 Cardiac baroreflex sensitivities assessed from the slope of the linear regression of pulse interval on mean arterial blood pressure during falls or rises of mean arterial blood pressure elicited by nitroprusside or methoxamine in conscious Long Evans rats ($n = 8$) in the absence or presence of N^G-nitro-L-arginine methyl ester (L-NAME) (1 mg kg^{-1} h^{-1})

	L-NAME absent		*L-NAME present*	
	ms mmHg^{-1}	*r* value	ms mmHg^{-1}	*r* value
Nitroprusside	1.321 ± 0.201	0.943 ± 0.01	1.655 ± 0.261	0.937 ± 0.02
Methoxamine	1.540 ± 0.267	0.958 ± 0.01	1.514 ± 0.207	0.946 ± 0.02

Values are mean ± s.e.mean.

Table 3 Peak changes in cardiac haemodynamic variables in response to arginine vasopressin (AVP, 0.7 or 7.0 pmol) or lysine vasopressin (LVP, 0.7 and 7.0 pmol) in the presence of N^G-nitro-L-arginine methyl ester (1 mg kg^{-1} h^{-1}) in conscious, Long Evans rats

	0.7 pmol		7.0 pmol	
	AVP	*LVP*	*AVP*	*LVP*
Heart rate (beats min^{-1})	−35 ± 8*	−63 ± 10*†	−98 ± 6*	−120 ± 12*
Mean arterial pressure (mmHg)	+20 ± 4*	+35 ± 6*†	+43 ± 4*	+48 ± 2*
Cardiac index (ml min^{-1} 100 g^{-1})	−3.1 ± 0.6*	−5.0 ± 1.1*†	−10.6 ± 1.0*	−13.3 ± 1.0*†
Peak aortic flow (ml min^{-1} 100 g^{-1})	−12 ± 2*	−21 ± 3*†	−35 ± 4*	−42 ± 4*†
$+dF/dt_{max}$ (l min^{-2} 100 g^{-1})	−75 ± 11*	−119 ± 18*†	−189 ± 17*	−208 ± 12*†
Total peripheral conductance (μl min^{-1} mmHg^{-1} 100 g^{-1})	−49 ± 7*	−73 ± 12*†	−116 ± 13*	−137 ± 10*†
Stroke index (μl beat^{-1} 100 g^{-1})	−3 ± 2	−7 ± 2*	−18 ± 3*	−25 ± 2*†
Central venous pressure (cmH_2O)	−0.76 ± 0.44	+0.93 ± 0.56	+1.07 ± 0.40*	+2.61 ± 0.53*†

Values are mean ± s.e.mean, $n = 8$; * $P < 0.05$ versus baseline; † $P < 0.05$ LVP versus AVP.

Cardiac haemodynamic effects of arginine vasopressin and lysine vasopressin in the absence and presence of L-NAME

In the absence of L-NAME, LVP (0.7 pmol) had significantly greater effects than AVP on heart rate, mean arterial blood pressure, cardiac index, peak aortic flow, $+dF/dt_{max}$ and total peripheral conductance (Figure 3). At the higher dose, LVP had greater effects than AVP on all variables (Figure 4).

In the presence of L-NAME (1 mg kg^{-1} h^{-1}) the differences in the cardiac haemodynamic effects of the lower dose of LVP and AVP persisted (Table 3), but the pressor effects of the higher dose of LVP and AVP were not different (Table 3). However, this was against a background of LVP still causing significantly greater reductions than AVP in cardiac index and total peripheral conductance (Table 3).

Regional haemodynamic effects of arginine vasopressin and lysine vasopressin in the absence and presence of indomethacin

In the absence of indomethacin the differences in the regional haemodynamic effects of AVP and LVP (Figure 5) were as described above in the other groups under control conditions. In the presence of indomethacin (5 mg kg^{-1}, 5 mg kg^{-1} h^{-1}) the responses to the lower dose of AVP and LVP were unaffected (data not shown). However, the renal vasoconstrictor effects of the high dose of AVP and LVP (−49 ± 4 and −77 ± 1% maximum changes in vascular conductance, respectively) were significantly enhanced relative to the maximum responses in the absence of indomethacin (AVP −40 ± 3%; LVP −66 ± 4%; Figures 5 and 6) whereas the difference in the effects of AVP and LVP on the hindquarters vascular bed was abolished (Figure 6).

Discussion

In the present work we performed four separate experiments involving different groups of conscious Long Evans rats (31 rats in total) and found that, under normal conditions, LVP had significantly greater regional and cardiac haemodynamic effects than AVP. Such a difference has not been reported previously (see van Dyke *et al.*, 1956); indeed Altura (1963) found that AVP was more potent than LVP in causing mesenteric vasoconstriction *in vivo*. Other workers have used LVP instead of AVP in the rat (e.g. Waeber *et al.*, 1983; Lote *et al.*, 1987), although AVP is the endogenous peptide in this species.

Treatment with L-NAME, which inhibits nitric oxide production and endothelium-dependent vasorelaxation *in vitro* (Moore *et al.*, 1990; Hecker *et al.*, 1990; Dubbin *et al.*, 1990; Mülsch & Busse, 1990; Ishii *et al.*, 1990), at a dose sufficient to increase mean arterial blood pressure by 23 ± 3 mmHg did not abolish the differences in the regional vasoconstrictor effects of AVP and LVP. However, in the presence of a lower dose of L-NAME (0.3 mg kg^{-1} h^{-1}), that did not change baseline cardiovascular status, there were significant increases in the regional haemodynamic effects of AVP and LVP indicating that nitric oxide release was probably acting under normal conditions to oppose vasoconstrictor effects of both peptides, rather than just the response to AVP (Randall *et al.*, 1988).

In all three experiments in which the responses to high doses of AVP and LVP were assessed in the presence of L-NAME, the difference in the pressor effects of the peptides was abolished. This was due to enhancement of the pressor effect of AVP, with no change in the response to LVP. While these findings appear to indicate a selective enhancement of the pressor effects of AVP in the presence of L-NAME, the regional and cardiac haemodynamic profiles were still different following AVP and LVP. Thus, as mentioned above, the more potent vasoconstrictor effects of LVP persisted in the presence of L-NAME but they were buffered by more marked reductions in cardiac function than were seen with AVP. Hence, it may be that the reductions in cardiac function were directly releated to the increases in afterload and the pressor response to LVP represented the maximum achievable under these conditions.

Although other factors, such as changes in coronary vascular conductances and cardiac baroreflex mechanisms, may have contributed to the differences in the cardiac effects of AVP and LVP, there were no significant changes in cardiac baroreflex sensitivities in the presence of L-NAME, making it unlikely that the latter modified baroreflex control in such a way as to obscure an important contribution of nitric oxide to the differences in the regional vascular effects of AVP and LVP.

In the presence of indomethacin the renal vasoconstrictor effects of the high dose of AVP and LVP were enhanced, consistent with vasodilator cyclo-oxygenase products opposing the renal vasoconstrictor actions of the peptides under normal conditions (Hofbauer *et al.*, 1983; Walker, 1985; Lote *et al.*, 1987; Walker *et al.*, 1988). In contrast, there was a relative attenuation of the hindquarters vasoconstrictor response to LVP but not of that to AVP in the presence of indomethacin. This indicates that vasoconstrictor cyclo-oxygenase products might have contributed preferentially to the greater hindquarters vasoconstrictor effects of LVP under normal conditions.

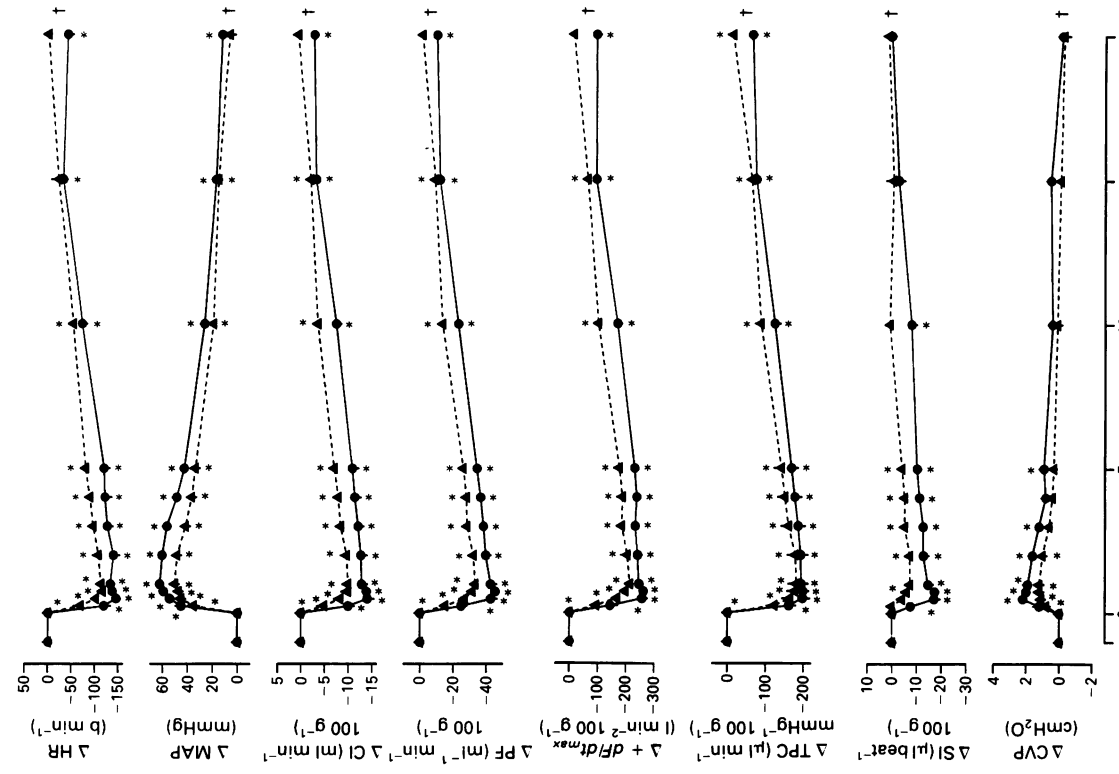

Figure 4 Cardiovascular changes (Δ) in conscious Long Evans rats ($n = 8$; the same animals as in Figure 3) following i.v. bolus injections of arginine vasopressin (AVP, ▲, 7.0 pmol) or lysine vasopressin (LVP, ●, 7.0 pmol). $^*P < 0.05$ versus baseline (Friedman's test); †$P < 0.05$ AVP versus LVP (Wilcoxon's test applied to areas under or over curves). Values are mean with s.e.mean shown by vertical lines; where the latter do not appear they lie within the area covered by the symbol. HR = heart rate; MAP = mean systemic arterial blood pressure; CI = cardiac index; PF = peak aortic flow; $+dF/dt_{max}$ = maximum rate of rise of aortic flow; TPC = total peripheral conductance; SI = stroke index; CVP = central venous pressure.

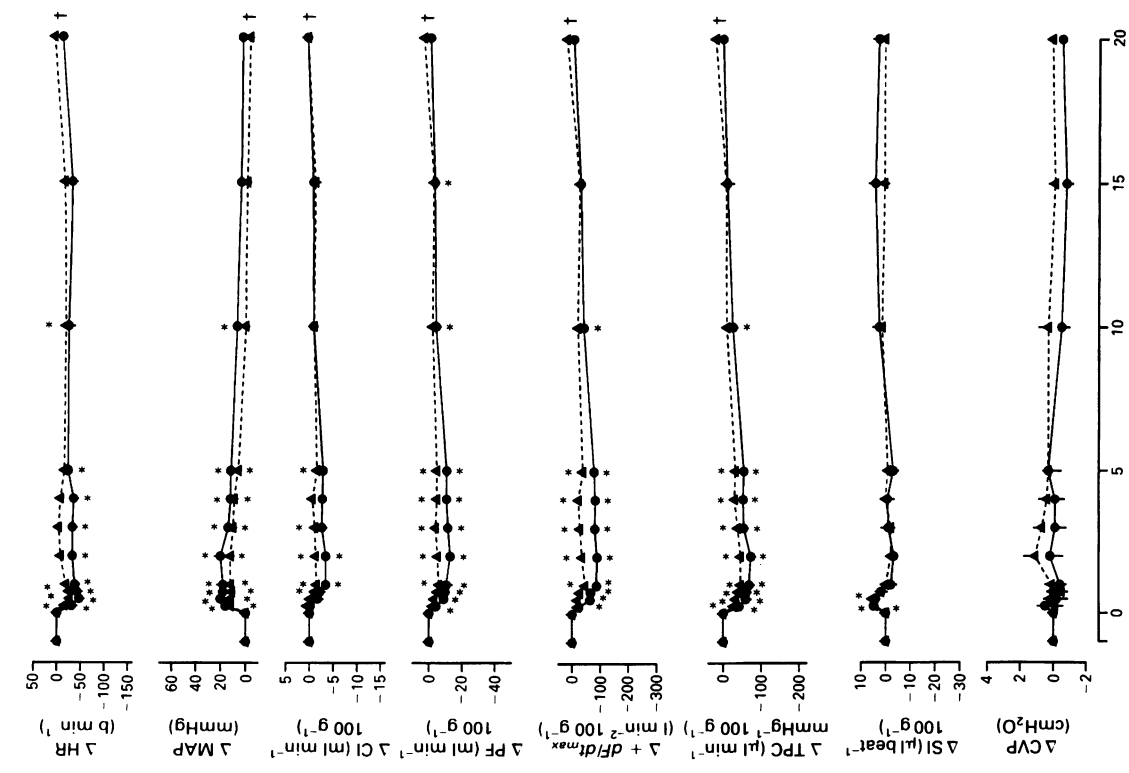

Figure 3 Cardiovascular changes (Δ) in conscious Long Evans rats ($n = 8$) following i.v. bolus injections of arginine vasopressin (AVP, ▲, 0.7 pmol) or lysine vasopressin (LVP, ●, 0.7 pmol). $^*P < 0.05$ versus baseline (Friedman's test) †$P < 0.05$ AVP versus LVP (Wilcoxon's test applied to areas under or over curves). Values are mean with s.e.mean shown by vertical lines; where the latter do not appear they lie within the area covered by the symbol. HR = heart rate; MAP = mean systemic arterial blood pressure; CI = cardiac index; PF = peak aortic flow; $+dF/dt_{max}$ = maximum rate of rise of aortic flow; TPC = total peripheral conductance; SI = stroke index; CVP = central venous pressure.

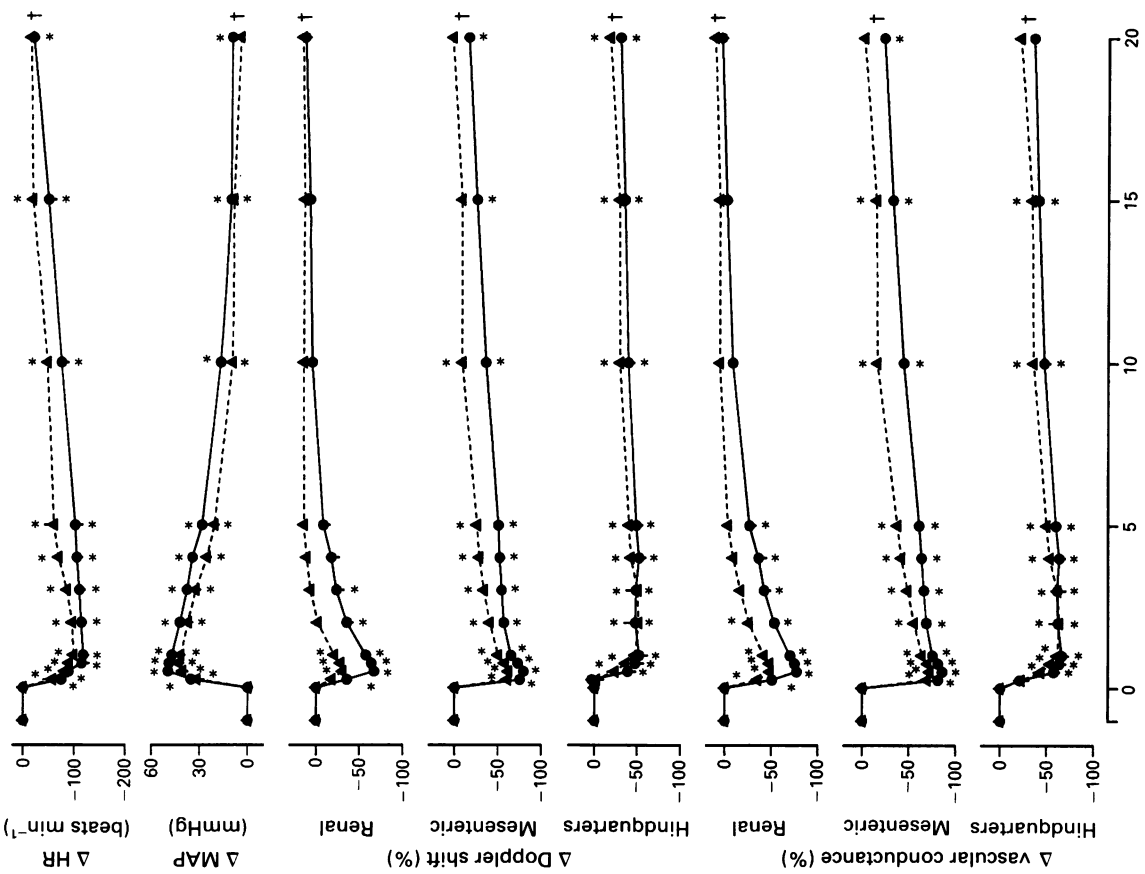

Figure 6 Cardiovascular changes (Δ) in conscious Long Evans rats ($n = 8$; the same animals as in Figure 5) following i.v. bolus injections of arginine vasopressin (AVP, ▲, 0.7 pmol) or lysine vasopressin (LVP, ●, 0.7 pmol) in the presence of indomethacin (5 mg kg^{-1} bolus followed by infusion at 5 mg kg^{-1} h^{-1}). * $P < 0.05$ versus baseline (Friedman's test); † $P < 0.05$ AVP versus LVP (Wilcoxon's test applied to areas under or over curves). Values are mean with s.e.mean shown by vertical lines; where the latter do not appear to lie within the area covered by the symbol. HR = heart rate; MAP = mean systemic arterial blood pressure.

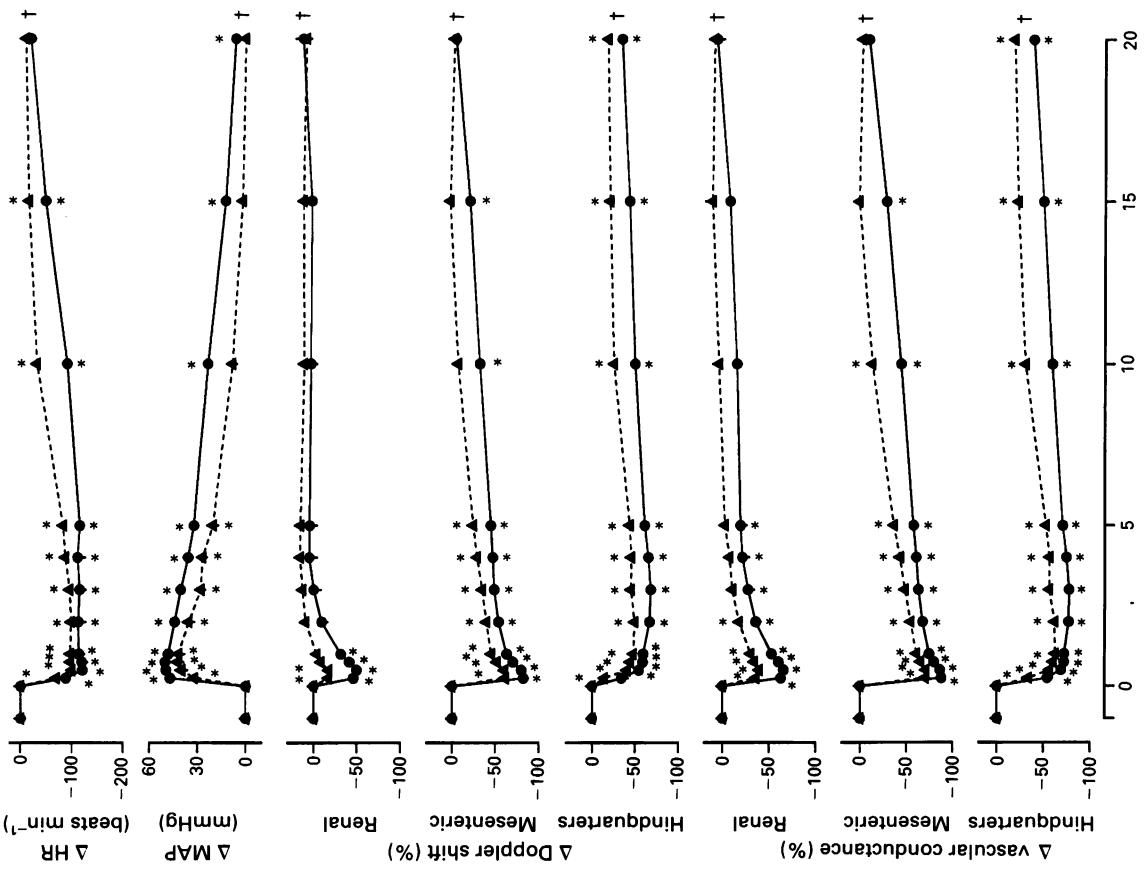

Figure 5 Cardiovascular changes (Δ) in conscious Long Evans rats ($n = 8$) following i.v. bolus injections of arginine vasopressin (AVP, ▲, 0.7 pmol) or lysine vasopressin (LVP, ●, 0.7 pmol). * $P < 0.05$ versus baseline (Friedman's test); † $P < 0.05$ AVP versus LVP (Wilcoxon's test applied to areas under or over curves). Values are mean with s.e.mean shown by vertical lines; where the latter do not appear they lie within the area covered by the symbol. HR = heart rate; MAP = mean systemic arterial blood pressure.

Nonetheless, in the presence of indomethacin the pressor and renal and mesenteric vasoconstrictor effects of LVP remained greater than those of AVP.

In conclusion, LVP exerts greater regional and cardiac haemodynamic effects than AVP and these differences persist in the presence of either L-NAME or indomethacin.

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Lack of correlation between the antiarrhythmic effect of L-propionylcarnitine on reoxygenation-induced arrhythmias and its electrophysiological properties

M. Barbieri, †P.U. Carbonin, *E. Cerbai, †G. Gambassi, Jr, †[2]P. Lo Giudice, *I. Masini, [1]A. Mugelli & †M. Pahor

Institute of Pharmacology, University of Ferrara; †Gerontology Division, Catholic University, Roma and *Department of Pharmacology, University of Firenze; Italy

1 The antiarrhythmic effect of L-propionylcarnitine (L-PC) was evaluated in the guinea-pig isolated heart; arrhythmias were induced with hypoxia followed by reoxygenation and by digitalis intoxication.

2 L-PC 1 μM, was found to be the minimal but effective antiarrhythmic concentration against reoxygenation-induced ventricular fibrillation. No antiarrhythmic effect was observed against digitalis-induced arrhythmias. D-Propionylcarnitine, L-carnitine and propionic acid did not exert antiarrhythmic effects.

3 During hypoxia and reoxygenation L-PC consistently prevented the rise of the diastolic left ventricular pressure, and significantly reduced the release of the cardiac enzymes creatine kinase (CK) and lactic dehydrogenase (LDH).

4 The electrophysiological effects of L-PC were then studied on either normal sheep cardiac Purkinje fibres or those manifesting oscillatory afterpotentials induced by barium or strophanthidin.

5 L-PC (1 and 10 μM) did not significantly modify action potential characteristics and contractility of normal Purkinje fibres, or the amplitude of OAP induced by strophanthidin or barium.

6 It is concluded that the antiarrhythmic action of L-PC on reoxygenation-induced arrhythmias is not correlated with its direct electrophysiological effects studied on normoxic preparations.

Introduction

Several studies have suggested that L-carnitine exerts protection of the ischaemic myocardium in both experimental animals and man (Folts *et al.*, 1978; Liedtke & Nellis, 1979; Thomsen *et al.*, 1979; Kamikawa *et al.*, 1984). It has been reported that exogenous administration of L-carnitine may counteract the myocardial depletion of endogenous carnitine stores, resulting in myocardial protection (Liedtke & Nellis, 1979; 1981; Liedtke *et al.*, 1982). L-Carnitine has also been reported to exert antiarrhythmic activity on ventricular arrhythmias caused by coronary ligation and/or reperfusion in the dog (Suzuki *et al.*, 1981; Kobayashi *et al.*, 1983; Imai *et al.*, 1984). Recently, it has been shown that L-propionylcarnitine (L-PC) protects the ischaemic myocardium more than L-carnitine or L-acetylcarnitine (Paulson *et al.*, 1986). An antiarrhythmic effect of L-PC on reperfusion-induced arrhythmias in isolated hearts from spontaneously hypertensive rats (Carbonin *et al.*, 1990) has also been observed.

Reperfusion- and reoxygenation-induced arrhythmias may be caused by a common electrophysiological arrhythmogenic stimulus. Some evidence suggests that oscillatory afterpotentials (OAPs) could represent this electrophysiological mechanism (Corr & Witkowsky, 1983; Manning & Hearse, 1984; Amerini *et al.*, 1985a; 1988). It is also well known that OAPs are responsible for digitalis-induced arrhythmias (Ferrier, 1977).

We thought that it would be interesting to evaluate the antiarrhythmic properties of L-PC (in comparison with those of equimolar concentrations of D-propionylcarnitine, L-carnitine, and propionic acid) on reoxygenation-induced arrhythmias. In an attempt to clarify the possible electrophysiological mechanism of its antiarrhythmic action, we evaluated the electrophysiological effects of antiarrhythmic concentrations of L-PC on the transmembrane potential characteristics of normal sheep Purkinje fibres and of preparations exposed to barium or strophanthidin and manifesting OAPs (Amerini *et al.*, 1985a; 1988). This approach has been extremely helpful in the understanding of the mechanism of class I antiarrhythmic drugs on reoxygenation-, reperfusion- and digitalis-induced arrhythmias (Amerini *et al.*, 1985a; 1988).

Thus, the aim of this study was to evaluate the antiarrhythmic effect of L-PC on a well characterized model of arrhythmias in order to obtain information on its possible mechanisms of action.

Methods

Perfusion of the isolated heart

Guinea-pigs (body weight: 350–400 g) were used. Thirty minutes after an intraperitoneal heparin injection (100 iu) the animals were killed by a sharp blow at the base of the skull. The hearts were rapidly excised and placed in ice-cold Tyrode solution and utilized for Langendorff perfusion. The details of the technique have been described elsewhere (Carbonin *et al.*, 1981). The hydrostatic aortic perfusion pressure was 8 kPa. The control medium was equilibrated at 37°C with 95% O_2 plus 5% CO_2 gas mixture and contained (in mM): NaCl 117.0, $NaHCO_3$ 23.0, KCl 4.6, NaH_2PO_4 0.8, $MgCl_2$ 1.0, $CaCl_2$ 2.0 and glucose 5.5. The O_2 and CO_2 partial pressures and pH values of the perfusion fluid were periodically determined by means of a gas analyser (Instrumentation Laboratories model 213).

Epicardial electrograms were recorded by means of an atraumatic electrode connected to an amplifier (E & M Instrument model V 1205). The left ventricular pressure was measured by inserting a 12 cm polyethylene catheter (0.5 mm diameter) into the left ventricle through the ventricular wall

[1] Author for correspondence at Institute of Pharmacology, Via Fossato di Mortara 64/b, 44100 Ferrara, Italy.

[2] Present address: Institute for Research on Senescence, Sigma Tau, Pomezia, Roma, Italy.

and was recorded by means of a pressure transducer (Statham P23) connected to a pressure amplifier (E & M Instrument model V 2203). All data were recorded on paper with an E & M Instrument model VR 12 Simultrace recorder. The coronary flow rate was measured by collecting the effluent.

Rhythm disturbances were subdivided into: conduction disturbances (sino-atrial and atrio-ventricular blocks) and ventricular tachyarrhythmias: (a) ventricular fibrillation (VF) and (b) ventricular arrhythmias (VA) including ventricular premature beats (VPB) and ventricular tachycardia (VT). A large and aberrant QRS complex and the absence of a preceding P wave identified VPB. More than 5 consecutive VPBs were considered VT. Complete morphological irregularity of at least 10 complexes was considered VF. VF and VA were quantified by counting the number of hearts that exhibited VF or VA over 1 min periods. As VPB or VT could obviously not be evaluated in the fibrillating hearts, the hearts manifesting VF were excluded from VA analysis.

After 20 min of control perfusion to obtain the stabilization of heart rate and ventricular function, the hearts in one group were exposed to hypoxia followed by reoxygenation and in a second group to perfusion with digitalis.

Hypoxia was produced by gassing the medium with a mixture of 95% N_2 plus 5% CO_2 (O_2 partial pressure < 1.33 kPa). During the hypoxic period the hearts were perfused with a glucose-free medium. After 15 min of hypoxia, the perfusion with the oxygenated and glucose-containing medium was rapidly restored and maintained for 15 min (reoxygenation phase). Experiments of hypoxia and reoxygenation were performed in 130 hearts. They were exposed to 9 different treatment protocols as follows: controls ($n = 20$), 0.1 μM L-PC ($n = 15$), 1 μM L-PC ($n = 20$), 10 μM L-PC ($n = 15$), 1 μM L-carnitine ($n = 10$), 1 μM D-propionylcarnitine ($n = 10$) and 1 μM propionic acid ($n = 10$) added to the medium during the whole hypoxic and reperfusion periods, 1 μM L-PC ($n = 15$) and 10 μM L-PC ($n = 15$) added to the medium only during reoxygenation.

Digitalis intoxication was obtained by perfusing the heart with 1 μM digoxin for 30 min. Four digitalis experiments were used as controls and in four experiments 1 μM L-PC was added to the medium 10 min before and during superfusion with digoxin.

Determination of creatine kinase and lactic dehydrogenase in the effluent

Samples of cardiac effluent were collected periodically and assayed for creatine kinase (CK) and lactic dehydrogenase (LDH) content with kits obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). All determinations were done on a Pye-Unicam spectrophotometer. The results were related to the ventricular wet mass.

Electrophysiological studies

Sheep cardiac Purkinje fibres were excised from the ventricles and kept in oxygenated Tyrode solution until used. One strand was mounted in a tissue bath and superfused with Tyrode solution at a rate of 8 ml min^{-1}. The composition of solution was (mM): NaCl 137.0, $NaHCO_3$ 11.9, KCl 4, NaH_2PO_4 0.42, $MgCl_2$ 0.5, $CaCl_2$ 2.7, and glucose 5. The Tyrode solution was equilibrated with 97% O_2 and 3% CO_2 (pH 7.4). One end of the preparation was fixed to the Sylgard floor of the tissue bath and the other was connected to an isometric force transducer (Mangoni GC01).

The preparations were stimulated with rectangular pulses (0.5 to 1 ms in duration and 1.5 times the threshold) through bipolar silver electrodes electrically insulated except for the tip. Action potentials were recorded by means of two 3 M KCl-filled glass microelectrodes, one of which was inserted intracellularly and the other placed in the solution close to the preparation. Microelectrodes were coupled to two high-input impedance guard electrometer amplifiers (Bigongiari, Firenze). The action potential was displayed on a Tektronix (model 5113) dual beam storage oscilloscope and recorded on an FM tape recorder (Racal 14 DS). The records were played back into a chart recorder (Gould Brush 2400). An automated analysis of action potential was performed, as previously described (Fusi *et al.*, 1984). Evaluation of the following parameters was carried out: action potential amplitude, overshoot, maximum diastolic potential, $\dot{V}_{max}$, action potential duration at −60 mV (APD_{-60}) and at 90% of repolarization (APD_{90}).

OAPs were induced by exposing the preparations to low barium concentration or to strophanthidin, as described elsewhere (Mugelli *et al.*, 1983; Amerini *et al.*, 1985a,b; 1988). The drive stimulus was interrupted periodically (usually every minute for 30 s) to assess the presence of OAPs. Drugs were superfused at increasing concentrations; the effect of each concentration was followed for at least 20 min.

The drugs used in this study were chemically pure: L-carnitine, L-propionylcarnitine, D-propionylcarnitine, propionic acid (Sigma Tau), digoxin (Boehringer Biochemia Robin), strophanthidin (Sigma).

Statistical analysis

Results were expressed as means ± standard error of the mean. Statistical analysis was performed by means of the Student's *t* test, the Fisher exact test, the chi-squared test and ANOVA corrected for multiple measures with Scheffes' procedure. To compare CK and LDH release during hypoxia and reoxygenation between control and treated hearts, ANOVA for multiple measures statistic was used (MANOVA procedure of the SPSS/PC+ software). A $P < 0.05$ (two tailed) was considered statistically significant.

Results

Effect of L-propionylcarnitine on reoxygenation-induced arrhythmias

Perfusion with a hypoxic glucose-free medium caused, as expected, a decrease in ventricular rate (Table 1) followed by conduction disturbances (Carbonin *et al.*, 1981; Amerini *et al.*, 1985a; 1988). Reoxygenation was associated with the rapid development of ventricular arrhythmias in all the control hearts and 55% of them developed ventricular fibrillation (VF) (Figure 1a,b). The lowest but effective concentration of L-PC on reoxygenation-induced VF was 1 μM when the drug was added to the medium during the hypoxic and reoxygenation phases (Figure 1). The effect was dose-dependent, with a higher concentration (10 μM) being more effective on VF and also reducing significantly ventricular arrhythmias (VA). L-PC (0.1 μM) did not significantly modify the incidence of VA or VF (Figure 1a,b). The rise of the diastolic left ventricular pressure which was consistently observed during hypoxia and

Table 1 Effect of L-propionylcarnitine (L-PC) on heart rate (beats min^{-1}) during hypoxia and reoxygenation

				L-PC	
	Time	*Control*	*0.1 μM*	*1 μM*	*10 μM*
Baseline	0	254 ± 14	255 ± 12	258 ± 10	259 ± 9
Hypoxia	5	142 ± 8	146 ± 11	146 ± 9	135 ± 11
	10	104 ± 14	108 ± 10	104 ± 11	101 ± 8
	15	85 ± 14	78 ± 9	80 ± 11	78 ± 11
Reoxygen.	2	187 ± 16	191 ± 15	194 ± 17	191 ± 16
	4	205 ± 10	218 ± 16	212 ± 18	214 ± 16
	6	227 ± 6	234 ± 11	225 ± 8	233 ± 9
	10	250 ± 7	253 ± 9	247 ± 6	249 ± 8
	15	249 ± 10	254 ± 8	253 ± 6	251 ± 9

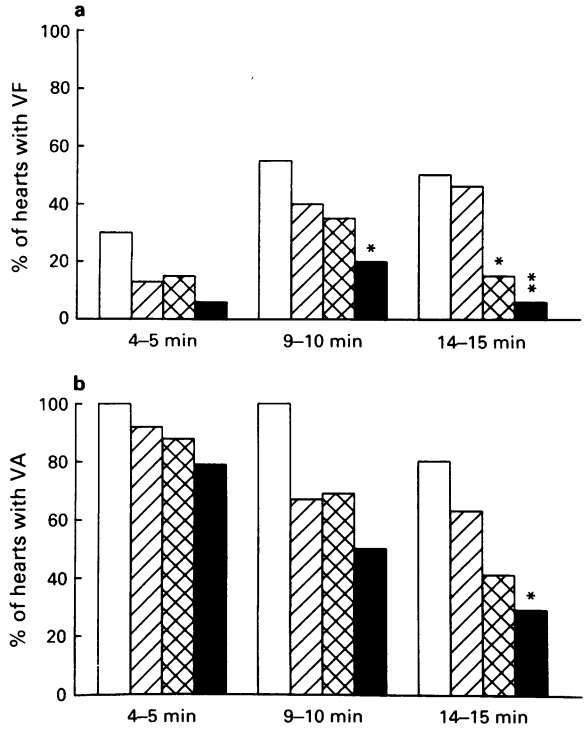

Figure 1 Guinea-pig isolated hearts: effect of increasing concentrations of L-propionylcarnitine (L-PC) (added during hypoxia and reoxygenation) on ventricular fibrillation (a) and on ventricular arrhythmias (b). The columns show the percentage of hearts in which ventricular fibrillation or ventricular arrhythmias were observed over 1 min periods of reoxygenation at the time indicated on the abscissa scale. (Control open columns; 0.1 μM L-PC hatched columns; 1 μM L-PC cross-hatched columns; 10 μM L-PC solid columns. * $P < 0.05$, ** $P < 0.01$ vs. control).

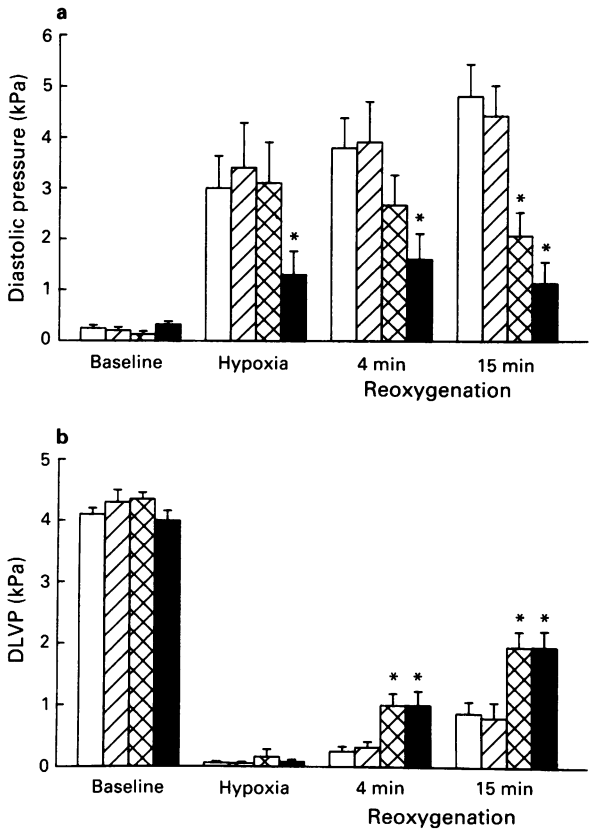

Figure 2 Guinea-pig isolated hearts: variations of the left ventricular diastolic pressure (a) and of developed left ventricular pressure (DLVP) (b) after baseline perfusion (0 min), during hypoxia and reoxygenation of control hearts $n = 20$ (open columns), and hearts perfused with 0.1 μM L-propionylcarnitine (L-PC), $n = 15$ (hatched columns); 1 μM L-PC, $n = 20$ (cross-hatched columns) and 10 μM L-PC, $n = 15$ (solid columns). Vertical bars show s.e.mean. L-PC was added during hypoxia and reoxygenation. * $P < 0.05$ vs. control.

reoxygenation was also prevented by L-PC in a dose-dependent manner (Figure 2a). Consequently, the recovery of the developed left ventricular pressure during reoxygenation was more pronounced in the groups of hearts perfused with L-PC (Figure 2b). The release of cardiac enzymes (CK and LDH) in the effluent was significantly reduced by 1 μM L-PC during hypoxia and reoxygenation (Figure 3); however, L-PC did not significantly influence the heart rate and the coronary flow rate (Tables 1 and 2).

The antiarrhythmic effect of L-PC is less pronounced when it is added to the medium only during the period of reoxygenation. In this case, in fact, the results obtained with 1 μM L-PC were not significantly different from controls whereas 10 μM L-PC still significantly inhibited VF (Figure 4).

At a dose of 1 μM, L-carnitine, D-propionylcarnitine and propionic acid added to the medium during hypoxia and reoxygenation had no significant effects on reoxygenation-induced VA or VF compared to controls. In fact, after 15 min of reoxygenation, the percentage of VA and VF were, respectively, 67% and 40% (L-carnitine, $n = 10$), 67% and 50% (D-propionylcarnitine, $n = 10$), 80% and 50% (propionic acid, $n = 10$), 80% and 50% (control, $n = 20$).

Experiments with digoxin in the isolated heart

Perfusion with 1 μM digoxin induced severe VA in all the isolated hearts ($n = 4$) within 4–7 min. This effect is consistent with our previous observations (Amerini *et al.*, 1985a; 1988). Addition of 1 μM L-PC ($n = 4$) to the medium 10 min before and during perfusion with digoxin did not modify the incidence and severity of VA. In fact in both groups, 3 out of 4 hearts developed VF within 4–10 min. Furthermore the time necessary for the appearance of VA was similar in control and L-PC-treated hearts (6 ± 2 min and 7 ± 3 min respectively, NS).

Electrophysiological effects of L-propionylcarnitine in Purkinje fibres

The electrophysiological and mechanical effects of L-PC (1 and to 10 μM) on electrically driven sheep Purkinje fibres are shown in Table 3. It is apparent that L-PC did not modify the action potential characteristics and the contractile force.

L-PC (1 and 10 μM) did not significantly modify OAP amplitude in strophanthidin-treated preparations ($n = 8$) (5.6 ± 3.6 and 5.8 ± 1.8 mV, respectively, vs 5.2 ± 2.2 mV of the control). In barium-treated preparations ($n = 16$), L-PC caused a reduction of OAP amplitude, from 7.8 ± 2.2 in control to 6.2 ± 1.4 mV with 1 μM L-PC and 3.9 ± 1.0 mV with 10 μM

Table 2 Effect of L-propionylcarnitine (L-PC) on coronary flow (ml min^{-1})

	Time	*Control*	*L-PC* 0.1 μM	1 μM	10 μM
Baseline	0	16.2 ± 0.7	15.6 ± 0.7	15.8 ± 0.6	15.4 ± 0.9
Hypoxia	5	15.5 ± 0.9	14.9 ± 0.8	15.1 ± 0.9	14.7 ± 1.0
	10	7.1 ± 0.7	6.4 ± 0.7	6.7 ± 0.5	6.7 ± 0.8
	15	4.8 ± 0.6	3.9 ± 0.8	3.9 ± 0.7	4.5 ± 0.6
Reoxygen.	2	8.2 ± 1.0	7.3 ± 0.9	7.5 ± 0.8	9.0 ± 1.3
	4	10.7 ± 0.8	10.5 ± 0.7	9.8 ± 0.7	11.1 ± 1.0
	6	13.3 ± 0.8	12.6 ± 0.8	12.7 ± 0.7	13.3 ± 0.9
	10	14.3 ± 0.6	13.6 ± 0.6	13.7 ± 0.5	14.4 ± 0.9
	15	14.7 ± 0.5	14.1 ± 0.6	14.0 ± 0.5	14.4 ± 0.6

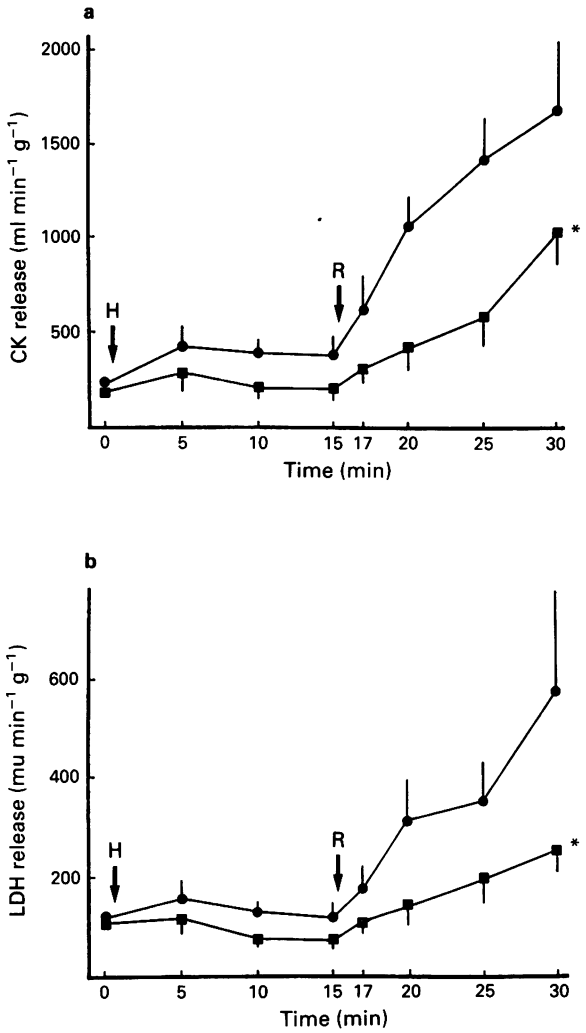

Figure 3 Guinea-pig isolated hearts: variations of creatine kinase (CK) (a) and of lactic dehydrogenase (LDH) (b) release in the effluent after baseline perfusion (0 min), during hypoxia (H) and during reoxygenation (R) in control hearts $n = 10$ (●) and in hearts perfused with 1 μM L-propionylcarnitine $n = 10$ (■). Vertical lines show s.e.mean. * $P < 0.05$ vs. control refers to the curve analysed by ANOVA for multiple measures.

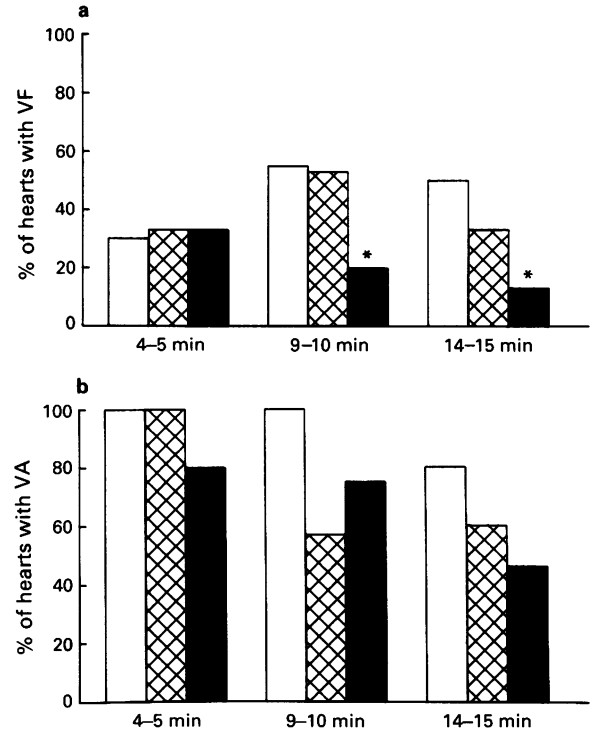

Figure 4 Guinea-pig isolated hearts: effect of increasing concentrations of L-propionylcarnitine (L-PC) added only during reoxygenation on ventricular fibrillation (VF) (a) and on ventricular arrhythmias (VA) (b). The columns show the percentage of hearts in which VF or VA was observed over 1 min periods of reoxygenation. Control (open columns), 1 μM L-PC (cross-hatched columns), 10 μM L-PC (solid columns). * $P < 0.05$ vs control.

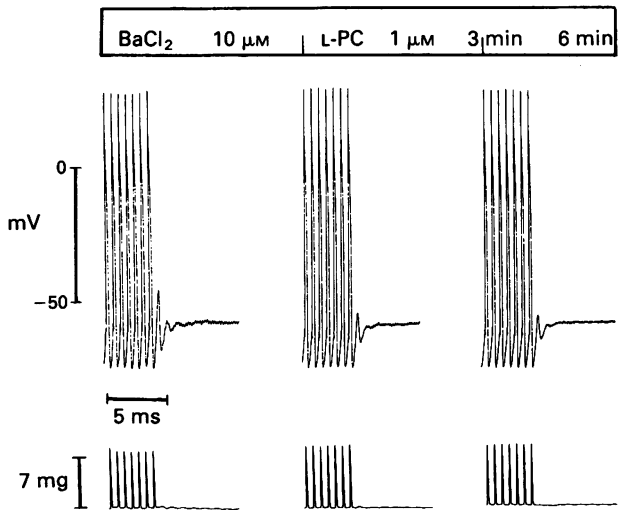

Figure 5 Effect of 1 μM L-propionylcarnitine (L-PC) on barium (10 μM)-induced oscillatory afterpotentials. Each panel shows the electrical (upper traces) and mechanical (lower traces) activity recorded during and after the interruption of the stimulation.

L-PC. The effect of L-PC, however, was not statistically significant due to its variability. In fact, while in 2 cases L-PC did not cause any change of the OAP amplitude, it decreased OAP amplitude in 50% of the remaining cases, and increased it in the others. A typical experiment in which L-PC caused a decrease of OAP amplitude is shown in Figure 5. It is apparent that superfusion with L-PC caused a reduction of the OAP amplitude despite the increase in contractile force. No clearcut correlation was observed between the effects of L-PC on OAP amplitude and contractility.

Table 3 Effect of L-propionylcarnitine (L-PC) on the transmembrane action potential and mechanical activity of driven (1 Hz) Purkinje fibres of sheep

	OS (mV)	*MDP* (mV)	*AP* (mV)	APD_{-60} (ms)	APD_{90} (ms)	$\dot{V}_{max}$ (V/s)	*Contraction* (%)
Control ($n = 7$)	40.0 ± 1.6	83.0 ± 0.8	123.0 ± 1.6	316 ± 27	352 ± 30	583 ± 81	100
L-PC 1 μM	39.7 ± 1.6	83.7 ± 0.7	123.5 ± 1.5	315 ± 33	351 ± 35	564 ± 80	103 ± 11
L-PC 10 μM	39.5 ± 1.5	83.8 ± 1.0	123.4 ± 1.2	323 ± 33	358 ± 33	570 ± 89	93 ± 6

Data are presented as means ± s.e.mean. The number in parentheses indicates the number of experiments. OS = overshoot, MDP = maximum diastolic potential, AP = action potential amplitude, APD_{-60} and APD_{90} = action potential duration at −60 mV and 90% repolarization, respectively, $\dot{V}_{max}$ = maximum rate of upstroke.

Discussion

The present results demonstrate that L-PC (and not L-carnitine) exerts antiarrhythmic effects versus reoxygenation-induced arrhythmias. This effect appears to be associated with a protection of the hypoxic and reoxygenated myocardium: in fact the release of CK and LDH is significantly reduced by L-PC and the rise of diastolic left ventricular pressure is prevented. As a consequence, developed left ventricular pressure after reoxygenation is greater in the L-PC-treated hearts than in controls. This 'protection' does not appear to be connected to an effect on heart rate or coronary flow, both of which are unaffected by L-PC-treatment.

We also evaluated the possibility that the antiarrhythmic action of L-PC could be due to a direct effect on the electrophysiological properties of the heart. However, concentrations of L-PC that are antiarrhythmic are devoid of any electrophysiological effect in normal Purkinje fibres. L-PC has recently been reported to affect action potential duration of canine Purkinje fibres but only at millimolar concentrations (Aomine *et al.*, 1989). Similar behaviour has been described in guinea-pig ventricular muscle under acidic conditions (Aomine & Arita, 1987) and after addition of amphiphilic lipids (Aomine *et al.*, 1988). Thus, it is unlikely that modifications of refractoriness could play a relevant role under our experimental conditions at micromolar concentrations of L-PC.

L-PC was able to reduce the rise of diastolic left ventricular pressure observed during hypoxia and reoxygenation; the increase in diastolic left ventricular pressure is considered an expression of the intracellular calcium overload (Poole-Wilson *et al.*, 1984). Calcium overload may result in OAPs and triggered activity (Manning & Hearse, 1984). One could consequently expect a reduction of the amplitude of OAPs as a result of the effect of L-PC. This is not the case, since L-PC is not able to affect significantly OAP amplitude in two different experimental conditions, i.e. in barium- and strophanthidin-treated preparations that we have described previously (Mugelli *et al.*, 1983; Amerini *et al.*, 1985b) and which are certainly suitable for the study of antiarrhythmic effects of drugs (Amerini *et al.*, 1985a,b; 1988). However, since OAPs play a fundamental role in digitalis-induced arrhythmias (Ferrier, 1977) and L-PC was completely ineffective against digitalis-induced arrhythmias, it appears unlikely that L-PC can exert its protective effect on reoxygenation-induced arrhythmias through action of OAPs induced by ischaemia/reperfusion. However, we did not study the effects of L-PC on the electrophysiological properties of the guinea-pig heart under ischaemic or hypoxic conditions; thus the possibility cannot be excluded that L-PC might exert some direct electrophysiological effect under those circumstances.

The effect of L-PC on diastolic tension and possibly on calcium overload appears to be operative only when the cause is an hypoxic (present results) or ischaemic (Paulson *et al.*, 1986) insult, followed by reoxygenation or reperfusion, respectively. However, the mechanism by which these effects occur remains unsettled.

Finally, these results confirm the data in the literature showing that L-PC is more effective than L-carnitine (Paulson *et al.*, 1986; Siliprandi *et al.*, 1987; Subramian *et al.*, 1987) and demonstrate that the action we described is specific for L-PC.

In conclusion, L-PC in micromolar concentrations is able to protect the heart from a period of hypoxia followed by reoxygenation, an antiarrhythmic effect which cannot be explained by its direct electrophysiological properties on normoxic preparations.

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The effects of calcitonin gene-related peptide on submucosal gland secretion and epithelial albumin transport in the ferret trachea *in vitro*

S.E. Webber, J.C.S. Lim & J.G. Widdicombe

Department of Physiology, St George's Hospital Medical School, Cranmer Terrace, London SW17 0RE

1 We have examined the effect of calcitonin gene-related peptide (CGRP) on basal mucus volume, lysozyme and albumin outputs from the ferret whole trachea *in vitro*, and on the outputs produced by methacholine and substance P (SP). We have also examined the effect of inhibiting neutral enkephalinase with thiorphan on the responses to CGRP.

2 CGRP (1–100 nM) produced small concentration-dependent increases in basal mucus volume, lysozyme and albumin outputs. These effects of CGRP were enhanced by thiorphan. The increases in basal outputs with CGRP and the potentiation by thiorphan were considerably less than previously observed with SP and neurokinin A (NKA). CGRP had no significant effect on potential difference (PD) across the trachea.

3 CGRP produced a concentration-dependent inhibition of methacholine- and SP-induced lysozyme output but a concentration-dependent increase in methacholine- and SP-induced albumin output. The effects of CGRP on methacholine-induced lysozyme and albumin outputs were enhanced by thiorphan. CGRP weakly inhibited methacholine-induced mucus volume output and weakly enhanced SP-induced mucus volume output.

4 Thus, CGRP weakly stimulates basal serous cell secretion and epithelial albumin transport, but does not alter epithelial integrity. CGRP inhibits the serous cell secretion due to methacholine or SP, but potentiates the epithelial albumin transport produced by these agents. The interaction between CGRP and other sensory neuropeptides or muscarinic agonists on airway submucosal glands and epithelium may be important in the normal airway and in inflammatory airway diseases where release of sensory neuropeptides is enhanced.

Introduction

Calcitonin gene-related peptide (CGRP) is a 37 amino acid peptide which is derived from alternative processing of the calcitonin gene mRNA transcript, and which was first characterized in neural tissue (Rosenfeld *et al.*, 1983). CGRP has been found in numerous species including man and has actions in many different organs e.g. neural tissue, striated muscle, cardiac muscle, vasculature (including that of the airways) and bone (Breimer *et al.*, 1988).

CGRP has been localized to the airways of a number of species including guinea-pig, rat, ferret and man (Palmer *et al.*, 1987). High affinity binding sites for CGRP have been demonstrated in rat visceral organs including lung (Nakamuta *et al.*, 1986). In human lung preparations CGRP was detected by radioimmunoassay, with the highest concentrations found in the cartilaginous airways. Autoradiographic studies have shown CGRP binding sites in the human lung which are particularly densely distributed over smooth muscle with some labelling over seromucous glands but no apparent binding over epithelium or smooth muscle (Mak & Barnes, 1988). CGRP has been localized to nerves and ganglia in human airways, by use of immunocytochemistry, and particularly in association with sensory nerves. In the rat, CGRP immunoreactive fibres have been localized to smooth muscle, seromucous glands, beneath and within the epithelium and in association with blood vessels (Cadieux *et al.*, 1986). From immunohistochemical distribution and capsaicin depletion studies, it is thought likely that CGRP co-exists with a number of other peptides, especially substance P (SP) and neurokinin A (NKA), in these primary sensory nerves (Lundberg *et al.*, 1985).

When released CGRP has potent effects on the airways. It produces a concentration-dependent contraction of human bronchi *in vitro*, and is more potent than either SP or carbachol (Palmer *et al.*, 1987). CGRP is also a potent and long-lasting vasodilator of tracheobronchial blood vessels *in vitro* (McCormack *et al.*, 1989) and *in vivo* (Salonen *et al.*, 1988) and can increase airway microvascular permeability (Aursudkij *et al.*, 1988). There is also evidence that CGRP can interact with other sensory nerve peptides such as SP in the skin (Gamse & Saria, 1985) and airways (Gatto *et al.*, 1989).

Thus, there is evidence that CGRP is localized to airway submucosal glands and epithelium, has potent effects on some airway tissues and can interact with other sensory transmitters such as SP. However, the effects of CGRP on submucosal gland secretion and epithelial transport mechanisms, and the interaction with other pharmacological agents including SP on these tissues have not yet been studied. It is also not known if inhibiting neutral enkephalinase enhances any action of CGRP on airway tissues. Therefore we have used the ferret whole trachea *in vitro* (Webber & Widdicombe, 1987) to examine the effect of CGRP on submucosal gland secretion including lysozyme secretion from serous cells, and on epithelial albumin transport (Webber & Widdicombe, 1989) in the presence and absence of thiorphan. We have also examined the interaction of CGRP with SP and methacholine on these parameters.

Methods

The ferret in vitro *trachea*

Ferrets of either sex, weighing 0.5–1.5 kg, were anaesthetized by an intraperitoneal injection of sodium pentobarbitone (Sagatal, May & Baker, 50 mg kg^{-1}). The trachea was exposed and cannulated about 5 mm below the larynx with a perspex cannula containing a conical collecting well (Webber & Widdicombe, 1987). The ferret was then killed with an overdose of sodium pentobarbitone injected into the heart. The chest was opened along the midline and the trachea exposed to the

carina, cleared of adjacent tissue, removed and cannulated just above the carina. The trachea was mounted, laryngeal end down, in a jacketed organ bath with Krebs-Henseleit buffer restricted to the submucosal side. The composition of the Krebs-Henseleit solution was (mM): NaCl 120.8, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ $7H_2O$ 1.2, $NaHCO_3$ 24.9, $CaCl_2$ 2.4, glucose 5.6. The buffer was maintained at 37°C and gassed with 95% O_2/5% CO_2. The lumen of the trachea remained air-filled. Secretions were carried by gravity and mucociliary transport to the lower cannula, where they pooled and could be withdrawn periodically into a polyethylene catheter which was inserted into the lower cannula to form an airtight seal. The catheters containing the secretions were sealed at both ends with bone wax, numbered and stored frozen until required.

After defrosting, the secretions were washed out of the catheters into labelled plastic vials with 0.5 ml distilled H_2O. The vials were frozen and stored for use in the albumin and lysozyme assays. Preliminary experiments had shown that frozen storage for up to 6 months does not affect the enzymatic activity of lysozyme or the albumin content. Secretion volumes were estimated by the differences in weights of the catheters with secretions and dried without secretions, and the secretion rates were expressed as $\mu l\,min^{-1}$ (assuming 1 g of secretion is equivalent to 1 ml).

The electrical potential difference (PD) across the tracheal wall was measured with two calomel reference electrodes. These were filled with 3.8 M KCl and placed in separate beakers of the same solution. Electrical contact was made with the preparation by use of two agar bridges. These were constructed from polyethylene tubing (0.5 mm internal diameter) filled with 3.8 M KCl in 2.5% w/v agar solution. One bridge was placed in the buffer on the submucosal side of the trachea and the second inserted into a second hole in the perspex cannula used to collect the mucus. Electrical contact between this bridge and the tracheal luminal wall was maintained by the mucus collecting in the perspex cannula. Output from the two electrodes was into a high input impedance buffer amplifier and then displayed on a digital voltmeter. The two agar bridges were initially placed together in 0.15 M NaCl to confirm that this produced a stable potential difference close to 0 V. Any residual voltage measured here was subtracted from subsequent measurements of potential difference made in the preparation.

Before the start of an experiment each trachea was allowed to equilibrate for 20 min, and during this time changes of bathing medium were made every 5 min.

Assay for lysozyme

The lysozyme concentrations of the mucus samples were measured by a turbidimetric assay which relies on the ability of lysozyme to break down the cell wall of the bacterium *Micrococcus lysodeikticus*. Addition of lysozyme to a solution of the bacteria reduces the turbidity of the solution, thereby leading to a fall in optical density (OD) measured at 450 nm.

A stock suspension of *M. lysodeikticus* of $3\,mg\,ml^{-1}$ was prepared. When diluted 10 fold (the dilution in the assay) this suspension gives an OD of approximately 0.6 at 450 nm. To produce a standard curve, various concentrations of hen egg white lysozyme (0.5 to $100\,ng\,ml^{-1}$) were incubated in duplicate in 1.5 ml potassium phosphate buffer (50 mM, pH 7.4) containing *M. lysodeikticus* ($0.3\,mg\,ml^{-1}$), sodium azide ($1\,mg\,ml^{-1}$) and bovine serum albumin (BSA, $1\,mg\,ml^{-1}$). The BSA was included in the assay for its protein stabilizing effects and the sodium azide was added to prevent the growth of bacteria in the incubating solutions. The reaction mixtures were incubated for 18 h at 37°C. After incubation the OD of each solution was measured at a wavelength of 450 nm with potassium phosphate buffer pH 7.4 containing BSA ($1\,mg\,ml^{-1}$) as a blank. The standard curve was constructed by plotting the fall in OD (reduction in turbidity) against the concentration of lysozyme in the solution.

To estimate the concentration of lysozyme in a mucus sample, 20 μl of sample was incubated in 1.5 ml potassium phosphate buffer (50 mM, pH 7.4), as described above for the known concentrations of lysozyme used in the preparation of the standard curve. The lysozyme concentrations (equivalent to hen egg white lysozyme) of the 20 μl samples and hence of the original mucus samples were estimated from the standard curve. The rate of output of lysozyme was then calculated by dividing the total amount of lysozyme in a mucus sample by the time over which the sample accumulated.

Albumin transport

To examine the effect of CGRP on methacholine-induced transport of albumin across the ferret trachea, BSA was added to the buffer bathing the submucosal surface of the trachea in a concentration of $4\,mg\,ml^{-1}$. Fluorescent BSA ($0.02–0.03\,mg\,ml^{-1}$) was also added to the buffer as a marker and enabled an estimate to be made of the total amount of albumin which appeared in the mucus samples.

The fluorescence of the mucus samples was measured with a fluorimeter, using an excitation wavelength of 550 nm and an emission wavelength of 490 nm. The fluorescent albumin concentration of the mucus samples was estimated from a standard curve relating fluorescence (arbitrary units) to the concentration of fluorescent albumin (range $25\,ng\,ml^{-1}$ to $3\,\mu g\,ml^{-1}$). The total concentration of albumin in the mucus samples was obtained by multiplying the fluorescent albumin concentration (estimated from the standard curve) by the ratio of non-fluorescent to fluorescent albumin used in the experiment. The rate of output of albumin was determined by dividing the total amount of albumin in a mucus sample by the time over which that sample accumulated.

Experimental protocol

Effect of CGRP on baseline secretion After a 30 min control period, three concentrations (1–100 nM) of CGRP were added to the buffer surrounding the trachea in a random sequence. Each concentration of CGRP was left in contact with the trachea for 30 min. After each 30 min, any secretion produced was withdrawn and processed as described above. One or two control periods of 30 min were allowed between each addition of peptide. After three concentrations of CGRP had been added to the trachea, the buffer surrounding the trachea was replaced with buffer containing thiorphan. This buffer was left in contact with the trachea for 30 min and any mucus produced was withdrawn and processed. The same three concentrations of CGRP were then added to the trachea in the same sequence as above, with the same number of control periods between peptide additions. The CGRP was always diluted and added to the trachea in buffer containing thiorphan. Changes in mucus volume output produced by CGRP were calculated as the difference in the mucus volume output obtained between the control period immediately before the peptide was added and the period when the peptide was in the organ bath. All mucus samples obtained in these experiments were assayed for lysozyme and albumin.

Effect of CGRP on methacholine- and SP-induced secretion Previous studies have shown that both methacholine and SP produce concentration-dependent increases in mucus volume, lysozyme and albumin outputs from the ferret trachea (Webber & Widdicombe, 1987; Webber, 1989). After a 30 min control period either methacholine (20 μM) or SP (0.1 μM) was added to the buffer bathing the trachea. These concentrations of methacholine and SP produce 70–80% of their respective maximum responses. Mucus was withdrawn every 30 min until a steady 'maintained' mucus volume output had been obtained. After each 30 min period the buffer surrounding the trachea was replaced with fresh buffer containing either methacholine or SP. When a maintained mucus volume output had been obtained, three concentrations of

Table 1 Mucus volume, lysozyme and albumin outputs in control periods before the addition of any drugs, immediately after addition of methacholine or substance P (SP) and the maintained outputs to methacholine and SP immediately before the addition of calcitonin gene-related peptide (CGRP, 2.5–3.5 h after first addition of methacholine or SP)

	Methacholine						*SP*	
	Control		*After methacholine*		*Before CGRP*		*Control*	*After SP*	*Before CGRP*
	− Thiorphan	+ Thiorphan	− Thiorphan	+ Thiorphan	− Thiorphan	+ Thiorphan			
Mucus volume ($\mu l\,min^{-1}$)	0.03 ± 0.02	0.04 ± 0.03	2.40 ± 0.24*	2.62 ± 0.50*	0.79 ± 0.05*	0.88 ± 0.19*	0.04 ± 0.02	0.80 ± 0.17*	0.54 ± 0.14*
Lysozyme ($ng\,min^{-1}$)	19 ± 6	21 ± 6	700 ± 84*	663 ± 109*	126 ± 30*	164 ± 21*	23 ± 5	381 ± 104*	168 ± 46*
Albumin ($\mu g\,min^{-1}$)	0.27 ± 0.11	0.30 ± 0.14	6.1 ± 1.0*	5.8 ± 1.4*	1.9 ± 0.4*	2.1 ± 0.4*	0.3 ± 0.2	2.2 ± 0.5*	1.6 ± 0.4*

Values shown are the means (± s.e.mean) of 6 determinations. * Significantly different ($P < 0.05$) from control value by Student's paired t test.

CGRP (1–100 nM) were added in ascending order to the secretagogue in the buffer surrounding the trachea. Each concentration of CGRP was left in contact with the trachea for 30 min. After 30 min the secretion produced was withdrawn and processed. The buffer surrounding the trachea was then replaced with fresh buffer containing the secretagogue and the next concentration of CGRP. After three concentrations of CGRP had been added, the buffer was replaced with buffer containing only methacholine or SP and the mucus volume output determined for two further periods of 30 min. The change in mucus volume output produced by CGRP was calculated as the difference in mucus volume output obtained between the period immediately before the peptide was added and the period when the peptide was in the organ bath, expressed as a percentage. SP only produced a satisfactory maintained secretion in the presence of thiorphan; therefore in all experiments with SP, thiorphan was present in the buffer throughout. With methacholine, thiorphan was present in the buffer in half and absent in half of experiments. All mucus samples obtained in these experiments were assayed for lysozyme and albumin.

Analysis of results

The effects of CGRP on baseline mucus volume, lysozyme and albumin outputs, and on the maintained outputs produced by methacholine and SP were analysed for statistical significance by one-way analysis of variance followed by Student's paired t tests. Significance was accepted for $P < 0.05$. Values shown are means ± s.e.mean.

Results

Effects of thiorphan

In experiments with methacholine, the mean mucus volume, lysozyme and albumin outputs in control periods before the addition of any drugs and in the absence and presence of thiorphan are shown in Table 1. There were no significant differences between the control values in the absence and presence of thiorphan suggesting that thiorphan has no effect on baseline mucus volume, lysozyme or albumin output. Similarly the control PD's across the trachea in the absence and presence of thiorphan were -8.6 ± 0.6 ($n = 18$) and -8.1 ± 0.5 mV ($n = 24$) respectively. These values are also not significantly different, suggesting thiorphan has no effect on PD.

Effects of CGRP on baseline parameters

In the absence of thiorphan, CGRP (1–100 nM) produced small, concentration-dependent increases in mucus volume, lysozyme and albumin outputs (Figure 1). In the presence of thiorphan, the concentration-response curves for CGRP-induced mucus volume, lysozyme and albumin outputs were all shifted upwards with significantly increased responsiveness at 1, 10 and 100 nM (Figure 1).

CGRP (1–100 nM) had no significant effect on PD across the trachea, in the presence or in the absence of thiorphan (Table 2).

Effects of CGRP on methacholine-induced responses

In the 30 min period immediately after addition of methacholine the mucus volume, lysozyme and albumin outputs increased significantly from preceding control values (Table 1). There were no significant differences between the outputs obtained in the presence and absence of thiorphan, suggesting thiorphan does not affect the responses to methacholine. On continued application of methacholine the mucus volume, lysozyme and albumin outputs declined but reached a steady 'maintained' level after 2.5–3.5 h (Table 1). There were no sig-

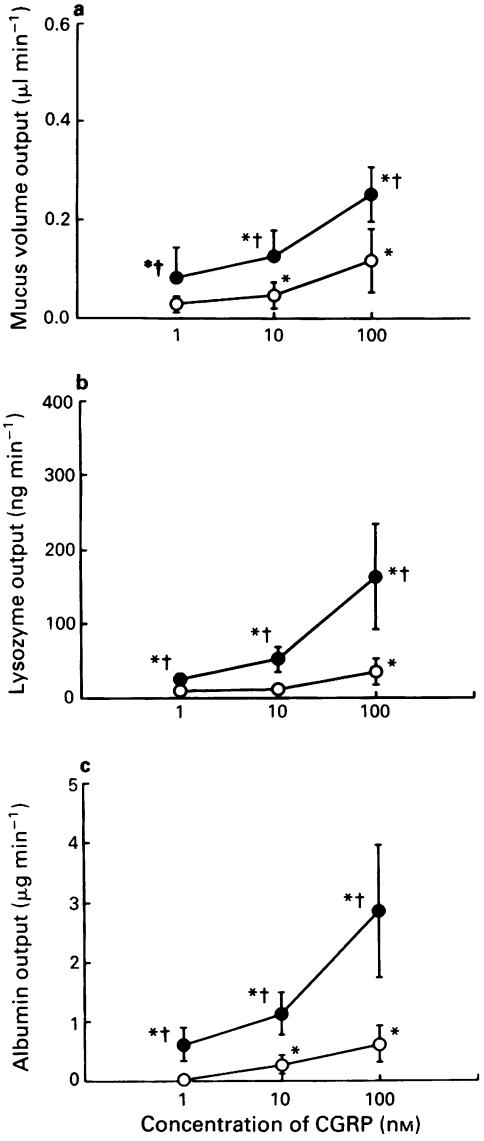

Figure 1 Concentration-response curves for the effect of calcitonin gene-related peptide (CGRP) on (a) mucus volume, (b) lysozyme and (c) albumin outputs from the ferret trachea *in vitro*. Responses to CGRP in the absence (○) and presence (●) of thiorphan (10 μM). Points are means of 4–6 determinations with s.e.means shown as vertical lines. * Response significantly ($P < 0.05$) different from zero. † Significantly different from response in the absence of thiorphan.

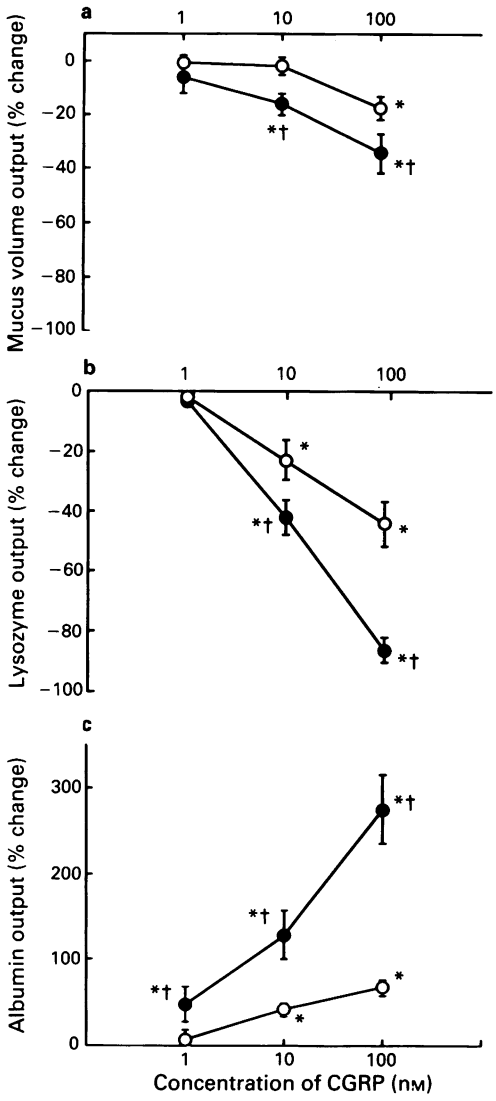

Figure 2 Concentration-response curves for the effect of calcitonin gene-related peptide (CGRP) on the maintained (a) mucus volume, (b) lysozyme and (c) albumin outputs produced by methacholine (20 μM). Responses to CGRP in the absence (○) and presence (●) of thiorphan. Points are means of 6 determinations with s.e.means shown as vertical lines. * Response significantly different from zero. † Significantly different from response in the absence of thiorphan.

nificant differences in the maintained outputs with or without thiorphan (Table 1).

In the absence of thiorphan, CGRP produced concentration-dependent reductions in the maintained methacholine-induced mucus volume and lysozyme outputs, but a concentration-dependent increase in maintained methacholine-induced albumin output (Figure 2). The effects of CGRP at 10 and 100 nM were all significantly enhanced in the presence of thiorphan (Figure 2).

Effects of CGRP on substance P-induced responses

SP significantly increased mucus volume, lysozyme and albumin outputs from preceding control values (Table 1). On continued application of SP the outputs fell slightly but reached a steady 'maintained' level after 2.5–3.5 h (Table 1). CGRP (1–100 nM) had no significant effect on SP-induced mucus volume output, but produced a concentration-dependent reduction in SP-induced lysozyme output and a concentration-dependent increase in SP-induced albumin output (Figure 3).

Table 2 The effect of calcitonin gene-related peptide (CGRP) on potential difference (PD) across the ferret trachea in the absence and presence of thiorphan

	Absence of thiorphan		*Presence of thiorphan*	
Concentration of CGRP (nM)	*Control*	*+ CGRP*	*Control*	*+ CGRP*
	PD (mV)			
1	−7.2 ± 0.8	−7.2 ± 0.8	−8.1 ± 0.6	−8.3 ± 0.7
10	−7.7 ± 0.8	−8.2 ± 0.9	−8.0 ± 0.8	−7.7 ± 0.7
100	−8.2 ± 0.8	−8.3 ± 0.8	−8.0 ± 0.6	−8.0 ± 0.5

Values shown are means ± s.e.mean. $n = 4$–6.

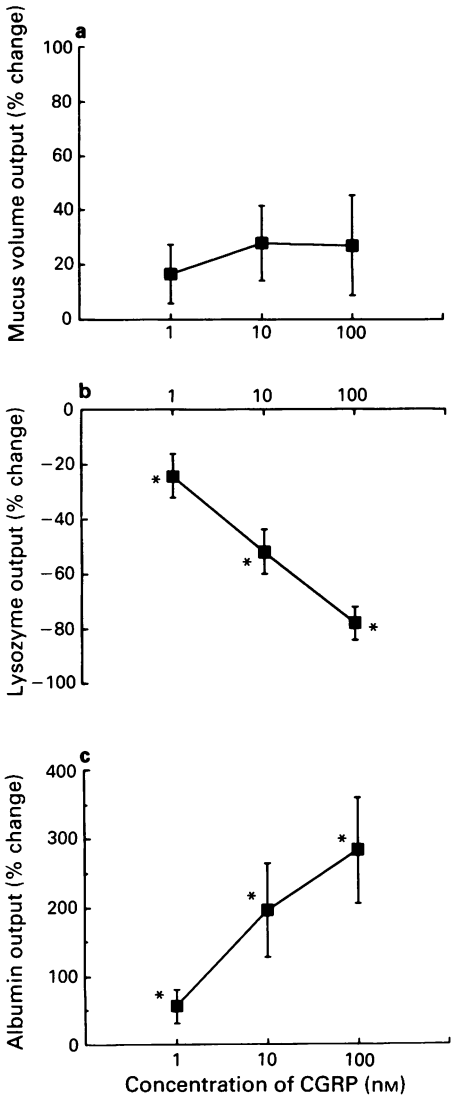

Figure 3 Concentration-response curves for the effect of calcitonin gene-related peptide (CGRP) on the maintained (a) mucus volume, (b) lysozyme and (c) albumin outputs produced by substance P (0.1 μM). All responses to CGRP were obtained in the presence of thiorphan. Points are the means of 4–6 determinations with s.e.means shown as vertical lines. * Response significantly different from zero.

Discussion

The ferret trachea has numerous submucosal glands, possibly in compensation for the notable paucity of goblet cells, the mucus secreting epithelial cells usually found in greatest density at the caudal end of the trachea and in the main bronchi of larger animals such as the cat and rabbit (Richardson & Somerville, 1988). There are two main secretory cells in the submucosal glands of the ferret trachea, mucous and serous cells. Mucous cells produce a thick viscous secretion rich in acidic glycoprotein. Unfortunately there is no specific marker for secretion from these cells. Serous cells produce a much thinner watery secretion which, as well as containing neutral glycoprotein, also contains the antibacterial enzymes lysozyme and lactoferrin. Lysozyme can be easily assayed turbidimetrically (Selsted & Martinez, 1980) and is therefore a useful specific marker for secretion from these cells. Additionally albumin is actively and specifically transported across the ferret trachea from the submucosa into the lumen (Webber & Widdicombe, 1989) and this transport is thought to occur across the epithelium (Price *et al.*, 1990).

In the present study, CGRP produced small concentration-dependent increases in baseline mucus volume, lysozyme and albumin outputs. The increase in basal lysozyme output with CGRP suggests stimulation of epithelial albumin transport. increase in basal mucus volume output may be due entirely to this stimulation but it may also stimulate secretion from mucous cells; however, it is not possible to determine this because of the lack of a specific marker for secretion from these cells. The increase in baseline albumin output with CGRP suggests a stimulation of epithelial albumin transport. Thus, CGRP has similar stimulatory actions on serous cell secretion and albumin transport as the other sensory peptides SP and NKA (Webber, 1989). However, it should be emphasized that the responses to CGRP at a particular concentration are considerably smaller than those produced by SP and NKA at the same concentration, although it was not possible to obtain a maximum response with CGRP due to the cost of this peptide. Thiorphan had no significant effect on any of the baseline outputs suggesting that there is no effective basal release of sensory neuropeptides from the ferret trachea *in vitro*, and that thiorphan has no direct effect on submucosal gland secretion or albumin transport. The effects of CGRP on all the basal outputs were enhanced in the presence of thiorphan (as would be expected if thiorphan prevented the degradation of CGRP by neutral enkephalinase). However, the enhancement of CGRP-induced effects was again much less than the enhancement of SP and NKA-induced outputs by thiorphan (Webber, 1989). For instance at 100 nM, CGRP-induced lysozyme and albumin outputs were increased 8 fold in the presence of thiorphan, whereas those due to SP (100 nM) were increased about 25 fold. Thus, it is likely that CGRP is a more stable peptide than SP or that there are more important enzymes than neutral enkephalinase responsible for the breakdown of CGRP, whereas this is the major enzyme responsible for the degradation of SP and NKA. CGRP had no effect on the potential difference across the trachea either in the absence or presence of thiorphan, suggesting it was not affecting mucosal integrity.

Methacholine- and SP-induced lysozyme outputs were inhibited by CGRP suggesting that CGRP inhibits the serous cell secretion produced by these two secretagogues. It is not clear why CGRP enhances baseline serous cell secretion whilst inhibiting the stimulated secretion due to methacholine or SP. It is possible that there are two different receptors for CGRP; the first is excitatory and leads to an increase in serous cell secretion and the second is inhibitory and is only activated by CGRP when secretion has already been stimulated for instance by SP or methacholine. Methacholine-induced mucus volume output was slightly reduced by CGRP, whilst that due to SP was slightly enhanced. These results, particularly those with SP, might be explained by CGRP exerting a stimulatory effect on mucous cell secretion whilst inhibiting secretion from serous cells (as indicated by the reduction in lysozyme output), the mucus volume output reflecting the net effect of these changes. However, it is difficult to gauge the nature of CGRP-induced effects on mucous cell secretion without a specific marker for secretion from these cells. Methacholine- and SP-induced albumin outputs were increased by CGRP suggesting enhancement of epithelial albumin transport produced by these agents. The effects of CGRP on methacholine-induced lysozyme, mucus volume and albumin outputs were enhanced by thiorphan, again suggesting that CGRP is at least partly being degraded by neutral enkephalinase in the ferret trachea.

CGRP interacts with SP on other tissues including some from the airways. It potentiates SP-induced microvascular leakage in rat skin (Gamse & Saria, 1985; Brain & Williams, 1985), which is consistent with its potentiation of SP-induced epithelial albumin transport in the present study. This increased responsiveness to SP may be due to CGRP preventing the breakdown of SP (Le Greves *et al.*, 1985); however, this seems unlikely in the present study as thiorphan was present throughout the experiments with CGRP and SP. In contrast CGRP had no significant effect on the increased

airway microvascular leakage produced by SP in guinea-pigs (Rogers *et al.*, 1988). In contrast to enhancing the action of SP, CGRP blocks the increased airway resistance produced by SP in anaesthetized guinea-pigs (Gatto *et al.*, 1989); this is consistent with the inhibitory action of CGRP on SP-induced serous cell secretion shown in the present study. The mechanism of action of CGRP in inhibiting SP-induced airway responses is not known. CGRP may bind to its own receptors and elicit responses which are opposite to those produced by SP, or may bind to SP receptors themselves preventing the access of SP. Clearly CGRP can either enhance, inhibit or have no effect on SP-induced airway responses. The type of response observed *in vivo* when the peptides are released together will depend on the amount of each peptide released and the tissue they are acting on.

Thus, CGRP has a weak stimulatory action on submucosal gland serous cell secretion and epithelial albumin transport in the ferret trachea. Even after inhibition of enkephalinase the effects of CGRP are considerably weaker than those produced by similar concentrations of other sensory neuropeptides such as SP or NKA, or by muscarinic agonists such as methacholine. Therefore, it is unlikely that CGRP is an important mediator of mucus secretion and epithelial transport mechanisms *in vivo*. However, in view of its potent modulatory actions demonstrated in this study it is likely that CGRP, when released *in vivo* with SP or at the same time as muscarinic receptor stimulation by acetylcholine, will modulate the serous cell secretion and epithelial albumin transport produced by these mediators. This may lead to considerable changes in the composition of airway surface liquid which could have important implications in health and inflammatory airway disease.

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Effect of endothelium removal on the vasoconstrictor response to neuronally released 5-hydroxytryptamine and noradrenaline in the rat isolated mesenteric and femoral arteries

Masanobu Urabe, [1]Hiromu Kawasaki & Koichiro Takasaki

Department of Pharmacology, Miyazaki Medical College, 5200 Kiyotake, Miyazaki 889-16, Japan

1 The role of the vascular endothelium in the vasoconstrictor response to transmural nerve stimulation (TNS) was studied in isolated ring segments of rat mesenteric and femoral arteries.

2 In both types of artery, TNS (1 to 16 Hz) produced frequency-dependent vasoconstriction, which was abolished by 100 nM tetrodotoxin, 10 μM guanethidine or 10 nM prazosin, indicating that the response was mediated by endogenous noradrenaline (NA) released from noradrenergic nerves. NA-mediated vasoconstriction in response to TNS was significantly potentiated by removal of the endothelium.

3 In the presence of 10 nM prazosin, the reduced vasoconstriction in response to TNS was restored by incubation with 10 μM 5-hydroxytryptamine (5-HT) for 20 min. Restoration of the response to TNS was markedly attenuated by treatment with 10 nM ketanserin, 100 nM tetrodotoxin, or 10 μM guanethidine, indicating that the restored response was mediated by 5-HT released from noradrenergic nerves. Vasoconstriction mediated by 5-HT in response to TNS was not modified by removal of the endothelium.

4 In both types of artery with intact endothelium, treatment with 3 μM methylene blue potentiated the NA-mediated contractile response to TNS, but did not potentiate the 5-HT-mediated response to TNS.

5 In both types of artery, the contractile responses to exogenous NA and 5-HT were potentiated by removal of the endothelium.

6 These results suggest that endothelial cells regulate neurogenic vasoconstriction by releasing endothelium-derived relaxing factor. Furthermore, it appears likely that the response to neuronally released 5-HT is not affected by the endothelium.

Introduction

It is widely recognized that the vascular endothelium plays an important role in the response of isolated arterial segments to several vasodilators and vasoconstrictors, including noradrenaline (NA), 5-hydroxytryptamine (5-HT), acetylcholine, bradykinin, histamine, adenosine 5'-triphosphate (ATP) and others (Furchgott & Zawadzki, 1980; Cocks & Angus, 1983; Furchgott, 1984; Griffith *et al.*, 1984; Lues & Schümann, 1984; Miyazaki & Toda, 1986). These substances apparently interact with the endothelium to cause the release of endothelium-derived relaxing factor (EDRF), which then has an inhibitory effect on vascular smooth muscle tone (Furchgott, 1984; Vanhoutte, 1989).

Although a vasodilator response to transmural nerve stimulation (TNS) of blood vessels with active tone has been observed in canine coronary arteries (Rooke *et al.*, 1982), feline cerebral arteries (Lee *et al.*, 1984) and the rat mesenteric vascular bed (Kawasaki *et al.*, 1988), this relaxation has been confirmed to be endothelium-independent. In contrast, electrical stimulation of contracted lung vessels releases EDRF to cause relaxation (Frank & Bevan, 1983). Furthermore, the endothelium has been shown to inhibit not only the vasoconstrictor response to noradrenergic nerve stimulation (Tesfamariam *et al.*, 1987; Hynes *et al.*, 1988) but also transmitter (NA) release from vascular noradrenergic nerves (Cohen & Weisbrod, 1988).

Vascular noradrenergic nerves can accumulate 5-HT as an alternative transmitter or co-transmitter, which is then released by noradrenergic nerve stimulation (Verbeuren *et al.*, 1983; Kawasaki & Takasaki, 1984; Saito & Lee, 1987; Jackowski *et al.*, 1989; Kawasaki *et al.*, 1989). The 5-HT released from nerves in the rat mesenteric artery has been shown to produce contraction of blood vessels via the activation of 5-HT_2-receptors (Kawasaki & Takasaki, 1984; 1986; Kawasaki *et al.*, 1989). In this context, a recent study has revealed that the endothelium can inhibit the penetration of 5-HT into the wall of the canine saphenous vein (Verbeuren *et al.*, 1988). The present study investigated the role of the endothelium in the vasoconstrictor response to 5-HT released from noradrenergic nerves by TNS, and also evaluated the response to neuronally released NA in the presence and absence of endothelium.

Methods

The superior mesenteric and femoral arteries were obtained from male Wistar rats weighing 270–400 g, which were maintained at the Experimental Animal Center of Miyazaki Medical College. The animals were anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, i.p.). Pairs of arteries were excised and cut into approximately 3.5 mm long ring segments under a microscope. One ring from each pair was left intact, and the other was stripped of its endothelial cells by rubbing with a smooth wooden rod. The preparations were suspended under a tension of 1 g in a 30 ml organ bath containing a modified Krebs-Ringer bicarbonate solution (mM): NaCl 127.0, KCl 5.0, $CaCl_2$ 2.4, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, EDTA-2Na 0.027 and glucose 11.0. The solution was aerated with a mixture of 95% O_2–5% CO_2 and maintained at 37°C. Two fine stainless steel wires were inserted through the lumen of the segment; one was anchored to the stationary support and the other was connected to an isotonic transducer (TD-112S, Nihon Kohden). Changes in vessel tone were recorded on a polygraph (Recti-Horiz, Sanei). Endothelium removal was confirmed by the absence of relaxation when 1 μM acetylcholine was applied to rings contracted with 100 nM U46619 (9,11-dideoxy-11α,9α-methano-epoxyprostaglandin $F_2\alpha$) before exposure of 80 mM KCl at the completion of each experiment.

After the preparations were allowed to equilibrate for 60 min and a stable tension was obtained, the first TNS series was performed at 1, 2, 4, 8 and 16 Hz (0.5 ms in duration, supramaximal voltage, for 30 s). TNS at each frequency was

[1] Author for correspondence.

performed at 8 min intervals via platinum electrodes which sandwiched the preparation, by use of a stimulator (DPS-160B, Sanei). The neurogenic nature of the TNS response was confirmed by the abolition of the vasoconstrictor response by 100 nM tetrodotoxin or 10 μM guanethidine. Methylene blue was added 60 min before the second TNS series.

In both the preparations with and without endothelium, the 5-HT-mediated contractile response to TNS was measured by use of the following schedule. After the first contractile response to TNS was obtained as a control, a second series of TNS was perfomed 20 min after 10 nM prazosin, and thereafter the preparation was exposed to 10 μM 5-HT for 20 min. The third series of TNS was performed in the presence of prazosin 30 min after repeated washing out of the 5-HT (2 or 3 times). In some experiments, the preparation treated with 5-HT was exposed to methylene blue for 60 min in combination with prazosin and then TNS was performed.

In the experiments with exogenous NA or 5-HT, concentration-response curves were obtained by use of a cumulative concentration schedule, and the second curve obtained was used as a control. The contractile response to 5-HT was always determined in the presence of 10 nM prazosin.

In order to normalize the data, the contractile forces were expressed as a percentage of the maximum force generated in response to 80 mM KCl in each tissue. The determination of pD_2 values (the negative logarithm of the concentration causing half maximum contraction: $-\log EC_{50}$) was performed as described by Van Rossum (1963). Data are expressed as the mean $\pm$ s.e.mean and were analysed by unpaired Student's t test for group mean comparisons and one way analysis of variance followed by Dunnett's test. A P value less than 0.05 was considered to be statistically significant.

The following drugs were used: (−)-NA HCl, 5-HT HCl, tetrodotoxin (all Sigma), prazosin HCl (Taito-Pfizer), ketanserin tartrate (Janssen), methylene blue (Wako Jyunyaku), guanethidine sulphate (Tokyo Kasei) and U46619 (Sigma). Both NA and 5-HT were dissolved in 0.9% saline containing 0.1% ascorbic acid and stored in a freezer. On the day of the experiment, final dilutions of NA and 5-HT were made with Krebs-Ringer bicarbonate solution. All other drugs were dissolved in 0.9% saline and then diluted in the physiological solution.

Results

Response to potassium chloride

The vasoconstrictor response induced by exposure of the preparations to KCl (80 mM) at the completion of the experiment was significantly greater in mesenteric arteries with intact endothelium (0.461 $\pm$ 0.018 mm, $n = 22$; $P < 0.01$) than in those without endothelium (0.382 $\pm$ 0.022 mm, $n = 22$). The response was also greater in femoral arteries with endothelium (0.613 $\pm$ 0.015 mm, $n = 21$; $P < 0.001$) than in those without endothelium (0.461 $\pm$ 0.019 mm, $n = 18$).

Vasoconstrictor response to TNS

As shown in Figures 1a and 2b, TNS of the femoral artery at 1 to 16 Hz produced a frequency-dependent contractile response, and a similar response to TNS was also observed in the mesenteric artery (Figure 2a). In both vessels, the response to TNS was abolished by 100 nM tetrodotoxin ($n = 3$) or 10 μM guanethidine ($n = 3$) (data not shown), and was markedly reduced by prazosin (Figures 1b and 2), indicating that the contraction was mediated by endogenous NA released by noradrenergic nerve stimulation (NA-mediated contractile response to TNS).

After incubation with 10 μM 5-HT for 20 min (Figure 1c), the contractile response to TNS reduced by prazosin was frequency-dependently and significantly restored (Figures 1d and 2). The restored contractile response to TNS in the presence of prazosin was completely inhibited by 10 nM ketanserin (Figures 1e and 2), 100 nM tetrodotoxin or 10 μM guanethidine (data not shown), indicating that this restored response was mediated by 5-HT released from noradrenergic nerve terminals (5-HT-mediated contractile response to TNS).

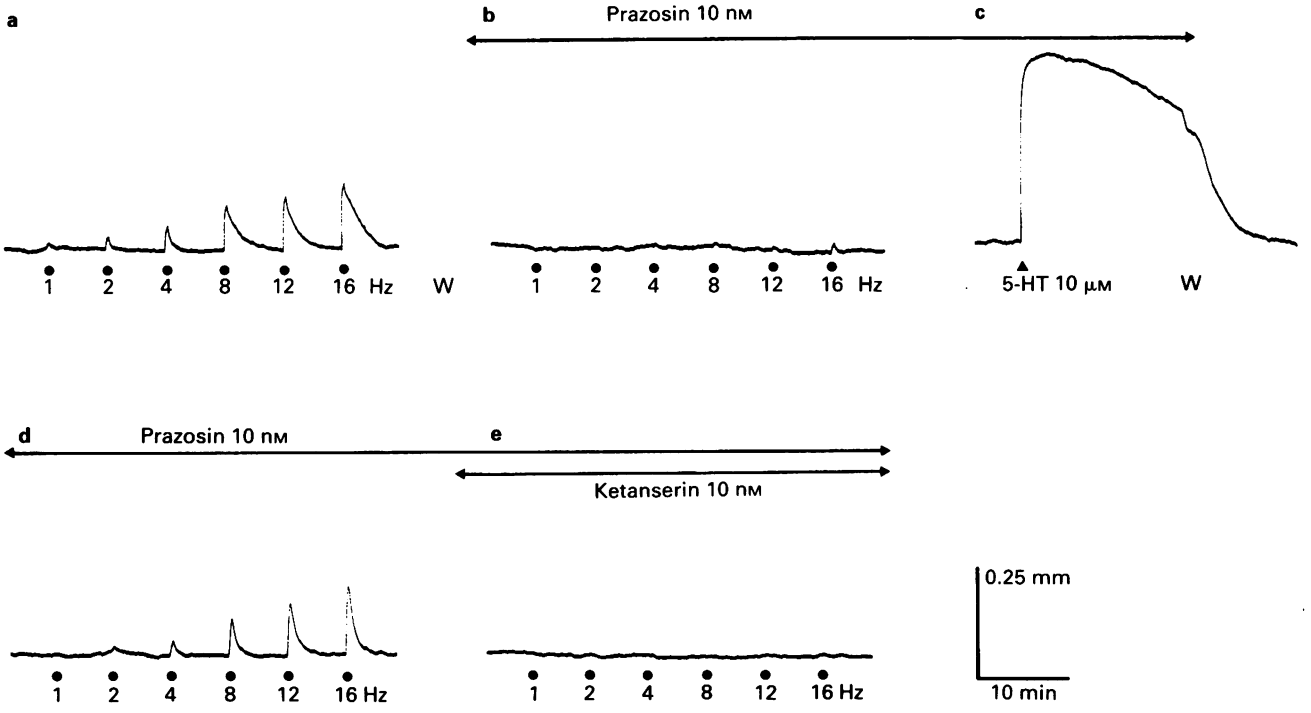

Figure 1 Typical record of the effect of 5-hydroxytryptamine (5-HT) treatment on the contractile response of a rat isolated femoral arterial ring segment to transmural nerve stimulation (TNS; 1–16 Hz) in the presence of prazosin or ketanserin. (a) Control response. (b) Response to TNS in the presence of 10 nM prazosin. (c) Response to 5-HT. (d) Response to TNS after 5-HT treatment in the presence of prazosin. (e) Response to TNS in the presence of prazosin and ketanserin. W, washout of the muscle chamber.

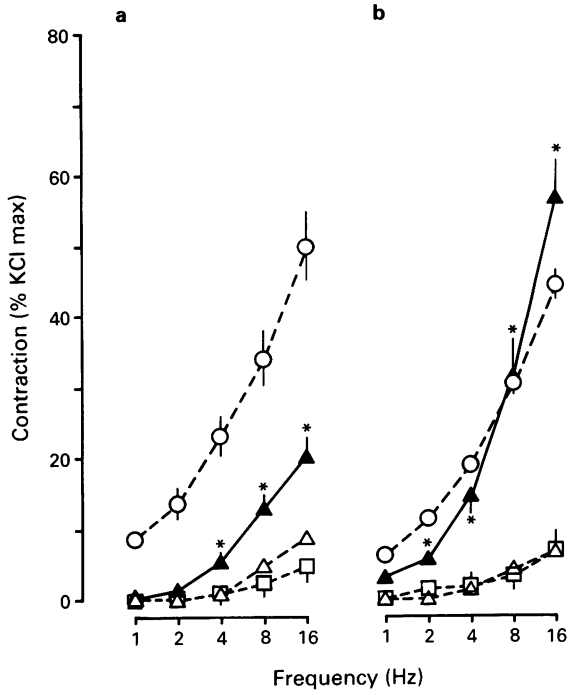

Figure 2 Effect of treatment with 5-hydroxytryptamine (5-HT, 10 μM) on the vasoconstriction of rat isolated mesenteric (a) and femoral (b) arteries induced by transmural nerve stimulation (TNS, 1–16 Hz) in the presence of 10 nM prazosin and prazosin plus 10 nM ketanserin. (○) Control response to TNS. (△) Response to TNS in the presence of prazosin. (▲) Response to TNS in the presence of prazosin after 5-HT treatment. (□) Response to TNS in the presence of prazosin and ketanserin after 5-HT treatment. The ordinate scale represents the % of the maximum contraction induced by 80 mM KCl. Each point indicates the mean of 5–8 experiments and vertical lines show s.e.mean. * $P < 0.05$, compared with the response to TNS in the presence of prazosin.

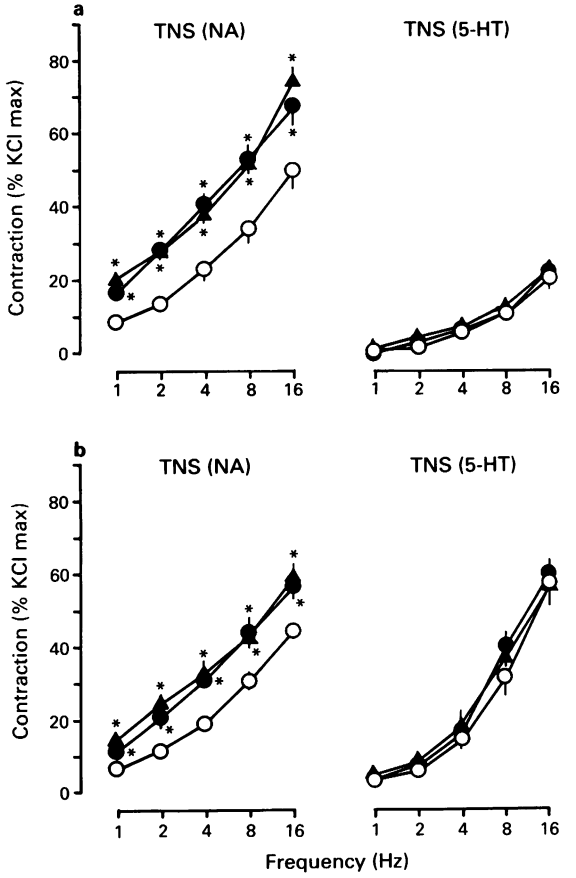

Figure 3 Effects of removal of the endothelium and pretreatment with 3 μM methylene blue on contractile response to transmural nerve stimulation (TNS, 1–16 Hz) in rat isolated mesenteric (a) and femoral (b) arteries. TNS (NA), noradrenaline (NA)-mediated contractile response to TNS. TNS (5-HT), 5-hydroxytryptamine (5-HT)-mediated contractile response to TNS. (○) Arteries with intact endothelium; (●) arteries without endothelium; (▲) pretreatment with methylene blue in arteries with intact endothelium. The ordinate scales represent the % of the maximum contraction induced by 80 mM KCl. Each point indicates the mean of 5–8 experiments and vertical lines show s.e.mean. * $P < 0.05$, compared with control response.

In the mesenteric artery with intact endothelium, incubation of 10 μM 5-HT for 20 min did not alter the dose-response curves for exogenous 5-HT (control pD_2, 5.93 ± 0.05; maximum response, 98.5 ± 10.5%; pD_2 after 5-HT incubation, 5.9 ± 0.06; maximum response, 88.5 ± 10.8%).

Effect of removal of the endothelium

As shown in Figure 3, removal of the endothelium from femoral and mesenteric arteries produced a two fold increase in the NA-mediated contractile response to TNS. However, the 5-HT-mediated contractile response to TNS was not altered by removal of the endothelium in either artery (Figure 3).

Effect of methylene blue

In order to characterize further potentiation of the vasoconstrictor response to TNS by removal of the endothelium, the effects of methylene blue on the response to TNS were examined in the mesenteric and femoral arteries. In both vessels with intact endothelium, 3 μM methylene blue, which had no effect on the resting tension, significantly potentiated the NA-mediated contractile response to TNS at all frequencies (Figure 3), but did not potentiate the 5-HT-mediated contractile response to TNS (Figure 3).

In both the mesenteric and femoral arteries, augmentation by methylene blue of the NA-mediated contractile response to TNS was not observed after removal of the endothelium (Figure 4).

Effect of endothelium removal on the vasoconstrictor response to exogenous NA and 5-HT

As shown in Figure 5, exogenous NA and 5-HT (in the presence of prazosin) caused concentration-dependent contraction in both mesenteric and femoral arteries. Removal of the endothelium caused marked potentiation of the contractile response to NA and 5-HT in both arteries. The pD_2 values and maximum contractile force induced by NA and 5-HT were significantly increased by removal of the endothelium in both arteries (Table 1).

Discussion

The present study demonstrated that removal of the endothelium increased the sensitivity (as defined by pD_2 values) and the maximum contractile response to exogenous NA and 5-HT in the rat superior mesenteric and femoral arteries. The present findings are consistent with many reports that removal of vascular endothelium in several species potentiates the direct vasoconstrictor effects of 5-HT and NA (Cocks & Angus, 1983; Garland, 1985; Connor & Feniuk, 1989). Therefore, it seems likely that the vasoconstrictor response to exogenous 5-HT or NA is attenuated by receptor-mediated

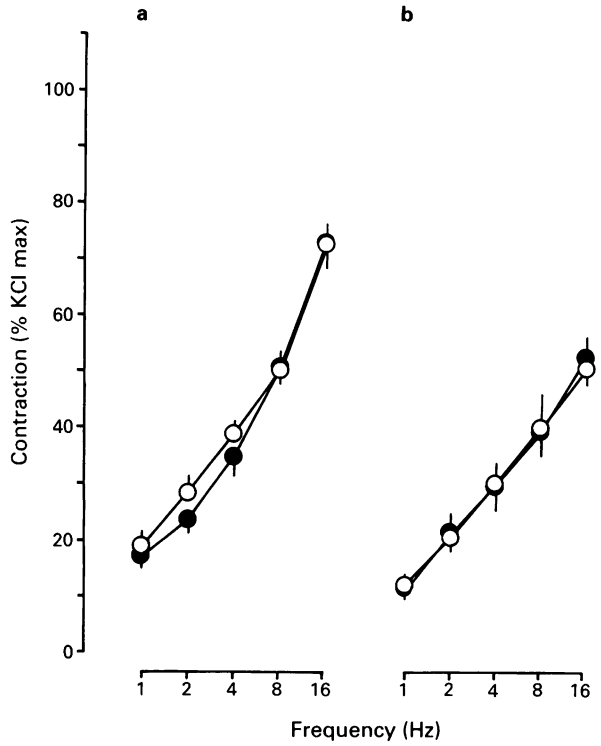

Figure 4 Effect of methylene blue on the noradrenaline (NA)-mediated contractile response to transmural nerve stimulation (TNS) in rat isolated mesenteric (a) and femoral (b) arteries without the endothelium. (○) Arteries without endothelium; (●) pretreatment with 3 μM methylene blue in arteries without endothelium. The ordinate scale represents the % of the maximum contraction induced by 80 mM KCl. Each point indicates the mean of 5–8 experiments and vertical lines show s.e.mean.

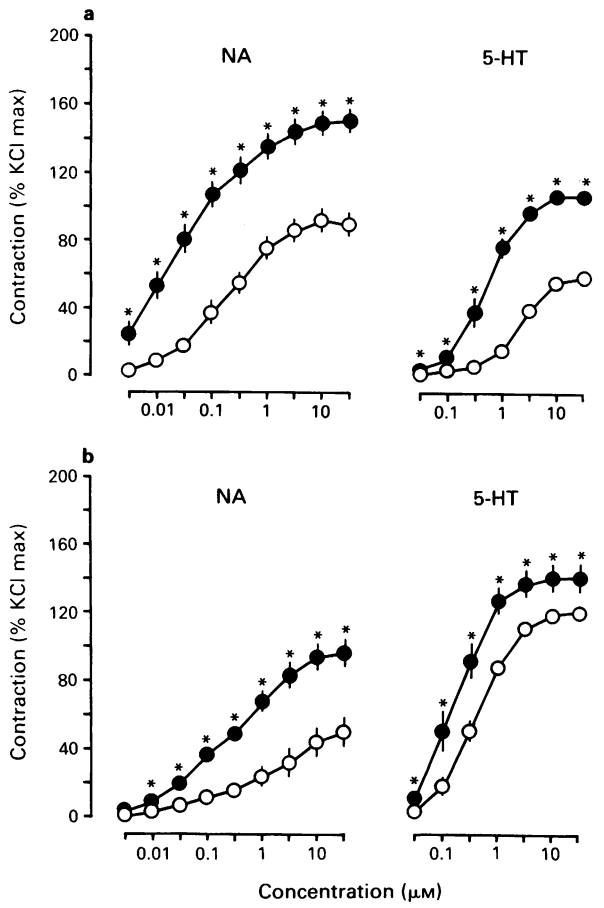

Figure 5 Effect of removal of the endothelium on the contractile response to exogenous noradrenaline (NA) and 5-hydroxytryptamine (5-HT) in rat isolated mesenteric (a) and femoral (b) arteries. (○) Arteries with endothelium intact; (●) arteries without endothelium. The ordinate scales represent the % of the maximum contraction induced by 80 mM KCl. Each point indicates the mean of 5–8 experiments and vertical lines show s.e.mean. * $P < 0.05$, compared with control arteries with intact endothelium.

release of EDRF. More recent studies have shown that a continuous basal release of EDRF from the endothelium may influence vasoconstriction by NA and 5-HT (Martin *et al.*, 1986; Connor & Feniuk, 1989).

In the present experiments, the maximum contraction induced by 80 mM KCl was significantly smaller in arteries without endothelium than in arteries with intact endothelium. Similar results have been observed in the dog and pig coronary artery (Cocks & Angus, 1983). However, Connor & Feniuk (1989) have demonstrated that there is no difference in the KCl-induced contraction between endothelium intact and denuded dog basilar artery. Although precise mechanisms remain unknown, this may be due to differences in the tissues and species used.

The present study showed that removal of the endothelium from isolated mesenteric and femoral arteries caused an enhancement of vasoconstriction in response to TNS, which is mediated by neurally released NA. These results are in accord with several previous reports that vasoconstriction in response to noradrenergic nerve stimulation is augmented in vessels with the endothelium removed (Tesfamariam *et al.*, 1987; Hynes *et al.*, 1988; Cohen & Weisbrod, 1988). These studies have suggested that the endothelium may inhibit the response to noradrenergic nerve stimulation via the spontaneous release of EDRF. A recent study has also suggested that the endothelium may attenuate neurogenic vasoconstriction by

Table 1 The pD_2 values and maximum contraction induced by noradrenaline (NA) and 5-hydroxytryptamine (5-HT) in rat mesenteric and femoral arteries with or without the endothelium

		pD_2 value[a]		*Maximum contraction*[b] (% KCl max)	
Blood vessel	*Agonist*	*Intact*	*Denuded*[c]	*Intact*	*Denuded*
Mesenteric artery	NA	6.74 ± 0.28	7.63 ± 0.31*	92.0 ± 6.3	150.4 ± 6.5*
	5-HT[d]	5.72 ± 0.05	6.33 ± 0.17*	58.5 ± 4.1	107.3 ± 3.4*
Femoral artery	NA	5.44 ± 0.42	6.56 ± 0.47*	69.2 ± 6.6	101.9 ± 9.4*
	5-HT	6.40 ± 0.14	6.80 ± 0.31*	120.8 ± 4.1	141.3 ± 8.2*

[a] The $-\log EC_{50}$ value is expressed as the pD_2 value, which represents the mean ± 95% confidence limits of 5 to 8 experiments.
[b] Maximum contraction relative to that induced by 80 mM KCl, represented as the mean ± s.e.mean of 5 to 8 experiments.
[c] Removal of the endothelium.
[d] Contraction induced by 5-HT in the presence of prazosin (10 nM).
* $P < 0.05$, compared with the intact preparation.

participating in the uptake and metabolism of NA released from nerves, as well as by inhibiting the release of transmitter NA from noradrenergic nerves (Tesfamariam *et al.*, 1987; Cohen & Weisbrod, 1988). However, Frank & Bevan (1983) demonstrated the endothelium-dependent release of EDRF by TNS in the pulmonary vessels of rabbit, cats and monkeys, and proposed that TNS itself may release EDRF to counteract TNS-induced vasoconstriction.

In contrast to the NA-mediated contractile response to TNS, the contractile response to TNS restored after treatment with 5-HT in the presence of prazosin was unaffected by removal of the endothelium in both the mesenteric and femoral arteries. Because the restored response is neurogenic and mediated by 5-HT released from noradrenergic nerves by TNS, it is unlikely that TNS itself releases EDRF to inhibit neurogenic vasoconstriction in the rat mesenteric and femoral arteries. Furthermore, the present findings suggest that there is little contribution by a continuous basal release of EDRF to the 5-HT-mediated contractile response to TNS.

In the present experiments, the arteries were exposed to a high concentration of 5-HT (10 μM) to allow its accumulation in the noradrenergic nerves. It is possible that this treatment could cause desensitization of the endothelium to 5-HT, damage to endothelial cells, or the depletion of EDRF. However, the dose-response curve for exogenous 5-HT was not altered by this treatment, and removal of the endothelium caused a marked enhancement of the vasoconstrictor response to exogenous 5-HT. Exposure of vessels to acetylcholine after their contraction by a second dose of 5-HT or U46619 following 5-HT treatment could produce relaxation, indicating that there was no depletion of EDRF following exposure to 5-HT (data not shown). Moreover, after incubation with 5-HT, the artery was washed with 5-HT-free Krebs solution two or three times and TNS was carried out without 5-HT in the medium. These results indicate clearly that the endothelium remained intact after 5-HT treatment.

The discrepancy between NA- and 5-HT-mediated contractile responses to TNS in the vessel with their endothelium removed may be explained by a quantitative difference in NA and 5-HT release by nerve stimulation. That is, the amount of 5-HT released by TNS might be sufficient to contract the vascular smooth muscle but insufficient to stimulate the 5-HT receptors (if any) on endothelial cells to release EDRF. However, this possibility seems unlikely because vasoconstriction in response to exogenous 5-HT at low concentrations (0.01–1 μM), which was smaller than the restored response to TNS, was significantly augmented by removal of the endothelium in both mesenteric and femoral arteries. In contrast, the 5-HT-mediated contractile response to TNS, even at 16 Hz, was not affected at all by removal of the endothelium. Another explanation for the discrepancy is that the 5-HT receptors of endothelial cells may be located on the luminal surface, with none or only a few facing the smooth muscle, while NA receptors may be located on both surfaces of the endothelial cells. According to this model, both neurally released NA and exogenous NA could stimulate endothelial cell receptors to release EDRF so that vasoconstriction was inhibited. On the contrary, neurally released 5-HT would be unable to release EDRF, even if 5-HT reached the endothelial cells, because of the paucity of endothelial cell receptors adjacent to the smooth muscle. It would be likely that neurally released 5-HT might be difficult to reach the vessel lumen to stimulate receptors and release EDRF, because the endothelium reduces the penetration of 5-HT (Verbeuren *et al.*, 1988). However, exogenous 5-HT could release EDRF by the stimulation of endothelial cell receptors located on the luminal surface. This model can also explain the discrepancy between the vasoconstriction in response to neurally released 5-HT and exogenous 5-HT in the vessel rings without endothelium. However, further studies are needed to clarify the actual situation.

Recently, EDRF has been postulated to be identical with nitric oxide (NO) (Ignarro *et al.*, 1987; Palmer *et al.*, 1987), although the existence of an additional EDRF, which relaxes blood vessels by hyperpolarizing membranes, has been proposed (Taylor & Weston, 1988). Because the effects of endothelium-dependent vasodilators and nitrovasodilators (including NO) are inhibited by methylene blue, a guanylate cyclase inhibitor, it appears that these vasodilators act by stimulating soluble guanylate cyclase to cause the accumulation of guanosine 3′:5′-cyclic monophosphate (cyclic GMP) (Gruetter *et al.*, 1981; Ignarro *et al.*, 1984; Griffith *et al.*, 1985). In the present experiments, methylene blue did not modify the 5-HT-mediated contractile response to TNS in intact mesenteric and femoral arteries. This result, together with the lack of alteration of the 5-HT-mediated contractile response to TNS in arteries with mechanical removal of the endothelium, provides further evidence that the endothelium did not inhibit the 5-HT-mediated response to TNS. In contrast, the NA-mediated contractile response to TNS was potentiated by methylene blue in arteries with intact endothelium, a result correlating with the increased NA-mediated response to TNS observed after removal of the endothelium. Furthermore, methylene blue has no effect on the NA-mediated contractile response to TNS in arteries without the endothelium, indicating that the action of methylene blue depended on the endothelium.

In conclusion, the present study suggests that the NA-mediated vasoconstriction in response to TNS but not the 5-HT-mediated response to TNS was modified by EDRF in a manner related to intracellular cyclic GMP formation. It seems likely that the endothelium acts as a physical barrier not only to neurally released NA and circulating NA but also to circulating 5-HT or 5-HT released from aggregated platelets. However, the endothelium provides no barrier to neurally released 5-HT, where 5-HT is taken up into noradrenergic nerves. This may cause local vasospasm due to an enhanced noradrenergic vasoconstriction, when 5-HT is accumulated in noradrenergic nerves.

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Evidence that part of the NANC relaxant response of guinea-pig trachea to electrical field stimulation is mediated by nitric oxide

[1]Chun Guang Li & Michael J. Rand

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

1 The nitric oxide (NO) synthesis inhibitors N^G-monomethyl L-arginine (L-NMMA) and L-nitroarginine methyl ester (L-NAME) reduced relaxations of guinea-pig tracheal smooth muscle elicited by stimulation of intramural non-adrenergic, non-cholinergic (NANC) nerves, but D-NMMA had no effect. L-NAME was 10–30 times more potent than L-NMMA. Relaxations produced by sodium nitroprusside and vasoactive intestinal polypeptide (VIP) were not affected by L-NMMA or L-NAME.

2 The inhibitory effect of L-NMMA on NANC-mediated relaxations was partially reversed by L-arginine but was not affected by D-arginine.

3 VIP antibody and α-chymotrypsin abolished or greatly reduced the relaxant action of VIP and reduced relaxations elicited by stimulation of NANC nerves; the residual NANC relaxation was further reduced by L-NAME.

4 The results suggest that NO and VIP are mediators of NANC-induced relaxations of guinea-pig tracheal smooth muscle. We propose the term 'nitrergic' to describe transmission processes which are mediated by NO.

Introduction

It has been demonstrated that there are noradrenergic, cholinergic and non-adrenergic non-cholinergic (NANC) components in the innervation of tracheal smooth muscle (Karlsson, 1986; Lundberg & Saria, 1987). Guinea-pig trachea has an inhibitory NANC innervation and there is some evidence suggesting that vasoactive intestinal polypeptide (VIP) may be the transmitter (Matzusaki *et al.*, 1980; Karlsson, 1986; Ellis & Farmer, 1989a,b). However, when responses to VIP were abolished by treatment with VIP antiserum or peptidases, responses to stimulation of the NANC nerves were reduced but not abolished, indicating that a non-VIP component may be involved in the NANC stimulation-induced relaxations (Ellis & Farmer, 1989a,b).

There is evidence that nitric oxide (NO) is a mediator of NANC stimulation-induced relaxations of the anococcygeus muscle of the rat (Li & Rand, 1989; Gillespie *et al.*, 1989; Ramagopal & Leighton, 1989) and mouse (Gibson *et al.*, 1990) and of rat gastric fundus strips (Li & Rand, 1990a). This is based on findings with L-N^G-monomethyl arginine (L-NMMA), which inhibits the synthesis of NO from L-arginine in endothelial cells and thereby blocks endothelium-dependent relaxations of vascular smooth muscle (Rees *et al.*, 1989). L-NMMA blocks relaxations of rat anococcygeus muscles and gastric fundus strips elicited by low frequencies of stimulation of NANC nerves, suggesting that they are mediated by NO.

Therefore, we used L-NMMA, and the more potent NO synthesis inhibitor L-nitroarginine methyl ester (L-NAME) (Palacios *et al.*, 1989) to investigate the possibility that NO might mediate the non-VIP component of NANC transmission in guinea-pig tracheal smooth muscle. A preliminary account of this work has been communicated to the Australian Neuroscience Society (Li & Rand, 1990b).

Methods

Guinea-pigs (Dunkin-Hartley) of either sex (300–500 g) were stunned by a blow on the head and decapitated. The trachea was rapidly removed and opened by cutting along the anterior wall. Alternate incomplete cuts were made at about 3 mm intervals between cartilage segments to create a zigzag strip, as described by Emmerson & Mackay (1979). The tracheal strip was set up in a 20 ml organ bath containing physiological salt solution (PSS) for isometric recording under a resting tension of 0.5 g.

The composition of the PSS was (mM): NaCl 118, KCl 4.7, $NaHCO_3$ 25, $MgSO_4$ 0.45, KH_2PO_4 1.03, $CaCl_2$ 2.5, D-(+)-glucose 11.1, disodium edetate 0.067 and ascorbic acid 0.14. The PSS was gassed with 5% CO_2 in O_2 and maintained at 37°C. The PSS contained atropine (3 μM) and guanethidine (5 μM) to block cholinergic and adrenergic involvement in responses to field stimulation of intramural nerves, and histamine (10 μM) to raise the tone of the smooth muscle. The preparation was allowed to equilibrate for at least 40 min, with changes of the PSS every 10 min, before making experimental observations.

After the equilibration period, NANC-mediated relaxations were elicited by stimulating the intramural nerves with square wave pulses of 1 ms duration and supramaximal field strength (17 V cm^{-1}) delivered from a Grass S88 stimulator through parallel platinum wire electrodes on either side of the strip; other stimulation parameters are given in Results. When agonists producing relaxation (VIP or sodium nitroprusside) were used, successive responses to a single or accumulated concentrations of each were elicited in each preparation. The responses produced by VIP (10 nM) and sodium nitroprusside (0.1 μM) were submaximal and approximately equal in amplitude to those elicited by field stimulation at 5 Hz. In some experiments, a range of concentrations of VIP (10–50 nM) was used. The tissue contact time for the agonists was 8–10 min, which was longer than the time taken for the peak effect to be reached. Subsequent responses to field stimulation or agonists were obtained in the presence of test drugs, or in their absence to provide time-controlled observations. L-NMMA (30 and 90 μM), D-NMMA (90 μM), L-NAME (3–90 μM), L-arginine (270 μM) and D-arginine (270 μM) were given 10 min before response to field stimulation or relaxant agonists were elicited. When the effects of L- or D-arginine (270 μM) on the action of L-NMMA (90 μM) were investigated, these agents were added 20 min after the administration of L-NMMA.

When VIP antibody or α-chymotrypsin was used, responses to VIP and field stimulation were elicited first, then the tissue was incubated with VIP antibody for 60 min or α-

[1] Author for correspondence.

chymotrypsin for 15 min before any further observation was made; thereafter, VIP antibody or α-chymotrypsin was replaced in the organ bath each time the PSS was changed.

Drugs used were: L- and D-arginine (Wellcome), atropine sulphate (Sigma), α-chymotrypsin (Sigma), ω-conotoxin GVIA (Peninsula), guanethidine sulphate (Ciba), histamine diphosphate (Sigma), L- and D-N^G-monomethyl arginine (L-NMMA, D-NMMA, Wellcome), L-nitroarginine methyl ester (L-NAME, Wellcome), sodium nitroprusside (Sigma), tetrodotoxin (Sigma), vasoactive intestinal polypeptide (VIP, human; Auspep, Australia), VIP antibody 7913, freeze-dried powder (Cure Radioimmunoassay Laboratory, U.S.A.). The VIP antibody has been described by Walsh & Wong (1987). It was made up in PSS in a concentration of 400 μg ml^{-1}, equivalent to a 1:170 dilution of the original serum.

Quantitative data are expressed as means ± standard error of mean (s.e.mean). Differences between means were compared by Student's t test, and values of $P < 0.05$ were taken to indicate statistical significance.

Results

Field stimulation (1 and 5 Hz for 1 min) produced relaxations of the tracheal strips (Figure 1). Tetrodotoxin (1 μM) abolished these NANC-induced responses. ω-Conotoxin GVIA (50 nM), after 30 min contact with the strips, abolished relaxations induced by stimulation at 1 Hz. Relaxations evoked at a pulse frequency of 5 Hz were significantly ($P < 0.01$) reduced to 30.3 ± 1.7% ($n = 3$) of control responses. The relaxations produced by VIP (10 nM) or sodium nitroprusside (0.1 μM) were not affected by tetrodotoxin (1 μM) or conotoxin (50 nM).

Effects of L-NMMA and L-NAME on NANC-induced relaxations

After exposure of tracheal strips for 10 min to L-NMMA (30 and 90 μM), relaxant responses to stimulation at 1 or 5 Hz for 1 min were reduced, as shown for one experiment in Figure 1a. In 5 experiments, the mean relaxations elicited by stimulation at 1 Hz after exposure to 30 and 90 μM L-NMMA for 10 min were 64.5 ± 3.4% and 37.7 ± 3.2%, respectively, of the control responses. Relaxations elicited by stimulation at 5 Hz were reduced to a lesser extent after exposure to 90 μM L-NMMA for 10 min, being 78.1 ± 2.2% ($n = 5$) of the control responses. D-NMMA (90 μM) had no effect (Figure 1a), the relaxations at 1 Hz being 100.4 ± 6.4% ($n = 3$) of control. In 6 time-control experiments, the relaxations at 1 and 5 Hz were 98.5 ± 3.0% and 100.3 ± 2.0% of the initial responses.

Exposure to L-NAME (90 μM) for 10 min abolished the response to stimulation at 1 Hz (Figure 1a) and significantly ($P < 0.01$) reduced that at 5 Hz, the mean relaxation being 48.7 ± 3.4% ($n = 6$) of the control responses.

The relative potencies of L-NMMA and L-NAME in inhibiting NANC relaxations were estimated with stimulation at 5 Hz. In these experiments the inhibitory effects of 3 and 10 μM of L-NAME bracketted that of 90 μM of L-NMMA (Figure 2). Thus L-NAME was about 10 to 30 times more potent than L-NMMA.

Neither L-NMMA (90 μM) nor L-NAME (90 μM) affected the resting tone of the tracheal strips or relaxations produced by sodium nitroprusside or VIP. The relaxations produced by sodium nitroprusside (0.1 μM) after strips had been exposed to 90 μM L-NMMA or L-NAME in 3 experiments with each were 98.2 ± 12.5% and 97.9 ± 12.2%, respectively, of control responses. The relaxations produced by 10, 20 and 50 nM VIP after strips had been exposed to L-NAME (90 μM) in 4 experiments were 99.2 ± 6.8%, 106.9 ± 6.3% and 93.8 ± 4.1%, respectively, of the control responses.

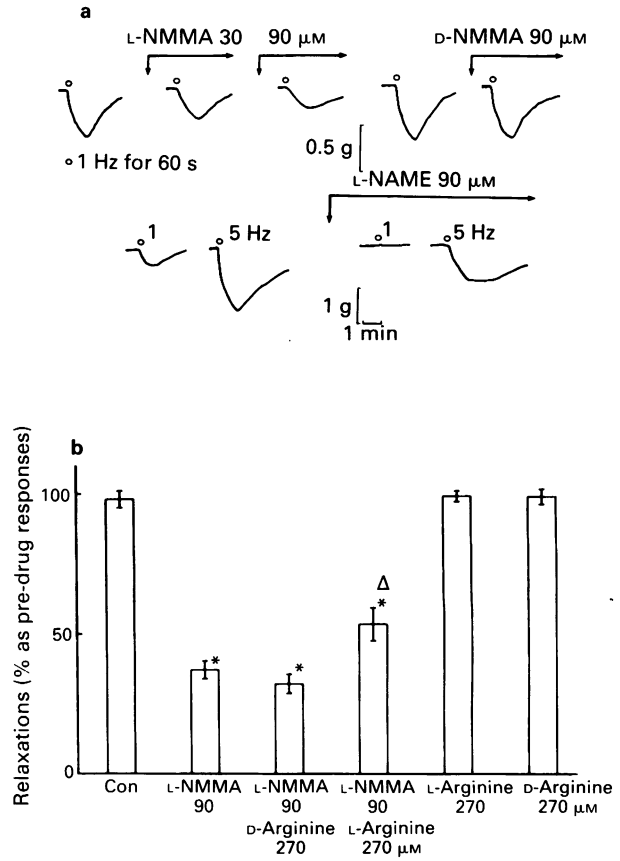

Figure 1 (a) Records of the effects of L- and D-N^G-monomethyl arginine (NMMA) and L-nitroarginine methyl ester (L-NAME) on NANC stimulation-induced (1 or 5 Hz for 60 s) relaxations of guinea-pig tracheal strips. Guanethidine (5 μM) and atropine (3 μM) were present to block responses to stimulation of adrenergic and cholinergic nerves and histamine (10 μM) was present to raise the tone. The contact time for L- or D-NMMA or L-NAME was 10 min. (b) Effects of L- and D-arginine on NANC stimulation-induced (1 Hz for 60 s) relaxations in the absence and presence of L-NMMA. The contact time for L-NMMA (90 μM) or L- or D-arginine (270 μM) was 10 min. In drug combination experiments, following 20 min of exposure to L-NMMA, tissues were exposed to L- or D-arginine for 10 min. The NANC relaxation in each preparation before the addition of L-NMMA or L-arginine was taken as 100%. Columns represent mean values ($n = 3$–6) and vertical bars indicate standard errors of means. * indicates $P < 0.05$ compared with control. Δ indicates $P < 0.05$ compared with L-NMMA alone.

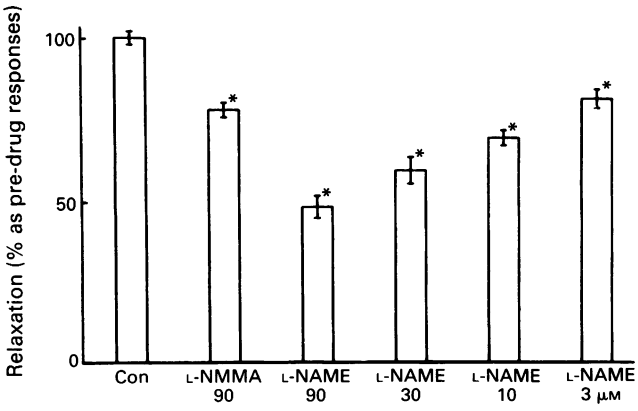

Figure 2 Effects of L-N^G-monomethyl arginine (NMMA) and L-nitroarginine methyl ester (NAME) on NANC stimulation-induced (5 Hz for 60 s) relaxations of guinea-pig tracheal strips. Columns represent mean values ($n = 3$–6) and vertical bars indicated standard errors of means. The contact time for L-NMMA or L-NAME was 10 min. * indicates $P < 0.05$ compared with control. Other details as in legend to Figure 1a.

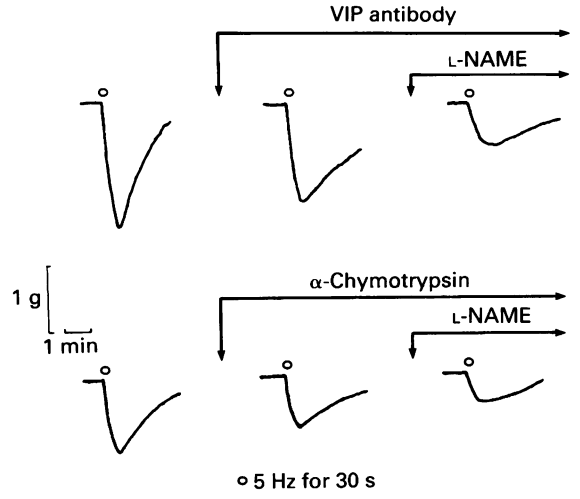

Figure 3 Records of the effects of VIP antibody 7913 (400 μg ml^{-1}, equivalent to serum dilution of 1:170), α-chymotrypsin (1 unit ml^{-1}) and L-nitroarginine methyl ester (NAME) (90 μM) on the NANC stimulation-induced (5 Hz for 30 s) relaxations of smooth muscle in guinea-pig tracheal strips. The tissue was incubated with VIP antibody for 60 min or α-chymotrypsin for 15 min before exposure to L-NAME for 10 min. Other details as in legend to Figure 1a.

The effect of L-NMMA in reducing stimulation-induced (1 Hz for 60 s) relaxations was partially reversed by L-arginine but not by D-arginine (Figure 1b). Neither D-arginine (270 μM) nor L-arginine (270 μM) modified NANC-induced relaxations (Figure 1b).

Effects of VIP antibody and α-chymotrypsin on NANC-induced relaxations

Incubation of guinea-pig tracheal strips with VIP antibody (400 μg ml^{-1}, equivalent to a serum dilution of 1:170) abolished relaxations produced by 10 and 20 nM VIP. (The relaxation produced by 10 nM VIP was of about the same magnitude as that produced by stimulation at 5 Hz.) The relaxation produced by 50 nM VIP, which was about 2.5 times larger in magnitude than that induced by stimulation at 5 Hz, was significantly ($P < 0.01$) reduced to 28.0 ± 2.3% ($n = 3$) of control. The relaxation elicited by stimulation at 5 Hz for 30 s was significantly ($P < 0.05$) reduced to 77.2 ± 2.8% ($n = 6$) of the control responses.

Incubation of tracheal strips with α-chymotrypsin (1 unit ml^{-1}) abolished the relaxant action of VIP (10–50 nM). The relaxation elicited by stimulation at 5 Hz for 30 s was significantly ($P < 0.05$) reduced to 63.4 ± 3.2% ($n = 6$) of the control responses.

The residual relaxant responses to stimulation after incubation of tracheal strips with VIP antibody or α-chymotrypsin were further reduced by L-NAME (Figure 3). In 3 experiments, the mean relaxations elicited by stimulation at 5 Hz after L-NAME (90 μM) were 38.4 ± 5.8% and 40.9 ± 7.2%, respectively, of the residual responses after incubation with VIP antibody or α-chymotrypsin.

Discussion

Incubation of guinea-pig tracheal strips with VIP antibody or α-chymotrypsin blocked VIP-induced relaxations and reduced NANC stimulation-induced relaxations, which is in accord with the suggestion that VIP is a possible candidate as a NANC inhibitory transmitter to guinea-pig tracheal smooth muscle (Ellis & Farmer, 1989a,b). However, it appears that a substantial part of the NANC relaxant response is mediated by a non-VIP component, as previously concluded by Ellis & Farmer (1989b).

L-NMMA, but not D-NMMA, markedly reduced NANC stimulation-induced relaxations of guinea-pig tracheal strips. The inhibitory effect of L-NMMA was partially reversed by L-arginine but not by D-arginine. These enantiomer-specific effects run parallel to those observed on endothelium-derived relaxing factor (EDRF)-mediated relaxations of vascular smooth muscle (Rees *et al.*, 1989), and to those observed in rat anococcygeus muscle (Li & Rand, 1989; Gillespie *et al.*, 1989) and gastric fundus (Li & Rand, 1990a). In addition, L-NAME was more potent (by a factor of 10–30) than L-NMMA in reducing NANC stimulation-induced relaxations, and is more potent (by a factor of 20) than L-NMMA as an inhibitor of NO synthesis (Schulz *et al.*, 1990). These findings suggest that a component of the NANC-induced relaxation of guinea-pig tracheal smooth muscle is mediated by NO.

The residual relaxations elicited by stimulation after incubation with the VIP antibody or α-chymotrypsin were further reduced by L-NAME, indicating that NO mediated a non-VIP component of NANC transmission. It is unlikely that NO release is stimulated by VIP since L-NAME did not affect VIP-induced responses.

The NANC-induced relaxation of guinea-pig tracheal strips was abolished by tetrodotoxin, as previusly found by Ellis & Farmer (1989a). The effect of ω-conotoxin GVIA on NANC-induced relaxations of guinea-pig tracheal smooth muscle was in accord with its effects on responses to cholinergic, noradrenergic and NANC nerve stimulation in a number of tissues (De Luca *et al.*, 1990): responses to stimulation at 1 Hz were abolished and those to stimulation at 5 Hz were reduced. Since similar frequency-dependent effects on NANC-induced relaxations of guinea-pig tracheal smooth muscle were produced by L-NMMA and L-NAME, it appears that ω-conotoxin GVIA selectively inhibited the component of the NANC-induced relaxation that was mediated by NO. Therefore, it may be suggested that a NO-generating system or a NO-yielding substance is released from NANC nerve terminals in guinea-pig tracheal smooth muscle, as has been suggested for the NANC terminals in the rat anococcygeus muscle (Li & Rand, 1989). The presence of a NO-generating system in nerve terminals (brain synaptosomes) has been demonstrated by Knowles *et al.* (1989).

Some differences exist between neurotransmission processes involving nitric oxide in the guinea-pig trachea and the rat anococcygeus. The inhibitory effect of L-NMMA on NANC-mediated relaxations of guinea-pig tracheal strips was only partially reversed by L-arginine, suggesting that L-NMMA is strongly bound to the NO-generating enzyme; in contrast, the inhibitory effect of L-NMMA on NANC-mediated relaxations of the rat anococcygeus muscle was more readily reversed (Li & Rand, 1989). Furthermore, L-NMMA (and L-NAME) did not affect the tone of the tracheal smooth muscle strip, whereas in rat anococcygeus muscle (and gastric fundus strips) L-NMMA produced small but clear increases in tone (Li & Rand, 1989; 1990a). The explanations for these differences remain to be elucidated.

The present experiments indicate that NO as well as VIP mediates NANC-induced relaxations of guinea-pig tracheal smooth muscle, but they do not preclude the possibility there may be yet another mediator. Moreover, they throw no light on the question of whether NO and VIP are released from different neurones or are components of a cotransmitter system.

There is now evidence to suggest that NO is a mediator of NANC inhibitory transmission in a number of tissues (see Introduction). It would therefore be appropriate to devise a term to describe this mode of transmission. We suggest 'nitrergic' to accord with the accepted terms cholinergic and (nor)adrenergic.

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Comparison of the effects of neuropeptide Y and noradrenaline on rat gastric mucosal blood flow and integrity

[1]B.L. Tepperman & [2]B.J.R. Whittle

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

1 The effects of neuropeptide Y (NPY) and noradrenaline on rat gastric mucosal blood flow, as estimated by laser Doppler flowmetry (LDF), have been examined. In addition, the ability of NPY and noradrenaline to induce acute mucosal haemorrhagic damage has also been assessed.

2 Close-arterial infusion of NPY (0.05–0.2 nmol kg^{-1} min^{-1}) for 10 min in the anaesthetized rat induced a dose-dependent fall in LDF, but had minimal effects on systemic arterial blood pressure. Higher doses of NPY did not produce any further reduction in LDF.

3 Close-arterial infusion (0.1–0.4 nmol kg^{-1} min^{-1}) of the structurally related peptide YY (PYY) or pancreatic polypeptide (PP), had inconsistent actions in decreasing LDF.

4 Close-arterial infusion of noradrenaline (30–90 nmol kg^{-1} min^{-1}) dose-dependently reduced gastric LDF.

5 Local infusion of NPY (0.1 and 0.2 nmol kg^{-1} min^{-1}) or noradrenaline (45 and 60 nmol kg^{-1} min^{-1}) resulted in dose-related increases in the area of mucosal hemorrhagic damage.

6 Pretreatment with the α_1-adrenoceptor antagonist, prazosin (0.1 mg kg^{-1}, i.v.) significantly reduced the effect of noradrenaline, but not NPY, on both LDF and mucosal damage.

7 These findings indicate that NPY and noradrenaline act directly on the gastric microvasculature to induce vasoconstriction and both can induce acute mucosal damage. Therefore endogenous NPY, like noradrenaline could play a modulatory role in regulating vascular tone and may influence mucosal integrity.

Introduction

In addition to their classical neurotransmitters, sympathetic neurones may also synthesize and release one or more biologically active peptides (Loren *et al.*, 1979; Hokfelt *et al.*, 1980). Among the many peptides which have been identified in these nerves is the 36 amino acid peptide, neuropeptide Y (NPY; Lundberg & Tatemoto, 1982).

NPY has been identified in postganglionic nerves supplying arteries and veins (Sundler *et al.*, 1983). In these nerves, the peptide co-exists with noradrenaline and is released together with noradrenaline by sympathetic stimulation (Lundberg *et al.*, 1984). In the gastrointestinal tract, NPY-like immunoreactivity has been found in sympathetic nerves associated with blood vessels (Lee *et al.*, 1985; Ekblad *et al.*, 1985) and in enteric neurones originating from the myenteric and submucosal plexus (Furness *et al.*, 1983; Lee *et al.*, 1985; Su *et al.*, 1987). In addition, peptides that are structurally similar to NPY including pancreatic polypeptide (PP) and polypeptide YY (PYY) have also been found in the gut, although these peptides have been identified primarily in endocrine cells (Lundberg *et al.*, 1982; El-Salhy *et al.*, 1983; Bottcher *et al.*, 1984).

The physiological role of NPY is as yet unclear. Exogenous administration of NPY has been reported to produce a direct pressor response in the systemic circulation which was not attenuated by α-adrenoceptor blockade (Lundberg & Tatemoto, 1982; Dahlof *et al.*, 1985). In addition, NPY has been found to enhance the contractile response of arterial segments to α-adrenoceptor stimulation both *in vitro* (Edvinsson *et al.*, 1984; Wahlestedt *et al.*, 1985) and *in vivo* (Aubert *et al.*, 1988). However, in the gastro-intestinal tract, investigations on the possible physiological role of NPY have been directed primarily at effects on non-vascular smooth muscle tone (Allen *et al.*, 1987; Hellstrom, 1987; Holzer *et al.*, 1987) or intestinal ion and water transport (Saria & Beubler, 1985).

In the present study, we have now compared the effects of NPY and noradrenaline on gastric mucosal blood flow using laser Doppler flowmetry in the anaesthetized rat. Furthermore, since adequate perfusion of the gastric microvasculature is necessary for maintaining mucosal integrity (see Whittle, 1989), we have also examined the effects of NPY and noradrenaline in inducing acute mucosal haemorrhagic damage.

Methods

Measurement of gastric mucosal blood flow

Male Wistar rats (230–260 g body weight) were deprived of food but not water for 18–20 h before the experiment. Animals were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.) and the stomach exposed by a mid-line incision. The left gastric artery was cannulated with a short 23 g teflon cannula as described by Esplugues & Whittle (1988). A small bore (8.5 mm o.d.) plastic cannula was then inserted via a small incision in the forestomach and tied in place, to allow free access to the gastric lumen. Gastric blood flow was recorded continuously with a laser Doppler blood flow monitor (Model MBF3D, Moor Instruments Ltd, Devon).

The principle of laser Doppler flowmetry for assessment of gastric blood flow has been described previously (Kiel *et al.*, 1985; Holm-Rutili & Berglindh, 1988). A stainless steel laser optic probe (1.65 mm o.d.; Moor Instruments) was inserted into the gastric lumen via the plastic cannula and was allowed to rest gently on the gastric fundic mucosa. Changes in laser Doppler flow (LDF) were assessed in response to intra-arterial infusions (10 μl min^{-1}) of isotonic saline or the compounds under investigation. Isotonic saline was infused for 5–10 min until LDF had stabilized, and NPY, related peptides or noradrenaline were then infused for 10 min. Average LDF values were determined for the 3 min period of saline infusion just prior to NPY or noradrenaline infusion. Similarly the average LDF was calculated for the final 3 min of the drug infusion period when values of LDF had stabilized. Furthermore, LDF values in the absence of blood flow were recorded after clamping the vascular pedicle. The small LDF value observed as a

[1] Present address: Department of Physiology, University of Western Ontario, London, Ontario, Canada, N6A 5C1.
[2] Author for correspondence.

result of non-specific light reflectance was subtracted from average LDF levels calculated for the control and test periods. In control experiments, saline infusion was continued throughout the experimental period.

In some experiments, agents were infused via the intravenous route with a 25 g hypodermic needle and 0.3 mm polyethylene cannula inserted into a tail vein. Furthermore, to evaluate any interaction between these vasoconstrictors, NPY and noradrenaline were infused simultaneously into the left gastric artery and LDF changes were monitored.

The mean systemic arterial blood pressure (BP) was also measured in some rats from a cannula inserted into a carotid artery and connected to a pressure transducer (Bell & Howell, Ashford, U.K.) and a chart recorder (Grass model 7D polygraph).

In a further series of studies, the α_1-adrenoceptor antagonist, prazosin (0.1 mg kg^{-1}), was injected intravenously 5 min before infusion of NPY or noradrenaline and the changes in LDF were subsequently determined. This dose was selected from preliminary dose-response studies with prazosin (0.05–0.5 mg kg^{-1}) on LDF and BP changes induced by noradrenaline.

Assessment of gastric mucosal damage

Gastric mucosal damage was assessed in further groups of rats in response to local arterial infusions of NPY or noradrenaline. The left gastric artery was cannulated as described above and after ligating the oesophagus and pylorus, 2.0 ml of 0.1M HCl in saline was instilled into the gastric lumen by injection through the non-glandular portion of the stomach. Either NPY or noradrenaline were then infused for 10 min via the left gastric artery. At the end of the infusion period the arterial cannula was clamped and 20 min later, the stomach was removed and opened along the greater curvature. The stomach was pinned out, mucosal side up, to a wax block and immersed in neutral buffered formalin and then photographed on colour transparency film. The extent of macroscopically visible damage, involving regions of both haemorrhagic lesions and visible exfoliation, was determined from these projected transparencies via computerized planimetry in a randomized manner. Damage was also assessed in NPY- or noradrenaline-treated rats which had also received an intravenous bolus injection of prazosin (0.1 mg kg^{-1}) 5 min prior to challenge.

For histological confirmation of the nature of the mucosal injury, two samples of the fundus were excised from standardized regions and were processed by routine techniques before embedding in paraffin. Sections (4 μm) were stained with haematoxylin and eosin and examined under a light microscope. Each section was assessed histologically for epithelial cell injury, glandular disruption, vasocongestion, and haemorrhagic damage.

Drugs

Neuropeptide Y (human, rat), pancreatic peptide (rat) and polypeptide YY (human), from Peninsula Labs (St Helens, Merseyside) were dissolved in distilled water and stored frozen in aliquots (−20°C). An aliquot was freshly thawed and diluted with isotonic saline when required. Noradrenaline (Sigma Chemical Co. Dorset) was freshly dissolved in isotonic saline. Prazosin (Pfizer, Sandwich, Kent) was freshly dissolved in a small volume of N,N-dimethylacetamide (Sigma; 10% of final volume) then brought up to volume with isotonic saline. This vehicle alone had no effect on BP or the response to noradrenaline.

Statistical analysis

Changes in mucosal blood flow were determined as % change in LDF from the initial control period during local infusion of isotonic saline. Mucosal injury was expressed as % of the total mucosal area. All data are expressed as mean ± s.e.mean. Comparisons between two groups were made by Student's t test for unpaired data, while multiple comparisons between different groups was by analysis of variance and Duncan's multiple range test. P values of less than 0.05 were taken as significant.

Results

Effects of neuropeptide Y and noradrenaline on laser Doppler flow

Close-arterial infusion of NPY (0.5–0.2 nmol kg^{-1} min^{-1}) resulted in a dose-dependent significant reduction in LDF (Figure 1). LDF was observed to decline within 3–5 min of the start of infusion and nadir values were achieved within 7 min, after which LDF stabilized at this level. The maximal fall in LDF (41 ± 5%, $n = 4$, $P < 0.05$) was obtained with NPY at 0.2 nmol kg^{-1} min^{-1}, and there was no further significant decrease in LDF with higher doses (0.4 and 0.6 nmol kg^{-1} min^{-1}) of this peptide (Figure 1). In contrast, close-arterial infusion of noradrenaline produced a progressive reduction in LDF over the entire narrow dose-range examined in the present study (30–90 nmol kg^{-1} min^{-1}), with a maximum fall of 84 ± 6% of control ($n = 5$; $P < 0.05$). The reduction in LDF was well-maintained throughout the infusion of these doses of noradrenaline. In control studies, close-arterial infusion of saline at 10 μl min^{-1} over similar periods of time did not result in significant changes in LDF ($n = 4$).

In contrast to local administration, intravenous administration of NPY (0.2 nmol kg^{-1} min^{-1}), in a dose significantly elevating BP (see below), did not significantly reduce LDF (5.5 ± 3.2% of control, $n = 4$). In other studies, simultaneous intra-arterial administration of near-threshold doses of NPY (0.05 nmol kg^{-1} min^{-1}) and noradrenaline (10 nmol kg^{-1} min^{-1}) produced no greater reduction in mean LDF than with each agent alone ($n = 3$).

Effects on systemic arterial blood pressure

Infusion of NPY (0.1–0.2 nmol kg^{-1} min^{-1}) via the gastric intra-arterial or intravenous routes resulted in small, dose-dependent increases in mean BP (Figure 2). At each dose examined, the increases in BP were significantly ($P < 0.05$) greater when NPY was infused intravenously than when given by the intra-arterial route.

Noradrenaline was less active than NPY in producing threshold increases in BP, when infused either by the gastric intra-arterial or intravenous route (Figure 2). As with NPY,

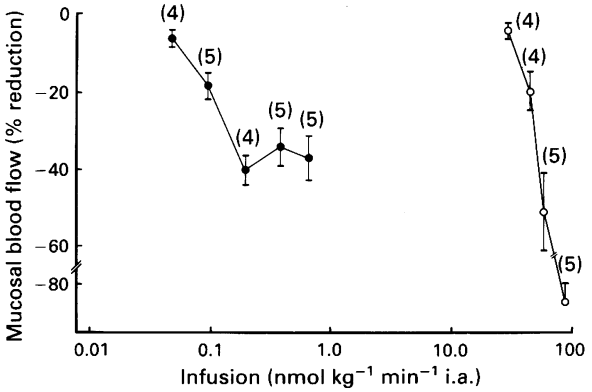

Figure 1 Effects of close-arterial infusion of neuropeptide Y (●, 0.05–0.8 nmol kg^{-1} min^{-1}) or noradrenaline (○, 30–90 nmol kg^{-1} min^{-1}) on gastric mucosal blood flow in the anaesthetized rat, as determined by laser Doppler flowmetry. Results are expressed as mean % reduction from control of (n) values, where vertical bars represent s.e.mean. All groups, except the lowest dose of noradrenaline, were significantly different ($P < 0.05$) from control.

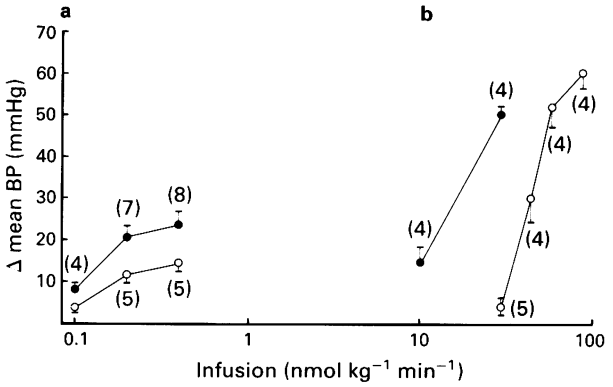

Figure 2 Effect of intravenous (●) or close-arterial (○) infusion of (a) neuropeptide Y (0.1–0.4 nmol kg^{-1} min^{-1}) or (b) noradrenaline (10–90 nmol kg^{-1} min^{-1}) on mean systemic arterial blood pressure (BP) in the anaesthetized rat. Results, expressed as BP (mmHg), are shown as the mean of (n) values, where vertical bars represent s.e.mean. All groups, except the lowest intra-arterial dose of NPY or noradrenaline, were significantly different ($P < 0.05$) from control.

noradrenaline was more active ($P < 0.05$) when infused intravenously and significant changes in BP were evident only when noradrenaline was infused intra-arterially in a dose greater than 30 nmol kg^{-1} min^{-1}. The observed maximal increase in BP in response to intra-arterial infusion of NPY (0.4 nmol kg^{-1} min^{-1}) was 15 ± 3 mmHg, whereas intra-arterial infusion of noradrenaline (90 nmol kg^{-1} min^{-1}) resulted in an increase in BP of 61 ± 4 mmHg ($n = 4$).

Effects of pancreatic polypeptide and peptide YY on laser Doppler flow

The effects of peptides which are structurally similar to NPY were also examined following close-arterial administration, PP infusion (0.1, 0.2 and 0.4 nmol kg^{-1} min^{-1}) resulted in small decreases in LDF of 10 ± 6% ($P > 0.05$), 19 ± 5% ($P < 0.05$), and 13 ± 8% ($P > 0.05$) of control, respectively ($n = 3$ for each), which were significantly smaller than those with equivalent doses of NPY. Close-arterial infusion of PYY (0.1–0.4 nmol kg^{-1} min^{-1}) reduced LDF to a comparable maximal level as NPY, although the dose-response relationship was less consistent. Thus, the changes in LDF with NPY (0.1, 0.2 and 0.4 nmol kg^{-1} min^{-1}) were 41 ± 2% ($P < 0.05$), 40 ± 6% ($P < 0.05$) and 28 ± 13% ($P < 0.05$) of control, respectively ($n = 3$ for each).

Gastric damage by neuropeptide Y and noradrenaline

Local intra-arterial infusion of NPY (0.1 and 0.2 nmol kg^{-1} min^{-1}) or noradrenaline (45 and 60 nmol kg^{-1} min^{-1}) resulted in significant dose-related increases in the area of mucosal damage when compared to control animals (Figure 3). The damage was observed as areas of exfoliation of surface tissue and distinct regions of haemorrhagic necrosis. Mucosal damage in response to NPY (0.2 nmol kg^{-1} min^{-1}) was not significantly different from that produced by noradrenaline (45 nmol kg^{-1} min^{-1}).

On histological examination, the mucosal injury induced by NPY (0.2 nmol kg^{-1} min^{-1}) involved loss of surface epithelial cells with deep glandular damage involving up to 50% of the length of the section, with areas of vasocongestion and haemorrhage, as shown in Figure 4. The mucosal damage induced by noradrenaline (45 nmol kg^{-1} min^{-1}) likewise involved distinct epithelial and glandular disruption (Figure 4).

Effect of α_1-adrenoceptor antagonist, prazosin

The effects of α_1-adrenoceptor blockade with prazosin (0.1 mg kg^{-1}) on LDF responses to NPY and noradrenaline

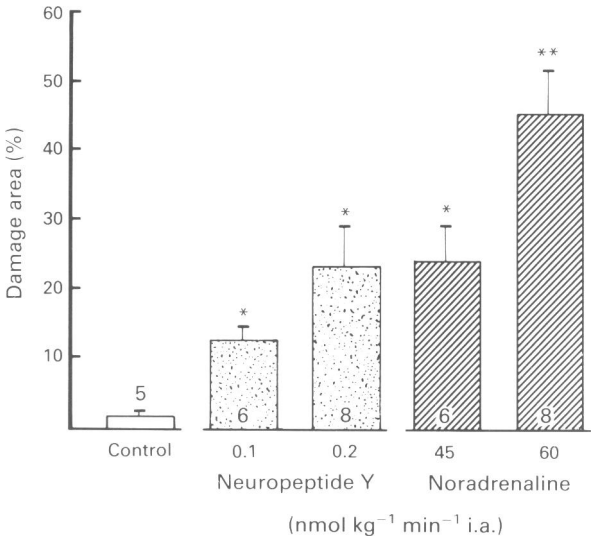

Figure 3 Induction of gastric mucosal damage following close-arterial infusion of neuropeptide Y (0.1 and 0.2 nmol kg^{-1} min^{-1}) or noradrenaline (45 and 60 nmol kg^{-1} min^{-1}) for 10 min in the anaesthetized rat. Results, expressed as macroscopic damage, % of total mucosal area, are the mean of (n) values, where vertical bars represent s.e.mean and significant difference from control is shown as * $P < 0.05$, ** $P < 0.01$.

were investigated. In these studies, prazosin was evaluated against doses of noradrenaline (60 nmol kg^{-1} min^{-1}) and NPY (0.2 nmol kg^{-1} min^{-1}) which produced statistically similar changes in LDF (Figure 1). Whereas prazosin did not significantly alter the effect of NPY infusion on LDF, the reduction in LDF observed after intra-arterial infusion of noradrenaline was eliminated, as shown in Figure 5. Prazosin administration alone did not significantly influence LDF ($n = 3$).

The effects of prazosin on the extent of mucosal haemorrhagic damage induced by intra-arterial infusion of NPY (0.2 nmol kg^{-1} min^{-1}) or noradrenaline (60 nmol kg^{-1} min^{-1}) were also investigated. Prazosin substantially ($P < 0.05$) reduced the extent of haemorrhagic damage induced by noradrenaline (69 ± 5% inhibition), yet did not significantly reduce the area of mucosal damage induced by NPY (Figure 6).

Discussion

The results of the present study indicate that NPY is a potent vasoconstrictor in the gastric mucosal microcirculation, as assessed by laser Doppler flowmetry. This finding extends previous studies demonstrating vasoconstrictor actions of NPY on splenic, renal, testicular, skeletal muscle, and large or small intestinal vascular beds (Lundberg & Tatemoto, 1982; Lundberg *et al.*, 1982; Hellstrom, 1987; Westfall *et al.*, 1987; MacLean & Hiley, 1990). The present study also demonstrates that local intra-arterial infusion of noradrenaline will reduce gastric mucosal blood flow in the rat. The vasoconstrictor activity of noradrenaline in the rat gastric microcirculation has been previously demonstrated by *in vivo* microscopy techniques (Guth & Smith, 1975; Oren-Wolman & Guth, 1984) following local application to the submucosal vessels. While that study demonstrated the phenomenon of vascular escape from the vasoconstriction, such an effect was not evident under the conditions of the present study, which may reflect the different methodologies used to measure the microvascular responses, the region of measurement, or the route of administration of noradrenaline.

NPY appeared to be more active in producing threshold vasoconstrictor responses in the gastric microcirculation than

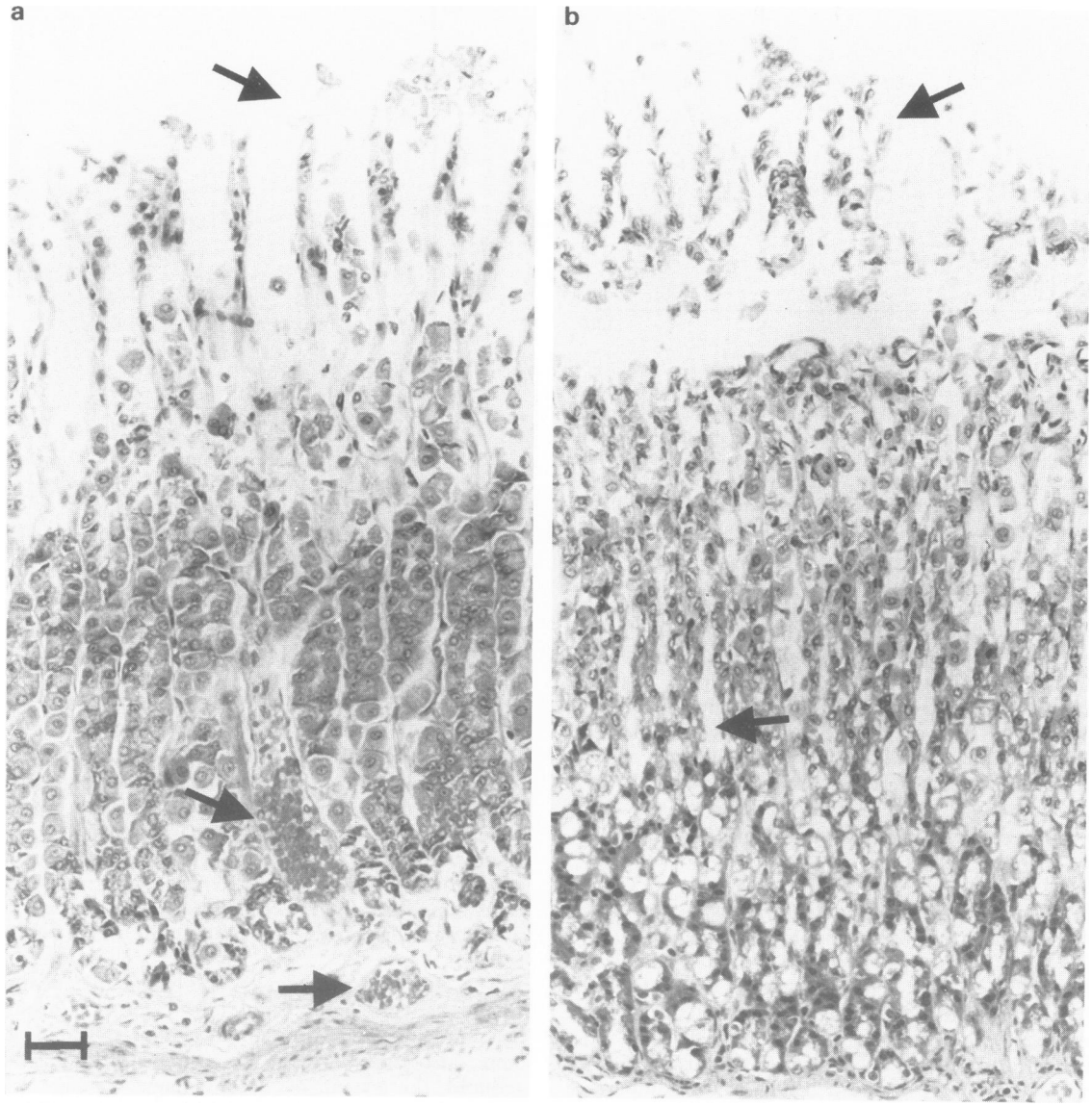

Figure 4 Histological appearance of the corpus region of the rat gastric mucosa following local intra-arterial infusion of (a) neuropeptide Y (0.2 nmol kg^{-1} min^{-1}) or (b) noradrenaline (45 nmol kg^{-1} min^{-1}) for 10 min. Sections (4 μm) were stained with haemotoxylin and eosin. The upper arrows denote epithelial disruption while the lower arrows denote areas of deeper damage and vasocongestion. Marker bar represents 40 μm.

noradrenaline, although noradrenaline did produce a significantly greater maximal reduction in LDF. Similarly, Lundberg & Tatemoto (1982) have demonstrated that NPY was a more potent vasoconstrictor than noradrenaline in the cat submandibular gland. These authors have also demonstrated that the structurally-related peptides PP and PYY were effective vasoconstrictors in that vascular bed. In that study, the relative molar potencies were found to be PYY > NPY > PP (Lundberg & Tatemoto, 1982). In other studies on the rabbit isolated femoral artery, the vasoconstrictor potencies of NPY and PYY have been found to be approximately equivalent, while PP appeared to be less potent (Wahlstedt *et al.*, 1985). In the present study, NPY and PYY produced similar maximal reductions in gastric mucosal blood flow although the responses to PYY were less consistent, while PP induced minimal changes following close-arterial infusion. In doses reducing LDF, local infusion of NPY induced small changes in systemic arterial BP. Furthermore, intravenous infusion of NPY induced significantly greater increases in BP than following close-arterial infusion, yet did not significantly alter mucosal blood flow. Likewise, in recent studies in the pithed rat, intravenous infusion of four fold greater doses of NPY which elevated BP and induced vasoconstriction in the mesenteric bed, did not increase vascular resistance in the stomach, as estimated by radiolabelled microspheres (MacLean & Hiley, 1990). The changes in gastric mucosal blood flow observed during local intra-arterial infusion of NPY are thus unlikely to be due primarily to alterations in systemic arterial BP.

Close-arterial infusion of NPY or noradrenaline both resulted in dose-dependent increases in the area of haemorrhagic damage to the oxyntic mucosa. Previous studies have shown that reduction in microvascular perfusion either by direct vasoconstriction or by removal of endogenous vasodilator tone can induce or enhance the development of mucosal damage, erosion or ulceration (Whittle, 1989). Thus, intravenous infusion of noradrenaline in the rat or intra-arterial infusion of vasopressin in the dog, potentiated mucosal damage induced by topical application of bile salts (Whittle, 1983; Ritchie, 1975). The possible role for endogenous NPY in the pathogenesis of mucosal damage is as yet unknown. However, Morris *et al.* (1987) and Lundberg *et al.* (1987) have shown that plasma NPY levels are increased after haemorrhage in both rats and pigs. Since haemorrhagic shock is often followed by gastric damage and bleeding (Guth & Leung, 1987), it could be speculated that NPY release plays some role in this type of gastric injury.

Prazosin treatment did not significantly reduce the effects of

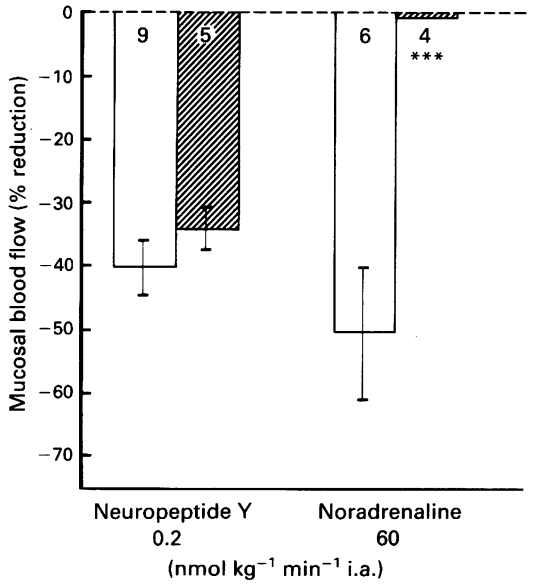

Figure 5 Effect of the α_1-adrenoceptor antagonist, prazosin (0.1 mg kg^{-1}, i.v., hatched columns) on the fall in gastric mucosal blood flow induced by close-arterial infusion of neuropeptide Y or noradrenaline (0.2 and 60 nmol kg^{-1} min^{-1} respectively), as determined by laser Doppler flowmetry; control, open columns. Results are shown as mean % reduction from control of (n) values, where vertical bars represent s.e.mean, and significant difference from the corresponding control groups is shown as *** $P < 0.01$.

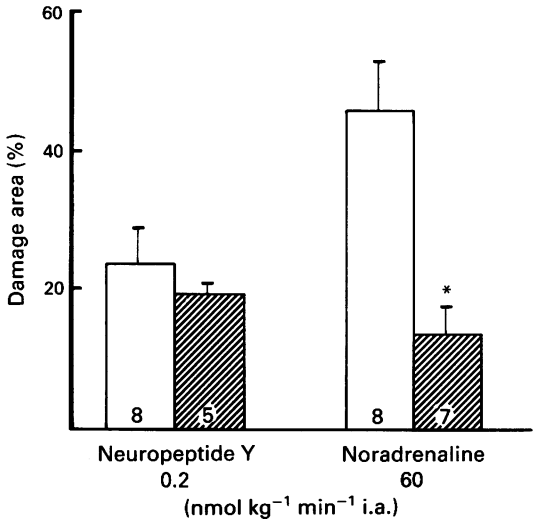

Figure 6 Effect of the α_1-adrenoceptor antagonist, prazosin (0.1 mg kg^{-1} i.v., hatched columns) on the gastric mucosal damage induced by a 10 min close-arterial infusion of neuropeptide Y or noradrenaline (0.2 and 60 nmol kg^{-1} min^{-1} respectively); control, open columns. Results, expressed as macroscopic damage, % of the total mucosal area, are shown as mean of (n) values, where vertical bars represent s.e.mean and significant difference from corresponding control is given as * $P < 0.05$.

NPY on either gastric mucosal blood flow or the induction of mucosal damage, in doses that substantially attenuated the responses to noradrenaline. The inability of α_1-adrenoceptor antagonism to overcome the effect of NPY in the gastric circulation is consistent with other findings in the mesenteric circulation of the rat (Westfall *et al.*, 1987) and circular muscle of guinea-pig intestine (Holzer *et al.*, 1987). While we have not investigated the effects of α_2-adrenoceptor antagonism on NPY-induced reduction in gastric mucosal blood flow, others have demonstrated that activation of α_2- or β-adrenoceptors does not seem to be a prerequisite for NPY vasoconstrictor activity (Aubert *et al.*, 1988; Westfall *et al.*, 1987). The present data demonstrate that NPY and noradrenaline potently affect the gastric microcirculation, probably by an action directly on gastric vascular smooth muscle, through activation of different receptor types.

Previous studies have demonstrated the close anatomical relationship between NPY and noradrenergic neurones in the rat stomach (Wang *et al.*, 1987). After treatment with 6-hydroxydopamine to deplete noradrenergic nerves, NPY-containing fibres around blood vessels also disappeared. Furthermore, it has been demonstrated that in some preparations, NPY may also modulate the actions of noradrenaline by acting prejunctionally to suppress noradrenaline release or postjunctionally to potentiate the noradrenaline response (Wahlstedt *et al.*, 1985; Hakanson *et al.*, 1988; Aubert *et al.*, 1988). However, in our preliminary studies, we have not been able to confirm a significant postjunctional interaction between exogenously administered threshold doses of NPY and noradrenaline in the gastric microcirculation, at least under the present experimental conditions. Much of the data in favour of such interactions between NPY and noradrenaline in different vascular beds has been obtained from *in vitro* studies, although facilitation of the systemic pressor actions of α-adrenoceptor agonists *in vivo* by NPY has been observed in pithed and unanaesthetized rats (Dahlof *et al.*, 1985; Aubert *et al.*, 1988). Such NPY-noradrenaline interactions have not been observed in all vascular tissue studied; NPY did not potentiate the vasoconstrictor effects of noradrenaline in segments of gastro-epiploic vein in contrast to its actions on gastro-epiploic artery (Wahlstedt *et al.*, 1985). Moreover, suppression of noradrenaline release by NPY in rabbit gastro-epiploic artery could not be demonstrated (Ekblad *et al.*, 1984). However, we cannot yet exclude the possibility that endogenous NPY does interact with noradrenaline under physiological conditions in the rat gastric microvasculature.

It is of interest that the cardiovascular profile of NPY shares some similarities to that of the endothelium-derived vasoconstrictor peptide, endothelin-1 (see MacLean & Hiley, 1990), and previous studies have shown that endothelin-1 can also induce damage to the rat gastric mucosa following local intra-arterial infusion (Whittle & Esplugues, 1988). The interaction of NPY with locally released endothelin-1 and other vasoactive mediators in the gastric microcirculation thus warrants investigation. The present findings that NPY induces vasoconstriction and damage in the gastric mucosa, therefore suggest that endogenous NPY, like noradrenaline may have a modulatory role in the regulation of gastric mucosal blood flow and mucosal integrity under physiological and pathophysiological conditions.

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Effects of cyclic nucleotides and phorbol myristate acetate on proliferation of pig aortic endothelial cells

Shonna Alexandra Moodie & [1]William Martin

Department of Pharmacology, University of Glasgow, Glasgow, G12 8QQ

1 The role of cyclic nucleotides and protein kinase C in controlling proliferation of pig aortic endothelial cells (PAEC) in culture was investigated.

2 Dibutyryl cyclic AMP (30 μM), added twice daily, inhibited proliferation but 8 bromo cyclic GMP (30 μM) had no effect. Two other stimuli known to increase PAEC cyclic GMP content by stimulating particulate and soluble guanylate cyclase respectively, atriopeptin II (10 nM) and sodium nitroprusside (1 μM), were also without effect on proliferation.

3 Two agents known to inhibit soluble guanylate cyclase and lower intercellular cyclic GMP content, haemoglobin (10 μM) and methylene blue (10 μM), each inhibited proliferation of PAEC.

4 The inhibitory effect of haemoglobin (10 μM) was mediated by inhibition of soluble guanylate cyclase since it was reversed by agents known to increase cyclic GMP content, i.e. atriopeptin II (10 nM), 8 bromo cyclic GMP (30 μM) or sodium nitroprusside (1 μM). The inhibitory effect of methylene blue (10 μM) was not reversed by these agents.

5 Phorbol 12-myristate 13-acetate (PMA, 0.1 nM–1 μM), which activates protein kinase C, inhibited proliferation in a concentration-dependent manner. No early stimulation of proliferation was seen wtih PMA. The inactive isomer, 4α-phorbol 12,13-didecanoate (0.3 μM), lacked the ability of PMA to inhibit proliferation of PAEC.

6 PMA-induced inhibition of proliferation appeared not to be due to stimulated production of destructive oxygen-derived free radicals since it was unaffected by the radical scavengers, vitamin E (30 μM) or butylated hydroxytoluene (30 μM). The antiproliferative actions of paraquat (10 μM), an agent which generates free radicals intracellularly, was, in contrast, inhibited by vitamin E or butylated hydroxytoluene. Furthermore, neither dibutyryl cyclic AMP (30 μM) nor 8 bromo cyclic GMP (30 μM) had any effect on the ability of PMA to inhibit proliferation.

7 This study suggests that cyclic AMP, cyclic GMP and protein kinase C play a role in controlling the proliferation of PAEC.

Introduction

Identification of the hormonal factors and intracellular pathways that control migration and proliferation of vascular endothelial and smooth muscle cells is important since dysfunction of both cell types is observed in several vascular diseases (Hoshi *et al.*, 1988a; Klagsbrun & Edelman, 1989). With the exception of cells from the microvasculature (Bar *et al.*, 1989), endothelial cells exhibit a growth independence from platelet-derived mitogens by being able to grow equally well in serum- or plasm-supplemented medium (Kazlauskas & DiCorleto, 1985). Several endothelial cell mitogens have been identified, including, basic fibroblast growth factor, epidermal growth factor and a family of acidic polypeptide mitogens, including acidic fibroblast growth factor, endothelial cell growth factor and eye-derived growth factor (Gospodarowicz *et al.*, 1978; 1986; Schreiber *et al.*, 1985; Sato & Rifkin, 1988). The mitogenic actions of growth factors are mediated by interactions with cell surface receptors, and protein phosphorylation is known to be an early biochemical signal initiated upon activation of these receptors. Furthermore, differences in protein phosphorylation patterns occur in proliferating and density-inhibited endothelial cells (Kazlauskas & DiCorletto, 1987).

Several studies indicate that cyclic nucleotides acting via specific protein kinases may regulate proliferation of cells (Friedman, 1981). Subsequent reports have shown that cyclic AMP has differing actions on proliferation of endothelial cells from different sources in culture: inhibition of proliferation was observed with bovine aortic and rat brain microvascular endothelial cells (Leitman *et al.*, 1986; Kempski *et al.*, 1987), but stimulation was observed in a foetal bovine aortic endothelial cell line (Presta *et al.*, 1989). In contrast to cyclic AMP, the role of cyclic GMP in endothelial cell proliferation has not been reported.

The role of the phospholipid/calcium-dependent kinase, protein kinase C, in signal transduction is well documented (Nishizuka, 1984; Blackshear, 1988). The activation of protein kinase C by tumour promoting phorbol esters has been shown to have differing actions on proliferation of endothelial cells from different sources in culture: inhibition of proliferation was observed with human aortic and bovine capillary endothelial cells (Doctrow & Folkman, 1987; Hoshi *et al.*, 1988b); no effect was seen on bovine aortic endothelial cells (Doctrow & Folkman, 1987); and stimulation was observed on foetal bovine aortic and bovine cerebral cortex capillary endothelial cells (Daviet *et al.*, 1989; Presta *et al.*, 1989). In pig aortic endothelial cells, phorbol esters have been reported to produce an initial stimulation of proliferation which is followed within hours by inhibition (Uratsuji & DiCorleto, 1988).

In this study we have attempted to evaluate more fully the influence of cyclic nucleotides and of protein kinase C stimulation upon proliferation of pig aortic endothelial cells in culture.

Methods

Endothelial cell culture

Pig aortae were obtained from a local abattoir and the endothelial cells isolated by collagenase (0.2%) treatment as previously described (Gordon & Martin, 1983). Pig aortic endothelial cells (PAEC) were grown in Dulbecco's modified

[1] Author for correspondence.

Eagle's medium (DMEM) supplemented with foetal calf serum (10%), newborn calf serum (10%), penicillin (100 units ml^{-1}), streptomycin (100 $\mu g\,ml^{-1}$), kanamycin (100 $\mu g\,ml^{-1}$) and glutamine (4 mM): this is subsequently referred to in the text as normal serum-supplemented DMEM. Cells were grown at 37°C under an atmosphere of 5% CO_2 in air.

Cells were seeded initially into 80 cm^2 tissue culture flasks and grown to confluence, attained in 6–8 days. The serum-supplemented DMEM was aspirated off and replaced every 2 or 3 days.

Cells were characterized as endothelial cells by their growth in a strict monolayer and typical cobblestone appearance. Furthermore, we have previously reported their ability to secrete prostacyclin and endothelium-derived relaxing factor (Martin *et al.*, 1988), and to fluoresce when incubated with the selective marker, acetylated low-density lipoprotein labelled with 1,1'dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchorate (CMD, UK Ltd) (Voyta *et al.*, 1984).

Proliferation studies

For proliferation studies, PAEC were seeded at a density of approximately 10^4 cells/cm^2 in six-well plates (9.6 cm^2). The effects of various treatments on proliferation were then examined over the following 6–10 days. PMA, vitamin E, butylated hydroxytoluene and 4α-phorbol 12,13-didecanoate (4α-PDD) were dissolved in 100% ethanol. At the dilutions used, the maximum concentration of ethanol (0.1% v/v) had no effect by itself on proliferation. All other drugs were dissolved in distilled water and sterilised by filtration through a Millipore filter (0.2 μM pore size). Solutions of haemoglobin were reduced to the ferrous form before use with sodium dithionite as previously described (Martin *et al.*, 1985). All drugs were added twice daily with the exception of methylene blue which was added once daily. At the various time points indicated in the Results, cells were detached with a solution of trypsin (0.05%) and EDTA (0.02%) and counted by haemocytometry.

[^3H]-thymidine incorporation experiments

PAEC were plated at a density of approximately 1.5×10^5 cells/cm^2 in six-well plates. The cells were grown in normal serum-supplemented DMEM for 24 h. After a further 24 h incubation in serum-free medium, the cells were challenged with drugs in low serum (2% foetal calf and 2% newborn calf serum) containing DMEM and pulsed with a mixture of [^{3}H]-thymidine (2 μCi/well) and unlabelled thymidine (1 μM) for various times as indicated in the Results. At the end of the incubation period, the cells were washed 3 times with 2 ml of 5% trichloroacetic acid and solubilised in 1 ml of 0.25 M NaOH. The radioactivity in the solubilised cells was determined by liquid scintillation counting and expressed in d.p.m.

Materials

Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, glutamine, foetal calf serum, newborn calf serum and trypan blue were purchased from Gibco Ltd. (Paisley, Scotland). Trypsin – EDTA and kanamycin were purchased from Flow Laboratories (Irvine, Scotland). Tissue culture flasks (80 cm^2) and six-well multidishes were supplied by Nunc (Denmark).

Atriopeptin II, 8 bromo guanosine 3':5'-cyclic monophosphate (8 bromo cyclic GMP), butylated hydroxytoluene, collagenase (type II), dibutyryl adenosine 3':5'-cyclic monophosphate (dibutyryl cyclic AMP), haemoglobin (bovine erythrocytes), methylene blue, paraquat (1,1'dimethyl-4-4'-bipyridinium dichloride), 4α-phorbol 12,13-didecanoate (4α-PDD), phorbol 12-myristate 13-acetate (PMA), sodium nitroprusside, vitamin E (D,L-α-tocopherol acetate) and thymidine were purchased from the Sigma Chemical Company Ltd. (Poole, Dorset). Sodium dithionite was purchased from BDH (Poole, Dorset). [Methyl-^{3}H]-thymidine was purchased from Amersham (Bucks).

Statistical analysis

Results are expressed as the mean ± s.e.mean and comparisons were made by use of Student's *t* test or the Mann-Whitney test when there was unequal variance between samples. A probability of 0.05 or less was considered significant. In the Results, *n* represents the number of wells of cells.

Results

Effect of phorbol 12-myristate 13-acetate on endothelial cell proliferation

Pig aortic endothelial cells (PAEC) seeded at a density of 10^4 cells/cm^2 in normal serum-supplemented DMEM grew to confluence within 6–8 days (Figure 1). The activator of protein kinase C, PMA (0.3 μM), when added twice daily, produced a marked inhibition of proliferation: 86 ± 2% ($n = 6$) inhibition was observed at day 8 (Figure 1). The ability of PMA to inhibit proliferation was observed over the concentration range 0.1 nM–1 μM (Figure 2), but the inactive phorbol ester, 4α-PDD (0.3 μM), lacked the ability of PMA to inhibit proliferation (Figure 1). PMA did not stimulate trypan blue uptake by cells (data not shown).

Phorbol esters are know to stimulate production of oxygen-derived free radicals in endothelial cells (Matsubara & Ziff, 1986). To determine if these radicals contribute to the ability of PMA to inhibit proliferation of PAEC, we examined the effects of vitamin E (30 μM) and butylated hydroxytoluene (BHT, 30 μM). Vitamin E and BHT, when added twice daily, each had no effect on proliferation by themselves and had no effect on the ability of PMA (0.3 μM) to inhibit proliferation (Figure 3a). Paraquat (10 μM), an agent which generates oxygen-derived free radicals intracellularly (Minakami *et al.*, 1990), when added twice daily, also inhibited proliferation of PAEC (Figure 3b). This inhibition was associated with accumulation of trypan blue, and was prevented by the presence of vitamin E (30 μM) or BHT (30 μM) (Figure 3b).

Effect of PMA on [^3H]-thymidine incorporation by endothelial cells

Phorbol esters have been reported initially to stimulate then inhibit proliferation of pig aortic endothelial cells (Uratsuji &

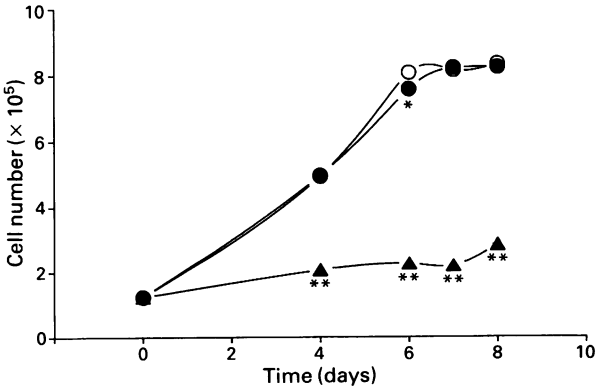

Figure 1 The effect of phorbol myristate acetate (PMA) and 4α-phorbol 12,13-didecanoate (4α-PDD) on the proliferation of pig aortic endothelial cells. Cells were seeded at a density of 10^4 cells/cm^2 in normal serum-supplemented DMEM and received either no drug (○), PMA (0.3 μM, ▲), or 4α-PDD (0.3 μM, ●) twice daily. At the time points indicated, cells were counted by haemocytometry. Points shown mean cell numbers ($n = 6$); all s.e.means are contained within the symbols. * $P < 0.05$; ** $P < 0.0005$; denotes a significant difference from untreated cells on that day.

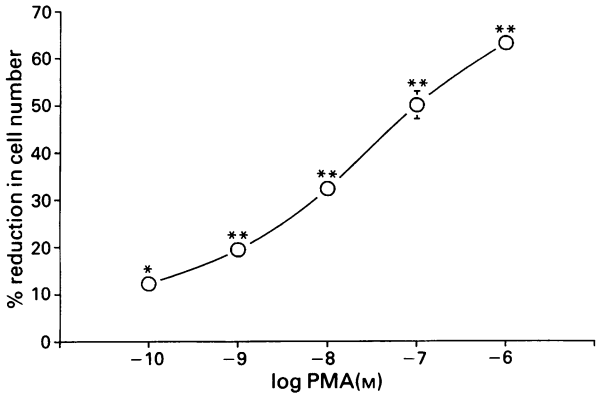

Figure 2 Concentration-effect curve showing the ability of phorbol myristate acetate (PMA) to inhibit proliferation of pig aortic endothelial cells (PAEC) in normal serum-supplemented DMEM. Cells were seeded at a density of 10^4 cells/cm^2. PMA (0.1 nM–1 μM) was added twice daily. The cells were allowed to grow for 4 days and then counted by haemocytometry. The results are expressed as the mean (%) of cell number (s.e.mean reduction shown by vertical bars) when compared with untreated cells ($n = 6$); where error bars are not seen they are contained within the symbols. * $P < 0.005$; ** $P < 0.0005$; denotes a significant difference from untreated cells.

DiCorleto, 1988). Our studies in which cells were counted by haemocytometry showed that PMA induced only inhibition of proliferation. By employing a more sensitive index of proliferation, i.e. [^{3}H]-thymidine incorporation, we attempted to determine if PMA induced an early stimulation of proliferation. PAEC were seeded at a density of 1.5×10^5 cells/cm^2 in 6 well-dishes and allowed to grow for 24 h in normal serum-supplemented DMEM. They were then grown in low serum (2% foetal calf and 2% newborn serum)-containing DMEM in the presence or absence of PMA (0.3 μM), and [^{3}H]-thymidine incorporation measured after 4 and 12 h.

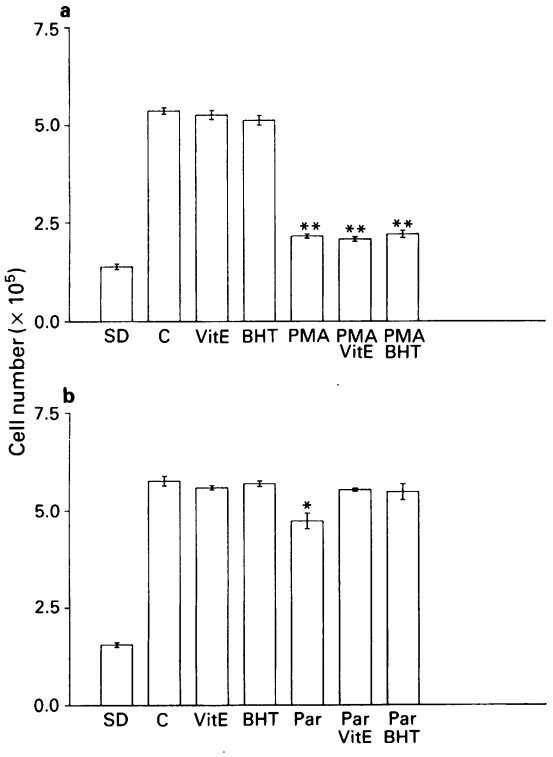

Figure 3 Effects of vitamin E and butylated hydroxytoluene on the antiproliferative actions of phorbol myristate acetate (PMA) and paraquat. Pig aortic endothelial cells were seeded at a density (SD) of $1.3–1.6 \times 10^5$ cells/cm^2 and grown for 4 days in normal serum-supplemented DMEM. (a) Cells received either no drug (C), PMA (0.3 μM), vitamin E (30 μM, VitE), butylated hydroxytoluene (30 μM, BHT), a combination of PMA and vitamin E, or a combination of PMA and BHT. (b) Cells received either no drug (C), paraquat (10 μM, Par), vitamin E (30 μM), BHT (30 μM), a combination of paraquat and vitamin E, or a combination of paraquat and BHT. Drugs were added twice daily and cells were counted by haemocytometry. Columns show the mean cell number with s.e.mean shown by vertical bars. * $P < 0.005$; ** $P < 0.0005$ denotes a significant difference from untreated cells.

Table 1 Effect of phorbol myristate acetate (PMA) on [^{3}H]-thymidine incorporation by pig aortic endothelial cells (PAEC)

Treatment	[^{3}H]-*thymidine incorporation* (d.p.m.) 4 h	12 h
No serum	6454 ± 553**	14259 ± 2865**
Low serum	12430 ± 1060	31380 ± 2520
Low serum and PMA (0.3 μM)	8530 ± 550**	17730 ± 1890**

PAEC were seeded at a density of 1.5×10^5 cells/cm^2 and grown for 24 h in normal serum-supplemented DMEM, followed by a further 24 h in serum-free DMEM. The serum-starved cells were then grown in either serum-free DMEM, low serum (2% foetal calf and 2% newborn calf serum)-containing DMEM, or a combination of low serum-containing DMEM and phorbol 12-myristate 13-acetate (PMA, 0.3 μM). All samples were pulsed with 2 μCi per well of [^{3}H]-thymidine for a period of 4 or 12 h. Results are expressed as the mean ± s.e.mean incorporation of [^{3}H]-thymidine (d.p.m.) ($n = 6$). ** $P < 0.01$ denotes a significant difference from incorporation by cells grown in low serum-containing DMEM.

PMA (0.3 μM) inhibited [^{3}H]-thymidine incorporation by PAEC at both time points (Table 1). In other experiments, PMA (0.3 μM) was found to inhibit [^{3}H]-thymidine incorporation by PAEC in normal serum-supplemented as well as in serum-free DMEM, when measured after 4 and 12 h (data not shown).

Effects of cyclic nucleotides on endothelial cell proliferation

The membrane permeant analogue of cyclic GMP, 8 bromo cyclic GMP (30 μM), added twice daily, had no effect on proliferation of PAEC in normal serum-supplemented DMEM (Figure 4a). The permeant analogue of cyclic AMP, dibutyryl cyclic AMP (30 μM), added twice daily, inhibited proliferation by 35 ± 2% ($n = 6$) at day 8 (Figure 4b). Neither 8 bromo cyclic GMP (30 μM) nor dibutyryl cyclic AMP (30 μM) had any significant effect on the ability of PMA (0.3 μM) to inhibit proliferation (Figure 4).

Two agents that inhibit the activation of soluble guanylate cyclase, methylene blue (10 μM), added once daily, and haemoglobin (10 μM), added twice daily, each inhibited proliferation of PAEC in normal serum-supplemented DMEM (Figure 5): methylene blue caused complete inhibition of proliferation and haemoglobin inhibited proliferation by 27 ± 3% ($n = 6$) and 44 ± 4% ($n = 6$) in two separate experiments. 8 Bromo cyclic GMP (30 μM), added twice daily, had no effect on the ability of methylene blue (10 μM) to inhibit proliferation, but reversed the ability of haemoglobin (10 μM) to inhibit proliferation (Figure 5). Two agents known to elevate endothelial cyclic GMP content, the atrial natriuretic factor, atriopeptin II (10 nM), and the nitrovasodilator, sodium nitroprusside (1 μM), when added twice daily, each had little effect on proliferation by itself, but reversed the ability of haemoglobin to inhibit proliferation (data not shown). These two agents had no effect on the ability of methylene blue to inhibit proliferation (data not shown).

Discussion

The aims of this study were two fold: firstly, to investigate the effects of cyclic nucleotides on proliferation of pig aortic endo-

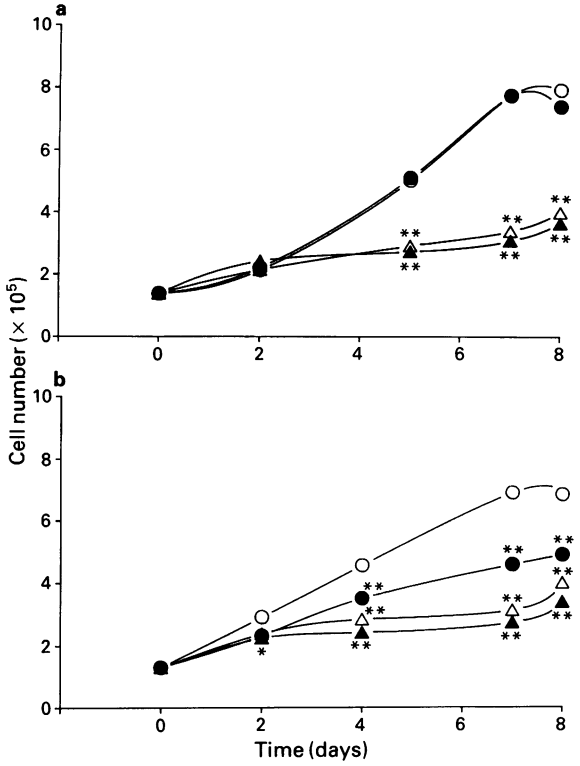

Figure 4 Effect of cyclic nucleotides and phorbol myristate acetate (PMA) on proliferation of pig aortic endothelial cells. Cells were seeded at a density of 10^4 cells/cm^2 and grown in normal serum-supplemented DMEM. (a) Cells received either no drug (○), phorbol myristate acetate (PMA, 0.3 μM, ▲), 8 bromo cyclic GMP (30 μM, ○) or a combination of PMA and 8 bromo cyclic GMP (△). (b) Cells received either no drug (○), PMA (0.3 μM, ▲), dibutyryl cyclic AMP (30 μM, ●) or a combination of PMA and dibutyryl cyclic AMP (△). Drugs were added twice daily, and at the time points indicated the cells were counted by haemocytometry. Points show the mean cell numbers ($n = 6$); all s.e.means are contained within the symbols. * $P < 0.005$; ** $P < 0.0005$; denotes a significant difference from untreated cells on that day.

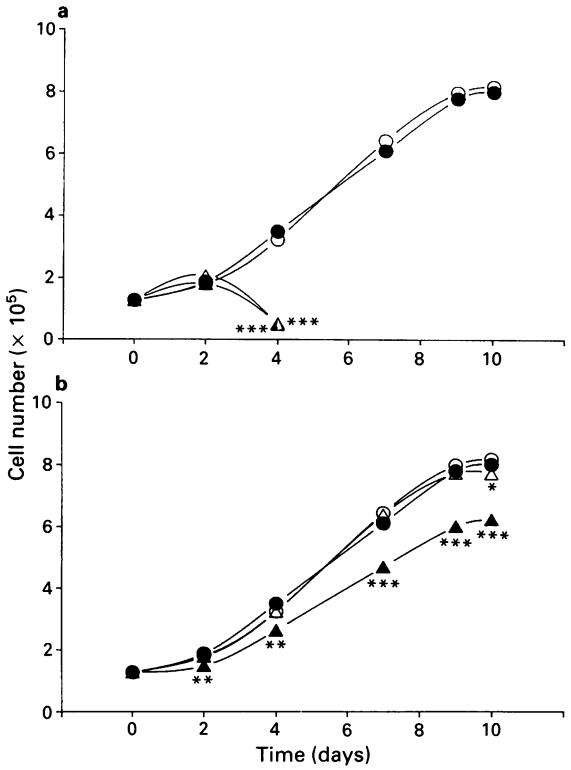

Figure 5 The ability of 8 bromo cyclic GMP to reverse the inhibitory effect of haemoglobin but not of methylene blue on pig aortic endothelial cell proliferation. Cells were seeded at a density of 10^4 cells/cm^2 and grown in normal serum-supplemented DMEM. (a) Cells received either no drug (○), methylene blue (10 μM, ▲), 8 bromo cyclic GMP (30 μM, ●) or a combination of methylene blue and 8 bromo cyclic GMP (△). Note that the symbols for methylene blue and for the combination of methylene blue and 8 bromo cyclic GMP are coincident. (b) Cells received either no drug (○), haemoglobin (10 μM, ▲), 8 bromo cyclic GMP (30 μM, ●) or a combination of haemoglobin and 8 bromo cyclic GMP (△). Drugs were added twice daily with the exception of methylene blue which was added once daily. At the time points indicated, the cells were counted by haemocytometry. Points show the mean cell numbers ($n = 6$); all s.e.means are contained within the symbols. * $P < 0.05$; ** $P < 0.0025$; *** $P < 0.0005$; denotes a significant difference from untreated cells on that day.

thelial cells (PAEC); and secondly, to investigate the effects of protein kinase C activation by phorbol esters on proliferation.

Cyclic nucleotides are known to modulate proliferation of many cell types in culture (Friedman, 1981). We found that the membrane permeant analogue of cyclic AMP, dibutyryl cyclic AMP, profoundly inhibited the proliferation of PAEC in serum-containing medium in culture. Our findings are therefore in agreement with those of Leitman *et al.* (1986) and of Kempski *et al.* (1987) who showed that elevated levels of cyclic AMP inhibit proliferation of bovine aortic and rat brain microvascular endothelial cells, respectively. Not all endothelial cells respond in this manner, however, since in a foetal bovine aortic endothleial cell line, cyclic AMP has been shown to stimulate proliferation (Presta *et al.*, 1989).

It has recently been reported that elevated levels of cyclic GMP inhibit proliferation of vascular smooth muscle cells in culture (Garg & Hassid, 1989). These workers further suggested that endothelium-derived relaxing factor (EDRF), which activates soluble guanylate cyclase, may have a role in inhibiting smooth muscle growth in the arterial wall. The possibility that EDRF or other stimulants of guanylate cyclase modulate proliferation of endothelial cells has not yet been tested. Vascular endothelial cells are known to contain both soluble and particulate guanylate cyclase (Leitman & Murad, 1986; Martin *et al.*, 1988), and measurements of cyclic GMP have shown that endothelial cells respond to the EDRF they produce (Martin *et al.*, 1988). We found that neither sodium nitroprusside, which activates endothelial soluble guanylate cyclase, nor atriopeptin II, which activates endothelial particulate guanylate cyclase (Martin *et al.*, 1988; Schini *et al.*, 1988) had any effect on the proliferation of PAEC in culture. Furthermore, the membrane permeant analogue, 8 bromo cyclic GMP, was also without effect on proliferation. These observations initially suggested that cyclic GMP had no effect on proliferation of PAEC. We found, however, that haemoglobin and methylene blue, which lower endothelial cyclic GMP content by inhibiting the ability of endogenously produced EDRF to stimulate soluble guanylate cyclase (Martin *et al.*, 1988), each inhibited proliferation of PAEC. In the case of haemoglobin, inhibition was specific since it was reversed by agents which increase endothelial cyclic GMP content, i.e. sodium nitroprusside, atriopeptin II or 8 bromo cyclic GMP. EDRF appears therefore to exert a permissive action in regulating proliferation of PAEC through stimulation of soluble guanylate cyclase. Whether or not EDRF exerts such a permissive action on proliferation of all endothelial cells remains to be determined. The inhibitory effect of methylene blue on proliferation was not reversed by sodium nitroprusside, atriopeptin II or 8-bromo cyclic GMP and is likely, therefore, to reflect an action unrelated to guanylate cyclase inhibition.

Activation of protein kinase C (PKC) is an important signal transduction mechanism in many cell types (Nishizuka, 1984; Blackshear, 1988). Recently, it has been reported that the activity of the endothelial cell mitogen, basic fibroblast growth factor, is, at least in part, related to its ability to activate PKC (Presta *et al.*, 1989). This suggests that PKC may play a regu-

latory role in the response of endothelial cells to mitogens. The effects of phorbol esters, known activators of PKC, have been investigated on the proliferation of several endothelial cell types. Phorbol ester-induced inhibition of proliferation was observed in bovine capillary and adult human aortic endothelial cells (Doctrow & Folkman, 1987; Hoshi *et al.*, 1988b), whereas stimulation of proliferation was observed in foetal bovine aortic and bovine cerebral cortex endothelial cells (Daviet *et al.*, 1989; Presta *et al.*, 1989). We found that PMA significantly inhibited proliferation of PAEC in serum-containing medium over a period of 1–8 days. Inhibition of growth is unlikely to result from cytotoxicity since phorbol esters do not affect plating efficiency or cell viability as assessed using the vital stains, trypan blue and fluorescein diacetate (Doctrow & Folkman, 1987; Hoshi *et al.*, 1988b; this study). Inhibition of proliferation by PMA was concentration-dependent and appeared to be due to activation of PKC since the inactive phorbol ester, 4α-phorbol 12,13-didecanoate, lacked antiproliferative activity. In one study of the effects of PMA on proliferation of PAEC, an early stimulation of proliferation was observed which was followed by inhibition (Uratsuji & DiCorleto, 1988). This dualistic action was considered to reflect the ability of phorbol esters to stimulate then down-regulate PKC. We observed no initial stimulation of proliferation by PMA when we counted our cells by haemocytometry. Measurement of [^{3}H]-thymidine incorporation represents a more sensitive index of proliferation rate, but even this technique indicated that PMA induced only inhibition of PAEC with no stimulatory phase even at the earliest time point tested (4 hours). Our results, utilising two independent methods, suggest that activation of PKC powerfully inhibits proliferation of PAEC.

It is generally accepted that PKC exerts its actions on proliferation through changes in the phosphorylation state of proteins and on the expression of oncogenes (Nishizuka, 1984; Blackshear, 1988; Colotta *et al.*, 1988). We considered, however, that the ability of PMA to inhibit the proliferation of PAEC could be mediated by alternative mechanisms. For example, PMA is known to stimulate production of destructive, oxygen-derived free radicals by endothelial cells (Matsubara & Ziff, 1986), and these could potentially contribute to the antiproliferative action. We found that paraquat, another agent known to generate free radicals intracellularly (Minakami *et al.*, 1990), also inhibited proliferation. This inhibition of proliferation was associated with increased uptake of trypan blue and was blocked in the presence of the radical scavengers, vitamin E or butylated hydroxytoluene. The antiproliferative action of PMA, in contrast, was not associated with increased uptake of trypan blue and was not reversed by vitamin E or butylated hydroxytoluene. It is unlikely, therefore, that generation of oxygen-derived free radicals contributes significantly to the ability of PMA to inhibit proliferation. Complex interactions are known to occur between cyclic nucleotides and PKC in the regulation of cell proliferation. For example, it has been reported that cyclic AMP inhibits phorbol ester-induced growth of a human T cell line (Goto *et al.*, 1988). We found, however, that neither dibutyryl cyclic AMP nor 8 bromo cyclic GMP had any effect on the ability of PMA to inhibit proliferation of PAEC. It is unlikely therefore that PMA exerts its antiproliferative action on PAEC by changing the intracellular levels of cyclic nucleotides.

In conclusion, our study shows that cyclic AMP inhibits and cyclic GMP exerts a permissive role on proliferation of PAEC. Furthermore, activation of protein kinase C by phorbol esters powerfully inhibits proliferation of these cells. The extent to which growth factors and regulators utilise each of these intracellular pathways in the control of endothelial growth remains to be determined.

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Further characterization, by use of tryptamine and benzamide derivatives, of the putative 5-HT_4 receptor mediating tachycardia in the pig

Carlos M. Villalón, Marien O. den Boer, Jan P.C. Heiligers & [1]Pramod R. Saxena

Department of Pharmacology, Faculty of Medicine and Health Sciences, Erasmus University Rotterdam, Post Box 1738, 3000 DR Rotterdam, The Netherlands

1 It has recently been shown that the tachycardic response to 5-hydroxytryptamine (5-HT) in the anaesthetized pig, being mimicked by 5-methoxytryptamine and renzapride and blocked by high doses of ICS 205-930, is mediated by the putative 5-HT_4 receptor. In the present investigation we have further characterized this receptor.

2 Intravenous bolus injections of the tryptamine derivatives, 5-HT (3, 10 and 30 $\mu g\,kg^{-1}$), 5-methoxytryptamine (3, 10 and 30 $\mu g\,kg^{-1}$) and α-methyl-5-hydroxytryptamine (α-methyl-5-HT; 3, 10, 30 and 100 $\mu g\,kg^{-1}$), resulted in dose-dependent increases in heart rate of, respectively, 25 ± 2, 48 ± 3 and 68 ± 3 beats min^{-1} (5-HT; $n = 35$); 15 ± 1, 32 ± 2 and 57 ± 3 beats min^{-1} (5-methoxytryptamine; $n = 30$); 6 ± 4, 18 ± 6, 34 ± 6 and 64 ± 11 beats min^{-1} (α-methyl-5-HT; $n = 3$).

3 The increases in heart rate following i.v. administration of certain substituted benzamide derivatives were genereally less marked and not dose-dependent: 1 ± 5, 11 ± 3 and 10 ± 5 beats min^{-1} after 300, 1000 and 3000 $\mu g\,kg^{-1}$ of metoclopramide, respectively, ($n = 8$); 21 ± 4, 19 ± 2 and 2 ± 2 beats min^{-1} after 100, 300 and 1000 $\mu g\,kg^{-1}$ of cisapride, respectively, ($n = 5$); 6 ± 2, 14 ± 2, 37 ± 6, 43 ± 8 and 34 ± 10 beats min^{-1} after 10, 30, 100, 300 and 1000 $\mu g\,kg^{-1}$ of zacopride, respectively, ($n = 6$); and 1 ± 1, 2 ± 1 and 5 ± 2 beats min^{-1} after 300, 1000 and 3000 $\mu g\,kg^{-1}$ of dazopride, respectively, ($n = 4$). These drugs behaved as partial agonists, antagonizing the responses to 5-HT and 5-methoxytryptamine dose-dependently.

4 The 5-HT_3 receptor agonist 1-phenyl-biguanide (100, 300 and 1000 $\mu g\,kg^{-1}$) induced only slight increases in heart rate of 1 ± 1, 6 ± 2 and 11 ± 1 beats min^{-1}, respectively, ($n = 3$). These effects were not antagonized by the selective 5-HT_3 receptor antagonist granisetron (3 $mg\,kg^{-1}$). In addition, 1-phenyl-biguanide (1000 $\mu g\,kg^{-1}$) did not modify the tachycardia induced by either 5-HT- or 5-methoxytryptamine.

5 High doses (3 $mg\,kg^{-1}$) of ICS 205-930, a 5-HT_3 receptor antagonist with an indole group and devoid of effects on porcine heart rate *per se*, antagonized the stimulatory effects of 5-HT, 5-methoxytryptamine, α-Me-5-HT, metoclopramide, cisapride, zacopride, dazopride and 1-phenyl-biguanide. However, the 5-HT_2 receptor antagonist ketanserin (0.5 $mg\,kg^{-1}$), the 5-HT_3 receptor antagonists granisetron (3 $mg\,kg^{-1}$) and MDL 72222 (3 $mg\,kg^{-1}$) and the dopamine D_2 receptor antagonist domperidone (3 $mg\,kg^{-1}$) had no antagonist activity.

6 The above results support our contention that 5-HT, 5-methoxytryptamine, α-Me-5-HT and the substituted benzamide derivatives increase porcine heart rate by a direct action on the cardiac pacemaker, via the activation of a putative 5-HT_4 receptor. The pharmacological profile of this novel 5-HT receptor is similar (neurones from mouse brain colliculi and human heart) or, perhaps, even identical (guinea-pig cholinergic neurones) to other putative 5-HT_4 receptors.

Introduction

5-Hydroxytryptamine (5-HT) can exert multiple cardiac effects including both increases and decreases in heart rate. In most species, bradycardia induced by 5-HT is mediated by 5-HT_3 receptors, via the activation of the von Bezold Jarisch reflex. In marked contrast, the mechanism of 5-HT-induced tachycardia is notoriously species-dependent and is mediated, directly or indirectly, either by 5-HT_1-like (cat), 5-HT_2 (rat, dog) or 5-HT_3 (rabbit, dog) receptors, or by tyramine-like (guinea-pig) or unidentified mechanisms (see Saxena, 1986; Saxena & Villalón, 1990). In the pig, we have reported that the 5-HT-induced tachycardia is mediated by a novel receptor type which differs from 5-HT_1-like, 5-HT_2 and 5-HT_3 receptors (Duncker *et al.*, 1985; Bom *et al.*, 1988), but resembles the putative 5-HT_4 receptor (Villalón *et al.*, 1990) mediating stimulation of adenylate cyclase in both mouse embryo colliculi neurones and guinea-pig hippocampal membranes (Dumuis *et al.*, 1988; 1989; Clarke *et al.*, 1989). In the present study, we have further characterized the porcine heart 5-HT receptor using several agonist and antagonist drugs, including some substituted benzamide derivatives.

Methods

General

After an overnight fast, 40 young Yorkshire pigs (15–20 kg) were sedated with azaperone (120 mg, i.m.) and metomidate (120–150 mg, i.v.). After intubation, the animals were connected to a respirator for intermittent positive pressure ventilation with a mixture of room air (70%) and O_2 (30%). The anaesthesia was maintained with a continuous infusion of pentobarbitone sodium (15–20 $mg\,kg^{-1}\,h^{-1}$, i.v.). Aortic blood pressure and heart rate were recorded with, respectively, a

[1] Author for correspondence.

Statham pressure transducer and a tachograph. All drugs were injected into the right jugular vein. The body temperature of the animals was maintained around 37°C by using an electric blanket and arterial blood gases and pH were kept within the normal limits (Po_2 > 90 mmHg; Pco_2 30–40 mmHg; pH 7.35–7.45) by adjusting respiratory rate and tidal volume or by infusing 4.2% sodium bicarbonate solution.

Experimental protocol

After the animals had been in a stable haemodynamic condition for at least 45 min, they received intravenous bolus injections of 5-HT (3, 10 and 30 $\mu g\,kg^{-1}$) and 5-methoxytryptamine (3, 10 and 30 $\mu g\,kg^{-1}$). Subsequently, several doses of α-methyl-5-HT (3, 10, 30 and 100 $\mu g\,kg^{-1}$), 1-phenyl-biguanide (30, 100, 300 and 1000 $\mu g\,kg^{-1}$), metoclopramide (300, 1000 and 3000 $\mu g\,kg^{-1}$), cisapride (30, 100, 300 and 1000 $\mu g\,kg^{-1}$), zacopride (10, 30, 100, 300 and 1000 $\mu g\,kg^{-1}$) or dazopride (300, 1000 and 3000 $\mu g\,kg^{-1}$) were given, and after each dose or the highest dose (1-phenyl-biguanide), the responses to 5-HT and 5-methoxytryptamine were elicited again (for number of experiments and other specifications, see Results). In addition, tachycardic responses to 5-HT- and 5-methoxytryptamine were induced before and after pretreatment with 3 $mg\,kg^{-1}$ of either MDL 72222, granisetron or domperidone. In another set of experiments, the tachycardic responses to 5-HT, 5-methoxytryptamine and α-methyl-5-HT were analyzed before and after pretreatment with either ketanserin (0.5 $mg\,kg^{-1}$) or ICS 205-930 (3 $mg\,kg^{-1}$). Lastly, the dose of each benzamide derivative eliciting the maximum increase in heart rate was chosen and given to a new group of animals (without previous administration of any of the benzamide derivatives) after pretreatment with 3 $mg\,kg^{-1}$ of ICS 205-930.

The interval between the different doses of the compounds used as agonists and/or antagonists depended on the duration of tachycardia produced by the preceding dose, as in each case we waited until heart rate had returned completely or nearly to baseline values. The dose-intervals for the different drugs were as follows; tryptamine derivatives, between 5 and 15 min; benzamide derivatives and 1-phenyl-biguanide, usually between 15 and 30 min, but sometimes even longer than 60 min (cisapride and zacopride); and other antagonists (ICS 205-930, MDL 72222, granisetron, ketanserin and domperidone), between 10 and 15 min. The dosing with ICS 205-930 was cumulative (given as 0.3, 0.7 and 2.0 $mg\,kg^{-1}$), whereas that with all other drugs was sequential.

Drugs

The drugs used in this study were: cisapride (gift: Dr J.A.J. Schuurkes, Janssen Pharmaceutica, Beerse, Belgium), (±)-dazopride (A.H. Robbins Co., Richmond, VA, U.S.A.), domperidone (gift: Dr J.A.J. Schuurkes, Janssen Pharmaceutica, Beerse, Belgium), granisetron (gift: Dr G.J. Sanger, Smith Kline Beecham, Harlow, U.K.), 5-hydroxytryptamine creatinine sulphate (Sigma Chemical Company, St. Louis, MO, U.S.A.), ketanserin tartrate (gift: Dr J.M. Van Nueten, Janssen Pharmaceutica, Beerse, Belgium), 5-methoxytryptamine hydrochloride (Janssen Chimica, Beerse, Belgium), (±)-S-α-methyl-5-HT (gift: Dr P.P.A. Humphrey, Glaxo Group Research, Ware, U.K.), metoclopramide hydrochloride (Pharmacy Department, Erasmus University, Rotterdam, The Netherlands), 1-phenyl-biguanide (Research Biochemicals Inc., Nathick, MA, U.S.A.), 1αH,3α,5αH-tropan-3yl-3,5-dichlorobenzoate (MDL 72222; gift: Merrel-Dow Research Institute, Strasbourg, France); (3α-tropanyl)-1H-indole-3-carboxylic acid ester (ICS 205-930), and (±)-zacopride (A.H. Robbins Co., Richmond, VA, U.S.A.). The doses of cisapride, dazopride, 5-HT, 5-methoxytryptamine, α-methy-5-HT, 1-phenyl-biguanide and zacopride are given as free base.

Data presentation and analysis

All data in the text, figures and tables are presented as mean ± s.e.mean. The peak changes in heart rate induced by the different doses of both tryptamine- and benzamide derivatives were determined. The increases in heart rate just before and after a particular antagonist drug were compared by Duncan's new multiple range test, once an analysis of variance (randomized block design) revealed that the samples represented different populations (Saxena, 1985). The effects of agonist drugs in the different groups of animals were compared by use of the unpaired Student's t test. A P value of 0.05 or less (two-tailed) was considered statistically significant.

Results

Initial blood pressure and heart rate changes by 5-HT agonist drugs

Baseline values of mean arterial blood pressure and heart rate in the 40 pigs were 84 ± 4 mmHg and 101 ± 4 beats min^{-1}, respectively. The changes induced in mean arterial blood pressure by each 5-HT agonist drug were: 5-HT (−18 ± 1, −18 ± 1 and −16 ± 1 followed by +2 ± 1 mmHg after 3, 10 and 30 $\mu g\,kg^{-1}$, respectively; n = 35), 5-methoxytryptamine (−20 ± 1, −18 ± 2 and −15 ± 2 followed by +2 ± 1 mmHg after 3, 10 and 30 $\mu g\,kg^{-1}$, respectively; n = 30), α-methyl-5-HT (−20 ± 1, −14 ± 2, +10 ± 3 and +41 ± 8 mmHg after 3, 10, 30 and 100 $\mu g\,kg^{-1}$, respectively; n = 3), metoclopramide (+5 ± 2, +8 ± 1 and +3 ± 3 mmHg after 300, 1000 and 3000 $\mu g\,kg^{-1}$, respectively; n = 8), cisapride (+4 ± 4, +11 ± 2, +5 ± 3 and −8 ± 5 mmHg after 30, 100, 300 and 1000 $\mu g\,kg^{-1}$, respectively; n = 5), zacopride (+5 ± 2, +10 ± 2, +14 ± 3 and +12 ± 2 mmHg after 10, 30, 100 and 300 $\mu g\,kg^{-1}$, respectively; n = 6), dazopride (+1 ± 1, +2 ± 1 and +4 ± 2 mmHg after 300, 1000 and 3000 $\mu g\,kg^{-1}$, respectively; n = 4), and 1-phenyl-biguanide (0 ± 0, 0 ± 0, +4 ± 1 and +9 ± 2 after 30, 100, 300 and 1000 $\mu g\,kg^{-1}$, respectively; n = 3). These effects were not evaluated further.

As shown in Figure 1, intravenous bolus injections of the above-mentioned 5-HT agonist drugs caused increases in heart rate of diverse magnitude; the order of potency was 5-HT ≥ 5-methoxytryptamine > α-methyl-5-HT > zacopride > cisapride > metoclopramide = 1-phenyl-biguanide >

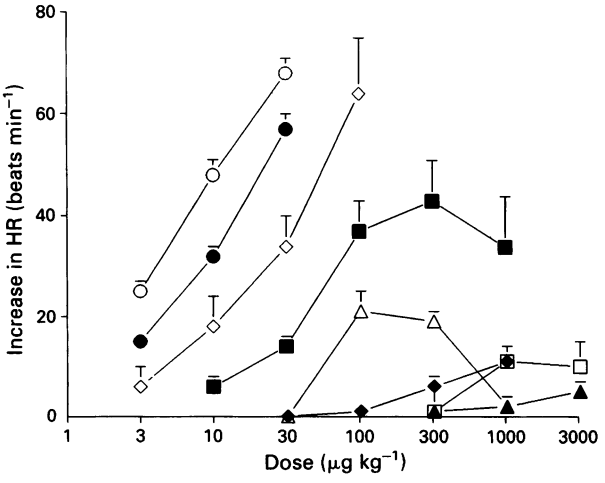

Figure 1 The tachycardic responses to 5-HT (○, n = 35), 5-methoxytryptamine (●, n = 30), α-methyl-5-HT (◇, n = 3), zacopride (■, n = 6), cisapride (△, n = 5), metoclopramide, (□, n = 8), 1-phenyl-biguanide (◆, n = 3) and dazopride (▲, n = 4) in the anaesthetized pig.

dazopride. At the doses used, the duration of action of cisapride (>60 min at 100 μg kg^{-1}) was longer than that of zacopride (17 ± 1, 23 ± 1, 43 ± 17 and 50 ± 8 min), metoclopramide (11 ± 1, 15 ± 2 and 21 ± 5 min), dazopride (1 ± 1, 5 ± 2 and >30 min), 1-phenyl-biguanide (0 ± 0, 0 ± 0, 5 ± 3 and 30 ± 3 min), 5-methoxytryptamine (5.9 ± 0.3, 9.6 ± 0.5 and 13.7 ± 0.6 min), 5-HT (2.2 ± 0.2, 4.5 ± 0.3 and 7.6 ± 0.7 min) or α-methyl-5-HT (0.4 ± 0.1, 0.9 ± 0.1, 4.3 ± 1.3 and 7.3 ± 1.3 min).

Modification of tachycardia in response to 5-HT and 5-methoxytryptamine induced by benzamide derivatives

In a previous publication, we have reported that the tachycardia induced by repeated administrations of 5-HT and 5-methoxytryptamine remained essentially unchanged in control animals receiving physiological saline (Villalón *et al.*, 1990). In marked contrast, the administration of several doses of either zacopride, cisapride, metoclopramide or dazopride antagonized the tachycardia induced by 5-HT (Figure 2) or 5-methoxytryptamine (Figure 3) in a dose-dependent manner; the order of potency for blockade of both 5-HT- and 5-methoxytryptamine-induced tachycardia was similar to that of their tachycardic response (see above): zacopride = cisapride ≫ metoclopramide > dazopride.

Tachycardia induced by benzamide derivatives after ICS 205-930

Because of the fact that the responses to the higher doses of the benzamide derivatives were usually less than the maximum response achieved (see Figure 1), the dose of each benzamide derivative eliciting the maximum increase in heart rate was administered to animals after treatment with 3 mg kg^{-1} of ICS 205-930. This dose of ICS 205-930 antagonizes the tachycardic responses to 5-HT, 5-methoxytryptamine and renzapride, but not that to isoprenaline (Villalón *et al.*, 1990). As shown in Figure 4, the increase in heart rate induced by either zacopride, cisapride, dazopride or metoclopramide was markedly antagonized by ICS 205-930.

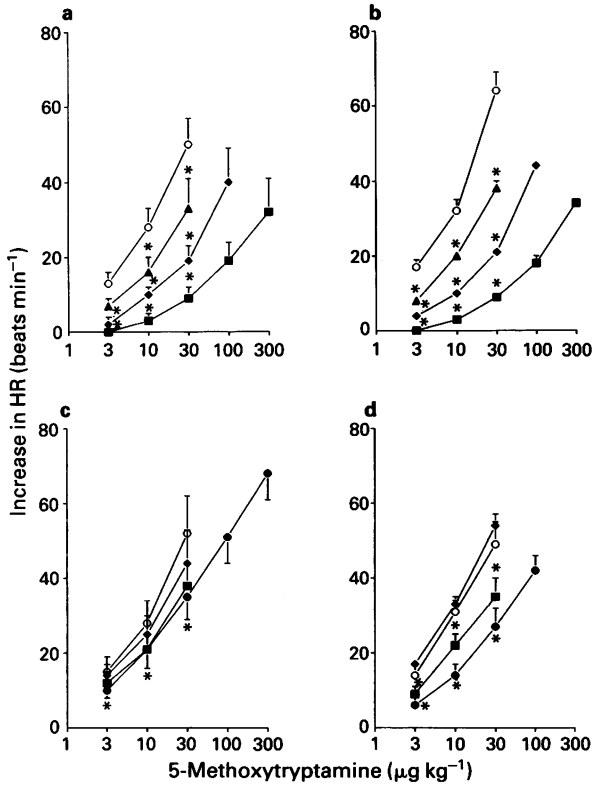

Figure 3 The effects of (a) zacopride (n = 4), (b) cisapride (n = 4), (c) dazopride (n = 4) and (d) metoclopramide (n = 5) on the tachycardic responses to 5-methoxytryptamine. The doses of the antagonists were: (○), 0 mg kg^{-1} (control); (▲) 0.1 mg kg^{-1}; (◆) 0.3 mg kg^{-1}; (■) 1.0 mg kg^{-1} and (●) 3.0 mg kg^{-1}. * Significantly different from the corresponding control response to 5-methoxytryptamine (P < 0.05).

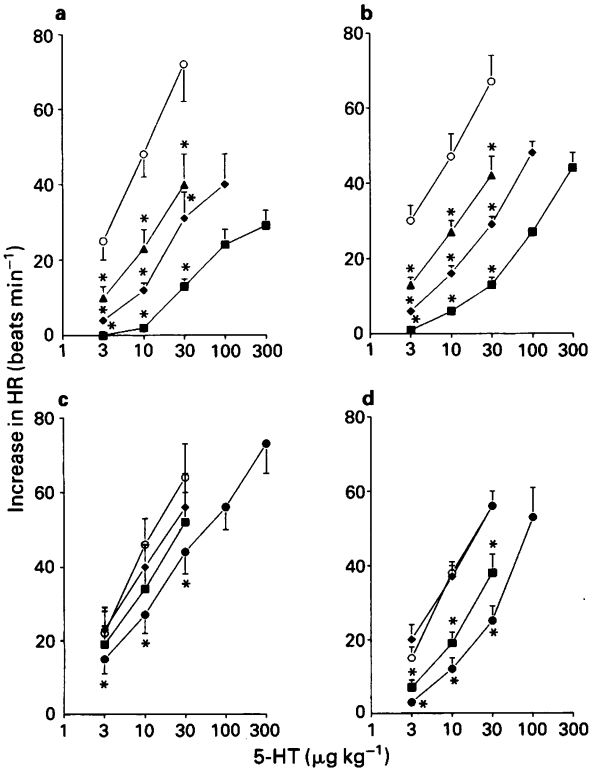

Figure 2 The effects of (a) zacopride (n = 5), (b) cisapride (n = 5), (c) dazopride (n = 4) and (d) metoclopramide (n = 8) on the tachycardic responses to 5-HT. The doses of the antagonists were: (○) 0 mg kg^{-1} (control); (▲) 0.1 mg kg^{-1}; (◆) 0.3 mg kg^{-1}, (■) 1.0 mg kg^{-1} and (●) 3.0 mg kg^{-1}. * Significantly different from the corresponding control response to 5-HT (P < 0.05).

Tachycardia induced by 5-HT or 5-methoxytryptamine after administration of some agonist and antagonist drugs

Inasmuch as all putative antagonists at this novel cardiac receptor also display high affinity for the 5-HT$_3$ receptors, we decided to investigate the effect of high doses of other selective 5-HT$_3$ receptor agonists and antagonists on the tachycardic responses induced by 5-HT or 5-methoxytryptamine; the

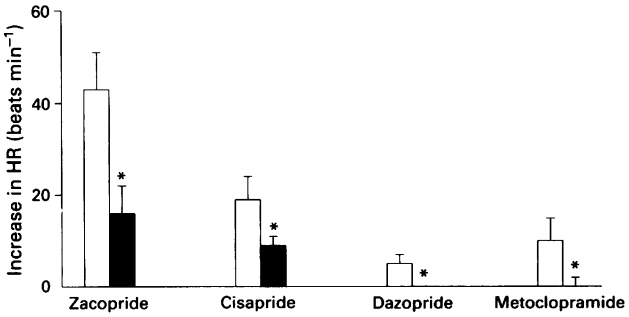

Figure 4 The tachycardic responses to zacopride (0.3 mg kg^{-1}), cisapride (0.3 mg kg^{-1}), metoclopramide (3 mg kg^{-1}) and dazopride (3 mg kg^{-1}) in untreated control pigs (open columns, n = 6, 5, 8 and 4, respectively) and pigs treated with ICS 205-930 (solid columns, n = 6, 6, 5 and 6, respectively). * Significantly different from the corresponding response in the untreated animals (P < 0.05).

Table 1 Effect of MDL 72222, granisetron, 1-phenyl-biguanide or domperidone on 5-HT- and 5-methoxytryptamine-induced increases in heart rate in the pig

			Increase in heart rate (beats min^{-1})					
	Dose		$3\,\mu g\,kg^{-1}$		$10\,\mu g\,kg^{-1}$		$30\,\mu g\,kg^{-1}$	
Antagonist	($mg\,kg^{-1}$)	n	Before	After	Before	After	Before	After
5-HT								
MDL 72222	3.0	6	26 ± 5	23 ± 6	49 ± 9	40 ± 8	69 ± 12	63 ± 11
Granisetron	3.0	3	32 ± 10	31 ± 10	58 ± 14	51 ± 13	77 ± 17	69 ± 16
1-Phenyl-biguanide	1.0	3	32 ± 10	33 ± 10	58 ± 14	56 ± 15	77 ± 17	74 ± 18
Domperidone	3.0	6	26 ± 5	29 ± 6	49 ± 9	50 ± 7	69 ± 12	69 ± 6
5-Methoxytryptamine								
MDL 72222	3.0	6	18 ± 4	18 ± 4	35 ± 6	33 ± 5	61 ± 10	57 ± 10
Granisetron	3.0	3	15 ± 2	14 ± 2	32 ± 7	28 ± 5	62 ± 8	54 ± 8
1-Phenyl-biguanide	1.0	3	15 ± 2	14 ± 3	32 ± 7	30 ± 7	62 ± 8	60 ± 8
Domperidone	3.0	6	18 ± 4	18 ± 5	35 ± 6	38 ± 5	61 ± 10	58 ± 7

All data are mean ± s.e.mean. None of the responses after antagonist drugs differed significantly from those before antagonist ($P > 0.05$).

selective dopamine$_2$ (D_2) receptor antagonist domperidone, as some benzamide derivatives (metoclopramide) also show affinity for D_2 receptors. As shown in Table 1, the responses to both 5-HT and 5-methoxytryptamine remained unchanged after administration of MDL 72222 ($3\,mg\,kg^{-1}$), granisetron ($3\,mg\,kg^{-1}$), 1-phenyl-biguanide ($1\,mg\,kg^{-1}$) or domperidone ($3\,mg\,kg^{-1}$).

Since α-methyl-5-HT (a 5-HT$_2$ and, to some extent, 5-HT$_1$-like receptor agonist) did induce quite consistent increases in heart rate (see Figure 1), we explored by pharmacological means the possible mechanisms involved in such an effect. In control animals where 5-HT and 5-methoxytryptamine were administered before and after the last set of injections of α-methyl-5-HT (3, 10, 30 and $100\,\mu g\,kg^{-1}$), the tachycardic responses induced by both 5-HT (3, 10 and $30\,\mu g\,kg^{-1}$) and 5-methoxytryptamine (3, 10 and $30\,\mu g\,kg^{-1}$) remained unchanged after α-methyl-5-HT [for 5-HT: 40 ± 9, 66 ± 11, and 88 ± 13 beats min^{-1} before and 36 ± 5, 62 ± 10 and 82 ± 12 beats min^{-1} after α-methyl-5-HT ($n = 3$), respectively; for 5-methoxytryptamine: 21 ± 7, 37 ± 8 and 67 ± 11 beats min^{-1} before and 17 ± 5, 32 ± 7 and 59 ± 11 beats min^{-1} after α-methyl-5-HT ($n = 3$), respectively]. Likewise, the increases in heart rate induced by 5-HT, 5-methoxytryptamine and α-methyl-5-HT were unaffected by ketanserin ($0.5\,mg\,kg^{-1}$), but were markedly antagonized by ICS 205-930 ($3\,mg\,kg^{-1}$) (Figure 5).

Lastly, it may be noted that the 5-HT$_3$ receptor agonist 1-phenyl-biguanide (30, 100, 300 and $1000\,\mu g\,kg^{-1}$) induced a small increase in porcine heart rate ($n = 3$); this effect was not blocked after administration of $3\,mg\,kg^{-1}$ of the selective 5-HT$_3$ receptor antagonist granisetron (0 ± 0, 1 ± 1, 6 ± 2 and 11 ± 1 beats min^{-1} before and 0 ± 0, 5 ± 3, 11 ± 3 and 16 ± 1 beats min^{-1} after granisetron, respectively). Notwithstanding, this effect appeared to be antagonized by $3\,mg\,kg^{-1}$ of ICS 205-930 (0 ± 0, 5 ± 3, 11 ± 3 and 16 ± 1 beats min^{-1} before and 0 ± 0, 3 ± 1, 4 ± 1 and 7 ± 1 beats min^{-1} after ICS 205-930, respectively).

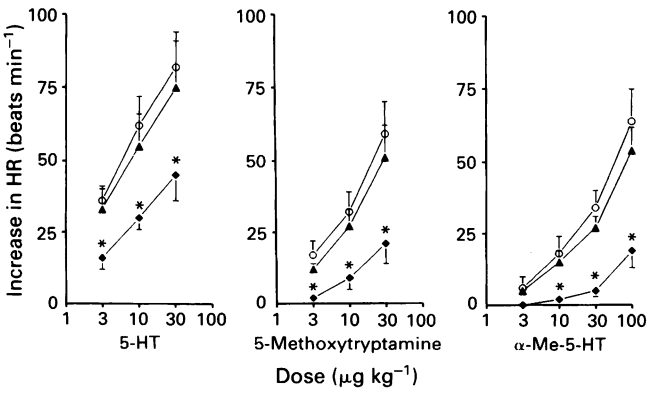

Figure 5 The tachycardic responses to 5-HT ($n = 3$), 5-methoxytryptamine ($n = 3$) and α-methyl-5-HT (α-Me-5-HT) ($n = 3$) before (control, ○) and after injections of ketanserin ($0.5\,mg\,kg^{-1}$, ▲), and ICS 205-930 ($3\,mg\,kg^{-1}$, ◆). * Significantly different from response in untreated animals ($P < 0.05$).

Discussion

We have shown that the 5-HT-induced tachycardia in the pig is neither mimicked by agonists at 5-HT$_1$-like (5-carboxamidotryptamine, 8-hydroxy-2-(di-n-propylamino)tetralin, RU 24969) and 5-HT$_3$ (2-methyl-5-HT) receptors, nor antagonized by drugs that act at various receptors: 5-HT$_1$ and/or 5-HT$_2$ (methiothepin, methysergide, ketanserin); 5-HT$_3$ (MDL 72222, ICS 205-930); adrenoceptors (phenoxybenzamine, propranolol); dopamine (haloperidol); histamine (mepyramine, cimetidine) (see Duncker *et al.*, 1985; Bom *et al.*, 1988). More recently, we found that the tachycardic effects of 5-HT in the pig, being mimicked by 5-methoxytryptamine and renzapride (Villalón *et al.*, 1990), but not by indorenate or sumatriptan (Villalón *et al.*, 1991), and blocked by high doses ($> 1\,mg\,kg^{-1}$) of ICS 205-930 (Villalón *et al.*, 1990), are mediated by a putative 5-HT$_4$ receptor which resembles the one mediating increases in adenosine 3':5'-cyclic monophosphate (cyclic AMP) in mouse embryo colliculi neurones and guinea-pig hippocampal membranes (Dumuis *et al.*, 1988; 1989; Clarke *et al.*, 1989). The present investigation extends these findings and clearly demonstrates that the porcine heart 5-HT receptor (i) can be stimulated by α-methyl-5-HT and some benzamide derivatives; (ii) does not resemble either 5-HT$_2$, 5-HT$_3$ or dopamine receptors; and (iii) resembles that present on the guinea-pig enteric neurones (Craig & Clarke, 1990) and human heart (Kaumann *et al.*, 1990).

Agonist action of α-methyl-5-HT and some benzamide derivatives on the porcine heart 5-HT receptor

Like 5-HT and 5-methoxytryptamine, it was observed that α-methyl-5-HT behaved as a potent agonist and elicited a dose-dependent tachycardia in the pig. The drug was also short-lasting in action and was devoid of any antagonist action against 5-HT or 5-methoxytryptamine. In contrast, the tachycardic action of the benzamide derivatives zacopride, cisapride, metoclopramide and dazopride, was less marked, but longer-lasting, and not strictly dose-dependent. In addition, each of these drugs antagonized the effects of 5-HT and 5-methoxytryptamine in a dose-dependent manner. It has to be emphasized that the tachycardic effects of 5-HT and 5-methoxytryptamine were not 'masked' by the increase in heart rate induced by the benzamide derivatives as the responses to 5-HT and 5-methoxytryptamine were elicited at the time when

the tachycardic effect of the benzamides had worn off (data not shown). Therefore, as previously found with renzapride (Villalón *et al.*, 1990), the benzamide derivatives employed here also behaved as partial agonists at the 5-HT$_4$ receptors in the porcine heart.

In our previous investigation, tachyphylaxis was observed with the tachycardic effect of renzapride (Villalón *et al.*, 1990). Though the present study was not designed for this purpose, we did observe tachyphylaxis in some preliminary experiments with the benzamide derivatives used here. Indeed, for this reason the antagonist effect of ICS 205-930 against the tachycardia induced by zacopride, cisapride, dazopride and metoclopramide was analyzed separately in control animals and in animals pretreated with ICS 205-930 (see Figure 4).

Lack of resemblance of the porcine heart 5-HT receptor with either 5-HT$_2$, 5-HT$_3$ or dopamine D$_2$ receptors

Both 5-methoxytryptamine and α-methyl-5-HT can interact with 5-HT$_2$ (and 5-HT$_1$-like) receptors (Richardson & Engel, 1986; Martin *et al.*, 1987; Hoyer, 1988). However, the tachycardic action of 5-methoxytryptamine and α-methyl-5-HT, as well as that of 5-HT (see also Bom *et al.*, 1988), was not modified after a dose of ketanserin (0.5 mg kg^{-1}) that is sufficient to block 5-HT$_2$ receptors (Van Nueten *et al.*, 1981; Saxena & Lawang, 1985). Therefore, the possibility that 5-HT$_2$ receptors might be involved in these effects is practically ruled out. Unlike ketanserin, 3 mg kg^{-1} of ICS 205-930 markedly antagonized the tachycardic responses to 5-HT, 5-methoxytryptamine and α-methyl-5-HT (see Figure 5), which again suggests the involvement of the 5-HT$_4$ receptor.

Admittedly, ICS 205-930 and the benzamide derivatives used here have the ability to block potently 5-HT$_3$ receptors (for references see Fozard, 1990). However, as previously discussed (Bom *et al.*, 1988; Villalón *et al.*, 1990), several results of this study clearly indicate that this novel 5-HT receptor in the pig heart does not belong to the 5-HT$_3$ receptor family. Firstly, besides the potency of 5-HT as a distinguishing factor, both α-methyl-5-HT and 5-methoxytryptamine are totally inactive at 5-HT$_3$ receptors (Richardson *et al.*, 1985; Richardson & Engel, 1986; Fozard, 1990). Secondly, the selective 5-HT$_3$ receptor agonists 2-methyl-5-HT and 1-phenyl-biguanide (Fozard, 1990) were practically inactive in the stimulation of 5-HT$_3$ receptors (Bom *et al.*, 1988; present results). Thirdly, high doses of other selective 5-HT$_3$ receptor antagonists (except ICS 205-930) such as granisetron (an indazole derivative; Sanger & Nelson, 1989; Fozard, 1990) or MDL 72222 (a dichlorobenzoate derivative; Fozard, 1984; 1990) were completely inactive (see Table 1). Lastly, it must be taken into consideration that the affinity of ICS 205-930 for the 5-HT$_4$ receptor involved in the stimulation of cyclic AMP production in mouse embryo colliculi neurones is much lower (pK$_i$ = 6–6.3; Dumuis *et al.*, 1989) than its affinity for 5-HT$_3$ receptors (pA$_2$ = 8–10; Richardson *et al.*, 1985; Richardson & Engel, 1986).

Most of the benzamide derivatives analyzed in the present study are currently used as prokinetic drugs (Schuurkes *et al.*, 1985; Alphin *et al.*, 1986; Cooper *et al.*, 1986; van Daele *et al.*, 1986; Sanger, 1987). Apart from metoclopramide, which also displays high affinity for central dopamine receptors (Cooper *et al.*, 1986), the other benzamide derivatives are devoid of important dopamine blocking activity. However, ICS 205-930, zacopride and other 5-HT$_3$ receptor antagonists are able to inhibit the release of dopamine by 5-HT and 2-methyl-5-HT in the central nervous system (Blandina *et al.*, 1988; Tricklebank, 1989). It is for these reasons, although haloperidol had been found ineffective (Bom *et al.*, 1988), that we decided to determine whether domperidone, a potent D$_2$ receptor antagonist (Kohli *et al.*, 1983) with gastrokinetic action, antagonizes the 5-HT-induced tachycardia or itself causes tachycardia in the pig. As shown in Table 1, domperidone (3 mg kg^{-1}) did not modify the tachycardic responses to either 5-HT or 5-methoxytryptamine. Therefore, the positive chronotropic effect induced by the tryptamine- and benzamide derivatives in the pig heart is unrelated to a possible action via dopaminergic pathways and/or receptors. Moreover, since domperidone failed to affect basal heart rate in the pig, the drug does not interact with the pig heart 5-HT$_4$ receptor.

Resemblance of the porcine heart 5-HT receptor to other putative 5-HT$_4$ receptors

At the 5-HT$_4$ receptor identified in the neurones from mouse embryo colliculi on the basis of increase in cyclic AMP, 5-methoxytryptamine, 5-carboxamidotryptamine (low affinity) and certain benzamide derivatives (renzapride, metoclopramide, cisapride), but not α-methyl-5-HT or 2-methyl-5-HT, are agonists; and ICS 205-930 (in high concentrations), but not MDL 72222, granisetron or ondansetron, acts as an antagonist (Dumuis *et al.*, 1988; 1989; Clarke *et al.*, 1989). The pharmacological characteristics of this receptor, though exhibiting several similarities, differ in some important respects. For example, 5-carboxamidotryptamine, apparently because of its low affinity, does not show activity in the pig heart (Duncker *et al.*, 1985; Bom *et al.*, 1988) in doses which are highly active in the cat heart (Saxena *et al.*, 1985; Connor *et al.*, 1986). Secondly, α-methyl-5-HT, which has little activity on the neurones from mouse embryo colliculi (Dumuis *et al.*, 1988; 1989), is highly active in our experiments. Thirdly, the agonist potency order reported by Dumuis *et al.* (1989) using mouse embryo colliculi (cisapride > renzapride > zacopride > 5-HT > metoclopramide), differs from that found in the pig heart (5-HT > 5-methoxytryptamine > α-methyl-5-HT > zacopride ≥ renzapride > cisapride > metoclopramide > dazopride; indorenate and sumatriptan, inactive at 1 and 3 mg kg^{-1}) (Villalón *et al.*, 1990; 1991; present results). Lastly, the benzamide derivatives cisapride and renzapride, which are full agonists at the mouse brain receptor, behaved as partial agonists at the pig heart receptor. Several possible explanations for these differences in agonist potencies may include: use of 'second messenger' (cyclic AMP) and functional (tachycardia) responses; tissue-dependent factors such as the number of receptors and coupling efficiency; and/or drug-dependent factors such as the affinity of 5-HT and related agonists for each of these novel receptors.

The 5-HT$_4$ receptor may also mediate the 5-HT-induced enhancement of cholinergic activity in the guinea-pig isolated ileum (Sanger, 1987; Craig & Clarke, 1990) and ascending colon (Elswood *et al.*, 1990), as well as relaxation of the rat oesophagus (Baxter & Clarke, 1990). As in the present experiments, the tryptamine derivatives 5-methoxytryptamine, α-methyl-5-HT and some benzamides mimic, and ICS 205-930 antagonizes 5-HT at the 5-HT$_4$ receptor in the guinea-pig gastrointestinal tract (Craig & Clarke, 1990; Elswood *et al.*, 1990) and the rat oesophagus (Baxter & Clarke, 1990). Moreover, the order of potency at the cholinergic neurones in the guinea-pig ileum (5-HT > 5-methoxytryptamine > renzapride > α-methyl-5-HT > zacopride = cisapride; Craig & Clarke, 1990) is practically identical to that found by us in the porcine heart.

The 5-HT$_4$ receptor is also apparently involved in the inotropic action of 5-HT, mediated via cyclic AMP increase in the human atria. The positive inotropic response to 5-HT is not modified by ketanserin, methysergide, lysergide, methiothepin, yohimbine (±)-propranol, (−)-pindolol or MDL 72222, but is blocked by a high concentration (2 μM) of ICS 205-930 (Kaumann *et al.*, 1990). The precise role of these receptors in cardiac function and cardiovascular pathologies remains to be determined.

In summary, the present investigation demonstrates that the tachycardic response to i.v. administered 5-HT in the anaesthetized pig can be mimicked by the tryptamine derivatives 5-methoxytryptamine and α-methyl-5-HT, and to a lesser extent by the partial agonist benzamide derivatives (in order of potency) zacopride, cisapride, metoclopramide and dazop-

ride. High doses of ICS 205-930, but not ketanserin, granisetron or MDL 72222, acted as an antagonist. These results further confirm the involvement of a putative 5-HT_4 receptor in the positive chronotropic action of 5-HT in the anaesthetized pig.

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Effects of glibenclamide on cytosolic calcium concentrations and on contraction of the rabbit aorta

Kiyonobu Yoshitake, Katsuya Hirano & [1]Hideo Kanaide

Division of Molecular Cardiology, Research Institute of Angiocardiology, Faculty of Medicine, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka, 812, Japan

1 Using fluorometry of fura-2 and rabbit aortic strips, we studied the effects of glibenclamide (GLB), a sulphonylurea anti-diabetic drug and an inhibitor of opening of K^+ channels, on cytosolic calcium concentrations ($[Ca^{2+}]_i$) and on force development.

2 Both high K^+-depolarization and noradrenaline (NA) increased $[Ca^{2+}]_i$ and force, in a concentration-dependent manner, in the presence of extracellular Ca^{2+} (1.25 mM). However, force development in relation to $[Ca^{2+}]_i$ ($[Ca^{2+}]_i$-force relationship) observed with NA was much greater than that observed with K^+-depolarization.

3 Pretreatment with GLB (10^{-6}–10^{-4} M) for 10 min partially inhibited, in a concentration-dependent manner, both $[Ca^{2+}]_i$ elevation and the force development induced by 118 mM K^+-depolarization or NA 10^{-5} M in the presence of extracellular Ca^{2+}. The $[Ca^{2+}]_i$-force relationship induced by both 118 mM K^+ physiological salt solutions and by NA 10^{-5} M in the GLB-treated strips overlapped that obtained in the non-treated strips, thereby suggesting that GLB has no effect on the Ca^{2+}-sensitivity of the intracellular contractile apparatus. Only high concentrations (10^{-4} M) of GLB decreased $[Ca^{2+}]_i$ and the force, when applied after the force induced by 118 mM K^+ PSS or NA 10^{-5} M reached the maximum level.

4 In the absence of extracellular Ca^{2+}, NA induced a transient increase in $[Ca^{2+}]_i$ and in the force and these increases were inhibited when the vascular strips were pretreated with GLB for 10 min. The $[Ca^{2+}]_i$-force relationship obtained in the GLB-treated strips overlapped that in the non-treated ones.

5 An ATP-sensitive K^+ channel opener, cromakalim (10^{-5} M) reduced the increased $[Ca^{2+}]_i$ and force induced by 25 mM K^+-depolarization and NA 10^{-5} M. Subsequent application of GLB concentration-dependently reversed this relaxant effect of cromakalim on the NA-induced contraction ($IC_{50} = 2 \times 10^{-7}$ M). Complete reversal of the effect was observed with 10^{-5} M GLB.

6 We suggest that GLB inhibits both high K^+-depolarization- and NA-induced contraction of the rabbit aorta, by decreasing $[Ca^{2+}]_i$ and with no effect on the $[Ca^{2+}]_i$-force relationship. However, when NA-induced contractions were inhibited by a K^+-channel opener, GLB reversed this inhibitory effect by inhibiting K^+-channel opening and increasing $[Ca^{2+}]_i$.

Introduction

K^+ channels inhibited by intracellular adenosine 5'-triphosphate (ATP) have been identified in vascular smooth muscle (Standen *et al.*, 1989), and these channels are also present in pancreatic β-cells (Ashcroft *et al.*, 1984), cardiac cells (Noma, 1983), skeletal muscle cells (Spruce *et al.*, 1985) and cortical neurones (Ashford *et al.*, 1988). The most potent and selective inhibitor of this channel known at present is the sulphonylurea hypoglycaemic agent, glibenclamide (GLB) (Schmidt-Antomarchi *et al.*, 1987). High-affinity sulphonylurea receptors have been noted on pancreatic cells (Gaines *et al.*, 1988) but no evidence of these receptors on vascular smooth muscle has been documented. In pancreatic β cell membranes, the sulphonylurea receptor may be closely linked to or be part of an ATP-sensitive K^+ channel (Gerich, 1989), and inhibition of the efflux of K^+ by GLB may lead to depolarization; as a consequence, voltage-dependent Ca^{2+} channels on the membrane would then open and permit entry of Ca^{2+} (Boyd, 1988). An increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and the resultant activation of myosin light chain kinase play a critical role in initiating contractions of smooth muscles. In the present study, we examined the effect of GLB on $[Ca^{2+}]_i$ and the force of rabbit aortic smooth muscle strips, using fluorometry of fura-2.

Methods

Tissue preparation

Japanese white rabbits (male, 16–20 weeks old, body weight 2.5–3.0 kg) were killed with sodium pentobarbitone (100 mg kg^{-1}, intravenously) and the abdominal aortae below the level of the renal arteries were immediately excised. Fat and adventitia were removed by dissection, under a binocular microscope. The endothelial cells were removed by rubbing the luminal surface with a cotton swab (Furchgott & Zawadzki, 1980). Medial preparations were cut into 1 × 3 mm circular strips 0.2 mm thick. The wet weight of the strips was 0.6 ± 0.1 mg ($n = 12$). Tissue density of the strips was assumed to be 1.05 g cm^{-3} (Murphy *et al.*, 1974) and the cross sectional area of each strip was calculated from the following equation: Cross-sectional area (m^2) = wet weight (mg)/length (mm)/1.05 (g cm^{-3}) × 10^{-6}. The mean value of cross-sectional area was $2.14 \pm 0.19 \times 10^{-7}$ m^2 ($n = 12$).

Fura-2 loading

The vascular strips thus obtained were loaded with the $[Ca^{2+}]_i$ indicator dye, fura-2 by incubation in medium containing 50 μM fura-2/AM (an acetoxymethyl ester form of fura-2) and 1.25% foetal bovine serum for 6–8 h at 37°C. Subsequently, the strips were washed in physiological salt solution (PSS) containing 1.25 mM Ca^{2+} at 37°C to remove the dye from the extracellular space and were then incubated with PSS for about 1 h before initiation of the measurements. Strips thus treated showed the specific peak of the fluorescence emission spectrum for fura-2 (500 nm) and the specific peak and the valley of the fluorescence excitation spectrum for fura-2, 340 nm and 380 nm, respectively, determined with a fluorescence spectrophotometer (model 650-40, Hitachi, Tokyo, Japan). Loading the vascular strips with fura-2 did not alter the time course or the maximum levels of force development during 118 mM K^+ depolarization, thereby suggesting that no tissue damage occurred by possible acidification of the cells due to formaldehyde released on AM-ester hydrolysis (Tsien *et al.*, 1982; Rink & Pozzan, 1985).

[1] Author for correspondence.

Measurement of tension

Strips were mounted vertically in a quartz organ bath, (strain gauge TB-612T, Nihon Koden, Tokyo, Japan). During a 1 h equilibration period, the strips were stimulated with 118 mM K^+ depolarization every 15 min, and the resting tension was increased in a stepwise manner. After equilibration, the resting tension was adjusted to 200 mg. The force development of the steady state was expressed as a % assuming the values in PSS (5.9 mM K^+) and 118 mM K^+ PSS to be 0% and 100%, respectively.

Fluorometry

Changes in the fluorescence intensity of the fura-2-Ca^{2+} complex were simultaneously monitored during measurement of the force. The fluorescence intensity was measured by use of a fluorometer specially designed for fura-2 fluorometry (CAM-OF-1), in collaboration with Japan Spectroscopic Co, Tokyo, Japan (Hirano *et al.*, 1989). In brief, the strips were illuminated by guiding the alternating (400 Hz), 340 nm and 380 nm, excitation light from a Xenon light source through quartz optic fibres arranged in a concentric inner circle (diameter = 3 mm). Surface fluorescence of the strips was collected by glass optic fibres arranged in an outer circle (diameter = 7 mm) and introduced through a 500 nm band-pass filter (full width at half maximum transmission 10 nm) into a photon-counting photomultiplier. Special care was taken to keep the distance between a strip and the end of the optic fibres short and constant during each measurement. The ratio of the 500 nm fluorescence at 340 nm excitation to that at 380 nm excitation was expressed as a %, assuming the values in PSS (5.9 mM K^+) and 118 mM K^+ PSS to be 0% and 100%, respectively. The absolute value of $[Ca^{2+}]_i$ was estimated as described by Grynkiewicz *et al.* (1985). The mean value of 7 different measurements of $[Ca^{2+}]_i$ at rest (0%) and during 118 mM K^+-depolarization (100%) were 121 ± 36 and 779 ± 125 nM, respectively.

Drugs and solutions

Physiological salt solution (PSS) was of the following composition (in mM): NaCl 123, KCl 4.7, $NaHCO_3$ 15.5, KH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 1.25, and D-glucose 11.5. High potassium PSS was identical, except for an equimolar substitution of KCl for NaCl. Ca^{2+}-free solution was identical except for a substitution of 2 mM ethylene glycol *bis* (β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) for 1.25 mM $CaCl_2$. PSS was gassed with a mixture of 95% O_2–5% CO_2 (pH adjusted to 7.4 at 37°C). Glibenclamide (GLB, 5-chloro-N-[2-[4 - [[[(cyclohexylamino)carbonyl]amino]sulphonyl]phenyl] - ethyl]-2-methoxybenzamide) and cromakalim (3,4-dihydro-3 - hydroxy - 2,2 - dimethyl - *trans* - 4 - (2 - oxo - 1 - pyrrolidinyl) - 2H-1-benzopyran-6-carbonitrile) were generous gifts from Hoechst Japan and Chugai (Japan), respectively. GLB was disolved in dimethylsulphoxide (DMSO) and diluted in the organ bath (final concentration of DMSO was 1%). Fura-2/AM was purchased from Molecular Probes (Eugene, OR, U.S.A.).

Statistical analysis

Values are expressed as means ± s.e.mean ($n = 3$). Student's t test was used to determine the statistical significance.

Results

Characteristics of changes in $[Ca^{2+}]_i$ and force induced by high external K^+-depolarization and noradrenaline

Representative recordings of changes in $[Ca^{2+}]_i$ and force induced by 118 mM K^+-depolarization followed by NA 10^{-5} M are shown in Figure 1. When the vascular strip was exposed to high external K^+ (118 mM) solution containing 1.25 mM

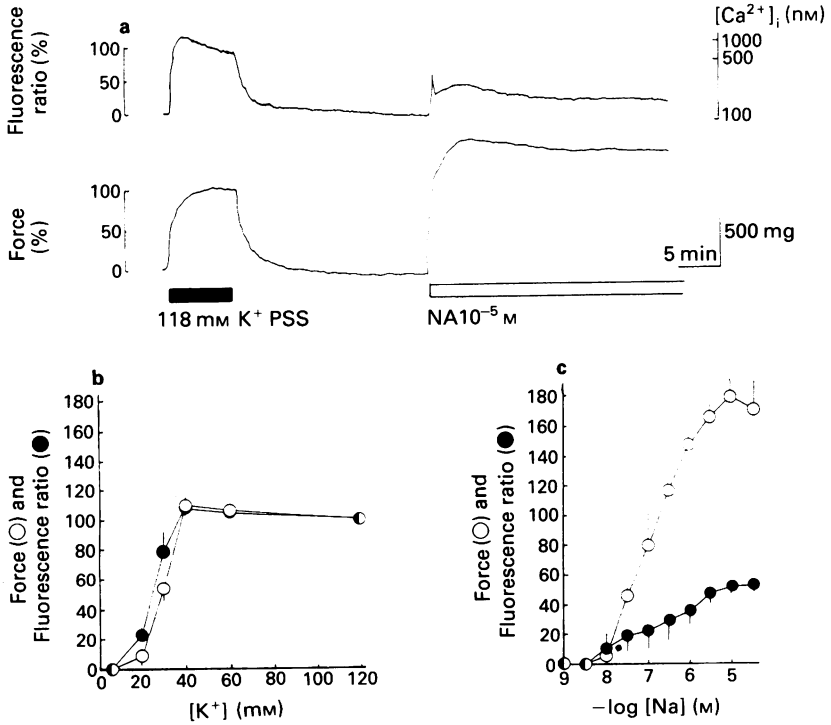


Figure 1 (a) Representative recordings of changes in fluorescence ratio (upper panel) and force development (lower panel) induced by high external K^+ (118 mM) physiological salt solution (PSS) followed by noradrenaline (NA) 10^{-5} M in the presence of 1.25 mM extracellular Ca^{2+}. (b) Dose-response curve for the maximum force development (○) and the fluorescence ratio (●) induced by high external K^+-depolarization in the presence of 1.25 mM Ca^{2+}. The abscissa scale is the concentration of K^+ in isosmotic high K^+ PSS. Measurements were done 5 min after application of high K^+. Ordinates indicate percentage fluorescence ratio and force. Vertical lines indicate s.e.mean ($n = 3$). Note that the maximum $[Ca^{2+}]_i$ and force induced by 40 mM K^+ are larger than those induced by 118 mM K^+. (c) Dose-response curve for fluorescence ratio (●) and the maximum force (○) induced by NA. Measurements were done at 6 min after NA application. The abscissa scale is −log[NA]. Each value is expressed as percentage fluorescence ratio or force, assuming the values obtained during 118 mM K^+ application to be 100%.

Ca^{2+}, $[Ca^{2+}]_i$ rose abruptly by 111% in the first 20s, and then, decreased slightly to reach a steady level at 6min; this level was assumed to be 100%. The force also rose rapidly and reached about 80% of the maximum level in the first 20s, the maximum level (100%) at about 5min, and remained at this level for at least 30min. When 118mM K^+ PSS was changed to normal PSS, both $[Ca^{2+}]_i$ and the force rapidly reverted to the normal, pre-depolarization level, 0%. Subsequent application of 10^{-5}M NA rapidly elevated $[Ca^{2+}]_i$, with a first peak ($67 \pm 18\%$, $n = 3$), reached in the first 15s. After a dip (to $38 \pm 7\%$, $n = 3$) at about 30s, $[Ca^{2+}]_i$ gradually reached a second peak ($57 \pm 9\%$, $n = 3$) at 3min, and was then sustained at a slightly decreased level ($42 \pm 11\%$, $n = 3$) for at least 20min. The force induced by NA 10^{-5}M also rose rapidly to reach a level of 115% in the first 15s, the maximum level ($178 \pm 12\%$, $n = 3$) at 6min, followed by the steady state level ($167 \pm 9\%$, $n = 3$), which was sustained for at least 20min. Figure 1b and c show that the elevations of $[Ca^{2+}]_i$ and force at the steady state induced by both external high K^+ PSS and NA application were dose-dependent. It should be noted that in the rabbit aorta, the dose-response curves for $[Ca^{2+}]_i$ and force induced by external high K^+ PSS showed somewhat bell-shaped curves; the maximum $[Ca^{2+}]_i$ and force were obtained with 40mM K^+ PSS, and concentrations of K^+ over 40mM caused less steady state $[Ca^{2+}]_i$ and force development. In addition, force development in relationship to the increase in $[Ca^{2+}]_i$ ($[Ca^{2+}]_i$-force relationship) observed with NA (Figure 1c) was much greater than observed with K^+-depolarization.

Effects of glibenclamide on increases in $[Ca^{2+}]_i$ and force induced by K^+-depolarization and noradrenaline in the presence of extracellular Ca^{2+}

When vascular strips were incubated with 10^{-4} GLB for 10min in PSS (5.9mM K^+, 1.25mM Ca^{2+}), there was no change in either $[Ca^{2+}]_i$ or force. To examine the effect of GLB on increases in the $[Ca^{2+}]_i$ and force induced by K^+-depolarization and NA, GLB was administered for 10min before and during application of these stimulations. Figures 2 and 3 show levels of $[Ca^{2+}]_i$ and force induced by K^+ 118mM and NA 10^{-5}M, respectively, in the presence of various concentrations of GLB. Treatment of the strips with GLB inhibited increases both in $[Ca^{2+}]_i$ and in force development at all time points of contractions induced by both 118mM K^+ PSS and 10^{-5}M NA, in a concentration-dependent manner within the range between 10^{-6}M and 10^{-4}M (Figure 2a,b and Figure 3a,b). We did not examine the effect of GLB in concentrations over 10^{-4}M, because it gave an optical artifact and interfered with the fura-2 fluorometry. The $[Ca^{2+}]_i$-force relationships obtained at the steady state of contraction are shown in Figure 2c and 3c. The $[Ca^{2+}]_i$-force relationships of contractions induced by 118mM K^+-depolarization (Figure 2c) and NA (Figure 3c) observed in the presence of GLB did not dissociate from those of the counterparts observed in the absence of GLB. These findings indicate that there was no change in Ca^{2+}-sensitivity of the intracellular contractile apparatus during the vasorelaxation induced by GLB.

When GLB was applied at the steady state of contraction induced by 118mM K^+-depolarization and 10^{-5}M NA, only higher concentrations of GLB (10^{-4}M) induced reduction of $[Ca^{2+}]_i$ and force ($[Ca^{2+}]_i$, from 42% to 35%, and force from 167% to 150%). Thus, differing from the results of pretreatment with GLB, application of GLB during K^+-depolarization- or NA-induced contractions had little effect on $[Ca^{2+}]_i$ and force (data not shown).

Effect of glibenclamide on the increases in $[Ca^{2+}]_i$ and force induced by noradrenaline (10^{-5}M) in the absence of extracellular Ca^{2+}

Typical recordings of increases in $[Ca^{2+}]_i$ and force induced by NA 10^{-5}M in the absence of extracellular Ca^{2+} are shown in Figure 4a. After recording the response to 118mM K^+ PSS, the vascular strip was exposed for 10min to Ca^{2+}-free PSS containing 2mM EGTA. In the absence of extracellular Ca^{2+}, $[Ca^{2+}]_i$ gradually decreased to a new steady level ($-17 \pm 2\%$, $n = 3$) within 10min, however, no change in force was observed. Subsequent application of 10^{-5}M NA induced a rapid and transient elevation of $[Ca^{2+}]_i$ and force: $[Ca^{2+}]_i$ rapidly rose to reach the maximum level ($30 \pm 11\%$, $n = 3$) in 12s, then declined to the pre-stimulation level within 8min (Figures 4a and 5a), and force developed rapidly to reach a maximum ($97 \pm 7\%$, $n = 3$), gradually declining to the pre-stimulation levels after about 30min (Figures 4b and 5b). Changes in $[Ca^{2+}]_i$ and force induced by NA were concentration-dependent (Figure 4b,c). When GLB was applied, in the absence of extracellular Ca^{2+}, for 10min before and during the application of 10^{-5}M NA, transient increases both in $[Ca^{2+}]_i$ and force induced by NA were concentration-dependently reduced. In the absence of extracellular Ca^{2+}, the $[Ca^{2+}]_i$-force relationship of the contraction induced by

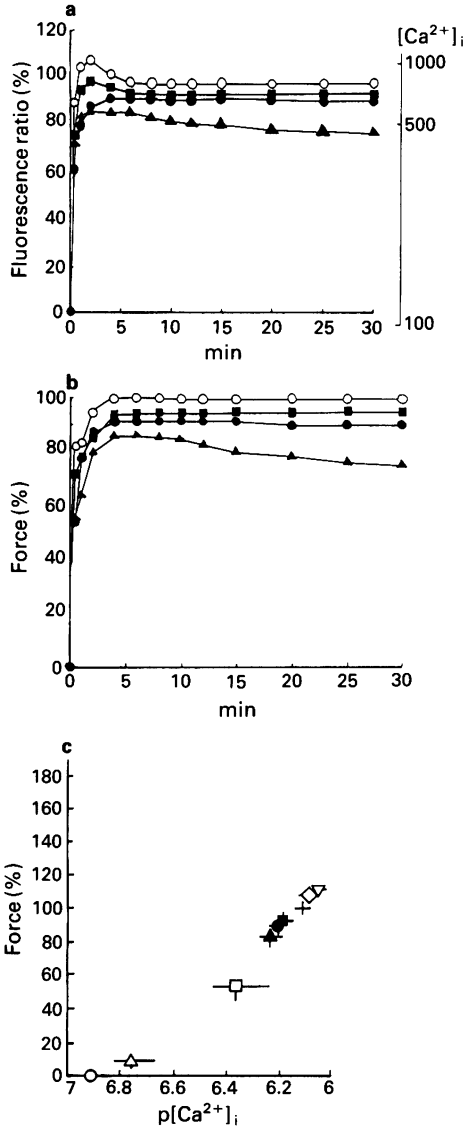


Figure 2 Effects of glibenclamide (GLB) on (a) fluorescence ratio and (b) force development induced by 118mM K^+. Vascular strips were incubated with GLB for 10min before and during 118mM K^+-application. GLB 10^{-6}M (■), 10^{-5}M (●), 10^{-4}M (▲), and without GLB (control, 118mM K^+ (○)). (c) Effects of GLB on $[Ca^{2+}]_i$-force relationship induced by high K^+. GLB 10^{-6}M (■), 10^{-5}M (●), 10^{-4}M (▲), and without GLB (control, 5.9mM K^+ (○), 20mM K^+ (△), 30 mM K^+ (□), 40mM K^+ (▽), 60mM K^+ (◇), 118mM K^+ (+)). $[Ca^{2+}]_i$-force relationship in the absence of GLB was obtained from the data in Figure 1b.

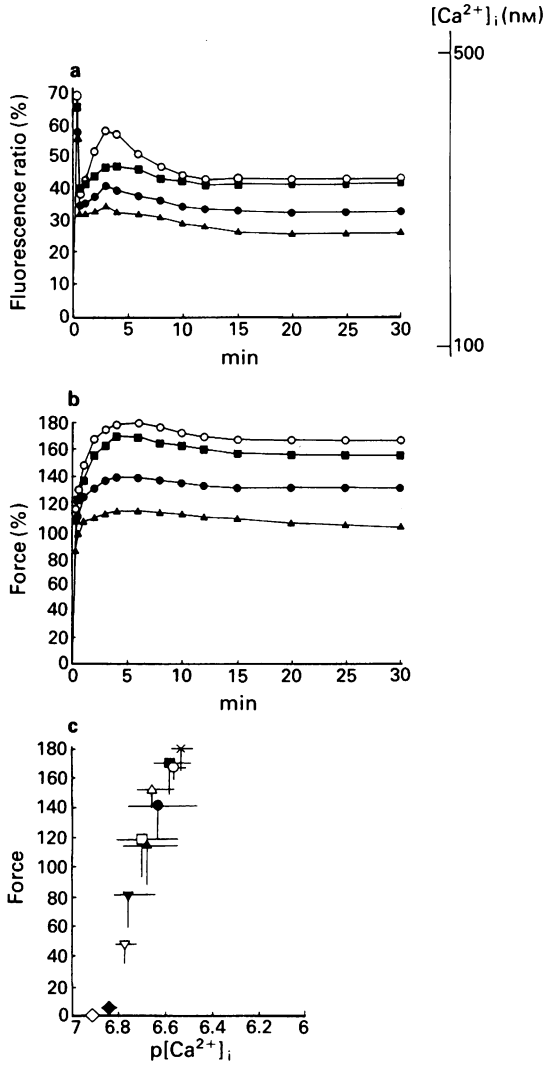


Figure 3 Effects of glibenclamide (GLB) on (a) fluorescence ratio and (b) force development induced by noradrenaline (NA). Vascular strips were incubated with GLB for 10 min before and during application of NA. GLB 10^{-6} M (■), 10^{-5} M (●), 10^{-4} M (▲), without GLB (control, NA 10^{-5} M (○)). (c) Effects of GLB on $[Ca^{2+}]_i$-force relationship induced by NA. GLB 10^{-6} M (■), 10^{-5} M (●), 10^{-4} M (▲), and without GLB (control, NA 3×10^{-9} M (◇), 10^{-8} M (◆), 3×10^{-8} M (▽), 10^{-7} M (▼), 3×10^{-7} M (□), 10^{-6} M (△), 3×10^{-6} M (○), 10^{-5} M (×)). Control $[Ca^{2+}]_i$-force relationship in the absence of GLB was obtained from the data in Figure 1c.

10^{-5} M NA with various concentrations of GLB did not differ from that induced by various concentrations of NA without GLB (Figure 5c).

Effects of glibenclamide on $[Ca^{2+}]_i$ and force in the presence of cromakalim

Cromakalim, an ATP-sensitive K^+ channel opener, decreased the increased levels of $[Ca^{2+}]_i$ and force, when applied at the steady state of contractions induced by 10^{-5} M NA (Figure 6a). Subsequent application of GLB, 10^{-5} M, completely reversed the effect of 10^{-5} M cromakalim on $[Ca^{2+}]_i$ and force in the NA-induced contraction (Figure 6a). There was no statistical difference ($P > 0.05$) between $[Ca^{2+}]_i$ and force before the relaxation induced by cromakalim and after reversal by GLB, 10^{-5} M. The reversal effect of GLB against cromakalim was concentration-dependent (Figure 6b) (IC_{50} of GLB = 2×10^{-7} M). However, 10^{-5} M cromakalim had no effect on the reduction in $[Ca^{2+}]_i$ and force induced by 10^{-4} M GLB applied at the steady state of contraction induced by 10^{-5} M NA (data not shown).

Cromakalim 10^{-5} M had no effect on $[Ca^{2+}]_i$ increase and force development during 118 mM K^+-depolarization. However, it decreased $[Ca^{2+}]_i$ and force during 25 mM K^+-depolarization; these changes were not reversed by GLB (data not shown).

Discussion

While using fura-2 fluorometry and rabbit aortic strips we found that both high K^+-depolarization and NA increased $[Ca^{2+}]_i$ and force, in a concentration-dependent manner, and that the force development in relation to the $[Ca^{2+}]_i$ observed with NA was much greater than that observed with K^+-depolarization. Also we found that GLB, a sulphonylurea antidiabetic drug known to be an ATP-sensitive K^+ channel blocker, inhibited the $[Ca^{2+}]_i$ increase and contraction induced both by K^+-depolarization and by NA, without altering the $[Ca^{2+}]_i$-force relationship. Thus, this inhibitory effect of GLB on contraction is not due to an effect on the Ca^{2+}-sensitivity of the intracellular contractile apparatus. However, we also found that when GLB was applied during the relaxation induced by a K^+ channel opener, GLB caused contraction with increasing $[Ca^{2+}]_i$, and thus, reversed the effect of cromakalim. This seems to be the first observation that the contraction of vascular strips induced by GLB is accompanied by an increase in $[Ca^{2+}]_i$. However, vasoconstriction with an increase in $[Ca^{2+}]_i$ is consistent with the report that GLB inhibited ATP-sensitive K^+ channel activity, activated by cromakalim and contracted rabbit mesenteric arterial strips (Standen *et al.*, 1989). It was suggested that a blockade of the K^+ channel depolarizes the membrane, and consequently activates voltage-dependent Ca^{2+} channels, followed by influx of extracellular Ca^{2+} and elevation of $[Ca^{2+}]_i$ (Peterson & Findlay, 1987). In pancreatic β cells, even in the absence of K^+ channel openers, GLB actively inhibited K^+ channel activity (Peterson & Findlay, 1987), depolarized the membrane and elevated $[Ca^{2+}]_i$, resulting in a secretion of insulin (Sturgess *et al.*, 1985, Schmid-Antomarchi *et al.*, 1987, Boyd, 1988). Conversely, in the present study, GLB decreased $[Ca^{2+}]_i$ and inhibited the force development of the rabbit aorta in the absence of cromakalim. When the K^+ channel was activated by cromakalim, GLB increased $[Ca^{2+}]_i$ and induced contraction. It was observed that the basal activity of the ATP-sensitive K^+ channel is high in pancreatic β cells (Findlay *et al.*, 1985) but low in vascular smooth muscle (Standen *et al.*, 1989). This may explain the difference in the response to GLB in the absence of K^+ channel openers between pancreatic β cells and smooth muscle cells, that is, the inhibitory effects of GLB on the K^+ channel and the resultant increase in $[Ca^{2+}]_i$ and contraction become apparent, when K^+ channel activity is kept high with cromakalim. In the present study, however, the reduction of 25 mM K^+-induced contraction by cromakalim was not reversed by GLB. This observation is consistent with the finding that cromakalim ($>3\,\mu$M) also inhibits Ca^{2+} flux through voltage-operated Ca^{2+} channels, independently of K^+ channel opening (Okabe *et al.*, 1990). It was shown that cromakalim caused hyperpolarization of the membrane potential at rest or during NA-induced depolarization, in the rat aorta (Doggrell *et al.*, 1989). In the present study, since GLB completely reversed the cromakalim-induced reduction of the NA-induced contraction, a mechanism independent of the K^+ channel opening might not be functioning in the cromakalim-induced reduction of the NA-induced contraction.

The vasorelaxation induced by GLB was accompanied by a decrease in $[Ca^{2+}]_i$ in the present study but the mechanism was not determined. Inhibition of contraction of rat uterine smooth muscle (Villar *et al.*, 1986) and reduction in myocardial contractility induced by GLB (Pogasta & Dubecz, 1977) has been reported. Since the $[Ca^{2+}]_i$-force relationship in GLB-induced inhibition of contraction was not affected, the inhibitory effect may be related to initial steps involved in

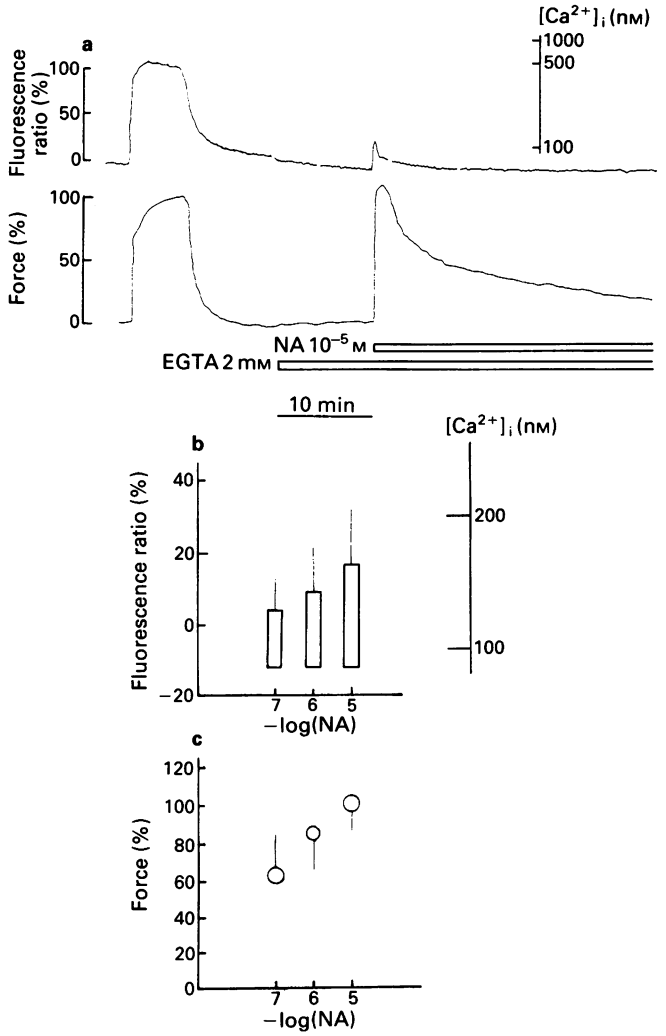


Figure 4 Changes in $[Ca^{2+}]_i$ and force induced by noradrenaline (NA) 10^{-5} M in the absence of extracellular Ca^{2+}. (a) Representative recordings of changes in fluorescence ratio and force development. After recording the responses of $[Ca^{2+}]_i$ and force to 118 mM K^+-application in the presence of 1.25 mM extracellular Ca^{2+}, the strips were incubated with Ca^{2+}-free PSS containing 2 mM EGTA for 10 min, and then NA was applied. (b) Dose-dependent responses of fluorescence ratio and (c) force development to NA. Data are means with s.e. shown by vertical lines ($n = 3$).

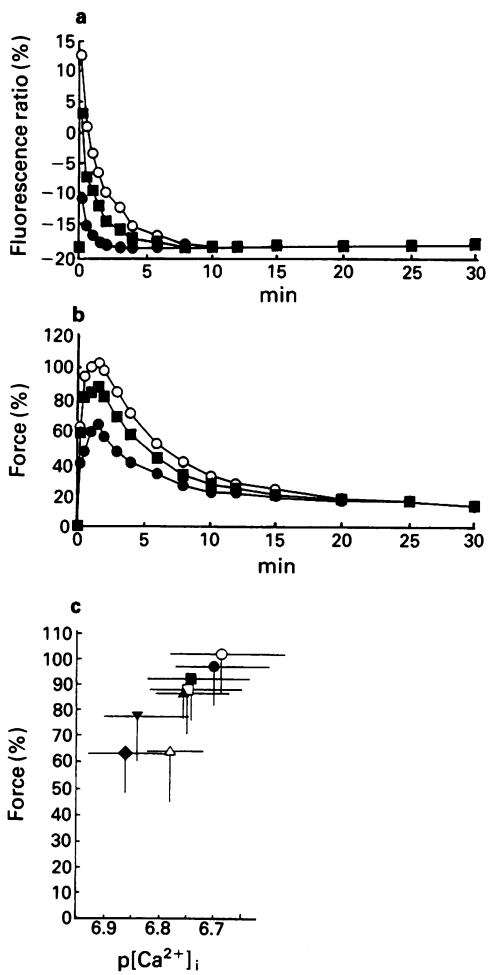
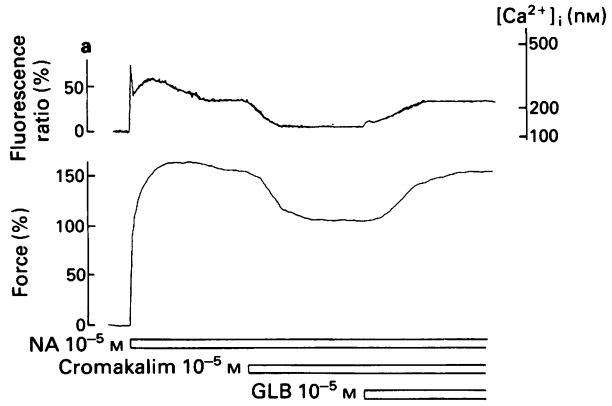


Figure 5 Effects of glibenclamide (GLB) on changes in (a) fluorescence ratio and (b) force development induced by noradrenaline (NA) 10^{-5} M in the absence of extracellular Ca^{2+}. GLB 10^{-6} M (■), 10^{-5} M (●), and without GLB (control, NA 10^{-5} M (○)). (c) Effects of GLB on $[Ca^{2+}]_i$-force relationship induced by NA in the absence of extracellular Ca^{2+}. GLB 10^{-7} M (●), 3×10^{-7} M (■), 10^{-6} M (▲), 3×10^{-6} M (▼), 10^{-5} M (◆), and without GLB (control, NA 10^{-7} M (△), 10^{-6} M (□), 10^{-5} M (○)). Control $[Ca^{2+}]_i$-force relationship with various concentrations of NA in the absence of GLB were obtained from the data in Figure 4b,c.



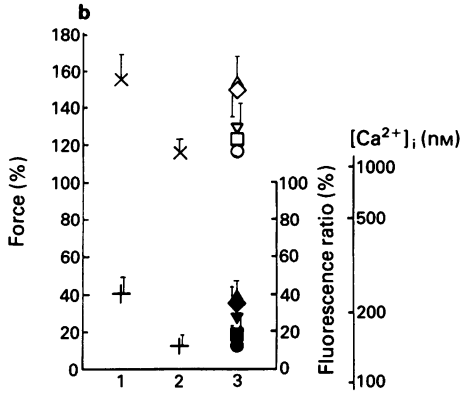


Figure 6 Effects of glibenclamide (GLB) and cromakalim on changes in $[Ca^{2+}]_i$ and force induced by noradrenaline (NA) 10^{-5} M (a) Representative recordings of changes in fluorescence ratio and force. Cromakalim was applied when the maximum force development was obtained in NA 10^{-5} M. Subsequently, GLB 10^{-5} M was applied when $[Ca^{2+}]_i$ and force were reduced to a steady level by cromakalim, 10^{-5} M. (b) Effects of GLB on $[Ca^{2+}]_i$ and force during cromakalim-induced relaxation and during NA-induced contraction. Fluorescence ratio (+) and force (×) of the steady level of NA-induced contraction (1), and following cromakalim-induced relaxation (2). The reversal effects of GLB against cromakalim were dose-dependent (3). GLB 10^{-9} M (○, ●), 10^{-8} M (□, ■), 10^{-7} M (▽, ▼), 10^{-6} M (◇, ◆), 10^{-5} M (△, ▲). Open and closed symbols are force and $[Ca^{2+}]_i$, respectively.

signal transduction. The reversal of cromakalim-induced relaxation was observed with 10^{-8} M to 10^{-5} M GLB (the concentration for the half maximum reversal effect was 2×10^{-7} M), while the inhibition of contraction was observed with 10^{-6} M to 10^{-4} M GLB. Because of the difference in the concentration-range between the two effects of GLB, the site of action of GLB for the inhibitory effect does not seem to be related to the K^+ channel which may be closely linked to receptors for sulphonylurea.

In conclusion, GLB inhibited contractions induced by K^+-depolarization and by NA in the rabbit aorta, by decreasing $[Ca^{2+}]_i$ and with no effect on the $[Ca^{2+}]_i$-force relationship. When contractions induced by NA were inhibited by a K^+-channel opener, GLB reversed this inhibitory effect by inhibiting K^+-channel opening and increasing $[Ca^{2+}]_i$.

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Protection induced by cholecystokinin-8 (CCK-8) in ethanol-induced gastric lesions is mediated via vagal capsaicin-sensitive fibres and CCK_A receptors

[1]S. Evangelista & C.A. Maggi

Pharmacology Department, Menarini Pharmaceuticals, via Sette Santi 3, 50131 Firenze, Italy

1 We have investigated the effect of intravenous injection of cholecystokinin-8 (CCK-8) and other peptides on gastric lesion formation in response to an intragastric perfusion with 25% ethanol in rats anaesthetized with urethane.

2 Intravenous injection of CCK-8 (50–100 nmol kg^{-1}), but not bombesin (1–100 nmol kg^{-1}), calcitonin gene-related peptide (1–50 nmol kg^{-1}), neurokinin A (1 μmol kg^{-1}) or substance P (100 nmol kg^{-1}), induced protection against gastric haemorrhagic lesions produced by ethanol.

3 The CCK_A-antagonist L-364,718 (2.45 μmol kg^{-1}, i.v.) increased the lesion index induced by ethanol and reversed the protective effect of CCK-8 (50 nmol kg^{-1}, i.v.). The CCK_B-antagonist L-365,260 (5 μmol kg^{-1}, i.v.) and a lower dose of L-364,718 (0.25 μmol kg^{-1}, i.v.) were ineffective.

4 The gastric protective effects afforded by CCK-8 (50 nmol kg^{-1}, i.v.) were not observed in vagotomized-rats and were reduced by capsaicin pretreatment. In capsaicin-pretreated rats there was a worsening of gastric lesions induced by ethanol-perfusion as compared to those observed in vehicle-pretreated rats.

5 These results demonstrate that the mucosal protective effect of CCK-8 involves, at least in part, the activation of CCK_A-receptors and is mediated by vagal capsaicin-sensitive fibres.

Introduction

Cholecystokinin-8 (CCK-8), the active fragment of CCK-33, is a peptide present in the gastrointestinal tract (Ekblad *et al.*, 1985) where it exerts an important role as physiological mediator of functions such as motility and emptying of the stomach (Raybould & Taché, 1988) and pancreatic secretion (Williams, 1982). These effects have been reported to be mediated through peripheral CCK_A receptors and blocked by the specific receptor antagonist L-364,718 (Evans *et al.*, 1986).

Recently, the inhibitory effect of CCK-8 on gastric motility and emptying has been shown to be partly mediated through vagal capsaicin-sensitive afferents (Raybould & Taché, 1988). Capsaicin is a selective neurotoxin that, when administered systemically to neonatal rats, induces a lifelong chemical sensory denervation (Holzer, 1988). Capsaicin-sensitive afferents are also involved in a local defence mechanism(s) against gastroduodenal ulcers (Holzer *et al.*, 1990a). Thus newborn rats treated with capsaicin are more prone than controls to develop gastric lesions in response to a variety of ulcerogenic stimuli (Evangelista *et al.*, 1988). The observation that the subcutaneous administration of CCK-8 reduced gastric lesions induced by ethanol (Evangelista *et al.*, 1987) prompted us to determine the possible involvement of capsaicin-sensitive afferents in the protective effects induced by CCK-8 and the influence of vagotomy on the same phenomenon. For comparison, similar experiments were carried out with other peptides contained in capsaicin-sensitive fibres such as bombesin, calcitonin gene-related peptide, neurokinin A and substance P (Holzer, 1988).

In view of the recent development of CCK_A- and CCK_B-receptor antagonists, namely L-364,718 and L-365,260 (Evans *et al.*, 1986; Bock *et al.*, 1989), these drugs were used to determine if the protective effects of CCK-8 on ethanol-induced gastric lesions might involve CCK_A- or CCK_B-receptors.

[1] Author for correspondence.

Methods

Experimental animals and procedures

Male albino rats, Sprague-Dawley Nossan strain, weighing 280–310 g, were housed at constant room temperature (21 ± 1°C), relative humidity (60%) and with 12 h light-dark cycle (light on 06 h 00 min). The animals were deprived of food for 20 h before the experiments but allowed free access to tap water.

Surgical preparation and induction of gastric lesions

The rats were anaesthetized by a subcutaneous injection of urethane (1.5 g kg^{-1}). The body temperature was maintained at 36–37°C by means of a heating lamp and tracheostomy was performed and the trachea cannulated to ensure a patent airway.

The gastric lumen was continuously perfused by a technique described previously (Holzer & Lippe, 1988). Briefly, a soft catheter (i.d. 0.8 mm) was inserted into the stomach through an incision in the cervical esophagus and held in place by a ligature. This catheter was connected to a peristaltic pump, and saline at pH 7 and 37°C was perfused through the gastric lumen at a rate of 0.7–0.8 ml min^{-1}. Gastric outflow was collected by means of a cannula (i.d. 3 mm) inserted into the stomach via an incision in the duodenum and held in place by two ligatures around the duodenum. At the beginning of the experiment the stomach was flushed with 50 ml of bodywarm saline to remove any solid contents. After a 60 min period, peptides under study were injected i.v. and 5 min later the gastric lumen was perfused with 25% ethanol.

In some experiments the vagi were cut bilaterally at cervical level, when the oesophagus was intubated (about 60 min before ethanol challenge), and the animals were artificially ventilated by means of a ventilator (Basile, Varese, Italy) for small rodents (60 strokes min^{-1}, 0.8 ml 100 g^{-1} body wt). At the end of the experiments (after 30 min of ethanol perfusion)

the animals were killed, their stomachs excised and examined for the presence of lesions by an observer unaware of the treatments. Each individual lesion (red streaks) was measured along its greatest length with the aid of a binocular microscope (magnification $\times 10$) to determine the lesion index (Holzer & Lippe, 1988). Lesions smaller than 1 mm were assigned a rating of 1, lesions measuring 1–2 mm were assigned a rating of 2 and lesions measuring more than 2 mm were given a rating according to their length in mm. The overall total was designated the 'lesion index'.

In order to assess the mechanisms of the antilesion effect of CCK-8, L-364,718 (0.25–2.45 μmol kg^{-1}, i.v.) or L-365,260 (5 μmol kg^{-1}, i.v.), at doses reported to antagonize specific *in vivo* receptor-mediated CCK-8 effects (Lotti *et al.*, 1987; Lotti & Chang, 1989), were administered 5 min before CCK-8.

Sensory denervation

On their second day of life, rats received capsaicin 50 mg kg^{-1}, by subcutaneous injection. This treatment is known to cause a permanent degeneration of unmyelinated afferent neurones (Holzer, 1988). Control animals received an equal volume of vehicle (10% ethanol, 10% Tween 80 and 80% saline v/v/v). All injections were performed under ether anaesthesia. The rats were then grown to adulthood and used for experiments 2–3 months after this treatment.

Drugs

L-364,718 (3S(−)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepine-3yl)-1H-indole-2-carboxamide) and L-365,260 ((3R(+)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepine-3yl)-N^1-(3-methyl-phenyl)mea) (Merck Sharp & Dohme) were dissolved in 1:1 dimethylsuphoxide (DMSO): Tween 80. The solution was sonicated and then diluted with saline to a final concentration of 8% DMSO and Tween 80. Controls were treated with the same vehicle. Peptides were purchased from Peninsula and dissolved in 0.9% saline. All drugs were administered i.v. in a volume of 1 ml kg^{-1} except for capsaicin (Sigma) which was given in 2 ml kg^{-1} by the subcutaneous route.

Statistics

All data related to the lesion index are expressed in the figures as mean ± s.e. and analyzed by means of the analysis of variance followed by Dunnett's test to determine differences among means.

Results

Effect of cholecystokinin-8 and other peptides

In the first series of experiments we investigated the effect of some peptides on the formation of haemorrhagic lesions in the glandular mucosa of the stomach produced by the intraluminal perfusion of 25% ethanol. The development of lesions was reduced by CCK-8 (Figure 1), but not by bombesin (1–100 nmol kg^{-1}), calcitonin gene-related peptide (1–50 nmol kg^{-1}), neurokinin A (1 μmol kg^{-1}) or substance P (100 nmol kg^{-1}), injected 5 min before the ethanol-perfusion ($n = 6$ for each peptide).

As shown in Figure 1 the protective effect induced by 50 nmol kg^{-1} i.v. of CCK-8 was not further enhanced by increasing the dose of CCK-8. The possibility was investigated that the effect of CCK-8 on ethanol-induced gastric lesions might have involved systemic changes in the cardiovascular system. Intravenous injection of 50 nmol kg^{-1} of CCK-8 produced a rapid and negligible increase in blood pressure (5 ± 0.7 mmHg at 5 min after CCK-8 injection; $n = 4$).

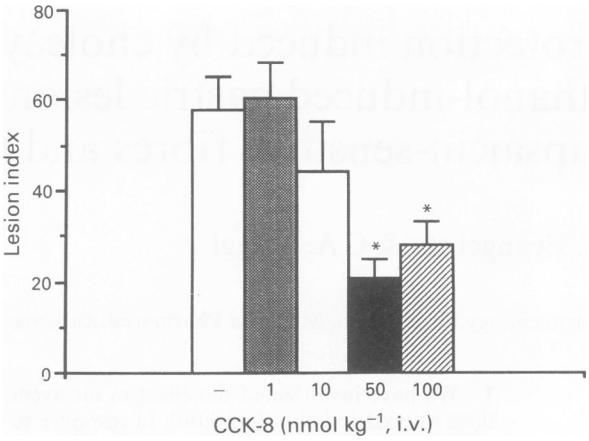

Figure 1 Effect of cholecystokinin-8 (CCK-8) (1–100 nmol kg^{-1}, i.v.) on gastric lesion formation caused by the intragastric perfusion of 25% ethanol for 30 min. CCK-8 was administered 5 min before the start of ethanol perfusion. Columns show mean values with s.e. indicated by vertical bars, $n = 8$; * $P < 0.05$ as compared to controls.

Effect of CCK receptor antagonists

Pretreatment with the CCK_A-receptor antagonist L-364,718 (2.45 μmol kg^{-1}, i.v.; see Lotti *et al.*, 1987) prevented the antiulcer effect of CCK-8 (Table 1) while a lower dose of L-364,718 (0.25 μmol kg^{-1}, i.v.) or the CCK_B-receptor antagonist L-365,260 (5 μmol kg^{-1}, i.v.) were without effect (Table 1). In the animals pretreated with L-364,718 (2.45 μmol kg^{-1}, i.v.). CCK-8 at a dose of 50 nmol kg^{-1} i.v. was unable to give any protective effect against the lesions induced by ethanol (Table 1).

It should be noted that pretreatment with L-364,718, at the dose that blocked the effect of exogenous CCK-8, aggravated the ethanol-induced gastric lesions, while pretreatments with L-364,718 at 0.25 μmol kg^{-1} i.v. or L-365,260 (5 μmol kg^{-1}, i.v.) were ineffective (Table 1).

Effect of vagotomy and capsaicin pretreatment

Protection against gastric lesions induced by CCK-8 (50 nmol kg^{-1}, i.v.) in vehicle-pretreated rats (63% reduction of lesion index as compared to controls) was reduced in capsaicin pretreated- and totally abolished in vagotomized rats (Figure 2). Treatment with CCK-8 (50 nmol kg^{-1}, i.v.) or vehicle in vagotomized rats produced almost similar values in lesion index following the intragastric perfusion with ethanol (Figure 2).

Table 1 Effect of L-365,260 or L-364,718 on cholecystokinin-8 (CCK-8) protection in gastric lesion formation caused by the intragastric perfusion of 25% ethanol

Treatments and dose	*Lesion index*
Vehicle + saline	58 ± 7
Vehicle + CCK-8 50 nmol kg^{-1}, i.v.	24 ± 4*
L-365,260 5 μmol kg^{-1}, i.v. + saline	65 ± 10
L-365,260 5 μmol kg^{-1}, i.v. + CCK-8 50 nmol kg^{-1}, i.v.	26 ± 8*§
L-364,718 0.25 μmol kg^{-1}, i.v. + saline	71 ± 12
L-364,718 0.25 μmol kg^{-1} i.v. + CCK-8 50 nmol kg^{-1} i.v.	32 ± 7*§
L-364,718 2.45 μmol kg^{-1}, i.v. + saline	99 ± 15**
L-364,718 2.45 μmol kg^{-1}, i.v. + CCK-8 50 nmol kg^{-1}, i.v.	79 ± 10*

Values are expressed as mean ± s.e., $n = 8$–10. * $P < 0.05$ and ** $P < 0.01$ as compared to vehicle + saline group; § $P < 0.05$ as compared to respective antagonist (L-365,260 or L-364,718) + saline group.
L-365,260 or L-364,718 and CCK-8 were injected 10 and 5 min respectively before the start of ethanol perfusion.

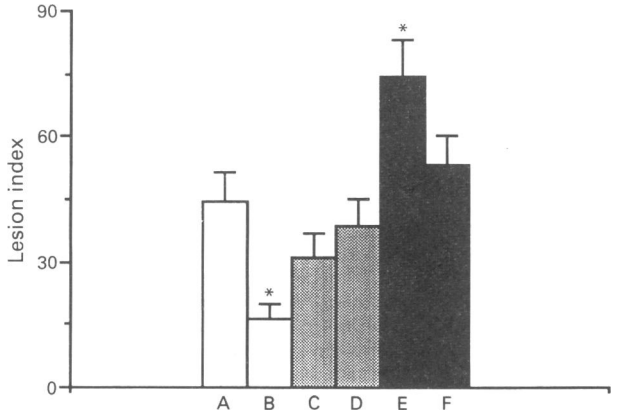

Figure 2 Gastric lesion formation caused by the intragastric perfusion of 25% ethanol for 30 min in controls (open columns), vagotomized (stippled columns) or capsaicin pretreated rats (solid columns). Groups refer to the following treatments carried out 5 min before the start of an ethanol perfusion: A, C and E = vehicle; B, D and F = CCK-8 50 nmol kg^{-1}, i.v. Columns show mean values with s.e. indicated by vertical bars, $n = 12$. * $P < 0.05$ and compared to group A (controls).

In rats pretreated with capsaicin, CCK-8 (50 nmol kg^{-1}, i.v.) produced only a 29% reduction of lesion index and was not significantly different as compared to capsaicin-pretreated vehicle treated rats (Figure 2). Capsaicin-pretreatment aggravated the lesion index induced by 25% ethanol as compared to vehicle-pretreated rats, while the effect of ethanol in vagotomized animals was not significantly different from that of sham-operated animals (Figure 2). Neither vagotomy nor capsaicin affected the lesion index in the absence of ethanol perfusion. When we increased the lesion index (by intragastric perfusion of 50% ethanol), to obtain a value similar to that found in capsaicin-pretreated rats (see Figure 2), CCK-8 (50 nmol kg^{-1}, i.v.) produced a significant reduction in lesion index (36 ± 8, $n = 4$) with a 59% reduction in this value as compared to controls (90 ± 5, $n = 4$). Therefore the reduction in the protective effect of CCK-8 observed in rats pretreated with capsaicin or the CCK_A-receptor antagonist is probably not ascribable to the aggravation of lesions produced by the pretreatments.

Discussion

The present results show that intravenous injection of CCK-8 affords protection against ethanol-induced gastric lesions in rats. This effect of CCK-8 is not shared by intravenous administration of other gut peptides such as bombesin, calcitonin gene-related peptide, neurokinin A or substance P, although some of the latter peptides have been shown to be effective after subcutaneous administration (Evangelista *et al.*, 1987; 1989) but the enzymatic breakdown strongly influences the effects of peptides when given intravenously. On the other hand the intravenous route for peptides is likely to be more suitable to obtain a specific effect as compared to the subcutaneous administration.

The protective effect of peptides given systemically could not be attributed to any systemic haemodynamic effect, since CCK-8 was devoid of significant effects on blood pressure up to a dose of 50 nmol kg^{-1} i.v.

The protective effect of CCK-8 is dependent on the integrity of the vagus nerve and, at least in part, mediated by capsaicin-sensitive afferents. Neurochemical studies have shown that sensory neurones supplying the stomach are of dual origin (vagal and spinal; Green & Dockray, 1988). Our results show that the protective properties of CCK seem to be restricted to the stimulation of sensory neurones of vagal origin.

Capsaicin-sensitive nerves appear to control the susceptibility of the gastric mucosa to ulcerogenic stimuli since their ablation aggravates (Evangelista *et al.*, 1988; Esplugues & Whittle, 1990; present results) and stimulation reduces (Holzer & Lippe, 1988) lesion formation induced by ethanol. A local release of sensory neuropeptides has been shown to play a pivotal role in these protective mechanisms (Holzer *et al.*, 1990b; Whittle *et al.*, 1990) through the regulation of local microvascular protective response to challenge (Holzer *et al.*, 1990a). In light of the above we cannot exclude the possibility that the protective effect of CCK-8 against mucosal damage might involve local release of sensory neuropeptides and increase in gastric blood flow. On the other hand, the complete failure of CCK-8 to afford ulcer protection within a short time from vagotomy, strongly suggests the involvement of reflex mechanisms. Indeed, it appears unlikely that the sensory neuropeptide content in vagal primary afferents in the stomach might have been depleted within 60 min from nerve section. It should be noted that acute vagotomy was more effective than capsaicin pretreatment in preventing the protective effect of CCK-8. Although the exact mechanism underlying this difference cannot be immediately understood on the basis of the present findings, a hypothesis to be considered is that CCK-8 stimulates certain vagal afferents which were unaffected by capsaicin pretreatment.

The recent development of potent and highly selective CCK receptor antagonists has made possible detailed studies on the physiological functions of CCK. Thus, peripheral CCK_A-receptors are involved in pancreatic secretion, stomach and gallbladder motility and anorexia (Evans *et al.*, 1986) and CCK_A binding sites have been specifically demonstrated on the vagus nerve and shown to be transported towards the periphery (Moran *et al.*, 1987). Receptors for CCK are well known to be present in the stomach (Sutliff *et al.*, 1987) and they have been considered to be mainly involved in the gastric motility effects of CCK. This study shows that CCK_A-receptors might also have a role in regulating the susceptibility of the gastric mucosa to injury. In fact the CCK_A-receptor-antagonist (L-364,718) prevented the protective effect of CCK-8 while the CCK_B-receptor antagonist (L-365,260) was ineffective. This effect was obtained with a dose of the CCK_A-receptor antagonist which has been shown to block selectively other *in vivo* peripheral effects mediated by CCK_A-receptors (Lotti *et al.*, 1987). Conversely L-365,260, which binds with high affinity to gastrin and central CCK_B-receptors (Lotti & Chang, 1989), did not affect the protective response to CCK-8 at the dose which selectively inhibits gastrin-stimulated gastric acid secretion (Lotti & Chang, 1989). Furthermore, administration of the CCK_A-receptor antagonist alone aggravated ethanol-induced lesions, implicating a possible role of endogenous CCK in the regulation of gastric mucosal integrity in response to injury.

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Mechanisms contributing to the differential haemodynamic effects of bombesin and cholecystokinin in conscious, Long Evans rats

[1]Paul J.J.M. Janssen, [2]Sheila M. Gardiner, Alix M. Compton & Terence Bennett

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

1 Long Evans rats were chronically instrumented with intravascular catheters and pulsed Doppler probes to assess changes in renal, mesenteric and hindquarters blood flows and vascular conductances in response to bombesin (2.5 $\mu g\,kg^{-1}$, i.v.) and cholecystokinin (CCK) (0.5 and 5.0 $\mu g\,kg^{-1}$, i.v.).

2 Bombesin caused an increase in heart rate and blood pressure, together with a transient renal vasoconstriction and prolonged mesenteric vasodilatation; there was an early hindquarters vasodilatation followed by vasoconstriction.

3 In the presence of phentolamine, bombesin caused a fall in blood pressure due to enhanced hindquarters vasodilatation; these effects were reversed by propranolol and hence were possibly due to circulating adrenaline acting on vasodilator β_2-adrenoceptors.

4 During concurrent administration of phentolamine, propranolol and atropine, bombesin caused prolonged tachycardia and a rise in blood pressure. The renal vasoconstrictor and mesenteric vasodilator effects of bombesin were not reduced under these conditions and thus probably were direct and/or indirect non-adrenergic, non-cholinergic (NANC) effects.

5 CCK caused dose-dependent increases in blood pressure accompanied by renal, mesenteric and hindquarters vasoconstrictions followed, after the higher dose, by vasodilatations. The lower dose of CCK increased heart rate but there was a bradycardia followed by a tachycardia after the higher dose.

6 Experiments with antagonists as described above indicated the pressor effect of CCK was mediated largely through α-adrenoceptors, as were the mesenteric and hindquarters vasoconstrictor effects; CCK exerted NANC negative chronotropic effects.

7 All the effects of CCK were markedly inhibited by L364,718. This observation, and the finding that L364,718 had no effect on the responses to bombesin, together with the dissimilarities in the regional haemodynamic effects of exogenous CCK and bombesin, indicate that the cardiovascular actions of the latter were not dependent on the release of endogenous CCK.

Introduction

Bombesin is a tetradecapeptide originally isolated from the skin of the frog *Bombina bombina* in 1971 (see Erspamer & Melchiorri, 1973). Gastrin-releasing peptide, a 27 amino acid peptide, is the mammalian homologue (Sunday *et al.*, 1988) and produces similar effects to bombesin after administration in mammals. Bombesin causes a reduction in food and water intake after central (Willis *et al.*, 1984) or peripheral administration (Gibbs *et al.*, 1981), which may be due to satiety-like effects, although it is difficult to exclude the possibility of nausea or malaise contributing to these actions. Bombesin also releases several gut peptides such as gastrin and cholecystokinin (CCK) (Ghatei *et al.*, 1982; Walsh, 1989).

CCK was originally described as a gut hormone causing gallbladder contraction. Subsequently the peptide was identified in neurones by use of anti-gastrin antibodies (CCK and gastrin share the same carboxyterminus), as described by Dockray (1988). Central or peripheral administration of CCK reduces food intake (Lukaszewski & Praissman, 1988; Griesbacher *et al.*, 1989; Rehfeld, 1989). Administration of CCK enhances the release of oxytocin, but not of vasopressin *in vivo* (Verbalis *et al.*, 1986; Carter & Lightman, 1987), although *in vitro* studies indicate that CCK can release both peptides (Bondy *et al.*, 1989).

Bombesin and CCK have been shown to exert cardiovascular actions. Intravenous or subcutaneous injection of bombesin in anaesthetized or conscious rats causes an increase in blood pressure and a tachycardia, although the latter may be slight and the pressor effect may not show clear dose-dependence (Melchiorri *et al.*, 1971; Erspamer *et al.*, 1972; Fisher *et al.*, 1985). Erspamer *et al.* (1972) obtained similar results in pithed rats, although Bayorh & Feuerstein (1985) observed dose-dependent pressor and tachycardic responses to bombesin under these conditions.

CCK, given i.v. in anaesthetized rats, causes dose-dependent bradycardia and complex blood pressure changes (Marker & Roberts, 1988). The ability of bombesin and CCK to overcome severe haemorrhagic shock in rats has been reported (Guarini *et al.*, 1987; 1988a,b; 1989), and recently it was claimed that the effects of bombesin were mediated by the release of endogenous CCK (Guarini *et al.*, 1989).

Since little information is available regarding the regional haemodynamic effects of bombesin or CCK administered i.v. in conscious rats, and because of the suggested relationship between these neuropeptides (Guarini *et al.*, 1989), the present study was designed to answer the following questions:

(1) Are the regional haemodynamic responses to i.v. injections of bombesin or CCK similar in conscious, unrestrained Long Evans rats?

(2) Are the regional haemodynamic responses to CCK influenced by L364,718, a selective antagonist of peripheral (A-type) CCK receptors?

(3) Given the possibility that bombesin releases endogenous CCK, are the regional haemodynamic responses to bombesin influenced by L364,718 at a dose that inhibits the effects of exogenous CCK?

(4) Since it is possible that CCK releases vasopressin, are the regional haemodynamic responses to CCK influenced by a vasopressin V_1-receptor antagonist?

(5) To what extent do the cardiovascular responses to bombesin or CCK involve adrenoceptors or muscarinic receptors?

[1] Present address: Department of Pharmacology, Faculty of Pharmacy, Rijks Universiteit Utrecht, 3511 GH, Catharijnesingel 60, Utrecht, The Netherlands.

[2] Author for correspondence.

Methods

Male, Long Evans rats (350–400 g) were used and throughout the experiments the rats were housed in cages with a solid floor, covered with sawdust. They had free access to water and food and the room temperature was kept between 18 and 21°C.

Animals were anaesthetized with sodium methohexitone (Lilly Ltd., 60 mg kg^{-1}, i.p., supplemented when required) prior to surgery. A midline abdominal incision was made and the connective tissue was carefully separated from the left renal and the superior mesenteric arteries and from the abdominal aorta below the ileocaecal artery. Appropriate sized pulsed Doppler probes (Haywood *et al.*, 1981; made in Nottingham but using DBF-120A-XS sub-assemblies from Crystal Biotech, Holliston, U.S.A.) were sutured around the vessels (with Ethicon 6/0 silk suture) and the leads of the probes were sutured to the abdominal wall (with Ethicon 3/0) in order to prevent possible changes in the position of the probes. The probe wires were tunnelled subcutaneously to exit at the back of the neck, where they were sutured in place. After replacement of the viscera and irrigating the abdominal cavity with sterile saline, the abdominal incision was closed with surgical silk (Ethicon 3/0). After surgery the rats were given an intramuscular injection of ampicillin (Penbritin, Beecham Ltd., 7 mg kg^{-1}) and were allowed to recover for at least 7 days.

After this period the rats were briefly re-anaesthetized with sodium methohexitone (40 mg kg^{-1}, i.p., supplemented when required), and prior to catheterization the leads of the implanted probes were soldered to a 6-way micro-connector (Microtech Inc., Boothwyn, U.S.A.) and the signals were checked on an oscilloscope (Telequipment DM64). All 3 phasic pulsed Doppler signals had to be of a good quality (signal: noise, 20 : 1); if this criterion could not be met the animal was rejected from the study. Between 2 and 4 catheters (Portex Ltd., i.d. 0.28 mm, o.d. 0.62 mm) were implanted in the right jugular vein and an intra-arterial catheter was implanted in the distal aorta, via the caudal artery, for measurement of blood pressure and heart rate (Gardiner & Bennett 1985; 1988).

The arterial catheter consisted of a 7 cm length of polyethylene catheter with a small diameter (Portex Ltd., i.d. 0.28 mm, o.d. 0.62 mm) fused to a more rigid, nylon catheter with a larger diameter (Portex Ltd., i.d. 0.58 mm, o.d. 1.02 mm). The catheters were tunnelled subcutaneously to the back of the neck where they emerged at the same point as the Doppler probe wires. The micro-connector, soldered to the latter, was clamped in a custom-made harness worn by the rat and the catheters ran through a flexible spring attached to the harness. The rats were allowed to recover for a day before experiments started (Gardiner & Bennett, 1988; Gardiner *et al.*, 1988a, b; 1990b).

At the end of every day the arterial catheter was filled with a strong saline/heparin solution (Monoparin, CP Pharmaceuticals Ltd., 450 units ml^{-1}, 1 ml) in order to prevent clots forming in the catheter. If a catheter became blocked a plasmin solution (porcine plasmin, Sigma Chemicals Ltd.; 1 unit ml^{-1}) was flushed down it in order to dissolve the blood clot.

The probes were connected to a pulsed Doppler flowmeter (Crystal Biotech, Holliston, U.S.A.), constructed to the original design by Hartley & Cole (1974) and Hartley *et al.* (1978), but operating with a pulse repetition frequency of 125 kHz (Gardiner *et al.*, 1990a). The mean Doppler signal represents the average velocity of the erythrocytes. The relationship between mean Doppler shift (kHz) and volume flow (ml min^{-1}) measured with an electromagnetic flowmeter, is linear (Haywood *et al.*, 1981) and hence the percentage change in mean Doppler shift relative to baseline was taken as an index of change in flow.

During the experiments 9 different variables were recorded (heart rate, phasic and mean blood pressure, and phasic and mean Doppler shift signals from renal, mesenteric and hindquarters probes). The purpose of recording phasic Doppler shift signals was to ensure that they were of an acceptable quality during the experiments. The pulsed Doppler flowmeter gives an electronic zero that corresponds to zero volume flow. Zero lines for all 3 regional flow signals were recorded continuously.

At selected time points heart rate, mean blood pressure and mean Doppler shifts were measured and related to the predrug baseline (absolute changes for the former two variables, percentages for the Doppler shifts). In addition, the Doppler shift was divided by mean blood pressure in order to obtain the vascular conductance changes (% relative to baseline; Gardiner *et al.*, 1990b). Before every experiment baseline measurements were made over a period of 30 min.

Experimental protocols

Three separate groups of rats were studied ($n = 8$ in each).

Group 1 Animals were given bombesin (2.5 $\mu g\,kg^{-1}$, i.v. bolus) on two occasions separated by at least 1.5 h.

The next day animals were given the vehicle for L364,718 (2% dimethylsulphoxide (DMSO) and 2% Tween 80 in isotonic saline; i.v. 0.1 ml bolus and 0.3 ml h^{-1} infusion), followed, after 10 min, by i.v. bombesin (2.5 $\mu g\,kg^{-1}$, i.v. bolus). Then, after at least 1.5 h, these animals were given L364,718 (Guarini *et al.*, 1989; 50 $\mu g\,kg^{-1}$, i.v. bolus, 150 $\mu g\,kg^{-1}\,h^{-1}$, infusion) followed, after 10 min, by bombesin (2.5 $\mu g\,kg^{-1}$, i.v. bolus).

Group 2 Animals were given 2 doses of CCK (0.5 and 5.0 $\mu g\,kg^{-1}$, i.v. bolus) separated by at least 1.5 h. Subsequently, (after at least 1.5 h) administration of the vehicle for L364,718 (see above) was started, and 10 min later CCK (5.0 $\mu g\,kg^{-1}$, i.v. bolus) was given.

The next day these animals were given L364,718 (50 $\mu g\,kg^{-1}$, i.v. bolus, 150 $\mu g\,kg^{-1}\,h^{-1}$, infusion) followed 10 min later by CCK (0.5 $\mu g\,kg^{-1}$, i.v. bolus). At least 1.5 h after this time the animals were given L364,718 (50 $\mu g\,kg^{-1}$, i.v. bolus, 150 $\mu g\,kg^{-1}\,h^{-1}$, infusion) followed 10 min later by CCK (5.0 $\mu g\,kg^{-1}$, i.v. bolus).

Group 3 Animals were given a vasopressin V_1-receptor antagonist (10 $\mu g\,kg^{-1}$, i.v. bolus) and after 10 min CCK (5.0 $\mu g\,kg^{-1}$, i.v. bolus) was administered. At least 1.5 h later phentolamine (1 mg kg^{-1}, i.v. bolus, 1 mg $kg^{-1}\,h^{-1}$, infusion) administration was started, followed, after 30 min, by bombesin (2.5 $\mu g\,kg^{-1}$, i.v. bolus) and, at least 45 min after bombesin administration, CCK (5.0 $\mu g\,kg^{-1}$, i.v. bolus) was given.

The next day phentolamine (1 mg kg^{-1}, i.v. bolus, 1 mg $kg^{-1}\,h^{-1}$, infusion) and propranolol (1 mg kg^{-1}, i.v. bolus, 0.5 mg $kg^{-1}\,h^{-1}$, infusion) administrations were started and 30 min after onset of the infusions, bombesin (2.5 $\mu g\,kg^{-1}$, i.v. bolus) was given. At least 45 min after bombesin administration CCK (5.0 $\mu g\,kg^{-1}$, i.v. bolus) was injected. Following a further 45 min during which the infusions of phentolamine and propranolol were continued, atropine (1 mg kg^{-1}, i.v. bolus, 1 mg $kg^{-1}\,h^{-1}$, infusion) administration was started, followed 30 min later by bombesin (2.5 $\mu g\,kg^{-1}$, i.v. bolus) and, after at least a further 45 min, by CCK (5.0 $\mu g\,kg^{-1}$, i.v. bolus).

Rats in group 1 were exposed to bombesin (2.5 $\mu g\,kg^{-1}$) on 4 occasions over 2 days and there were no systematic differences in the responses. Hence in those experiments where the responses to bombesin were affected by pretreatment (see Results), these differences must have been due to the pretreatment rather than the repeated exposure to bombesin.

Rats in group 2 were exposed to CCK (5.0 $\mu g\,kg^{-1}$) on 2 occasions during one day; no systematic differences in responses were observed. So, the different responses to CCK, observed in animals in groups 2 and 3 following administration of different antagonists (see Results), are likely to have

Table 1 Peak cardiovascular changes following increasing i.v. doses of bombesin in conscious, Long Evans rats

	Dose of bombesin		
	0.25 μg kg⁻¹	*2.5 μg kg⁻¹*	*25 μg kg⁻¹*
Heart rate (beast min⁻¹)	24 ± 10*	94 ± 10*	83 ± 11*
Mean blood pressure (mmHg)	3 ± 2	18 ± 3*	20 ± 2*
Flow (%)			
Renal	−13 ± 7	−76 ± 5*	−77 ± 4*
Mesenteric	23 ± 11	43 ± 3*	47 ± 4*
Hindquarters	26 ± 10*	55 ± 16*	51 ± 9*
Conductance (%)			
Renal	−15 ± 8	−77 ± 5*	−78 ± 3*
Mesenteric	21 ± 12	23 ± 4*	24 ± 4*
Hindquarters	23 ± 10*	57 ± 13*	45 ± 14*

Values are mean ± s.e.mean; $n = 8$.
* $P < 0.05$ versus baseline (Friedman's test).

been caused by those pretreatments rather than the repeated exposure to CCK.

Peptides and drugs

The following peptides and drugs were used during the experiments: bombesin (Bachem Ltd); CCK (26–33), sulphated (Bachem Ltd.); L364,718 (3S(−)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepine-3-yl)-1H-indole-2-carboxamide), (Merck, Sharp and Dohme Ltd.); vasopressin V_1-receptor antagonist, $d(CH_2)_5$[Tyr(Et)]DAVP (Prof. Manning, Medical College of Ohio); phentolamine mesylate (Ciba Geigy Ltd.); propranolol hydrochloride (Imperial Chemical Industries Ltd.); atropine methyl nitrate (Sigma Chemicals Ltd.).

Bombesin and CCK (26–33) were dissolved in 1% BSA in isotonic saline. L364,718 was dissolved in 2% DMSO and 2% Tween 80 in isotonic saline. The vasopressin V_1-receptor antagonist was dissolved in 0.5 ml glacial acetic acid, diluted to the working concentration with isotonic saline; phentolamine, propranolol and atropine were dissolved in saline.

The dose of bombesin was determined in pilot experiments that showed a ten fold lower dose had little effect, whereas a ten fold higher dose had no greater effect than the dose employed (Table 1). The range of the CCK concentrations was based on the studies by Marker & Roberts (1988), while the dose of L364,718 was that used by Guarini *et al.* (1989). The concentrations of the vasopressin antagonist, phentolamine, propranolol and atropine used were based on published findings (Gardiner & Bennett, 1985; Winn *et al.*, 1985; Gardiner & Bennett, 1988; Fisher *et al.*, 1985, respectively).

Statistical analysis

Two different statistical tests were applied to the data. For intra-group comparisons Friedman's test (Theodorsson-Norheim, 1987) was used; for paired comparisons Wilcoxon's rank sums test was used, and for unpaired, inter-group comparisons the Mann-Whitney U-test was applied.

A P value < 0.05 was taken to indicate a significant difference. In the results, time values given in parentheses indicate points at which values were significantly different from baseline or significantly different from values obtained in rats not pretreated with antagonists. Values at time points other than those mentioned were not significantly different.

Results

Cardiovascular effects of bombesin

Haemodynamic responses to bombesin (2.5 μg kg⁻¹) (Figures 1 and 2) The cardiovascular effects of bombesin in a single rat are shown in Figure 1, and Figure 2 shows the group mean results.

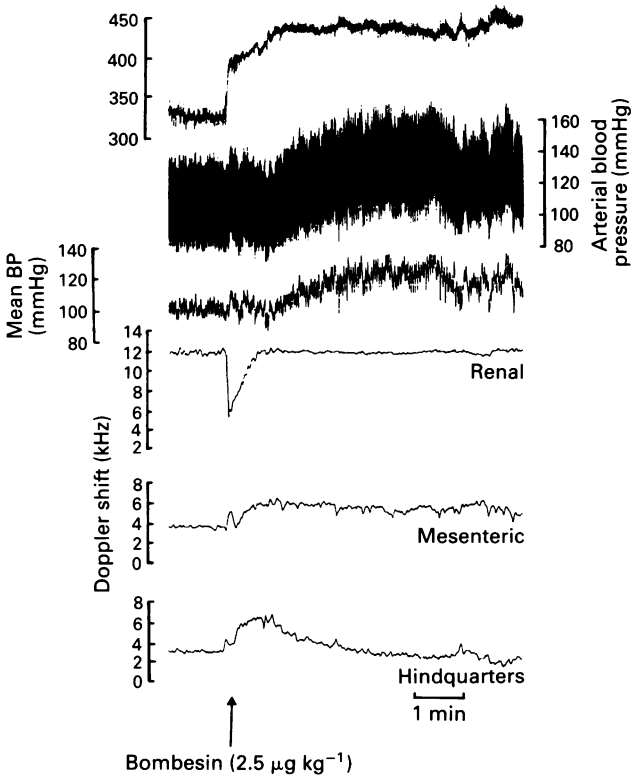

Figure 1 Haemodynamic responses to bombesin (2.5 μg kg⁻¹, bolus, i.v.) in a conscious, unrestrained, Long Evans rat.

A marked tachycardia (significant at 1–5 min, $P < 0.05$) and a small increase in blood pressure (significant at 2–5 min, $P < 0.05$) occurred after administration of bombesin. Renal flow decreased (significant at 0.5 min, $P < 0.05$), while mesenteric (significant at 0.5–30 min, $P < 0.05$) and hindquarters (significant at 0.5–2 min, $P < 0.05$) flows increased. There was a profound, but very short-lasting (significant at 0.5 min, $P < 0.05$) renal vasoconstriction. However, the mesenteric vascular bed showed a prolonged (significant at 1–30 min, $P < 0.05$) vasodilatation, while the hindquarters vascular bed dilated initially (significant at 0.5–1 min, $P < 0.05$) and constricted thereafter (significant at 5 min, $P < 0.05$).

Haemodynamic responses to bombesin (2.5 μg kg⁻¹) in the presence of vehicle or L364,718 Pretreatment with vehicle or with the peripheral CCK receptor antagonist, L364,718, did not change baseline levels or the haemodynamic responses to bombesin (Table 2).

Table 2 Peak cardiovascular changes following administration of vehicle or L364,718, or bombesin in the presence of L364,718

	Vehicle	*L364,718*	*Bombesin in the presence of L364,718*
Heart rate (beats min⁻¹)	+5 ± 4	+19 ± 12	+108 ± 13*
Mean blood pressure (mmHg)	+1 ± 1	+5 ± 3	+16 ± 3*
Flow (%)			
Renal	+2 ± 1	+1 ± 2	−58 ± 4*
Mesenteric	+1 ± 3	−6 ± 5	+44 ± 6*
Hindquarters	+1 ± 4	−4 ± 5	+74 ± 15*
Conductance (%)			
Renal	+1 ± 1	−3 ± 3	−62 ± 4*
Mesenteric	+1 ± 3	−10 ± 5	+32 ± 7*
Hindquarters	+1 ± 4	−8 ± 5	+60 ± 16*

Values are mean ± s.e.mean; $n = 8$.
* $P < 0.05$ versus baseline; compare with Figure 2.

Haemodynamic responses to bombesin (2.5 μg kg^{-1}) in the presence of phentolamine (Figure 2) Thirty min after onset of phentolamine administration the fall in blood pressure was not significant, but there was a persistent tachycardia, a reduction in renal flow, an increase in hindquarters flow, a constriction of the renal and dilatation of the hindquarters vascular beds (all significant, $P < 0.05$).

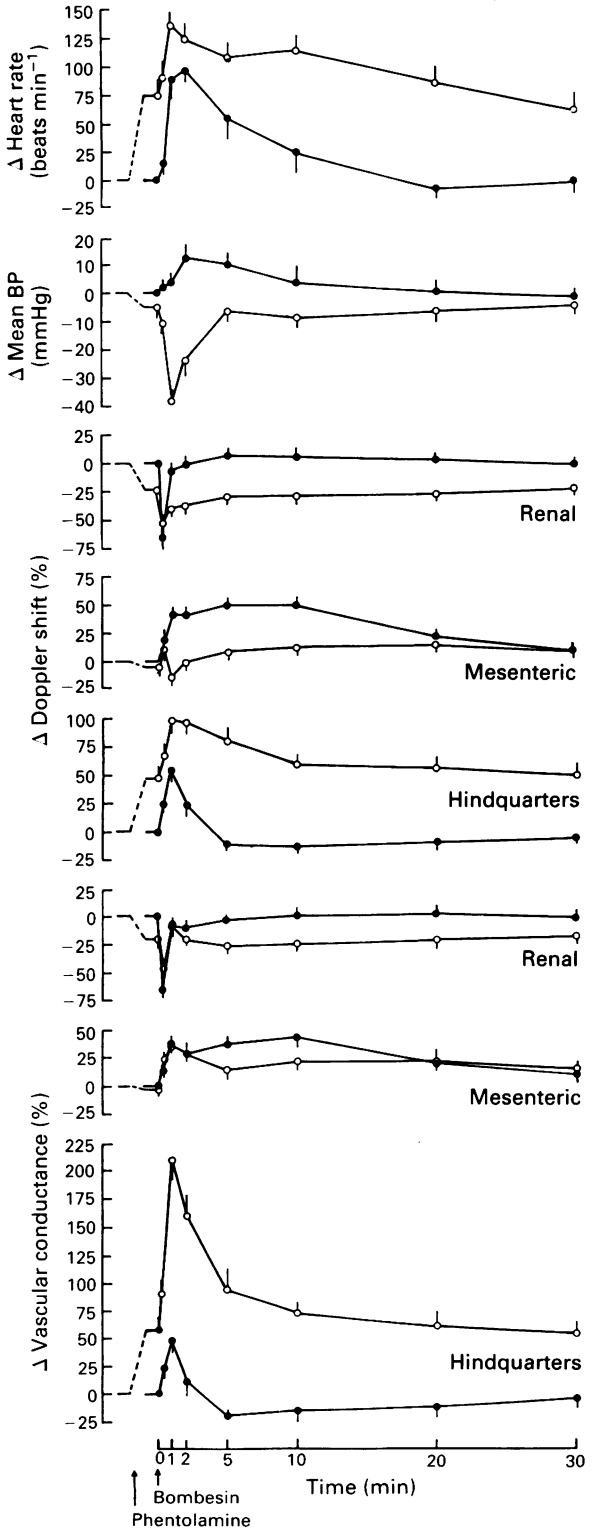

Figure 2 Haemodynamic responses to bombesin (2.5 μg kg^{-1}, bolus, i.v.) in conscious, unrestrained, Long Evans rats, in the absence, (●), or in the presence of phentolamine, (○). The dotted line represents the response to phentolamine. Values are mean ($n = 8$) with s.e.mean shown by vertical lines. All statistics are given in the text.

In the presence of phentolamine, bombesin caused a tachycardia (significant at 0.5–5 min, $P < 0.05$), which was less than in untreated animals (significant at 0.5–5 min, $P < 0.05$) even though the blood pressure decreased (significant at 0.5–2 min, $P < 0.05$), i.e. an effect opposite (significant at 0.5–5 min, $P < 0.05$) to that seen in untreated rats. There was a renal vasoconstriction (significant at 0.5 min, $P < 0.05$), followed by vasodilatation (significant at 1 min, $P < 0.05$); the vasoconstriction was less than that in untreated rats (significant at 0.5 min, $P < 0.05$). The mesenteric vascular bed showed a long-lasting vasodilatation (significant at 0.5–30 min, $P < 0.05$) which was not different from that in untreated rats. However, there was a hindquarters vasodilatation (significant at 0.5–2 min, $P < 0.05$) which was greater (significant at 1–5 min, $P < 0.05$) than the response observed in untreated rats, and there was no subsequent vasoconstriction.

Haemodynamic responses to bombesin (2.5 μg kg^{-1}) in the presence of phentolamine and propranolol (Figure 3) Thirty min after the onset of phentolamine and propranolol adminis-

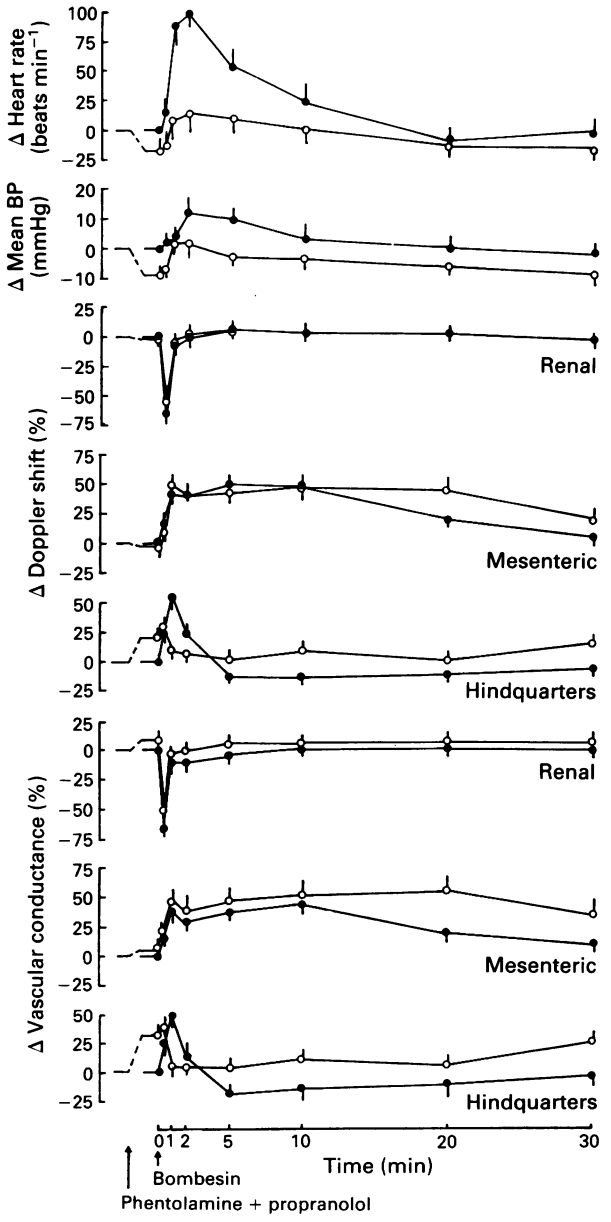

Figure 3 Haemodynamic responses to bombesin (2.5 μg kg^{-1}, bolus, i.v.) in conscious, unrestrained, Long Evans rats, in the absence, (●), or in the presence of phentolamine and propranolol, (○). The dotted line represents the response to infusions of phentolamine and propranolol. Values are mean ($n = 8$) with s.e.mean shown by vertical lines. All statistics are given in the text.

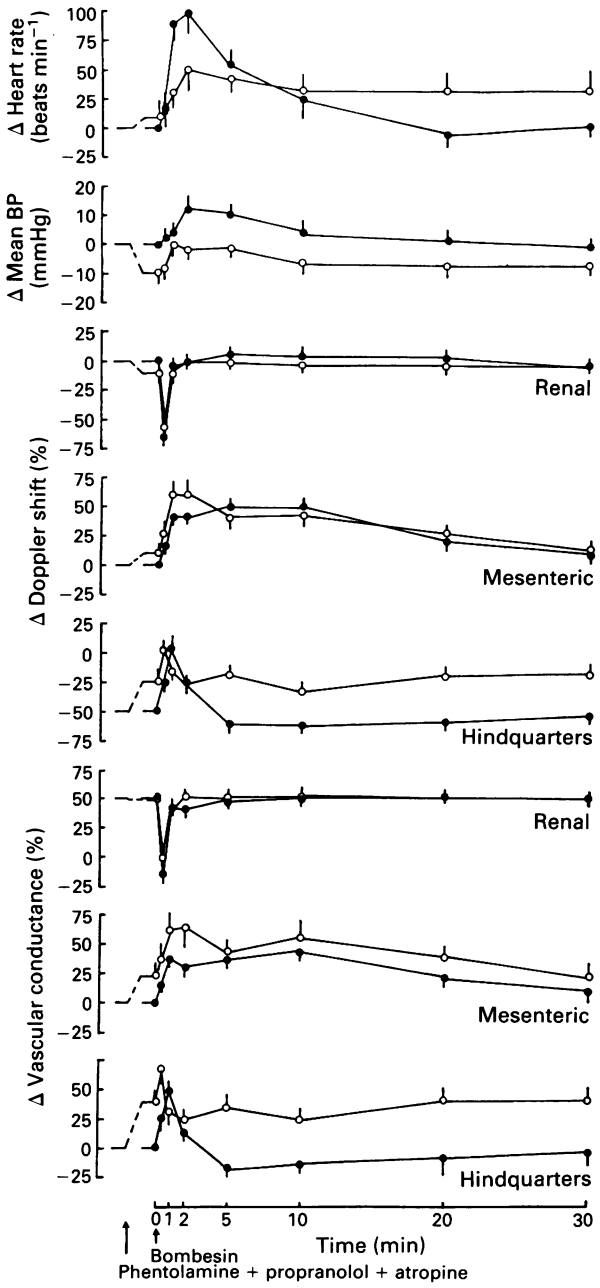

Figure 4 Haemodynamic responses to bombesin (2.5 $\mu g\,kg^{-1}$, bolus, i.v.) in conscious, unrestrained, Long Evans rats, in the absence, (●), or in the presence of phentolamine, propranolol and atropine, (○). The dotted line represents the response to phentolamine, propranolol and atropine administration. Values are mean ($n = 8$) with s.e.mean shown by vertical lines. All statistics are given in the text.

tration there was a persistent bradycardia, a decrease in blood pressure, an increase in hindquarters flow, and renal, mesenteric and hindquarters vasodilatations (all significant, $P < 0.05$).

In the presence of phentolamine and propranolol, bombesin caused a tachycardia (significant at 1–10 min, $P < 0.05$) which was less (significant at 1–2 min, $P < 0.05$), and a pressor response (significant at 1–10 min, $P < 0.05$) which was greater (significant at 1 min, $P < 0.05$), than in rats receiving bombesin alone. The renal vasoconstriction (significant at 0.5 min, $P < 0.05$) was similar to that in untreated animals, but the mesenteric vasodilatation (significant at 0.5–30 min, $P < 0.05$) was larger (significant at 20 min, $P < 0.05$). The transient hindquarters vasodilatation seen in untreated rats was absent, but a prolonged hindquarters vasoconstriction (significant at 1–20 min, $P < 0.05$) occurred.

Haemodynamic responses to bombesin (2.5 $\mu g\,kg^{-1}$) in the presence of phentolamine, propranolol and atropine (Figure 4) Thirty min after the onset of atropine administration, in the presence of phentolamine and propranolol, blood pressure was decreased ($P < 0.05$), but heart rate was not different from baseline. There was a decrease in renal and an increase in hindquarters flow, together with mesenteric and hindquarters vasodilatations (all significant, $P < 0.05$).

In the presence of phentolamine, propranolol and atropine, bombesin caused a sustained tachycardia (significant at 2–30 min, $P < 0.05$), but the maximum tachycardic response was smaller (significant at 1–2 min, $P < 0.05$) than in untreated rats. However, subsequently the tachycardia was greater (significant at 20 min, $P < 0.05$) than in untreated rats. The pressor response (significant at 1–10 min, $P < 0.05$) was slightly greater (significant at 1 min, $P < 0.05$), the renal vasoconstriction (significant at 0.5 min, $P < 0.05$) smaller, and the mesenteric vasodilatation (significant at 1–2 and 10 min, $P < 0.05$) not different, from those in untreated rats. The hindquarters showed a vasodilatation (significant at 0.5 min, $P < 0.05$) followed by a vasoconstriction (significant at 2 and 10 min, $P < 0.05$). The change in hindquarters vascular conductance was less (significant at 1 min, $P < 0.05$) than in untreated rats.

Cardiovascular effects of cholecystokinin

Haemodynamic responses to CCK (0.5 $\mu g\,kg^{-1}$) in the absence or the presence of L364,718 (Figure 5) After administration of CCK there was a tachycardia (significant at 1–10 min, $P < 0.05$) and a short-lived rise in blood pressure (significant at 0.25–0.5 min, $P < 0.05$). Renal (significant at 0.25–1 min, $P < 0.05$) and hindquarters (significant at 1–2 min, $P < 0.05$) flows were increased slightly, whereas mesenteric flow initially showed a decrease (significant at 0.25–1 min, $P < 0.05$) followed by a small increase (significant at 5–10 min, $P < 0.05$). Vasoconstrictions occurred in the renal (significant at 0.5 min, $P < 0.05$), mesenteric (significant at 0.25–1 min, $P < 0.05$) and hindquarters (significant at 0.5 min, $P < 0.05$) vascular beds. Subsequently there were mesenteric (significant at 5 min, $P < 0.05$) and hindquarters (significant at 1–2 min, $P < 0.05$) vasodilatations.

Pretreatment with the CCK receptor antagonist did not affect baseline values, but in its presence CCK (0.5 $\mu g\,kg^{-1}$) had no significant effects ($n = 8$, data not shown).

Haemodynamic responses to CCK (5.0 $\mu g\,kg^{-1}$) alone or in the presence of vehicle or L364,718 (Figures 6 and 7) Figure 6 shows the effects of CCK in a single rat, and Figure 7 shows the group mean results.

CCK caused a bradycardia (significant at 0.5–1 min, $P < 0.05$) followed by a tachycardia (significant at 5–10 min, $P < 0.05$). The bradycardia was accompanied by a marked increase in blood pressure (significant at 0.25–1 min, $P < 0.05$). Renal flow was increased slightly (significant at 1–2 min, $P < 0.05$; maximum $+6 \pm 2\%$) while initial decreases in mesenteric (significant at 0.25–1 min, $P < 0.05$) and hindquarters (significant at 0.25–0.5 min, $P < 0.05$) flows were followed by increases (mesenteric significant at 5–10 min, $P < 0.05$; hindquarters significant at 1–2, 10 min, $P < 0.05$). There were vasoconstrictions in renal (significant at 0.25–0.5 min, $P < 0.05$), mesenteric (significant at 0.25–2 min, $P < 0.05$) and hindquarters (significant at 0.25–0.5 min, $P < 0.05$) vascular beds, followed by vasodilatations (renal (significant at 2–5 min, $P < 0.05$); mesenteric (significant at 5–10 min, $P < 0.05$); hindquarters (significant at 2 min, $P < 0.05$)).

Pretreatment with vehicle did not cause any changes in baseline values or in the responses to CCK ($n = 8$, data not shown).

In rats pretreated with L364,718, CCK caused an initial tachycardia (significant at 1 min, $P < 0.05$), i.e. the opposite of that which was seen in untreated rats (significant at 0.25–

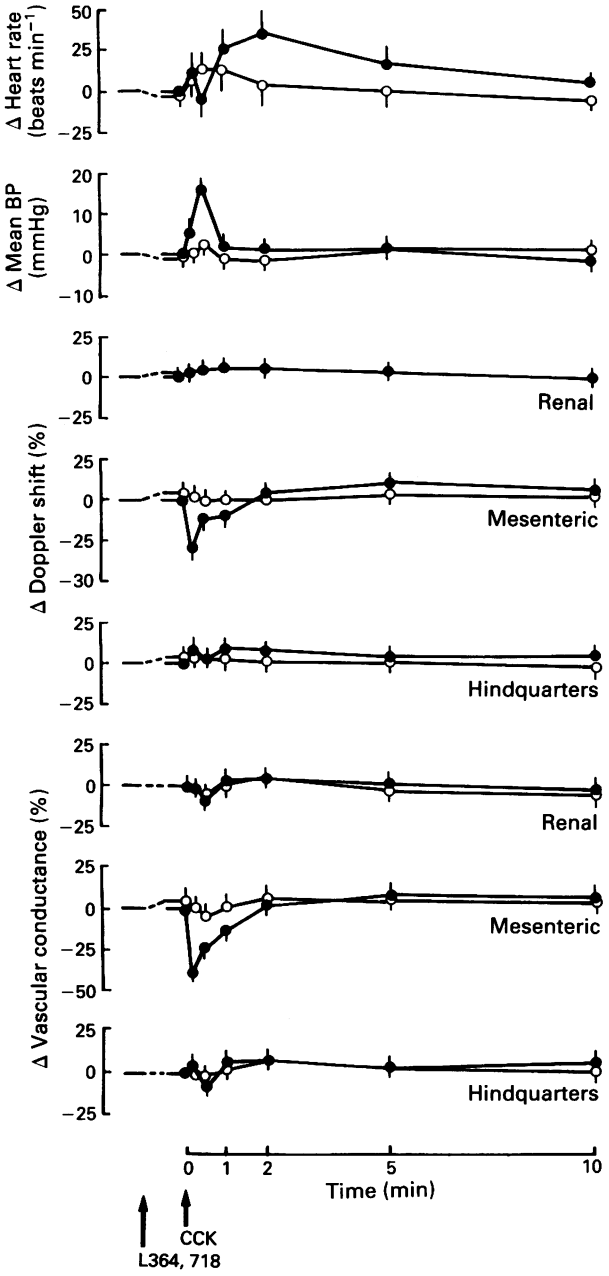

Figure 5 Haemodynamic responses to cholecystokinin (CCK, 0.5 $\mu g\,kg^{-1}$, bolus, i.v.) in conscious, unrestrained, Long Evans rats, in the absence, (●), or in the presence of L364,718, (○). The dotted line shows the response to L364,718. Values are mean ($n = 8$) with s.e.mean shown by vertical lines. All statistics are given in the text.

1 min, $P < 0.05$). The pressor response (significant at 0.5 min, $P < 0.05$) was smaller (significant at 0.25–1 min, $P < 0.05$) than in untreated rats. Changes in renal and hindquarters vascular conductances were not significant, while the mesenteric vasoconstriction (significant at 0.5 min, $P < 0.05$) was smaller (significant at 0.25–1 min, $P < 0.05$) than in untreated rats, and the subsequent mesenteric vasodilatation, seen in untreated rats, did not occur.

Haemodynamic responses to CCK (5.0 $\mu g\,kg^{-1}$) in the presence of a vasopressin V_1-receptor antagonist Pretreatment with the vasopressin V_1-receptor antagonist did not change baseline values or the haemodynamic responses to CCK ($n = 8$, data not shown).

Haemodynamic responses to CCK (5.0 $\mu g\,kg^{-1}$) in the presence of phentolamine (Figure 8) Seventy-five min after the onset of phentolamine administration there was a marked tachycardia

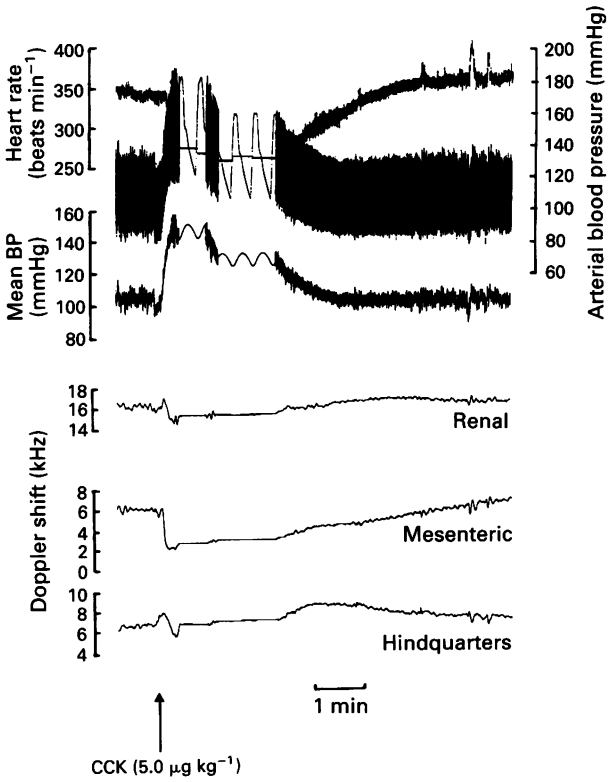

Figure 6 Haemodynamic responses to cholecystokinin (CCK, 5.0 $\mu g\,kg^{-1}$, bolus, i.v.) in a conscious, unrestrained, Long Evans rat.

and reductions in blood pressure and in renal and mesenteric flows. There were renal and mesenteric vasoconstrictions, and a hindquarters vasodilatation (all significant, $P < 0.05$).

In the presence of phentolamine, CCK caused a bradycardia (significant at 0.5 min, $P < 0.05$) and an increase in blood pressure (significant at 0.5 min, $P < 0.05$) that were less (significant at 0.5 min, $P < 0.05$) than in untreated rats. Furthermore, there was a subsequent hypotension (significant at 1–2 min, $P < 0.05$), which did not occur in untreated rats. The renal vasoconstriction observed in untreated rats was absent, but a vasodilatation (significant at 1–2 min, $P < 0.05$) occurred. Changes in mesenteric vascular conductance were absent, and there was no hindquarters vasoconstriction, but the hindquarters vasodilatation (significant at 1–2 min, $P < 0.05$) was greater (significant at 1–2 min, $P < 0.05$) than in untreated rats.

Haemodynamic responses to CCK (5.0 $\mu g\,kg^{-1}$) in the presence of phentolamine and propranolol (Figure 9) Seventy-five min after the onset of phentolamine and propranolol administration there was bradycardia, hypotension, and decreases in renal and mesenteric flows, together with hindquarters hyperaemia and vasodilatation, (all significant, $P < 0.05$).

In the presence of phentolamine and propranolol, CCK caused a rise in mean blood pressure (significant at 0.5–1 min, $P < 0.05$) and a bradycardia (significant at 0.5–1 min, $P < 0.05$) followed by a tachycardia (significant at 5–10 min, $P < 0.05$); the bradycardia and the pressor effect were less (significant at 0.5–1 min and 0.25–1 min, respectively $P < 0.05$) than in untreated animals. All vascular beds showed vasoconstrictions but the mesenteric (significant at 0.25–0.5 min, $P < 0.05$) and the hindquarters (significant at 0.5 min, $P < 0.05$) vasoconstrictions were less than in untreated rats. The late hindquarters vasodilatation seen in untreated rats was absent.

Haemodynamic responses to CCK (5.0 $\mu g\,kg^{-1}$) in the presence of phentolamine, propranolol and atropine (Figure 10) Seventy-five min after the onset of atropine adminis-

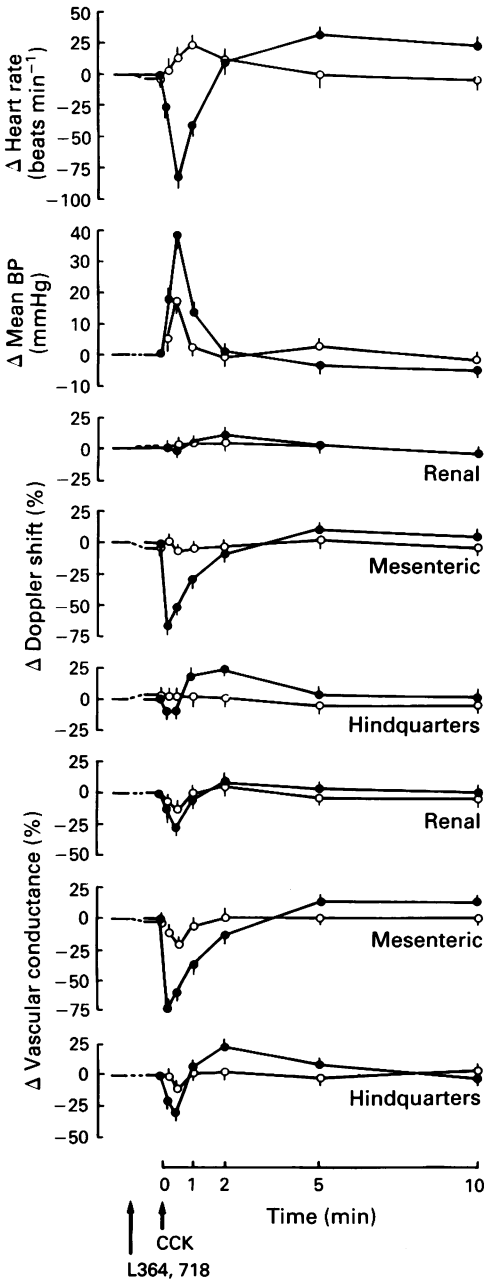

Figure 7 Haemodynamic responses to cholecystokinin (CCK, 5.0 $\mu g\,kg^{-1}$, bolus, i.v.) in conscious, unrestrained, Long Evans rats, in the absence, (●), or in the presence of L364,718, (○). The dotted line represents the response to L364,718 before CCK was given. Values are mean ($n = 8$) with s.e.mean shown by vertical lines. All statistics are given in the text.

tration in the presence of phentolamine and propranolol, heart rate and blood pressure were not different from baseline, but there were rises in mesenteric and hindquarters flows, together with vasodilatations (all significant, $P < 0.05$).

In the presence of phentolamine, propranolol and atropine, CCK caused a bradycardia (significant at 0.5–1 min, $P < 0.05$) and a rise in blood pressure (significant at 0.5 min, $P < 0.05$) that were smaller (significant at 0.5–1 min and 0.5 min, respectively $P < 0.05$) than in untreated rats. Renal (significant at 0.5 min, $P < 0.05$) and mesenteric (significant at 0.5–2 min, $P < 0.05$) vasoconstrictions occurred, but the latter was smaller (significant at 0.25–0.5 min, $P < 0.05$) than in untreated rats. The hindquarters vascular bed showed no initial vasoconstriction or subsequent vasodilatation, unlike untreated rats (significantly different at 0.5 and 2 min, $P < 0.05$).

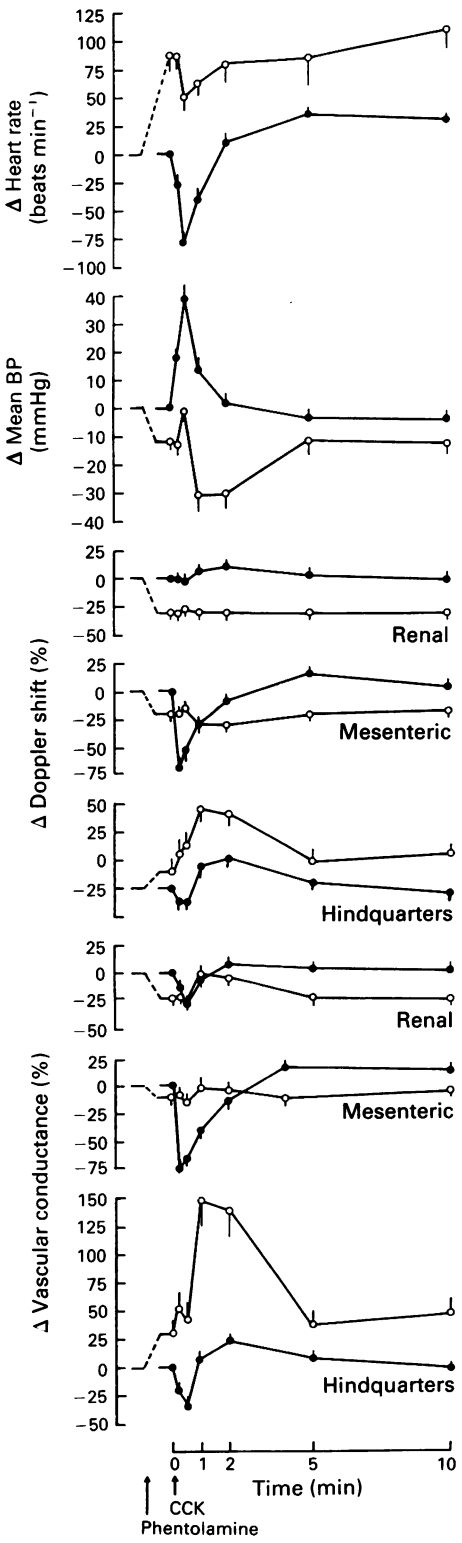

Figure 8 Haemodynamic responses to cholecystokinin (CCK, 5.0 $\mu g\,kg^{-1}$, bolus, i.v.) in conscious, unrestrained, Long Evans rats, in the absence, (●), or in the presence of phentolamine, (○). The dotted line represents the response to phentolamine. Values are mean ($n = 8$) with s.e.mean shown by vertical lines. All statistics are given in the text.

Discussion

The present work has shown that i.v. administration of the neuropeptides, bombesin and CCK, can have substantial haemodynamic effects in conscious Long Evans rats. However, such experiments do not necessarily provide information about putative physiological effects of bombesin and CCK.

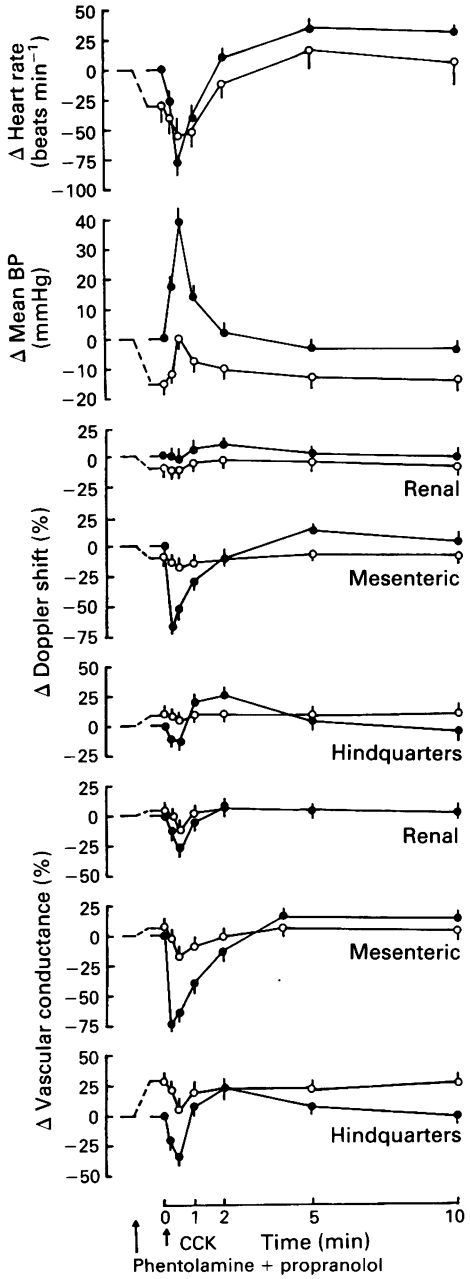

Figure 9 Haemodynamic responses to cholecystokinin (CCK, 5.0 $\mu g\,kg^{-1}$, bolus, i.v.) in conscious, unrestrained, Long Evans rats, in the absence, (●), or in the presence of phentolamine and propranolol, (○). The dotted line represents the response to phentolamine and propranolol. Values are mean ($n = 8$) with s.e.mean shown by vertical lines. All statistics are given in the text.

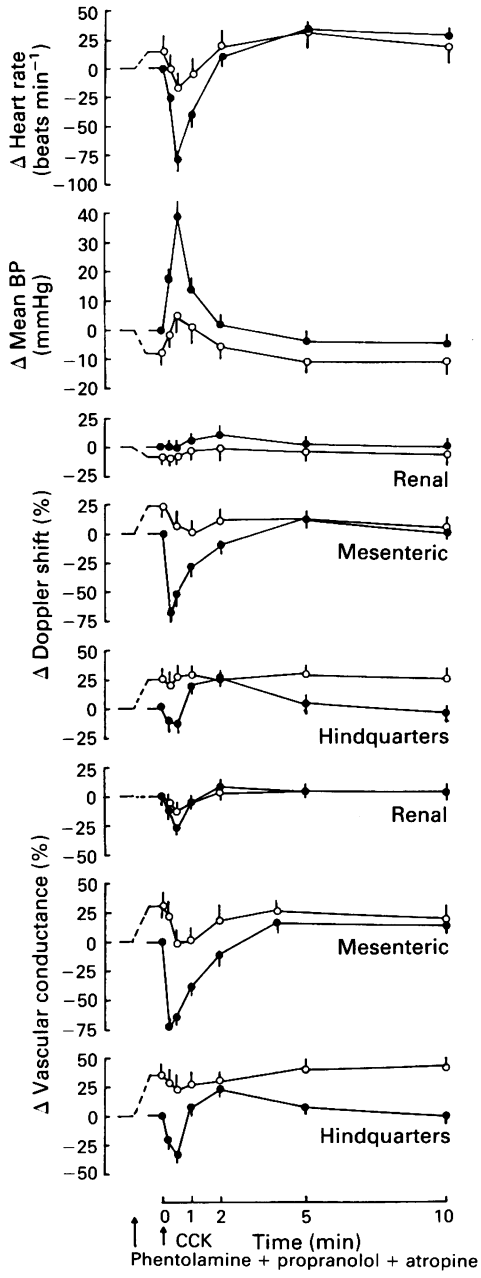

Figure 10 Haemodynamic responses to cholecystokinin (CCK, 5.0 $\mu g\,kg^{-1}$, bolus, i.v.) in conscious, unrestrained, Long Evans rats, in the absence, (●), or in the presence of phentolamine, propranolol and atropine, (○). The dotted line represents the response to phentolamine, propranolol and atropine. Values are mean ($n = 8$) with s.e.mean shown by vertical lines. All statistics are given in the text.

Indeed, there is no evidence that CCK or the mammalian equivalent of bombesin are circulating cardiovascular hormones. Furthermore, it is not possible to assess the extent to which any paracrine effects of such endogenous peptides would be simulated by i.v. administration of the exogenous peptides. Thus, the major aim of our study was to characterize the regional haemodynamic profiles of the peptides given i.v. and then, with the use of pharmacological interventions, to attempt to delineate the mechanisms contributing to the effects seen.

Haemodynamic effects of bombesin

In this study, in conscious, Long Evans rats, bombesin (2.5 $\mu g\,kg^{-1}$, i.v.) caused a tachycardia, an increase in blood pressure, a short-lasting renal vasoconstriction and a sustained mesenteric vasodilatation; there was an initial hindquarters vasodilatation followed by a vasoconstriction (Table 3). As noted above (see Methods and Table 1), bombesin did not cause dose-dependent effects possibly because, in the conscious intact rats investigated, baroreflex mechanisms were acting to oppose its actions (see below). However, Erspamer *et al.* (1972) also noted a variable dose-dependency in the pressor effects of bombesin in anaesthetized rats.

Phentolamine attenuated the tachycardic response to bombesin, and converted its pressor action into a depressor effect (Table 3). The latter would have been expected to cause a greater baroreflex-mediated tachycardia, and hence the reduced tachycardia observed may have been due to the fact that in the presence of phentolamine heart rate was increased already. It is possible also that cardiac α-adrenoceptors (Bennett & Kemp, 1978; Flavahan & McGrath, 1982; Tung *et al.*, 1982; 1985) were involved in the tachycardic responses to bombesin.

Table 3 Patterns of cardiovascular changes elicited by bombesin (2.5 $\mu g\,kg^{-1}$) under the conditions of the experiments described in the Results

Condition	*Bombesin alone*	*Phentolamine + bombesin*	*Phentolamine + propranolol + bombesin*	*Phentolamine + propranolol + atropine + bombesin*
Heart rate	↑+++	↑++	↑+	↑++
Mean blood pressure	↑++	↓+++	↑++	↑++
Renal flow	↓+++	↓++	↓+++	↓+++
Mesenteric flow	↑+++	↑+	↑+++	↑+++
Hindquarters flow	↑++	↑++	↓++	↑+
Renal conductance	↓+++	↓+	↓+++	↓++
Mesenteric conductance	↑++	↑++	↑+++	↑++
Hindquarters conductance	↑++↓+	↑+++	↓++	↑+↓+

The directions of the arrows for each variable represent the directions of the changes; biphasic changes are represented by a sequence of arrows in different directions. The relative magnitudes of the changes are indicated by + signs.

The increase in heart rate following bombesin was further attenuated by propranolol in the presence of phentolamine (Figure 3, Table 3), consistent with the findings of Bayorh & Feuerstein (1985). However, the duration of the tachycardia was prolonged by atropine in the presence of phentolamine and propranolol, possibly due to suppression of reflex vagal influences. Bearing in mind the changes in the blood pressure profiles in these various conditions (see below), it is likely that the heart changes elicited by bombesin were influenced by circulating catecholamines (Fisher *et al.*, 1985) and by cardiac baroreflexes exerting actions through adrenoceptors and muscarinic receptors (to different extents in different experiments). Following antagonism of these receptors (accepting competitive antagonists were used) there remained a sizeable (about half that seen in the unblocked conditions) and prolonged tachycardic response to bombesin (Table 3). It is feasible that this was due to a direct cardiac action of the peptide and/or an indirect effect mediated through non-adrenergic, non-cholinergic (NANC) mechanisms, possibly involving histamine (Bayorh & Feuerstein, 1985).

The pressor response to bombesin in the presence of phentolamine and propranolol was similar to that seen in the unblocked state (Table 3) (albeit against a different profile of haemodynamic changes). Thus, it appears likely that the marked hypotensive response to bombesin in the presence of phentolamine alone (Table 3) was due to an enhancement of β-adrenoceptor-mediated vasodilator responses (as a consequence of antagonism of prejunctional, α-adrenoceptor autoinhibitory effects, in the presence of suppression of post-junctional α-adrenoceptor vasoconstrictor influences), rather than to unmasking of the normal degree to which vasodilator β-adrenoceptor-mediated mechanisms were involved in the unblocked state. This is consistent with the substantial augmentation of the hindquarters vasodilator response to bombesin seen in the presence of phentolamine (Table 3). However, it is clear also that the hindquarters response to bombesin alone involved an early vasodilator component that was susceptible to blockade by propranolol (Table 3). It is feasible this was due to neurally released and/or circulating catecholamines (Fisher *et al.*, 1985) acting on post-junctional β_2-adrenoceptors, since there is evidence that in the hindquarters vascular bed this dilator mechanism is particularly well-developed (Gardiner & Bennett, 1988).

As mentioned above, in the presence of phentolamine and propranolol the pressor effect of bombesin was little different from normal. However, this was against a background of diminished tachycardia and an abolition of the hindquarters vasodilatation, together with persistence of the vasoconstrictor response to bombesin in this vascular bed (Table 3). Thus, it appears bombesin could have had NANC vasoconstrictor effects in the hindquarters vascular bed, although, since flow did not fall below baseline (in the presence of phentolamine, propranolol and atropine) it is feasible the vasoconstriction was autoregulatory. However, skeletal muscle does not usually show well-developed autoregulation (Heistad & Abboud, 1974).

In the renal vascular bed phentolamine alone caused some attenuation of the vasoconstriction following administration of bombesin, but the degree to which this reflected the involvement of α-adrenoceptor mechanisms in the change in renal vascular conductance following bombesin alone is difficult to assess since the marked fall in blood pressure following bombesin administration in the presence of phentolamine (Table 3) would have enhanced baroreflex-mediated sympathetic efferent outflow to the kidney. However, in the presence of phentolamine, propranolol and atropine there was only slight attenuation of the bombesin-induced renal vasoconstriction (even though blood pressure did not fall; Table 3), so NANC mechanisms must have featured large in this response. While it is feasible the renin-angiotensin system was involved (Melchiorri *et al.*, 1971), the time course of change of renal flow and conductance following bombesin administration makes it more likely these changes were due to a direct renal action of bombesin (Melchiorri *et al.*, 1971; Erspamer & Melchiorri, 1973) and/or to the involvement of neural mechanisms influencing the vasculature of the kidney through NANC pathways. The marked actions of bombesin on renal blood flow could contribute to its reported antidiuretic effect (Melchiorri *et al.*, 1971; Erspamer & Melchiorri, 1973).

Under all conditions the relatively slow-onset, persistent, mesenteric vasodilator response to bombesin was unaffected (Table 3) (although the increase in flow was reduced when bombesin caused a fall in blood pressure in the presence of phentolamine). These observations are consistent with bombesin exerting direct, and/or indirect, NANC effects to increase mesenteric vascular conductance. It is feasible that such an influence was mediated through release of other gut hormones (Walsh, 1989) and a possible candidate is corticotropin-releasing hormone (CRH), since this peptide exerts profound superior mesenteric vasodilator effects (Gardiner *et al.*, 1988b). However, CRH is not likely to have been responsible for the putative NANC effects of bombesin in the renal (or hindquarters) vascular beds, since exogenous CRH has little effect in these regions (Gardiner *et al.*, 1988b).

Overall, the present results are consistent with bombesin influencing cardiac activity through sympatho-adrenal mechanisms and also exerting NANC effects on the heart and renal (constriction), mesenteric (dilatation) and , possibly, hindquarters (constriction) vascular beds. In the hindquarters vascular bed early vasodilatation was probably due to circulating adrenaline (Fisher *et al.*, 1985) acting on β_2-adrenoceptors.

The work of Guarini and colleagues (1989) indicated that, following haemorrhage, the pressor effects of bombesin were due to CCK release since the CCK antagonist, L364,718,

Table 4 Patterns of cardiovascular changes elicited by cholecystokinin (CCK, 5.0 $\mu g kg^{-1}$) under the conditions of the experiments described in the Results

Condition	*CCK alone*	*Phentolamine + CCK*	*Phentolamine + propranolol + CCK*	*Phentolamine + propranolol + atropine + CCK*
Heart rate	↓+++ ↑++	↓+	↓++ ↑++	↓+
Mean blood pressure	↑+++	↑+ ↓++	↑++	↑++
Renal flow	↑+	→	→	→
Mesenteric flow	↓+++ ↑+	→	→	↓+
Hindquarters flow	↓+ ↑++	↑+++	→	→
Renal conductance	↓++ ↑+	↑++	↓+	↓+
Mesenteric conductance	↓+++ ↑++	→	↓+	↓++
Hindquarters conductance	↓++ ↑++	↑+++	↓+	↓+

The directions of the arrows for each variable represent the directions of the changes; biphasic changes are represented by a sequence of arrows in different directions. The relative magnitudes of the changes are indicated by + signs. Where an arrow is shown horizontal there was no significant change in that variable under that condition.

abolished the influence of bombesin on blood pressure. In the present work, in conscious normotensive rats, L364,718, in the same dose as used by Guarini *et al.* (1989), had no effect on the cardiovascular actions of bombesin. Hence it is unlikely that endogenous CCK was responsible for the NANC effects of bombesin in the present experiments. However, these results do not preclude the possibility that bombesin releases CCK (Ghatei *et al.*, 1982; Guarini *et al.*, 1989; Walsh, 1989) in amounts insufficient to influence haemodynamic status in conscious, Long Evans rats.

Haemodynamic effects of CCK

CCK caused dose-dependent pressor effects associated with renal, mesenteric and hindquarters vasoconstrictions, followed (after the higher dose) by vasodilatations. After the low dose, heart rate increased, but after the high dose there was a bradycardia followed by a tachycardia (Table 4).

The bradycardic effect of the high dose of CCK was attenuated in the presence of phentolamine, as was the pressor action of the peptide (Table 4) indicating the bradycardia may have been dependent, in part, on baroreflex mechanisms as observed in the pentobarbitone-anaesthetized dog (Koyama *et al.*, 1990). However, the occurrence of a bradycardia in the presence of phentolamine, propranolol and atropine (Table 4) suggests that, in the rat, CCK has direct, and/or indirect, NANC, negative chronotropic effects, as reported by Marker & Roberts (1988). The late tachycardic effect of the higher dose of CCK occurred independently of changes in blood pressure, and it was blocked by atropine (Table 4), thus it is feasible that the increase in heart rate was due to a non-baroreflex-mediated inhibition of vagal tone. Since the low dose of CCK had only a tachycardic effect and since only tachycardia was seen following the higher dose of CCK in the presence of L364,718, it seems that a direct and/or indirect action of CCK to inhibit cardiac vagal tone was exerted at a lower dose than its NANC negative chronotropic effects. Central administration of CCK causes tachycardia in chloralose-anaesthetized cats (Pagani *et al.*, 1982), so may be the atropine-sensitive tachycardia seen here was mediated centrally, although it is equally feasible it was due to an action of CCK on vagal afferent function (Zarbin *et al.*, 1981).

The marked pressor effect of CCK was substantially reduced in the presence of phentolamine and there was a pronounced secondary depressor response that was absent in the additional presence of propranolol (Table 4). As with bombesin, the likely explanation of these findings is that β-adrenoceptor-mediated vasodilator effects (see below) were augmented in the presence of phentolamine and the cardiovascular effects of CCK under these conditions probably were not a true representation of the contribution of β-adrenoceptor-mediated mechanisms to the responses to CCK alone. However, the attenuation of the pressor effects of CCK in the presence of phentolamine and propranolol indicates that a large part of the increase in blood pressure following CCK administration alone was probably due to increased sympathetic efferent activity (Koyama *et al.*, 1990) causing α-adrenoceptor-mediated vasoconstriction, in contrast to the mechanisms involved in the pressor response to bombesin (see above).

In response to CCK, the hindquarters showed a vasoconstriction followed by a vasodilatation, associated with a reduction and an increase in flow, respectively (Table 4). In the presence of phentolamine, CCK caused a marked hyperaemic vasodilatation in the hindquarters that was absent in the additional presence of propranolol (Table 4). It is likely the hindquarters vasodilator response to CCK was responsible for the fall in blood pressure seen in the presence of phentolamine. The hindquarters vasoconstriction that occurred in response to CCK in the presence of phentolamine and propranolol was not associated with a reduction in blood flow below resting levels (Table 4) and hence it could have been an autoregulatory change. Thus it appears CCK did not exert NANC effects in the hindquarters vascular bed. Collectively, these findings indicate CCK caused hindquarters vasoconstriction through activation of postjunctional α-adrenoceptor-mediated mechanisms and, following blockade of these and prejunctional, autoinhibitory α-adrenoceptors, β-adrenoceptor-mediated hindquarters vasodilatation was augmented. The different patterns of hindquarters response to CCK (vasoconstriction followed by vasodilatation) and bombesin (vasodilatation followed by vasoconstriction) are consistent with the possibility that the effects of CCK on the hindquarters were mediated largely through activation of sympathetic efferent outflow to that vascular bed whereas those of bombesin were due in greater part to adrenal medullary adrenaline release (Fisher *et al.*, 1985).

Under all conditions, there were only slight changes in renal blood flow following administration of CCK (Table 4) and, therefore, all the changes in vascular conductance seen could have been autoregulatory. This picture contrasts sharply with that observed following bombesin administration and indicates that CCK did not exert any specific direct and/or indirect effects on the renal vasculature as assessed here. However, it is feasible that CCK influenced intrarenal blood flow, for example, without affecting total renal blood flow.

In the presence of phentolamine, CCK caused no significant reductions in mesenteric blood flow or vascular conductance (Table 4). Therefore, the mesenteric vasoconstriction following CCK administration in the absence of phentolamine (Table 4) was probably mediated through α-adrenoceptors. However,

there was a slight enhancement of the mesenteric vasoconstrictor effect of CCK in the presence of phentolamine and propranolol, compared to that in the presence of phentolamine alone. This might indicate that any non-adrenergic vasoconstrictor effects of CCK in the latter condition were masked by β-adrenoceptor-mediated vasodilatation. In the presence of phentolamine, propranolol and atropine, CCK caused mesenteric vasoconstriction (Table 4) that was likely to have been responsible for the increase in blood pressure, since there was a bradycardia and the changes in renal and hindquarters vascular conductances were probably autoregulatory (see above). The possibility that this NANC mesenteric vasoconstrictor effect of CCK was due to release of vasopressin seems unlikely since pretreatment with a V_1-receptor antagonist did not significantly affect the haemodynamic responses to CCK. However, it is feasible under those conditions other mechanisms adjusted for the absence of vasopressin. In this context it is of interest that there was a tendency towards a reduction in the peak mesenteric vasoconstriction following CCK administration in the presence of the V_1-receptor antagonist (data not shown). A definite answer should come from a comparison of the cardiovascular responses to CCK in the presence of phentolamine, propranolol and atropine with those in the additional presence of a V_1-receptor antagonist, or with those in Brattleboro rats.

The CCK receptor antagonist, L364,718, abolished the cardiovascular effects of the low dose of CCK and reduced substantially the effects of the higher dose. In fact, there were minimal changes in renal, mesenteric or hindquarters blood flows in response to the higher dose of CCK in the presence of L364,718 and, hence, all changes in vascular conductances could have been autoregulatory. Under these conditions the pressor effect of CCK was most likely due to an increase in cardiac output consequent upon inhibition of cardiac vagal tone (see above). The effectiveness of the dose of L364,718 used against the cardiovascular actions of CCK indicates a major involvement of peripheral A-type receptors (Dourish *et al.*, 1989), although it is feasible the atropine-sensitive, tachycardic effects of CCK were mediated through central B-type receptors (Lotti & Chang, 1989). The substantial effects of L364,718 on the cardiovascular actions of exogenous CCK, and the failure of L364,718 to modify the actions of exogenous bombesin are not consistent with the proposal that release of endogenous CCK is responsible for the cardiovascular actions of exogenous bombesin (Guarini *et al.*, 1989). In addition, the markedly different haemodynamic changes following administration of exogenous bombesin and CCK do not support that proposal.

Finally, during the course of the present experiments several important observations were made regarding the influence of phentolamine, propranolol and atropine on cardiovascular status in conscious Long Evans rats. Administration of phentolamine caused a marked tachycardia, but only a slight fall in blood pressure, accompanied by renal and mesenteric vasoconstrictions and hindquarters vasodilatation. In the additional presence of propranolol the marked tachycardia gave way to a bradycardia, in spite of a further reduction in blood pressure; renal and mesenteric vasoconstrictions were abolished while the hindquarters vasodilatation was unaffected. These results are consistent with antagonism of prejunctional autoinhibitory α-adrenoceptors causing augmentation of sympathetic efferent effects on cardiac β-adrenoceptors. The apparent lack of such a component in the hindquarters vasodilator response to phentolamine is consistent with the hindquarters β_2-adrenoceptor-mediated vasodilator mechanisms (discussed above) being more dependent on adrenal medullary activation than on sympathetic efferent input to that vascular bed. The selective hindquarters vasodilatation following phentolamine indicates the existence of α-adrenoceptor-mediated tone in that vascular bed, but it is likely that any renal and mesenteric vasodilatations due to inhibition of α-adrenoceptor-mediated tone were masked by concurrent vasoconstrictions consequent upon activation of the renin-angiotensin system (i.e. effects antagonized by propranolol). This proposition is consistent with the potent renal and mesenteric vasoconstrictor effects of angiotensin II and its relative lack of effect on the hindquarters vascular bed (Gardiner *et al.*, 1988a).

The resting bradycardia seen in the presence of phentolamine and propranolol was abolished by atropine in spite of the fact that blood pressure was below baseline. Hence it appears that some cardiac vagal tone existed in conscious, Long Evans rats even in the presence of a relative hypotension.

Administration of atropine in the presence of phentolamine and propranolol caused mesenteric vasodilatation (compare Figures 9 and 10). It is possible this was due to activation of an endothelium-dependent mechanism (Thomas *et al.*, 1988), and in this context it is notable that inhibition of nitric oxide (the major endothelium-dependent relaxing factor: Moncada *et al.*, 1988) has particularly potent effects on the mesenteric vascular bed (Gardiner *et al.*, 1990b). An alternative, or additional, explanation for the mesenteric vasodilator effects of atropine is that the latter inhibited vasopressin release (Iitake *et al.*, 1986; Bisset & Chowdrey, 1988; Shoji *et al.*, 1989). Although atropine methyl nitrate does not cross the blood-brain-barrier readily, it is feasible that an inhibitory effect on vasopressin release could have been exerted centrally at a site outside the blood-brain-barrier (Gregg, 1985). Hence, it is likely that in the presence of phentolamine, propranolol and atropine the relative maintenance of blood pressure was dependent largely on NANC activation of the renin-angiotensin system together with vasopressin release (Winn *et al.*, 1985).

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Modes of hexamethonium action on acetylcholine receptor channels in frog skeletal muscle

*[1]D.J. Adams, †[2] S. Bevan & **[3]D.A. Terrar

Departments of *Pharmacology, and †Zoology, University College London, London WC1E 6BT, and of **Pharmacology and Therapeutics, The Middlesex Hospital Medical School, London W1

1 The antagonism between hexamethonium and cholinoceptor agonists was investigated in frog skeletal muscle fibres with voltage-clamp techniques. Hexamethonium caused a voltage-dependent reduction in the amplitude of endplate currents. For neurally evoked endplate currents, the reduction increased e-fold with a 38 mV membrane hyperpolarization.

2 The effect of hexamethonium on the time course of endplate currents was small, and was most apparent as a slight prolongation of the decay phase at hyperpolarized potentials (more negative than −100 mV). A similar small prolongation of single channel lifetime was detected with fluctuation analysis techniques. Hexamethonium produced a voltage-dependent reduction in apparent single channel conductance as the membrane was hyperpolarized.

3 Log (concentration-response) curves for acetylcholine (ACh)-induced currents, determined either from currents accompanying ramp changes in membrane potential or from steady state currents in voltage-jump experiments, were less steep for responses in the presence of hexamethonium. This reduction in slope became more pronounced at more negative membrane potentials. Observations at +50 mV suggested that the equilibrium constant for competitive antagonism was approximately 200 μM.

4 In voltage-jump experiments with a two-microelectrode voltage clamp, the current evoked by ACh in the presence of hexamethonium differed from that recorded with ACh alone. In the presence of hexamethonium, the expected 'instantaneous' ohmic increase in membrane current in response to a hyperpolarizing step was not detected; instead a decrease in current was observed. This problem was further investigated with a vaseline-gap voltage-clamp technique which provides improved temporal resolution. With this method a rapid decrease in the ACh-induced inward current was observed with step hyperpolarizations in the presence of hexamethonium.

5 When the membrane potential was stepped back to its resting level from a more hyperpolarized potential in the presence of hexamethonium, there was a surge of ACh-induced inward current that decayed with a time constant of less than 100 μs.

6 The slow relaxation in the ACh-induced current that followed a voltage step recorded in the presence of hexamethonium was slower than that recorded with ACh alone. In the presence of hexamethonium the time constant of this relaxation increased e-fold for a 67 mV hyperpolarization.

7 The results are consistent with a rapid voltage-dependent block of ACh-activated channels by hexamethonium with hyperpolarization, and voltage-dependent unblock with depolarization. The voltage-dependent block is combined with competitive antagonism at the ACh receptors. However, not all observations appear to be compatible with a simple sequential block of open ion channels, but rather suggest that occupation of the channel by hexamethonium may not prevent channel closure.

Introduction

Hexamethonium has been known since the experiments of Paton & Zaimis (1949) to block transmission both at autonomic ganglia and at the neuromuscular junction. The principle factor in the blocking action at ganglia has been shown to be a block of ion channels associated with acetylcholine (ACh) receptors rather than a competitive antagonism at the receptors (Blackman, 1959; Ascher *et al.*, 1979; Rang, 1982; Skok *et al.*, 1983; Gurney & Rang, 1984). At the neuromuscular junction, where hexamethonium is less potent, the action of the drug is less well understood. From investigations of the interactions between tubocurarine and hexamethonium at the rat neuromuscular junction Ferry & Marshall (1973), Blackman *et al.* (1975) and Rang & Rylett (1984) concluded that the two drugs interacted at the same sites and that at least a part of the action of hexamethonium was at ACh receptors. Milne & Byrne (1981) investigated the effects of hexamethonium on endplate currents in frog muscle in which neuromuscular transmission had been blocked with high concentrations of magnesium and concluded that hexamethonium blocked open ion channels. More recently, Brenner & Micheroli (1985) concluded from a study on normal and vagus-reinnervated frog endplates that hexamethonium acted both as a competitive antagonist and as a channel blocking agent, although direct evidence for channel block could not be demonstrated.

We have investigated the effects of hexamethonium on neurally evoked endplate currents and have examined the interaction between bath applied agonists and hexamethonium. The major effect of hexamethonium was to reduce the amplitude of endplate currents in a voltage-dependent manner. From observations on the relaxations of ACh-induced currents evoked by voltage steps, we conclude that hexamethonium blocks ion channels associated with ACh receptors. The observations cannot, however, be accounted for by a simple sequential block of open ion channels of the type previously advanced for local anaesthetic block of ACh-operated channels (Ruff, 1977; Neher & Steinbach, 1978). One possibility consistent with the results is that hexamethonium can remain in the channel without substantial inhibition of the normal process of channel closure, in the way suggested for hexamethonium at ganglion cells (Gurney & Rang, 1984).

[1] Present address: Department of Pharmacology, University of Miami School of Medicine, Miami, FL 33136, U.S.A.
[2] Author for correspondence at present address: Sandoz Institute for Medical Research, 5 Gower Place, London WC1E 6BN.
[3] Present address: University Department of Pharmacology, South Parks Road, Oxford OX1 3QT.

A preliminary account of some of these results has been presented to the British Pharmacological Society (Adams et al., 1983).

Methods

Intracellular microelectrode experiments

The initial series of experiments was made on endplates of the cutaneous pectoris muscle of *Rana temporaria*. Endplate regions were located using Nomarski optics; the location was confirmed by focal recording of miniature endplate potentials (m.e.p.ps) with an intracellular electrode filled with 3M KCl. The endplate membrane was voltage clamped with a second (current passing) microelectrode filled with 2M K acetate inserted adjacent to the voltage recording electrode. Current was measured either as the voltage drop across a 1 MΩ resistor in series with the current-passing electrode or with a virtual earth current to voltage converter.

Muscles were bathed in a Ringer solution of the following composition (mM): NaCl 115, KCl 2, $CaCl_2$ 1.8, buffered to pH 7.2 with either Na-HEPES 5 or Na_2HPO_4 1.92 plus NaH_2PO_4 0.48. To minimize electrode displacement upon muscle contraction, muscles were stretched to 1.5 times their resting length (Terrar, 1978). Tetrodotoxin (100 nM) was added to the external solution except when neurally evoked e.p.ps were under investigation. In some experiments a high magnesium Ringer solution was used; this contained no added calcium and 10 mM $MgCl_2$. For dose-response experiments, muscles were superfused with Ringer solution containing either carbamylcholine chloride (CCh, Sigma) or acetylcholine chloride (ACh, Sigma). In these experiments acetylcholinesterase was inhibited by the addition of neostigmine methylsulphate 3 μM to the external solutions. Hexamethonium bromide (Sigma) was added to the Ringer solution, at the concentration stated. Experiments were usually carried out at room temperature (20–23°C), although in some experiments the temperature was lowered to 13°C.

Endplate currents were recorded on FM tape (Racal Store 4) at $7\frac{1}{2}$ ips (bandwidth d.c. to 2.5 kHz) and later photographed. In some experiments agonist induced-current during changes of membrane potential was determined by subtracting current in the absence of agonist from that in its presence. This was done both for experiments where the membrane potential was changed gradually by applying 'ramp' commands to the voltage-clamp, and in other experiments where the membrane potential was stepped from one potential to another ('voltage-jumps'). In some voltage-jump experiments a Neurolog NL750 averager was used to give the average current during a number of similar changes in membrane potential, and thus to increase the signal-to-noise ratio.

Vaseline-gap voltage clamp experiments

Acetylcholine-induced current fluctuation and current relaxation measurements were made on single muscle fibres dissected from innervated and denervated (14–21 days) semitendinosus muscles of *Rana temporaria*. Fibres were voltage-clamped by the vaseline-gap technique as previously described (Hille & Campbell, 1976; Adams et al., 1981). The ends of the muscle fibres were cut in an unbuffered 'internal' solution of 80 mM K EGTA (pH 7.2) and the A-pool (volume 0.1 ml), which contained the endplate membrane or extrajunctional region of denervated fibres, was continuously perfused at $>200\,\mu l\,s^{-1}$ with control (normal Ringer) or agonist-containing external solutions. The external Ringer solution used for these experiments contained (mM) NaCl 114, KCl 2.4, $CaCl_2$ 1.5, Na-HEPES buffer 10 pH 7.2. The effective rate of exchange of the external solution composition at the membrane face was approximately $1.5\,s^{-1}$ (Adams & Colquhoun, unpublished). The cholinomimetic agonists, acetylcholine chloride (Sigma) and suberyldicholine diodide, (kind gift of Dr B. Sakmann) were bath-applied to active ACh receptor-channels.

Analysis of both current fluctuations and relaxations was done by the methods described by Colquhoun et al. (1979) and Colquhoun & Sheridan (1981). For acetylcholine-induced current fluctuations, high gain a.c. current records were filtered through an eight-pole Butterworth bandpass filter (Barr & Stroud, EF3-02) set at 0.05 Hz and 1 kHz. Net power spectra, obtained from the difference of spectra recorded in the presence and absence of agonist, were calculated at 0.5 Hz resolution and fitted by a least-squares fitting routine on LSI 11/03 computer. The routine calculated estimates of the parameters of the spectra and the standard deviations of these estimated parameters.

A PDP 11/40 computer with a laboratory interface (Cambridge Electronic Design 502) was used to supply command potentials for the vaseline-gap voltage-jump experiments, each voltage-jump being followed by on-line sampling of endplate currents, filtered at between 2 and 80 kHz (−3 dB point, four-pole Bessel filter). Details of the sampling and fitting of current relaxations are described by Colquhoun & Sheridan (1982). The reversal (zero-current) potential for open ACh receptor-channels was obtained by direct measurement and occasionally used to constrain the fit of the relaxation(s) to intercept the estimated instantaneous (zero-time) current. The membrane potential, clamp current and variance of the current fluctuations (rms noise) were monitored throughout experiments with a flat-bed chart recorder (Kipp & Zonen, BD 41).

All vaseline-gap experiments were carried out at a maintained temperature of 10–12°C, as measured by a thermistor probe located close to the muscle fibre.

Results

Effects of hexamethonium on the amplitude of endplate currents

Figure 1 shows endplate currents recorded before (a1 to a3) and after 3 min exposure to 1 mM hexamethonium (b1 to b3) at three membrane potentials, −60 mV, −90 mV and −120 mV in an external solution containing physiological concentrations of calcium and magnesium. It can be seen that hexamethonium reduced the amplitude of endplate currents at −60 mV (a1,b1) and that the extent of this reduction was greater when the fibre was hyperpolarized to −90 mV (a2,b2) or −120 mV (a3,b3). This voltage-dependent reduction in the amplitude of the endplate currents by hexamethonium is illustrated graphically in Figure 2 where endplate current amplitude is plotted against membrane potential (squares before, circles during hexamethonium). To assess the voltage-dependence of the reduction in peak amplitude of endplate currents, the value of R-1 for various membrane potentials was calculated (Acher et al., 1979) where

$$R = \frac{\text{amplitude of control e.p.c.}}{\text{amplitude of e.p.c. in pres. of hexamethonium}}$$

Based on results like those shown in Figure 1, an e-fold change in $R - 1$ was produced by a 38 mV change in membrane potential. This is similar to the reduction of synaptic current amplitude by hexamethonium in rat submandibular ganglia (Rang, 1982) and at the rat neuromuscular junction (Rang & Rylett, 1984). Voltage-dependent antagonism of this kind was consistently seen in all fibres studied with hexamethonium concentrations in the range of 100 μM to 1 mM.

Hexamethonium also reduced the amplitude of the current evoked by bath-applied carbachol in a voltage-dependent manner. This is illustrated in Figure 3 which shows the results of an experiment in which the membrane potential at the endplate of a voltage-clamped muscle fibre was made to follow a slow ramp command which depolarized or hyperpolarized the

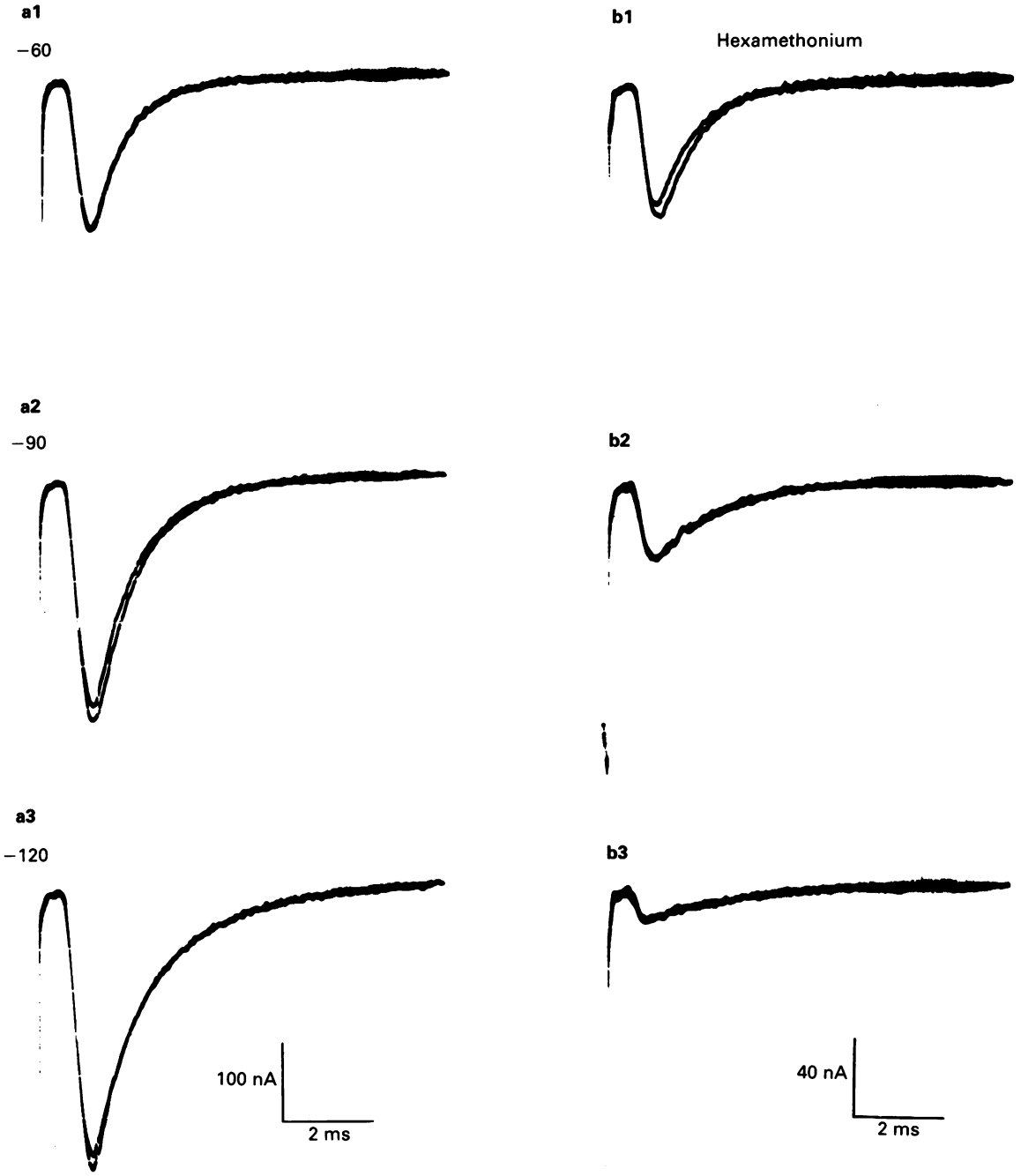

Figure 1 Effect of hexamethonium on the amplitude of endplate currents recorded from one muscle fibre before (a1, a2 and a3) and in the presence of hexamethonium, 1 mM, applied in the solution bathing the muscle for at least 3 min (b1, b2 and b3). Membrane potential: −60 mV (a1, b1): −90 mV (a2, b2): or −120 mV (a3, b3).

membrane to potentials between −60 mV and −120 mV. Current measurements were made in the absence and presence of carbachol and the difference between the currents was taken as the carbachol-induced current. At least two doses of carbachol were tested in this way; the complete procedure was then repeated for several carbachol concentrations in the presence of hexamethonium (100 μM to 1 mM). For the example in Figure 3, while the carbachol-induced current increased with hyperpolarization in the absence of hexamethonium, the agonist-evoked current decreased with hyperpolarization when hexamethonium was present.

Log(concentration)-response curves to carbachol in the absence and presence of hexamethonium were determined from currents at three levels of membrane potential, −60 mV, −90 mV and −120 mV, and examples are shown in Figure 4. It can be seen that the log(concentration)-response curve for carbachol at −120 mV was much less steep in the presence than in the absence of hexamethonium, and that the flattening effect of hexamethonium on the carbachol log(concentration)-response curve was reduced at less negative membrane potentials.

To avoid diffusion problems associated with the presence of the nerve terminal and the narrow synaptic cleft, equilibrium responses were also studied at extrajunctional regions of single denervated muscle fibres. Responses to bath applied 10 μM ACh were recorded in the presence and absence of 200 μM hexamethonium at membrane potentials of +50 mV and −50 mV (Figure 5). In this experiment, the peak amplitude of the agonist-induced response (145 nA) at +50 mV was reduced to 45% of control by 200 μM hexamethonium, while at −50 mV, the response was reduced to 15% of the control value (−172 nA). At a membrane potential of +50 mV, the influence of the electrochemical gradient to drive hexamethonium into the open ion channel would be greatly

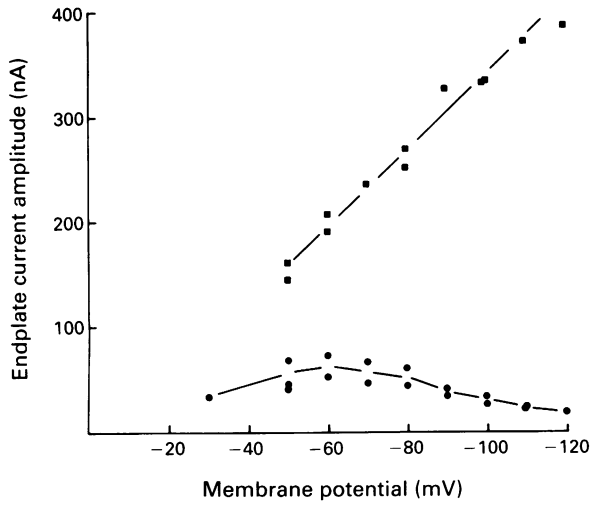

Figure 2 Effect of hexamethonium on the relation between amplitude of endplate currents and membrane potential. Currents were recorded from one endplate in the absence (■) and presence (●) of hexamethonium 1 mM, applied in the solution bathing the muscle for at least 3 min.

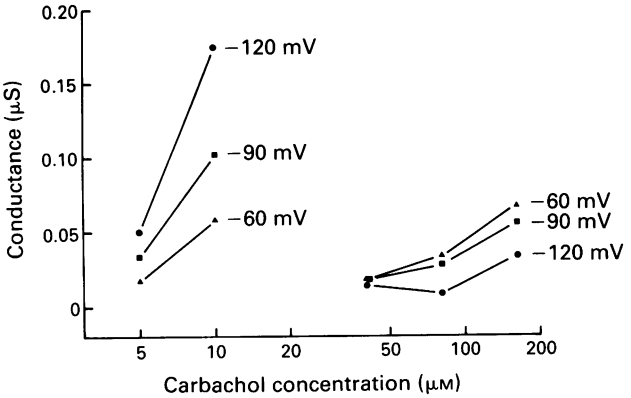

Figure 4 Log(concentration)-response curves determined from carbachol-induced currents of the kind shown in Figure 3. Conductance was calculated from the carbachol-induced current assuming a reversal potential of 0 mV. The curves were determined at three membrane potentials: −60 mV (▲), −90 mV (■) and −120 mV (●). Note that hexamethonium, 1 mM, shifted the curves to higher carbachol concentrations, and that, in particular at −120 mV, the curve in the presence of hexamethonium was less steep than in its absence.

reduced, and so any reduction of the ACh-induced conductance change may be attributed primarily to voltage independent antagonism.

Time course of endplate currents

Figure 1 also shows that while the amplitude of endplate currents was greatly reduced by hexamethonium, there was little change in their time course under these conditions (21°C, 1 mM hexamethonium). Semilogarithmic plots of the amplitude of endplate currents from Figure 1 a3 and b3 against time during their decay are illustrated in Figure 6a. There was no sign of a detectable fast phase of decay of the endplate current in the presence of 1 mM hexamethonium, although a small prolongation of the decay was observed at this membrane potential (−120 mV). The observation that hexamethonium did not increase the rate of decay of endplate currents under these conditions contrasts with observations of the effects of other drugs which show a voltage-dependent reduction in the amplitude of endplate currents (e.g. procaine, Katz & Miledi, 1975). It also differs from the findings of Milne & Byrne (1981) who observed biphasic decays of endplate currents in high magnesium (7.5–10 mM) Ringer solution at 13°C, and found that hexamethonium increased the rate constant of the early phase under these conditions. Since the observations shown in Figure 3 were made at room temperature (23°C), we repeated the experiment at 13°C to determine whether the difference in temperature could account for the discrepancy between our observations and those of Milne & Byrne (1981). There was no detectable increase in the initial rate of decay of endplate currents in the presence of hexamethonium (either 100 μM or 1 mM), and 1 mM hexamethonium caused a small

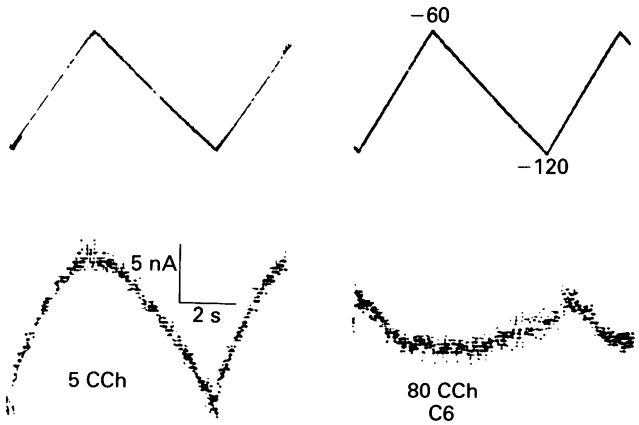

Figure 3 Effect of membrane potential on carbachol (CCh)-induced current in the presence and absence of hexamethonium (C6). The membrane potential was made to change from −60 mV to −120 mV and back to −60 mV at a steady rate by applying 'ramp' commands to the voltage-clamp, and is shown in the upper two panels. The current induced by 5 μM carbachol is shown in the lower left panel, and was obtained by subtraction of the current in the absence of carbachol from that in its presence (see Methods). The lower right panel shows that current induced by carbachol (80 μM) in the presence of hexamethonium (1 mM). Note that in the absence of hexamethonium the carbachol-induced current becomes more inward (downward deflection) with hyperpolarization, whereas in the presence of hexamethonium it becomes more outward with hyperpolarization.

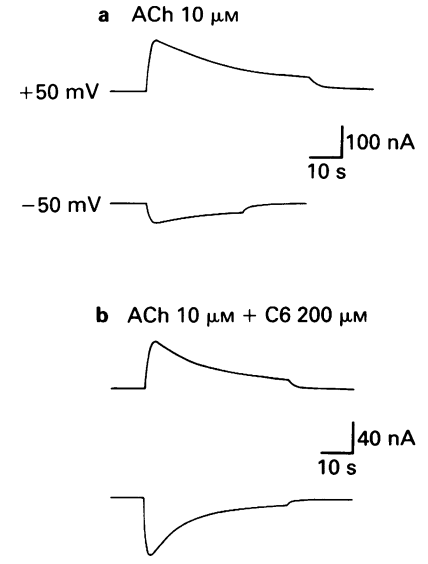

Figure 5 Effect of hexamethonium (C6) on equilibrium responses to acetylcholine (ACh) 10 μM. (a) Agonist-induced currents in response to bath application of ACh 10 μM at +50 mV (upper trace) and −50 mV (lower trace). Reversal (zero-current) potential, +3.1 mV. (b) Responses to bath application of ACh 10 μM and hexamethonium 200 μM at +50 mV (upper trace) and −50 mV (lower trace). Inward current plotted downwards. Note the calibration scale in (b).

prolongation similar to that observed at room temperature. The prolonging effect of hexamethonium on endplate current decay observed either at room temperature or at 13°C was dependent on the membrane potential. This is illustrated in Figure 6b where the time constant for decay of endplate currents recorded at 23°C is plotted semi-logarithmically against membrane potential for currents in the presence (squares) or absence (circles) of 1 mM hexamethonium. It can be seen that the prolonging effect of hexamethonium increased as the membrane potential was made more negative. A similar voltage-dependent prolongation of the decay of endplate currents by hexamethonium was observed consistently in all fibres studied over the membrane potential range of +60 mV to −140 mV. No component with an increased rate of decay was detected over this range of membrane potentials under the conditions of our experiments.

Fluctuation analysis of endplate currents

The mechanism underlying the prolongation of endplate current decay by hexamethonium was further investigated by measurement of agonist-induced current fluctuations (Anderson & Stevens, 1973) to distinguish between an effect of hexamethonium on acetylcholinesterase activity and/or the mean channel open-time ('burst length'; Colquhoun & Sakmann, 1983). Current fluctuation measurements were made on the endplate voltage-clamped with the vaseline-gap method. The spectrum of current noise induced by acetylcholine alone is shown in Figure 7a; the noise was recorded during the plateau of response (an inward current of −30 nA) produced by bath application of 1 μM ACh at a membrane potential of −100 mV. The theoretical Lorentzian curve fitted to the observed points related the single-sided spectral density, G(f), to the frequency, f, by the relation

$$G(f) = \frac{G(0)}{1 + (f/f_c)^2}$$

which is defined by the parameters G(0), the spectral density at zero frequency and the corner frequency, f_c, which is the frequency at which the spectral density is one half of G(0). The noise spectrum (Figure 7a) is fitted by a single Lorentzian curve with a corner frequency, f_c, of 48.2 Hz which corresponds to a time constant, tau, of 3.3 ms for the mean channel open-time.

The presence of hexamethonium (500 μM) produced a distortion of the spectrum of high frequencies (Figure 7b). The spectrum obtained at a membrane potential of −100 mV was fitted by a single Lorentzian curve (f_c = 400 Hz) giving a time constant, tau, of 4.0 ms; this corresponds to a 28% increase in the mean channel open time in the presence of 500 μM hexamethonium. There was, however, a deviation of noise spectra from a single Lorentzian curve in the presence of hexamethonium, suggesting a second kinetic component of current

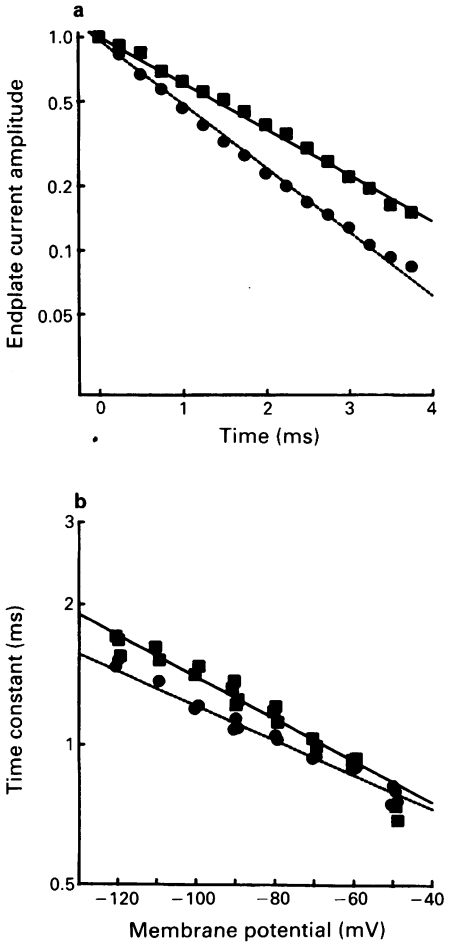

Figure 6 (a) Decay of endplate currents plotted semilogarithmically as a function of time; (●) in the absence of drugs; (■) in the presence of hexamethonium 1 mM, applied in the solution bathing the muscle for more than 3 min. (b) Voltage-dependence of the time course of endplate currents in the absence (●) and presence (■) of hexamethonium, 1 mM. Semilogarithmic plot of the time constant of decay of endplate current amplitude as a function of membrane potential. Temperature 20°C.

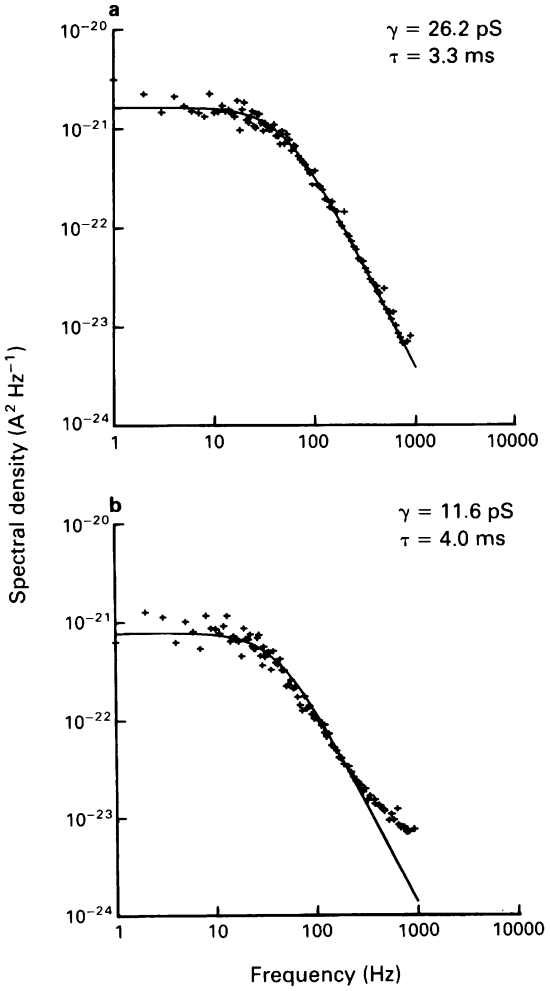

Figure 7 Power spectral density of endplate current fluctuations measured at −100 mV in the presence of acetylcholine (ACh) 1 μM (a) and ACh 10 μM plus hexamethonium 500 μM (b). Each spectrum was fitted up to 1 kHz by a single Lorentzian curve (solid line) with the time constants calculated from the corner frequency indicated.

Table 1 Voltage dependence of the endplate channel 'apparent' conductance (γ) and mean open time (τ) in the presence of (10 μM ACh and 500 μM hexamethonium) and absence of (1 μM ACh) hexamethonium

Membrane potential (mV)	*ACh 1 μM* γ(pS)	τ(ms)	*ACh 10 μM + C6 500 μM* γ(pS)	τ(ms)	$\tau_{drug}/\tau_{control}$
−70	26 ± 1	2.3 ± 0.16 (6)	19 ± 1	2.6 ± 0.3 (6)	1.13
−100	22 ± 1	3.6 ± 0.2 (5)	10 ± 1	4.8 ± 0.3 (4)	1.33
−130	25 ± 2	6.0 ± 0.3 (5)	6 ± 1	9.6 ± 1.4 (4)	1.60

Results of current fluctuation measurements made at 10–12°C given as mean ± s.e.mean. (n = number of experiments).
Ratios of mean channel open time (τ) in the presence and absence of hexamethonium are given in the right hand column.

fluctuations which is not adequately resolved in the present experiments. The effect of a fixed concentration of hexamethonium on the apparent mean open time became more pronounced as the membrane was hyperpolarized (Table 1). There was also an apparent reduction in single channel conductance in the presence of hexamethonium (Table 1), which may be attributed to an underestimation of the variance by failing to account for a second spectral component (see Figure 7b).

Effect of hexamethonium on agonist-induced currents in voltage-jump experiments

Microelectrode experiments The effects of membrane potential on agonist-induced currents (determined during the plateau phase of response) were examined by applying step hyperpolarizations from −70 mV to between −100 and −160 mV. Net agonist-induced currents obtained after subtraction of the control currents from those obtained in the presence of agonist are illustrated in Figure 8 for responses to 5 μM acetylcholine alone (a) or for 15 μM acetylcholine in the presence of 1 mM hexamethonium (b). Neostigmine 3 μM was present throughout to inhibit acetylcholinesterase. In the absence of hexamethonium, the acetylcholine-induced current at the beginning of the step increased with hyperpolarization as if there was an approximately ohmic increase of current through a fixed number of open ion channels as the driving force on ion flow was increased. This instantaneous current was followed by an exponential relaxation of the current to a new steady level, which corresponds to the new equilibrium number of open ion channels at the hyperpolarized membrane

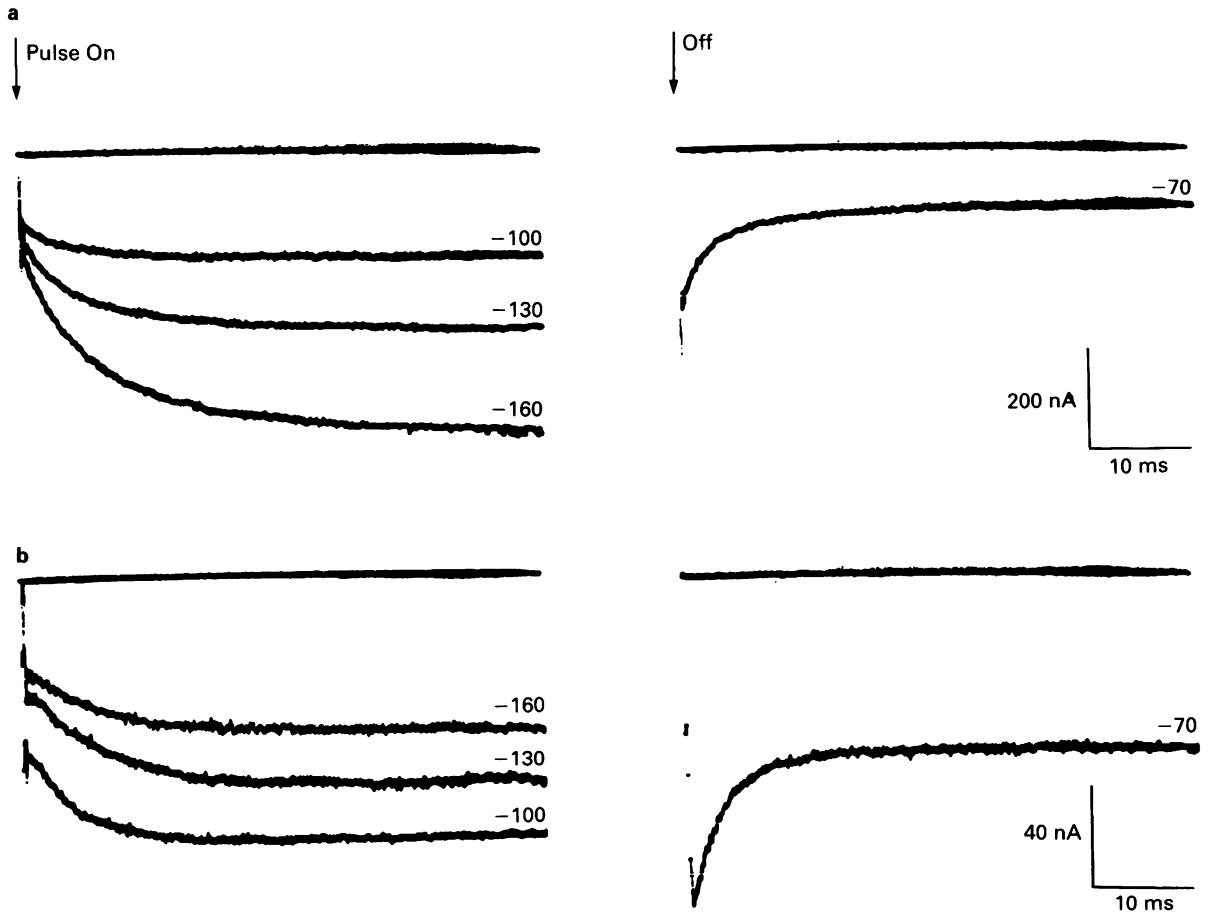

Figure 8 Acetylcholine (ACh)-induced currents recorded from a single endplate immediately following a series of step changes in membrane potential. At the arrow indicating 'pulse on' the membrane potential was hyperpolarized from −70 mV to the potential shown with the current; at the arrow indicating 'pulse off' the membrane potential was returned to −70 mV. The top panels (a) show currents induced by acetylcholine, 5 μM. The bottom panels show currents induced by acetylcholine 15 μM in the presence of hexamethonium, 1 mM. Zero current is shown by the horizontal line at the top of each panel. Time constants for the slow component of relaxation were: 3.8 ms (−100 mV), 6.4 ms (−130 mV), and 7.9 ms (−160 mV) in the absence of hexamethonium; 4.4 ms (−100 mV), 6.6 ms (−130 mV) and 7.3 ms (−160 mV) in the presence of hexamethonium.

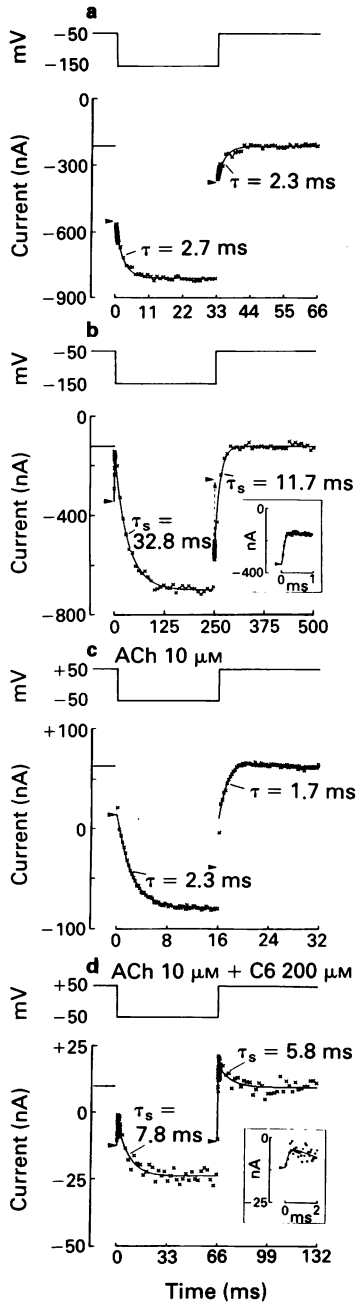

Figure 9 Relaxations of net agonist-induced current following step changes in membrane potential. Current relaxations for a 100 mV jump from and back to −50 mV holding potential during bath application of acetylcholine (ACh) 10 μM (a) and ACh 10 μM plus hexamethonium, 500 μM (b). Insert in (b): fast inverse relaxation following ohmic jump in current observed with voltage-jump to −150 mV in the presence of hexamethonium (tau_f 90 μs). Relaxations for voltage-jumps from +50 mV to −50 mV and back in the presence of ACh 10 μM (c) and ACh 10 μM plus hexamethonium (C6) 200 μM (d). Insert in (d): small amplitude, inverse relaxation recorded in response to a hyperpolarizing jump to −50 mV in the presence of hexamethonium 200 μM (tau_f 200 μs). The arrowheads indicate the (ohmic) instantaneous current.

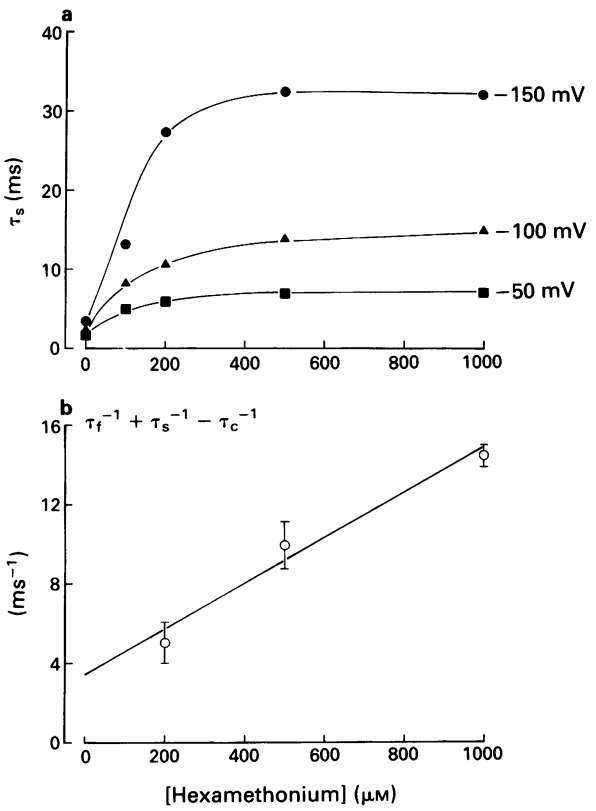

Figure 10 (a) Slow relaxation time constant(s) as a function of hexamethonium concentration at three different membrane potentials. (b) plot of ($tau_f^{-1} + tau_s^{-1} - tau_c^{-1}$) against hexamethonium concentration at −150 mV. Pooled data obtained from voltage-jump experiments. Straight line is a regression fit to the data.

potential. In the presence of hexamethonium the acetylcholine-induced current did not show an ohmic increase at the beginning of the step hyperpolarization, but rather decreased as the voltage was stepped to more negative potentials. The current then relaxed to a new equilibrium level, but this was reduced with more negative steps, in contrast to that observed in the absence of hexamethonium. For the example in Figure 8, the time constant for the slow relaxation in the presence of hexamethonium was increased from a control value of 3.8 to 4.4 ms at −100 mV, whereas little or no prolongation was observed with steps to more hyperpolarized levels. It should be borne in mind that 'buffered diffusion' of acetylcholine in the synaptic cleft may complicate the estimation of such rate constants under the conditions of these experiments (see for example, Armstrong & Lester, 1979).

Figure 8 also shows the response evoked when the membrane potential was stepped back from −160 mV to −70 mV. In the presence of ACh alone, there was an initial ohmic step that was followed by an exponential decay of current back to the original holding level. However, in the presence of ACh plus hexamethonium, the current immediately after the depolarizing step was greater than the equilibrium level seen at the hyperpolarized level. The current then decayed to the new steady level.

Vaseline gap experiments It seemed possible that the non-ohmic behaviour of the ACh-induced current at the beginning of the voltage jumps in the presence of hexamethonium might reflect a voltage-dependent antagonism (block) which was too rapid to resolve with the two-electrode voltage clamp. Similarly the initial large current seen immediately after the hyperpolarizing pulse could reflect a rapid unblocking of membrane channels. This hypothesis was tested using the vaseline-gap voltage clamp method which allows resolution of current transitions in the microsecond range. These experiments were done on the extrajunctional region of denervated muscle fibres to avoid concentration transients which could arise within the synaptic cleft during a hyperpolarizing jump (Armstrong & Lester, 1979; Colquhoun & Sheridan, 1982). The use of denervated muscle also avoids the need to inactivate the enzyme acetylcholinesterase, which is absent in extrajunctional regions of frog muscle (Katz & Miledi, 1964; Miledi *et al.*, 1984).

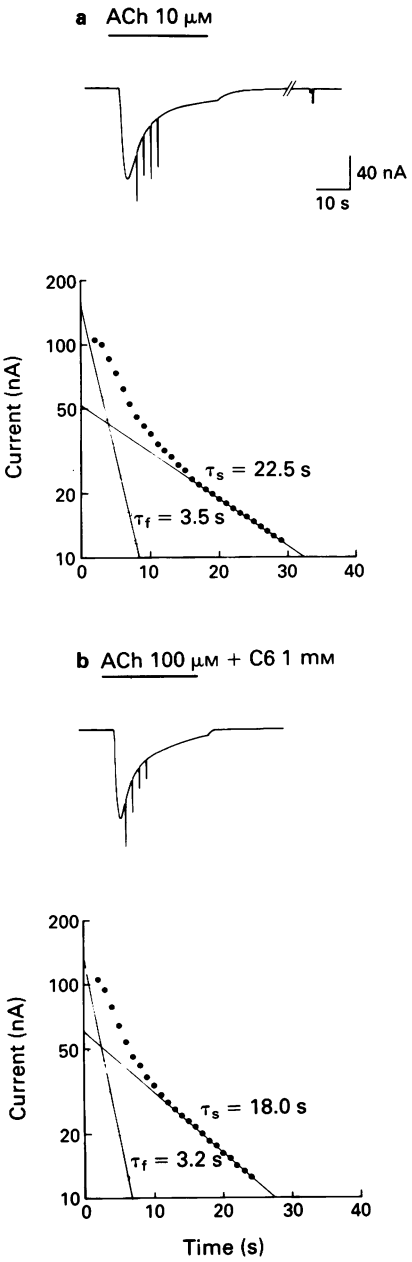

Figure 11 Inward currents in response to acetylcholine (ACh) 10 μM (a) and ACh 100 μM plus hexamethonium (C6) 1 mM (b) at −50 mV. The duration of bath application of the agonist is indicated by the line above the current records. The time lag between application of the agonist and the start of the response is due to the 'dead-space' time between the solution inlet valve and for the solution change to reach the muscle membrane. Superimposed downward deflection on current records are responses (attenuated by pen recorder) to hyperpolarizing voltage-jumps to −100 mV and 150 mV. In the upper trace of (a) the position of the control voltage jumps, imposed in the absence of agonist, are also shown. Semilogarithmic plot of the decay of the current in (a) and (b) are shown below the corresponding record. Time constants of each exponential component of the current decay (desensitization) observed in (a) and (b) are given.

The relaxations of the net current induced by 10 μM ACh under these conditions (Figure 9a) were fitted by a single exponential curve with a time constant of 2.7 ms at 150 mV and 2.3 ms at −50 mV. In this, and other experiments of this series, the acetylcholine concentration was kept constant in the presence and absence of hexamethonium. In the presence of hexamethonium (500 μM) the shape of the relaxations is changed (Figure 9b). Hyperpolarization caused the expected ohmic jump (which had not been detected with the two-microelectrode clamp described above) followed by an initial rapid decrease in inward current and then an increase which was slower than that in the control. The current during the first millisecond following each voltage step was sampled at a higher rate (64 kHz) and is shown on an expanded time scale in the insert of Figure 9b to show clearly the rapid, inverse relaxation following the ohmic jump in current at −150 mV. The relaxation following the voltage jump to −150 mV in Figure 9b was well fitted by two exponentials with time constants, tau_f = 90 μs and tau_s = 32.8 ms.

When the membrane potential was stepped back from −150 to −50 mV, the current seen in the presence of hexamethonium did not show the exponential decrease seen with the agonist alone (Figure 9a) but rather a rapid increase in current followed by an exponential decay in the normal direction. The rate of decay was again slower (tau_s = 11.7 ms) than in the control (tau_s = 2.3 ms). The amplitude and rate constant of the fast relaxation were dependent on the hexamethonium concentration and increased with higher concentrations of hexamethonium. When the concentration of hexamethonium was >500 μM, the time course of the relaxation following the 100 mV depolarizing step was invariably too rapid to be resolved, even when sampled at 64 kHz.

Similar observations were also made when hyperpolarizing voltage-jumps were applied from a holding potential of +50 mV (Figure 9c and d).

The effect of hexamethonium concentration on the slow relaxation time constant at membrane potentials −50 mV, −100 mV and −150 mV is shown in Figure 10a. Acetylcholine concentration was kept constant in the presence and absence of hexamethonium. As the hexamethonium concentration was increased above 200 μM the slow relaxation time constant measured at all voltages was independent of antagonist concentration. A similar observation has been made for the concentration-dependence of the slow relaxation time constant recorded in the presence of decamethonium (Adams & Sakmann, 1978; Adams & Colquhoun, unpublished). Over the voltage range illustrated, an e-fold increase of the rate constant of the slow relaxation (tau_s^{-1}) required an approximately +67 mV change in membrane potential.

The sequential model for open channel block (Adams, 1976) predicts that, in the presence of a drug, the agonist-induced current relaxes as the sum of fast and slow exponential components whose rates are, respectively, increased and decreased linearly with increasing drug concentration. As with the observations on endplate currents reported above, this prediction was not fulfilled for the relationship between tau_s and hexamethonium concentration (Figure 10a). A second prediction of the model is that the sum of the fast and slow rate constants (reciprocal time constants) minus the control rate constant should depend linearly on hexamethonium concentration according to:

$$tau_f^{-1} + tau_s^{-1} - tau_c^{-1} = [H]k_{+1} + k_{-1},$$

where tau_f = time constant for the fast component of decay, tau_s = time constant for the slow component of decay in the presence of hexamethonium, tau_c = time constant of decay in the absence of blocking agent, [H] = concentration of hexamethonium, and k_{+1} and k_{-1} are the forward and backward rate constants for the blocking reaction. A plot of (tau_f^{-1} + tau_s^{-1} − tau_c^{-1}) against hexamethonium concentration is shown in Figure 10b for results obtained at a membrane potential of −150 mV. The data, though dominated by the value obtained for tau_f^{-1}, were fitted by a linear regression line; the slope of the line gives an estimate of k_{+1} of $1.2 \times 10\,M^{-1}s^{-1}$, and the intercept yields a value, k_{-1}, of $3400\,s^{-1}$. The apparent equilibrium constant, K, for the binding of hexamethonium to its channel blocking site is given by: $K = k_{-1}/k_{+1} = 283\,\mu M$ at −150 mV. (This estimate should be regarded with some caution since a more complex model in which blocked channels may close would not be expected to give a linear relationship for Figure 10b). The rate constants of the two kinetic components (and consequently the equilibrium constant also) showed a strong dependence on

membrane potential (see Figure 10a). However, our inability to resolve the fast, inverse relaxations during voltage-jumps to potential less negative than −150 mV for all hexamethonium concentrations tested precluded any quantitative analysis of the voltage-dependence of the association (k_{+1}) and dissociation (k_{-1}) rate constants and hence equilibrium constants (K). If we assume similar voltage-dependent rates of association and dissociation to those measured for open channel block by gallamine (Colquhoun & Sheridan, 1981) or QX222 (Neher & Steinbach, 1978), the equilibrium constant for the binding of hexamethonium to the ACh-activated channel at membrane potential of −70 mV would be expected to be approximately 2 to 3 mM. These values for the equilibrium constant are slightly greater than the concentrations required for 50% block of endplate currents at −70 mV (see Figure 2).

Desensitization in the presence of hexamethonium

Another feature of ACh responses in the presence of hexamethonium was noticed during the course of these experiments. In the records shown in Figure 11 the peak amplitude of the response (−110 nA) to 10 μM ACh was mimicked by increasing the ACh concentration to 100 μM in the presence of 1 mM hexamethonium. These comparable currents may reflect similar receptor occupancy by ACh, and comparison can be made between the rates of desensitization of the response in the presence and absence of hexamethonium. The decay of these inward current from their peaks was biphasic and best fit by the sum of two exponentials. The similarity between these rates of onset of desensitization in the presence and absence of hexamethonium at comparable peak inward currents is consistent with the suggestion that hexamethonium does not significantly enhance or diminish the degree of desensitization (cf. Rang & Ritter, 1970; Terrar 1974).

Discussion

The main findings of the present study are that hexamethonium reduces the amplitude of endplate currents in a voltage-dependent manner while only minor changes are detectable in the time course of endplate currents. The observations from 'voltage-jump' studies are compatible with a rapid and voltage-dependent blocking action by hexamethonium of ACh-activated channels, and the question arises whether this action would be sufficient to account for the observed effects on endplate currents.

In mammalian ganglion cells, there is good evidence that hexamethonium blocks ion channels associated with nicotinic receptors (Ascher *et al.*, 1979; Gurney & Rang, 1984; see also Blackman, 1959 and Blackman & Purves, 1968). The action of hexamethonium in skeletal muscle is less clear. Milne & Byrne (1981) concluded that part of the action of hexamethonium on frog skeletal muscle was similar to some other drugs that are thought to block ion channels, for example procaine (Adams, 1977), in that the decay of endplate currents in the presence of hexamethonium showed two exponential components, the faster of which is though to reflect the forward reaction of binding to the open channel. However, with up to 1 mM hexamethonium under the conditions of our experiments, we have not detected a component of decay of endplate currents which is faster than that in the absence of drugs. Raising the external magnesium ion concentration has been shown to prolong e.p.c. decay as a consequence of an increase in the mean channel open time at the amphibian motor endplate (Takeda *et al.*, 1981; see also McLarnon & Quastel, 1983). This effect of high magnesium on end-plate channel kinetics may contribute to the appearance of a biphasic e.p.c. decay observed in the presence of hexamethonium by Milne & Byrne (1981).

The most direct evidence from our experiments for a channel blocking action of hexamethonium comes from the voltage-jump experiments, where the rapid reduction in agonist-induced current following a step hyperpolarization in the absence of hexamethonium can be interpreted as a block by hexamethonium of open ion channels. Channel-block models also predict the observed rapid surge of inward current at the end of a step hyperpolarization (cf gallamine, Colquhoun & Sheridan, 1981), the interpretation being that this represents rapid unblocking of the channels as the membrane potential is restored to its initial level.

Additional evidence consistent with channel-block is provided by further analysis of voltage-jump experiments where different concentrations of hexamethonium were used: it was shown in Figure 10 that the expression, $\text{tau}_f^{-1} + \text{tau}_s^{-1} - \text{tau}_c^{-1}$, was approximately linearly related to the concentration of hexamethonium. According to the sequential channel block model (in which the blocking drug can only bind to and dissociate from open channels, see e.g. Adams, 1977), the slope of this relationship should be equal to the forward rate constant, k_{+1}, for the blocking reaction, and the intercept on the ordinate should be the backward rate constant, k_{-1}, for unblock. The estimates for hexamethonium were: $k_{+1} = 12\,\mu\text{M}^{-1}\text{s}^{-1}$ and $k_{-1} = 3400\,\text{s}^{-1}$. The equilibrium constant for the reaction between hexamethonium and ion channels, derived from the simple sequential model as the ratio of these two figures, is 280 μM at −150 mV.

There are, however, some discrepancies between our observation and the predictions of a simple sequential channel block model. If single channel behaviour is considered, the sequential model predicts that a blocking drug which dissociates rapidly from the channel (e.g. gallamine) would be expected to cause substantial prolongation of the time from first opening to final closure during a cluster of openings (see Colquhoun & Sheridan, 1981; and Ogden & Colquhoun, 1985). This in turn leads to a slow phase in decay of endplate currents, which was not pronounced in our experiments in which 1 mM hexamethonium caused less than a twofold prolongation even at −100 mV, and at −50 mV no prolongation was detectable. More complex models for channel block in which channel closure is not inhibited by occupation of the channel by the blocking drug have been described in detail by Colquhoun & Ogden (1985; see also Adams, 1977). One point arising from their discussion is that the mean open time per cluster is no longer independent of the concentration of blocking agent (since blocked-open channels may convert to blocked-shut channels, and then dissociation of the blocking drug may occur without re-opening). This in turn means that the prolongation discussed above need not be as great as would otherwise be the case. A further extension of this argument is that the total charge crossing the membrane during an endplate current appears to be reduced more in our experiments than would be expected on the basis of a sequential block. In addition, in the voltage-jump experiments the concentration dependence of the slow relaxation time constant is non-linear which is at variance with that predicted from the sequential model of channel block (see Adams & Sakmann, 1978).

If a more complex model for channel block (in which channels may close from a blocked state, see Ogden & Colquhoun, 1985) applies under the conditions of our experiments, the estimated equilibrium constant of 280 μM calculated above for the binding of hexamethonium to the channel would have to be revised.

An interesting point in this context is the route taken by hexamethonium when it dissociates from the channel; in the case of the open channel complex it seems reasonable that following dissociation the hexamethonium would normally escape to the exterior of the fibre, but it remains possible that the dissociation could come about by entry of hexamethonium into the fibre. Permeation of ion channels by hexamethonium is consistent with the observations of Dwyer *et al.* (1980) and of Creese & England (1970). In the case of the closed channel complex with hexamethonium, it seems possible that the hexamethonium may not easily escape to the

exterior and that dissociation might normally be accompanied by entry of hexamethonium into the fibre. Gurney & Rang (1984) have discussed a similar cyclic model for block of ganglionic neurones by methonium compounds, and concluded that for compounds with nine or more carbons between the charged nitrogens, the blocking drug may inhibit channel closure, whereas with smaller compounds (e.g. hexamethonium) the drug may become trapped in the closed channel unless the drug is so small that it can easily escape to the interior of the cell. It seems possible that in the case of hexamethonium at the neuromuscular junction the blocking drug is small enough not to inhibit substantially channel closure, and yet has a low enough permeability to cause block, while still being able to dissociate slowly by entry into the fibre. If this were the case for hexamethonium, it would account for the reduction in amplitude of endplate currents without substantial prolongation. It also seems possible that the forward rate constant for channel block with 1 mM hexamethonium (approximately $10{,}000\,s^{-1}$, see above) may lead to a development of block during the rising phase of an endplate current (approximately 500 μs), so that no fast phase of endplate current decay reflecting channel block is detectable. This is unlike the majority of blocking drugs e.g. procaine (Katz & Miledi, 1975) or gallamine (Colquhoun & Sheridan, 1981), and might be attributed, at least in part, to the low potency of hexamethonium: k_{+1} for hexamethonium is not substantially greater than k_{+1} for gallamine (Colquhuon & Sheridan, 1981), but the effective forward rate is much larger for hexamethonium because the concentration required for an action is so high (more than 100 times greater than the concentration of gallamine).

There may of course be an additional effect of hexamethonium on receptors associated with ion channels. An upper estimate of such a possible effect can be derived from experiments at a membrane potential of +50 mV. At this potential the influence of the electric field would be to reduce the entry of hexamethonium into ion channels; however, there would still be a concentration gradient tending to drive hexamethonium into the channel and so even at this potential any residual antagonism may include a component of channel block. At +50 mV, the concentration of hexamethonium required to reduce the agonist-induced current to approximately 50% was 200 μM: this may be compared with the dissociation constant of hexamethonium binding to acetylcholine receptors measured from the rate of inhibition of α-bungarotoxin binding to receptors in denervated rat muscle, which is approximately 100 μM (Colquhoun & Rang, 1976). Brenner & Micheroli (1985) also concluded that the action of hexamethonium at the frog endplate was in part a competitive block of receptors, combined with an action at ion channels which was not a simple sequential block.

Another question which deserves attention is to what extent there may be an anticholinesterase effect of hexamethonium under the conditions of our experiments. Such an effect of hexamethonium on mammalian skeletal muscle has been described by Rang & Rylett (1984). In our experiments on frog skeletal muscle, a small prolongation of the decay of endplate currents was seen at a membrane potential of −120 mV, but unlike the effects of anticholinesterases such as neostigmine (Magleby & Stevens, 1972), the prolongation became less at more positive potentials, and was not detectable at −50 mV. The small prolonging effect of hexamethonium on endplate currents which we observed, and its dependence on membrane potential, may be compared with observations in experiments where acetylcholine-induced current 'noise' was measured: hexamethonium caused a small shift in the power spectrum of the noise at −100 mV towards lower frequencies (the reduction in the corner frequency, like the prolongation of endplate current decay at this potential, was slightly less than a factor of two), but no such shift was observed at −50 mV. The increased concentration of acetylcholine in the presence of hexamethonium might lead to an abbreviation of channel lifetime (when ACh concentrations are greater than the 'low concentration limit', see e.g. Anderson & Stevens, 1973); if such an effect were to occur it would lead to underestimation of the apparent prolongation in the presence of hexamethonium. Inhibition of acetylcholinesterase would not be expected to modify the power spectrum for acetylcholine-induced current noise because the lifetime of occupied open channels would not be expected to be lengthened by the action of an anticholinesterase (which would be expected only to influence the time for which acetylcholine molecules are present in the synaptic cleft). It seems unlikely that an anticholinesterase effect of hexamethonium is a major cause of the small prolongation of endplate current decay and the shift in the power spectrum at more negative potentials. It may be that the small prolongation at negative potentials reflects an interference with the normal closure of ion channels, in the manner suggested above for the action of larger channel blocking drugs.

In summary, the results are consistent with a block by hexamethonium of ACh-activated ion channels, and it appears that this block may not prevent the presumed conformational change in the channel which in the absence of hexamethonium normally leads to channel closure. Thus, it appears that hexamethonium can cause a voltage-dependent reduction in the amplitude of endplate currents without a substantial effect on their time course of decay.

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Phorbol dibutyrate enhances local anaesthetic action

S. Austin, J. McGivern & [1]C.N. Scholfield

School of Biomedical Science, Queen's University, 97, Lisburn Road, Belfast, BT9 7BL

1 Synaptically-evoked field responses were elicited by stimulation of the lateral olfactory tract of rat olfactory cortex slices maintained *in vitro*.

2 Various concentrations of lignocaine (5–500 μM) were applied to the solution bathing the slices. These produced dose-dependent depressions of the synaptically-evoked potential over the concentration range 20–500 μM. The responses completely recovered on washing out the lignocaine. Similar depressions were also noted for procaine (100–1000 μM).

3 In the 47 slices tested, application of β-phorbol 12,13-dibutyrate (1 μM) increased the amplitude of the synaptic response (from 0.99 $\pm$ 0.05 to 1.36 $\pm$ 0.06 mV). β-Phorbol 13-monbutyrate (1 μM) had no effect.

4 In the presence of phorbol dibutyrate the depressant effect of lignocaine was increased: the EC_{50} changed from 91 $\pm$ 10 to 24 $\pm$ 2 μM (a mean potency increase of 3.47 $\pm$ 0.14). A similar increase in potency for procaine was observed with phorbol dibutyrate (from 264 $\pm$ 23 to 49 $\pm$ 9 μM: a 5.49 $\pm$ 0.82 increase in potency). If the tissue was pre-equilibrated in a concentration of lignocaine which produced a 60–80% depression, addition of phorbol ester caused a complete abolition of the evoked potential.

5 β-Phorbol 13-monobutyrate (1 μM) had no effect on the potency of lignocaine.

6 The Na and K currents generating the action potential in the presynaptic nerve terminals were unaffected by phorbol dibutyrate. The depressant effect of lignocaine on these currents was not modified by phorbol dibutyrate.

7 The potentiation of lignocaine could not be accounted for by membrane depolarization or by non-specific actions of phorbol dibutyrate, and was distinct from the action on transmitter release. Therefore, it seems likely that protein kinase C activation was responsible for the modified action of lignocaine, although the mechanism for this is unclear.

Introduction

In the nervous system, one of the most clear and consistent effects of β-phorbol diesters is the increased transmitter release reported by several authors using a variety of techniques (Publicover, 1985; Zurgil & Zisapel, 1985; Eusebi *et al.*, 1986; Shapira *et al.*, 1987; Murphy & Smith, 1987; Malenka *et al.*, 1987; Haimann *et al.*, 1987; Scholfield & Smith, 1989). The implication of these observations is that a protein kinase C may regulate transmitter release.

In a previous study on phorbol esters (Scholfield & Smith, 1989) and in some of our unpublished experiments, several agents which are considered to act on protein kinase C or influence Ca mobilisation were tested. We considered that some of the observed effects might have been nonspecific or local anaesthetic-like actions. To control for such possible actions, the effects of these agents were compared with those of lignocaine. Most agents previously tested which reduced synaptic transmission potentiated the effect of phorbol esters, whereas the contrary was observed in the presence of lignocaine: there was a more intense depressant effect of lignocaine on synaptic transmission in the presence of phorbol ester. We now report the results of further experiments on this initial observation. A preliminary account of these effects has appeared in abstract form. (Austin & Scholfield, 1990).

Methods

Pial surface slices of periamygdaloid area of the olfactory cortex were cut from freshly excised brains of rats. The slices were either 150–250 μm or 500 μm thick for recording presynaptic axonal currents or synaptically-evoked responses, respectively. The slices were trimmed to leave a pial surface area of 2 × 6 mm and included the periamygdaloid cortex and a 3 mm length of lateral olfactory tract (LOT). These preparations were placed on a nylon mesh within a recording bath having an effective volume of 0.5 ml (Scholfield, 1980; 1990). Fifty or 20 ml of Krebs solution equilibrated with 95% O_2/5% CO_2 was recirculated through the bath at 5 ml min^{-1}. The caudal part of the LOT was continually stimulated with supramaximal stimuli of 0.2 ms duration (normally 20–30 V at 0.05 Hz) via a pair of tungsten 125 μm diameter wires with lacquer insulation.

Synaptically-evoked potentials were recorded from the pial surface of the thicker slices via a single glass micropipette filled with Krebs solution (resistance 1 MΩ). This was connected via a silver electrode to a capacitively coupled amplifier having an input resistance of 10^{10} ohm and a time constant of 3 s. Responses were captured, stored and replayed using an IBM-AT type computer equipped with an analogue-to-digital converter run by our own software.

To record the axonal Na currents which generate the action potential, use is made of the tight aggregation of the unmyelinated axons into a layer beneath the pial surface (Scholfield, 1990). These axons give off synapses as varicosities *en passant* and are derived from the myelinated axons within the LOT. A transverse section was cut through the thinner slices perpendicular to the radiation of the unmyelinated axons and a suction electrode with a 50 μm internal tip diameter was offered up to the cut face of the slice in the region containing the *en passant* synapses. Gentle suction was applied to draw some of the slice into the electrode (Scholfield, 1990). The electrode was connected to a current amplifier and the waveforms captured, stored and processed by computer. K currents were revealed as a negative after current on polarizing the electrode by +50 to +200 mV (Scholfield, 1990).

The β-phorbol esters, phorbol 12,13-dibutyrate or phorbol 13-monobutyrate were dissolved in anhydrous dimethylsulphoxide (DMSO). For a final concentration of 1 μM phorbol ester, the concentration of DMSO added to the 50 ml of normal solution was 10 μM. The diacylglycerols were also added in DMSO. In most experiments, local anaesthetic was added directly to the bathing solution in sequentially increasing concentrations. The drug was then washed out for twice the time required for a complete recovery of the response.

[1] Author for correspondence.

The peak amplitudes of the evoked potential or presynaptic currents were measured. To assess the effects of anaesthetics, the measured peak amplitude was expressed as a proportion of the amplitude observed before adding anaesthetic. Comparison of potentials before and after drug applications were statistically tested using Students t test (paired t test with data from the same slices, unpaired with different slices). Multiple measurements were summarised as mean $\pm$ s.e.mean. Comparisons of potencies were made by constructing log-concentration amplitude graphs for each slice, reading off the EC_{50} values and then averaging these.

Drugs and solutions

The normal solution used had the following composition (mM): Na 144, K 5.9, Ca 2.5, Mg 1.3, Cl 128, HCO_3 25 and D-glucose 10. The lignocaine came from the Astra Laboratories, Watford; β-phorbol 12,13-dibutyrate, β-phorbol 13-monobutyrate, and procaine were obtained from Sigma Chemical Co., Poole, Dorset.

Results

Stimulation of slices of rat olfactory cortex submerged in Krebs solution evokes a compound synaptic potential of around 1 mV (Figure 1). Most of this response is monosynaptically generated, although the small, longer latency components are mediated through further synapses (Gilbey & Wooster, 1979). The present work studied the shortest latency monosynaptic response. Application of 50 μM lignocaine produced a partial and reversible depression of the evoked potential. After the recovery of the preparation, phorbol dibutyrate was added and this increased the amplitude of the response. Reapplication of the same concentration of lignocaine then produced a much greater depression of the evoked potential (Figure 1).

Figure 2 shows a time-course of the peak amplitude of the monosynaptically-evoked potential. After recovery of the slice following preparation, this amplitude remains fairly constant for several hours. Again application of 1 μM phorbol dibutyrate increased the amplitude of the synaptic response (by 50%) and normally this effect would be sustained: for example, in normal solution the evoked potential was increased from 0.99 $\pm$ 0.05 to 1.36 $\pm$ 0.06 mV in 47 slices (the mean increase was 40.0 $\pm$ 4.4%, $P < 0.0001$). In Figure 2, 100 μM lignocaine was applied periodically for 15 min periods. In normal solution, these repeated applications of lignocaine depressed the evoked potential by a constant amount each time: by 0.63 $\pm$ 0.01 mV in this experiment. In the presence of phorbol dibutyrate, the depression produced by lignocaine was substantially increased such that very little of the response remained (Figure 2). On washing out the phorbol dibutyrate, the enhanced depressant action of lignocaine persisted as did the enhancement of transmitter release (Scholfield & Smith, 1989). Similar results were obtained in three other experiments of this type.

In 7 experiments a different protocol was used involving a prolonged single application of lignocaine (200 μM; Figure 3a). When the depression due to lignocaine had equilibrated, the application of phorbol dibutyrate (1 μM) transiently increased the evoked potential. With continued application of the lignocaine-phorbol dibutyrate mixture, the response then declined until it was completely depressed (Figure 3a). After prolonged washing out of lignocaine (60 min), the amplitude of the evoked potential increased: the amplitude finally

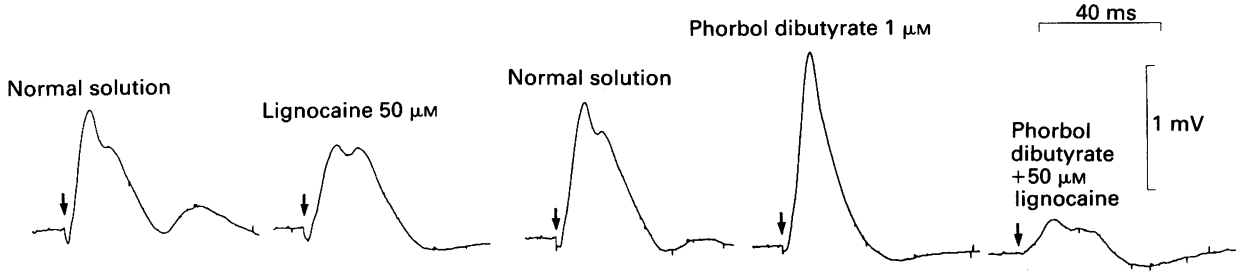

Figure 1 Compound synaptically-evoked potentials recorded from a glass pipette electrode placed on the pial surface of a slice of rat olfactory cortex. For each of the responses from left to right, the preparation was bathed sequentially in: normal solution, lignocaine 50 μM (10 min), normal solution (55 min), phorbol dibutyrate (30 min) and lignocaine 50 μM and phorbol dibutyrate. The traces shown are averages of 10 records. At the end of the experiment, 1 mM lignocaine was added to abolish completely all biologically generated electrical activity, and this trace was used to subtract out the stimulus artefact from the other traces. The arrow indicates the point of stimulus application.

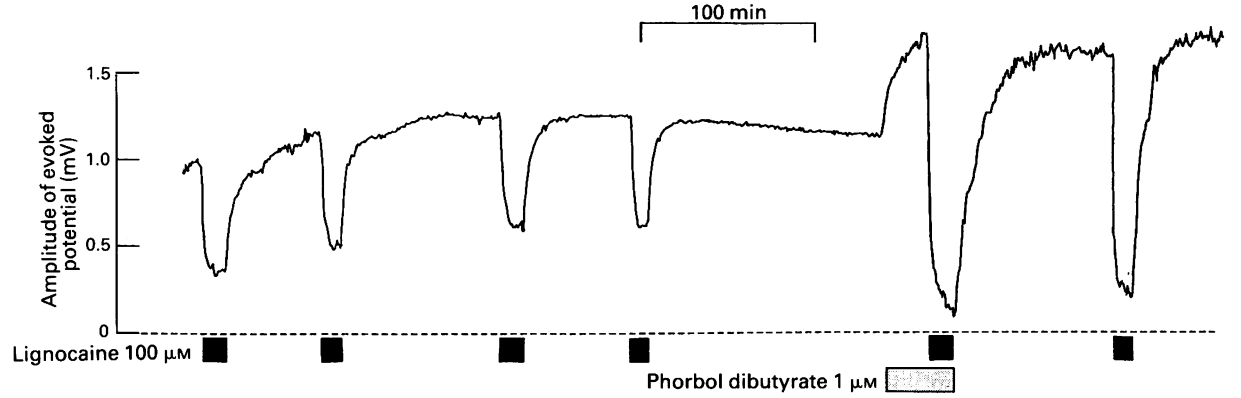

Figure 2 A time course for the height of the monosynaptically-evoked potential showing the effect of repeated applications of lignocaine 100 μM during the periods marked by (■). During the period marked by the stippled bar, 1 μM phorbol dibutyrate was added to the bathing solution. The slice was placed in the recording bath 90 min after preparation, a time when normally the synaptic response has not fully developed.

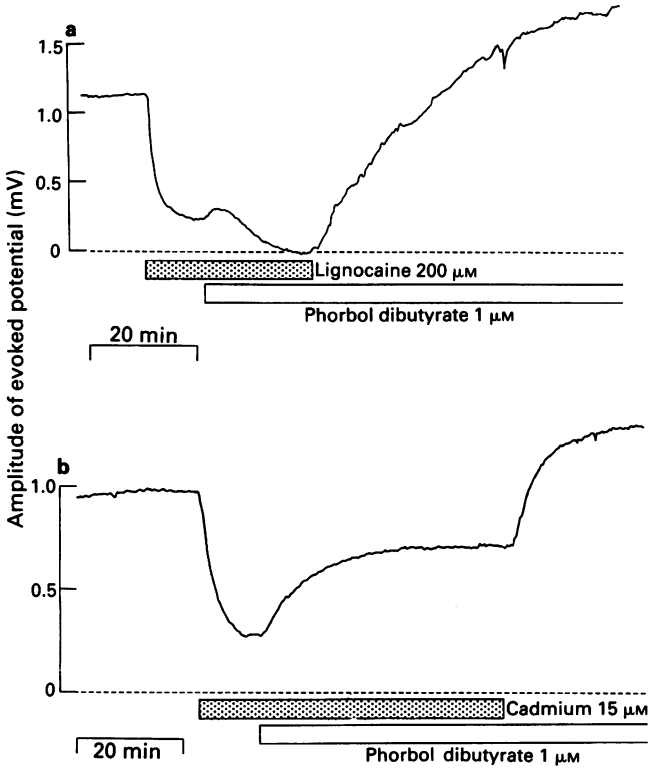

Figure 3 (a) A time-course of the peak height of the synaptically-evoked potential showing the effect of application of lignocaine 200 μM followed by the addition of phorbol dibutyrate 1 μM during the periods marked by the bars. (b) A similar time-course for another slice showing the effect of phorbol dibutyrate 1 μM applied in the presence of Cd 15 μM.

attained was greater than that seen before lignocaine application. The final amplitude was similar to that in experiments where phorbol dibutyrate was applied without the intervention of lignocaine (a 58% increase in amplitude compared to that in normal solution in Figure 3a; compare Figure 2 and see below).

In 3 experiments (see also Scholfield & Smith, 1989), the synaptic response was depressed by Cd, an agent which reduces Ca influx into nerve terminals (Kuan & Scholfield, 1986). Figure 3b is an example where the response was reduced by 15 μM Cd. When phorbol dibutyrate was added there followed a large and sustained enlargement of the evoked potential compared to the transient effect in the presence of lignocaine (Figure 3a). When the Cd was washed out, the evoked potential again increased to a level greater than that before the addition of Cd.

The above observations indicate an increased potency of lignocaine in the presence of phorbol dibutyrate. Therefore, to assess the potency of lignocaine, concentration-depression curves were constructed. Lignocaine was applied in serially increasing concentrations from 20–500 μM producing a 13% to 100% depression (Figure 4a). On washing out the anaesthetic, the amplitude of the evoked potential recovered over a period of 20–60 min. After a 30 min application of 1 μM phorbol dibutyrate, lignocaine was again serially applied starting at a concentration of 5 μM. Figure 4a shows a concentration-effect curve for lignocaine. In all the 10 slices tested, the potency of lignocaine was increased. Thus in normal solution, the concentration producing a 50% depression (EC_{50}) was 93 ± 12 μM and this was decreased to 25 ± 2.4 μM in the presence of phorbol dibutyrate ($P < 0.0001$). This represents an averaged increase in potency of 3.47 ± 0.14 fold. Both concentration-effect curves had the same shape. Because of the prolonged action of phorbol dibutyrate (>12 h, see also Scholfield & Smith, 1989), it was impossible to observe a recovery.

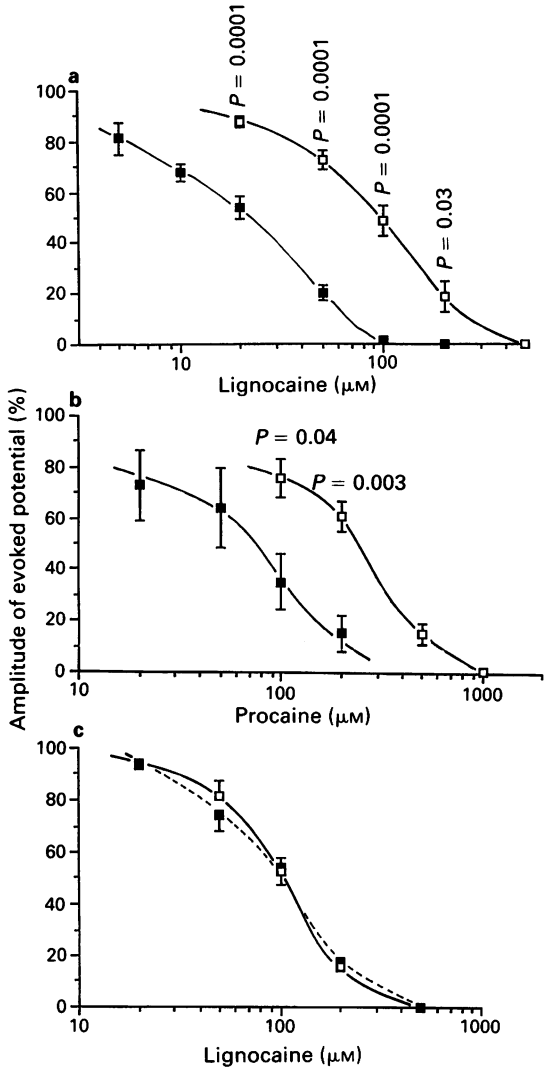

Figure 4 Dose-response curves to (a) lignocaine ± phorbol dibutyrate, (b) procaine ± phorbol dibutyrate and (c) lignocaine ± phorbol monobutyrate: (□) experiments in normal solution and (■) in the presence of phorbol ester 1 μM. The ordinate scale is the amplitude of the peak of the evoked potential expressed as a percentage of that in the same solution before adding local anaesthetic.

When the phorbol diester was added, the elevation of the evoked response was always sustained as in Figure 3b (see also Scholfield & Smith, 1989). In contrast, the elevated response to phorbol dibutyrate showed fade if lignocaine had been previously applied and washed out. Although the preparation appeared to recover completely from the effect of lignocaine, a concentration of anaesthetic which is normally subthreshold may remain in the tissue which in the presence of phorbol diester becomes effective. Therefore a minimum washout time for lignocaine of twice the recovery time was used to minimise this problem.

Procaine

To verify that the enhanced potency was not unique to lignocaine, we also tested another local anaesthetic, procaine, over the concentration range 100–1000 μM (Figure 5). Again there was a substantial increase in the potency of procaine in the presence of 1 μM phorbol dibutyrate. To obtain the same depressant effect compared to those obtained before adding phorbol dibutyrate, the concentration range of procaine used was 20–200 μM (Figure 4b). Thus the EC_{50} for procaine in normal solution was 264 ± 23 and in phorbol ester 49 ± 9 μM, a 5.49 ± 0.82 fold increase in potency ($P = 0.0002$, $n = 5$). As with lignocaine, there was a parallel shift of the concentration-

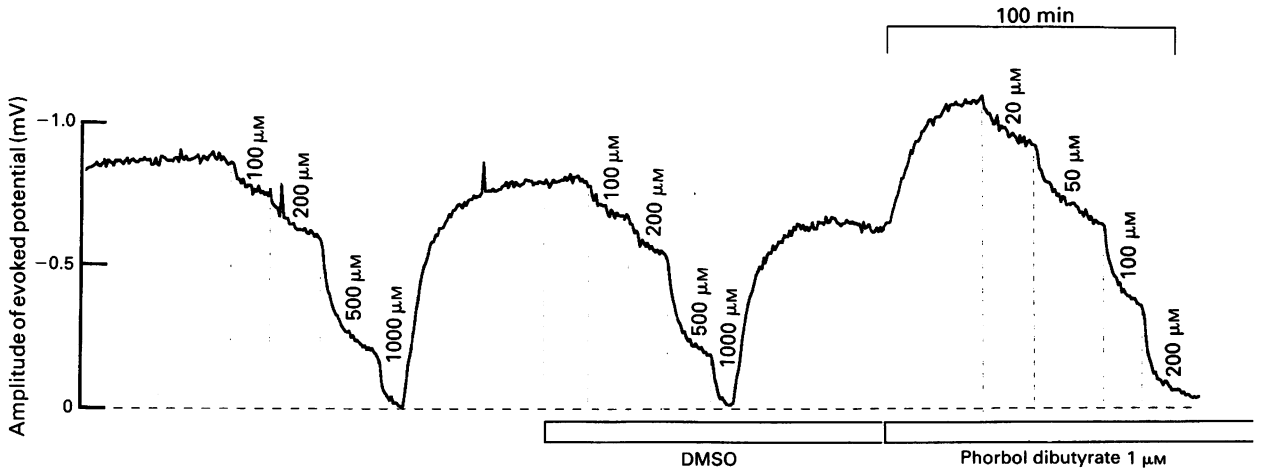

Figure 5 A time-course for the application of sequentially increasing concentrations of procaine. The first four concentrations (100–1000 μM) were applied in normal solution. The procaine was washed out and dimethylsulphoxide (DMSO, 0.2 μl ml^{-1} of bathing solution) added and the same concentrations of procaine applied. After recovery, 1 μM phorbol ester in DMSO was added. After the effect of phorbol dibutyrate on the amplitude of the evoked response had equilibrated, procaine was added (20–200 μM).

effect curve to the left. There was no change in the response to procaine when DMSO was added without the phorbol dibutyrate (Figure 5).

The action of 0.1 μM phorbol dibutyrate

In many systems, 1 μM phorbol dibutyrate is considered a relatively high concentration. Further experiments were performed with lignocaine and 0.1 μM phorbol dibutyrate. In two experiments, after 30 min incubation in 0.1 μM phorbol dibutyrate when the increase in the amplitude of the evoked potential had reached an equilibrium (see also Scholfield & Smith, 1989), there was little apparent change in lignocaine potency. A further 5 slices were preincubated in 0.1 μM phorbol dibutyrate for 120 min before retesting lignocaine. Under these conditions, lignocaine potency was substantially increased: the EC_{50} was 78.4 $\pm$ 13.6 μM in normal solution and 31.6 $\pm$ 3.1 μM in 0.1 μM phorbol dibutyrate, a 2.7 $\pm$ 0.6 fold increased potency ($P = 0.03$).

Effect of temperature

Most of the experiments were performed at 25°C. It is likely that both lignocaine and phorbol diester (via protein kinase C) interact at the plasma membrane (see Discussion) and since lipid membranes undergo phase changes with temperature, it was important to verify the effect of lignocaine on this preparation at more physiological temperatures. At 36°C, the EC_{50} in normal solution was 124.3 $\pm$ 30.8 mM and in phorbol dibutyrate 1 μM it was 31.8 $\pm$ 6.5 μM, a 4.0 $\pm$ 0.7 fold increase in lignocaine potency ($P = 0.001$, $n = 5$). The increase in potency was not significantly different from that at 25°C ($P = 0.36$).

Lignocaine and phorbol monobutyrate

Of the phorbol esters, only the β-phorbol 12,13-diesters activate protein kinase C. Therefore β-phorbol 13-monobutyrate was tested in 5 slices and this had no effect on the evoked response ($P = 0.14$) nor did it influence the potency of lignocaine ($P = 0.9$) (Figure 4c).

Lignocaine and Cd

In a previous study, the response to phorbol diester was enhanced by procedures which reduce nerve terminal Ca influx (Scholfield & Smith , 1989). Since reduced Ca itself can increase lignocaine potency (Hille, 1977), further experiments were performed in a concentration of Cd (10–20 μM) which produced about a 70% depression of the evoked potential. Serial concentrations of lignocaine were then applied alone and after a 30 min application of phorbol dibutyrate. The potency of lignocaine was the same or slightly less than in the absence of Cd: the EC_{50} in the presence of Cd was 67.2 $\pm$ 6.3 and 24.0 $\pm$ 5.8 μM with phorbol dibutyrate, a 3.2 $\pm$ 0.5 fold increase in potency after phorbol dibutyrate application ($P = 0.0036$, $n = 5$).

Relationship between increased lignocaine potency and enhanced transmitter release

To test this, two types of experiment were performed: (a) The increased transmitter release induced by phorbol dibutyrate appears to result from increased availability or increased effectiveness of intracellular Ca (Scholfield & Smith, 1989). Therefore to compensate for this, the Ca concentration of the bathing solution was adjusted from the usual 2.5 mM to 1.1–1.5 mM until the amplitude of the evoked potential was the same or slightly less than that seen before the addition of phorbol dibutyrate. Under these conditions, phorbol dibutyrate increased the effectiveness of lignocaine at least as effectively as it did without Ca compensation. The EC_{50} concentration for lignocaine in normal solution was 90.8 $\pm$ 8.7 μM and 18.2 $\pm$ 2.9 μM in low Ca and 1 μM phorbol dibutyrate, a 5.8 $\pm$ 1.5 fold increased potency ($P = 0.003$, $n = 5$). (b) Although the exact nature of synaptic transmission in the rat olfactory cortex is uncertain, it probably relies on an acidic amino acid or small peptide (Collins, 1986). Since phorbol esters increase both stimulated and resting transmitter release (Pozzan *et al.*, 1984), the possibility exists that the postsynaptic cells are continuously depolarized in the presence of phorbol dibutyrate. To test this, lignocaine was applied in the presence of glutamate (0.2–5 mM, 3 slices). Glutamate itself produced up to a 50% depression of the evoked response presumably as a result of its depolarizing action. The potency of lignocaine was unaffected by any of the concentrations of glutamate. (c) All experiments with lignocaine (irrespective of temperature) or procaine (see below) were pooled but no significant correlation between the effect of transmitter release and increased local anaesthetic potency was found ($r = 0.20$ by linear regression).

Interaction of lignocaine and phorbol dibutyrate on presynaptic axonal currents

Na and K currents were measured in thin slices. Five preparations were equilibrated with 100 μM lignocaine. On adding 1 μM phorbol dibutyrate there was no change in the amplitude of either the Na or K currents.

Discussion

The present experiments clearly show that phorbol dibutyrate increased the potency of the two local anaesthetics tested. Four pieces of evidence indicate that this was independent of the enhancement of the evoked potential: (i) Compensation for the phorbol ester induced transmitter release by reducing the Ca did not interfere with the enhanced lignocaine potency. (ii) There was a temporal dissociation between the enhanced transmitter release and the effect on the potency of lignocaine. (iii) In the presence of 100 nM phorbol dibutyrate, there was a period when the evoked potential was fully enhanced whereas the increase in the potency of lignocaine was at that stage, poorly developed. (iv) The effect of phorbol dibutyrate on the potency of lignocaine could not be replicated by glutamate, the putative excitatory transmitter in this preparation.

The increased synaptic response in the presence of phorbol dibutyrate appears to result from increased transmitter release (Scholfield & Smith, 1989). Because this effect was increased with low Ca concentrations and with Cd, it was considered to result from increased Ca influx or increased Ca effectiveness. The present results suggest that the phorbol ester-lignocaine interaction was unaffected by Cd and this also distinguishes it from the enhanced transmitter release.

In the present study, the site at which lignocaine and phorbol dibutyrate were interacting is unclear. However, the slower onset of the enhanced local anaesthetic action would be compatible with a postsynaptic action since dendrites in this brain area have a smaller surface area/volume ratio than the presynaptic axons (Gracey & Scholfield, 1990).

The mechanism by which local anaesthetics interact with axonal membrane proteins has been a matter of some dispute. They may act directly on membrane lipids thereby interfering with membrane proteins (Mullins, 1954; Lee, 1976; Lipnick, 1989). On the other hand, local anaesthetics might interact directly with the lipophilic parts of membrane proteins (Weber & Changeux, 1974; Franks & Lieb, 1984; Greenberg & Tsong, 1984). Considering the mass of data on the stimulation of protein kinase C by phorbol diesters, it would be reasonable to conclude that phorbol dibutyrate was also acting on protein kinase C in the present study. The protein kinase C may become membrane bound and phosphorylate membrane proteins including those forming ion channels and receptors (Messing *et al.*, 1986; Caratsch *et al.*, 1986; Baranyi *et al.*, 1988). But how this phosphorylation might influence the effectiveness of a local anaesthetic remains a matter of speculation.

Protein kinase C isozymes are particularly abundant and widely distributed in brain. The actions of substances acting like local anaesthetics could therefore be profoundly affected by these and perhaps by other kinases. The present experiments suggest that axonal conduction is unlikely to be affected by protein kinase C activation. However, lignocaine is widely used to reduce cardiac excitability and phorbol diesters and protein kinase C activation have a negative inotropic and chronotropic effect on the heart (Yuan *et al.*, 1987). Thus the state of these enzymes in the heart may profoundly affect the effect of some anti-arrhythmics.

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α-Adrenoceptor subtypes in dog saphenous vein that mediate contraction and inositol phosphate production

[1]Peter E. Hicks, Martine Barras, *Geneviève Herman, *Philippe Mauduit, J. Michael Armstrong & *Bernard Rossignol

Department of Pharmacology, Recherche Syntex France, 91310 Leuville-sur-Orge and *Laboratoire de Biochimie des transports cellulaires (CNRS VRA 1116), Université de Paris-Sud, 91405 Orsay, France

1 Studies have been made of the contractile responses to the α-adrenoceptor agonists phenylephrine (Phen), cirazoline (Cir) or BHT-920 (BHT) in dog isolated saphenous vein (DSV) rings, using the antagonists yohimbine (Yoh), idazoxan (Idaz), prazosin (Praz), WB-4101 (WB) and nitrendipine or zero Ca^{2+} medium.

2 Contractile concentration-response curves to Phen or BHT were displaced to the right of controls by Yoh (0.01–3 μM) with mean apparent antagonist dissociation constants (pK_Bs) of 7.9 and 8.6 respectively. Yoh did not show simple competitive antagonism against either agonist, since the Schild plot slopes were significantly less than unity. Neither the antagonist affinity of Yoh against Phen, nor the slope of the Schild plot was modified in the presence of catecholamine uptake inhibitors, nor in the presence of α,β-methylene ATP, which desensitizes P_2-purinoceptors, suggesting that Phen does not release ATP, or noradrenaline to cause contraction in DSV. In the presence of Praz (0.3 μM) the antagonist potency of Yoh (mean pK_B 7.4) against Phen was slightly decreased. Yoh had low potency against responses induced by Cir (pK_B 6.3).

3 WB (0.001–1.0 μM) was a very potent antagonist of Phen-induced contractions, however, the biphasic Schild plot against Phen could be separated into two affinity sites, a high pK_B of 9.3 (equivalent to that obtained using Cir as the agonist; pK_B 9.6) and a lower affinity (pK_B 8.6). WB showed an even lower antagonist affinity (pK_B 7.4) against BHT-induced contractions, suggesting that these effects might be mediated by α_{2A}-adrenoceptors. Praz also appeared to identify two sites using Phen-induced contractions, a high pK_B of 8.4 was equivalent to that obtained with Cir (pK_B 8.2) and a lower affinity site (pK_B 7.7; pA_2 7.6; slope 1.1) at which Praz showed competitive antagonism. Higher concentrations of Praz were required to antagonize contractions to BHT (pK_B 5.9).

4 Idaz was a weak partial agonist in this tissue with threshold contractile effects at concentrations in excess of 3 μM. Idaz (0.1–1 μM) competitively antagonized the contractile effects of BHT, but showed low antagonist affinity against Phen at these concentrations.

5 Contractions to Phen were slightly antagonized by nitrendipine (1 μM), with a 36% decrease in E_{max}. Contractions to Phen and Cir were also markedly attenuated in zero calcium medium (with EGTA), but maximum responses of 4.2 ± 0.1 and 3.6 ± 0.1 g, could be obtained with these agonists respectively. Only part of the contractile effects to Phen or Cir are therefore due to calcium influx (but L-type channels are not totally implicated), while the contractile effects of BHT were abolished in zero Ca^{2+} medium. Yoh (0.1 μM) retained its antagonist effects on Phen-induced responses in zero Ca^{2+} medium.

6 The formation of inositol phosphates (InsPs) in the presence of lithium (10 mM) was measured after incubation of intact DSV strips with myo-2-[^{3}H]-inositol. Phen (1–1000 μM) and Cir (0.01–10 μM) induced concentration-dependent increases in total labelled $InsP_{1-3}$, but BHT showed minimal InsP stimulation. InsPs were recovered after Phen (100 μM) stimulation (10 min) as labelled $InsP_1$ (71%), $InsP_2$ (25%) and $InsP_3$ (4%). Phen (100 μM)-stimulated $InsP_{1-3}$ formation was significantly antagonized by Praz (10 nM), but was not fully inhibited even after Praz 1 μM. Yoh and Praz (0.1 and 1.0 μM) were equipotent inhibitors of this response, while Idaz (0.3 μM) showed no effects.

7 The receptors in DSV which are stimulated by Phen to cause contraction show characteristics of the α_{1A}-adrenoceptor (high pM antagonist affinity for WB-4101 and extracellular calcium sensitivity) and the α_{1B}-adrenoceptor (contraction in calcium-free medium, increase in InsP and low nM antagonist affinity of WB). The paradoxical results obtained with Yoh (potent antagonist effects on Phen-stimulated PI and pK_B 7.9 on contraction) and Praz (low affinity competitive antagonist of Phen-induced contraction, pK_B 7.7 and failure to inhibit completely the PI response at 1 μM), cannot fully exclude an α_{2B}-subtype characterization of these responses. These pharmacological differences suggest that the adrenoceptor involved in the contractile and in particular the second messenger effects of Phen in DSV is not typically an α_{1B}-adrenoceptor.

Introduction

The dog saphenous vein (DSV) is known to contain a mixed population of postsynaptic α_1- and α_2-adrenoceptors (Sullivan & Drew, 1980; De Mey & Vanhoutte, 1981; Shepperson & Langer, 1981; Constantine *et al.*, 1982; Fowler *et al.*, 1984; Alabaster *et al.*, 1985; Flavahan & Vanhoutte, 1986a; Guimarães *et al.*, 1987; Eskinder *et al.*, 1988) and as such is a widely used preparation for the study of α-adrenoceptors. In addition, the α-adrenoceptor pharmacology in DSV shows striking similarities to human saphenous vein (Beckeringh *et al.*, 1987; Eskinder *et al.*, 1988).

The majority of evidence for an α-adrenoceptor heterogeneity in DSV comes from the use of selective α_1 and α_2-adrenoceptor agonists and antagonists. However, contractions evoked by phenylephrine (Phen) (usually considered as α_1; McGrath, 1982) are only weakly antagonized by prazosin (Praz) in DSV (Shoji *et al.*, 1983; Alabaster *et al.*, 1985; Akers

[1] Author for correspondence.

et al., 1987; Hicks et al., 1987, Guimarães et al., 1987; Eskinder et al., 1988). Furthermore Guimarães et al. (1987) have concluded that Phen (and also methoxamine) can stimulate α_2-adrenoceptors to cause contraction in DSV, since after partial receptor occlusion with the irreversible antagonist phenoxybenzamine, the contractions to these agonists were potently antagonized by yohimbine (Yoh), at concentrations which also blocked the contractions induced by the α_2-selective agonist UK-14304 (Cambridge, 1981). Stimulation of α_2-adrenoceptors by Phen has recently been implicated in human resistance vessels (Hair et al., 1988). However, a number of observations are not consistent with Phen acting as an α_2-adrenoceptor agonist in DSV. Rauwolscine (pA_2 8.7 vs BHT-920 or xylazine; Flavahan et al., 1984, or UK-14304; pA_2 8.5; Alabaster et al., 1985) was shown to be less potent against Phen-induced contractions in this tissue (pA_2 6.9; Alabaster et al., 1985).

It is known that Phen increases $^{45}Ca^{2+}$ uptake in DSV (Janssens & Verhaeghe, 1984), which is resistant to blockade by nifedipine (Jim et al., 1985). Furthermore calcium antagonists are less effective as antagonists of Phen-induced responses in this tissue relative to their marked inhibitory effects against responses induced by α_2-adrenoceptor agonists such as UK-14304 (Flavahan & Vanhoutte, 1986a; Guimarães et al., 1987), BHT-920 (Cooke et al., 1985; Hicks et al., 1988) or the aminotetralin M7 (Cavero et al., 1983). Phen-induced contractions in DSV are therefore at least partly dependent on calcium entry, although not entirely through dihydropyridine-sensitive calcium channels. In contrast to the α_2-adrenoceptor agonist BHT-920, Phen can also stimulate intracellular calcium release as shown by $^{45}Ca^{2+}$ efflux (Janssens & Verhaeghe, 1984; Jim & Matthews, 1985).

At least two second messenger systems are known to be involved in agonist-mediated intracellular Ca^{2+} release. G protein(s) functionally couple receptors to phospholipase C, which subsequently hydrolyses membrane bound phosphatidylinositol-4,5-bisphosphate to form inositol 1,4,5-trisphosphate (Ins[1,4,5]P_3) which then releases calcium from the endoplasmic reticulum (Berridge & Irvine, 1984; 1989; Abdel-Latif, 1986). A second pathway involves the formation of diacylglycerol which activates protein kinase C and may be responsible for a sustained response and/or promotion of calcium entry. A number of investigations on phosphoinositide (PI) hydrolysis stimulated by α-adrenoceptor agonists, including Phen, have been reported in blood vessels (Zeleznikar et al., 1983; Campbell et al., 1985; Fox et al., 1985; Heagerty et al., 1986; Chiu et al., 1987; Ollerenshaw et al., 1988; Eid & De Champlain, 1988; Tsujimoto et al., 1989); however, it is difficult to ascertain the absolute affinity of Praz in antagonizing these effects, since many workers have only used high concentrations of the antagonist to block the PI-response.

In the course of our studies on DSV we have routinely observed Yoh to be a potent antagonist of Phen-mediated contractions in this tissue, consistent with reported literature (see above), but with higher antagonist affinity than usually associated with Yoh at classical α_1-adrenoceptors in other tissues (Weitzell et al., 1979; Drew, 1985), with the possible exception of rat aorta (Ruffolo et al., 1981). Phen can readily contract DSV in calcium-free medium (Janssens & Verhaeghe, 1984) suggesting intracellular calcium release. These observations, coupled with the recent proposal that phenylethylamine agonists such as Phen or methoxamine can apparently stimulate α_2-adrenoceptors (Guimarães et al., 1987) in DSV particularly under conditions of low receptor reserve, has therefore prompted further analysis of the contractile and second messenger (InsP formation) effects of this agonist with the aim of clarifying the identity of the α-adrenoceptor subtype involved in these effects.

Part of this work has been presented in abstract form to the British Pharmacology Society (Barras et al., 1989) and the 7th meeting on Adrenergic mechanisms, PORTO, Oct. 1989, (Hicks et al., 1989).

Methods

Preparation

Saphenous veins were obtained from mongrel dogs of either sex under pentobarbitone (35 mg kg^{-1}, i.v.) anaesthesia. Tissues were either used fresh or after 24 h at 4°C storage in oxygenated Krebs bicarbonate. The contractions induced by either KCl (80 mM), or Phen (0.1–100 μM) were not significantly different between fresh or 24 h-stored tissues as previously reported (Eskinder & Gross, 1986). Veins cleared of connective tissue were cut into rings of approximately 5 mm length and were denuded of endothelium by carefully rubbing with forceps. The success of this technique was shown in selected tissues by demonstrating the failure of acetylcholine (ACh) to relax a Phen-mediated contraction. Although ACh-induced endothelium-dependent relaxations are smaller in DSV than in other arteries, they can nevertheless be demonstrated in the presence of an intact endothelium.

Functional responses

Preparations were mounted in 10 ml organ baths on steel hooks, under 2 g resting tension in Krebs bicarbonate solution (PSS) at 37°C bubbled with 95% O_2 and 5% CO_2. The PSS was of the following composition (mM): NaCl 117, KCl 4.7, $NaHCO_3$ 25, $MgSO_4$ 1.2, KH_2PO_4 1.0, $CaCl_2$ 2.5, glucose 11 and contained propranolol (1 μM). Except for a specific study, inhibitors of neuronal or extraneuronal uptake were not routinely included since the contractile effects of Phen or BHT-920 were shown not to be modified in DSV by desipramine or corticosterone. Isometric contractile concentration-response curves were obtained in separate preparations using Phen, BHT-920 (BHT) or cirazoline (Cir) and responses displayed on a Gould BS-274 chart recorder. Two consecutive concentration-response curves were obtained to either BHT or Phen before incubating tissues with the antagonist. Antagonists were evaluated at varying concentrations, only one concentration being used in each preparation ($n = 4$–7 tissues/group). Appropriate concentrations of antagonists were added to the bath for a period of 30 min before repeating a second or third agonist-response curve. As the effects of cirazoline washed out slowly, only one concentration-response curve per preparation was obtained to this agonist in vehicle or antagonist-treated DSV rings after an initial contraction to KCl (80 mM) which served as a 100% reference response.

For studies in Ca^{2+}-free medium, preparations were first contracted with KCl (80 mM) in Krebs containing [Ca^{2+}] 2.5 mM, followed by 1 h incubation in zero Ca^{2+}-Krebs in the presence of EGTA (2 mM). Phen, Cir and BHT were then studied under these conditions. In a further series of experiments, concentration-response curves to Phen were constructed in separate preparations ($n = 6$/group) incubated for 30 min with or without Yoh (0.1 μM).

Incorporation of [^{3}H]-inositol into phospholipids

DSV rings were prepared as described above and then cut open into rectangular strips (weighing about 10–17 mg). These were incubated at 37°C for 3 h in PSS buffer supplemented with 5.5 mM glucose and propranolol (1 μM), containing myo-2-[^{3}H]-inositol (60 μCi ml^{-1}) and equilibrated in 95% O_2; 5% CO_2.

A preliminary study on the time course of incorporation of [^{3}H]-inositol into membrane phospholipids, shows that equilibrium between intracellular medium and phospholipids is reached after 3 h of incubation.

After this time DSV strips were washed three times with unlabelled PSS buffer containing 10 mM LiCl and further incubated in PSS for 10 min. Results from this study show that a significant increase of the label (about 30%) into total phosphatidylinositol phosphates (PtdInsPs) occurred after stimulation with Phen (100 μM; for 10 min; Table 1).

Table 1 Incorporation of [^{3}H]-inositol into membrane phospholipids and the recovery of labelled $InsP_1$, $InsP_2$, $InsP_3$ and total $InsP_{1-3}$ in control (non-stimulated) or phenylephrine (Phen 100 μM, for 10 min)-stimulated dog saphenous vein strips in the presence of 10 mM LiCl

	Total PtdIns	*$InsP_{1-3}$*	*$InsP_1$* (c.p.m. mg^{-1} tissue)	*$InsP_2$*	*$InsP_3$*
Control (n = 10)	6422 ± 1518	270 ± 85 (100%)	183 ± 27 (67%)	58 ± 11 (21.5%)	29 ± 6 (11%)
Phen(100 μM)(n = 11)	8638 ± 2397	1760 ± 203* (100%)	1257 ± 154* (71%)	432 ± 65* (25%)	72 ± 17 (4%)
Stim. over basal	29%	552%	587%	645%	148%

Values shown in parentheses are the percentage recovery of individual InsPs with respect to total $InsP_{1-3}$. Stimulation over basal is the % increase with respect to non stimulated controls. Total PtdInsPs are PtdIns, PtdInsP and $PtdInsP_2$.
* Significantly greater than control (P less than 0.05)

Measurement of labelled inositol phosphate production

Separate groups of tissues were then treated for an additional 30 min with either vehicle or antagonists under study. In these experiments, preparations were stimulated with either Phen (1–1000 μM), Cir (0.1–10 μM) or BHT (0.1–10 μM) for 10 min periods. Total recovery of [^{3}H]-InsPs comprised $InsP_1$ (71%), $InsP_2$ (25%) and $InsP_3$ (4%) after stimulation with Phen (100 μM; for 10 min; Table 1). No measurement of InsP isomers was attempted. Each incubation was stopped by filtration and plunging each tissue (in 1 ml of ice-cold H_2O) into liquid N_2. Strips were then lyophilised and weighed. After addition of TCA (7.5% wt/vol), acid extraction of water-soluble inositol metabolites was performed and each tube further frozen at −20°C for 30 min. Subsequent thawing of frozen tissue was sufficient to liberate all the InsPs. This simple manoeuvre obviated the tissue homogenization which is extremely difficult in blood vessels, due to the large proportion of elastic/fibrous components.

After centrifugation for 15 min at 20,000 g at 0°C, the inositol phosphate-containing supernatants were treated (5 × 4 ml) with water-saturated diethylether to remove TCA and then neutralized at pH between 6 and 7 with 100 mM Tris base. The supernatants were applied to anion-exchange columns containing 800 mg of Bio-Rad AG 1-X8, (200–400 mesh, formate form, Richmond, Ca). Inositol and the different inositol phosphates were eluted stepwise according to Berridge (1983) by the successive addition of solutions containing increasing amounts of ammonium formate. An aliquot of each fraction was counted for radioactivity after addition of an equal amount of scintillant (Ready gel, Beckmann). The 20,000 g pellets containing the [^{3}H]-inositol-rich phospholipids were dissolved in 2 ml of 1N NaOH, and the radioactivity content was determined by liquid scintillation.

Calculation of results

Functional responses Concentration-contractile response curves to the various agonists were obtained in the presence of various concentrations of antagonists and the results represented graphically.

Antagonist concentration-ratios (CR) were then calculated at the EC_{50} level of the agonist in the presence of each concentration of antagonist. Apparent antagonist dissociation constants (pK_B) were calculated for each concentration of antagonist by the methods of Furchgott (1972). Where quoted, mean pK_B refers to the averaged values over a given antagonist concentration-range. For the experiments conducted with Cir, EC_{50} values calculated for control tissues were used for the calculation of K_B.

$$\text{where } K_B = \frac{\text{concentration of antagonist } [-\log \text{M}]}{\text{CR} - 1}$$

Where relevant, pA_2 values were calculated by the method of Arunlakshana & Schild (1959). Due to the biphasic nature of some of the Schild plots, differences in pK_B values were also statistically evaluated between treatment groups at different concentrations of the antagonist using a non-paired Student's t test. Significance was accepted at 5%.

Calculation of labelled inositol phosphates The amounts of each labelled inositol phosphate ($InsP_1$, $InsP_2$ and $InsP_3$) were cumulated and expressed as c.p.m. mg^{-1} lyophilised tissue. Total [^{3}H]-InsP formation in response to increasing concentrations of agonist was calculated and expressed as stimulation over basal in non-treated or antagonist treated preparations. In some experiments tissue dry weight was correlated with protein content by the method of Lowry *et al.* (1951). One mg lyophilized tissue was equivalent to 0.4 mg protein and represented about 24% of wet tissue weight.

Drugs

The following drugs were used: yohimbine HCl (Sigma); WB-4101 (*N*-[2-(2,6-dimethoxyphenoxy)-ethyl]1,4-benzodioxane-2-methylamine, Research Biochemicals Inc.); (−)-phenylephrine HCl (Sigma); BHT-920 HCl ([2-amino-6-allyl-5,6,7,8-tetrahydro-4*H*-thiazolo-(4,5-d)azepine] Boehringer Ingleheim); idazoxan HCl and prazosin HCl (synthesized by Syntex Palo Alto); (±)-propranolol HCl (Sigma), cirazoline HCl (Synthelabo); nitrendipine (synthesized by Syntex France); myo-2-[^{3}H]-inositol (Amersham); α,β-methylene adenosine triphosphate HCl (α,β-MeATP) and β,γ-methylene adenosine triphosphate HCl (β,γ-MeATP) (Sigma); desipramine HCl (Sigma); corticosterone acetate (Sigma). Corticosterone was dissolved in ethanol, all other compounds were solubilized and diluted in distilled water.

Results

Functional responses in dog saphenous vein

Concentration-dependent contractile-response curves obtained to Phen or BHT-920 were progressively displaced to the right of controls by Yoh (0.001–3 μM) with little change in the maximum responses to these agonists (Figure 1). In control tissues three subsequent response curves to these agonists were superimposible. High concentrations of Yoh (10 μM) were, however required to displace the Cir-induced contractile response curves to the right of controls (Figure 1c). The calculated mean pK_B value for all concentrations of Yoh against Phen was 7.9 ± 0.2 and against BHT was 8.6 ± 0.1. Schild analysis of these data gave apparent pA_2 values of 8.2 and 8.8 respectively, although the slope of each Schild plot was less than unity (Figure 2a; Table 2). Log(CR − 1) data calculated for either agonist in the presence of Yoh were significantly different at each concentration of antagonist tested (Figure 2a). However, strict competitive antagonism was not demonstrated by Yoh against either Phen or BHT.

Incubation of DSV rings with desipramine (1 μM) and corticosterone (40 μM) did not modify the concentration-response curve to Phen, the antagonist affinity or the slope of the Schild plot for Yoh against Phen (Table 2).

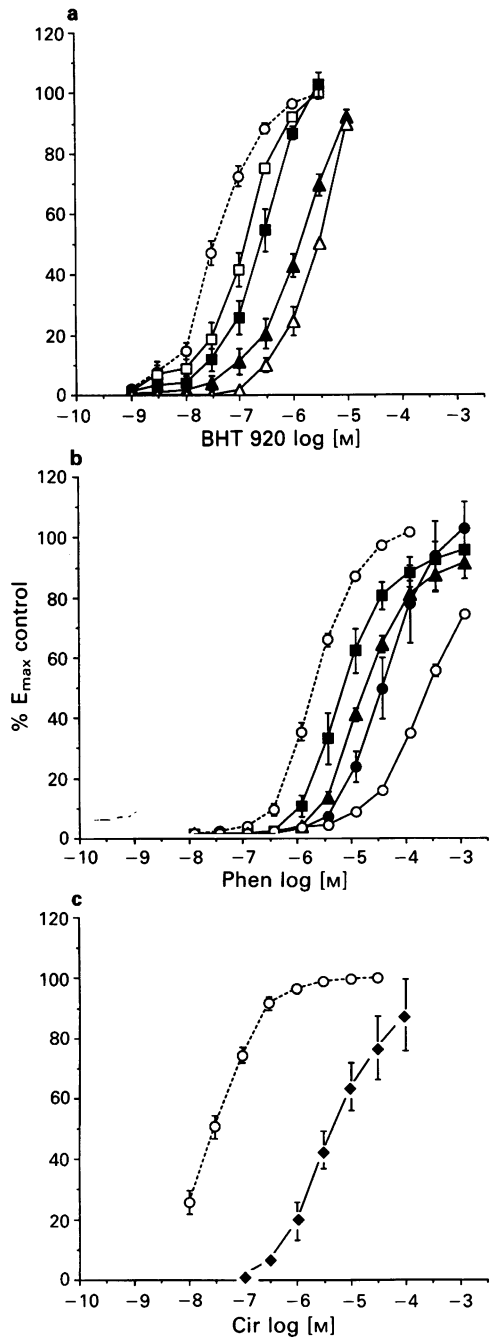

Figure 1 Contractile concentration-response curves for (a) BHT 920, (b) phenylephrine (Phen) and (c) cirazoline (Cir) in dog isolated saphenous vein rings in the absence (○- - - -○), or presence of different concentrations of yohimbine (μM): 0.001 (□- - - -□); 0.01 (■———■); 0.1 (▲———▲); 0.3 (△———△); 1 (●———●); 3 (○———○), and 10 (◆———◆). Results are expressed as % max response to each agonist with s.e. mean shown by vertical bars. n = 4–7 preparations/curve.

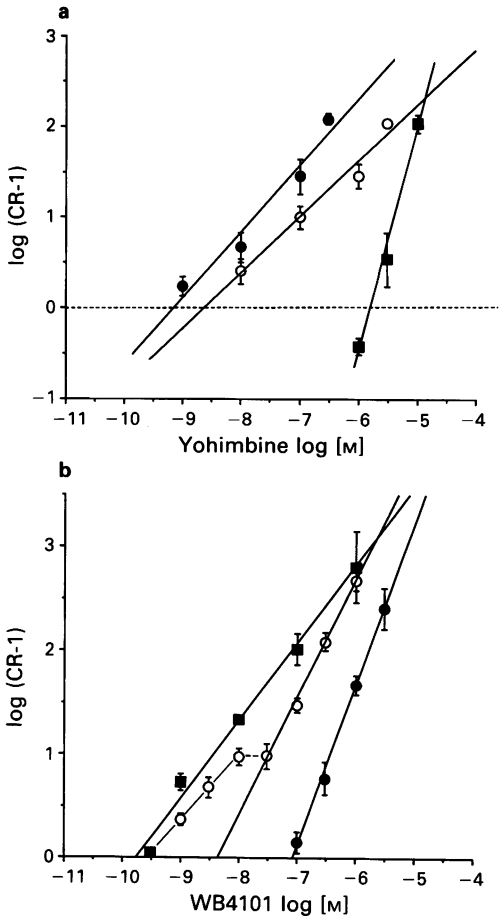

Figure 2 Schild plots (log[concentration-ratio] − 1 versus negative log[M] of antagonist) for yohimbine (a) or WB-4101 (b) against contractile responses induced by cirazoline (■), phenylephrine (○) or BHT-920 (●). Each point represents the mean of 4–7 preparations at each concentration; vertical bars show s.e.mean.

Yoh was a weak affinity antagonist (mean pK_B of 6.3 ± 0.2; Figure 2a; Table 2) against Cir-induced contractions, but a greater antagonist affinity value was obtained after Yoh (10 μM; 6.9 ± 0.1), than at 3 μM (pK_B 6.2 ± 0.1).

Praz (1 μM) failed to antagonize the contractile effects of BHT and Praz (0.01 − 1 μM) showed relatively weak antagonist effects against Phen-induced contractions. A 'high' affinity antagonist effect on Phen-induced responses was seen with Praz at 0.01 μM (pK_B 8.4) with a lower affinity antagonist effect over the concentration-range 0.1–1 μM, which was apparently competitive (pK_B 7.7, pA_2 7.6; slope 1.1; Table 2). Against responses to Cir, only one antagonist affinity value for Praz could be derived (mean pK_B 8.2 ± 0.1; pA_2 8.4, slope 0.8; Table 2).

In the presence of Praz (0.3 μM), the antagonist effects of Yoh (mean pK_B 7.4) against Phen were slightly decreased, however, the antagonist potency of Yoh against BHT-920 was not altered by Praz (1 μM; Table 2).

The α_2-adrenoceptor antagonist Idaz (0.1–1 μM) caused concentration-dependent, parallel rightward displacements of the BHT response curve (not shown). The calculated antagonist affinity for Idaz against BHT responses (mean pK_B 7.5; pA_2 7.6; slope 0.9; Table 2) was higher than against Phen (pK_B 6.2) or Cir (pK_B 5.9)-induced responses. At higher concentrations Idaz (3–100 μM) caused weak contractile effects in DSV.

WB-4101 (WB: 0.001–1 μM) progressively displaced the Phen-induced contractile response curve to the right of controls (Figure 3) and was the most potent antagonist tested. Over the range 0.001–0.03 μM WB, the response curves to Phen were not displaced proportionally to the increases in antagonist concentration. Close inspection of the Schild plot for WB showed two apparent slopes for this antagonist against Phen (Figure 2b) and allowed the calculation of a high affinity (mean pK_B 9.3; interpolated pA_2 9.4) and a lower affinity (mean pK_B 8.6; interpolated pA_2 8.4). WB also showed high antagonist affinity against responses induced by Cir and was a competitive antagonist against this agonist over the concentration-range 0.0003–1 μM (mean pK_B 9.6; pA_2 9.7; slope 0.8; Table 2).

Responses induced by BHT were antagonized by WB over the concentration-range 0.1–3 μM (Figure 2b) with a mean pK_B

Table 2 Relative antagonist affinities (pA_2 or mean pK_B) of α-adrenoceptor antagonists against contractions elicited by BHT-920, cirazoline (Cir) or phenylephrine (Phen) in dog saphenous vein

Agonist		*Yoh*	*Praz* High	*Praz* Low	*Yoh + Praz*	*Idaz*	*WB-4101* High	*WB-4101* Low
BHT-920	pA_2	8.8	—	—	—	7.6	7.1	
	slope	0.7a	—	—	—	0.9	1.4a	
	pK_B	8.6 ± 0.1*	—	5.9 ± 0.2b	8.7 ± 0.1b	7.5 ± 0.2	7.4 ± 0.2*	
Cir	pA_2	6.1	8.4	—	—	—	9.7	—
	slope	1.4	0.8	—	—	—	0.8	—
	pK_B	6.3 ± 0.2*	8.2 ± 0.1*	—	—	5.9 ± 0.2	9.6 ± 0.2	—
Phen	pA_2	8.2		7.6	8.1	—	9.4	8.4
	slope	0.6a	—	1.1	0.6a	—	0.9	0.9
	pK_B	7.9 ± 0.2	8.4 ± 0.1e	7.7 ± 0.2	7.4 ± 0.2c	6.2 ± 0.1	9.3 ± 0.2e	8.6 ± 0.1
Phen/desipramine/corticosterone								
	pA_2	8.6						
	slope	0.5a						
	pK_B	7.9 ± 0.2		NT	NT	NT	NT	
Phen/α,β-MeATP								
	pK_B	7.9 ± 0.2d		NT	7.6 ± 0.2c	NT	NT	

Yoh = yohimbine, Praz = prazosin, Idaz = idazoxan, α,β-MeATP, α,β-methylene ATP
* Significantly different from Phen, P less than 0.05.
(a) Significantly different from unity, P less than 0.05.
(b) Prazosin at 1 μM; (c) prazosin at 0.3 μM; (d) yohimbine at 0.1 μM. (e) Significantly different from Phen low, P less than 0.05.
Slope calculated from log(CR − 1) vs −log[M] antagonist; pK_B = −log M antagonist dissociation constant.
NT, not tested.

7.4. The slope of the Schild plot was however, greater than unity (Table 2).

Incubation of DSV rings with the P_2-purinoceptor desensitizing agent α,β-MeATP (10 μM) or the P_2-receptor agonist β,γ-MeATP (100 μM), evoked contractile responses (5.0 ± 0.5 g, and 6.2 ± 0.6 g respectively; n = 5/group). These contractile effects waned to base-line tension within 10 min. Incubation of a further group of DSV (n = 5) with α,β-MeATP (10 μM) abolished the contractile effects induced by β,γ-MeATP (100 μM).

In the presence of α,β-MeATP (10 μM) and Praz (0.3 μM) the contractile response curves to Phen were unchanged from those obtained in the absence of the P_2-receptor desensitizing agent. The apparent antagonist affinity of Yoh against Phen (0.1 μM; pK_B 7.9 ± 0.2) was not significantly modified by α,β-MeATP and Praz (Table 2).

Effects of nitrendipine and zero calcium medium on responses to phenylephrine

Concentration-dependent contractile effects of Phen were displaced slightly to the right of controls by nitrendipine (1 μM) in a non-competitive manner with a 36% decrease in the maximum effect of Phen (Figure 4b). Nitrendipine (10 μM) failed to modify further the contractions induced by Phen (not shown). These concentrations of nitrendepine were far greater than those required to antagonize voltage-operated calcium channels in this tissue, since the pIC_{50} for nitrendipine to relax KCl (80 mM)-induced contractions was 8.7 ± 0.2; n = 8.

In zero calcium medium, Phen caused concentration-related contractions over the range 1 to 1000 μM and although these responses were markedly reduced with respect to control tissues, Phen was capable, under these conditions, of evoking contractions of 4.2 ± 0.8 g tension (Figure 4b). Under the same conditions of zero calcium, Cir was also capable of contracting DSV (2.8 ± 0.5g), but BHT responses were abolished (Figure 4a). Under normal incubation conditions, Yoh (0.1 μM) displaced the Phen-induced contractile response curve to the right of controls (Figure 4c). In the presence of Yoh (0.1 μM) the Phen-induced contractile response curves in zero calcium medium were also displaced to the right of control non-Yoh-treated tissues, albeit with depression of the maximum response (Figure 4d).

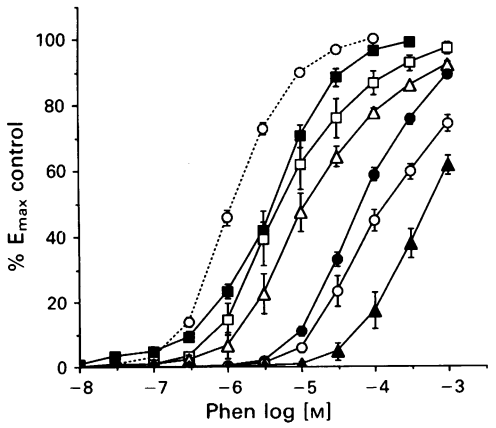

Figure 3 Contractile concentration-response curves for phenylephrine (Phen) in dog isolated saphenous vein rings in the absence (○- - - -○; n = 26), or presence of different concentrations of WB-4101. Concentrations (μM) are 0.001 (■———■); 0.003 (□———□); 0.03 (△———△); 0.1 (●———●); 0.3 (○———○); 1 (▲———▲). Results are expressed as % max response to Phen; vertical bars show s.e.mean. n = 4–7 preparations for each concentration of WB.

Measurement of labelled inositol phosphate production

Preparations of DSV incubated with [^{3}H]-inositol for 3 h readily incorporated the label into membrane polyphosphoinositides (PtdIns). A typical elution profile for labelled $InsP_1$, $InsP_2$ and $InsP_3$ is shown in two DSV strips after 10 min stimulation with 100 μM Phen or under non-stimulated conditions (Figure 5).

Small amounts of glycerophosphoinositol (GroPIns) were measured under basal conditions, which remained unchanged after Phen stimulation. $InsP_1$ and $InsP_2$ increased about 7 fold after Phen (100 μM), while $InsP_3$ increased 2.5 times at 10 min (Table 1).

Phen (1 to 1000 μM) and Cir (0.1–10 μM) caused concentration-dependent increases above base-line in total [^{3}H]-$InsP_{1-3}$ formation (10 min stimulation), while BHT showed only weak InsP stimulating effects, which were not concentration-related (Figure 6b). The comparative contractile and InsP stimulating effects of Phen, Cir and BHT are also shown in Figure 6. Cir and BHT were more potent contractile agonists than Phen in DSV, while Cir showed greater intrinsic contractile effects than the other agonists.

A single concentration of Phen (100 μM) was used to stimulate total [^{3}H]-$InsP_{1-3}$ formation in antagonist studies. Praz (0.01 μM) inhibited by 34% the Phen-stimulated PI response,

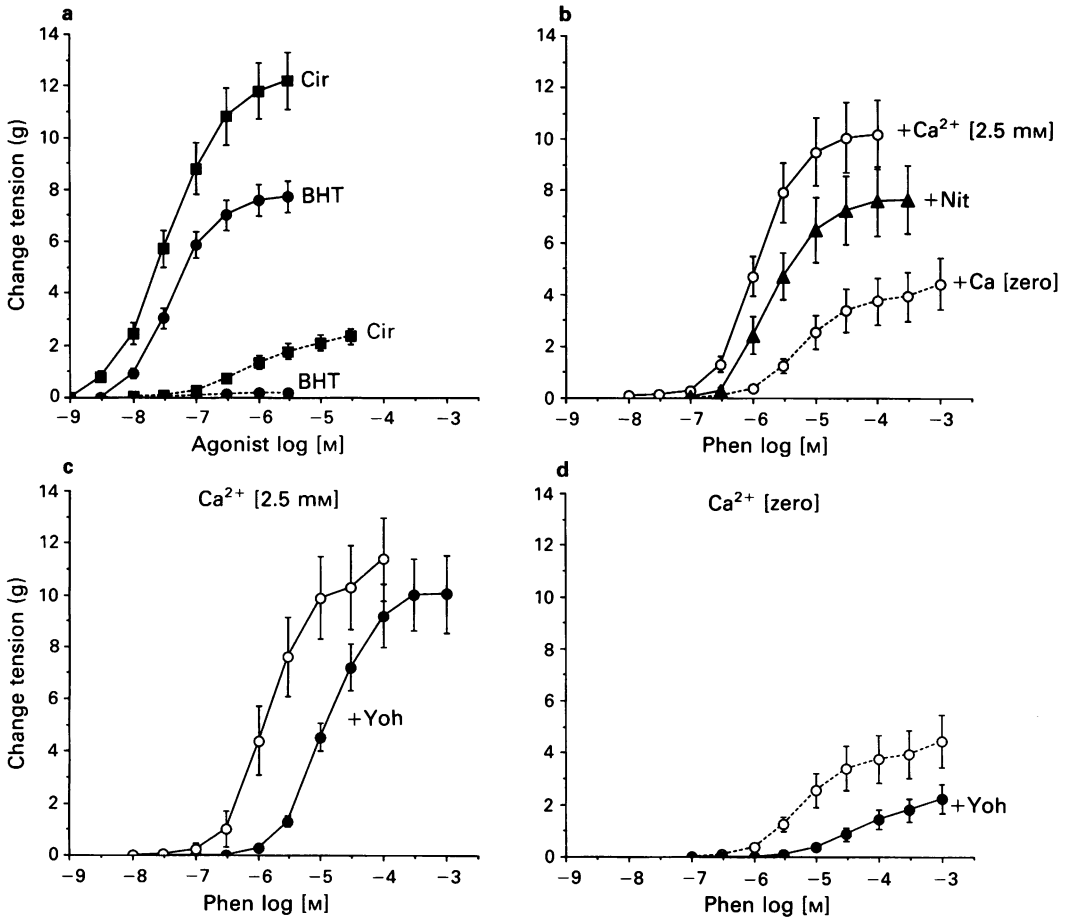

Figure 4 (a) Concentration effect response curves (change tension, g) for cirazoline (Cir, ■) or BHT-920 (●) in dog saphenous vein in calcium 2.5 mM Krebs (———) or calcium-free medium containing EGTA (- - - -). (b) Contractile concentration-response curves (change tension, g) for phenylephrine (Phen) in dog isolated saphenous vein rings in calcium [2.5 mM] in the absence (O———O) or presence of nitrendipine (Nit 1.0 μM; ▲———▲), or in zero calcium medium containing EGTA (Ca[zero]: O- - - -O). (c) Concentration-response curves (change tension, g) for Phen in calcium 2.5 mM Krebs, before (O———O) and after 30 min treatment with yohimbine (Yoh; 0.1 μM; ●———●). (d) Concentration-response curves (change tension, g) for Phen in zero calcium medium (Ca^{2+} [zero]), before (O- - - -O) or after 30 min treatment with yohimbine (Yoh; 0.1 μM; ●———●). Vertical bars indicate s.e.mean.

but even the highest concentration of Praz (1 μM), failed to inhibit completely these effects of Phen (Figure 7). Yoh (0.01 μM), showed no inhibitory effects on Phen-stimulated InsP formation; however, at 0.1 μM and 1.0 μM both Praz and Yoh showed equivalent antagonist effects on this response (Figure 7). Although Idaz showed contractile effects in DSV at concentrations above 3 μM, Idaz showed no effects on basal InsP formation at 0.3 μM and at this concentration failed to inhibit Phen-stimulated InsP formation (Figure 7).

Discussion

The results of this study confirm previously published data that Phen-induced contractions of the DSV are potently antagonized by Yoh (Sullivan & Drew, 1980; Guimarães *et al.*, 1987). However, the rather weak antagonist effects of Praz previously reported against this agonist in DSV (Alabaster *et al.*, 1985, Akers *et al.*, 1987; Eskinder *et al.*, 1988; Shi *et al.*, 1989), comprise high and low affinity sites. In contrast to Yoh, idazoxan was a weak antagonist of Phen-induced contractions. WB-4101 was a very potent antagonist of Phen and Cir-induced contractile responses in DSV and demonstrated two apparent affinity values against Phen, which were both higher than the affinity against BHT-induced contractile responses. Contractions to Phen were only partly dependent on extracellular Ca^{2+}, since responses (although much reduced) were still obtained in zero calcium medium. Nitrendipine showed weak inhibitory effects on Phen-induced contractions, suggesting a minimal influence of L-type calcium channels in these responses. Phen and Cir (at high concentrations), but not BHT, increased InsP production in a concentration-dependent manner in DSV and the effects of Phen were potently antagonized by Yoh, but not by Idaz.

Antagonism by Yoh of both Phen-induced contractions and PtdIns hydrolysis, might imply that these effects are mediated through α_2-adrenoceptors, as suggested by Guimarães *et al.* (1987). However, preliminary results in DSV which show rauwolscine to be inactive on Phe-stimulated InsP formation (Rees & Matthews, 1986) and weakly active on contraction (Alabaster *et al.*, 1985) and failure of Idaz to block either Phen (present study) or NA-stimulated InsP production in rat femoral vein (Stubbs *et al.*, 1988) strongly argue against an α_2-adrenoceptor involvement in PtdIns hydrolysis in this or other tissues.

Can the Phen-induced contractile effects in DSV be considered an α_2-response? The current nomenclature identifies at least two subtypes of α_2-adrenoceptors (Bylund, 1988). α_{2A}-Sites are labelled with high affinity in various tissues with antagonists which include Yoh, rauwolscine and Idaz. Human α_2-platelet clone (α_2-C10; Regan *et al.*, 1988) is defined as α_{2A}. Affinity values for Praz obtained at α_{2A}-adrenoceptors are usually in the high nM to low micromolar range, consistent with pA_2 determinations in functional tests. The contractile responses of DSV to agonists such as BHT (and UK-14304; Alabaster *et al.*, 1985) are, therefore, likely to be α_{2A}-adrenoceptor mediated.

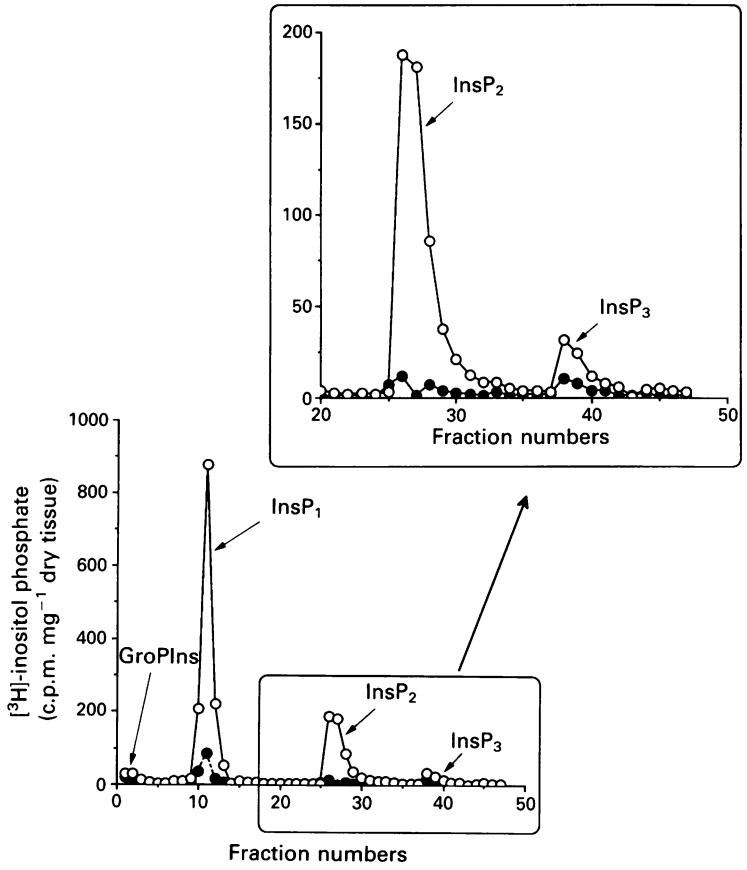

Figure 5 Typical elution profile of [^{3}H]-inositolphosphates (glycerophosphoinositol; GroPIns; $InsP_1$; $InsP_2$ and $InsP_3$; c.p.m. mg^{-1} dry tissue) under basal conditions (●) or after 10 min stimulation with phenylephrine (○, 100 μM) in dog isolated saphenous vein strips.

The failure of Praz to block contractions to BHT (pK_B 5.9 present study) except at high concentrations, is consistent with the potency of Praz at presynaptic α_2-sites in functional studies (see Drew, 1985 and refs therein) and on [^{3}H]-rauwolscine or yohimibine binding in cerebral cortex and other tissues (Cheung *et al.*, 1982; Bylund *et al.*, 1988; Bylund, 1988; Michel *et al.*, 1989b), or human α_2-platelet clone (α_2-C10; Regan *et al.*, 1988). While Yoh showed higher antagonist potency against contractile responses to BHT in DSV, than normally associated with its presynaptic α_2-adrenoceptor antagonist effects (Ruffolo *et al.*, 1981), Idaz showed considerable selectivity for BHT-induced contractile responses in DSV (pK_B 7.5) compared with Phen (pK_B 6.2). The adrenoceptors which are stimulated by Phen to cause contraction and InsP formation in DSV do not therefore appear to be α_{2A}-adrenoceptors.

α_{2B}-Adrenoceptors have been characterized by use of [^{3}H]-yohimbine (or rauwolscine) in kidney from several animal species (Summers, 1984, Michel *et al.*, 1989b) and neonatal rat lung (Latifpour *et al.*, 1982). cDNA human kidney clone (α_2-C4; Regan *et al.*, 1988) is considered representative of the α_{2B}-adrenoceptor. Affinity values for Praz in the low nM range obtained with labelled Yoh or rauwolscine as ligands, also characterize an α_{2B}-site, although no functional correlate has so far been identified. To date, no postsynaptic α_2-adrenoceptor has been demonstrated to be coupled through phospholipase C and it therefore appears unlikely that the inhibitory effects of Yoh on Phen-stimulated PI are mediated by α_2-adrenoceptors. Finally, the relative agonist selectivities of both BHT-920 and Phen at α_{2A} or α_{2B}-adrenoceptors respectively, would need to be very high to account for the antagonist selectivity found with WB (80 fold selectivity for Phen over BHT contractions) and with Idaz, which retained a 15 fold selectivity for BHT over Phen. The relative selectivity/affinity of these agonists for α_{2A} or α_{2B}-sites, assessed on human α C_{10}/C_4 cloned receptors (Regan *et al.*, 1988), showed a 2 fold selectivity for Phen at the α_{2B}-site while BHT was not selective for either site.

How then can the receptors which mediate contraction and InsP formation to Phen in DSV be characterized? Firstly, the antagonist effects of Yoh against Phen occurred at slightly higher concentrations than those required to antagonize responses to BHT (5–8 fold difference in mean pK_B). The antagonist effects of Yoh against both of these agonists did not, however, show simple competitive antagonism, since the slopes of the Schild plots were less than unity. These observations have important implications, since it clearly shows the necessity of evaluating antagonists using small concentration increments over a wide range, particularly when heterogeneous receptor populations are present. Schild plots with slopes significantly less than unity, or which are biphasic can arise for a number of reasons (Kenakin, 1982; Milnor, 1986), although receptor heterogeneity remains a probability when other factors are controlled. The fact that inclusion of desipramine and corticosterone in the bathing medium did not modify the contractile-response curve to Phen, or change the antagonist affinity or the slope of the Schild plot for Yoh, suggests that Phen is not a good substrate for either neuronal or extraneuronal uptake in DSV. Phen does not appear to have an indirect sympathomimetic action, which by releasing endogenous noradrenaline, might account for the low Schild slopes obtained. The routine absence of uptake blockers in the present study should not therefore confound the results obtained. Nevertheless, a surprising finding in this study was that Praz, although weak (relative to its α_1-antagonist affinity in other tissues) as a blocker of Phen-induced responses in DSV (Alabaster *et al.*, 1985; Akers *et al.*, 1987; Eskinder *et al.*, 1988; Shi *et al.*, 1989), did not increase the antagonist affinity of Yoh against Phen when the two drugs were combined. This might have been expected if only two subtypes of α-

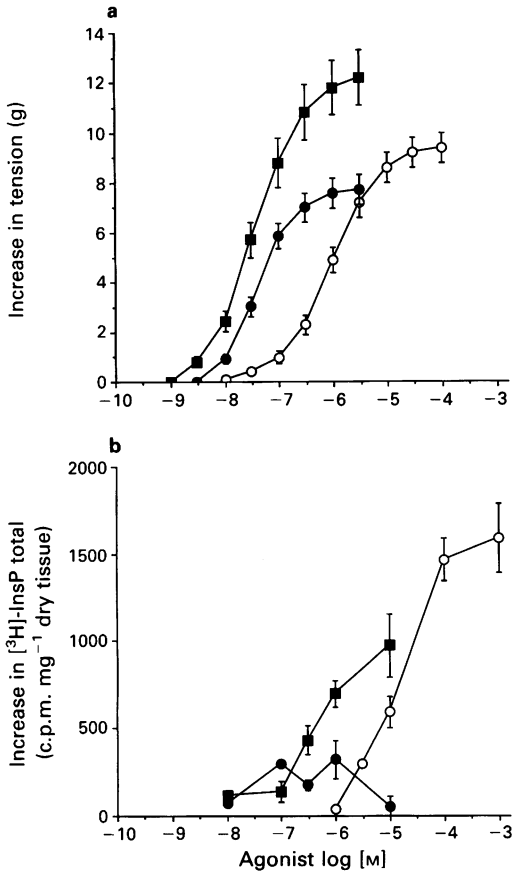

Figure 6 (a) Cumulative concentration-response curves (change tension, g) for cirazoline (■), phenylephrine (○) or BHT-920 (●) in dog saphenous veins in calcium 2.5 mM medium. (b) Concentration-response curves (increase in total [^{3}H]-InsP$_{1-3}$; c.p.m. mg^{-1} dry tissue) for cirazoline (■), phenylephrine (○) or BHT-920 (●) in dog saphenous vein in calcium 2.5 mM medium. Each point represents the mean value obtained from separate dog saphenous vein strips (n = 4–5/group); vertical bars show s.e.mean.

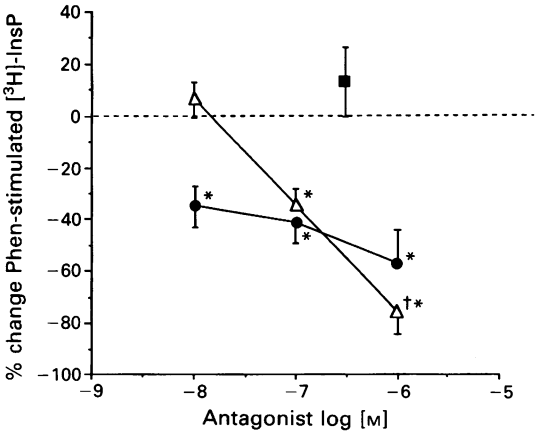

Figure 7 Inhibitory effects of yohimbine (△), prazosin (●) or idazoxan (■) on phenylephrine (Phen 100 μM)-stimulated total [^{3}H]-inositolphosphate production (InsP$_{1-3}$) in separate groups of dog saphenous vein strips. Inhibitory effects were calculated with respect to non-treated Phen-stimulated strips after correction for basal InsP values. 100% control values for Phen were 1887 ± 161 c.p.m. mg^{-1} for the yohimbine- and prazosin-treated groups and 1666 ± 104 c.p.m. ms^{-1} for the idazoxan group. Vertical bars indicate s.e.mean; n = 3–6 strips/point.
* Significant reduction from control tissues (P less than 0.05).
† Significant difference from yohimbine 10^{-7} M data (P less than 0.05).

adrenoceptor are present in this tissue and Phen was acting as a non-selective agonist. Indeed in rabbit saphenous vein where both α_1- and α_2-subtypes can be clearly identified using functional responses (Daly *et al.*, 1988a,b,c), the combination of rauwolscine and Praz did increase the potency of the α_2-adrenoceptor antagonist. It remains plausible that in DSV, the contractions to Phen which occur over the concentration-range 1–300 μM in the presence of both Praz (0.3 μM) and a high concentration of Yoh (3 μM) are not adrenoceptor-mediated.

The question of the 5-HT$_{1A}$ affinity of WB has previously been addressed by Morrow & Creese (1986), in considering the nanomolar affinity of this antagonist at α_{1B}-sites. Certainly WB has relatively high affinity for 5-HT$_{1A}$ receptors (Norman *et al.*, 1985); however, in DSV 5-HT-induced responses are mediated by 5-HT$_1$ like-receptors which are apparently not 5-HT$_{1A}$ (Humphrey *et al.*, 1988). Further, Phen has not been reported to have affinity at 5-HT sites to our knowledge.

The possibility that high concentrations of Phen might release ATP to cause contraction was also considered. Thus incubation of DSV rings with a combination of Praz (0.3 μM) and the P$_2$-receptor desensitizing agent α,β-MeATP (Sneddon & Burnstock, 1984) at a concentration that blocked the contractions of the P$_2$-receptor agonist β,γ-MeAPT, failed to modify either the contractile-response curve to Phen in the presence of Praz, or the antagonist affinity of Yoh. Phen does not therefore indirectly release ATP to cause sustained contraction through P$_2$-receptor mechanisms in DSV.

The range of antagonist dissociation constants reported in the literature for Yoh is considerable (Ruffolo *et al.*, 1981; Drew, 1985; Flavahan & Vanhoutte, 1986b) and it is clear that this antagonist shows higher potency in DSV against selective α_2-adrenoceptor agonists (pA$_2$ or pK$_B$ 8.2–8.7) than at prejunctional α_2-adrenoceptors (pA$_2$ or pK$_B$ 7.3–7.8) in other preparations. On the other hand Yoh was a weak antagonist against contractions induced by Cir (pK$_B$ 6.3; present study), in close agreement with values obtained by Cavero *et al.* (1983). This indicates further differences in the α-adrenoceptor antagonist affinities of Yoh and suggests that the receptor mediating Phen-induced contractions is not a 'classical' α_1-subtype. The contractile effects of Phen in DSV have been reported to be more sensitive to inhibition by phenoxybenzamine (Constantine *et al.*, 1982; Hicks *et al.*, 1988) than those induced by α_2-adrenoceptor agonists (Flavahan *et al.*, 1984; Ruffolo & Zeid, 1985; Guimarães *et al.*, 1987) and a greater receptor reserve exists for both Phen and other α_1-adrenoceptor agonists (Cooke *et al.*, 1985; Ruffolo & Zeid, 1985) compared with α_2-adrenoceptor agonists in this vessel. These findings would be entirely consistent with the greater α_1-adrenoceptor selectivity for phenoxybenzamine (Dubocovitch & Langer, 1974).

Phen-induced contractions in DSV are at least partly dependent on calcium entry, although not entirely through dihydropyridine-sensitive (L-type) calcium channels (Jim *et al.*, 1985). However, unlike the α_2-agonist BHT-920, Phen can also stimulate intracellular calcium release as evidenced by increased $^{45}Ca^{2+}$ efflux (Janssens & Verhaeghe, 1984; Jim & Matthews, 1985) and responses of DSV to these agonists in calcium-free medium (Janssens & Verhaeghe, 1984; present study). The present results which show that nitrendipine (1 μM) had minimal inhibitory effects on Phen-induced contractions entirely support these data. The fact that Phen-induced contractions can be evoked in calcium-free medium, confirms the significant role of receptor-mediated intracellular Ca^{2+} release in these responses. The relatively high antagonist potency of Yoh (0.1 μM) shown on Phen-induced contractile responses in zero calcium was not anticipated, but was difficult to quantify in terms of affinity, since the Phen-induced response curves in the presence of Yoh were displaced in a non parallel manner in these experiments. Nevertheless Yoh (0.1 μM) clearly antagonized the effects of Phen under zero extracellular calcium conditions.

The demonstration that Phen caused a concentration-

dependent increase in total InsP formation, suggests that this agonist stimulates a receptor which could be coupled through phospholipase C in DSV. Our assumptions are based on the increase in $InsP_1$ which makes up the largest percentage increase in total InsPs measured (71%) after 10 min of Phen stimulation in the presence of lithium. We have not attempted in this study to measure $Ins(1,4,5)P_3$, since the methodology of Berridge (1983), does not allow the separation of $InsP_3$ isomers. Preliminary results obtained with high performance liquid chromatography (h.p.l.c.), indicate that 74% of total $InsP_3$ was recovered as $Ins(1,4,5)P_3$ and 26% as $Ins(1,3,4)P_3$ following 10 min stimulation with Phen(100 μM) in DSV.

α_1-Adrenoceptors have recently been subclassified from functional studies (Han *et al.*, 1987) into α_{1A} (rat vas deferens) or α_{1B} (rat spleen), based on different molecular mechanisms used to evoke these responses. The high pM antagonist effects of WB on contractions to Phen and Cir are consistent with the presence of α_{1A}-sites in DSV and are close to the reported affinity of WB at this site as defined by binding studies (Minneman *et al.*, 1988; Michel *et al.*, 1989a). Although previous reports on Praz in DSV indicate a rather weak antagonist effect on Phen contractions, our present data indicate that Praz also identifies two sites in this vessel, with a high pK_B (8.4 at 10 nM) which is close to the pK_B of 8.2 calculated using Cir as the agonist. The high nM antagonist effects of Praz on Phen contractions (pK_B 7.7) appear to be competitive in nature and are close to previously reported values (Alabaster *et al.*, 1985; Akers *et al.*, 1987). It is tempting to speculate that the antagonist effects of both WB and Praz on Cir reflect an α_{1A}-selectivity of this agonist in this tissue.

In DSV the α-adrenoceptor stimulated by Phen to increase InsP shows similarities to the α_{1B} subtype in rat spleen (Han *et al.*, 1987), except that in DSV the increase in InsP formation was antagonized by yohimbine. Where tested, α-adrenoceptor-mediated InsP production (Minneman & Johnson, 1984; Fox *et al.*, 1985; Chiu *et al.*, 1987) or [^{3}H]-P incorporation into PtdIns (Zeleznikar *et al.*, 1983), were antagonized by Praz, but data reported thus far show that Yoh has low potency as an antagonist of α-adrenoceptor-mediated PI (Legan *et al.*, 1985; Han *et al.*, 1987) consistent with its affinity (pK_D 5.6) at cloned α_1 sites from the hamster (Cotecchia *et al.*, 1988). The absolute affinity of Praz for PI responses is, however, not easy to ascertain from the literature, since relatively high concentrations of Praz have often been used. Recently Michel *et al.* (1990), have reported complete inhibition by Praz (0.1 μM) of NA-stimulated PI in rat cerebral cortex. In DSV the inhibitory effects of Praz on Phen-stimulated PI were weaker than would be expected. Thus a 30% inhibition of this effect was obtained with 10 nM Praz, but even at 1 μM, Praz only inhibited this response by 60%. Furthermore, at concentrations of 0.1 and 1 μM, Praz and Yoh were equipotent (see Figure 7).

The antagonist potency of WB reported at α_{1B}-adrenoceptors in rat spleen against NA-induced contraction or InsP formation (pK_B 8.2: Han *et al.*, 1987) are close to the intermediate affinity values shown in DSV against Phen-induced contractions (Akers *et al.*, 1987; present study pA_2 8.4) and are also close to the low nM affinity WB sites identified with [^{3}H]-BE-2254 in other peripheral tissues i.e. vas deferens, liver. All of these affinity estimates for WB are intermediate between the high pM affinity WB sites (Minneman *et al.*, 1988; Michel *et al.*, 1989a) and the currently reported low potency α_{2A}-antagonist effects in DSV (pK_B 7.1) and suggest that α_{1B}-receptors are indeed present in DSV. In functional tests the α_2-adrenoceptor antagonist effects of WB (pA_2 6.6 against inhibition of the twitch response of rat proximal vas deferens by clonidine; Pigini *et al.*, 1988, or facilitation of stimulation-evoked release of [^{3}H]-NA in cat isolated spleen; IC_{50}: 0.2 μM: Massingham *et al.*, 1981) closely approximate the pK_B calculated on BHT-induced contractions in the present study. The effects of WB on Phen-stimulated PI are currently being examined in DSV.

The present results therefore agree with previously published data in DSV, which show that Phen-induced contractions are antagonized by Yoh at concentrations similar, but not identical to those required to block responses induced by α_2-adrenoceptor agonists. Idaz was significantly less potent against Phen that BHT-mediated contractions. WB was the most potent and selective antagonist tested against the contractile effects of Phen and identified two high affinity sites. The high pM affinity value was close to that identified when Cir was used as the agonist and probably represents effects at the α_{1A}-adrenoceptor. The low nM affinity site identified for WB on Phen could therefore be the α_{1B}-adrenoceptor. Prazosin has previously been reported to show low potency against Phen-stimulated contractions in DSV, but like WB also appears to identify two sites in this tissue. Both antagonists demonstrate competitive antagonism against the low affinity site stimulated by Phen. Finally, BHT-920 appears to be a relatively selective α_{2A}-adrenoceptor agonist in DSV. These data provide little support for the contention that Phen mediates contraction in DSV by stimulating 'classical' α_2-adrenoceptors (Guimarães *et al.*, 1987), although a receptor which shows some homology with the α_{2B}-subtype cannot yet be excluded, since the nM antagonist potency of Praz was similar to the potency of Yoh on Phen-stimulated contraction. Nevertheless, only high concentrations of Idaz antagonized the contractile effects of Phen and neither Yoh nor Idaz antagonized the effects of Cir at concentrations less than 1 μM.

The increase in InsPs in response to Phen and Cir indicates that InsPs are involved in adrenoceptor-response coupling in this tissue. By use of the PI response, further differentiation can be made between Phen and the α_{2A}-adrenoceptor agonist BHT. The fact that contractions evoked by BHT, but not Phen, were abolished in the absence of extracellular calcium, demonstrates an important role for receptor-mediated intracellular Ca^{2+}-release in the contractile effects of Phen. Current theory would predict that α-adrenoceptor-mediated increases in InsP are mediated by α_{1B}-adrenoceptors.

The results imply that multiple α-adrenoceptor subtypes exist in DSV. The receptor which is stimulated by Cir to cause contraction is competitively antagonized by WB with a pA_2 of 9.6 and by Praz with a pA_2 of 8.2 and is probably the α_{1A}-adrenoceptor. Yoh and Idaz both show antagonist effects in the μM range at this site. Contractile effects of BHT-920 are antagonized by Yoh and Idaz at concentrations consistent with known α_2-affinities of these compounds, while Praz was inactive at 1 μM. These data indicate the presence of α_{2A}-adrenoceptors on DSV. The receptors that are stimulated by Phen to evoke contraction in this tissue show certain characteristics of the α_{1A}-adrenoceptor, since WB demonstrated high pM antagonist affinity against these responses and the contractions were only partially inhibited by the L-channel calcium antagonist nitrendipine. Although a low nM antagonist affinity was shown with Praz on Phen and Cir-induced contractions, the predominant antagonist effect of Praz on Phen-induced responses, although competitive in nature, occurred at higher concentrations of the antagonist and was equivalent to the antagonist affinity of Yoh. Both the contractile effects of Phen in the absence of extracellular calcium and the InsP stimulating effects of Phen were antagonized by Yoh at 0.1–1 μM, but not by the imidazoline α_2-adrenoceptor antagonist Idaz. The inhibitory effects seen with Praz (10 nM) on Phen-stimulated InsP formation in DSV could represent α_{1B}-adrenoceptor antagonist effects of Praz; however, the fact that higher concentrations of Praz (0.1–1 μM) only inhibited this response up to 60%, suggests that these latter effects of Praz and those of Yoh on PI cannot readily be equated to the α_{1B}-receptor as currently defined. We consider that this site could represent an 'atypical' α-adrenoceptor. Further characterization of this site requires the identification of more selective antagonists.

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Effects of metabolic inhibitors on endothelium-dependent and endothelium-independent vasodilatation of rat and rabbit aorta

Cameron J. Weir, Ian F. Gibson & [1]William Martin

Department of Pharmacology, University of Glasgow, Glasgow G12 8QQ

1 Basal release of endothelium-derived relaxing factor (EDRF) rendered endothelium-containing rings of rat aorta 4.7 fold less sensitive to the contractile actions of phenylephrine and depressed the maximum response when compared with endothelium-denuded rings. The responsiveness and maximum response to phenylephrine was, however, similar in rings of rabbit aorta with or without endothelium.

2 Rotenone (1 nM–0.1 μM), an inhibitor of oxidative phosphorylation, induced a profound, irreversible blockade of phenylephrine-induced tone in endothelium-containing and endothelium-denuded rings of rat aorta, but induced only slight inhibition of tone in rings of rabbit aorta.

3 2-Deoxy glucose (10 mM), an inhibitor of glycolysis, had no effect on phenylephrine-induced contraction in endothelium-denuded rings of rat aorta, but inhibited reversibly the endothelium-dependent depression of contraction in endothelium containing rings. 2-Deoxy glucose had no effect on phenylephrine-induced contraction in rings of rabbit aorta with or without endothelium.

4 Rotenone (0.1 μM) inhibited acetylcholine-induced, endothelium-dependent relaxation of phenylephrine-contracted rings or rat and rabbit aorta. In endothelium-denuded rings of rat aorta, relaxation induced by glyceryl trinitrate of isoprenaline was also inhibited, but relaxation induced by 8-bromo cyclic GMP or dibutyryl cyclic AMP was not. Relaxation induced by verapamil on KCl-contracted, endothelium-denuded rings of rat aorta was also unaffected.

5 2-Deoxy glucose (10 mM) inhibited acetylcholine-induced, endothelium-dependent relaxation of phenylephrine-contracted rings of rat and rabbit aorta. In endothelium-denuded rings of rat aorta, relaxation induced by glyceryl trinitrate and by isoprenaline was also inhibited, but relaxation induced by 8-bromo cyclic GMP or dibutyryl cyclic AMP was not. Relaxation induced by verapamil on KCl-contracted, endothelium-denuded rings of rat aorta was also unaffected.

6 These data suggest that in rabbit and in rat aorta, rotenone inhibits acetylcholine-induced relaxation by inhibiting EDRF production, and by depressing smooth muscle sensitivity to EDRF, respectively. They further suggest that 2-deoxy glucose inhibits acetylcholine-induced relaxation in both tissues by depressing the sensitivity to EDRF, probably as a result of reduced synthesis of cyclic GMP. The additional possibility that 2-deoxy glucose inhibits EDRF production warrants further investigation.

7 The blockade by 2-deoxy glucose of the endothelium-dependent depression of phenylephrine-induced tone in rat aorta probably reflects blockade of the actions of spontaneously released EDRF.

Introduction

The vascular endothelial cell produces a powerful vasodilator substance, endothelium-derived relaxing factor (EDRF; Furchgott & Zawadzki, 1980), which relaxes vascular smooth muscle by stimulating soluble guanylate cyclase (Forstermann *et al.*, 1986; Ignarro *et al.*, 1986) and elevating cellular gua nosine 3′:5′-cyclic monophosphate (cyclic GMP) content (Holzmann, 1982; Rapoport & Murad, 1983). It has recently been shown that EDRF is nitric oxide (Palmer *et al.*, 1987; Ignarro *et al.*, 1987; Furchgott, 1988) the precursor of which in the endothelium is L-arginine (Palmer *et al.*, 1988).

Little is known of how endothelial production of EDRF is controlled. Production occurs in the resting state (Griffith *et al.*, 1984; Martin *et al.*, 1985) and can be increased further following chemical (Furchgott, 1984) or physical (Holtz *et al.*, 1984) stimulation. Calcium is clearly involved in basal as well as stimulated production of EDRF (Singer & Peach, 1982; Furchgott, 1983; Long & Stone, 1985; Griffith *et al.*, 1986), and this probably relates to the calcium-sensitivity of the enzyme, nitric oxide synthase (Palacios *et al.*, 1989). It has been proposed that stimulated, but not basal, production of EDRF requires metabolic energy (Griffith *et al.*, 1986; 1987). This conclusion was drawn from the finding that metabolic inhibitors such as rotenone powerfully inhibit stimulated but not basal endothelium-dependent vasodilatation in rabbit aorta. The inhibitor of glycolysis, 2-deoxy glucose, was much less effective suggesting that the required metabolic energy was derived from oxidative rather than glycolytic metabolism.

We wished to establish whether the dependence of EDRF production on oxidative metabolism was a generalised phenomenon by comparing the actions of rotenone and 2-deoxy glucose on endothelium-dependent relaxation in rabbit and rat aorta. We also examined the effects of these metabolic inhibitors on the sensitivity of the vascular smooth muscle of these preparations to the constrictor effects of phenylephrine and to the dilator effects of endothelium-independent relaxants.

Methods

Preparation of aortic rings and tension recording

The preparation of aortic rings was similar to that originally described by Furchgott & Zawadzki (1980). Briefly, male Wistar rats weighing 300–400 g and male New Zealand white rabbits weighing 2–3 kg were killed by stunning and exsanguination. The aorta was removed, cleared of adhering fat and connective tissue and cut into 2.5 mm wide transverse rings with a razor blade slicing device. Endothelial cells were removed from some rings by gently rubbing the intimal surface with a moist wooden stick for 30–60 s. Successful

[1] Author for correspondence.

removal of endothelial cells from aortic rings was confirmed later by the inability of acetylcholine (1 μM) to induce relaxation and in some experiments histological examination of endothelial integrity was performed by use of a silver staining technique (Poole *et al.*, 1958). Rat and rabbit aortic rings were then mounted under 1 g and 2 g resting tension, respectively, on stainless steel hooks in 12 ml organ baths, and bathed at 37°C in Krebs solution containing (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24, glucose 11 and disodiumedetate 0.03, and gassed with 95% O_2 and 5% CO_2. In experiments in which 2-deoxy glucose was used, the Krebs solution was identical to that described above except that glucose was omitted and replaced with 2-deoxy glucose (10 mM). Tension was recorded isometrically with Grass FTO3 transducers and displayed on Linseis chart recorders. Tissues were allowed to equilibrate for 90 min before experiments were begun, during which time the resting tension was maintained at the pre-set level.

For relaxation studies, submaximal (30–70%) tone was first induced with phenylephrine or KCl (60 mM). Relaxations were then expressed as percentage relaxation of phenylephrine- or KCl-induced tone.

Drugs

Acetylcholine chloride, 8-bromo cyclic GMP, 2-deoxy-D-glucose, dibutyryladenosine 3':5'-cyclic monophosphase (db cyclic AMP), (±)-isoprenaline hydrochloride, phenylephrine hydrochloride, rotenone and verapamil hydrochloride were obtained from Sigma. Glyceryl trinitrate was obtained from Napp Laboratories. All drugs were dissolved in twice distilled water except for rotenone which was dissolved in ethanol to give a stock solution of 1 mM.

Statistical analysis

Results are expressed as the mean ± s.e.mean and comparisons were made by means of Student's *t* test. A probability of 0.05 or less was considered significant.

Results

Phenylephrine-induced tone

Following contraction with phenylephrine (0.3 μM), rotenone (1 nM–0.1 μM) produced similar concentration-dependent relaxations in rings of rat aorta with or without endothelium (Figure 1). At concentrations above 0.1 μM, rotenone produced contractions that were mimicked by the solvent (ethanol) alone. The ability of rotenone to inhibit phenylephrine-induced tone in rat aorta was not reversed even with extensive washing. In endothelium-containing and endothelium-denuded rings of rabbit aorta, rotenone (1 nM–0.1 μM) produced only small relaxations of phenylephrine (0.3 μM)-induced tone (Figure 1).

Rings of rat aorta without endothelium were 4.7 fold more sensitive to the contractile actions of phenylephrine than endothelium-containing rings and displayed a significantly greater maximum contraction (Figure 2.). Following incubation for 20 min in glucose-free Krebs containing 2-deoxy glucose (10 mM), the maximum phenylephrine-induced contraction in endothelium-containing rings increased significantly with no significant change in the EC_{50} concentration (Figure 2). This augmentation of contraction was reversed when tissues were returned to normal glucose-containing Krebs and washed extensively. In endothelium-denuded rings of rat aorta the maximum phenylephrine-induced contraction and EC_{50} concentration were not significantly different from those obtained in normal glucose-containing Krebs (Figure 2).

Phenylephrine (1 nM–10 μM) induced similar concentration-dependent contractions in rings of rabbit aorta with or without endothelium. Incubation of endothelium containing or endothelium-denuded rings in glucose-free Krebs containing 2-deoxy glucose (10 mM) had no significant effect on phenylephrine-induced contractions (Figure 2).

A combination of rotenone (0.1 μM) and 2-deoxy glucose (10 mM) completely blocked, irreversibly, the ability of phenylephrine to contract both rabbit and rat aorta.

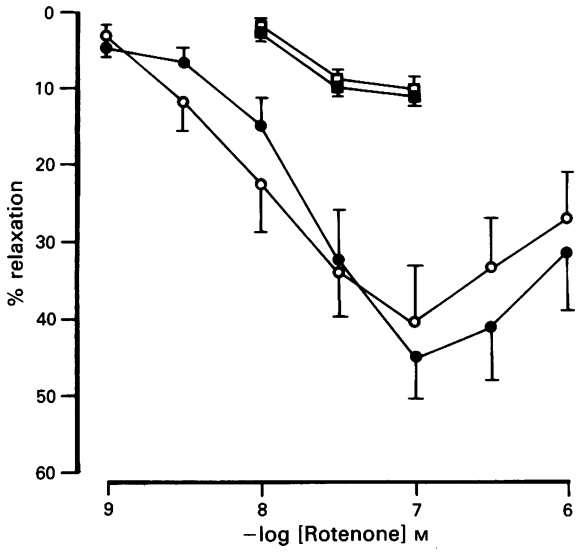

Figure 1 Concentration-response curves showing the ability of rotenone to inhibit phenylephrine (0.3 μM)-induced tone in endothelium-containing (●) and endothelium-denuded (○) rings of rat aorta and endothelium-containing (■) and endothelium-denuded (□) rings of rabbit aorta. Each point is the mean and vertical bars indicate the s.e.mean of 5–9 observations.

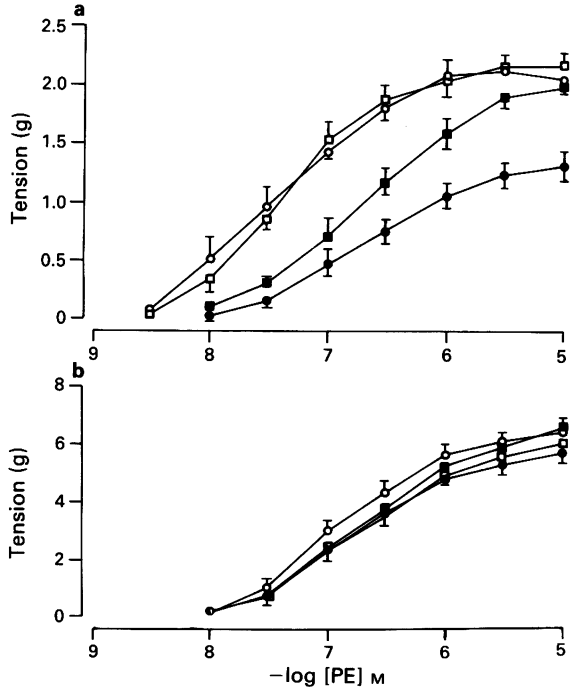

Figure 2 Concentration-response curves showing the contractile effects of phenylephrine (PE) on untreated endothelium-containing (●) and endothelium-denuded (○) rings of rat (a) and rabbit (b) aorta and responses obtained following treatment with 2-deoxy glucose (10 mM) on endothelium-containing (■) and endothelium-denuded (□) rings. Each point is the mean and vertical bars indicate the s.e.mean of 5–12 observations. * $P < 0.05$, indicates a significant difference in the maximum response from untreated, endothelium-containing rings.

Acetylcholine-induced relaxation

Following contraction with phenylephrine (0.3 μM), acetyl choline (3 nM–3 μM) induced concentration-dependent relaxation of endothelium-containing but not endothelium-denuded rings of rat and rabbit aorta (Figure 3).

Following treatment with rotenone (0.1 μM) for 20 min, acetylcholine-induced relaxation was inhibited in rings of rat and rabbit aorta (Figure 3). The ability of rotenone to depress acetylcholine-induced relaxation was only partially reversed following extensive washing. Following incubation for 20 min in glucose-free Krebs containing 2-deoxy glucose (10 mM), acetylcholine-induced relaxation was inhibited in endothelium-containing rings of rat and rabbit aorta (Figure 3). The ability of 2-deoxy glucose to depress acetylcholine-induced relaxation was reversed when tissues were returned to normal glucose-containing Krebs and washed extensively.

Glyceryl trinitrate and 8-bromo cyclic GMP

Treatment with rotenone (0.1 μM), inhibited glyceryl trinitrate (1 nM–1 μM)-induced relaxation in endothelium-denuded rings of rat but not rabbit aorta (Figure 4): in rat aorta the EC_{50} concentration was increased 4.3 fold, ($n = 4$), but the maximum relaxation was not affected (Figure 4). Rotenone (0.1 μM) had no effect, however, on the relaxation of endothelium-denuded rings of rat aorta induced by 8-bromo cyclic GMP (1–100 μM, Figure 4). The rotenone-induced depression of sensitivity to glyceryl trinitrate was reversed following extensive washing. Following incubation for 20 min in glucose-free Krebs solution containing 2-deoxy glucose (10 mM), glyceryl trinitrate-induced relaxation was inhibited in endothelium-denuded rings of rat and rabbit aorta (Figure 4): The EC_{50} concentration was increased 5.6 fold ($n = 8$), and 2.0 fold ($n = 6$), in rat and rabbit aorta, respectively, but only in rat aorta was there a significant reduction in the maximum relaxation (Figure 4). Treatment with 2-deoxy glucose (10 mM) had no significant blocking effect on relaxation of rat aortic rings induced by 8-bromo cyclic GMP except at a concentration of 30 μM (Figure 4).

In both rat and rabbit aorta the ability of 2-deoxy glucose to depress glyceryl trinitrate-induced relaxation was completely reversed when tissues were returned to normal glucose-containing Krebs and washed extensively.

Isoprenaline and dibutyryl cyclic AMP

Treatment with rotenone (0.1 μM), inhibited isoprenaline (10 nM–10 μM)-induced relaxation of endothelium-denuded

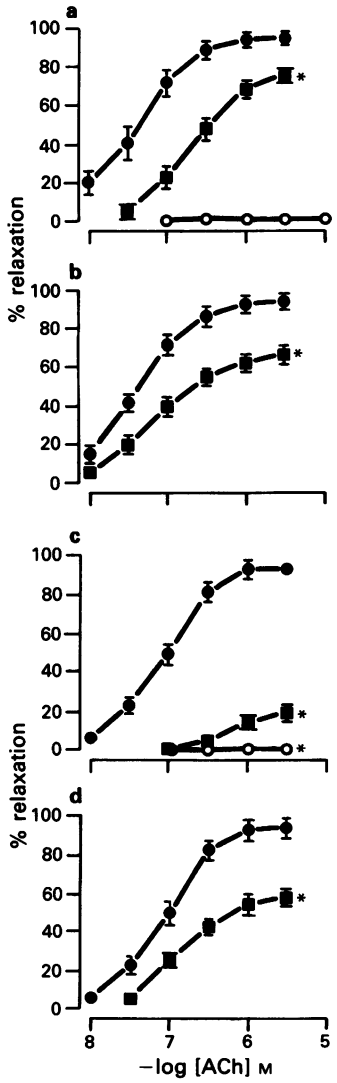

Figure 3 Concentration-response curves showing the relaxant effects of acetylcholine (ACh) on endothelium-containing (●) and on endothelium-denuded (○) rings of rat (a, b) and rabbit (c, d) aorta and on endothelium-containing rings following treatment (■) with rotenone (0.1 μM, a, c) or 2-deoxy glucose (10 mM, b, d). Each point is the mean and vertical bars indicate the s.e.mean of 4–8 observations. *$P < 0.05$ indicates a significant difference from the maximum relaxation obtained on untreated, endothelium-containing rings.

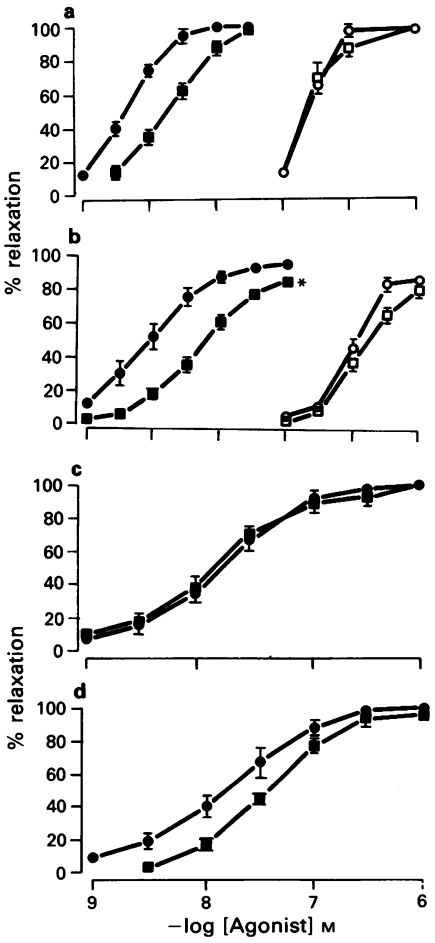

Figure 4 Concentration-response curves obtained on endothelium-denuded rings of rat (a,b) and rabbit (c,d) aorta showing the relaxant effects of glyceryl trinitrate (●) and 8-bromo cyclic GMP (○) before, and of glyceryl trinitrate (■) and 8 bromo cyclic GMP (□) after treatment with rotenone (0.1 μM, a,c) or 2-deoxy glucose (10 mM, b,d). Each point is the mean and vertical bars indicate the s.e.mean of 4–8 observations. *$P < 0.05$ indicates a significant difference from the maximum relaxation obtained on untreated rings.

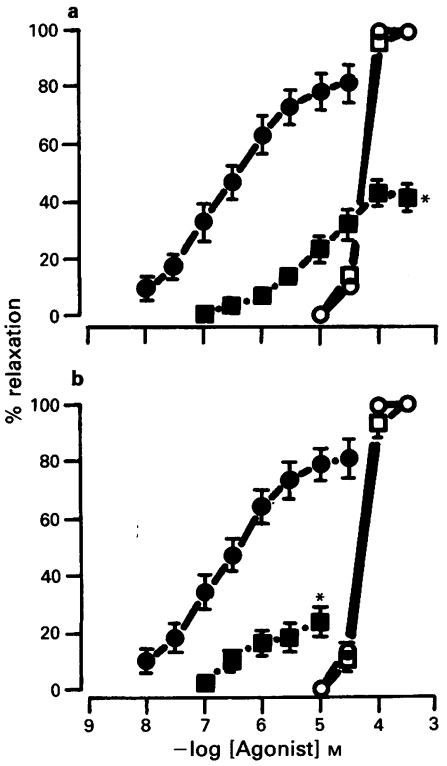

Figure 5 Concentration-response curves obtained on endothelium-denuded rings of rat aorta showing the relaxant effects of isoprenaline (●) and dibutyryl cyclic AMP (○) before, and of isoprenaline (■) and dibutyryl cyclic AMP (□) after treatment with rotenone (0.1 μM, a) or 2-deoxy glucose (10 mM, b). Each point is the mean and vertical bars indicate the s.e.mean of 4–9 observations. * $P < 0.05$ indicates a significant difference from the maximum relaxation obtained on untreated rings.

rings of rat aorta, but had no effect on relaxation induced by db cyclic AMP (10–300 μM, Figure 5). Incubation for 20 min in glucose-free Krebs solution containing 2-deoxy glucose (10 mM) inhibited isoprenaline-induced relaxation, but had no effect on relaxation induced by db cyclic AMP (Figure 5).

Verapamil

Treatment with rotenone (0.1 μM), or with 2-deoxy glucose (10 mM) in glucose-free Krebs solution, had no effect on the ability of verapamil (1–100 nM) to relax KCl (60 mM)-contracted, endothelium-denuded rings of rat aorta (data not shown).

Discussion

Our results confirm those of previous reports (Griffith *et al.*, 1986; 1987) that rotenone, an inhibitor of oxidative phosphorylation, rapidly and almost irreversibly, blocks endothelium-dependent relaxation in rabbit aorta. This blockade is probably due to inhibition of stimulated EDRF production since cascade bioassay experiments show that responsiveness of the vascular smooth muscle in rabbit aorta to EDRF is unaffected (Griffith *et al.*, 1986). Our finding that glyceryl trinitrate-induced relaxation, which occurs by the same cyclic GMP-dependent mechanism as EDRF-induced relaxation (Rapoport *et al.*, 1983), was unaffected by rotenone supports this conclusion. The possibility that rotenone inhibits EDRF production by lower levels of NADPH, a co-factor for the enzyme nitric oxide synthase (Palacios *et al.*, 1989), warrants further study. In rat aorta, however, rotenone inhibits acetylcholine-induced, endothelium-dependent relaxation to a lesser degree, and this is associated with a depression of the relaxant effects of glyceryl trinitrate. In contrast to rabbit aorta, therefore, rotenone-induced inhibition of endothelium-dependent relaxation involved a depression in sensitivity of the vascular smooth muscle. A possible additional inhibition of EDRF production by rotenone in rat aorta is suggested by the reversibility of blockade of glyceryl trinitrate-induced, but not acetylcholine-induced relaxation, and this warrants further investigation.

We confirmed also, the finding of Griffith *et al.*, (1986) that 2-deoxy glucose, an inhibitor of glycolysis, induces a slight inhibition of acetylcholine-induced relaxation in rabbit aorta, and found a similar inhibition in rat aorta. This inhibition is seen only in the absence of glucose and can be reversed following the addition of glucose (Griffith *et al.*, 1986; Richards *et al.*, 1990). Rather than being due to inhibition of EDRF production (Griffith *et al.*, 1986), the blockade probably results from a reduction in sensitivity of the vascular smooth muscle to EDRF since glyceryl trinitrate-induced relaxation was also inhibited in both tissues. Whether 2-deoxy glucose had an additional effect of blocking EDRF production could not be determined in our experiments.

The mechanisms by which rotenone and 2-deoxy glucose inhibit responsiveness of vascular smooth muscle to EDRF and glyceryl trinitrate might be due to lowered levels of the high energy phosphates, adenosine 5'-triphosphate (ATP) and guanosine 5'-triphosphate (GTP). ATP is required for smooth muscle contraction and relaxation, and a fall in cellular content may prevent relaxation by, for example, preventing the phosphorylation of myosin light chain kinase (Hathaway *et al.*, 1985). A more likely explanation, however, is that lowered levels of ATP and GTP result in a reduced capacity to form the second messengers cyclic AMP and cyclic GMP, respectively. Consistent with this explanation are our findings that metabolic inhibitors block relaxations induced by isoprenaline and glyceryl trinitrate, but not those induced by membrane permeant analogues of the second messengers that mediate their respective relaxations, i.e., db cyclic AMP and 8-bromo GMP. This ability of metabolic inhibitors to block relaxations mediated via cyclic nucleotides appears to be selective, since relaxation induced by the calcium channel blocking agent, verapamil, which acts independently of cyclic nucleotides, was completely unaffected.

The effects of metabolic inhibitors on phenylephrine-induced tone were complex. In rabbit aorta rotenone had only a slight depressant action and 2-deoxy glucose had none, but a combination of the two led to a complete and irreversible reduction of tone. This would suggest the smooth muscle of rabbit aorta derives energy from both oxidative and glycolytic metabolism. The profound depressant action of rotenone on tone in rat aorta suggests here the smooth muscle derives most of its energy from oxidative metabolism, and the lack of effect of 2-deoxy glucose on tone in endothelium-denuded rings is consistent with this. In endothelium-containing rings of rat aorta vasoconstrictor responses are depressed by the tonic vasodilator actions of spontaneously released EDRF (Egleme *et al.*, 1984; Martin *et al.*, 1986). The ability of 2-deoxy glucose to augment phenylephrine-induced tone in endothelium-containing rings of rat aorta is likely to be due to inhibition of this depressant action resulting from the reduced sensitivity of the smooth muscle to EDRF discussed above.

In conclusion, in rat aorta rotenone and 2-deoxy glucose each block acetylcholine-induced, endothelium-dependent relaxation by reducing the sensitivity of the vascular smooth muscle to EDRF. In rabbit aorta inhibition of acetylcholine-induced relaxation by rotenone and 2-deoxy glucose results from an inhibition of EDRF production and reduction in smooth muscle sensitivity to EDRF, respectively. Thus, depending on the tissue studied, metabolic inhibitors can block endothelium-dependent vasodilatation by affecting endothelial production of EDRF, smooth muscle sensitivity to EDRF, or both.

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Probing the molecular dimensions of general anaesthetic target sites in tadpoles (*Xenopus laevis*) and model systems using cycloalcohols

S. Curry, G.W.J. Moss, R. Dickinson, W.R. Lieb & N.P. Franks

Biophysics Section, Blackett Laboratory, Imperial College of Science, Technology & Medicine, Prince Consort Road, London, SW7 2BZ

1 The series of cycloalcohols C6, C7, C8 and C10 have been used to probe the molecular dimensions of a variety of general anaesthetic target sites.

2 The general anaesthetic EC_{50} concentrations of the cycloalcohols were determined for tadpoles (*Xenopus laevis*). All of the cycloalcohols tested were found to be potent general anaesthetics (on average $EC_{50}/C_{sat} = 0.03$).

3 The effects of the cycloalcohols on highly purified luciferase enzymes from fireflies (*Photinus pyralis*) and bacteria (*Vibrio harveyi*) were also investigated. Both enzymes were inhibited competitively, with the cycloalcohols competing with firefly luciferin for binding to the firefly enzyme and with *n*-decanal for binding to the bacterial enzyme.

4 The binding site on the firefly enzyme could accommodate two molecules of cycloalcohols C6 and C7 but only a single molecule of the larger cycloalcohols (C8 and C10), implying a volume of the binding site of about $250\,cm^3\,mol^{-1}$. In contrast, the binding site on the bacterial luciferase could bind only a single cycloalcohol molecule between C6 and C10.

5 While all of the cycloalcohols were potent inhibitors of the firefly luciferase enzyme (on average $EC_{50}/C_{sat} = 0.015$), they were very weak inhibitors of the bacterial luciferase enzyme (on average $EC_{50}/C_{sat} = 0.12$). Since both enzymes bind long-chain aliphatic *n*-alcohols tightly, the differing affinities of the cycloalcohols for the two enzymes is probably a consequence of geometrical factors.

6 The cycloalcohols produced very small effects on lipid bilayers. At EC_{50} concentrations which produce general anaesthesia, lipid bilayer phase transitions were shifted, on average, by only 0.43°C.

7 We conclude that the general anaesthetic effects of the cycloalcohols can most economically be explained by assuming that the cycloalcohols act at protein binding sites in the central nervous system. These target sites would have binding properties similar to those of the anaesthetic-binding site on firefly luciferase, but their average volume would be somewhat smaller than $250\,cm^3\,mol^{-1}$.

Introduction

Despite many years of effort, there is no agreement as to how general anaesthetics act or even as to the molecular nature of their target sites in the central nervous system (for reviews, see Richards, 1980; Franks & Lieb, 1982; Dluzewski *et al.*, 1983; Miller, 1985). Most attention has been focused on one or other of two extreme views: that anaesthetics act by binding directly to protein molecules (Richards, 1980; Franks & Lieb, 1982) or that anaesthetics exert their effects by first disrupting the lipid portions of nerve membranes (Miller, 1985; Elliott & Haydon, 1989). (Anaesthetics might, of course, act at a site composed of both protein and lipid, but there is, as yet, no evidence to support this position of compromise.) Considerable effort has been directed towards 'fine tuning' lipid theories (Janoff & Miller, 1982), and suggestions (Miller & Pang, 1976; Harris & Groh, 1985) have been made as to the most appropriate lipid composition of a membrane in order for it to constitute a 'good' model for anaesthetic target sites. On the other hand, there have been surprisingly few studies on the interactions between general anaesthetics and proteins and there is no precise idea of what is required of a protein binding site for it to be able to accommodate general anaesthetic drugs and accurately account for their potencies in animals.

Probably the best currently available protein model for general anaesthetic target sites is the anaesthetic-binding pocket on the light-emitting enzyme firefly luciferase (Franks & Lieb, 1984; 1985). This enzyme normally binds a relatively hydrophobic aromatic substrate, firefly luciferin, but this can be displaced by the binding of a wide variety of simple general anaesthetic agents. This competitive binding inhibits enzyme activity at anaesthetic concentrations which closely mimic those which induce anaesthesia in animals. Moreover, cut-offs in the anaesthetic potencies of homologous series of compounds which are observed in animals (Meyer & Hemmi, 1935) are also observed with the firefly enzyme (Franks & Lieb, 1985). However, other proteins which bind hydrophobic aromatic substrates are very poor models of anaesthetic target sites Chymotrypsin, for example is sensitively inhibited (Hymes *et al.*, 1969) by anaesthetic agents such as benzyl alcohol that closely resemble its natural substrates, but is virtually unaffected by many aliphatic anaesthetics such as diethyl ether (Miles *et al.*, 1962) and *n*-alcohols (Smith & Hansch, 1973) at concentrations which produce general anaesthesia. Recently, Curry *et al.* (1990) have shown that the substrate binding pocket on the purified bacterial luciferase enzyme from *Vibrio harveyi*, which normally binds a long-chain aldehyde substrate, preferentially binds narrow aliphatic inhibitors and tends to exclude larger and bulkier anaesthetics.

The principal aim of the present work was to probe the molecular architecture of general anaesthetic target sites in tadpoles (*Xenopus laevis*) and various model systems by choosing a set of compounds that would strongly discriminate between lipid bilayers and protein binding sites of different dimensions. In this paper, we describe the effects of cycloalcohols (from cyclohexanol to cyclodecanol) on the luciferase enzymes from the firefly (*Photinus pyralis*) and a bacterium (*Vibrio harveyi*) and on lipid bilayers, and we compare their

effectiveness as enzyme inhibitors and perturbants of lipid bilayer structure with their potencies as general anaesthetics.

Methods

Purification and assay of luciferase enzymes

Details of the procedures used to obtain pure preparations of the firefly (*Photinus pyralis*) luciferase enzyme have been published elsewhere (Branchini *et al.*, 1980; Franks & Lieb, 1984; 1986). The bacterial luciferase enzyme was purified from *Vibrio harveyi* (strain MB20). The preliminary stages of purification were essentially the same as those described by Hastings *et al.* (1978). Further purification was achieved (Curry *et al.*, 1990) by use of three additional chromatography columns (a DEAE-Sephacel ion-exchange column, an ACA 34 gel filtration column, and finally a Pharmacia FPLC ion-exchange column loaded with Mono Q monobeads). The highly purified enzymes were stored as stock solutions in 0.4 M ammonium sulphate, 1 mM EDTA, pH 7.8 at 4°C (firefly enzyme) or 50 mM potassium phosphate, 0.1 mM dithiothreitol, pH 7.0 at −20°C (bacterial enzyme). The activities of the enzymes were assayed (at 25 ± 1°C) as follows. For the firefly enzyme, the reaction was initiated by the rapid injection of ATP into a vial containing the enzyme, the substrate firefly luciferin, magnesium ions and variable concentrations of the cycloalcohols. The solutions were buffered at pH 7.8 with 25 mM glycylglycine. Typical final concentrations of the reactants were 10 nM enzyme, 2 mM ATP, 6.7 mM magnesium ions and 2–20 μM luciferin. For the bacterial luciferase, the reaction was initiated by the rapid injection of flavin (catalytically reduced by bubbling H_2 through an FMN solution containing palladium on activated charcoal) into a vial containing the enzyme, the substrate *n*-decanal and variable concentrations of the cycloalcohols. (Before entering the injection syringe, the $FMNH_2$ solution passed through a 3 μm polycarbonate filter, which removed all the charcoal particles.) The solutions were buffered at pH 7.0 with 50 mM potassium phosphate. Typical final concentrations were 0.4 nM enzyme, 100 μM $FMNH_2$ and 0.2–1.7 μM *n*-decanal. For both enzymes, the light from the reaction was detected with a photomultiplier, amplified with a current-voltage converter and stored on a digital oscilloscope before being plotted on a chart recorder. Luciferase activity was taken as the peak in the light intensity. The cycloalcohols were obtained from Lancaster Synthesis (cyclohexanol to cyclooctanol) and K & K Ltd. (cyclodecanol). (Cyclononanol was not used, since it was not available commercially.)

Analysis of enzyme inhibition data

For a competitive inhibitor it is convenient to define a function f(A), which is the factor by which the apparent Michaelis constant K_m^{app} changes with anaesthetic concentration [A], i.e. $K_m^{app} = f(A)K_m$, where K_m is the Michaelis constant (Franks & Lieb, 1984). At a fixed substrate (luciferin or *n*-decanal) concentration [S], f(A) is given by:

$$f(A) = \left[\frac{S + K_m}{K_m}\right]\left[\frac{v_o}{v_i} - \frac{S}{S + K_m}\right], \quad (1)$$

where v_o/v_i is the ratio of control to inhibited luciferase activities at an anaesthetic concentration [A].

If only a single molecule is involved in the inhibition, then it is easy to show that:

$$f(A) = 1 + \frac{[A]}{K_i}, \quad (2)$$

where K_i is the inhibition constant (i.e. the enzyme-inhibitor dissociation constant). To obtain a value for K_i and its associated standard error, f(A) was plotted against [A] and a straight line fitted using the method of weighted least squares (Cleland, 1967). The weights used were derived by assuming a constant percentage error in the measurement of enzyme activity and are given by:

$$w(A) = \frac{1}{\left\{f(A) + \frac{[S]}{K_m}\right\}^2}. \quad (3)$$

K_i is then given by the ratio of the intercept on the y-axis (c) and the slope (m) of the straight line (Eqn. 2). Thus:

$$K_i = \frac{c}{m}, \quad (4)$$

with a standard error given by:

$$\text{s.e.}(K_i) = \frac{1}{m}\sqrt{[\text{Var}(c) - 2K_i\text{Cov}(c,m) + K_i^2\,\text{Var}(m)]}, \quad (5)$$

where the variance and covariance terms in c and m are the diagonal and off-diagonal elements, respectively, of the variance-covariance matrix.

If two molecules are involved in the inhibition, then it can be shown (Franks & Lieb, 1984) that, if each molecule binds with the same inhibition constant K_i, and if only one molecule is necessary to cause inhibition, f(A) is given by:

$$f(A) = \left\{1 + \frac{[A]}{K_i}\right\}^2. \quad (6)$$

In this case, $\sqrt{f(A)}$ was plotted against [A] and a straight line fitted using weights given by:

$$w(A) = \frac{f(A)}{\left\{f(A) + \frac{[S]}{K_m}\right\}^2}. \quad (7)$$

Thereafter, the procedure to obtain a value for K_i and its associated standard error was exactly as above. Typically, each K_i determination involved about ten enzyme assays.

Determination of aqueous solubilities

The maximum aqueous solubilities of the cycloalcohols were determined in pure water by comparing inhibition of the bacterial luciferase enzyme by known concentrations of the cycloalcohol with that caused by variable known dilutions of a saturated solution. The saturated solutions were prepared by adding approximately a two fold excess of cycloalcohol to a 20 ml volume of pure water and intermittently vortexing vigorously over a period of 2 h. The excess alcohol was removed by centrifugation (C6–C8) or rapid filtration (C10). The cycloalcohol solutions were prepared in pure water at 25 ± 1°C but the assays were all performed under the standard conditions with final concentrations of 6.4 μM *n*-decanal and 50 mM potassium phosphate at pH 7.0. Typically, each solubility determination involved about twenty enzyme assays.

Determination of general anaesthetic potencies

General anaesthetic EC_{50} concentrations were determined for 1–2 week old *Xenopus laevis* tadpoles (average length about 1 cm) at 23 ± 1°C. Eight tadpoles were placed in each of six beakers containing 300 ml of tap water. The anaesthetic endpoint was defined as the lack of a sustained swimming response following a gentle prod with a smooth glass rod. After equilibration (this was complete after 20–60 min), the number of anaesthetized tadpoles was recorded and the tadpoles were returned to fresh tap water. Even after exposure to the highest concentrations of cycloalcohols tested, normal

swimming activity was rapidly restored. The dose-response curves were analysed by the method of Waud (1972).

Lipid phase transitions

An aqueous suspension of vesicles of dipalmitoyl lecithin (dipalmitoyl-L-α-phosphatidylcholine from Sigma Chemical Company) was made by adding 30 μl of a stock suspension to 3 ml of water or cycloalcohol solution in a 4 ml cuvette. The stock suspension (15 mg ml^{-1}) was prepared by vigorously mixing dried lipid with water above the chain-melting phase transition temperature (41–42°C). The cuvette was placed in the heated stage of a Beckman DU8 spectrophotometer and the absorbance at 450 nm measured as the temperature was increased at a rate of about 2°C per min (Hill, 1974). The temperature was recorded with a thermocouple and a digital thermometer. The outputs from the spectrophotometer and thermometer were stored on a computer. The transition temperature was defined as the mid-point in the abrupt step in absorbance. For the larger cycloalcohols (C8 and C10), determinations were made over a range of lipid concentrations in order to check for depletion of the aqueous concentration of cycloalcohol. The maximum depletion observed was 6% (for C10), and the quoted values have been corrected to account for this effect. Triplicate determinations were usually made.

Results

Each of the cycloalcohols tested caused a dose-related depression in light output from both the firefly and bacterial luciferase enzyme reactions. As might have been expected from previous work (Adey *et al.*, 1976; Middleton & Smith, 1976; Franks & Lieb, 1984; Curry *et al.*, 1990), the inhibition is competitive in nature. The alcohols compete with the luciferin substrate for binding to the firefly enzyme and with the decanal substrate for binding to the bacterial enzyme. (In both cases, the concentrations of the other substrate, ATP and $FMNH_2$, respectively, were close to saturating.) Typical double-reciprocal plots are presented in Figures 1a and b. In all cases there were substantial changes in the apparent Michaelis constants, K_m^{app}, but no significant changes in the maximum velocity of the enzyme, V_{max}. In order to determine the inhibition constants for binding to the enzymes, inhibition was measured as a function of cycloalcohol concentration at a fixed substrate (firefly luciferin or *n*-decanal) concentration. Thus the function f(A), which for a competitive inhibitor is simply the factor by which the apparent K_m^{app} increases as a function of anaesthetic concentration [A], could be calculated using equation 1. As described in Methods, plots of f(A) or $\sqrt{f(A)}$ versus [A] yield both the inhibition constant K_i as well as the number *n* of anaesthetic molecules interacting with the enzyme.

The firefly data show that two molecules of the smaller cycloalcohols (C6 and C7: see Figure 2a) but only one molecule of the larger cycloalcohols (C8 and C10; see Figure 2b) could bind to the firefly enzyme. For the bacterial enzyme, on the other hand, only a single molecule of any of the cycloalcohols could bind to the enzyme (see Figure 2c). Values for the inhibition constants K_i derived from plots of f(A) versus [A] (for $n = 1$) or $\sqrt{f(A)}$ versus [A] (for $n = 2$) are given in Table 1. These data show that the cycloalcohols bind between about 3 and 40 times tighter to the firefly luciferase enzyme than to the bacterial luciferase enzyme.

We determined the aqueous solubilities of the cycloalcohols (C6, C7, C8 and C10), and these values together with standard errors are given in Table 1.

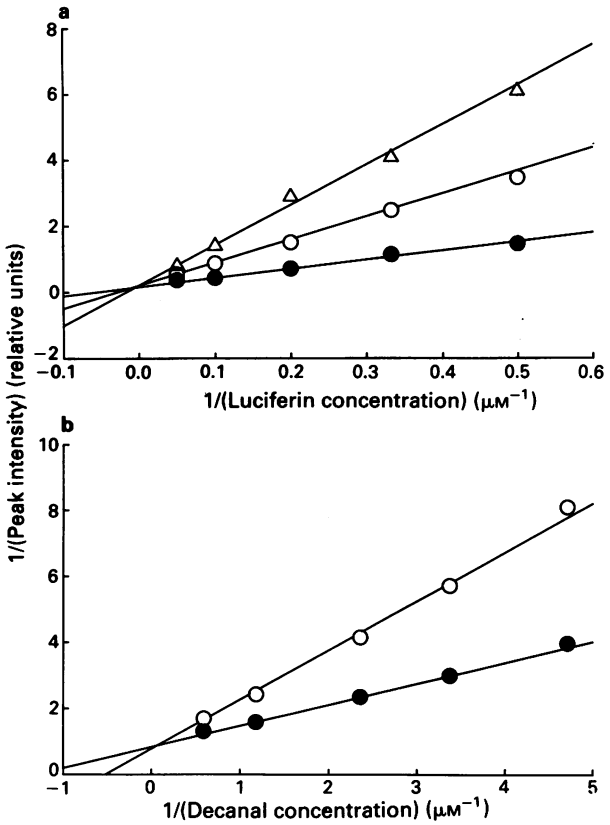

Figure 1 Cycloalcohols inhibit luciferase enzymes by competing for the binding of their hydrophobic substrates. (a) Double reciprocal plots showing the inhibition of firefly luciferase by cyclooctanol (control, ●; 170 μM, ○; 340 μM, △). The alcohol competes with firefly luciferin for binding to the enzyme and causes a large change in the apparent Michaelis constant with no significant change in V_{max}. (b) Double reciprocal plots showing the inhibition of bacterial luciferase by cyclooctanol (control, ●; 7.2 mM, ○). The alcohol competes with *n*-decanal for binding to the enzyme and causes a large change in the apparent Michaelis constant with no significant change in V_{max}. The straight lines were fitted by the method of weighted least squares using weighting factors which were proportional to the squares of the enzyme activities. Each data point represents the mean of two or three determinations. Essentially identical lines were obtained using distribution-free procedures (Cornish-Bowden, 1979). The error bars (± s.e.) are smaller than the size of the symbols.

Table 1 Cycloalcohol inhibition constants, aqueous solubilities, and concentrations to shift a lipid phase transition 1°C

	Firefly luciferase		*Bacterial luciferase*		*Aqueous solubility*	*Concentration for*
Cycloalcohol	K_i (mM)	*n*	K_i (mM)	*n*	C_{sat} (mM)	ΔT = 1°C (mM)
C6	2.5 ± 0.4	2	9.80 ± 1.09	1	166 ± 21	8.4 ± 0.4
C7	0.77 ± 0.02	2	5.16 ± 0.57	1	107 ± 17	3.8 ± 0.1
C8	0.07 ± 0.004	1	2.65 ± 0.38	1	44.8 ± 4.8	1.66 ± 0.08
C10	0.022 ± 0.003	1	0.068 ± 0.007	1	1.03 ± 0.17	0.152 ± 0.006

Values are given as means ± s.e. The procedures and numbers of assays are given in Methods. K_i = inhibition constant, *n* = number of molecules involved in inhibition, C_{sat} = maximum aqueous solubility, and ΔT = depression of lipid phase transition temperature.

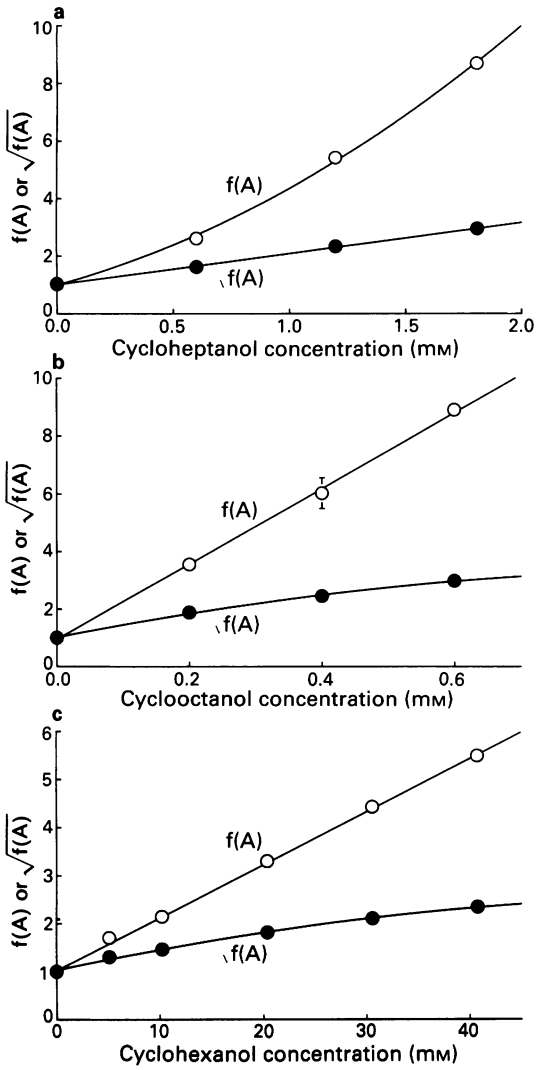

Figure 2 Inhibition of luciferase activity plotted as the function f(A), the factor by which the apparent Michaelis constant changes with alcohol concentration, [A]. (a) For the firefly luciferase, the cycloalcohols C6 and C7 gave f(A) plots which increased parabolically with [A], such that a plot of the square root of f(A) was linear. This indicates that two molecules were involved in the inhibition (Franks & Lieb, 1984). This figure shows data for cycloheptanol. (b) For the larger alcohols, C8 and C10, plots of f(A) vs. [A] were linear for firefly luciferase. This shows that for these compounds only a single inhibitor molecule was involved. This figure shows data for cyclooctanol. (c) For the bacterial luciferase, plots of f(A) vs. [A] were linear for all the cycloalcohols. This figure shows data for cyclohexanol. Most data points represent the mean of two or three determinations. Where error bars (± s.e.) are not shown they are smaller than the size of the symbols. The straight lines were used to obtain values for K_i as described in Methods.

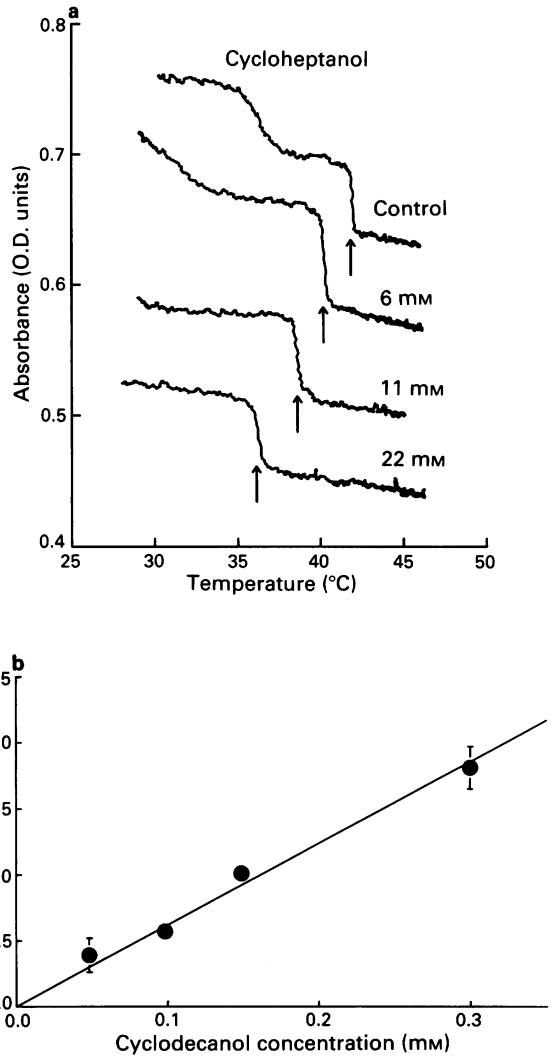

Figure 3 Cycloalcohols reduce the chain-melting phase transition temperature of dispersions of dipalmitoyl lecithin. (a) The optical absorbance at 450 nm shows an abrupt decrease as the temperature is raised through the phase transition temperature T_m (arrows). Increasing concentrations of cycloalcohols (these data are for cycloheptanol) shifted T_m to lower temperatures. The cycloalcohol traces have been offset in the vertical direction for clarity. (b) The shift in phase transition temperature increased linearly with concentration of cycloalcohol. This figure shows data for cyclodecanol. The line was fitted by the method of least squares. The error bars are s.e. for three determinations and when not shown are less than the size of the symbols.

All of the cycloalcohols shifted the main chain-melting phase transition of dipalmitoyl lecithin to lower temperatures. Typical data (for cycloheptanol) are shown in Figure 3a. The shift in phase transition temperature (ΔT) was directly proportional to alcohol concentration (see Figure 3b) and remained linear over the range of concentrations used. As a convenient measure (Hill, 1974) of the effectiveness of each cycloalcohol in reducing the phase transition temperature, we have calculated the concentrations required to reduce the transition temperature by one degree, and these are given in Table 1 together with their standard errors.

All of the cycloalcohols were potent anaesthetics as judged by their ability to inhibit the swimming activity of *Xenopus laevis* tadpoles. Our observed dose-response curves are shown in Figure 4. The dose-response curves gave EC_{50} values with

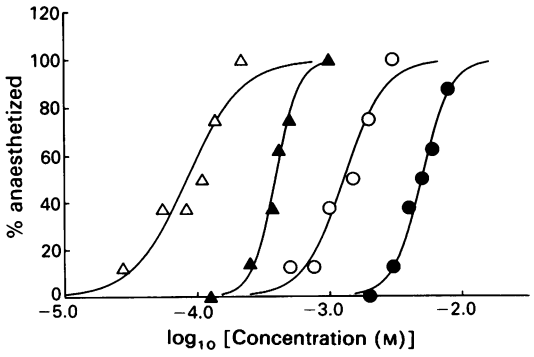

Figure 4 The cycloalcohols are potent general anaesthetics as judged by their ability to inhibit the swimming activity of tadpoles. The percentage of animals anaesthetized is plotted versus the logarithm of cycloalcohol concentration. The lines have been drawn according to the theoretical treatment of Waud (1972). Data are for C10 (△); C8 (▲); C7 (○); C6 (●).

standard errors of typically about 10% of the mean (see Table 2). [We also determined an EC_{50} value for *n*-hexanol and found it to be 0.73 ± 0.05 mM which is close to the value of 0.57 ± 0.04 mM published by Alifimoff *et al.* (1989) for tadpoles of *Rana pipiens*. This suggests that if species differences exist, they are likely to be small.]

Discussion

The range of compounds which cause general anaesthesia is truly remarkable, yet despite this diversity, most work on anaesthetic mechanisms has tended to concentrate on a fairly small selection of conventional agents. It is probable, however, that more will be learned about mechanisms of general anaesthesia by studying unusual molecules, which may exhibit unconventional behaviour (Miller, 1985). For example, the anomalous solubility properties of perfluorinated compounds were instrumental in disproving theories which supposed that the anaesthetic target site was structured water (Miller *et al.*, 1967), and the exceptional behaviour of long-chain compounds which are not anaesthetic (see, e.g. Meyer & Hemmi, 1935) should be an important clue as to how general anaesthetics act.

Our choice of cycloalcohols was based on the idea that their relative bulk and rigidity might make them discriminate between a variety of different anaesthetic target sites (compared to the long-chain *n*-alcohols, for example, which might be sufficiently flexible to be accommodated in binding sites of very different sizes and shapes).

Our results show that this is, indeed, the case. The cycloalcohols discriminate strongly between the binding sites on the two luciferase enzymes, being very potent inhibitors of the firefly luciferase but not very effective at inhibiting the bacterial luciferase. This can be contrasted with the fact that both enzymes bind flexible long-chain alcohols very tightly (Franks & Lieb, 1985; Curry *et al.*, 1990). The cycloalcohols inhibit both enzymes by competing for the binding of hydrophobic substrates, a bulky heterocyclic compound (firefly luciferin) in the case of the firefly enzyme, and a narrow aliphatic aldehyde (*n*-decanal) in the case of the bacterial luciferase. It seems reasonable to suppose, therefore, that the cycloalcohols bind much tighter to the firefly enzyme simply because they can be more easily accommodated by the geometry of its binding pocket. An approximate volume for this pocket can be estimated from the change in cycloalcohol-enzyme stoichiometry between C7 (molar volume ≡ molecular weight/density = $120\,cm^3\,mol^{-1}$) and C8 (molar volume = $132\,cm^3\,mol^{-1}$). This suggests a 'break point' at about $126\,cm^3\,mol^{-1}$, which in turn implies a binding site with a volume of about $252\,cm^3\,mol^{-1}$. This is essentially the same as the value ($250\,cm^3\,mol^{-1}$) deduced (Franks & Lieb, 1987) from the binding behaviour of *n*-alcohols to firefly luciferase.

How do the concentrations of the cycloalcohols required to inhibit the luciferase enzymes compare with those needed to induce general anaesthesia? The concentration of a given cycloalcohol required to half-inhibit luciferase activity depends upon the concentration of the competing substrate (since the inhibition is competitive in nature). Therefore, in order that a fair comparison can be made with general anaesthetic EC_{50} concentrations, we shall define an EC_{50} for the enzymes as the concentration of inhibitor required to half-inhibit enzyme activity when the concentration of the competing substrate is equal to its K_m. When this is done, and the EC_{50} concentrations for the enzymes are compared with those for general anaesthesia in tadpoles (see Figure 5 and Table 2), it is clear that the cycloalcohols are much less effective at inhibiting the bacterial luciferase enzyme, and somewhat more effective at inhibiting the firefly enzyme, than they are as general anaesthetics. Some idea as to the relative effectiveness of these compounds as general anaesthetics can be had by comparing their general anaesthetic EC_{50} concentrations with their aqueous solubilities C_{sat}. For compounds with low solubilities, this ratio approximates to the thermodynamic activity and can thus be considered to be an 'effective concentration' (Brink & Posternak, 1948). For most simple agents the ratio of EC_{50}/C_{sat} is between 0.02 and 0.04 (Brink & Posternak, 1948); using the data given in Tables 1 and 2 we can calculate that for the cycloalcohols (between C6 and C10), this ratio is, on average, 0.03. This is to be compared to a value of 0.015 for inhibiting firefly luciferase and 0.12 for inhibiting the bacterial luciferase. This comparison emphasizes the relatively weak binding of the cycloalcohols to the bacterial luciferase enzyme. That this is largely a steric factor is strongly suggested by the fact that the long-chain *n*-alcohols bind very tightly to the bacterial enzyme (Curry *et al.*, 1990). Indeed, Curry *et al.* (1990) have concluded that a relatively narrow anaesthetic-binding pocket is the reason for the insensitivity of the bacterial luciferase enzyme to bulky inhalational anaesthetics.

Our data also show, however, that lipid bilayer structure is disrupted by the presence of the cycloalcohols, and many have argued (see, for example, Janoff & Miller, 1982; Elliott &

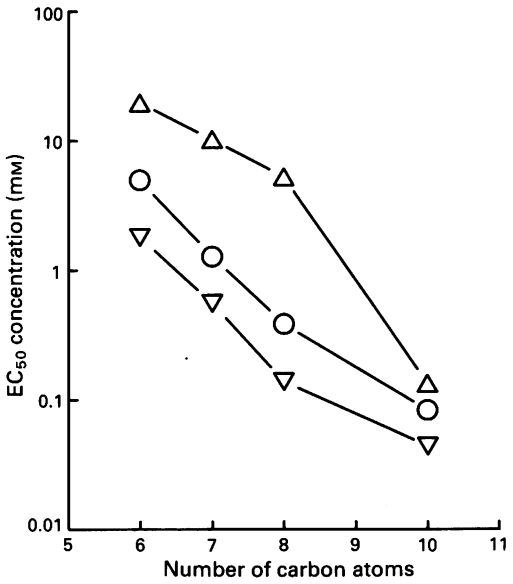

Figure 5 A comparison of EC_{50} concentrations of cycloalcohols for causing general anaesthesia (○) and for inhibiting luciferase enzymes from fireflies (▽) and bacteria (△). The data are tabulated in Table 2.

Table 2 Cycloalcohol EC_{50} concentrations for anaesthesia, inhibiting luciferase enzymes and the corresponding effect on lipid bilayers

	Tadpole anaesthesia		*Firefly luciferase*	*Bacterial luciferase*	*ΔT at tadpole*
Cycloalcohol	EC_{50} (mM)	Slope	EC_{50} (mM)	EC_{50} (mM)	*anaesthesia* (°C)
C6	4.98 ± 0.47	3.9 ± 1.2	1.8 ± 0.3	19.6 ± 2.2	0.59 ± 0.06
C7	1.29 ± 0.17	2.8 ± 0.8	0.56 ± 0.015	10.3 ± 1.1	0.34 ± 0.05
C8	0.39 ± 0.033	4.9 ± 1.7	0.14 ± 0.008	5.30 ± 0.76	0.23 ± 0.02
C10	0.084 ± 0.014	2.1 ± 0.7	0.044 ± 0.006	0.135 ± 0.014	0.55 ± 0.09

Values are given as means ± s.e. The procedures and numbers of assays are given in Methods. Tadpole EC_{50} concentrations and slopes were determined using the method of Waud (1972). Enzyme EC_{50} concentrations were calculated as $2K_i$ or $0.732K_i$ for $n = 1$ or 2, respectively (see Table 1 and Franks & Lieb, 1984). ΔT is the depression of the lipid phase transition temperature when the free concentration of anaesthetic is equal to the EC_{50} concentration needed to produce general anaesthesia in tadpoles.

Haydon, 1989) that this might underlie general anaesthesia. Although few workers would now support early suggestions (Hill, 1974; Jain *et al.*, 1975; Kamaya *et al.*, 1979) that anaesthetics act by disrupting lipid phase transitions, measurements of this sort are nonetheless a very useful guide as to the overall degree of bilayer perturbation caused by a given agent. In order to attempt correlations with data for general anaesthesia, probably the simplest comparison that can be made is to ask how large a shift in lipid phase transition temperatures would be observed at the concentrations of the cycloalcohols which cause general anaesthesia. Table 2 shows the results of these calculations. It can be seen that, on average, the cycloalcohols between C6 and C10 cause a shift in phase transition temperature of only 0.43°C. This is close to the average value for the reduction in phase transition temperature (Hill, 1974) of 0.46°C caused by general anaesthetic concentrations (Alifimoff *et al.*, 1989) of similar *n*-alcohols (C4 to C8). One might have expected that the relative bulk and rigidity of the cycloalcohols would have substantially reduced lipid bilayer partitioning although, of course, these same factors would also result in these compounds being particularly effective at perturbing lipid structure once they partition into the bilayer.

As regards their effects on bilayer structure at general anaesthetic concentrations, therefore, the cycloalcohols do not seem to be an exception to the large number of anaesthetic agents that have been tested, and this might be taken as qualitative support for the idea that membrane perturbations underlie anaesthetic action. Quantitatively, however, as discussed elsewhere (Franks & Lieb, 1978; 1982; Richards *et al.*, 1978), the changes in bilayer structure involved correspond to an increase in temperature of less than 1°C. Although it has been argued (Kita & Miller, 1982; Elliott & Haydon, 1989) that the effects of temperature on lipid bilayers should not be equated with the effects of general anaesthetics, the similarities are sufficiently close to be able to conclude that, in absolute terms, the perturbation of bilayer structure by the cycloalcohols at anaesthetizing concentrations is very small indeed. It seems unrealistic, therefore, to suppose that the general anaesthetic activities of the cycloalcohols can be accounted for in terms of their effects on lipid bilayers.

Given the data shown in Figure 5, it is obviously very much easier to account for the general anaesthetic activity of the cycloalcohols on tadpoles in terms of direct effects on proteins. Assuming that this is the case, these data can be interpreted as giving some information on the overall architectures of the critical target sites involved in general anaesthesia. First, the poor agreement between the animal and the bacterial data suggests that the animal sites are not as narrow as that on the bacterial enzyme. Secondly, the roughly parallel changes in the EC_{50} concentrations for inhibiting the firefly enzyme and anaesthetizing animals suggest target sites of similar hydrophobicities, while the slightly lower potencies for producing general anaesthesia could be interpreted as reflecting binding to animal sites which are slightly smaller in at least one critical dimension. This is consistent with the observation that, for the *n*-alcohols, the cut-off for anaesthetizing animals occurs slightly earlier (at about C13: Alifimoff *et al.*, 1989) than the cut-off for inhibiting the firefly luciferase enzyme (which occurs at about C15: Franks & Lieb, 1985).

Although it seems unlikely that anaesthetics exert their effects by acting only on lipids, it has been suggested that general anaesthetic target sites may be located at protein/lipid interfaces (Elliott & Haydon, 1989). It seems more probable, however, that protein sites underlying general anaesthesia will be exposed to water rather than to lipid. This is because protein sites exposed to lipid which are capable of binding hydrophobic anaesthetics would (presumably) be equally capable of binding lipid hydrocarbon chains; however, the effective membrane concentration of these apolar chains would be roughly two orders of magnitude higher than that of the anaesthetics at clinically relevant concentrations, and thus they would successfully compete for binding.

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Effects of a novel smooth muscle relaxant, KT-362, on contraction and cytosolic Ca^{2+} level in the rat aorta

Kiyoshi Sakata & Hideaki Karaki

Department of Veterinary Pharmacology, Faculty of Agriculture, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

1 Inhibitory effects of a novel smooth muscle relaxant, KT-362 (5-[3-([2-(3,4-dimethoxyphenyl)-ethyl]amino)-1-oxopropyl]-2,3,4,5-tetrahydro-1,5-benzothiazepine fumarate), on contraction and the cytosolic Ca^{2+} level ($[Ca^{2+}]_{cyt}$) in isolated vascular smooth muscle of rat aorta were examined.

2 KT-362 inhibited the contractions induced by high K^+ and noradrenaline. The inhibitory effect was antagonized by an increase in external Ca^{2+} concentration. A Ca^{2+} channel activator, Bay K 8644, did not change the effect of KT-362 on high K^+-induced contraction.

3 $[Ca^{2+}]_{cyt}$, measured with fura-2-Ca^{2+} fluorescence, increased during the contractions induced by high K^+ or noradrenaline. KT-362 decreased $[Ca^{2+}]_{cyt}$ and muscle tension stimulated by high K^+ or noradrenaline. By contrast, a Ca^{2+} channel blocker, verapamil, inhibited the noradrenaline-induced increase in $[Ca^{2+}]_{cyt}$ with only partial inhibition of the noradrenaline-induced contraction and KT-362 inhibited the verapamil-insensitive portion of the contraction without changing $[Ca^{2+}]_{cyt}$.

4 In a Ca^{2+}-free solution, noradrenaline and caffeine induced a transient contraction following a transient increase in $[Ca^{2+}]_{cyt}$. KT-362 inhibited the increments due to noradrenaline but not those induced by caffeine.

5 These results suggest that KT-362 inhibits vascular smooth muscle contraction by inhibiting Ca^{2+} channels, receptor-mediated Ca^{2+} mobilization, and receptor-mediated Ca^{2+} sensitization of contractile elements.

Introduction

Contraction of smooth muscle is due to an increase in the cytosolic Ca^{2+} level ($[Ca^{2+}]_{cyt}$) resulting from cellular Ca^{2+} release and opening of Ca^{2+} channels (Bolton, 1979; Karaki & Weiss, 1984; 1988). The compounds termed Ca^{2+} channel blockers have diverse chemical structures and still show a common mechanism of action, selectively inhibiting stimulated Ca^{2+} influx (Fleckenstein, 1977; Flaim, 1982; Hof & Vuorela, 1983; Karaki & Weiss, 1984; Godfraind *et al.*, 1986). These blockers include N-methyl-N-homoveratrylamines such as verapamil (Fleckenstein *et al.*, 1969), benzothiazepines such as diltiazem (Nagao *et al.*, 1975), 1,4-dihydropyridines such as nifedipine (Schumann *et al.*, 1975), and diphenylalkylamines such as cinnarizine (Godfraind & Kaba, 1969) and prenylamine (Fleckenstein *et al.*, 1969).

KT-362 (5-[3-([2-(3,4-dimethoxyphenyl)-ethyl]amino)-1-oxopropyl]-2,3,4,5-tetrahydro-1,5-benzothiazepine fumarate) is a newly synthesized antiarrhythmic agent with vasodilator effects (Wakabayashi *et al.*, 1986). This compound has a benzothiazepine radical as does diltiazem (*cis*-(+)-3-(acetyloxy)-5-[2-(dimethylamino)ethyl]-2,3-dihydro- 2-(4-methoxyphenyl)-1,5-benzothiazepine-4(5H)-one) and also has a (3,4-dimethoxyphenylethyl)amino radical as does verapamil (5-[(3,4-dimethoxyphenylethyl)methylamino] - 2 - (3,4 - dimethoxy - phenyl)2-isopropylvaleronitrile). Shibata *et al.* (1987) reported that KT-362 has effects which are different from those of Ca^{2+} channel blockers and concluded that the primary action of this compound is to inhibit release of intracellular Ca^{2+}. Eskinder *et al.* (1989) also showed that KT-362 inhibits phosphatidyl inositol turnover. In the present experiments, we further characterized the inhibitory effect of this compound by measuring contraction and $[Ca^{2+}]_{cyt}$ in isolated vascular smooth muscle.

Methods

Muscle preparations

Male Wistar rats (200–300 g) were killed by a blow on the neck and exsanguination. The thoracic aorta was rapidly removed and cut into spiral strips, 2–3 mm wide and 10–15 mm long. Endothelium was removed by gently rubbing the intimal surface with a finger moistened with normal physiological salt solution. In a preliminary experiment, it was confirmed that the inhibitory effect of KT-362 was not modified by vascular endothelium.

Solutions

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 ethylenediamine tetraacetic acid (EDTA) 0.01. In some experiments, the concentration of $CaCl_2$ was changed to 0.3 mM or 7.5 mM. Isosmotic 65.4 mM K^+ solution was made by substituting 60 mM NaCl in the normal solution with equimolar KCl. Ca^{2+}-free solution was made by omitting $CaCl_2$ and adding 1 mM ethyleneglycol *bis*(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA). These solutions were saturated with a mixture of 95% O_2 and 5% CO_2 at 37°C (pH 7.4).

Contractile tension

Muscle tension was recorded isometrically with a force-displacement transducer connected to a polygraph (Nihon Kohden). Passive tension of 1 g was initially applied and tissues were allowed to equilibrate in a 20 ml bath for 60 min before beginning the experimental period. KT-362 was cumulatively applied when the contractile tension induced by a stimulant reached a steady level or applied before the addition of stimulant. In some experiments, the Ca^{2+} concentration was changed (to 0.3 mM or 7.5 mM) or 0.1 μM Bay K 8644 was added 10 min before the addition of a stimulant. The concentration of KT-362 required to induce a 50% inhibition of contraction (IC_{50}) was calculated from the concentration-inhibition curves. IC_{50} is shown by −log M. The noradrenaline- or caffeine-induced transient contraction was obtained by the method described previously (Karaki, 1987). After exposure of the muscle strips to a 65.4 mM K^+ for 3 min, the muscle was washed with a Ca^{2+}-free solution followed by the addition of 1 μM noradrenaline or 20 mM caffeine. Following a wash with normal physiological salt solution, high K^+ was added for 3 min to load the Ca^{2+} store in the muscle.

Muscle strips were then rinsed with a Ca^{2+}-free solution followed by the applications of noradrenaline or caffeine. This procedure was repeated until the transient contraction became constant. Then, the experiment was repeated in the presence of KT-362 (for further details see Figure 4).

Cytosolic Ca^{2+} levels

Cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) was measured simultaneously with muscle contraction as described by Ozaki *et al.* (1987) and Sato *et al.* (1988) using a fluorescent Ca^{2+} indicator, fura-2 (Grynkiewicz *et al.*, 1985). The muscle strip was loaded with 5 μM acetoxymethyl ester of fura-2 for 3 h in the presence of 0.02% Cremophor EL at room temperature (23–25°C) and then placed in a tissue bath at 37°C. The muscle strip was illuminated alternately (48 Hz) with 340 nm and 380 nm light and 500 nm emission was detected with a fluorimeter (CAF-100, JASCO, Tokyo, Japan). The amount of the 500 nm fluorescence induced by the 340 nm excitation (F_{340}) and that induced by the 380 nm excitation (F_{380}) was measured and the ratio of these two fluoresence ($R_{340/380}$) was calculated. The absolute amount of $[Ca^{2+}]_{cyt}$ was calculated as described by Scanlon *et al.* (1987) and Malgaroli *et al.* (1987). Maximum and minimum $R_{340/380}$ was obtained in the presence of 10 μM ionomycin and 4 mM EGTA, respectively. We used the dissociation constant of fura-2 for Ca^{2+} of 224 nM (Grynkiewicz *et al.*, 1985). With this method, absolute $[Ca^{2+}]_{cyt}$ can be calculated even in the presence of background fluorescence. Resting and high K^+-stimulated $[Ca^{2+}]_{cyt}$ calculated by this method were 98 nM and 508 nM, respectively. However, the dissociation constant in cytoplasm may be greater than 224 nM (Karaki, 1989) and, therefore the calculated $[Ca^{2+}]_{cyt}$ may be an underestimate. Furthermore, the maximum $R_{340/380}$ obtained in the presence of ionomycin may be an underestimate. Because of these uncertainties and because there is an almost linear relationship between $R_{340/380}$ and logarithmic $[Ca^{2+}]_{cyt}$ when $[Ca^{2+}]_{cyt}$ was changed from approximately 50 nM to 1000 nM (Mitsui *et al.*, 1990), we used the relative $R_{340/380}$ value as an indicator of $[Ca^{2+}]_{cyt}$, taking $R_{340/380}$ in resting muscle as 0% and that in high K^+-stimulated muscle as 100%.

Statistics

Results of the experiments are expressed as mean ± s.e.mean. Values were considered to be significantly different when P value was less than 0.05 by use of Student's t test.

Drugs and chemicals

The following drugs and chemicals were used: KT-362 (Kotobuki Pharmaceutical, Nagano, Japan), (−)-noradrenaline bitartrate (Wako Pure Chemicals, Osaka, Japan), caffeine (Wako), sodium nitroprusside (Wako), Bay K 8644 (4-[2-(trifluoromethyl)phenyl] - 1,4 - dihydro - 2,6 - dimethyl - 3 - nitro - pyridine-5-carboxylic acid methylester, donated by Bayer AG, Leverkusen, FRG), verapamil hydrochloride (Sigma Chemical Co., St. Louis, MO, U.S.A.), acetoxymethyl ester of fura-2 (Dojindo Laboratories, Kumamoto, Japan), EDTA (Dojindo), and Cremophor EL (Nakarai Chemicals, Kyoto, Japan). In the preliminary experiments, we confirmed that the test drugs did not interfere with the fura-2-Ca^{2+} fluorescence.

Results

Sustained contractions

Addition of 1–100 μM KT-362 did not change the resting tone of rat aortic strips. High K^+ (65.4 mM), or noradrenaline 1 μM induced sustained increase in muscle tension. Cumulative addition of KT-362 inhibited the sustained contractions induced by 65.4 mM K^+ and 1 μM noradrenaline with similar IC_{50} values (Figure 1 and Table 1). The inhibitory effect on high K^+-induced contraction was slightly ($P < 0.05$) potentiated by the decrease in external Ca^{2+} concentration and was antagonized ($P < 0.01$) by the increase in external Ca^{2+} concentration (Table 1). Although the changes in external Ca^{2+} concentration similarly modified the inhibitory effect on noradrenaline-induced contraction, a statistically significant difference was not obtained. Bay K 8644 (0.1 μM) did not change the effect of KT-362 on high K^+-induced contraction.

The inhibitory effect of KT-362 was completely reversed by washing the muscle with normal physiological salt solution for 30 min ($n = 4$).

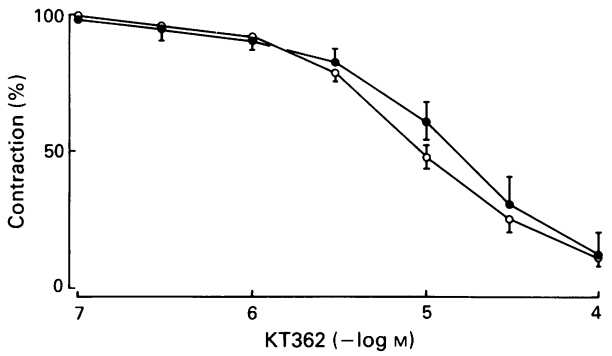

Figure 1 Inhibitory effect of KT-362 on the contractions in rat aorta stimulated with K^+ 65.4 mM (○) or noradrenaline 1 μM (●). KT-362 was cumulatively added during the sustained contraction; 100% represents the level of sustained contraction before the addition of KT-362. Each point represents mean of 4 to 8 experiments and s.e.mean is shown by vertical bars. Contractile tension before the addition of KT-362 and IC_{50} values are listed in Table 1.

Table 1 Concentrations of KT-362 required to induce a 50% inhibition of contraction in rat aorta

	K^+ (65.4 mM)		*Noradrenaline (1 μM)*	
Condition	*Contractile tension* (g)[1]	*IC_{50}* (−log M)	*Contractile tension* (g)[1]	*IC_{50}* (−log M)
Ca^{2+} 0.3 mM	0.81 ± 0.09*	5.22 ± 0.02*	0.71 ± 0.10	4.87 ± 0.04
Ca^{2+} 1.5 mM	0.99 ± 0.03	4.93 ± 0.06	0.90 ± 0.04	4.66 ± 0.15
Ca^{2+} 7.5 mM	0.99 ± 0.07	4.26 ± 0.04**	1.04 ± 0.08	4.50 ± 0.11
Ca^{2+} 1.5 mM + Bay K 8644 0.1 μM	1.10 ± 0.03*	4.79 ± 0.04		

Values are mean ± s.e.mean.
[1] Contractile tension before the cumulative addition of KT-362.
Significantly different from the value in the presence of 1.5 mM Ca^{2+} with * $P < 0.05$ and ** $P < 0.01$, respectively. Each value was obtained from 4 to 8 experiments.

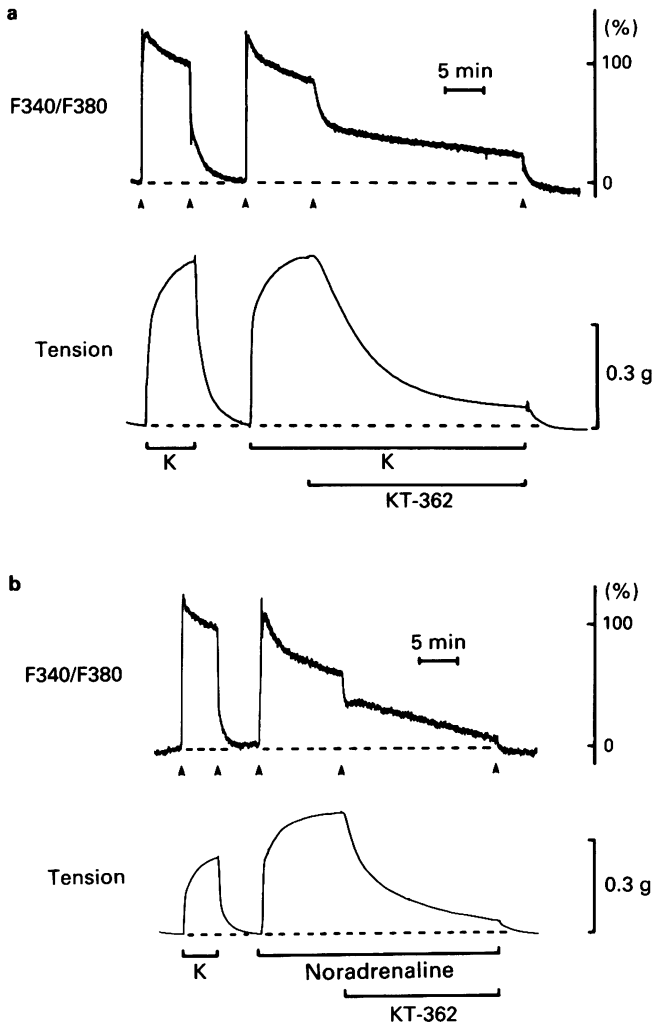

Figure 2 Inhibitory effect of KT-362 100 μM on $[Ca^{2+}]_{cyt}$ (indicated by F340/F380 ratio; upper trace) and muscle tension (lower trace) in rat aorta stimulated by KCl 72.7 mM (a) or noradrenaline 1 μM (b). Traced from typical experimental result.

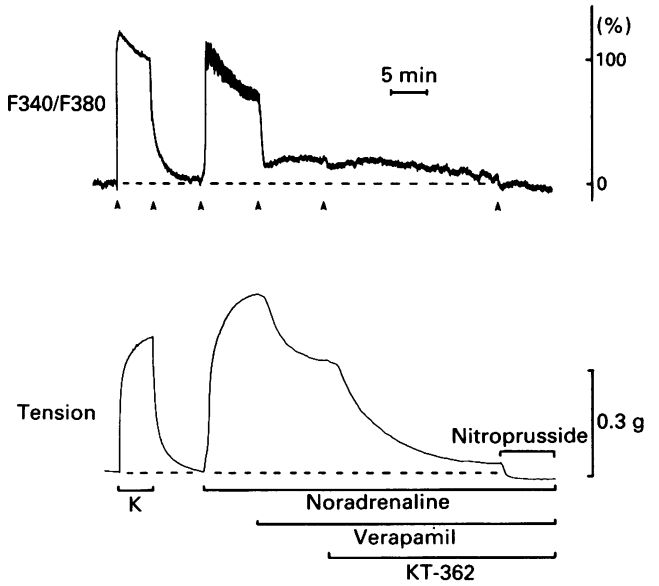

Figure 3 Inhibitory effect of verapamil 10 μM, KT-362 100 μM and sodium nitroprusside 1 μM on $[Ca^{2+}]_{cyt}$ (indicated by F340/F380 ratio; upper trace) and muscle tension (lower trace) in rat aorta stimulated by noradrenaline (1 μM). Traced from typical experimental result.

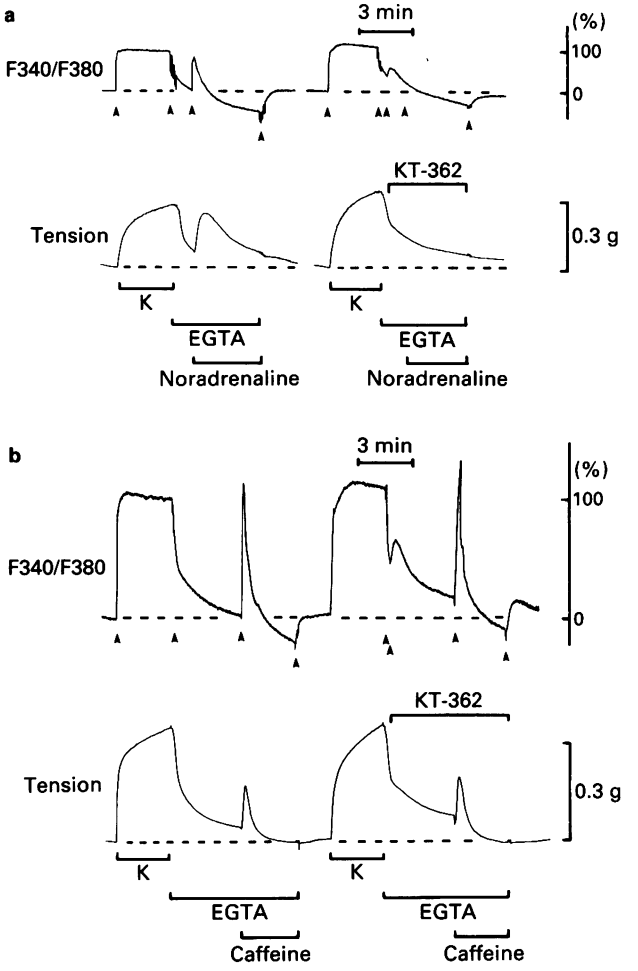

Figure 4 Inhibitory effect of KT-362 on transient increase in $[Ca^{2+}]_{cyt}$ and muscle tension induced by noradrenaline 1 μM (a) and caffeine 20 mM (b) in rat aorta in Ca^{2+}-free solution. After observing the effects of KCl 72.7 mM, the muscle was washed with Ca^{2+}-free solution containing EGTA 1 mM followed by an application of noradrenaline or caffeine. The muscle was then washed with normal solution, stimulated with high K^+, washed with Ca^{2+}-free solution and KT-362 (100 μM for noradrenaline and 300 μM for caffeine) was added. Traced from typical experimental result.

Cytosolic Ca^{2+} levels

Addition of high K^+ and noradrenaline induced rapid increase followed by a gradually declining plateau in $[Ca^{2+}]_{cyt}$ and an increase in muscle tension (Figure 2). Similar time-dependent dissociation between $[Ca^{2+}]_{cyt}$ and muscle tension has been reported (Sato *et al.*, 1988; Karaki, 1989) although the reason for this discrepancy has not been clarified yet. Addition of 100 μM KT-362 inhibited the high K^+-stimulated muscle tension to 32.7 $\pm$ 4.8% and $[Ca^{2+}]_{cyt}$ to 23.3 $\pm$ 6.9% ($n = 7$, each), and the noradrenaline-stimulated muscle tension to 7.5 $\pm$ 2.5% and $[Ca^{2+}]_{cyt}$ to 8.8 $\pm$ 4.1% ($n = 4$, each). Pretreatment of the muscle with 100 μM KT-362 showed similar inhibitory effects on the increments in muscle tension and $[Ca^{2+}]_{cyt}$ ($n = 4$ each, data not shown).

As shown in Figure 3, verapamil 10 μM, which completely inhibits the high K^+-induced increments in $[Ca^{2+}]_{cyt}$ and muscle tension, strongly inhibited the noradrenaline-stimulated $[Ca^{2+}]_{cyt}$ (to 25.1 $\pm$ 5.6%, $n = 4$) although noradrenaline-induced contraction was only partially inhibited (to 65.7 $\pm$ 6.5%, $n = 4$), as has been reported (Ozaki *et al.*, 1990). Sequential addition of 100 μM KT-362 inhibited the verapamil-resistant portion of the noradrenaline-induced contraction (to 13.1 $\pm$ 5.4%, $n = 4$) with little effect on $[Ca^{2+}]_{cyt}$ (to 15.1 $\pm$ 6.9%, $n = 4$, $P > 0.05$ compared to the value in the

presence of verapamil). Sodium nitroprusside (1 μM) completely inhibited the remaining portion of $[Ca^{2+}]_{cyt}$ and muscle tension ($n = 4$).

As shown in Figure 4, the transient contraction induced by 1 μM noradrenaline or 20 mM caffeine in a Ca^{2+}-free solution was accompanied by an increase in $[Ca^{2+}]_{cyt}$. Addition of 100 μM KT-362 induced a small increase in $[Ca^{2+}]_{cyt}$ (by 7.7 $\pm$ 3.0% of the high K^+-stimulated change, $n = 4$) and a small increase in muscle tension in Ca^{2+}-free solution (by 11.9 $\pm$ 2.4%, $n = 4$), suggesting that KT-362 releases a small amount of Ca^{2+} from a storage site. The transient increase in $[Ca^{2+}]_{cyt}$ and muscle tension induced by noradrenaline were completely inhibited in the presence of 100 μM KT-362 ($n = 4$). However, the increments due to caffeine were not inhibited by KT-362 even at a higher concentration (300 μM) ($[Ca^{2+}]_{cyt}$ to 94.2 $\pm$ 4.2% and muscle tension to 100.8 $\pm$ 5.6%, $n = 4$).

Discussion

In isolated aorta of the rat, KT-362 inhibited the high K^+-stimulated muscle tension and $[Ca^{2+}]_{cyt}$. The inhibitory effect of KT-362 on high K^+-induced contraction was antagonized by the increase in external Ca^{2+} concentration. These results are similar to those obtained with verapamil and other Ca^{2+} channel blockers (Flaim, 1982; Hof & Vuorela, 1983; Karaki & Weiss, 1984). A 1,4-dihydropyridine compound, Bay K 8644, did not modify the effect of KT-362. Bay K 8644 has been shown to stimulate Ca^{2+} entry through Ca^{2+} channels (Schramm *et al.*, 1983) and to antagonize the effects of verapamil, diltiazem and 1,4-dihydropyridine Ca^{2+} channel blockers but not the diphenylalkylamine Ca^{2+} channel blockers (Spedding & Berg, 1984). These results may suggest that although KT-362 has an effect similar to the Ca^{2+} channel blockers, the site of action of KT-362 is different from that of diltiazem or verapamil.

In vascular smooth muscle, it has been shown that Ca^{2+} channel blockers strongly inhibit the high K^+-induced contraction with less inhibitory effect on the noradrenaline-induced contraction (Flaim, 1982; Hof & Vuorela, 1983; Karaki & Weiss, 1984). By contrast, KT-362 inhibited the contractions induced by high K^+ and noradrenaline at similar concentrations. This result indicates that the inhibitory effect of KT-362 is not solely attributable to a Ca^{2+} channel blocker-like action. Recently, it has been shown that the noradrenaline-induced contraction is due to an increase in $[Ca^{2+}]_{cyt}$ and also to an increase in Ca^{2+} sensitivity of contractile elements (Sato *et al.*, 1988; Karaki *et al.*, 1988; Nishimura *et al.*, 1988; Karaki, 1989; Kitazawa *et al.*, 1989; Ozaki *et al.*, 1990). Verapamil inhibited $[Ca^{2+}]_{cyt}$ more strongly than muscle tension stimulated by noradrenaline, as reported previously (Sato *et al.*, 1988; Ozaki *et al.*, 1990) possibly because verapamil inhibits the increase in $[Ca^{2+}]_{cyt}$ but not the increase in Ca^{2+} sensitivity. In the present experiments, KT-362 inhibited the noradrenaline-stimulated muscle tension and $[Ca^{2+}]_{cyt}$ in parallel. Furthermore, KT-362 inhibited the verapamil-insensitivie portion of the noradrenaline-induced contraction with little effect on $[Ca^{2+}]_{cyt}$. This effect is not attributable to the direct inhibition of the smooth muscle contractile elements because a higher concentration of KT-362 did not inhibit the caffeine-induced contraction. These results suggest that KT-362 inhibits noradrenaline-induced increase in both $[Ca^{2+}]_{cyt}$ and Ca^{2+} sensitivity of contractile elements.

Noradrenaline induced a transient increase in muscle tension and $[Ca^{2+}]_{cyt}$ in Ca^{2+}-free solution which is attributable to the release of Ca^{2+} from intracellular storage sites. The transient changes induced by noradrenaline were inhibited by KT-362. KT-362 itself released a small amount of Ca^{2+} from a storage site. However, the inhibition of noradrenaline-induced changes does not seem to result from the depletion of the Ca^{2+} store because KT-362 did not inhibit the transient changes due to caffeine. It has been suggested that the noradrenaline-induced release of Ca^{2+} is attributable to receptor-mediated formation of inositol 1,4,5-trisphosphate whereas the caffeine-induced Ca^{2+} release is due to Ca^{2+}-induced Ca^{2+}-release mechanism (for review see Karaki & Weiss, 1988). Thus, KT-362 may relatively selectively inhibit the Ca^{2+} release due to inositol 1,4,5-trisphosphate in rat aorta. Previously, Shibata *et al.* (1987) showed that KT-362 inhibits the caffeine-induced contraction in rabbit aorta. The reason for this difference was not examined in the present experiments.

The inhibition by KT-362 of noradrenaline-stimulated Ca^{2+} influx, Ca^{2+} release and Ca^{2+} sensitization may be due to antagonism of α-adrenoceptors or inhibition of receptor-mediated signal transduction. However, KT-362 does not seem to be a selective inhibitor of α-adrenoceptors because this inhibitor also inhibited the contractions induced by histamine and prostaglandin $F_{2\alpha}$ in vascular smooth muscle and carbachol in intestinal smooth muscle (unpublished observations). KT-362 has been shown to inhibit the phosphatidyl inositol turnover in canine femoral artery (Eskinder *et al.*, 1989). This may be the reason why KT-362 inhibits the receptor-mediated changes.

In summary, the vasodilator effect of KT-362 may be explained as follows. KT-362 inhibits high K^+-induced contraction by inhibiting Ca^{2+} channels to decrease the $[Ca^{2+}]_{cyt}$. Furthermore, KT-362 inhibits noradrenaline-induced contraction by inhibiting Ca^{2+} influx, Ca^{2+} release and Ca^{2+} sensitization of contractile elements possibly by inhibiting the receptor-mediated signal transduction.

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Effects of histamine H_1-, H_2- and H_3-receptor selective drugs on the mechanical activity of guinea-pig small and large intestine

R. Leurs, M.M. Brozius, M.J. Smit, A. Bast & H. Timmerman

Department of Pharmacochemistry, Faculty of Chemistry, Vrije Universiteit, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands

1 In this study we have evaluated the possible contribution of acetylcholine release in histamine-induced contractions of guinea-pig large and small intestinal smooth muscle. Moreover, the presence of the histamine receptor types involved in smooth muscle relaxations and inhibition of electrically-induced twitches was studied by use of several selective agents.

2 Histamine-induced contractions appeared to be a pure H_1-receptor-mediated effect. Responses were not attenuated by the presence of 0.1 μM atropine and were competitively and stereoselectively inhibited by the two enantiomers of chlorpheniramine with pA_2 values of 6.73 ± 0.08, 7.30 ± 0.06, 6.93 ± 0.03 and 7.19 ± 0.04 for the L-isomer and 8.63 ± 0.09, 8.85 ± 0.09, 9.01 ± 0.16 and 8.98 ± 0.11 for the D-isomer in the duodenum, jejunum, ileum and colon, respectively.

3 There appeared to be a marked regional difference in sensitivity to histamine. In ileal and jejunal preparations pD_2 values of 6.24 ± 0.06 ($n = 22$) and 6.37 ± 0.07 ($n = 22$) were found, whereas the pD_2 values in the duodenum and colon were 5.55 ± 0.05 ($n = 36$) and 5.68 ± 0.06 ($n = 31$) respectively.

4 This regional difference in sensitivity to histamine was not due to variations in receptor affinity since pA_2 values for the two enantiomers of chlorpheniramine did not differ markedly among the four tested preparations. Since a similar variation in sensitivity was found for methacholine, it is likely that the signal transfer mechanism in guinea-pig ileum and jejunum is more efficient than in the duodenum and colon.

5 The H_2-agonists dimaprit and impromidine relaxed methacholine-precontracted (± 70% of maximum contraction) intestine at high concentrations (pD_2 values of 3.79 ± 0.03 and 4.44 ± 0.09 for the jejunum). These relaxations could not be antagonized by 0.1 μM tiotidine, famotidine or mifentidine and were observed in all parts of the intestine investigated.

6 The dimaprit analogues nordimaprit and homodimaprit (inactive at H_2-receptors) were equipotent in relaxing the methacholine-precontracted smooth muscle. Since several H_2-antagonists were also able to produce relaxations, we do not consider these relaxations to be mediated by a H_2-receptor subtype, but to be due to some nonspecific effects at the high concentrations used.

7 The histamine receptor involved in the inhibition of electrically-induced contractions in the presence of atropine could be classified as an H_3-receptor effect. In all parts of the intestine the H_3-agonist R-α-methylhistamine inhibited the twitches with pD_2 values ranging from 8.10 ± 0.06 (ileum) to 8.27 ± 0.03 (colon). This effect was competitively antagonized with the selective H_3-antagonist thioperamine (pA_2 values are 8.09 ± 0.07, 8.13 ± 0.05, 8.15 ± 0.04 and 8.36 ± 0.04 in duodenum, jejunum, ileum and colon, respectively.

8 The guinea-pig intestine is a suitable preparation for the evaluation of either H_1- or H_3-receptor effects. H_2-receptors, causing smooth muscle relaxation appear not to be present in our preparations. At high concentrations of H_2-receptor agents (agonists and antagonists) relaxations might be observed, due to unknown nonspecific effects.

Introduction

In their original paper, describing the subclassification of histamine receptors into H_1- and H_2-receptors, Ash & Schild (1966) used the *in vitro* contraction of guinea-pig isolated ileum as a test system for H_1-receptor activity. Although the intestinal preparation has become a very valuable tool for the screening of H_1-receptor selective drugs, the original notion of guinea-pig ileum as an H_1-receptor preparation (Ash & Schild, 1966) has been complicated by the outcome of several investigations.

It has been suggested that the contractile effects of histamine on guinea-pig ileum are partly dependent on a tetrodotoxin-sensitive release of acetylcholine (Rubenstein & Cohen, 1985). Moreover, using selective H_2-receptor agents Barker & Ebersole (1982) reported the presence of H_2-receptors on guinea-pig myenteric plexus neurones, mediating the release of acetylcholine. To complicate the situation even more, Bertaccini & Zappia (1981) described the presence of an H_2-receptor subtype on guinea-pig duodenum, mediating relaxations of precontracted muscle. H_2-receptor agonists, like dimaprit, impromidine and clonidine (also an α_2-agonist), caused relaxations with a potency lower than that usually found for e.g. the positive chronotropic effect on guinea-pig right atrium (Bertaccini & Zappia, 1981). The relaxations could however not be inhibited by the H_2-antagonists metiamide, cimetidine, ranitidine or oxmetidine, nor by tetrodotoxin, propranolol or phentolamine (Bertaccini & Zappia, 1981). Moreover, Fjalland (1979) also suggested the existence of an H_2-receptor subtype in guinea-pig ileum because histamine was shown to suppress electrically-induced twitches of ileal smooth muscle, although the order of potency of H_2-antagonists in inhibiting this action of histamine did not correlate with the known activity of the compounds at the H_2-receptor of guinea-pig right atrium (Fjalland, 1979). Recently the classification of histamine receptors has been extended (Arrang *et al.*, 1983) and the presence of an H_3-receptor, which is involved in the regulation of neurotransmitter release in both brain and peripheral tissues (Timmerman, 1990), is currently accepted. Since some H_2-antagonists can also act as H_3-antagonists (Schwartz *et al.*, 1986) the findings of Fjalland (1979) were re-evaluated by Trzeciakowski (1987) and from the latter study it was concluded that the observed inhibition of the electrically-induced

twitches was due to an H_3-receptor-mediated reduction of acetylcholine release (Trzeciakowski, 1987). Previously it has been reported that histamine could also inhibit electrically-induced twitches of guinea-pig ileum in the presence of atropine (Ambache & Zar, 1970; Ambache *et al.*, 1970). Based upon the inhibitory action of burimamide Ambache *et al.* (1973) also suggested the involvement of an H_2-receptor subtype. However, a pharmacological characterization of this effect in view of the currently accepted histamine receptor subclassification has not been provided.

In the present study we examined several of the problems outlined above, using highly selective histamine receptor agonists and antagonists. In both small and large intestine of the guinea-pig we investigated the possible indirect contractile effects of histamine (Rubenstein & Cohen, 1985), the existence of an H_2-receptor subtype, possibly mediating relaxations (Bertaccini & Zappia, 1981), and the inhibition of electrically-induced twitches of the intestinal smooth muscle in the presence of atropine, as originally described by Ambache & Zar (1970).

Methods

Tissue bath studies

Male guinea-pigs (350–450 g) were killed by a blow on the head. Small and large intestine were immediately excised and placed in Krebs-buffer (composition mM: NaCl 117.5, KCl 5.6, $MgSO_4$ 1.18, $CaCl_2$ 2.5, NaH_2PO_4 1.28, $NaHCO_3$ 25 and glucose 5.5). The first 12 cm of the small intestine was taken as duodenum and the middle portion was used as jejunum. The portion of the small intestine that lies 5–15 cm from the ileo-caecal valve was considered to be the ileum. The colonic smooth muscle was taken from the ascending colon. Intestinal smooth muscle strips (10 mm long, 2 mm wide) were prepared by cutting the intestine in the longitudinal direction; the mucosa was not removed. Muscle strips were mounted in 20 ml Krebs buffer, continuously gassed with 95% O_2:5% CO_2 and maintained at 37°C. Contractions were recorded isotonically under 0.4 g tension with a Hugo Sachs Hebel-Messvorsatz (TL-2)/HF-modem (Hugo Sachs Electronik, Hugstetten, West Germany) connected to a pen-recorder.

After equilibration for at least 45 min, with replacement of fresh Krebs-buffer every 10 min, cumulative dose-response curves for histamine or methacholine were recorded using half-log increments in agonist concentration in the organ bath until stable responses towards the contractile agent used were obtained.

To investigate the effect of the smooth muscle relaxants, smooth muscle strips were contracted to 70–80% of the maximal methacholine or histamine response, using single doses. In some experiments muscle strips were contracted with 40 mM KCl. This concentration of KCl also yielded 70–80% of the maximal methacholine response. The smooth muscle relaxants were subsequently added cumulatively.

In some experiments the muscle strips were mounted between two platinum electrodes. Rectangular-wave electrical pulses (Grass stimulator S-88, Grass Instruments Co., Quincy, U.S.A.) were delivered to the preparations at a frequency of 50 Hz and 0.5 ms duration in 10-pulse trains every 33 s. Muscle strips were stimulated supramaximally (50 V).

Antagonists were usually equilibrated for 5 min after which contractile or relaxant responses were retested; this period of time was usually sufficient to reach equilibrium as shown by Schild slope values that did not deviate from unity. For the antagonists atropine and D-chlorpheniramine, concentrations of 3, 10, 30 and 100 nM were tested, whereas for L-chlorpheniramine, 0.1, 0.3, 1 and 3 μM concentrations were used. Thioperamide was tested at concentrations of 30, 100 and 300 nM. Antagonistic activity (pA_2) was determined by use of a Schild-plot.

Statistics

All data shown are expressed as mean ± s.e.mean. EC_{50} values were transformed to logarithmic values (pD_2 values) for calculation of a mean value and statistical comparison. Tissue preparations from at least three different animals were used for each drug treatment. Statistical analysis was carried out with Student's t test; P values <0.05 were considered to indicate a significant difference.

Chemicals

Histamine dihydrochloride, procaine hydrochloride, 4-aminopyridine, cimetidine and atropine sulphate were purchased from Sigma Chemical Company Ltd. (St. Louis, U.S.A.). Ranitidine hydrochloride and tetraethylammonium chloride were obtained from Research Biochemicals Incorporated (Natick, U.S.A.). Gifts of famotidine (Merck Sharp & Dohme), tiotidine (Imperial Chemical Industries), mifentidine (De Angeli), thioperamide, R-α-methylhistamine maleate (Dr J.-C. Schwartz, Centre Paul Broca de l'INSERM, Paris), impromidine trihydrochloride (Smith Kline & French), 2-amino-6-dimethylamino-4,5,6,7-tetrahydrobenzo[d]thiazole (SND 861 C12, Boehringer Ingelheim), D- and L-chlorpheniramine (Dr A.J. Beld, Catholic University Nijmegen, The Netherlands) and pinacidil (Leo Pharmaceuticals) are gratefully acknowleged. Dimaprit dihydrobromide, nordimaprit dihydrobromide and homodimaprit dihydrobromide were taken from laboratory stock (Sterk *et al.*, 1984). All other reagents were of analytical grade.

Results

Histamine contracts smooth muscle of guinea-pig large and small intestine in a concentration-dependent way. There appeared to be a significant difference in sensitivity of the various intestinal preparations to histamine (Table 1). Ileal and jejunal smooth muscle have an approximately equal sensitivity to histamine, but are almost 5 fold more sensitive than the duodenal and colonic preparations under the present conditions (Table 1). Similar observations were made for the muscarinic agent methacholine, which was also more potent in contracting ileal and jejunal smooth muscle than duodenal and colonic preparations (Table 1).

The histamine-induced contractions were competitively inhibited by the two chlorpheniramine enantiomers. Schild-slope values did not significantly differ from unity (Table 2). In all tissue preparations the D-isomer of this H_1-antagonist appeared to be the most potent agent (50–80 fold more active than the L-isomer, Table 2). The contractions elicited by methacholine were competitively antagonized by atropine; Schild slope values did not deviate significantly from unity (Table 2).

Based on the results of the antagonism of methacholine-induced contractions a concentration of 0.1 μM atropine was chosen for the subsequent experiments, which were undertaken to elucidate a possible indirect contractile mechanism for histamine. As can be seen in Table 1, there was no indication of a contribution of histamine-induced acetylcholine release to the observed contractions of intestinal smooth muscle after histamine application. The pD_2 values for histamine in the parts of guinea-pig intestine examined were not influenced by the presence of 0.1 μM atropine under our experimental conditions (Table 1). Also the maximal response to histamine was not attenuated in the presence of atropine (Table 1).

To study the presence of an H_2-receptor subtype mediating relaxation of intestinal smooth muscle, we extended the original study of Bertaccini & Zappia (1981). As can be seen in Figure 1, dimaprit induced relaxation of methacholine-precontracted duodenal muscle strips. However, the potency of dimaprit was rather weak. High concentrations of dimaprit as well as of impromidine were necessary to relax the intesti-

Table 1 Characteristics of histamine- and methacholine-induced contractions of guinea-pig large and small intestinal smooth muscle

	Duodenum	*Jejunum*	*Ileum*	*Colon*
pD_2 Histamine	5.55 ± 0.05 (36)	6.37 ± 0.07 (22)	6.24 ± 0.06 (22)	5.68 ± 0.06 (31)
pD_2 Methacholine	6.09 ± 0.12 (13)	6.73 ± 0.09 (7)	6.74 ± 0.10 (8)	6.17 ± 0.05 (12)
pD_2 Histamine + atropine	5.53 ± 0.14 (8)	6.52 ± 0.14 (4)	6.26 ± 0.03 (6)	5.59 ± 0.12 (8)
% maximal response histamine + atropine	99 ± 3 (8)	99 ± 2 (4)	100 ± 9 (6)	108 ± 5 (8)

The pD_2-values for histamine and methacholine for each part of the intestine are shown. Histamine-induced contractions were also measured in the presence of 0.1 μM atropine. The pD_2 values and maximal responses (expressed as the percentage of the control responses) are shown for each part of the intestine. Data shown are mean ± s.e.mean. Numbers in parentheses are the numbers of independent experiments.

nal smooth muscle completely (Figure 1, Table 3, Table 4). We also tried to antagonize this relaxant effect of dimaprit with the H_2-antagonists tiotidine, mifentidine and famotidine. In concentrations that can be expected to inhibit H_2-receptor responses (0.1 μM) none of these H_2-antagonists affected the dimaprit-induced relaxations of guinea-pig duodenum. To characterize the receptor subtype involved more clearly, two dimaprit analogues, devoid of H_2-receptor activity, were examined. Both nordimaprit and homodimaprit were able to relax the methacholine-precontracted duodenum completely and were equipotent with dimaprit (Figure 1). The pD_2 values for the relaxations of the duodenum were 3.34 ± 0.12 ($n = 9$), 3.42 ± 0.05 ($n = 4$) and 3.28 ± 0.11 ($n = 4$) for dimaprit, homodimaprit and nordimaprit respectively.

These findings were not confined to guinea-pig duodenum. Dimaprit was able to relax methacholine-precontracted muscle strips of guinea-pig ileum as well as jejunum and colon with similar potency (Table 3). Homodimaprit proved to be as effective as dimaprit in this respect (Table 3). Guinea-pig colon was the least sensitive to dimaprit and homodimaprit; contractions were not completely relaxed in this preparation (Table 3). Moreover, dimaprit was also able to relax methacholine-precontracted rat jejunal muscle strips (pD_2 = 3.60 ± 0.09, $n = 2$).

Table 2 Characteristics of the antagonism of the histamine- and methacholine-induced contractions respectively by the two stereoisomers of chlorpheniramine and atropine in the various parts of the intestine

	Duodenum	*Jejunum*	*Ileum*	*Colon*
pA_2 L-Chlorpheniramine vs histamine	6.73 ± 0.08 (1.2 ± 0.1) $n = 6$	7.30 ± 0.06 (1.0 ± 0.1) $n = 4$	6.93 ± 0.03 (1.1 ± 0.1) $n = 6$	7.19 ± 0.04 (0.9 ± 0.1) $n = 3$
pA_2 D-Chlorpheniramine vs histamine	8.63 ± 0.09 (1.2 ± 0.1) $n = 5$	8.85 ± 0.09 (1.1 ± 0.2) $n = 5$	9.01 ± 0.16 (1.2 ± 0.1) $n = 6$	8.98 ± 0.11 (0.9 ± 0.1) $n = 6$
pA_2 Atropine vs methacholine	8.49 ± 0.06 (1.1 ± 0.1) $n = 6$	8.44 ± 0.09 (1.3 ± 0.1) $n = 4$	8.72 ± 0.12 (1.3 ± 0.1) $n = 6$	8.85 ± 0.04 (1.1 ± 0.1) $n = 6$

pA_2-values and Schild slopes are the mean ± s.e.mean of several independent experiments. Numbers in parentheses are the Schild slopes. n: number of experiments.

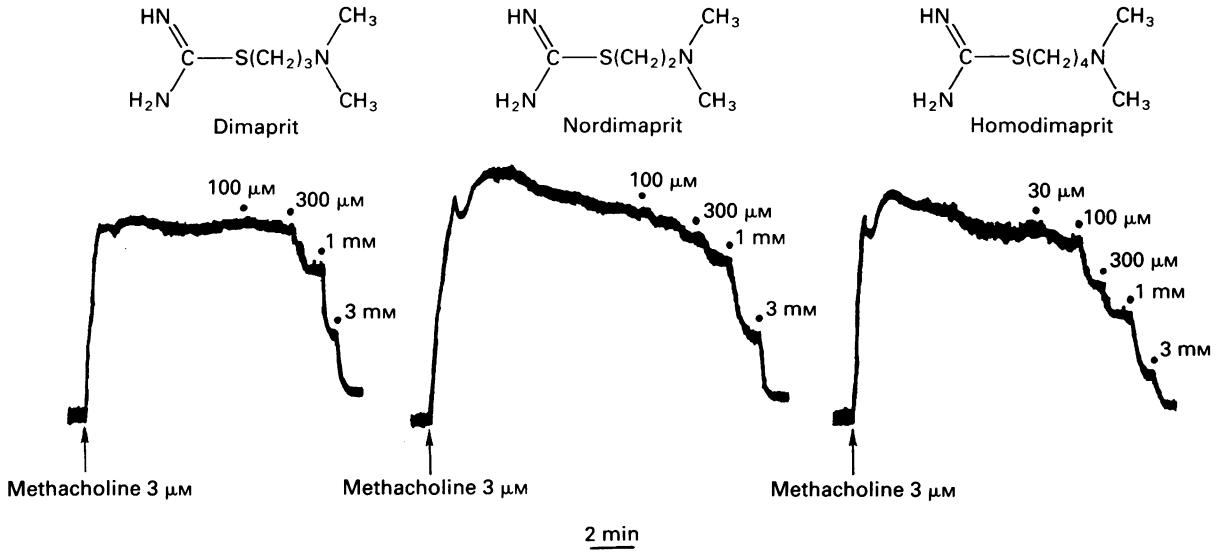

Figure 1 Representative recorder tracings of relaxations of guinea-pig duodenum induced by dimaprit, nordimaprit or homodimaprit. The duodenal preparation was precontracted with methacholine 3 μM and relaxations were recorded by cumulative applications of the relaxing agents.

Table 3 pD_2 values and maximal responses of dimaprit and homodimaprit for relaxing methacholine-precontracted muscle strips from guinea-pig duodenum, ileum, jejunum and colon

	Dimaprit			*Homodimaprit*		
Preparation	pD_2	% relaxation	*n*	pD_2	% relaxation	*n*
Duodenum	3.34 ± 0.12	95 ± 4	9	3.43 ± 0.05	109 ± 2	4
Ileum	3.67 ± 0.14	108 ± 10	7	3.83 ± 0.05	104 ± 3	7
Jejunum	3.79 ± 0.03	102 ± 1	62	4.13 ± 0.12	99 ± 4	7
Colon	3.34 ± 0.04	22 ± 3	7	3.38 ± 0.03	48 ± 2	3

Muscle strips of duodenum and colon were precontracted with methacholine 3 μM, whereas ileal and jejunal strips were stimulated with methacholine 1 μM. Data shown are mean ± s.e.mean of at least three independent experiments.
n: number of experiments.

Another dimaprit analogue, SND 861 C12, which comprises the complete structure of dimaprit in a cyclic molecule, behaved as a weak competitive H_2-antagonist against the positive chronotropic actions of histamine on guinea-pig right atrium ($pA_2 = 5.49 \pm 0.16$, $n = 2$, unpublished data from our laboratory). Nevertheless, despite its H_2-antagonistic properties this compound was 10 fold more potent than dimaprit in relaxing methacholine-precontracted jejunal smooth muscle strips (Table 4). Several other H_2-antagonists were also evaluated for their relaxant activity. Cimetidine, tiotidine, mifentidine and famotidine all appeared to be relaxing agents with a potency comparable to dimaprit (Table 4).

Dimaprit not only relaxed methacholine-precontracted intestinal smooth muscle but also relaxed jejunal strips precontracted with 1 μM histamine. The pD_2 value for relaxing these contractions was 3.83 ± 0.23 ($n = 5$). This value was not significantly different from the pD_2 values for the relaxation of methacholine-precontracted preparations (Table 4). Yet, if jejunal muscle strips were precontracted with KCl, using a concentration leading to an approximately similar contraction to that obtained with 1 μM methacholine, most of the histamine receptor agonists and antagonists were not able to relax the smooth muscle. Only at the highest tested concentrations (usually 3 mM) some small relaxations were sometimes noticed. Also the potassium channel opener, pinacidil, was more potent in relaxing methacholine-precontracted preparations ($pD_2 = 5.12 \pm 0.12$, $n = 10$) compared with 40 mM KCl-precontracted jejunal muscle strips ($pD_2 = 3.93 \pm 0.08$, $n = 11$) (Figure 2). Pinacidil concentration-dependently inhibited the spontaneous activity of the jejunal preparations. In contrast the potassium channel blockers tetraethylammonium (TEA, 0.3–10 mM), procaine (0.3–10 mM) and 4-aminopyridine (0.1 mM) concentration-dependently increased the amplitude of the basal rhythmic activity. Spontaneous activity induced by 1 mM TEA was, depending on the preparation, completely reduced by 5–20 μM pinacidil (Figure 3). However, none of the tested compounds affected basal jejunal activity and neither dimaprit (Figure 3) nor tiotidine could inhibit TEA-induced spontaneous activity at a concentration of 1 mM.

Table 4 pD_2 values and maximal responses of several compounds acting at histamine receptors for relaxing methacholine-precontracted muscle strips from guinea-pig jejunum

Drug	*pD_2*	*% relaxation*	n
Dimaprit	3.79 ± 0.03	102 ± 1	62
Impromidine	4.44 ± 0.09	95 ± 3	7
SND 861 C12	4.97 ± 0.18	100 ± 1	4
Cimetidine	3.61 ± 0.15	95 ± 3	7
Tiotidine	3.67 ± 0.02	98 ± 2	3
Famotidine	3.53 ± 0.05	93 ± 3	4
Mifentidine	3.97 ± 0.08	102 ± 1	4

Jejunal strips were precontracted with methacholine 1 μM. Data shown are mean ± s.e.mean of at least three independent experiments.
n: number of experiments.

Finally, we tried to characterize the reported effects of histamine on electrically-stimulated intestinal preparations in the presence of 0.1 μM atropine (Ambache & Zar, 1970; Ambache *et al.*, 1970; 1973). Tetanic stimulation of guinea-pig small

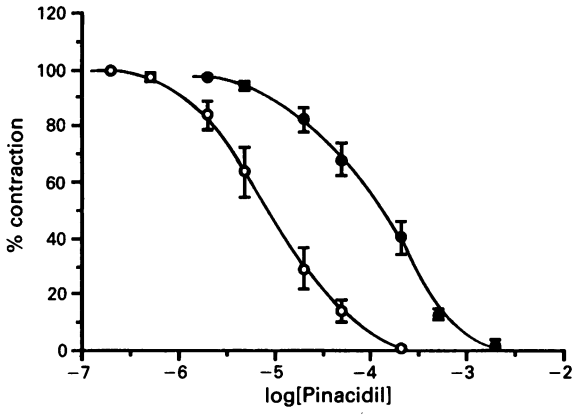

Figure 2 Dose-response curves for the pinacidil-induced relaxations of jejunal smooth muscle. The jejunal preparations were precontracted with 1 μM methacholine (○, $n = 10$) or 40 mM KCl (●, $n = 11$). Results are expressed as mean with s.e.mean shown by vertical bars.

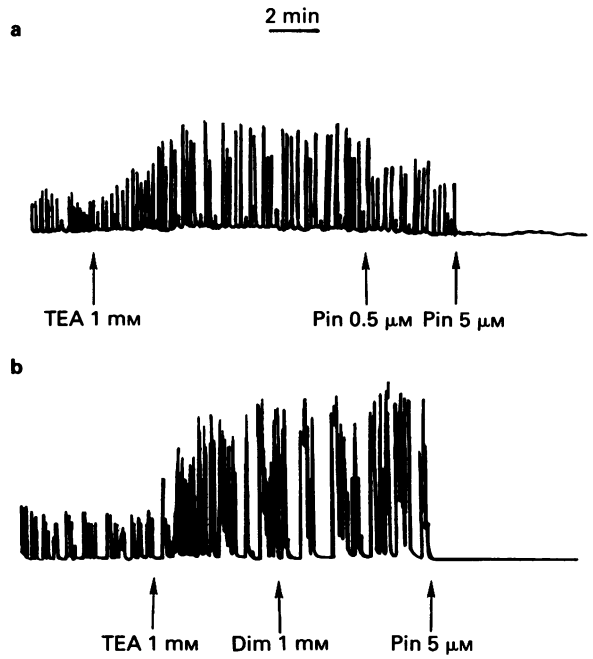

Figure 3 Effects of tetraethylammonium (TEA), pinacidil (Pin) and dimaprit (Dim) on the spontaneous motility of guinea-pig jejunum. Basal activity was enhanced by the addition of TEA 1 mM and was inhibited by pinacidil (a and b). Dimaprit did not attenuate the TEA effects (b).

intestine induced an atropine-resistant spasm, which consisted of several components (Figure 4a). Stimulation of colonic muscle strips resulted in a biphasic pattern: first a fast relaxation, which was followed by a rather slowly developing contraction (Figure 4b). In the present study we used the highly selective and potent H_3-receptor agonist **R**-α-methylhistamine to modulate the observed responses. The H_3-agonist was able to reduce the twitch height of the muscle strips at concentrations of 30 nM; maximal effect was usually noticed at a 0.3 μM concentration of **R**-α-methylhistamine (Figures 4 and 5). In the experiments with the colonic muscle strips only the contractile component of the response was modulated by **R**-α-methylhistamine (Figure 4b). The pD_2 value for **R**-α-methylhistamine did not differ markedly among the four preparations tested and was approximately 8.15 in all intestinal tissues (Table 5). The observed contractions could be inhibited by 60–70% with **R**-α-methylhistamine (Figure 5, Table 5).

The inhibitory effects of **R**-α-methylhistamine were competitively inhibited by the highly selective H_3-receptor antagonist thioperamide (Figure 5 inset). The potency of this compound was similar in all preparations; a pA_2 value of approximately 8.2 was found for all tissues (Table 5). The Schild-slope values did not deviate from unity in the various preparations (Table 5).

Discussion

The stimulatory action of histamine on intestinal smooth muscle has been known since the initial work of Dale & Laidlaw (1910, 1911). The development of the classical antihistamines and the observation that the histamine-induced effects on rat isolated uterus were refractory to inhibition by these drugs led to the hypothesis that histamine acted via at least two distinct receptor populations (Ash & Schild, 1966). After the discovery of burimamide (Black *et al.*, 1972) and related H_2-antagonists, this initial subclassification into H_1- and H_2-receptors was widely accepted.

Recently a new histamine receptor subtype has been described. The histamine H_3-receptor has been shown to affect [^{3}H]-histamine release from preloaded rat brain slices after potassium-depolarization (Arrang *et al.*, 1983) or electrical stimulation (Van der Werf *et al.*, 1987). This new receptor subtype shows remarkably different pharmacological characteristics; highly selective agonists (**R**-α-methylhistamine) and antagonists (thioperamide) have already been described (Arrang *et al.*, 1987). Due to the development of these selective H_3-receptor agents, investigations on the possible physiological role of the H_3-receptor have been conducted. It is now becoming clear that the H_3-receptor forms a general presynaptic regulatory system, which is not restricted to the brain (Timmerman, 1990).

Although the guinea-pig ileum was the first tissue used as an H_1-receptor preparation (Ash & Schild, 1966), there are now several reports indicating that histamine can act via several histamine receptor subtypes in the intestinal preparation. Histamine contracts both ileal longitudinal and circular smooth muscle layer via activation of H_1-receptors (Barker, 1985; Yamanaka & Kitamura, 1987), but can probably also relax circular muscle via an H_2-receptor-induced

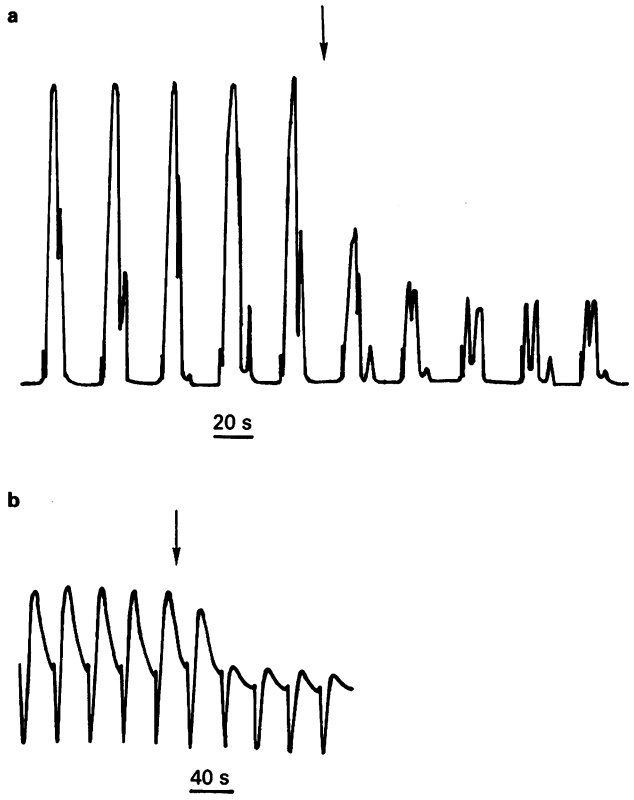

Figure 4 Representative recorder tracings of electrically-stimulated ileal (a) and colonic (b) smooth muscle strips. The arrow indicates the addition of **R**-α-methylhistamine 1 μM.

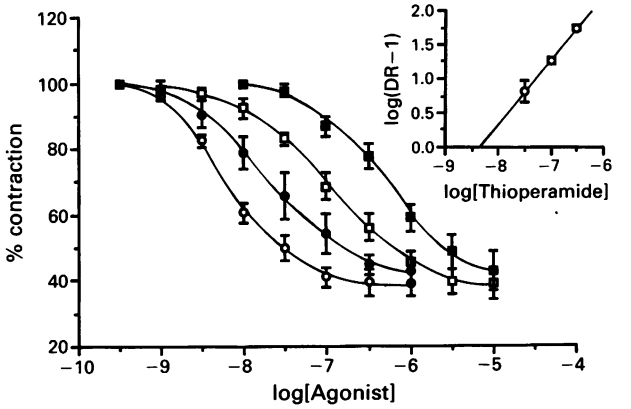

Figure 5 Dose-responses curve for the inhibition of electrically induced twitches of guinea-pig colonic muscle strips by the H_3-agonist, **R**-α-methylhistamine. A dose-response curve for **R**-α-methylhistamine in the absence (○) and presence of 30 (●), 100 (□) and 300 nM thioperamide (■) is shown. The inhibitory effects could be competitively antagonized by thioperamide. The inset shows the transformation of the data in the presence of thioperamide into a Schild plot. Results are expressed as mean of $n = 7$; s.e.mean shown by vertical bars.

Table 5 Activities of the H_3-receptor agonist **R**-α-methylhistamine and H_3-receptor antagonist thioperamide on electrically-stimulated muscle strips of guinea-pig large and small intestine

	R-α-methylhistamine			*Thioperamide*		
Preparation	pD_2	% inhibition	*n*	pA_2	Schild slope	*n*
Duodenum	8.09 ± 0.07	69 ± 4	6	8.09 ± 0.07	0.9 ± 0.1	6
Ileum	8.10 ± 0.06	65 ± 3	6	8.15 ± 0.04	0.9 ± 0.1	6
Jejunum	8.18 ± 0.06	65 ± 4	7	8.13 ± 0.05	1.0 ± 0.1	7
Colon	8.27 ± 0.03	57 ± 4	7	8.36 ± 0.04	0.9 ± 0.1	7

Data shown are mean ± s.e.mean of several independent experiments.
n: number of experiments.

hyperpolarization of the membrane (Yamanaka & Kitamura, 1987). Besides, H_2-receptor agonists have been reported to be both contractile and relaxant agents of guinea-pig intestinal longitudinal smooth muscle (Bertaccini & Zappia, 1981; Barker & Ebersole, 1985). Dimaprit has been reported to release acetylcholine from guinea-pig ileum myenteric plexus neurones in a tiotidine-sensitive manner (Barker & Ebersole, 1985), whereas acetylcholine release is also reported to contribute to the histamine-mediated contraction of the longitudinal smooth muscle (Rubenstein & Cohen, 1985).

In the present study such a contribution of acetylcholine could not be found in any of the intestinal preparations tested. Histamine proved to be a potent contractile agent, but the pD_2 value for histamine was not affected by the presence of 0.1 μM atropine. Based upon the measured pA_2 values for atropine against methacholine-induced responses (8.44–8.85), it can be concluded that in our preparations, 0.1 μM atropine is a suitable concentration for the study to a contribution of acetylcholine release to the histamine-mediated contractions. Such a contribution is therefore rather unlikely.

Finally, the histamine-induced contractions were competitively and stereoselectively antagonized by the two stereoisomers of the H_1-receptor antagonist chlorpheniramine. The pA_2 values for the two stereoisomers correspond well with their reported affinity for the H_1-receptor (Hill & Young, 1980) and do not differ markedly among the different intestinal preparations. These data suggest that the histamine-induced contraction is only due to the activation of H_1-receptors. Based upon the pA_2 values of the H_1-antagonists, there appeared to be no marked regional variation in H_1-receptor affinity. Previously similar findings have been reported by Barker (1985) for guinea-pig ileum and colon. In contrast to the pA_2 values for the H_1-receptor, a remarkable regional variation in sensitivity to histamine was noticed among the four preparations tested. This difference might be due to differences in H_1-receptor density or the stimulus transfer mechanism. Since methacholine-induced responses show a similar variation in sensitivity we assume that in contrast to the duodenum and colon, the guinea-pig ileum and jejunum possess an efficient stimulus transfer mechanism leading to a receptor reserve.

As already mentioned, it has been reported that H_2-receptor agonists can relax duodenal longitudinal smooth muscle (Bertaccini & Zappia, 1981). According to these authors, the relaxations could not be antagonized by a number of H_2-antagonists such as cimetidine, metiamide, ranitidine and oxmetidine and it was therefore suggested that they were mediated by an H_2-receptor subtype (Bertaccini & Zappia, 1981). In the present study these relaxations were observed in guinea-pig ileum, jejunum, colon and rat jejunum and several agents acting on histamine receptors were used for a more detailed pharmacological characterization. The recently developed H_2-receptor antagonists famotidine, mifentidine and tiotidine were all unable to antagonize the dimaprit-induced relaxations. Besides dimaprit, impromidine also relaxed the precontracted intestinal muscle at relatively high concentrations. The length of the alkyl chain in dimaprit is rather essential for H_2-receptor agonistic activity as both nordimaprit and homodimaprit possess hardly any H_2-receptor activity (Ganellin, 1982). Yet, these two compounds are approximately equipotent with dimaprit in relaxing the guinea-pig duodenum. Moreover, SND 861 C12, which comprises the structure of dimaprit in a cyclic molecule, is the most potent relaxant agent observed in this study. Nevertheless, this compound is devoid of any H_2-agonistic activity and behaves even as a weak competitive H_2-receptor antagonist against histamine-induced positive chronotropic effects in guinea-pig right atrium ($pA_2 = 5.49$). Moreover, other H_2-receptor antagonists also proved to be able to relax intestinal smooth muscle at high concentrations. Based on these results, the presence of an H_2-receptor subtype, involved in smooth muscle relaxation, seems rather unlikely. Despite their activity at the H_2-receptor (agonist, antagonist or inactive) all tested compounds were able to produce relaxations of visceral smooth muscle. Since α_2-adrenoceptor agonists (clonidine and tolazoline) are also relaxant agents in this preparation (Bertaccini & Zappia, 1981) the observed effects are probably related to a nonspecific effect at the high concentrations used.

In this study it was observed that muscle strips, when precontracted with KCl, were rather resistant to relaxation by agents acting on histamine receptors. This might indicate that the observed relaxations are due to an action at the potassium channels in the plasma membrane. It has been shown that some recently developed smooth muscle relaxants act via the opening of potassium channels (Weir & Weston, 1986). These drugs have been reported to be able to relax vascular pulmonary and also intestinal smooth muscle (Matsui et al., 1986; Weir & Weston, 1986; Buchheit & Bertholet, 1988; Mellemkjaer et al., 1989). The potassium channel opener, pinacidil, bears some structural similarity to some H_2-receptor antagonists and indeed proved to be more effective in relaxing methacholine-precontracted muscle strips than KCl-precontracted preparations. However, pinacidil also concentration-dependently inhibited jejunal spontaneous motility. This activity is not shared by any of the agents acting on histamine receptors. The inhibition of basal motor activity can probably be related to interference with the potassium channels, since similar findings have been published for another potassium channel opener, cromakalim (Buchheit & Bertholet, 1988). Moreover, in the present study, several potassium channel blockers (tetraethylammonium, 4-aminopyridine, procaine) proved to enhance the intestinal motility. This effect could be inhibited by pinacidil, but was not affected by dimaprit or tiotidine. These data indicate that intereference at the level of potassium channels cannot explain the relaxant effects of the agents acting on histamine receptors. The mechanism of action involved therefore remains to be elucidated.

Finally we studied the involvement of the H_3-receptor in the inhibitory response of histamine on electrically stimulated intestinal smooth muscle strips (Fjalland, 1979; Ambache & Zar, 1970; Ambache et al., 1970; 1973). Based on the inhibitory effects of burimamide, this effect was suggested to be mediated by an H_2-receptor subtype (Ambache et al., 1973; Fjalland, 1979). Recently Trzeciakowski (1987) showed that under conditions of electrical stimulation, that release acetylcholine, the histamine-induced inhibition could be denoted as an H_3-receptor effect. However, the myenteric plexus contains a rather heterogenous neuronal population, which might release various substances (Sternini, 1988). Besides acetylcholine, noradrenaline, 5-hydroxytryptamine, several neuropeptides and excitatory aminoacids are probably also involved in the regulation and modulation of intestinal motility (Sternini, 1988; Nemeth & Gullikson, 1989; Shannon & Sawyer, 1989). Indeed, Ambache & Freeman (1968) observed that tetanic stimulation of guinea-pig ileum produced a contraction due to the release of a non-cholinergic substance(s) from the myenteric plexus. The nature of the contractile substance(s) is not clear yet, but is now under investigation in our laboratory. The contractions after tetanic stimulation (in the presence of 0.1 μM atropine) were reduced by histamine (Ambache et al., 1970; Ambache & Zar, 1970) via a suggested H_2-receptor subtype (Ambache et al., 1973). By use of the highly selective H_3-receptor agonist **R**-α-methylhistamine, twitch responses in the presence of 0.1 μM atropine could be inhibited in all intestinal preparations. These effects are effectively and competitively antagonized by thioperamide, a highly selective H_3-receptor antagonist. The pA_2 values for this compound correlate well with previous findings (Arrang et al., 1987) and do not differ among the preparations tested, indicating a homogeneous population of H_3-receptors in the intestinal tract. These receptors appear to modulate the release of acetylcholine (Trzeciakowski, 1987), but can also inhibit the release of other non-cholinergic contractile substances. The H_3-receptor appears therefore to be a rather general regulatory mechanism in the intestine.

In conclusion, our study indicates that the guinea-pig intestine contracts in response to histamine via a pure H_1-receptor-activated mechanism. In the preparations we used, no contribution of acetylcholine release was observed. At high concentrations of certain histamine agonists and antagonists relaxation of the smooth muscle unrelated to H_2-receptor stimulation occurs via a yet unidentified nonspecific action. Moreover, in all parts of the intestine H_3-receptors appear to be present. Besides acetylcholine, release of unidentified, non-cholinergic substances are also inhibited via H_3-receptor stimulation. The intestinal smooth muscle might therefore be a suitable preparation for the evaluation of either H_1-receptor or H_3-receptor effects.

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Prejunctional nicotinic receptors involved in facilitation of stimulation-evoked noradrenaline release from the vas deferens of the guinea-pig

†*L. Todorov, *K. Windisch, **H. Shersen, **A. Lajtha, †*M. Papasova & [1]*E.S. Vizi

*Institute of Experimental Medicine, Hungarian Academy of Sciences, 1450 Budapest, P.O.B. 67, Hungary; **Center of Neurochemistry, Ward's Island, New York 10035, U.S.A. and †Institute of Physiology, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria

1 In guinea-pig prostatic vas deferens loaded with [^{3}H]-noradrenaline ([^{3}H]-NA), nicotinic receptor agonists, nicotine and dimethylphenylpiperazinium (DMPP) enhanced the resting and facilitated the stimulation-evoked release of [^{3}H]-NA in a concentration-dependent fashion. The effect of nicotine on both contraction of vas deferens and release of NA in response to field stimulation was stereospecific in favour of the naturally occurring (−)-enantiomer. Prolonged (15 min) exposure to (−)-nicotine resulted in a cessation of the facilitatory effect on NA release and on responses of the vas deferens to field stimulation.

2 The rank order of agonist potency in facilitating NA release was DMPP = (−)-nicotine > (+)-nicotine. Cytisine had no agonistic activity. The dissociation constants (K_D) of antagonists were 9.3 ± 0.6 and 31.4 ± 2.4 μM for (+)-tubocurarine and hexamethonium, respectively, when (−)-nicotine was used as agonist. α-Bungarotoxin had no antagonistic activity. These findings suggest that nicotinic receptors located on noradrenergic axon terminals are different from those located postsynaptically in striated muscle or ganglia but seem similar to those present on cholinergic axon terminals at the neuromuscular junction.

3 Cotinine, the breakdown product of nicotine failed to have any agonistic activity indicating that nicotine itself is responsible for the effects observed on axon terminals.

4 Stimulation of presynaptic muscarinic receptors by oxotremorine prevented the nicotine-induced facilitation of [^{3}H]-NA release, indicating the presence of both inhibitory muscarinic and facilitatory nicotinic receptors on noradrenergic axon terminals.

Introduction

A well-established property of nicotine in the peripheral nervous system is its agonist activity at nicotinic cholinoceptors in autonomic ganglia which give rise to the release of noradrenaline and acetylcholine from postganglionic sympathetic and parasympathetic axon terminals, respectively. In addition, there is universal agreement (cf. Westfall *et al.*, 1987) that nicotinic receptor stimulation of the nerve terminals of the nigrostriatal pathway enhances the resting release of dopamine from both synaptosomal (cf. de Belleroche & Bradford, 1978; Rapier *et al.*, 1988) and slice preparations (Westfall, 1974; Giorgueieff *et al.*, 1976). The effect of nicotinic receptor agonists on stimulation-evoked release of transmitters from axon terminals is rather controversial. While nicotine and other nicotinic receptor agonists have no effect on noradrenaline (NA) release from rat heart (Fuder *et al.*, 1982) and brain vessels (Edvinsson *et al.*, 1977), evidence is available that activation of presynaptic nicotinic receptors results in a facilitation of stimulation-evoked transmitter release in guinea-pig heart (NA, Lindmar *et al.*, 1968; Westfall & Brasted, 1972) and in skeletal muscle (acetylcholine, Vizi *et al.*, 1987; Vizi & Somogyi, 1989).

The purpose of the present investigation was to determine whether nicotine itself releases NA and/or facilitates electrical stimulation-evoked release. In addition, an attempt was made to study the mechanism of this action by examining the effects of pharmacological agents known to influence the action of nicotine on both contractions of, and [^{3}H]-NA release from, guinea-pig isolated vas deferens in response to field stimulation.

[1] Author for correspondence.

Methods

Guinea-pigs of either sex, weighing 300–500 g, were killed by a blow to the head under light ether anaesthesia. The excised vas deferens was incubated for 40 min in Krebs solution (composition in mM: NaCl 113, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, Mg_2SO_4 1.2, $NaHCO_3$ 25.0 and glucose, 11.5) at 37°C containing [1-7,8-^{3}H]-noradrenaline (490 kBq ml^{-1}, 555 GBq mmol^{-1} sp. act., Amersham). During the incubation, the medium was bubbled with 95% O_2 and 5% CO_2. After incubation the vasa were washed several times with Krebs solution and suspended in 2.5 ml organ baths. The preparations were superfused (1 ml min^{-1}) for 90 min with Krebs solution at 37°C containing ascorbic acid (3×10^{-3} M) Na_2EDTA (10^{-4} M) and prednisolone (10^{-4} M) and the effluent was discarded. Subsequently, 3 min fractions were collected. The total radioactivity of tritiated compounds ([^{3}H]-NA and ^{3}H-metabolites) released from the preparations was monitored by adding 1 ml samples of the perfusate to 7 ml of liquid scintillation fluid prepared by mixing 6 g 1,4-*bis*-(5-phenyloxazolyl-2)-benzene (POPOP), 2 g of 2,5-diphenyloxazole (PPO), 2000 ml toluene, and 100 ml of Triton X-100. Radioactivity was determined in a liquid scintillation counter (Packard 544) and the counts were converted to absolute activity by the external standard method. Release of tritium was expressed in Bq g^{-1} and as a percentage of the amount of radioactivity in the tissue at the time when the sample was collected (fractional release). A computer programme was used for calculation of fractional release. For assay of residual radioactivity, the tissues were blotted with filter paper, weighed, homogenized in 1 ml of ice-cold 10% trichloroacetic acid, and centrifuged at 1500 *g* for 10 min. An aliquot of the supernatant was assayed for radioactivity.

The tissues were stimulated through platinum ring electrodes, one located above and the other below the suspended vas deferens. Supramaximal ($>10\,V\,cm^{-1}$) field stimuli of 0.5 ms duration were applied at 8 Hz, for 1 min or 20 s periods by means of an Eltron (Budapest, Hungary) stimulator. Drugs were added to the organ bath 30 s before the second stimulation unless stated otherwise.

In some experiments contraction was measured with a force displacement transducer and recorded on a potentiometric recorder (Goerz Servogor). The EC_{50} value (concentration needed to enhance the response by 50%), was calculated from concentration-response curves. S_1 and S_2 refer to first and second periods of stimulation, respectively.

To determine the proportion of the released radioactivity attributable to [^{3}H]-NA the perfusate was analysed by high performance liquid chromatography (h.p.l.c.) combined with radiochemical detection (Vizi *et al.*, 1985). In agreement with others (Roffler-Tarlov & Langer, 1971), the release of radioactivity in response to electrical stimulation alone or with nicotine receptor stimulation was due largely to [^{3}H]-NA.

In some experiments the apparent equilibrium dissociation constant (K_D) for antagonists was determined by the dose-ratio method described by Furchgott (1972). The following equation was used to relate the dissociation constant (K_D) to the dose-ratio and the antagonist concentration

$$K_D = \frac{a}{DR - 1}$$

Where DR is the concentration-ratio, i.e. the EC_{50} value for agonist (nicotine) in the presence of the antagonist divided by the EC_{50} value in the absence of antagonist and a is the concentration of antagonist. EC_{50} indicates the concentration of agonist needed to produce a 50% increase of S_2/S_1 value. Three different concentrations of agonists were used to establish a concentration-response curve and each point was derived from three different experiments.

Statistics

Statistical analysis was carried out with one way analysis of variance followed by Dunn test. All data in the text are expressed as means ± s.e.mean.

Drugs

The following drugs were used: (−)-[7,8-^{3}H]-noradrenaline (Amersham); (−)-nicotine hydrogen tartrate (Sigma); (+)-nicotine hydrogen tartrate (Sigma); cytisine (Sigma); (−)-cotinine (Sigma); oxotremorine (Loba Chemie, Vienna); dimethylphenylpiperazinium (DMPP, Sigma); hexamethonium bromide (Sigma); (+)-tubocurarine (Wellcome); cocaine hydrochloride (Chinoin, Hungary); desipramine (EGIS, Hungary), prazosin hydrochloride (Bayer); prednisolone (Di-Adreson, Organon); α-bungarotoxin (Sigma).

Results

After 40 min loading with [^{3}H]-NA, followed by a 90 min washout, the tissues contained 3,850,000 ± 152,500 Bq g^{-1} ($n = 16$) radioactivity. By use of h.p.l.c. combined with radiochemical detection it was established that 87.5 ± 4.2% of this was [^{3}H]-NA.

At rest, during the 3 min collection periods 0.70 ± 0.05% (25,500 ± 1082 Bq g^{-1}) ($n = 16$) of the total content of radioactivity was released. In response to field stimulation 1.16 ± 0.06% of radioactivity present in the tissue at the time of stimulation, was released over basal release ($n = 24$). The stimulation-evoked release was fairly constant: the ratio (S_2/S_1) between the fractional amounts of radioactivity released by two consecutive stimulations (27 min elapsed between the two stimulations) was 0.98 ± 0.04 ($n = 26$). During the experiments only 11.60 ± 0.50% ($n = 56$) of the total radioactivity taken up and stored was released.

Effect on resting release of [^{3}H]-noradrenaline

When the tissue was exposed for 1 min to (−)-nicotine (50 μM) or DMPP (50 μM) the resting release of radioactivity was transiently enhanced by 16,200 ± 1150 ($n = 4$) and 18,150 ± 1330 Bq g^{-1} ($n = 4$) respectively, representing 0.41 ± 0.03% and 0.47 ± 0.04% of tissue radioactivity content. When the exposure time was longer (5 min) the total amount of radioactivity released was not further increased. (−)-Nicotine (10 μM), DMPP (10 μM) or cytisine (100 μM) had no effect on resting release. Of the radioactivity released by nicotinic receptor stimulation, 71.5 ± 8.3% was [^{3}H]-NA. (+)-Tubocurarine (100 μM) completely prevented the effect of (−)-nicotine (50 μM) on resting release of [^{3}H]-NA.

Effect on stimulation-evoked release of [^{3}H]-noradrenaline

(−)-Nicotine or DMPP enhanced stimulation-evoked release of radioactivity in a concentration-dependent manner (Table 1). However (+)-nicotine was 38 times less effective than (−)-nicotine and cytisine, (10–100 μM), a compound with high affinity for nicotinic binding sites, failed to affect the release. The nicotine metabolite, cotinine (Jacob *et al.*, 1988) even in 100 μM concentration did not facilitate the release.

Prolonged exposure (15 min) to (−)-nicotine (50 μM) resulted in a cessation of the facilitatory effect on the stimulation-evoked release of [^{3}H]-NA (Table 2).

Nicotinic receptor antagonists (α-bungarotoxin hexamethonium or (+)-tubocurarine) alone did not affect the S_2/S_1 ratio for [^{3}H]-NA release (Table 3). However, hexamethonium and (+)-tubocurarine, but not α-bungarotoxin, prevented or reduced the effect of (−)-nicotine or DMPP on the stimulation-evoked release (Table 3). K_D values were calculated to be 9.3 ± 0.6 μM and 31.4 ± 2.4 μM for (+)-tubocurarine and hexamethonium, respectively, when (−)-nicotine was the agonist, and 41.8 ± 1.8 and 60.0 ± 0.6 respectively when DMPP was the agonist.

Oxotremorine decreased [^{3}H]-NA release in an atropine-sensitive manner, and completely inhibited the facilitatory effect of (−)-nicotine on stimulation-evoked release of [^{3}H]-

Table 1 Effect of nicotinic receptor agonists on stimulation-evoked release of [^{3}H]-noradrenaline ([^{3}H]-NA)

Drugs		*[^{3}H]-NA release* S_2/S_1	
Control		0.98 ± 0.04	(26)
(−)-Nicotine	1 μM	0.97 ± 0.06	(4)
(−)-Nicotine	2 μM	1.19 ± 0.02*	(4)
(−)-Nicotine	10 μM	1.72 ± 0.20*	(4)
(−)-Nicotine	50 μM	2.85 ± 0.45*	(4)
(−)-Nicotine	100 μM	3.12 ± 0.08*	(4)
(+)-Nicotine	100 μM	0.89 ± 0.06	(3)
(+)-Nicotine	200 μM	0.96 ± 0.07	(3)
(+)-Nicotine	500 μM	1.63 ± 0.11*	(3)
DMPP	1 μM	1.03 ± 0.07	(4)
DMPP	10 μM	1.57 ± 0.02*	(4)
DMPP	50 μM	2.97 ± 0.56*	(4)
Cytisine	100 μM	1.07 ± 0.08	(3)
Cotinine	100 μM	0.94 ± 0.05	(5)

Twenty seven minutes elapsed between the two stimulations (8 Hz, 480 shocks). Drugs were added to the organ bath 30 s prior to second stimulation (S_2). Note that rank order of activity of nicotinic agonists: DMPP = (−)-nicotine > (+)-nicotine ≫ cytisine. Mean ± s.e.mean. Number of experiments is in parentheses. The EC_{50} for (−)-nicotine and (+)-nicotine was 10.2 and 410 μM, respectively.
* Significant difference from control, $P < 0.05$.
DMPP = dimethylphenyl piperazinium.

Table 2 Effect of prolonged exposure to (−)-nicotine on [^{3}H]-noradrenaline release

	Control	(−)-Nicotine[1] (50 μM) 30 s exposure	15 min exposure
S_2/S_1	0.97 ± 0.07 (4)	2.48 ± 0.12* (4)	0.99 ± 0.12 (4)

[1] (−)-Nicotine was added to the organ bath 30 s or 15 min before the second stimulation (S_2). * Significant difference from control, $P < 0.01$. Twenty seven minutes elapsed between the two stimulations (8 Hz, 480 shocks).

NA (Table 4). Oxotremorine failed to affect the enhanced resting release induced by (−)-nicotine (50 μM).

Effect on mechanical responses of vas deferens to field stimulation

The response to field stimulation is biphasic: an initial fast contraction is followed by a slow response (Figures 1 and 2, left side). While (−)-nicotine (20 μM) produced a transient contraction and potentiated both phases of the response to stimulation, (+)-nicotine was much less effective (Figure 2 middle). Prazosin mainly reduced the second phase and slightly affected the initial phase of the muscle response to stimulation but prevented the transient contraction produced by (−)-nicotine (50 μM) (Figures 1 and 2, right). If the 1 min nicotine applications were repeated at intervals no shorter than 30 min, contractions and potentiation of responses to stimulation were reproducible. The EC_{50} value for (−)-nicotine potentiating the slow, NA-mediated response was 11 μM ($n = 4$).

Table 3 Hexamethonium and (+)-tubocurarine prevent the effect of nicotinic receptor agonists on [^{3}H]-noradrenaline ([^{3}H]-NA) release

Drugs		*[^{3}H]-NA release* S_2/S_1	
Control		0.97 ± 0.06	(6)
Hexamethonium,	300 μM	0.89 ± 0.08	(3)
(−)-Nicotine	50 μM	3.01 ± 0.21*	(6)
DMPP	50 μM	3.21 ± 0.21*	(3)
Hexamethonium, + (−)-nicotine	300 μM 50 μM	1.12 ± 0.10	(3)
(+)-Tubocurarine,	100 μM	0.84 ± 0.12	(3)
(+)-Tubocurarine, + (−)-nicotine	100 μM 50 μM	1.02 ± 0.22	(4)
(+)-Tubocurarine, + DMPP	100 μM 50 μM	1.55 ± 0.22*	(4)
α-Bungarotoxin,	3 μg ml^{-1}	1.01 ± 0.04	(4)
α-Bungarotoxin, + (−)-nicotine	3 μg ml^{-1} 50 μM	2.75 ± 0.15*	(4)

Twenty seven minutes elapsed between the two stimulations (8 Hz, 480 shocks).
Antagonists were added into the perfusion fluid 15 min prior to second stimulation. (−)-Nicotine or DMPP were added 30 s prior to second stimulation. Number of experiments is in parentheses. * Significant difference from control, $P < 0.05$.
DMPP = dimethylphenylpiperazinium.

Table 4 Interaction between muscarinic inhibitory and nicotinic stimulatory action on stimulation-evoked release of [^{3}H]-noradrenaline ([^{3}H]-NA)

	Drugs		*[^{3}H]-NA release* S_2/S_1		*Signif. $P < 0.05$*
1	Control		0.95 ± 0.06	(6)	
2	(−)-Nicotine,	50 μM	2.91 ± 0.18	(4)	2:1
3	Oxotremorine,	1 μM	0.54 ± 0.03	(4)	3:1
4	Oxotremorine, + (−)-nicotine,	1 μM 50 μM	1.04 ± 0.09	(4)	4:2 4:3
5	Atropine	1 μM	1.02 ± 0.08	(4)	
6	Atropine + oxotremorine, + (−)-nicotine,	1 μM 1 μM 50 μM	3.50 ± 0.21	(4)	6:4

Twenty seven minutes elapsed between the two stimulations (8 Hz, 480 shocks).
(−)-Nicotine and DMPP were added 30 s prior to second stimulation. Atropine and oxotremorine were added 15 min prior to second stimulation. Significance is calculated between groups as indicated in final column. Number of experiments in parentheses.
DMPP = dimethylphenylpiperazinium.

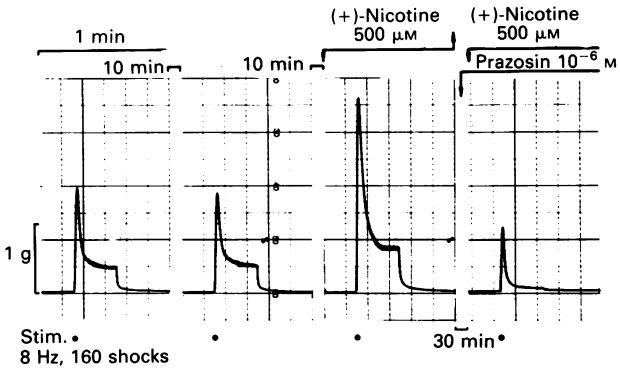

Figure 1 Effect of (−)-nicotine on contraction of prostatic part of guinea-pig vas deferens in response to stimulation. Field stimulations as indicated. Intervals between stimulation 10 and 30 min as indicated. Note that (−)-nicotine (20 μM) potentiated both phases of contraction in response to stimulation. The transient contraction and the second phase were mainly prazosin-sensitive.

Discussion

The presence of prejunctional nicotinic receptors on noradrenergic axon terminals in vas deferens of the guinea-pig is supported by our observations that the nicotinic receptor agonists, (−)-nicotine or DMPP, facilitated both the resting and stimulation-evoked release of [^{3}H]-NA and that these facilitations of release were reduced by hexamethonium or (+)-tubocurarine. It is unlikely that the elevated S_2/S_1 ratio in the presence of nicotine or DMPP was due simply to an additive effect of the increase in basal overflow to the release due to electrical stimulation. Thus, (−)-nicotine or DMPP at a

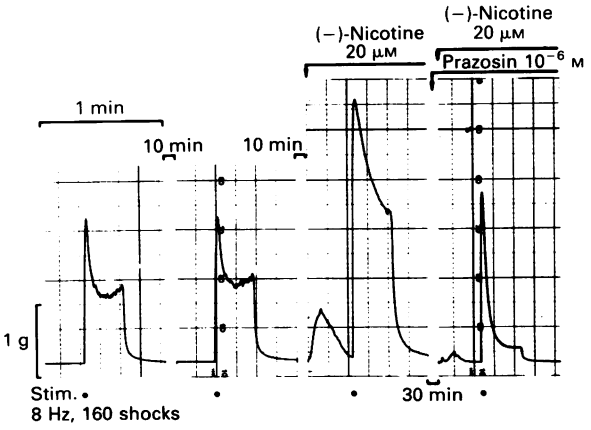

Figure 2 Effect of (+)-nicotine on contraction of prostatic part of guinea-pig vas deferens in response to stimulation. Field stimulation was indicated. Intervals between stimulations 10 and 30 min as indicated. Note that (+)-nicotine (500 μM) also potentiated the contraction, but in much higher concentrations than (−)-nicotine.

concentration (10 μM) that did not affect resting release, significantly enhanced stimulation-evoked release. Furthermore, when the transient contraction to (−)-nicotine had ceased the contraction of the muscle in response to stimulation was still potentiated (Figure 1).

The effect of nicotine on both contraction of vas deferens and release of NA in response to field stimulation was stereospecific with (+)-nicotine being 40 times less effective in facilitating NA release than (−)-nicotine. A similar observation was made by Rapier *et al.* (1988) in measuring dopamine release from striatal synaptosomes. The rank order of potency for presynaptic agonistic activity in our experiments was DMPP = (−)-nicotine > (+)-nicotine ≫ cytisine, whereas that obtained by Wonnacott (1987) using rat brain with binding techniques was cytisine > (−)-nicotine > DMPP > (+)-nicotine. Our observation for the rank order of antagonists ((+)-tubocurarine > hexamethonium > α-bungarotoxin) indicates that the presynaptic nicotinic receptors located on the axon terminals of sympathetic neurones are different from the well-characterized receptors located somatodendritically (e.g., in the ganglion, where the rank potency order of antagonists is hexamethonium ≫ (+)-tubocurarine), (Paton & Zaimis, 1952) or postjunctionally on skeletal muscle (the rank order: (+)-tubocurarine > α-bungarotoxin > hexamethonium), but are similar to those located on cholinergic axon terminals at the neuromuscular junction (Vizi *et al.*, 1987; Vizi & Somogyi, 1989). The nicotinic receptors of electric organs of the skate and eel are different from those of muscle and ganglia (Loring & Zigmond, 1988). The existence of multiple nicotinic receptors is supported by recent molecular biology studies in which different subtypes of nicotinic receptor were identified in brain (Goldman *et al.*, 1987; Colquhoun *et al.*, 1987).

The second, tonic part of the motor response to field stimulation of this preparation is noradrenergic (Bentley & Sabine, 1963; Vizi & Burnstock, 1988) and is mediated via α_1-adrenoceptors (Figure 1). The first phasic contraction was also potentiated by (−)-nicotine and the extent of potentiation of the first rapid twitch and the tonic second contraction was similar (Figures 1 and 2).

For guinea-pig vas deferens it was suggested (Birmingham & Wilson, 1963; Robinson, 1969; Fukushi & Wakui, 1987) that cholinergic input exerts a dual modulatory effect on sympathetic neuro-effector transmission: through presynaptic muscarinic receptors acetylcholine (ACh) would reduce the release of NA, whereas, on the effector cells, also through muscarinic receptors, ACh would enhance the response (Stjärne, 1975). In our study oxotremorine, a muscarinic receptor agonist, reduced the release of [^{3}H]-NA and antagonized the nicotinic receptor-mediated facilitation of [^{3}H]-NA release evoked by field stimulation. Thus if muscarinic receptors are stimulated, the nicotinic receptor-mediated potentiation of NA release is not operative. But the fact that both muscarinic and nicotinic receptors are involved in the modulation of NA release indicates that the cholinergic innervation of the vas deferens (Birmingham & Wilson, 1963; Knoll *et al.*, 1972) may play a role in presynaptic modulation of NA release from the sympathetic axon terminals. The concentration of nicotine achieved during smoking (0.3–2 μM, Russell *et al.*, 1980; Jacob *et al.*, 1988) is comparable to that which affects prejunctional nicotinic receptors in the guinea-pig isolated vas deferens.

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Vascular 5-HT_1-like receptors that mediate contraction of the dog isolated saphenous vein and carotid arterial vasoconstriction in anaesthetized dogs are not of the 5-HT_{1A} or 5-HT_{1D} subtype

[1]Marion J. Perren, Wasyl Feniuk & Patrick P.A. Humphrey

Pharmacology Division, Glaxo Group Research Limited, Park Road, Ware, Hertfordshire SG12 0DP

1 There is controversy about whether 5-HT_{1A} receptors mediate contraction of isolated cerebral blood vessels. We have therefore compared the vascular actions of the 5-HT_{1A} receptor agonist, 8-hydroxy-2-(di-n-propyl-amino)-tetralin (8-OH-DPAT) with those of the 5-HT_1-like receptor agonist, sumatriptan, on the dog isolated saphenous vein, which contains a 5-HT_1-like receptor similar to those on cerebral blood vessels, and in the carotid circulation of the anaesthetized dog.

2 5-Hydroxytryptamine (5-HT), sumatriptan and 8-OH-DPAT each caused contraction of dog isolated saphenous vein with a rank order of agonist potency of 5-HT > sumatriptan > 8-OH-DPAT and EC_{50} values (95% confidence limits) of 0.06 (0.04–0.08), 0.3 (0.1–0.8) and 3.9 (2.0–7.5) μM respectively. The maximum contractile effect produced by each agonist was similar.

3 The contractile effects of 5-HT, sumatriptan and 8-OH-DPAT in the dog isolated saphenous vein were resistant to antagonism by the 5-HT_{1A} receptor antagonists spiperone, spiroxatrine and pindolol (all 1 μM). The 5-HT_{1D} receptor ligands, metergoline (0.1 μM) rauwolscine (1 μM) and yohimbine (1 μM) had little or no antagonist activity. In contrast, the non-selective 5-HT_1-like receptor blocking drug, methiothepin (0.03–0.3 μM) potently antagonized the contractile effects of 5-HT, sumatriptan and 8-OH-DPAT to a similar degree, suggesting that all three agonists act at the same receptor.

4 In ganglion-blocked, anaesthetized dogs, intra-carotid administration of 8-OH-DPAT (0.3–3 $\mu g\,kg^{-1}$) and sumatriptan (0.1–1 $\mu g\,kg^{-1}$), caused dose-dependent carotid arterial vasoconstriction. The two agonists were approximately equipotent in this respect.

5 The carotid arterial vasoconstrictor actions of 8-OH-DPAT and sumatriptan were not modified by spiperone (1 $mg\,kg^{-1}$, i.v.) but were antagonized to a similar extent by the subsequent administration of methiothepin (1 $mg\,kg^{-1}$, i.v.).

6 These results suggest that 8-OH-DPAT contracts the dog isolated saphenous vein and constricts the carotid arterial circulation of anaesthetized dogs by activation of 5-HT_1-like receptors which are not of the 5-HT_{1A} subtype, nor, on the basis of data with metergoline in the dog isolated saphenous vein, of the 5-HT_{1D} subtype. The receptor involved in these actions appears to be the same as that mediating the vasoconstrictor effects of sumatriptan. This receptor does not appear to be like any known 5-HT_1 ligand binding site; hence the current description, 5-HT_1-like, remains the most appropriate.

Introduction

The existence of 5-HT_1 receptor subtypes has been proposed based on the subdivision of the 5-HT_1 binding site (Pedigo *et al.*, 1981; Pazos & Palacios, 1985; Heuring & Peroutka, 1987). However, the lack of sufficiently selective compounds to discriminate between the different 5-HT_1 recognition sites has often limited their correlation to functional subtypes of the 5-HT_1-like receptor group (Bradley *et al.*, 1986; Humphrey & Richardson, 1989). Functional 5-HT_1 and 5-HT_1-like receptor subtypes have been largely characterized by use of selective agonists. One such agonist is 8-hydroxy-2-(di-n-propyl-amino)-tetralin (8-OH-DPAT) which has both high affinity and selectivity for the 5-HT_{1A} recognition site compared to other 5-HT_1 recognition sites (Middlemiss & Fozard, 1983; Hoyer *et al.*, 1985; Heuring & Peroutka, 1987).

Amongst its many pharmacological actions, 8-OH-DPAT causes contraction of dog basilar artery by a mechanism which has been proposed to involve activation of 5-HT_{1A} receptors (Taylor *et al.*, 1986; Peroutka *et al.*, 1986). However, this interpretation is controversial since the potent and highly selective 5-HT_1-like receptor agonist, sumatriptan GR43175, causes contraction of dog isolated saphenous vein (Humphrey *et al.*, 1988) as well as primate, dog and human basilar artery (Connor *et al.*, 1989; Parsons & Whalley, 1989) by a receptor which is not of the 5-HT_{1A} type but has been termed 5-HT_1-like. Studies *in vivo* have shown that sumatriptan causes selective vasoconstriction within the carotid artery bed of anaesthetized dogs by activation of similar 5-HT_1-like receptors, with the effect being specifically antagonized by methiothepin (Feniuk *et al.*, 1989). The carotid arterial vasoconstrictor action of sumatriptan, like that recently described for 8-OH-DPAT, appears to be largely restricted to an action on carotid arteriovenous anastomoses (Perren *et al.*, 1989a; Bom *et al.*, 1989). The precise 5-HT_1-like receptor subtypes involved in these vasoconstrictor actions of sumatriptan and 8-OH-DPAT have therefore been investigated further.

A preliminary account of these findings has been presented to the British Pharmacological Society (Perren *et al.*, 1989b).

Methods

Dog isolated saphenous vein preparation

Saphenous veins were removed from anaesthetized beagle dogs, spirally cut into strips and suspended in modified Krebs solution (Apperley *et al.*, 1976) bubbled with 95% O_2, 5% CO_2 at 37°C under an initial resting tension of 0.5 g. Isometric tension changes were recorded with Dynamometer UF1 2 oz strain gauges. Preparations were allowed to equilibrate for at least 1 h and then the contractile response to a submaximal concentration of potassium chloride (30 mM) was determined.

[1] Author for correspondence.

Preparations were then allowed to equilibrate for a further period of at least 30 min following washout of potassium chloride.

Determination of agonist potency

These experiments were conducted in the presence of atropine (1 μM), ketanserin (1 μM) and mepyramine (1 μM) to exclude any possible effects of the agonists at muscarinic cholinoceptors, 5-HT_2 receptors and α_1-adrenoceptors and histamine H_1 receptors, respectively.

Cumulative concentration-effect curves to 5-HT (10 nM–1 μM) were obtained on all preparations. 5-HT was then washed from the bath over a period of 30 min and tissues were then left for a further 30 min before re-challenging with either 5-HT (control preparation), or test agonist (10 nM–5 μM or 0.1–200 μM for sumatriptan and 8-OH-DPAT, respectively). The control preparation was used to monitor any changes in sensitivity to 5-HT.

Relative potencies were determined by dividing the EC_{50} (molar concentration required to produce 50% maximum contractile effect) for the test compound by the EC_{50} value for 5-HT in the same preparation. This value was then corrected for spontaneous change in sensitivity to 5-HT by dividing it by the ratio of the EC_{50} values for 5-HT in the control preparation. Maximum contractile responses to the test agonists were compared with the maximum contractile response to 5-HT in the same tissue and corrected for time-related changes by dividing by the ratio of the relative maximum responses to 5-HT in the control preparation.

Antagonist studies in the dog isolated saphenous vein

These experiments were conducted in the presence of ketanserin (1 μM) to negate any actions of the agonists at 5-HT_2 receptors or α_1-adrenoceptors. Agonist cumulative concentration-effect curves were obtained in each of four saphenous vein preparations from the same vessel. Concentration-effect curves were then repeated after 30 min incubation with an antagonist on three of the preparations, whilst a fourth preparation was again challenged with test agonist in the absence of antagonist, and therefore acted as a control.

Agonist concentration-ratios were determined by comparing the EC_{50} values in the absence and presence of antagonist, with corrections being made for spontaneous changes in agonist sensitivity in the control preparation as described above. Agonist sensitivity in the control preparation varied by less than two fold during each experiment.

When more than one concentration of a particular antagonist was examined, the results were analysed according to the method of Arunlakshana & Schild (1959) and pA_2 values and slopes of the regression determined.

Anaesthetized dog

Beagle dogs (7.4–9.7 kg) of either sex were anaesthetized with barbitone (300 mg kg^{-1}, i.p.) following induction with thiopentone (25 mg kg^{-1}, i.v.) and pentobarbitone (60 mg, i.v.) and artificially respired with room air at a rate of 20 strokes min^{-1} and a stroke volume of 13–16 ml kg^{-1}, adjusted to maintain arterial pH, $Paco_2$ and Pao_2 within normal physiological limits. Body temperature was maintained at 39–40°C. Arterial blood pressure and right common carotid artery blood flow were recorded as described previously (Feniuk *et al.*, 1989). In addition, the right cranial thyroid artery was cannulated retrogradely for the intra-carotid administration of sumatriptan or 8-OH-DPAT. In order to exclude any effects of the agonists mediated by the autonomic nervous system, dogs were treated with mecamylamine (5 mg kg^{-1}, i.v.) and atropine (0.5 mg kg^{-1}, i.v.) administered in small doses over approximately 30 min and then allowed a further 30 min to equilibrate. Bolus intra-carotid doses of sumatriptan (0.1–1.0 μg kg^{-1}) or 8-OH-DPAT (0.1–3.0 μg kg^{-1}) were then administered in volumes of less than 0.1 ml injected into the common carotid artery with 0.25 ml 0.9% saline solution. The interval between each dose was at least 15 min such that full recovery of each response occurred before subsequent doses were administered. In view of our previous finding, that the carotid arterial vasoconstrictor action of intravenously administered sumatriptan is long lasting (Feniuk *et al.*, 1989), carotid arterial vasoconstrictor responses produced by intra-arterial administration were studied over a narrow submaximal dose-range and reproducibility of the vasoconstrictor action of sumatriptan was assessed by repeating an intermediate dose. Reproducibility of the vasoconstrictor responses produced by 8-OH-DPAT was assessed by repeating the full dose-effect curve. Agonist dose-effect curves were then repeated 15 min after spiperone (1 mg kg^{-1}, i.v.) and subsequently, 15 min after methiothepin (1 mg kg^{-1}, i.v.).

Changes in carotid arterial vascular resistance were determined from the resting value immediately prior to each dose of agonist. Dose-ratios were calculated from the linear portion of the agonist dose-effect curves.

Statistics

Unless otherwise stated, values are the arithmetic means ± s.e.mean or geometric means with 95% confidence limits in parentheses from n observations.

The pA_2 values and slopes of the Schild regression obtained for methiothepin against 5-HT-, sumatriptan- and 8-OH-DPAT-induced contractions of dog isolated saphenous vein were compared by an unpaired Student's t test. Differences were considered significant when the P value was <0.05.

Drugs used

The following compounds were purchased: atropine sulphate (Sigma), 8-OH-DPAT (8-hydroxy-2-[di-n-propylamino]-tetralin) hydrogen bromide (RBI), 5-HT (5-hydroxytryptamine) creatinine sulphate (Sigma), mepyramine maleate (May and Baker), metergoline (Farmitalia), (±)-pindolol (Sigma), rauwolscine hydrochloride (Carl Roth) and yohimbine hydrochloride (Sigma).

The following compounds were gifts: mecamylamine hydrochloride (Merck, Sharpe and Dohme), methiothepin maleate (Hoffman La Roche), methoxamine hydrochloride (Wellcome), spiroxatrine (Janssen) and we acknowledge the generosity of the companies.

Spiperone and sumatriptan succinate (GR43175; 3-[2-(dimethylamino)ethyl] - N - methyl - 1H - indole - 5 - methane sulphonamide) were synthesized by the Chemistry Research Department at Glaxo Group Research Limited, Ware.

All drugs, with the exception of ketanserin, methiothepin, pindolol and spiperone, were initially dissolved in distilled water and diluted with 0.9% w/v saline. Ketanserin and spiperone were initially dissolved in 0.1 M tartaric acid and diluted with 0.9% w/v saline. Methiothepin was dissolved in 10% ethanol in distilled water. Pindolol was initially dissolved in 2 M hydrochloric acid and diluted with distilled water to give a final solution in approximately 0.1 M HCl.

For studies *in vivo*, all doses of drugs refer to the free base.

Results

In vitro *studies in dog isolated saphenous vein*

Agonist-relative potency determinations 5-HT (10 nM–1 μM), sumatriptan (30 nM–5 μM) and 8-OH-DPAT (1 μM–100 μM) caused concentration-dependent contractions of the dog isolated saphenous vein with EC_{50} values (95% confidence limits) of 0.06 (0.04–0.08), 0.3 (0.1–0.8) and 3.9 (2.0–7.5) μM, respectively. The maximum contractile responses produced by each agonist were similar (Figure 1). The relative potencies (5-

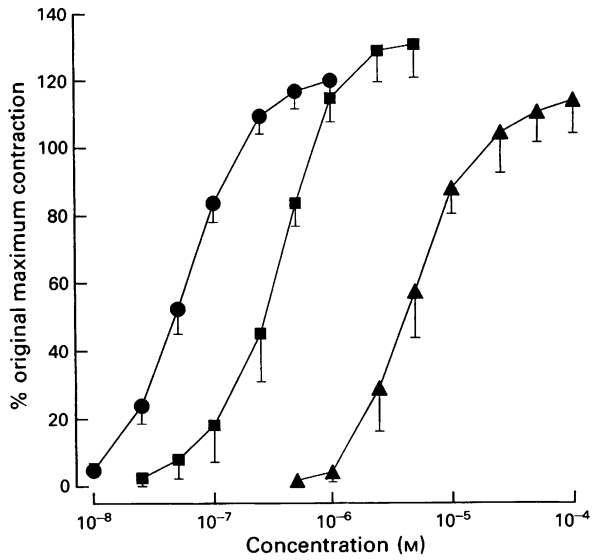

Figure 1 Concentration-effect curves to 5-hydroxytryptamine (5-HT, ●; $n = 9$), sumatriptan (■; $n = 4$) and 8-hydroxy-2-(di-n-propyl-amino)-tetralin (8-OH-DPAT, ▲; $n = 5$) in dog isolated saphenous vein in the presence of atropine, mepyramine and ketanserin (all 1 μM). Each point represents the mean from n experiments with s.e.mean shown by vertical bars. Agonist equipotent molar ratios (5-HT = 1) were 4.6 and 67 for sumatriptan and 8-OH-DPAT respectively (see text).

HT = 1) were 4.6 (2.5–8.2) and 67 (27–164) for sumatriptan and 8-OH-DPAT, respectively (mean with 95% confidence limits in parentheses).

Antagonist studies The contractile actions of 5-HT, sumatriptan and 8-OH-DPAT in the dog isolated saphenous vein were resistant to antagonism by the non-selective 5-HT_{1A} receptor antagonists, spiperone, spiroxatrine and pindolol (each at a single concentration of 1 μM). The 5-HT_{1D} receptor ligands, metergoline (0.1 μM) rauwolscine (1 μM) and yohimbine (1 μM) also had little or no antagonist activity (see Table 1). A higher concentration of metergoline (1 μM) produced a small displacement of the concentration-effect curve produced by 5-HT in the dog isolated saphenous vein. The concentration-ratio for 5-HT in the presence of this higher concentration of metergoline (1 μM) was 3.5 (1.9–6.3) corresponding to a pK_B value of 6.4 ± 0.1. However, metergoline (1 μM) also caused a similar attenuation of contractile responses elicited by the α_1-adrenoceptor agonist, methoxamine (mean concentration-ratio 3.3, $n = 2$). In five out of twelve experiments, metergoline (0.1 or 1 μM) also caused a small contraction of the dog isolated saphenous vein. However, the magnitude of these contractile responses was less than 10% of the maximum contractile response produced by the test agonists.

In contrast, methiothepin (0.03–0.3 μM) was a potent antagonist, producing concentration-dependent antagonism of the contractile effect of 5-HT, sumatriptan and 8-OH-DPAT in the dog isolated saphenous vein (Figure 2). Analysis of the data according to the method of Arunlakshana & Schild (1959) showed that the pA_2 values obtained for methiothepin against each agonist were similar in the range of 8.3–8.6 (Table 2). Additionally, the antagonism produced by methiothepin of the 5-HT- and sumatriptan-induced contractions of the dog isolated saphenous vein was competitive since slopes of the Schild regression were not significantly different from unity. The slope obtained with 8-OH-DPAT however, was significantly less than 1.0. These results are shown in Figure 2 and Table 2.

Table 1 Agonist concentration-ratios for 5-hydroxytryptamine (5-HT), sumatriptan and 8-hydroxy-2-(di-n-propyl-amino)-tetralin (8-OH-DPAT) following 30 min incubation with spiperone, pindolol, spiroxatrine, rauwolscine or yohimbine (all at 1 μM) or metergoline (0.1 μM, see text) in the dog isolated saphenous vein

	Agonist concentration-ratio		
	5-HT	*Sumatriptan*	*8-OH-DPAT*
Spiperone	1.1 (0.8–1.4)	1.0 (0.3–3.0)	0.8 (0.4–1.6)
Pindolol	0.9 (0.8–1.1)	0.9 (0.4–1.8)	1.2 (0.8–1.6)
Spiroxatrine	0.7 (0.3–1.8)	1.2 (0.8–1.6)	1.1 (0.7–1.7)
Metergoline	2.5 (1.7–3.7)	1.5 (0.5–4.2)	1.3 (0.7–2.5)
Rauwolscine	2.1 (1.0–4.5)	2.1 (0.7–6.6)	1.8 (0.5–6.4)
Yohimbine	2.2 (0.9–5.2)	2.1 (0.9–5.1)	2.0 (0.2–1.9)

The experiments were conducted in the presence of ketanserin (1 μM). Values shown are geometric means (95% confidence limits) from at least 3 experiments.

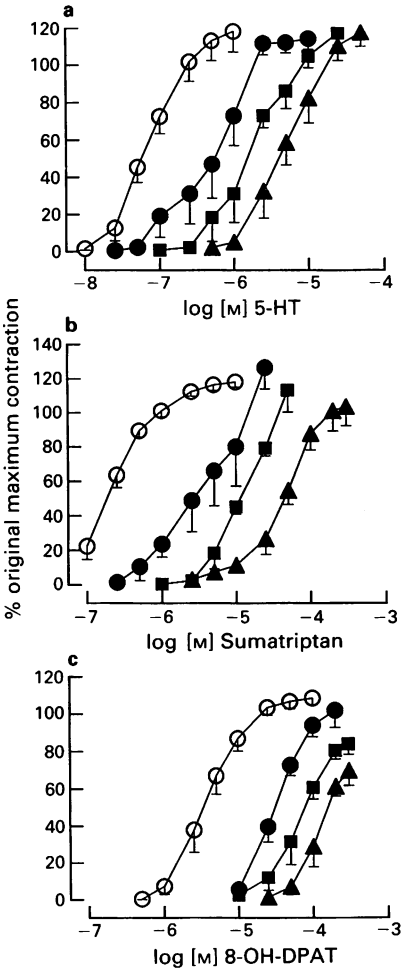

Figure 2 Effect of methiothepin on concentration-effect curves produced by (a) 5-hydroxytryptamine (5-HT), (b) sumatriptan and (c) 8-hydroxy-2-(di-n-propyl-amino)-tetralin (8-OH-DPAT) in the dog isolated saphenous vein in the presence of ketanserin (1 μM). Control concentration-effect curves are shown by (○) and concentration-effect curves in the presence of (●) 0.03, (■) 0.1 and (▲) 0.3 μM methiothepin are shown. Each point represents the mean from four experiments with s.e.mean shown by vertical bars. Calculated pA_2 values and Schild slopes from these data are shown in Table 2.

Table 2 Data from experiments in dog saphenous vein shown in Figure 2 were analysed by Schild analysis to give pA_2 values and slopes for methiothepin against 5-hydroxytryptamine, sumatriptan or 8-hydroxy-2-(di-n-propyl-amino)- tetralin (8-OH-DPAT) as agonists

Agonist	pA_2 values and slopes for methiothepin pA_2	Slope
5-Hydroxytryptamine	8.26 ± 0.20	1.03 ± 0.06
Sumatriptan	8.56 ± 0.26	1.15 ± 0.19
8-OH-DPAT	8.41 ± 0.06	0.78 ± 0.03*

Values are the mean ± s.e.mean from four experiments.
* Significantly different from unity ($P < 0.05$).

Anaesthetized dog studies

Effect of sumatriptan and 8-OH-DPAT following intra-arterial administration to the common carotid artery bed The intra-carotid administration of bolus doses of sumatriptan (0.1–1.0 $\mu g\,kg^{-1}$) or 8-OH-DPAT (0.3–3.0 $\mu g\,kg^{-1}$) to anaesthetized ganglion-blocked dogs, produced dose-dependent decreases in common carotid arterial blood flow. These reductions in blood flow were a consequence of increases in carotid arterial vascular resistance. Arterial blood pressure was not modified by either agonist. The vasoconstrictor responses produced by 8-OH-DPAT in the carotid artery bed of the anaesthetized dog were of shorter duration than those produced by sumatriptan. The percentage increase in carotid arterial vascular resistance produced by sumatriptan and 8-OH-DPAT to a bolus dose of 1 $\mu g\,kg^{-1}$ i.a. was 69 ± 4 and 76 ± 6% respectively, suggesting that the two agonists were approximately equipotent in this action. ED_{50} values for the agonists were not calculated since maximum vasoconstrictor responses were not achieved at the doses used (see Methods). Experimental recordings illustrating these actions of sumatriptan and 8-OH-DPAT in the anaesthetized dog are shown in Figure 3.

Antagonist studies

Effect of spiperone The intravenous administration of spiperone (1 $mg\,kg^{-1}$) to anaesthetized ganglion-blocked dogs caused a small fall in diastolic blood pressure and carotid arterial blood flow in both groups of dogs. However, carotid arterial vascular resistance was little changed from pre-dose levels. These effects are summarised in Table 3. Spiperone had no effect on carotid arterial vasoconstrictor responses elicited by either sumatriptan or 8-OH-DPAT; agonist dose-response curves in the presence of spiperone were superimposable with controls, with agonist dose-ratios close to unity in each case (Figure 4).

Effect of methiothepin The subsequent intravenous administration of methiothepin (1 $mg\,kg^{-1}$) had little effect on diastolic blood pressure or carotid arterial blood flow in either group of dogs. Consequently, carotid arterial vascular resistance was little changed from pre-dose levels. These effects are summarised in Table 3. However, methiothepin caused a marked attenuation of carotid arterial vasoconstrictor responses produced by close intra-arterial administration of both sumatriptan and 8-OH-DPAT to the anaesthetized dog. This attenuation was seen as both a rightward displacement of the agonist dose-effect curves to both sumatriptan and 8-OH-DPAT and a slight reduction in the slope (see Figure 4). The mean agonist dose-ratios (95% confidence limits, $n = 4$) produced by methiothepin for sumatriptan- and 8-OH-DPAT-induced carotid vasoconstrictor responses were similar, being 15 (8–27) and 17 (5–54) for sumatriptan and 8-OH-DPAT, respectively.

Discussion

It has been shown that 8-OH-DPAT has carotid arterial vasoconstrictor activity *in vivo* (Bom *et al.*, 1989). The aim of this study has been to investigate the effects of 8-OH-DPAT, in

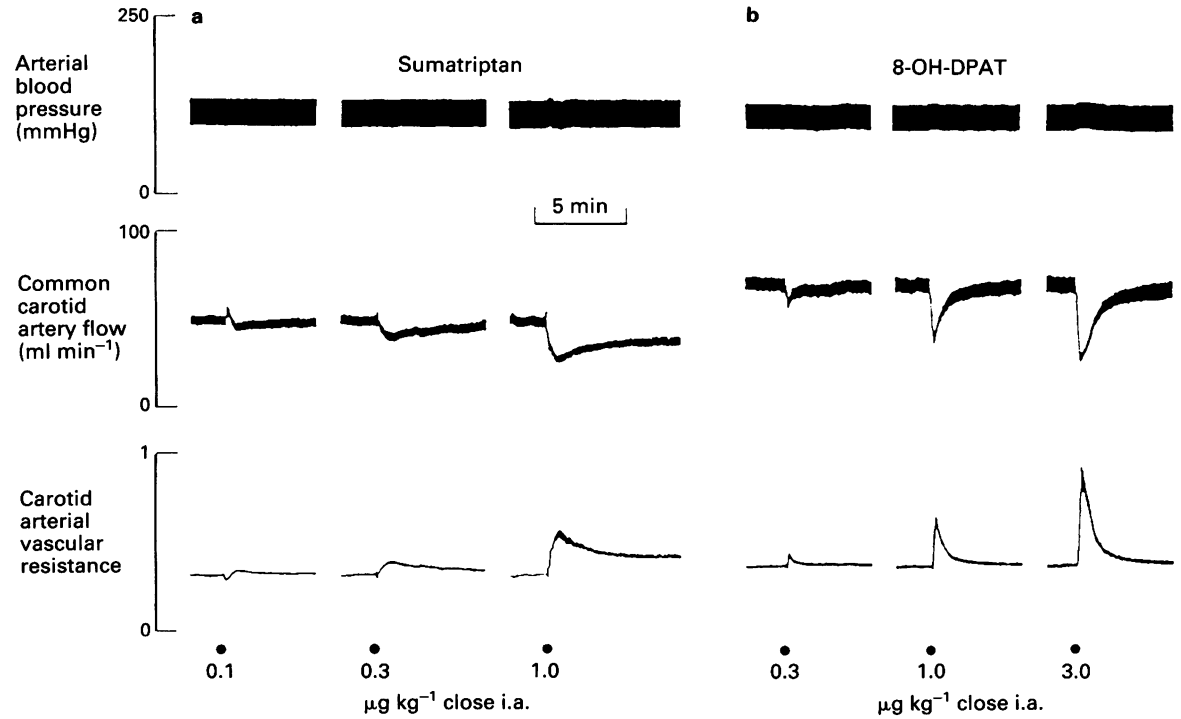

Figure 3 Experimental tracing illustrating the effects of (a) sumatriptan and (b) 8-hydroxy-2-(di-n-propyl-amino)-tetralin (8-OH-DPAT) when injected into the common carotid artery of ganglion-blocked, anaesthetized dogs. Vascular resistance in the carotid arterial bed was electronically determined and displayed continuously as arbitrary units. Both agonists produced transient carotid arterial vasoconstrictor responses (increases in vascular resistance) with little or no effect on arterial blood pressure.

Table 3 Haemodynamic effects of spiperone (1 mg kg^{-1}, i.v.) and methiothepin (1 mg kg^{-1}, i.v.) in barbitone anaesthetized, ganglion-blocked dogs

	Sumatriptan group	*8-OH-DPAT group*
Resting levels before spiperone		
Diastolic blood pressure (mmHg)	75 ± 7	75 ± 5
Carotid flow (ml min^{-1})	53 ± 12	57 ± 7
Carotid vascular resistance (mmHg min ml^{-1})	1.84 ± 0.30	1.59 ± 0.14
15 min after spiperone (1 mg kg^{-1})		
Diastolic blood pressure (mmHg)	63 ± 6	68 ± 4
Carotid flow (ml min^{-1})	49 ± 12	49 ± 6
Carotid vascular resistance (mmHg min ml^{-1})	1.78 ± 0.33	1.71 ± 0.19
Resting levels before methiothepin		
Diastolic blood pressure (mmHg)	61 ± 7	100 ± 4
Carotid flow (ml min^{-1})	38 ± 10	45 ± 6
Carotid vascular resistance (mmHg min ml^{-1})	2.17 ± 0.40	1.81 ± 0.25
15 min after methiothepin (1 mg kg^{-1})		
Diastolic blood pressure (mmHg)	56 ± 7	64 ± 4
Carotid flow (ml min^{-1})	40 ± 9	44 ± 6
Carotid vascular resistance (mmHg min ml^{-1})	1.89 ± 0.30	1.79 ± 0.24

8-OH-DPAT = 8-hydroxy-2-(di-n-propyl-amino)-tetralin.
Various haemodynamic parameters were measured as described in the text. Values are the mean ± s.e.mean from 4 dogs in each group.

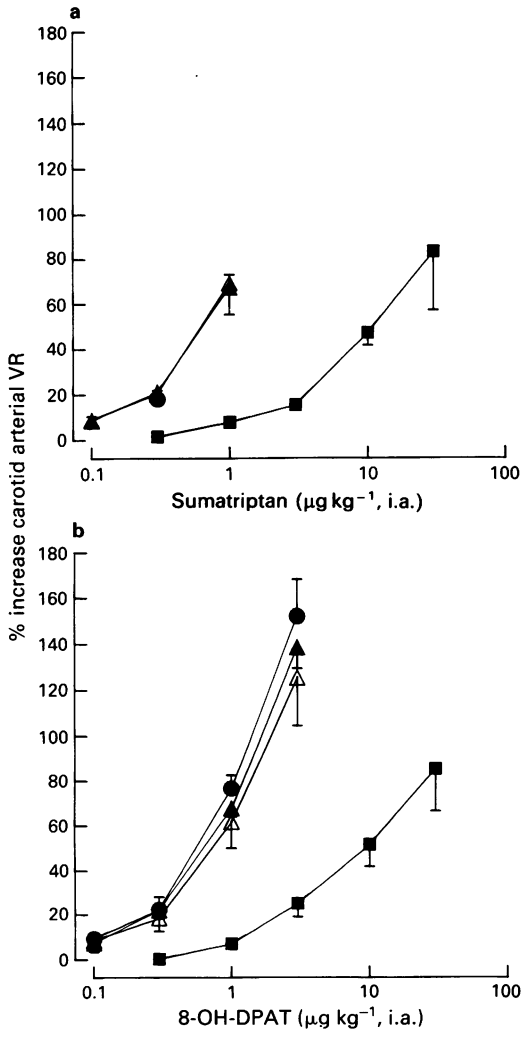

Figure 4 Carotid arterial vasoconstrictor responses to (a) sumatriptan and (b) 8-hydroxy-2-(di-n-propyl-amino)-tetralin (8-OH-DPAT) in the ganglion-blocked anaesthetized dog in the absence, (△, ●) and presence of 1 mg kg^{-1}, i.v. spiperone (▲) and finally after the subsequent administration of 1 mg kg^{-1} methiothepin (■). Note that dose-effect curves produced by both sumatriptan and 8-OH-DPAT were not affected by spiperone but were markedly displaced by methiothepin to the right to a similar extent. Values shown are mean (with s.e.mean shown by vertical bars) from four dogs.

the dog isolated saphenous vein and in the carotid artery bed of anaesthetized dogs which contain the same 5-HT$_1$-like receptor (Feniuk *et al.*, 1989). We have also compared the actions of 8-OH-DPAT with those of the selective 5-HT$_1$-like receptor agonist, sumatriptan, and attempted to characterize the receptor mechanism(s) involved in these actions by use of selective receptor antagonists.

8-OH-DPAT caused a concentration-dependent contraction of the dog isolated saphenous vein and a similar maximum contractile effect to that produced by both 5-HT and sumatriptan. 8-OH-DPAT was approximately 65 times weaker than 5-HT and 15 times weaker than sumatriptan in this respect. The ability of methiothepin to antagonize the contractile effects of 5-HT, sumatriptan and 8-OH-DPAT to the same degree suggests that all three agonists activate the same, 5-HT$_1$-like receptor mechanism. We have previously shown that at a concentration of 0.1 μM the antagonist action of methiothepin in the dog isolated saphenous vein is specific since concentration-effect curves to the thromboxane A$_2$ receptor agonist, U-46619 are unaffected (Humphrey *et al.*, 1988). The reason for the slightly shallow slope of the Schild regression obtained with 8-OH-DPAT is not known. However, it is possible that higher concentrations of 8-OH-DPAT may additionally cause contraction of the dog isolated saphenous vein by activation of receptors other than 5-HT receptors.

The contractile actions of 8-OH-DPAT, sumatriptan and 5-HT in the dog isolated saphenous vein were resistant to antagonism by the 5-HT$_{1A}$ and 5-HT$_2$ receptor antagonist, spiperone, suggesting that the 5-HT$_1$-like receptor which mediates these effects is not of the 5-HT$_{1A}$ subtype. The concentration of spiperone used (1 μM) is approximately 50 times greater than that required to inhibit 5-HT receptor-mediated inhibition of forskolin-stimulated adenylate cyclase in the guinea-pig and rat hippocampus (De Vivo & Maayani, 1985; 1986) or to inhibit 8-OH-DPAT-induced inhibition of the contraction produced by transmural electrical field stimulation of the guinea-pig whole ileum preparation (Fozard & Kilbinger, 1985). These systesms have been characterized as functional models of 5-HT$_{1A}$ receptor activation. In this study, we have also examined the effects of other putative 5-HT$_{1A}$ receptor antagonists; pindolol and spiroxatrine on 8-OH-DPAT-, sumatriptan- and 5-HT-induced contraction of dog isolated saphenous vein and failed to demonstrate any antagonistic effects, thus further reinforcing the view that 5-HT$_{1A}$ receptors are not involved.

These results support our previous findings, where the contractile effects of 5-HT and sumatriptan in the dog isolated

saphenous vein and cerebral blood vessels from dog and primate were shown to be resistant to antagonism by the 5-HT_{1A} and 5-HT_{1B} ligand, cyanopindolol (Humphrey *et al.*, 1988; Connor *et al.*, 1989). Furthermore, the results from our study seriously question the conclusions reached by others (Taylor *et al.*, 1986; Peroutka *et al.*, 1986) that 5-HT_{1A} receptors mediate contraction of canine cerebral blood vessels. The results from the present study would suggest that the contractile actions of 8-OH-DPAT and sumatriptan in such vessels are mediated via the activation of the same 5-HT_1-like receptor which is not of the 5-HT_{1A} subtype.

In the second part of the study we have compared the effects of 8-OH-DPAT with those of sumatriptan in the carotid arterial circulation of ganglion-blocked anaesthetized dogs. The animals were treated with mecamylamine and atropine in order to exclude the marked cardiovascular effects of 8-OH-DPAT which occur as a consequence of activation of central 5-HT_{1A} receptors (Ramage & Fozard, 1987). The intra-arterial administration of 8-OH-DPAT and sumatriptan to the carotid artery bed of anaesthetized dogs caused dose-dependent, reproducible vasoconstrictor responses with little or no effect on arterial blood pressure or heart rate. The carotid arterial vasoconstrictor responses produced by 8-OH-DPAT were transient in nature, whilst those produced by sumatriptan tended to be of longer duration. In marked contrast to the dog isolated saphenous vein where 8-OH-DPAT was approximately 15 times weaker than sumatriptan in causing contraction, the two agonists were approximately equipotent in causing carotid arterial vasoconstriction in anaesthetized dogs. The reason for this apparent difference in sensitivity is not known.

As was seen *in vitro*, spiperone had no antagonistic action on carotid arterial vasoconstrictor responses produced by either 8-OH-DPAT or sumatriptan in the anaesthetized dog, suggesting that the responses do not involve activation of either 5-HT_{1A} or 5-HT_2 receptors. The dose of spiperone used in our studies (1 mg kg^{-1}, i.v.) is approximately 40 times higher than that required to inhibit the flat body posture and forepaw treading components of the behavioural response to 8-OH-DPAT in the reserpinized rat, effects which have been shown to be mediated by activation of 5-HT_{1A} receptors (Tricklebank *et al.*, 1984). The carotid arterial vasoconstrictor action of 8-OH-DPAT and indeed that of sumatriptan was markedly attenuated by the subsequent administration of methiothepin. The extent of the attenuation produced by methiothepin against each agonist was similar, suggesting that both 8-OH-DPAT and sumatriptan cause carotid arterial vasoconstriction by activation of the same (5-HT_1-like) receptor. This result confirms our previous finding with sumatriptan (Feniuk *et al.*, 1989), where we also demonstrated the specificity of action of methiothepin as an antagonist of sumatriptan.

In summary, the results from the present study have clearly demonstrated that 8-OH-DPAT, like sumatriptan, causes contraction of dog isolated saphenous vein and vasoconstriction within the carotid arterial circulation of anaesthetized dogs. The receptors involved in these actions of 8-OH-DPAT are clearly 5-HT_1-like since they are antagonized by methiothepin but are not of the 5-HT_{1A} subtype since the effects of 8-OH-DPAT are unaffected by spiperone (and additionally spiroxatrine and pindolol *in vitro*). It would seem that the receptors involved in these actions of 8-OH-DPAT in the dog isolated saphenous vein and carotid artery bed of anaesthetized dogs are apparently the same as those mediating the effects of sumatriptan in the two systems. The results from our *in vitro* experiments demonstrate that 8-OH-DPAT is a relatively weak agonist (EC_{50} 3.9 (2.0–7.5) μM) in the dog isolated saphenous vein which is in marked contrast to its high potency at the 5-HT_{1A} receptor in the guinea-pig ileum (Fozard & Kilbinger, 1985). However, our studies *in vivo* show that 8-OH-DPAT activates these non 5-HT_{1A} receptors in the carotid artery bed of anaesthetized dogs at low doses, (0.3–3 μg kg^{-1}, i.a.) similar to those at which it reduces carotid arteriovenous anastomotic blood flow in anaesthetized pigs (Bom *et al.*, 1989) and causes 5-HT_{1A} receptor-mediated reductions in arterial blood pressure and heart rate in the rat (Fozard *et al.*, 1987) and cat (Ramage & Fozard, 1987). Thus, caution should be exercised in ascribing some of the *in vivo* actions of 8-OH-DPAT to 5-HT_{1A} receptor activation unless such studies are accompanied by additional experiments with selective 5-HT_{1A} receptor blocking drugs.

It has been suggested that the anti-migraine effects of sumatriptan may be mediated by the activation of 5-HT_{1D} receptors (see Hoyer, 1989; Hoyer *et al.*, 1989; Schoeffter & Hoyer, 1989; Peroutka & McCarthy, 1989). We have, therefore, considered whether the vasoconstrictor effects of 8-OH-DPAT and sumatriptan in the dog isolated saphenous vein and carotid circulation of anaesthetized dogs demonstrated in this study are mediated via the activation of 5-HT_{1D} receptors. In ligand binding studies, 8-OH-DPAT has an affinity at 5-HT_{1D} recognition sites which is some 20 fold less than that observed with sumatriptan (Hoyer *et al.*, 1989). Thus the relative affinity of the two compounds for the 5-HT_{1D} binding site is similar to their relative potency in causing contraction of the dog isolated saphenous vein. However, the 5-HT_{1D} ligand, metergoline, had no antagonistic activity in the dog saphenous vein against either 8-OH-DPAT- or sumatriptan-induced contraction even at a concentration which exceeded the estimated K_D value in calf caudate by a factor of 100 fold (Hoyer, 1989). A 10 fold higher concentration of metergoline (1 μM), caused weak antagonism of contractile responses elicited by 5-HT in the dog isolated saphenous vein (calculated pK_B value 6.4 $\pm$ 0.1). However, metergoline (1 μM) also antagonized methoxamine-induced contraction of the preparation. Evidence is emerging that there may be variations in the affinity of metergoline for 5-HT_{1D} recognition sites between different species (Waeber *et al.*, 1988; Xiong & Nelson, 1989). However, even a comparison of the lowest estimate for the 5-HT_{1D} binding affinity of metergoline (pK_i 7.24 $\pm$ 0.06, rabbit caudate nucleus; Xiong & Nelson, 1989) with its pK_B value in the dog isolated saphenous vein (6.4 $\pm$ 0.1) shows that there is still almost a 10 fold separation, suggesting that the 5-HT_1-like receptor site in the dog isolated saphenous vein is different to any 5-HT_{1D} recognition site so far identified. It would clearly be of interest to determine the affinity of metergoline for 5-HT_{1D} recognition sites in dog brain tissue.

Other 5-HT_{1D} ligands, rauwolscine and yohimbine, have been found to be similarly poor as antagonists in the dog isolated saphenous vein in concentrations as high as 1 μM which is at least ten times higher than their affinities for 5-HT_{1D} recognition sites in calf caudate (Hoyer, 1989). Admittedly these ligands have even lower affinity for the 5-HT_{1D} recognition site in human caudate, but this only serves to illustrate their limitations as drug tools (Waeber *et al.*, 1988). Importantly too, Sumner & Humphrey (1989) have provided evidence that the 5-HT_{1D} recognition site in porcine brain is heterogeneous which must confound attempts to determine the affinities of antagonists for the 5-HT_{1D} site. It would, therefore, seem reasonable to conclude, in the absence of better, more selective antagonists, that the receptor which mediates 5-HT-induced contraction of the dog isolated saphenous vein and presumably also cranial vessels from higher species, is not of the 5-HT_{1D} type.

Clearly, the definitive characterization of the receptor involved in the cranial vasoconstrictor effects of 8-OH-DPAT and sumatriptan and its relation to 5-HT_{1D} receptors identified in ligand binding studies awaits the identification of a selective receptor antagonist. Until then, the description '5-HT_1-like' still seems the most appropriate. The findings in this paper also raise questions about the degree of selectivity of 8-OH-DPAT for 5-HT_{1A} receptors, most particularly *in vivo*.

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L-N^G-nitro arginine methyl ester exhibits antinociceptive activity in the mouse

[1]P.K. Moore, A.O. Oluyomi, R.C. Babbedge, P. Wallace & S.L. Hart

Smooth Muscle Pharmacology Group, Biomedical Sciences Division, King's College, University of London, Manresa Road, London SW3 6LX

1 L-N^G-nitro arginine methyl ester (L-NAME, 1–75 mg kg^{-1}) administered intraperitoneally (i.p.) elicits dose-related antinociception in the mouse assessed by the formalin-induced paw licking procedure. Antinociceptive activity is still present 24 h after injection. L-NAME (75 mg kg^{-1}, i.p.) is also antinociceptive in the acetic acid-induced abdominal constriction and hot plate procedures.

2 L-NAME additionally produces a dose-related inhibition of formalin-induced paw licking following intracerebroventricular (i.c.v., 0.1–100 μg per mouse) and oral (p.o., 75–150 mg kg^{-1}) administration.

3 L-Arginine (600 mg kg^{-1}, i.p.) but not D-arginine (600 mg kg^{-1}) or naloxone (5 mg kg^{-1}) reverses the antinociceptive effect of L-NAME in the formalin test.

4 High doses of L-NAME (37.5–600 mg kg^{-1}) but not D-NAME (75 mg kg^{-1}) administered i.p. produce dose-related increases in blood pressure of the urethane-anaesthetized mouse whilst i.c.v. injected L-NAME (0.1 and 100 μg per mouse) is inactive.

5 L-NAME (75 mg kg^{-1}, i.p.) did not inhibit oedema formation in the formalin-injected mouse hindpaw.

6 L-NAME (75 mg kg^{-1}) did not produce any overt behavioural changes in treated mice and failed to influence locomotor activity or the incidence of dipping, crossing, rearing or circling behaviour assessed by a modified 'head-dipping' board procedure. A high dose of L-NAME (600 mg kg^{-1}) reduced dipping behaviour and locomotor activity suggesting a possible sedative effect. D-NAME (600 mg kg^{-1}) was inactive.

7 These results suggest that L-NAME produces an opioid-independent and long-lasting antinociception in the mouse most probably by a direct effect within the central nervous system.

Introduction

Guanidino-monosubstituted derivatives of L-arginine including L-N^G-nitro arginine (L-NOARG) and its methyl ester (L-NAME), L-N^G-monomethyl arginine (L-NMMA) and L-N^G-amino arginine selectively inhibit nitric oxide (NO) biosynthesis by vascular endothelial cells (Rees *et al.*, 1989; Fukuto *et al.*, 1990; Moore *et al.*, 1990). Recently, it has become evident that NO biosynthesis is a widespread phenomenon in the body occurring not only in the vascular endothelium but also in leucocytes, Kuppfer cells, hepatocytes, neuroblastoma cells, fibroblasts and the adrenal gland (Moncada *et al.*, 1989; Ishii *et al.*, 1990). In addition, NO or a closely chemically related substance, is released from peripheral non-adrenergic, non-cholinergic (NANC) nerves innervating the mouse and rat anococcygeus (Gillespie *et al.*, 1989; Gibson *et al.*, 1990), guinea-pig trachea (Tucker *et al.*, 1990) and dog ileocolonic junction (Bult *et al.*, 1990). The discovery that NO is also released from rat cerebellar synaptosomes stimulated with N-methyl-D-aspartate or kainate (Garthwaite *et al.*, 1988; 1989) and the identification of a brain synaptosomal enzyme which converts L-arginine into L-citrulline and NO (Knowles *et al.*, 1989) has raised the possibility that NO may additionally function as a neurotransmitter in the central nervous system. However, the biological role or roles of NO in the brain have yet to be investigated. Using L-NAME as a pharmacological tool we report here that NO may have an important function in pain perception in the mouse. Some of these results have been published in preliminary form (Hart *et al.*, 1990).

Methods

Male LACA mice (30–35 g) were used in this study. Animals were allowed food and water *ad libitum* until transported to the laboratory 1 h before the experiment. All experiments were performed in the period between 13 h 00 min and 17 h 00 min and, except where otherwise indicated, were conducted in normal room light and temperature (22 ± 2°C).

Assessment of antinociceptive effect of L-NAME

The antinociceptive activity of L-NAME was assessed by use of 3 separate procedures: (i) Formalin-induced paw-licking was determined essentially as described by Hunskaar & Hole (1987). Briefly, animals were injected sub-plantar in one hindpaw with formalin (5%, 10 μl). The duration of paw-licking (an index of nociception) was measured 0–5 min and 15–30 min after formalin administration. The first phase response is believed to represent a direct irritant effect of formalin on sensory C fibres whilst the latter phase response is most likely secondary to the development of an inflammatory response and the release of algesic mediators (Hunskaar & Hole, 1987). L-NAME (1–75 mg kg^{-1}) or D-NAME (600 mg kg^{-1}) were injected intraperitoneally (i.p.). In separate experiments, L-NAME was administered either intracerebroventricularly (i.c.v.) as described by Oluyomi (1989) or orally (p.o.). The time interval between L-NAME and formalin administration was 15 min in each case. In order to determine the time course of effect some animals were injected with L-NAME (i.p.) 24 h before the formalin injection and assessment of licking time. Control animals received an equal volume (0.2 ml i.p. and p.o.; 5 μl i.c.v.) of saline (0.9% w/v NaCl). In some experiments, mice injected i.p. with L-NAME (75 mg kg^{-1}) were additionally pretreated (i.p.) with either L-arginine (150–600 mg kg^{-1}), D-arginine (600 mg kg^{-1}) or naloxone (5 mg kg^{-1}) 20 min prior to formalin administration. Such a dose of naloxone has previously been reported to abolish morphine-induced antinociception in the formalin test in mice (Vaccarino *et al.*, 1989). In control experiments, additional animals received either L- or D-arginine (600 mg kg^{-1}) alone. For comparison with other known antinociceptive agents mice were injected i.p. with morphine (2.5–10 mg kg^{-1})

[1] Author for correspondence.

or indomethacin (10–40 mg kg^{-1}) 15 min prior to formalin injection and assessment of licking time. (ii) Acetic acid (0.6%, 10 ml kg^{-1}, i.p.) induced abdominal constrictions were counted over a 10 min period 25 min following i.p. injection of L-NAME (75–600 mg kg^{-1}), D-NAME (600 mg kg^{-1}) or saline (0.2 ml) and 10 min after acetic acid administration. (iii) Change in foot withdrawal reaction time on a hot plate set to 56°C. Animals were injected with L-NAME (75 mg kg^{-1}, i.p.) or saline (0.2 ml) 15 or 130 min before assessment of reaction time.

Effect of L-NAME on formalin-induced hindpaw oedema

The possibility that the antinociceptive activity of L-NAME in the formalin-induced paw-licking assay may be secondary to an anti-oedema effect was assessed by comparing the weight of the formalin-injected and contralateral (untreated) hindpaws excised at the knee joint at the end of the experiment. Animals were pretreated (i.p.) with L-NAME (75 mg kg^{-1}), indomethacin (40 mg kg^{-1}) or saline (0.2 ml) 15 min prior to subplantar formalin (5%, 10 μl) injection. Groups of mice were killed 45 min thereafter.

Effect of L-NAME on mouse blood pressure

Since changes in paw blood flow might be expected to influence the antinociceptive effect of L-NAME assessed by the formalin-induced paw-licking and hot plate assays the effect of L-NAME administration on mouse blood pressure was also investigated. For these experiments, mice were anaesthetized with urethane (10 g kg^{-1}, i.p.) and a cannula inserted into the carotid artery. Blood pressure was monitored continuously with a Bell & Howell pressure transducer connected to a Grass 4-channel pen recorder, L-NAME (1–75 mg kg^{-1}, i.p.; 0.1 and 100 μg per mouse, i.c.v.), D-NAME (75 mg kg^{-1}, i.p.) or saline (0.5 ml, i.p.: 5 μl, i.c.v.) were administered and mean arterial blood pressure (MABP) determined at intervals thereafter.

Effect of L-NAME on animal behaviour

In preliminary experiments, mice were housed singly and observed for any overt changes in behaviour for up to 3 h following i.p. injection of L-NAME (75 or 600 mg kg^{-1}) or saline (0.2 ml). A more objective estimate of the effect of L-NAME on mouse behaviour was performed by use of a modified head dipping board procedure as described by Davis & Wallace (1976). Briefly, mice were injected with L-NAME (75 and 600 mg kg^{-1}), D-NAME (600 mg kg^{-1}) or saline (0.2 ml) and placed 60 min thereafter onto the central square of a 16 square dipping board apparatus positioned approximately 1 metre above the ground. All experiments were performed in neutral red light in a closed and quiet room. Four behavioural parameters (dips, rears, crosses, circles) were monitored over a 3 min test period. In separate experiments, mouse locomotor activity was determined 60 min after L-NAME, D-NAME or saline administration in automated activity cages (Ugo Basile Ltd., Italy). Results are expressed as counts obtained over an initial 10 min period.

Drugs and chemicals

All drugs were purchased from Sigma with the exception of D-NAME which was obtained from Bachem Ltd. Indomethacin was dissolved in 5% (w/v) Na_2CO_3. All other drugs were dissolved in saline.

Statistical analysis

Results indicate mean ± s.e.mean with the exception of dipping board and locomotor activity data which are expressed as medians. Statistical significance of differences between groups was determined by unpaired Student's t test unless otherwise indicated.

Results

Antinociceptive activity of L-NAME

Formalin-induced paw licking In saline-treated mice the duration of paw licking over the first time period was 103.3 ± 1.8 s ($n = 8$) and over the second period, 146.7 ± 3.8 s ($n = 8$), D-NAME (600 mg kg^{-1}) failed to reduce paw licking time in either the early or late phase indicating the absence of significant antinociceptive effect. In contrast, L-NAME (1–75 mg kg^{-1}) injected i.p. caused a dose-related reduction in paw licking in the second phases of measurement whilst only the highest dose of L-NAME (75 mg kg^{-1}) significantly reduced first phase paw licking time (Figure 1a). L-NAME (75 mg kg^{-1}) reduced paw licking by 30.8 ± 4.5% in the first phase and 96.3 ± 3.0% (both $n = 8$, $P < 0.001$) in the second phase. This effect of L-NAME (75 mg kg^{-1}) was not influenced by pretreatment of mice with naloxone (5 mg kg^{-1}) (first phase licking time: 68.5 ± 2.3 s, c.f. 71.2 ± 1.2 s, second phase licking time: 9.1 ± 1.1 s, c.f. 5.6 ± 3.4 s, both $n = 8$, $P > 0.05$). Interestingly, L-NAME (75 mg kg^{-1}) retained both first phase (licking time, 90.9 ± 1.3 s, c.f. 101.9 ± 1.6 s, $n = 6$, $P < 0.05$) and second phase antinociceptive activity 24 h after i.p. administration (licking time, 69.8 ± 16.3 s, c.f. 133.0 ± 11.4 s, $n = 6$, $P < 0.01$).

The antinociceptive potency of L-NAME was also compared to that of indomethacin and morphine (Figure 1b,c). Clearly, L-NAME exhibited greater second phase antinociceptive activity in this model than indomethacin. In contrast to L-NAME, indomethacin did not influence first phase

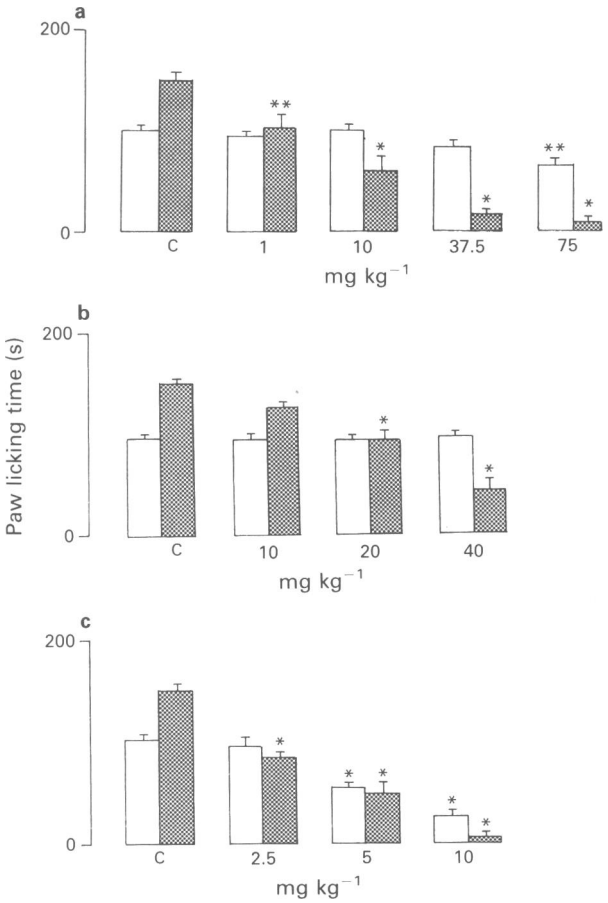

Figure 1 Antinociceptive effect of L-N^G-nitro arginine methyl ester (L-NAME) (a), indomethacin (b) and morphine (c) in the mouse following i.p. administration. C represents control results obtained in saline-treated animals. Open columns indicate licking time in the first phase (0–5 min) whilst stippled columns indicate licking time in the second phase (15–30 min). Results show mean of $n = 8$; s.e.mean shown by vertical bars. ** $P < 0.05$; * $P < 0.001$.

licking time. Morphine was a more potent antinociceptive agent than L-NAME in both the first and second phases of the response.

L-NAME also exhibited antinociceptive activity following i.c.v. and p.o. administration (Figure 2). For example, 100 μg L-NAME injected i.c.v. or 150 mg kg^{-1} administered p.o. abolished paw-licking in the second phase. Additionally, L-NAME (100 μg, i.c.v.) reduced first phase licking time by 72.2 $\pm$ 7.9% ($n = 8$). In contrast, oral administration of L-NAME (75 mg kg^{-1}) did not significantly influence first phase licking time but did reduce the second phase licking.

L-Arginine (150–600 mg kg^{-1}, i.p.) but not D-arginine (600 mg kg^{-1}, i.p.) pretreatment completely reversed the first phase antinociceptive effect of L-NAME (75 mg kg^{-1}, i.p.) and partially reversed second phase antinociception (Figure 3). Interestingly, L-arginine (600 mg kg^{-1}, i.p) administered to mice in the absence of L-NAME itself produced a small but significant reduction in paw licking time in the second phase (111.4 $\pm$ 8.3 s, c.f. 148.6 $\pm$ 12.4 s, $n = 8$, $P < 0.05$) without affecting the first phase response. D-Arginine (600 mg kg^{-1}, i.p.) was without effect in both phases (data not shown).

Acetic acid-induced abdominal constriction Acetic acid administration in saline-injected mice resulted in the production of 28.0 $\pm$ 3.5 abdominal constrictions in the 10 min test period. L-NAME pretreatment reduced the incidence of abdominal constrictions by approximately 40% and 90% at doses of 75 and 600 mg kg^{-1} respectively (Figure 4).

Hot plate assay The latency of foot withdrawal of naive mice on the hot plate set to 56°C was 5.7 s (median). Saline injection failed to influence the latency of foot withdrawal 15 min post-injection (4.3 s; median, $P > 0.05$, Mann-Whitney U test). In contrast, L-NAME (75 mg kg^{-1}) significantly prolonged the time for foot withdrawal both 15 min and 130 min post-injection (Figure 4).

Effect of L-NAME on mouse hindpaw weight

Hindpaw weight of saline-injected mice injected subplantar with formalin increased by 190.7 $\pm$ 6.9 mg ($n = 12$) compared with the contralateral (untreated) hindpaw indicating significant oedema formation. Indomethacin (40 mg kg^{-1}) pretreatment reduced oedema formation by approximately 26%

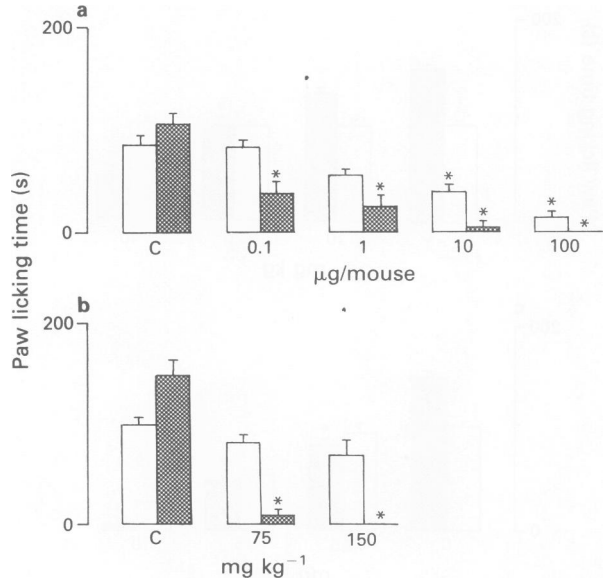

Figure 2 Antinociceptive effect of L-N^G-nitro arginine methyl ester (L-NAME) in the mouse following i.c.v. (a) or p.o. (b) administration. C represents control results obtained in saline-treated animals. Open columns indicate licking time in the first phase (0–5 min) whilst stippled columns indicate licking time in the second phase (15–30 min). Results show mean of $n = 8$ (a) or $n = 6$ (b); s.e.mean shown by vertical bars. * $P < 0.001$.

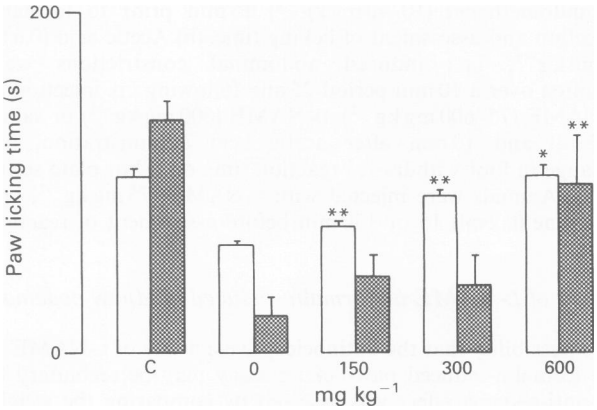

Figure 3 Reversibility of the antinociceptive effect of L-N^G-nitro arginine methyl ester (L-NAME, 75 mg kg^{-1}, i.p.) by L-arginine in the mouse. Figures beneath each pair of histograms indicate dose of L-arginine (mg kg^{-1}) administered i.p. 5 min prior to subplantar formalin administration. C represents control results obtained in saline-treated animals. Open columns indicate licking time in the first phase (0–5 min) whilst stippled columns indicate licking time in the second phase (15–30 min). Results show mean of $n = 6$; s.e.mean shown by vertical bars. ** $P < 0.05$, * $P < 0.001$ (compared to results obtained in mice pretreated with L-NAME).

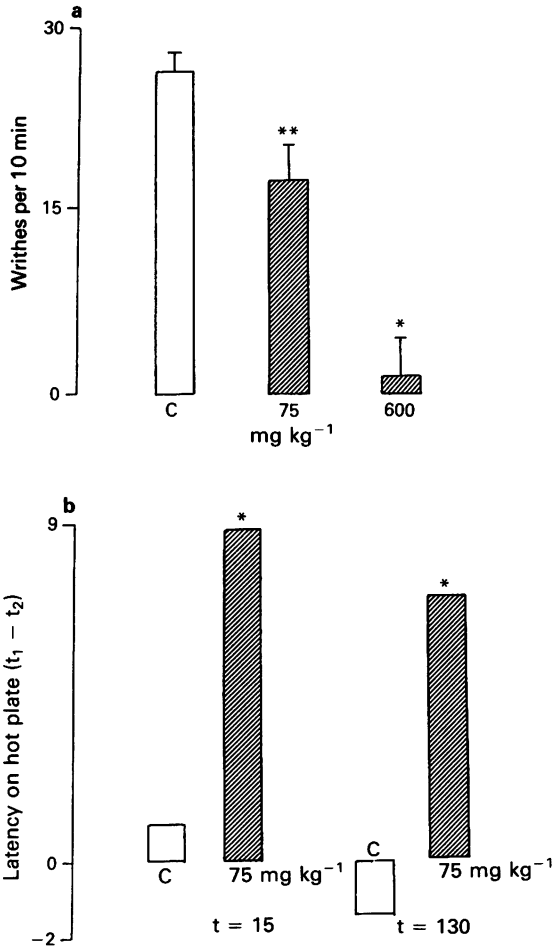

Figure 4 Antinociceptive effect of L-N^G-nitro arginine methyl ester (L-NAME) in the mouse assessed by the acetic acid-induced abdominal constriction assay (a) and the hot plate assay (b). In both cases C represents control results obtained in saline-pretreated animals. Results in (a) indicate number of writhes in a 10 min period and are mean of $n = 6$–9; s.e.mean shown by vertical bars. ** $P < 0.05$, * $P < 0.001$. Results in (b) indicate difference in hot plate latency of mice measured before (t_1) and either 15 min ($t = 15$) or 130 min ($t = 130$) after treatment with L-NAME (t_2). Results show median values, $n = 5$–20, * $P < 0.001$ (Mann-Whitney U test).

Table 1 Effect of L-N^G-nitro arginine methyl ester (L-NAME) on blood pressure of the urethane-anaesthetized mouse

	MABP (mmHg) $t = 0$	$t = 15$
L-NAME (i.p.)		
1.0	50.5 ± 2.4	49.7 ± 2.8
10.0	62.0 ± 7.7	78.6 ± 10.6
37.5	46.2 ± 4.0	65.7 ± 7.1*
75.0	68.3 ± 6.9	91.8 ± 10.9*
600.0	61.2 ± 5.4	115.0 ± 5.3*
D-NAME (i.p.)		
75.0	46.7 ± 1.6	50.2 ± 3.5
L-NAME (i.c.v.)		
0.1	51.2 ± 3.8	55.5 ± 4.2
100.0	64.0 ± 6.4	62.4 ± 5.3

Urethane-anaesthetized mice were injected i.p. (mg kg^{-1}) or i.c.v. (μg per mouse) with L-NAME or i.p. (mg kg^{-1}) with D-NAME. MABP was determined before ($t = 0$) and 15 min ($t = 15$) after drug injection. In control experiments no increase in MABP was detected in mice injected with saline (0.5 ml i.p.; 10 μl i.c.v.). Results show mean ± s.e.mean, $n = 3$–6, * $P < 0.05$.

(140.8 ± 10.3 mg, $n = 6$, $P < 0.01$). In contrast, L-NAME (75 mg kg^{-1}) failed to influence the weight of formalin-injected hindpaws (170.2 ± 18.4 mg, $n = 12$, $P > 0.05$).

Effect of L-NAME on mouse blood pressure

The resting MABP of urethane-anaesthetized mice was 55.0 ± 2.3 mmHg ($n = 32$). Intravenous injection of L-NAME (1–600 mg kg^{-1}) elicited a dose-related increase in MABP (Table 1). At the highest dose of L-NAME used in this study, MABP was approximately doubled. The threshold dose for vasopressor activity was 10 mg kg^{-1}. In contrast, L-NAME (0.1 and 100 μg per mouse) injected i.c.v. failed to influence MABP (Table 1).

Effect of L-NAME on animal behaviour

Subjective observation of mice injected i.p. with L-NAME (up to 75 mg kg^{-1}) revealed no obvious changes in animal behaviour over a period of 3 h when compared with control animals injected with saline. A high dose of L-NAME (600 mg kg^{-1}) produced no change in animal behaviour until 60 min post-injection. Thereafter, mice remained fixed and motionless although they appeared alert and could be easily aroused. No loss of righting reflex was observed.

L-NAME (75 mg kg^{-1}, i.p.) failed to influence rearing, crossing, circling or dipping forms of behaviour as assessed by the dipping board assay. In addition, no change in locomotor behaviour in activity cages was detected with this dose of L-NAME. A high dose of L-NAME (600 mg kg^{-1}) did not significantly change rearing, crossing or circling but did reduce dipping behaviour by approximately 50% and additionally depressed overall locomotor count by approximately 35%. These results are summarised in Table 2.

Table 2 Effect of L-N^G-nitro arginine methyl ester (L-NAME) on dipping board and locomoter activity of mice

	Dips	*Rears*	*Crosses*	*Circles*	*LA*
Saline	24	39	10	2	1340
L-NAME (75)	16	49	15	2	1229
L-NAME (600)	9*	31	8	5	654*
D-NAME (600)	22	34	12	4	1512

Mice were injected i.p. with L-NAME or D-NAME at doses (mg kg^{-1}) indicated in parentheses. Dips, rears, crosses and circles were monitored over a 3 min period 60 min post-injection. Results show median values, $n = 5$–12, * $P < 0.05$ (Mann-Whitney U test). Locomotor activity (LA) was monitored in activity cages immediately post-injection and represents arbitrary counts per 10 min. Results show median values, $n = 8$, $P < 0.05$ (Mann-Whitney U test) subsequent to Kruskal-Wallis analysis of variance.

Discussion

The results of the present study indicate that L-NAME (but not D-NAME) produces a potent antinociceptive effect in the mouse assessed by use of three different experimental procedures. Moreover, L-NAME is effective following i.p., i.c.v. or p.o. administration in the formalin assay and its antinociceptive action is reversed by L- but not D-arginine. Interestingly, L- but not D-arginine at a high dose itself elicits a weak antinociceptive action in the formalin test. The rationale underlying this direct effect of L-arginine is unknown but is clearly unlikely to be consequent upon NO production. Since L-NAME is a selective inhibitor of NO biosynthesis in a variety of mammalian tissues and cells including both peripheral (Gibson *et al.*, 1990) and central (Murad *et al.*, 1990) nerves we propose that a similar inhibition of NO formation underlies its observed antinociceptive action.

The precise site of the antinociceptive effect of L-NAME in the mouse remains unclear. That i.c.v. administered L-NAME reduces formalin-induced paw licking time strongly suggests an effect within the central nervous system. Furthermore, L-NAME is antinociceptive in the hot plate assay which is widely believed to be sensitive solely to drugs acting supraspinally (Yaksh & Rudy, 1977).

Since animal models of nociception may be indirectly influenced by drugs acting non-specifically on other body systems we considered it important to exclude other effects of L-NAME in the antinociception observed. For example, intravenous administration of L-NAME reportedly increases blood pressure in the rat (Rees *et al.*, 1990; Dubbin *et al.*, 1990). Thus, it is conceivable that a similar effect in the mouse may contribute to the observed antinociceptive effect of L-NAME in at least two of the three assays employed by altering hindpaw blood flow. We show here that L-NAME administered i.p. elicits a dose-related vasopressor response in the anaesthetized mouse. Interestingly, the threshold dose for significant vasopressor effect was >10 mg kg^{-1} even though antinociception was observed at doses of L-NAME as low as 1 mg kg^{-1}. Furthermore, i.c.v. injection of L-NAME failed to increase mouse blood pressure although did produce antinociception in the formalin test when injected by this route. Thus, it seems unlikely that the cardiovascular effect of L-NAME contributes significantly to the antinociception.

In addition, the potent antinociceptive effect of L-NAME in the second phase of the formalin test is not secondary to an anti-oedema action since L-NAME pretreatment failed to prevent the increase in hindpaw weight following formalin injection. In contrast, indomethacin significantly inhibited inflammatory oedema formation in the formalin-injected mouse hindpaw. These results seem at variance with those of Antunes *et al.* (1990) who reported that L-NMMA inhibited polyarginine-induced inflammation in the rat hindpaw. The reason for the discrepancy between the two studies is not clear although it may reflect differences between species, nociceptive stimulus or inhibitor of NO formation used. Based upon the present results it therefore seems unlikely that L-NAME exhibits antinociceptive activity in the formalin model secondary to an anti-inflammatory effect.

At doses of L-NAME which produce pronounced antinociceptive activity (up to 75 mg kg^{-1}) no changes in animal behaviour were observed or could be detected by the dipping board procedure. Furthermore, no reduction in locomotor activity was apparent. Thus, at doses within the antinociceptive range, L-NAME has no detectable sedative or

other behavioural effects which might contribute to its antinociceptive effect.

The present results raise two intriguing possibilities: (i) that NO, or a like substance formed from L-arginine, has a physiological role to play in the mouse to promote the pain response most probably by an action within the central nervous system although some effect on peripheral sensory nerves cannot be discounted. (ii) L-NAME, or a like inhibitor of NO biosynthesis, may at appropriate dose, have clinical application as an analgesic. In this context it is of interest that antinociception in the mouse due to L-NAME is not antagonized by naloxone and is thus independent of endogenous opioid release. Moreover, in contrast to the present generation of clinically used analgesics, the antinociceptive effect of L-NAME, at least in the mouse, persists for up to 24 h.

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Effects of hypoxia and metabolic inhibitors on production of prostacyclin and endothelium-derived relaxing factor by pig aortic endothelial cells

Joanna M. Richards, *Ian F. Gibson & *[1]William Martin

Department of Cardiology, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN and *Department of Pharmacology, University of Glasgow, Glasgow G12 8QQ

1 The content of adenosine triphosphate (ATP) and basal and bradykinin-stimulated production of prostacyclin and endothelium-derived relaxing factor (EDRF) was measured in primary cultures of porcine aortic endothelial cells under normoxic (14.4% O_2) and hypoxic (2.8% O_2) conditions, and following treatment with rotenone and 2-deoxy glucose, which inhibit oxidative and glycolytic metabolism, respectively.

2 ATP content and basal and bradykinin-stimulated production of prostacyclin were similar under normoxic and hypoxic conditions. EDRF production, assessed as endothelial guanosine 3':5'-cyclic monophosphate (cyclic GMP) content, was also similar under both conditions.

3 Treatment with rotenone (0.3 μM) had no effect on ATP content or basal or bradykinin-stimulated production of prostacyclin or of EDRF, measured as endothelial cyclic GMP content. Elevation of cyclic GMP content by atriopeptin II was also unaffected.

4 Treatment with 2-deoxy glucose (20 mM) in glucose-free Krebs solution lowered ATP content, reduced bradykinin-stimulated production of prostacyclin and abolished the bradykinin-stimulated elevation of cyclic GMP content. Resting production of prostacyclin was unaffected but basal content of cyclic GMP was lowered in some experiments. Elevation of cyclic GMP content by atriopeptin II was abolished.

5 Combined treatment with rotenone (0.3 μM) and 2-deoxy glucose (20 mM) lowered ATP content more than with 2-deoxy glucose alone. Basal production of prostacyclin rose slightly and bradykinin-stimulated production was powerfully inhibited. Basal content of cyclic GMP was unaffected, but bradykinin-stimulated production was abolished. Elevation of cyclic GMP by atriopeptin II was also abolished.

6 Cascade bioassay experiments using endothelium-denuded rings of rabbit aorta as a detector system confirmed that bradykinin-stimulated production of EDRF was blocked by 2-deoxy glucose, but not by rotenone.

7 These data indicate that porcine aortic endothelial cells in culture operate under mainly glycolytic metabolism and this probably explains why production of prostacyclin and EDRF is unaffected under hypoxic conditions. They also indicate that glycolytic metabolism is required for agonist-stimulated production of prostacyclin and EDRF by these cells.

Introduction

Hypoxia has profound effects on tone of the vasculature *in vivo*, producing, in general, vasodilatation in the systemic circulation (Hilton & Eichholtz, 1925), and vasoconstriction in the pulmonary circulation (Fishman, 1976). The factors regulating these responses to hypoxia are complex, but include interference with respiratory chain function (Fay, 1971; Hellstrand *et al.*, 1977), and production of tissue-derived (Berne & Rubio, 1977) or blood vessel-derived vasoactive metabolites (Busse *et al.*, 1983; Rubanyi & Vanhoutte, 1985).

As the vascular endothelium is in intimate contact with the circulating blood, many studies have attempted to determine its role in regulating vascular responsiveness to hypoxia. For example, *in vitro* studies have shown that hypoxia-induced vasoconstriction or augmentation of vasoconstrictor tone is endothelium-dependent in porcine pulmonary artery (Holden & McCall, 1984), in canine femoral and cerebral artery (De Mey & Vanhoutte, 1983; Katusic & Vanhoutte, 1986), and in canine, porcine, bovine and ovine coronary artery (Rubanyi & Vanhoutte, 1985; Rubanyi & Paul, 1984; Kwan *et al.*, 1989). In contrast, in branches of canine femoral artery, and in rabbit aorta, the endothelium has been shown to mediate, at least in part, hypoxia-induced vasodilatation (Busse *et al.*, 1983; Coburn *et al.*, 1986; Bassenge *et al.*, 1988). These hypoxia-induced vasodilator responses may result from increased production of prostanoids (Kälsner, 1977), or a combination of prostanoids and endothelium-derived relaxing factor (EDRF; Bassenge *et al.*, 1988). In other studies, mild or severe hypoxia has been shown to inhibit endothelium-dependent vasodilatation of rabbit aorta and pulmonary artery (Furchgott & Zawadzki, 1980; Johns *et al.*, 1989) and canine femoral and cerebral artery (De Mey & Vanhoutte, 1983; Katusic & Vanhoutte, 1986). Blockade of oxygen utilization by inhibitors of oxidative phosphorylation has, like hypoxia, been shown to inhibit agonist-induced endothelium-dependent vasodilatation in rabbit aorta (Griffith *et al.*, 1986; 1987). Furthermore, these studies showed that basal release of EDRF was unaffected by inhibition of oxidative metabolism.

It is clear, therefore, that hypoxia induces a complex array of endothelium-dependent changes in vascular responsiveness in different blood vessels, and in some, the effects of hypoxia can be mimicked by inhibition of oxidative metabolism. The above studies were conducted on isolated blood vessels containing a heterogeneous mixture of cell types, and where the responsiveness of the endothelium is often difficult to discern. Studies on endothelial cells in isolation from other vascular cells may provide a greater insight into the responsiveness of this cell type to hypoxia and metabolic inhibition. For example, it has recently been shown that hypoxia inhibits agonist-induced production of EDRF by bovine isolated pulmonary artery endothelium (Warren *et al.*, 1989), and this, rather than production of a constrictor factor may account for pulmonary hypoxic vasoconstriction. By examining the production of prostacyclin and EDRF by porcine cultured aortic endothelial cells, we hoped to determine how hypoxia and

[1] Author for correspondence.

metabolic inhibition regulate production of these two vasodilator substances in a systemic artery.

Methods

Endothelial cell culture

Endothelial cells were isolated from porcine aorta as previously described (Gordon & Martin, 1983). Briefly, after ligating the intercostal arteries, collagenase (0.2%, type II, Sigma) was introduced into the aortic lumen and the vessel incubated at 37°C for 20 min. The collagenase solution containing aortic endothelial cells was spun (50 *g*, 3 min, room temperature) and the cells resuspended in 60 ml of Medium E199 supplemented with: foetal bovine serum (10%); newborn bovine serum (10%); glutamine (4 mM); benzyl penicillin (100 units ml^{-1}); streptomycin (100 $\mu g\,ml^{-1}$) and kanamycin (100 $ng\,ml^{-1}$).

The endothelial cells were characterized by several criteria: they grew as a strict monolayer and in randomly selected cultures, no fewer than 98% of cells accumulated acetylated low-density lipoprotein labelled with a fluorescent marker (C.M.D. U.K. Ltd; Voyta *et al.*, 1984). This study shows also that prostacyclin and EDRF are produced by these cells.

For monolayer studies the cells from each aorta were seeded into 3 Linbro plates each containing 6 wells (9.6 cm^2) and grown in an incubator at 37°C under an atmosphere of 5% CO_2 in air. The medium was changed every 2 days and the cells used within 3–7 days.

For experiments in which cells were used on microcarrier beads, the cells from each of four aortae were seeded into a T 75 Falcon flask. When confluent, cells were dispersed with a solution of trypsin (0.025%) in ethylenediamine tetra acetic acid (EDTA, 0.02%, disodium salt), spun (50 *g*, 3 min, room temperature), resuspended in 100 ml of Medium E199 supplemented as listed above, seeded onto 3 ml of Biosilon microcarrier beads (Nunc, 200 μm diameter) in a sterile siliconised Techne microcarrier flask, and grown at 37°C under an atmosphere of 5% CO_2 in air. The beads were stirred at 30 r.p.m. for 2.5 min every 30 min for 3–5 days during which time the cells grew to confluence, which was confirmed by microscopic examination after staining a sample of the cells with methyl violet (0.1%, B.D.H.).

All tissue culture media and supplements were obtained from Flow Laboratories.

Endothelial monolayer studies

The tissue culture medium was removed and the endothelial cells rinsed twice with 2 ml of Krebs solution containing (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24 and glucose 11. The cells were then incubated in 2 ml of Krebs solution at 37°C under an atmosphere of 5% CO_2 in air for a minimum of 60 min.

In experiments in which the effects of oxygen tension were studied, cells were placed in an 'Atmos Bag' (Sigma) in an incubator at 37°C. The bag was then inflated with either 5% CO_2 in air (normoxic conditions) or with 95% N_2, 5% CO_2 (hypoxic conditions). Five minutes after inflation, the saturation of oxygen in the Krebs solution, measured with a Clark-type oxygen electrode, under normoxic and hypoxic conditions was 14.4 ± 0.4% and 2.8 ± 0.8% ($n = 6$), respectively and these values remained constant throughout the experiment. All subsequent procedures were carried out under these controlled atmospheres using gloves incorporated into the 'Atmos Bag'. After 30 min the Krebs solution bathing the cells was removed and retained for analysis of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), and the cells were immediately extracted with 1 ml of ice-cold trichloroacetic acid (TCA, 6%) for analysis of guanosine 3':5'-cyclic monophosphate (cyclic GMP), ATP and DNA content.

All studies with metabolic inhibitors were performed under normoxic conditions. In these experiments rotenone (0.3 μM), 2-deoxyglucose (20 mM) or a combination of rotenone (0.3 μM) and 2-deoxyglucose (20 mM) was present throughout the 30 min incubation period. When 2-deoxyglucose was used, glucose was omitted from the Krebs solution.

Following extraction with TCA (6%), cells were scraped from the multiwells and transferred to Eppendorf tubes and spun at 13,000 r.p.m. for 6 min at room temperature. The DNA content of the pellets was measured by the fluorescence technique of Kissane & Robins (1958). The supernatants were neutralised (to pH 5.5–6) by adding 2 ml of 0.5 M tri-n-octylamine in freon (1, 1, 2 trichlorotrifluoroethane) and vortex mixing for 90 s. The aqueous layer (upper) was retained for cyclic GMP and ATP analysis.

Measurement of prostacyclin

The prostacyclin content of Krebs samples was determined by radioimmunoassay of the stable breakdown product 6-keto-$PGF_{1\alpha}$ using an antiserum kindly supplied by Dr A.C. Newby, Department of Cardiology, University of Wales College of Medicine. The cross-reactivity of the antiserum at 50% displacement with PGE_2, PGE_1 and $PGF_{2\alpha}$ was 5.0%, 1.3% and 1.4%, respectively. Prostacyclin production was expressed as ng 6-keto-$PGF_{1\alpha}\,\mu g^{-1}$ DNA.

Measurement of cyclic GMP

The cyclic GMP content of neutralised cell extracts was measured by radioimmunoassay with New England Nuclear kits as previously described (Martin *et al.*, 1985). Cyclic GMP content was expressed as fmol μg^{-1} DNA.

Measurement of ATP

The ATP content of neutralised cell extracts was measured in a luminometer (Products For Research Inc., U.S.A.) using the luciferin-luciferase reagent (Sigma). ATP content was expressed as pmol μg^{-1} DNA.

Cascade bioassay

EDRF release from pig aortic endothelial cells grown on microcarrier beads and perfused in columns was detected by bioassay on an endothelium-denuded ring of rabbit aorta. The preparation of endothelial cell columns was as previously described (Gordon & Martin, 1983). Briefly, microcarrier beads containing endothelial cells were packed on top of a bed of glass wool in 1 ml syringes. The packed volumes of beads was 0.3–0.7 ml. After replacing the syringe plunger, through which a PP_{30} polythene delivery tube had been inserted, the columns were perfused from the bottom upwards at a rate of 4 $ml\,min^{-1}$ at 37°C with Krebs solution containing superoxide dismutase (30 units ml^{-1}), to potentiate the actions of EDRF and gassed with 5% CO_2 in air. The perfusate was passed over an endothelium-denuded ring of rabbit aorta that had been suspended under 2 g resting tension and contracted sub-maximally with phenylephrine (0.1 μM). Where indicated in the Results, the aortic ring was perfused simultaneously by a second circuit that had no contact with the endothelial cells. Tension was recorded isometrically with grass FTO3C transducers and displayed on a Grass recorder.

Drugs

Atriopeptin II (rat synthetic), bradykinin triacetate, 2-deoxy-D-glucose, haemoglobin (bovine Type 1), phenylephrine hydrochloride, rotenone and superoxide dismutase (bovine erythrocyte) were obtained from Sigma. Glyceryl trinitrate was obtained from Napp Laboratories. All drugs were dissolved in twice-distilled water except for rotenone, which was dissolved in ethanol.

Solutions of haemoglobin were reduced to the ferrous form with dithionite before use, as previously described (Martin *et al.*, 1985).

Statistical analysis

As the resting content of cyclic GMP and basal production of prostacyclin varied in batches of cells, all experiments were performed with their own internal controls. In the results, n represents the number of replicate dishes of cells randomly taken from different aortae. Results are expressed as the mean ± s.e.mean and comparisons were made by means of Student's t test. A probability of 0.05 or less was considered significant.

Results

Hypoxia

Under normoxic conditions (14.4% O_2) the resting production of 6-keto-$PGF_{1\alpha}$ by primary cultures of pig aortic endothelial cells, measured over 30 min, was 0.24 ± 0.02 ng μg^{-1} DNA ($n = 18$). The resting content of cyclic GMP was 24.9 ± 3.4 fmol μg^{-1} DNA ($n = 11$), and the resting content of ATP was 500 ± 35 pmol μg^{-1} DNA ($n = 8$, Table 1). When placed under hypoxic conditions (2.8% O_2), resting endothelial production of 6-keto-$PGF_{1\alpha}$, and the resting content of cyclic GMP and ATP were unaffected (Table 1).

When bradykinin (0.1 μM) was added to endothelial cells under normoxic conditions for the final 5 min of the 30 min incubation period, 10.1 fold ($n = 16$) and 44.4 fold ($n = 7$) increases in 6-keto-$PGF_{1\alpha}$ production and cyclic GMP content were obtained, respectively. When placed under hypoxic conditions, the bradykinin-induced increases in 6-keto-$PGF_{1\alpha}$ production and cyclic GMP content were unaffected.

When oxyhaemoglobin (10 μM), an EDRF blocking agent, was present throughout the 30 min incubation period, resting production of 6-keto-$PGF_{1\alpha}$ was unaffected under both normoxic and hypoxic conditions (Table 2). The resting content of cyclic GMP was, however, lowered under both normoxic and hypoxic conditions (Table 2).

When superoxide dismutase (30 units ml^{-1}), an EDRF potentiating agent, was present throughout the 30 min incubation period, resting production of 6-keto-$PGF_{1\alpha}$ was unaffected under either normoxic or hypoxic conditions (Table 2). However, the resting content of cyclic GMP was elevated under both normoxic and hypoxic conditions (Table 2).

Table 1 Effects of hypoxia, rotenone, 2-deoxy glucose and a combination of rotenone and 2-deoxy glucose on the ATP content of pig aortic endothelial cells

Treatment	*ATP content* (pmol μg^{-1} DNA)	n
Normoxia (control)	500 ± 35	8
Hypoxia	538 ± 27	8
Rotenone	555 ± 40	6
2-DOG	200 ± 6***	6
Rotenone and 2-DOG	9 ± 2***	6

The ATP content of pig aortic endothelial cells was determined following 30 min incubation in normoxic (14.4% O_2) and hypoxic conditions (2.8% O_2), and following treatment with rotenone (0.3 μM), 2-deoxy glucose (2-DOG 20 mM) and a combination of rotenone and 2-deoxy glucose in normoxic conditions. Results are expressed as the mean ± s.e.mean. ***$P < 0.001$ indicates a significant difference from control.

Metabolic inhibitors

When rotenone (0.3 μM), an inhibitor of oxidative phosphorylation, was present throughout the 30 min incubation period under normoxic conditions, resting and bradykinin (0.1 μM)-stimulated production of 6-keto-$PGF_{1\alpha}$ and cyclic GMP content were unaffected. Levels of ATP were also unaffected (Table 1).

When 2-deoxy glucose (20 mM), an inhibitor of glycolysis, was present throughout the 30 min incubation period, in glucose-free Krebs solution, resting production of 6-keto-$PGF_{1\alpha}$ was unaffected, but bradykinin (0.1 μM)-stimulated production was significantly inhibited (Figure 1). The bradykinin-stimulated rise in cyclic GMP content was abolished, and in some but not all experiments, the resting content fell (Figure 1, Table 3). The ability of superoxide dismutase (30 units ml^{-1}) to elevate cyclic GMP content was inhibited (Table 3) and levels of ATP fell by 60% ($n = 6$, Table 1). The bradykinin-stimulated rise in cyclic GMP content was unaffected when cells were incubated in glucose-free Krebs or glucose-containing Krebs solution in the presence of 2-deoxy glucose.

A combination of rotenone (0.3 μM) and 2-deoxy glucose (20 mM) enhanced slightly resting production of 6-keto-$PGF_{1\alpha}$ and blocked bradykinin (0.1 μM)-stimulated production (Figure 2). Resting levels of cyclic GMP were unaffected, but the bradykinin-stimulated elevation was blocked and levels of ATP fell by 98% (Figure 2, Table 1).

Atriopeptin II

When a sub-maximal concentration of atriopeptin II (10 nM) was added to endothelial cells for the final 1.5 min of the 30 min incubation period under normoxic conditions, a 2.6 fold ($n = 6$) increase in cyclic GMP content was obtained (Figure 3). The atriopeptin II-induced elevation of cyclic GMP content was unaffected by treatment with rotenone (0.3 μM), but was blocked following treatment with 2-deoxy

Table 2 Effects of haemoglobin and superoxide dismutase on prostacyclin production and cyclic GMP content of pig aortic endothelial cells in normoxic and hypoxic conditions

O_2 tension	*Treatment*	*6-keto-$PGF_{1\alpha}$* (ng μg^{-1} DNA)	*Cyclic GMP* (fmol μg^{-1} DNA)		n
Normoxia	None	0.24 ± 0.02	19.8 ± 3.5	*	12
Normoxia	Hb	0.25 ± 0.03	7.8 ± 1.5		12
Hypoxia	None	0.32 ± 0.04	24.3 ± 2.6	*	11
Hypoxia	Hb	0.42 ± 0.07	14.1 ± 4.0		11
Normoxia	None	0.25 ± 0.03	20.7 ± 3.3	**	6
Normoxia	SOD	0.26 ± 0.03	58.9 ± 8.4		6
Hypoxia	None	0.26 ± 0.02	29.0 ± 6.2	*	6
Hypoxia	SOD	0.26 ± 0.03	94.2 ± 19.8		6

The production of 6-keto-$PGF_{1\alpha}$ and content of cyclic GMP was measured at the end of a 30 min incubation period in the presence of oxyhaemoglobin (Hb, 10 μM) or superoxide dismutase (SOD, 30 units ml^{-1}) in normoxic (14.4% O_2) or hypoxic (2.8% O_2) conditions. Results are expressed as the mean ± s.e.mean. *$P < 0.05$, **$P < 0.005$ indicates a significant difference between two groups joined by a bracket.

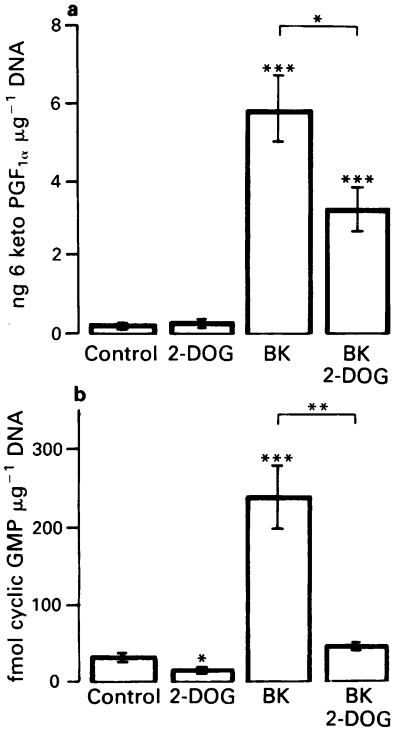

Figure 1 Production of 6-keto-$PGF_{1\alpha}$ (a) and content of cyclic GMP (b) under resting conditions (Control) and following stimulation with bradykinin (BK, 0.1 μM), measured both in normal Krebs and in glucose-free Krebs solution containing 2-deoxy glucose (2-DOG, 20 mM). Values indicate the mean of 6–12 observations and vertical bars indicate the s.e.mean. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, indicates a significant difference from control or between two groups joined by a bracket.

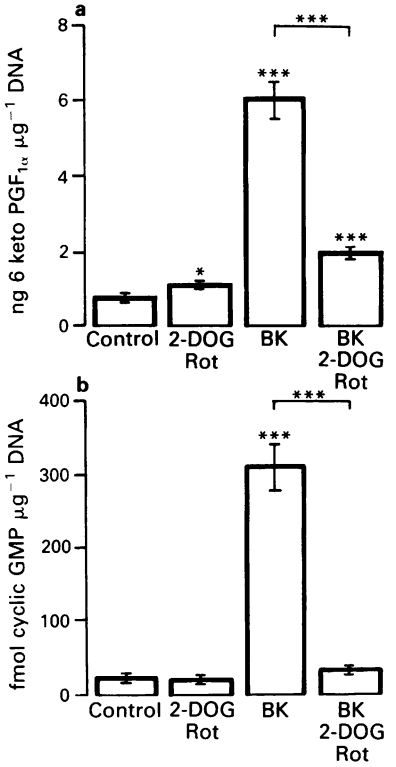

Figure 2 Production of 6-keto-$PGF_{1\alpha}$ (a) and content of cyclic GMP (b) under resting conditions (Control) and following stimulation with bradykinin (BK, 0.1 μM), measured in the absence and presence of a combination of rotenone (Rot, 0.3 μM) and 2-deoxy glucose (2-DOG, 20 mM). Values indicate the mean of 6 observations and vertical bars indicate the s.e.mean. * $P < 0.05$, *** $P < 0.001$, indicates a significant difference from control or between two groups joined by a bracket.

glucose (20 mM) or a combination of 2-deoxy glucose and rotenone (Figure 3).

Cascade bioassay

In cascade bioassay experiments, bradykinin (10 nM) infused into columns of pig aortic endothelial cells for 3 min periods, induced relaxation of phenylephrine (0.1 μM)-contracted, endothelium-denuded rings of rabbit aorta (Figure 4). When rotenone (0.3 μM) was added to the Krebs solution perfusing both the endothelial cells and bioassay tissue, bradykinin-induced relaxation was completely unaffected (Figure 4). In a separate series of experiments in which the bioassay tissues were perfused jointly by Krebs solution containing 2-deoxy glucose (20 mM) first passed into the endothelial column and by a separate circuit with normal glucose-containing Krebs, the relaxant effect of bradykinin (10 nM) was significantly reduced (Figure 4). However, the relaxant effect of a sub-maximal concentration of glyceryl trinitrate (10 nM) was completely unaffected (Figure 4).

Discussion

Our finding that ATP levels fell following treatment with 2-deoxy glucose in glucose-free Krebs but not with rotenone, suggest that porcine aortic endothelial cells in culture derive most of their energy from glycolytic metabolism. They probably derive some energy from oxidative phosphorylation, since a combination of rotenone and 2-deoxy glucose induced a greater fall in ATP levels than treatment with 2-deoxy glucose alone. These findings are consistent with a previous study on the metabolic properties of endothelial cells in culture (Dobrina & Rossi, 1983).

In rabbit aorta, agonist-induced production of EDRF is blocked by a variety of different inhibitors of oxidative metabolism, including rotenone, but 2-deoxy glucose is much less effective (Griffith *et al.*, 1986). If, as this study suggests, EDRF production is dependent upon the availability of ATP, it follows that in cells such as porcine aortic endothelial cells which derive most of their energy from glycolytic metabolism, EDRF production should be insensitive to inhibitors of oxida-

Table 3 Effects of 2-deoxy glucose and superoxide dismutase on prostacyclin production and cyclic GMP content of pig aortic endothelial cells

Stimulus	*Pretreatment*	*6-keto-$PGF_{1\alpha}$* (ng μg^{-1} DNA)	*Cyclic GMP* (fmol μg^{-1} DNA)	n
None (control)	None	0.15 ± 0.02	31.6 ± 3.6	6
None	2-DOG	0.15 ± 0.02	20.9 ± 3.4	6
SOD	None	0.16 ± 0.02	63.0 ± 5.2***] *	6
SOD	2-DOG	0.20 ± 0.04	42.3 ± 6.2]	6

The production of 6-keto-$PGF_{1\alpha}$ and content of cyclic GMP was measured at the end of a 30 min incubation in normal (control) or in glucose-free Krebs containing 2-deoxy glucose (2-DOG, 20 mM), with and without superoxide dismutase (SOD, 30 units ml^{-1}). Results are expressed as the mean ± s.e.mean. * $P < 0.05$, *** $P < 0.001$ indicates a significant difference from control or between two groups joined by a bracket.

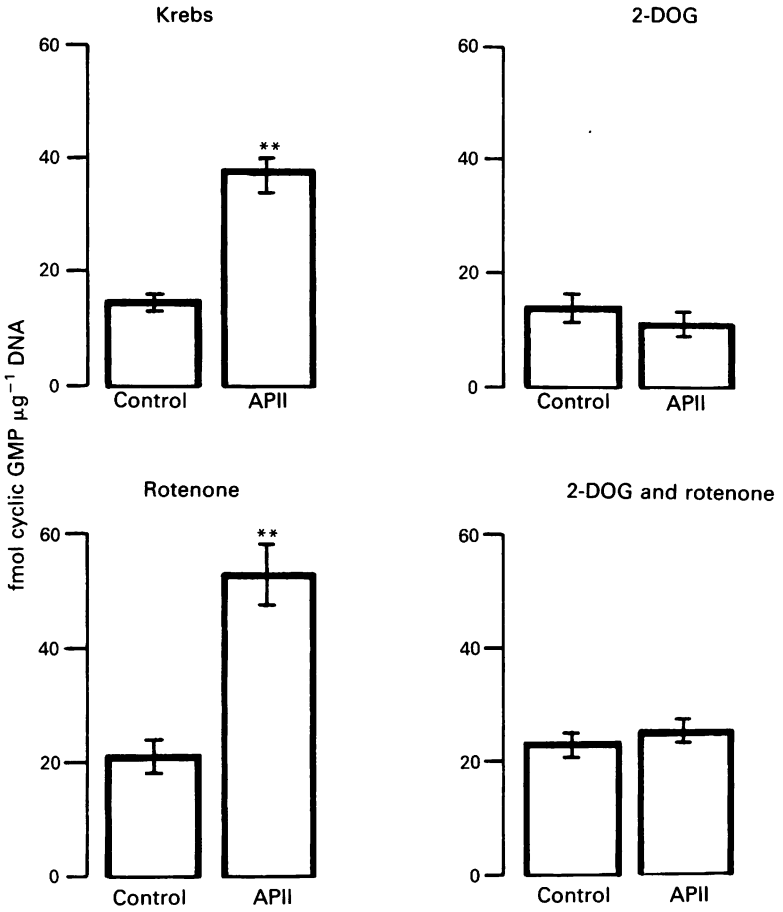

Figure 3 Content of cyclic GMP under resting conditions (Control) and following stimulation with atriopeptin II (APII, 10 nM), measured in normal Krebs solution (Krebs) or following treatment with rotenone (0.3 μM), 2-deoxy glucose (2-DOG, 20 mM) or a combination of rotenone and 2-deoxy glucose. Values indicate the mean of 4–6 observations and vertical bars indicate the s.e.mean. ** $P < 0.01$, indicates a significant difference from control.

tive phosphorylation. Using endothelial cyclic GMP content as an index of EDRF production (Martin *et al.*, 1988; Boulanger *et al.*, 1990), we found this to be the case; rotenone had no effect on resting or bradykinin-stimulated content of cyclic GMP, indicating a lack of effect on spontaneous and agonist-induced production of EDRF. According to the same scheme, inhibition of glycolytic metabolism with 2-deoxy glucose should inhibit agonist-induced production of EDRF by porcine aortic endothelial cells. In keeping with this hypothesis, we found that treatment with 2-deoxy glucose in glucose-free Krebs solution abolished bradykinin-stimulated elevations of cyclic GMP content. Furthermore, in some but not all experiments, treatment with 2-deoxy glucose lowered resting levels of cyclic GMP and in experiments where the effect of spontaneously released EDRF was potentiated with superoxide dismutase, this too was inhibited. These data indicated a possible additional action of 2-deoxy glucose in inhibiting basal EDRF production. This was unlikely, however, since basal production of EDRF has been shown to be unaffected by metabolic inhibition (Griffith *et al.*, 1987). We therefore considered an alternative possibility that 2-deoxy glucose reduced endothelial cyclic GMP content not by inhibiting EDRF production, but by lowering the levels of the high energy phosphate, GTP, from which cyclic GMP is formed (Weir *et al.*, 1990). This suspicion was confirmed by our finding that atriopeptin II-induced elevations of endothelial cyclic GMP content, which occur independently of EDRF production (Martin *et al.*, 1988), were also inhibited following treatment with 2-deoxy glucose. At the concentration used (20 mM), 2-deoxy glucose is only effective in the absence of glucose (Griffith *et al.*, 1986). Using a cascade bioassay system in which porcine aortic endothelial cells were perfused with glucose-free Krebs containing 2-deoxy glucose and the bioassay endothelium-denuded ring of rabbit aorta was perfused both with this and with a separate circuit of glucose-containing Krebs to protect it from metabolic inhibition, we found bradykinin-stimulated production of EDRF to be inhibited. Under these conditions, glyceryl trinitrate-induced vasodilatation was unaffected, indicating that soluble guanylate cyclase, the effector pathway for EDRF (Rapoport & Murad, 1983), was intact. Cascade bioassay experiments, like those in which endothelial cyclic GMP was measured, showed that rotenone had no effect on bradykinin-stimulated production of EDRF by porcine aortic endothelial cells. Thus, our data agree with those of Griffith *et al.* (1986) that ATP is required for agonist-induced production of EDRF, but shows, in contrast to rabbit aortic endothelium, that pig aortic endothelium derives this from glycolytic metabolism. The precise mechanism by which ATP contributes to agonist-induced EDRF production is unknown, but lowered levels of ATP might reduce the availability of NADPH, an important co-factor for the enzyme nitric oxide synthase which converts L-arginine to nitric oxide (Palacios *et al.*, 1989).

In common with EDRF, we found that bradykinin-stimulated production of prostacyclin was inhibited following treatment with 2-deoxy glucose but not rotenone. Inhibition was even more intensive following combined treatment with 2-deoxy glucose and rotenone. Agonist-induced production of prostacyclin, like EDRF, appears therefore to require metabolic energy, but the energy-dependent step is unknown at present.

As expected of cells operating mainly under glycolytic

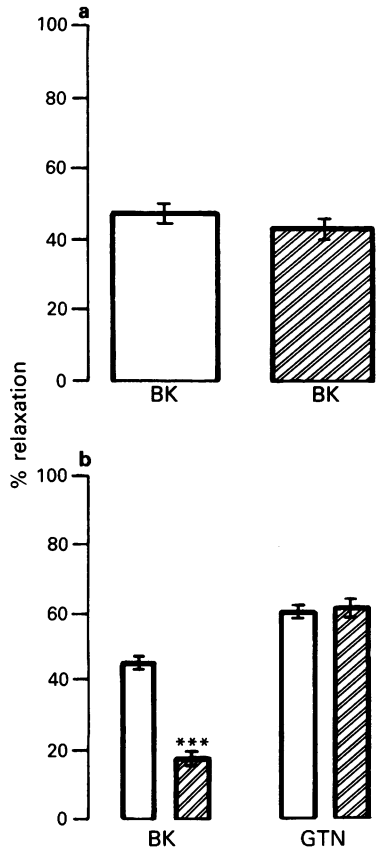

Figure 4 Cascade bioassay experiments in which the ability of bradykinin (BK, 10 nM) to elicit EDRF production by porcine aortic endothelial cells grown on microcarrier beads and perfused in columns was measured on phenylephrine (0.1 μM)-contracted, endothelium-denuded rings of rabbit aorta. (a) Rotenone (0.3 μM, hatched column) infused through both the endothelial cells and bioassay tissue had no effect on the ability of bradykinin to elicit relaxation. Open column, control. (b) When the bioassay tissues were perfused jointly with glucose-free Krebs containing 2-deoxy glucose (2-DOG, 20 mM, hatched column) first passed into the endothelial column, and by a separate circuit with normal glucose-containing Krebs solution, the relaxant effect of bradykinin was inhibited but that of glyceryl trinitrate (GTN, 10 nM) was unaffected. Open column, control. Values are the mean of 4–6 observations and vertical bars indicate the s.e.mean. *** $P < 0.001$, indicates a significant difference from control.

metabolism, the ATP content of pig aortic endothelial cells did not fall when incubated under hypoxic conditions (2.8% O_2). Under these conditions both basal and bradykinin-stimulated production of prostacyclin was identical to the obtained under normoxic conditions. Our data therefore differ from previous reports on bovine coronary artery and canine femoral artery where stimulation of prostanoid formation was obtained (Kalsner, 1977; Busse *et al.*, 1983), and on bovine cultured pulmonary artery endothelium where inhibition was obtained (Madden *et al*, 1986). Furthermore, in contrast to the stimulation of EDRF production induced by hypoxia in canine femoral artery (Bassenge *et al.*, 1988), or the inhibition induced in bovine pulmonary artery (Warren *et al.*, 1989), we found no effect on bradykinin-stimulated production of EDRF, as assessed by measuring the cyclic GMP content of porcine aortic endothelial cells. Basal production of EDRF, indicated by haemoglobin-sensitive cyclic GMP content in unstimulated cells (Martin *et al.*, 1988), the action of which is potentiated following treatment with superoxide dismutase (Gryglewski *et al.*, 1986; Rubanyi & Vanhoutte, 1986), was also unaffected under hypoxic conditions. Porcine aortic endothelial cells in culture do not therefore appear to be a suitable model with which to study hypoxia-induced vasodilatation or inhibition of EDRF production.

Why endothelial cells from rabbit freshly isolated aorta should utilize mainly oxidative metabolism (Griffith *et al.*, 1986) while porcine cultured aortic endothelial cells utilize glycolytic metabolism is unclear. One possibility is that in culture, where the availability of oxygen is lower than in arterial blood, cells may switch from oxidative to glycolytic metabolism and this warrants further investigation. It is clear, however, that endothelial cells in culture sustain damage when grown in high Po_2 (Ody & Junod, 1985) due to increased production of oxygen-derived free radicals. Alternatively, since glutaminolysis is an important energy source in the endothelium (Leighton *et al*, 1987), it is possible that removal from tissue culture medium containing 4 mM glutamine to Krebs solution containing none might induce a change in cellular metabolism.

In conclusion, our study supports the concept of Griffith *et al.* (1986) that ATP is required for agonist-induced production of EDRF. In contrast to rabbit isolated aorta which obtains ATP from oxidative metabolism (Griffith *et al.*, 1986), porcine aortic cells in culture derive this from glycolytic metabolism. This reliance on glycolytic rather than oxidative metabolism may explain why production of prostacyclin and EDRF by porcine aortic endothelial cells is insensitive to hypoxia.

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Relation between adrenergic neurogenic contraction and α_1-adrenoceptor subtypes in dog mesenteric and carotid arteries and rabbit carotid arteries

Ikunobu Muramatsu

Department of Pharmacology, Fukui Medical School, Matsuoka, Fukui 910-11, Japan

1 We examined the distribution of α_1-adrenoceptor subtypes and their relation to adrenergic neurogenic contraction induced by electrical transmural stimulation in the dog mesenteric and carotid arteries and the rabbit carotid artery.

2 In the dog mesenteric artery, contraction to noradrenaline was competitively inhibited by HV723 ($pK_B = 9.37$) and prazosin ($pK_B = 8.40$). Pretreatment with chlorethylclonidine (CEC) slightly attenuated only the contractions induced by low concentrations of noradrenaline. Contraction induced by electrical transmural stimulation was inhibited at lower concentrations of HV723 than of prazosin.

3 In the dog carotid artery, contraction to noradrenaline was inhibited with higher affinity by prazosin ($pK_B = 9.82$) than by HV723 ($pK_B = 8.47$). Prozosin was also more potent than HV723 in inhibiting the contraction to electrical stimulation. Pretreatment with CEC markedly attenuated or abolished contraction to noradrenaline and electrical stimulation.

4 In the rabbit carotid artery, prazosin inhibited noradrenaline-induced contraction biphasically ($pK_B = 9.91$ and 8.60). After CEC pretreatment, contraction to noradrenaline was attenuated moderately and the high affinity site for prazosin was abolished. HV723 competitively inhibited the noradrenaline response with a similar pK_B value (approximately 8.5) regardless of CEC treatment. Contraction to electrical stimulation was inhibited by prazosin more effectively than by HV723 in preparations not treated with CEC, while it was equipotently inhibited by both antagonists in CEC-treated preparations.

5 These results suggest that the contractions induced by endogenous and exogenous noradrenaline are mediated through the same subtypes of α_1-adrenoceptor distributed in each artery; according to our recent subclassification: α_{1N} subtype in the dog mesenteric artery, α_{1H} subtype in the dog carotid artery and α_{1H} and α_{1L} subtypes in the rabbit carotid artery. Different susceptibility to α_1-adrenoceptor antagonists of sympathetic adrenergic responses in various blood vessels may be related to heterogeneous involvement of distinct α_1-adrenoceptor subtypes in the sympathetic response.

Introduction

Blood vessels are innervated by sympathetic nerves and the muscle tonus is predominantly regulated through α_1-adrenoceptors by noradrenaline released from the nerve terminals (McGrath, 1982; Bülbring & Tomita, 1987). Recently, α_1-adrenoceptors of blood vessels have been classified into three subtypes (α_{1H}, α_{1L} and α_{1N}) by their affinity for α_1-adrenoceptor antagonists (prazosin, HV723) and by their susceptibility to chlorethylclonidine (CEC) (Muramatsu *et al.*, 1990b). The α_{1H}-subtype is the most sensitive to prazosin (pA_2: >9.5) and is selectively susceptible to CEC. The α_{1N}-subtype has a higher affinity for HV723 (pA_2: >9.0) than for prazosin (pA_2: 8.0–9.0), while the α_{1L}-subtype shows low affinity for both the α_1-adrenoceptor antagonists (pA_2: 8.0–9.0). These α_1-adrenoceptor subtypes may co-exist in a single tissue, involved in a contraction induced by exogenous α_1-adrenoceptor agonists (Holck *et al.*, 1983; Flavahan & Vanhoutte, 1986; Flavahan *et al.*, 1987; Muramatsu *et al.*, 1990a). However, it is uncertain which subtype (or subtypes) is activated by endogenous noradrenaline and is involved in adrenergic neurogenic contraction. The present study was carried out to demonstrate the relationship between α_1-adrenoceptor subtypes and sympathetic adrenergic contraction in three arteries (dog mesenteric and carotid arteries and rabbit carotid arteries), in which the noradrenaline-induced contraction has been reported to be caused predominantly through the postulated three different α_1-adrenoceptor subtypes (Muramatsu *et al.*, 1990b).

Methods

Mongrel dogs of either sex (7–15 kg) and male rabbits (2.5–4.0 kg) were used in the present experiments. After being killed under pentobarbitone anaesthesia, dog mesenteric and carotid arteries and rabbit carotid artery were isolated from the animals and cut helically under a dissecting microscope (Muramatsu *et al.*, 1990b). In order to avoid the possible involvement of endothelium-derived relaxing factor in the mechanical response (Furchgott, 1981), the endothelial cells of blood vessels were removed by rubbing them with filter paper. The functional loss of endothelial cells was confirmed by the loss of the relaxant response to acetylcholine (1 μM) in noradrenaline-precontracted arteries (Muramatsu, 1987). Each strip was mounted vertically in an organ bath containing 20 ml Krebs-Henseleit solution of the following composition (mM): NaCl 112, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 2, $NaHCO_3$ 25, $NaHPO_4$ 1.2 and glucose 11.5. The medium was maintained at 37°C, pH 7.4, and was equilibrated with a gas mixture consisting of 95% O_2 and 5% CO_2. The tension was recorded isometrically through a force-displacement transducer. The preparations were equilibrated for 90 min before starting the experiments.

Concentration-response curves for noradrenaline were obtained by adding the drug directly to the bathing media in cumulative concentrations. Desmethylimipramine (0.1 μM), deoxycorticosterone acetate (5 μM), and propranolol (3 μM) were present throughout this series of experiments in order to block neuronal and extraneuronal uptake of noradrenaline and to block β-adrenoceptors, respectively. The concentration-response curves were obtained 6 times in the same strip at 90 min intervals and the third concentration-response curve was used as control. In preliminary experiments, the reproducibility of the concentration-response curves obtained by the third to sixth trials in the absence of α-adrenoceptor antagonist was confirmed (Muramatsu *et al.*, 1990b). The reproducibility was also often checked in paired vehicle experiments. α-Adrenoceptor antagonists were present

for 30 min before and during the concentration-response curves were obtained. The concentrations of α-antagonist were chosen randomly but in sequence from a given concentration to higher concentrations in individual preparations. With chlorethylclonidine (CEC) treatment, the preparations were treated once for 20 min with CEC 50 μM and then washed with the drug-free solution.

The pK_B value was estimated according to Arunlakshana & Schild (1959). Briefly, the concentration of noradrenaline necessary to give a half-maximal contraction in the presence of α-adrenoceptor antagonist was divided by the concentration giving a half-maximal response in the control to determine the agonist concentration-ratio (CR). Data were plotted as the −log antagonist concentration (M) vs the log (CR − 1), and pA_2 values were calculated from Schild plots along mean slope and 95% confidence limits (95% CL) and straight lines were drawn by least square linear regression. When the straight line yielded a slope with unity, the pA_2 value estimated was represented as the pK_B (Arunlakshana & Schild, 1959). The pK_B value for α-adrenoceptor antagonist was also determined for single concentrations of antagonist by concentration-ratio method (Furchgott, 1972).

Electrical transmural stimulation was applied through a pair of platinum-wire electrodes at 10–15 min intervals (Muramatsu *et al.*, 1989). The preparation was placed in parallel between the electrodes. The distance between the electrodes was approximately 2 mm. Stimulus parameters were 0.3 ms duration, frequencies of 20 Hz and supramaximum voltage (10 V) for 10 s. In this series of experiments, DG-5128 (10 μM) and propranolol (1 μM) were added to the bath medium to block prejunctional α_2-adrenoceptors and postjunctional β-adrenoceptors, respectively (Muramatsu *et al.*, 1983; 1989). DG-5128 (10 μM) had no effect on the contractile response to noradrenaline in each preparation. In the experiments with the dog mesenteric artery, α,β-methylene ATP (10 μM) was present throughout the experiments in order to block the sympathetic purinergic component (Muramatsu *et al.*, 1989). No effect of α,β-methylene ATP on noradrenaline-induced contraction was confirmed in the dog mesenteric artery (Muramatsu, 1987).

Experimental values are given as a mean ± s.e.mean or means with 95% confidence limits. Results were analysed by Student's *t* test (unpaired or paired comparison) and a probability of less than 0.05 was considered significant.

Drugs used were: (−)-noradrenaline bitartrate, desmethylimipramine hydrochloride, α,β-methylene ATP (Sigma, St Louis, U.S.A.), deoxycorticosterone acetate, (±)-propranolol (Nacalai, Kyoto, Japan), prazosin (Taito-Pfizer, Tokyo, Japan), chlorethylclonidine dihydrochloride (CEC: Funakoshi, Tokyo, Japan), HV723 (Oshita *et al.*, 1988; α-ethyl-3,4,5- trimethoxy-α-(3- ((2-(2-methoxyphenoxy) ethyl)-amino)-propyl) benzeneacetonitrile fumarate, Hokuriku Seiyaku, Katsuyama, Fukui, Japan), tetrodotoxin (Sankyo, Tokyo, Japan), guanethidine sulphate (Tokyo-Kasei, Tokyo, Japan) and DG-5128 (2-(2-(4,5-dihydro- 1H-imidazol-2-yl)- 1-phenylethyl) pyridine dihydrochloride sesquihydrate, Daiichi Seiyaku, Tokyo, Japan).

Results

Effects of HV723 and prazosin on response to noradrenaline

Noradrenaline produced concentration-dependent contractions in the dog mesenteric and carotid arteries and the rabbit carotid arteries. The concentration-response curves were attenuated by HV723 and prazosin, resulting in a rightward displacement of the curves. Figure 1 shows the Schild plots in three tissues. The slopes of the Schild plots for HV723 were close to unity, indicating that HV723 competitively inhibited the contractile responses induced by noradrenaline in three tissues. The pK_B value for HV723 was 9.37 ± 0.06 in the dog mesenteric artery, which was approximately 1 log unit higher than those in the dog and rabbit carotid arteries (Table 1).

Prazosin also inhibited the noradrenaline response in competitive manner in the dog mesenteric and carotid arteries (Figure 1). The pK_B value for prazosin was greater in the carotid artery than in the mesenteric artery. On the other hand, Schild plots for prazosin in the rabbit carotid artery showed a deviation from a straight line (slope = 0.676, 95% CL = 0.592–0.761), suggesting that the inhibition by prazosin occurred biphasically. Therefore, two different pK_B values (9.91 ± 0.10 and 8.60 ± 0.14) for prazosin were estimated in the rabbit carotid artery (Table 1).

In the preparations pretreated with CEC (50 μM), the noradrenaline-induced contraction was attenuated. However, the extent of the attenuation varied between the tissues tested (Table 2). In the dog mesenteric artery, the contractile response to noradrenaline at concentrations less than a medium effective concentration was significantly reduced. In the dog carotid artery, CEC pretreatment abolished or markedly inhibited the responses to noradrenaline at all concentrations tested. On the other hand, the extent of inhibition in the rabbit carotid artery was intermediate between that in the dog mesenteric and carotid arteries.

HV723 and prazosin competitively inhibited the noradrenaline responses in the dog mesenteric and rabbit carotid arteries which had been pretreated with 50 μM CEC (Figure 1, open symbols), resulting in a pK_B value for an antagonist in each tissue (Table 1). Each value was approximately the same as that in CEC-untreated preparations (except for the high value in the rabbit carotid artery). The antagonist experiments were not carried out in the CEC-treated dog carotid artery

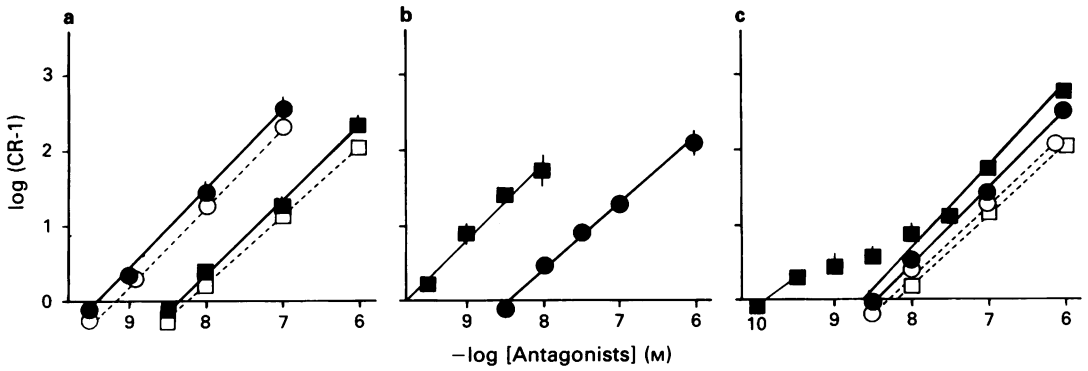

Figure 1 Schild plots for competitive inhibition of noradrenaline contractions by HV723 (circles) and prazosin (squares) in dog mesenteric artery (a), dog carotid artery (b) and rabbit carotid artery (c). Closed symbols, control; open symbols, chlorethylclonidine-treated tissues. Each point is the mean of data obtained from five to six preparations and vertical lines show s.e.mean. For pK_B values and slopes see Table 1.

Table 1 pK_B values and slope factors for HV723 and prazosin in the dog mesenteric and carotid arteries and rabbit carotid artery

	pK_B and slope (95% CL)			
	HV723		*Prazosin*	
	CEC-untreated	*CEC-treated*[a]	*CEC-untreated*	*CEC-treated*[a]
Dog mesenteric artery	9.37 ± 0.06 1.068 (0.978–1.158)	9.29 ± 0.05 0.997 (0.913–1.080)	8.40 ± 0.09 0.954 (0.827–1.082)	8.22 ± 0.05 0.919 (0.837–1.002)
Dog carotid artery	8.47 ± 0.09 0.870 (0.696–1.099)	—[b]	9.82 ± 0.12 1.016 (0.773–1.258)	—[b]
Rabbit carotid artery	8.50 ± 0.05 0.995 (0.921–1.070)	8.33 ± 0.04 0.934 (0.867–1.000)	9.91 ± 0.10[c]	
			8.60 ± 0.14 1.090 (0.920–1.260)	8.25 ± 0.10 0.912 (0.786–1.039)

[a] Experiments were carried out in the preparations which had been treated with 50 μM chlorethylclonidine (CEC) for 20 min beforehand and washed out repeatedly.
[b] Not examined.
[c] Determined from the inhibitory effects of 0.1 and 0.3 nM prazosin.

because of a marked reduction of noradrenaline-induced contraction after CEC-pretreatment (Table 2).

Effects of HV723 and prazosin on adrenergic nerve-mediated contraction

Electrical transmural stimulation at 20 Hz for 10 s produced transient contractions in the dog mesenteric artery (in the presence of 10 μM α,β-methylene ATP), dog and rabbit carotid arteries. These responses were abolished by guanethidine (3 μM) or tetrodotoxin (0.5 μM) ($n = 5$, in each drug and each artery). Pretreatment with 50 μM CEC in the presence of 10 μM DG-5128 and 1 μM propranolol attenuated the contractile response slightly in the dog mesenteric artery, moderately in the rabbit carotid artery and markedly in the dog carotid artery (Figure 2).

HV723 and prazosin inhibited the contractions induced by electrical stimulation in a concentration-dependent manner (Figure 3). In the dog mesenteric artery, the inhibition by HV723 was more potent than that by prazosin, and their inhibitory potencies were not influenced by CEC-pretreatment (Table 3). In the rabbit carotid artery, prazosin was more effective in inhibiting the neurogenic response than HV723 in CEC-untreated preparations, but the inhibition by both drugs was equipotent in CEC-treated arteries. In the CEC-untreated dog carotid artery, prazosin attenuated the contractile response to electrical stimulation at approximately 10 times lower concentrations than HV723.

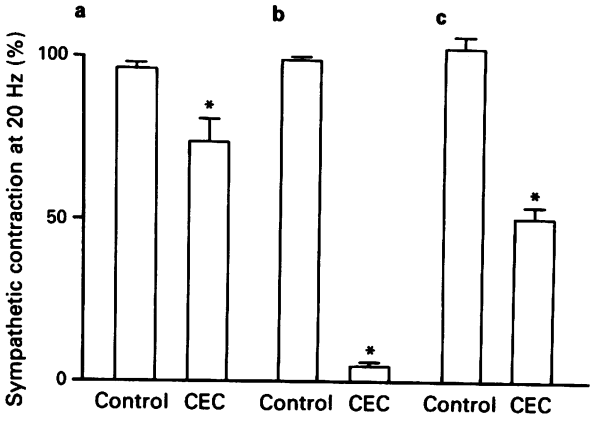

Figure 2 Effects of pretreatment with chlorethylclonidine (CEC) on sympathetic adrenergic contractions induced by electrical transmural stimulation in the dog mesenteric artery (a), dog carotid artery (b) and rabbit carotid artery (c). Contractile amplitude induced by electrical transmural stimulation (20 Hz, for 10 s) is represented as a percentage of the contraction before treatment with CEC (50 μM, 20 min). DG-5128 (10 μM) and propranolol (1 μM) were present throughout this series of experiments. α,β-Methylene ATP (10 μM) was also present in the experiments with dog mesenteric artery. * Significantly different from the time control (control) ($P < 0.05$, unpaired t test). Each value is the mean of 5–6 experiments with s.e.mean shown by vertical lines.

Discussion

As mentioned in the Introduction, α_1-adrenoceptors of blood vessels were recently subclassified into three subtypes (α_{1H}, α_{1L} and α_{1N}) based on the different affinities for α_1-adrenoceptor antagonists (prazosin and HV723) and on the susceptibility to CEC (Muramatsu *et al.*, 1990b). According to the criteria proposed, noradrenaline-induced contractions of the dog mesenteric and carotid arteries seem to be predominantly mediated

Table 2 Effects of chlorethylclonidine (CEC)-pretreatment on noradrenaline contraction in the dog mesenteric and carotid arteries and rabbit carotid artery

Tissue	*Noradrenaline concentration*	*% contraction*[a] *Before CEC*	*After CEC*	*P*[b]
Dog mesenteric artery	0.3 μM	48.8 ± 4.3	35.7 ± 3.8	<0.01
	10 μM	97.5 ± 1.8	94.8 ± 2.1	
Dog carotid artery	0.1 μM	46.0 ± 2.8	0.8 ± 0.4	<0.001
	10 μM	99.3 ± 0.3	24.8 ± 5.6	<0.001
Rabbit carotid artery	0.1 μM	48.4 ± 2.1	3.2 ± 0.9	<0.001
	10 μM	96.3 ± 0.4	88.8 ± 3.8	<0.1

[a] Relative contraction compared with a maximum contraction induced by 100 μM noradrenaline before pretreatment with 50 μM CEC.
[b] Comparison between the values before and after CEC-pretreatment (Student's t test, paired comparison).
Mean ± s.e. of 5 to 6 experiments.

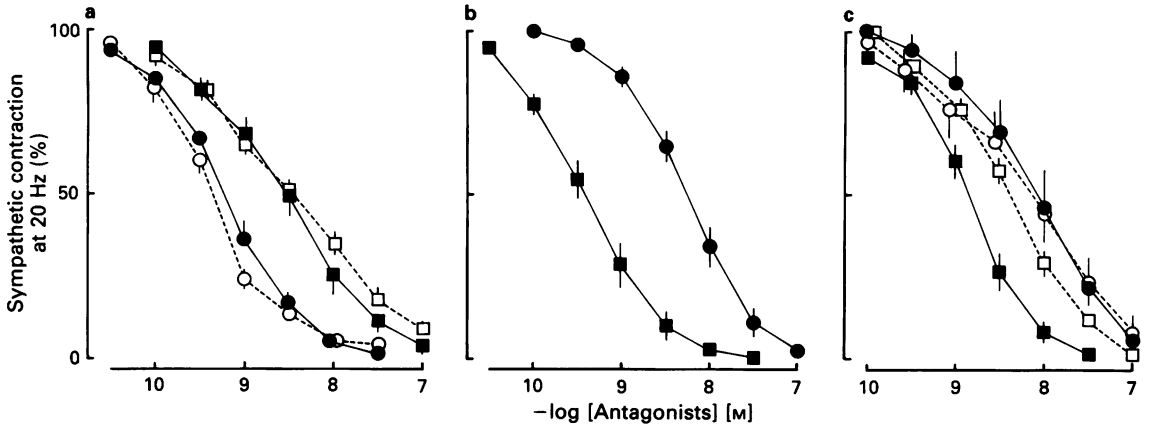

Figure 3 Concentration-response curves for HV723 (circles) and prazosin (squares) in inhibiting the sympathetic adrenergic contraction of the dog mesenteric artery (a), dog carotid artery (b) and rabbit carotid artery (c). Sympathetic adrenergic contraction was elicited by the application of electrical transmural stimulation (20 Hz, 10 s). The contractile amplitude before addition of HV723 or prazosin was taken as 100%. Closed symbols, control; open symbols, CEC-treated tissues. Each value is the mean of 5–6 experiments with s.e.mean shown by vertical lines.

through α_{1N} and α_{1H} subtypes, respectively, because of the high pK_B values for HV723 (9.37) in the mesenteric artery and for prazosin (9.82) in the carotid artery. However, a minor contribution of α_{1H} adrenoceptors cannot be ruled out in the mesenteric artery, where CEC, an inactivating drug of the α_{1H} subtype, slightly but significantly attenuated the contractions induced by low concentrations of noradrenaline. On the other hand, the response to noradrenaline in the rabbit carotid artery was biphasically inhibited by prazosin. Two distinct pK_B values for prazosin (9.91 and 8.60) and a low affinity for HV723 (8.50) suggest that the noradrenaline contraction is mediated through α_{1H} and α_{1L} subtypes in this artery. In fact, CEC selectively inactivated the high affinity site (α_{1H}) detected by prazosin. These results are reminiscent of our recent observations in the rabbit thoracic aorta where noradrenaline causes a contraction through both α_{1H} and α_{1L} subtypes (Muramatsu *et al.*, 1990a). Therefore, it is likely that exogenous noradrenaline acts on the co-existing α_1-adrenoceptor subtypes resulting in a contraction.

Which α_1-adrenoceptor subtypes are involved in the sympathetic contraction induced by endogenous noradrenaline? We analysed sympathetic adrenergic contractions elicited by electrical stimulation in three arteries. Prejunctional α_2-adrenoceptors were blocked by DG-5128 (Muramatsu *et al.*, 1983). The sympathetic purinergic component of the dog mesenteric artery was inhibited by desensitization of P_{2x}-purinoceptor with α,β-methylene ATP (Machaly *et al.*, 1988; Muramatsu *et al.*, 1989). Lack of effects of DG-5128 or α,β-methylene ATP on the responses to exogenous noradrenaline was confirmed. Therefore, under such conditions the contractions elicited by electrical stimulation seem to reflect the adrenergic component of the sympathetic response. In fact, the contractions were completely inhibited not only by tetrodotoxin or guanethidine but also by α_1-adrenoceptor antagonists, prazosin and HV723. However, the inhibitory potencies of prazosin and HV723 varied between the arteries tested and were differently affected by CEC.

In the mesenteric artery, HV723 was several times more potent than prazosin in inhibiting the adrenergic contraction, and the inhibitory potencies of both antagonists were not affected by CEC pretreatment. In contrast, sympathetic adrenergic contractions of the dog carotid artery were inhibited by prazosin with a higher affinity than HV723. Electrical transmural stimulation failed to cause a significant contraction in the CEC-pretreated carotid artery. These results suggest that the sympathetic adrenergic contractions of the dog mesenteric and carotid arteries are predominantly mediated through α_{1N} and α_{1H} subtypes, respectively.

The case of the rabbit carotid artery seems to be more complex. In preparations not treated with CEC, prazosin was an antagonist more potent than HV723. However, a half of the adrenergic contraction in amplitude was inhibited by CEC and the residual response was equipotently inhibited by prazosin and HV723. Therefore, it is likely that the sympathetic adrenergic contraction of the rabbit carotid artery is caused through α_{1H} and α_{1L} subtypes before CEC treatment but only through the α_{1L} subtype after CEC treatment.

In conclusion, the present pharmacological study confirms the presence of three distinct α_1-adrenoceptor subtypes in vascular smooth muscles and, at the same time, shows that such α_1-adrenoceptor subtypes are activated not only by exogenous but also by endogenous noradrenaline. Different sensitivity of sympathetic adrenergic responses to various α_1-adrenoceptor antagonists may reflect heterogeneous involvement of distinct α_1-adrenoceptor subtypes in the sympathetic response.

We thank N. Aoki for secretarial assistance. This work was supported in part by a grant from the Ministry of Education, Science and Culture, Japan and a grant from the Smoking Research Foundation of Japan.

Table 3 EC_{50} values for HV723 and prazosin in inhibiting the sympathetic contraction induced by electrical transmural stimulation

	EC_{50} (nM)			
	HV723		*Prazosin*	
Tissue	*CEC-untreated*	*CEC-treated*	*CEC-untreated*	*CEC-treated*
Dog mesenteric artery	0.53 ± 0.07	0.43 ± 0.05	3.80 ± 1.58[b]	2.98 ± 0.54[b]
Dog carotid artery	6.38 ± 1.41	—[a]	0.48 ± 0.14[b]	—[a]
Rabbit coronary artery	10.3 ± 3.04	9.92 ± 3.81	1.54 ± 0.33[b]	4.33 ± 0.77[c]

[a] Not examined.
[b] Significantly different from the corresponding value for HV723 ($P < 0.05$, unpaired comparison).
[c] Significantly different from the value for prazosin in CEC-untreated preparations ($P < 0.05$, unpaired comparison).
Mean ± s.e. of 5 to 6 experiments.

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Existence of two components in the tonic contraction of rat aorta mediated by α_1-adrenoceptor activation

Kanami Nishimura, Mikio Ota & [1]Katsuaki Ito

Department of Veterinary Pharmacology, Faculty of Agriculture, Miyazaki University, Miyazaki 889-21, Japan

1 The mechanisms involved in the contraction of rat aorta induced by the activation of α_1-adrenoceptors were studied. Phenylephrine induced a phasic contraction in the aorta incubated in Ca^{2+}-free medium containing 0.5 mM EGTA. Subsequent addition of Ca^{2+} induced a tonic contraction, which exhibited a stepwise development, an initial phase lasting 3 to 6 min (tonic-I) followed by a superimposing second phase (tonic-II).

2 2-Nitro-4-carboxyphenyl-N,N-diphenylcarbamate, which has been reported to inhibit phosphatidylinositol turnover, and H-7, a protein kinase C inhibitor, inhibited the tonic-I phase more effectively than the tonic-II phase. On the other hand, the tonic-II phase was more sensitive to nifedipine and cromakalim.

3 The rate of $^{45}Ca^{2+}$ influx during the tonic-I phase in phenylephrine-treated muscles was not different from that in untreated muscles, while that during the tonic-II phase was significantly greater. Nifedipine inhibited the increased $^{45}Ca^{2+}$ influx during the tonic-II phase, whereas H-7 did not affect the uptake during either phase.

4 These results suggest that the tonic contraction of rat aorta following α_1-adrenoceptor activation involves two different mechanisms: one is directly related to consequences of the polyphosphoinositide cascade, probably to protein kinase C, and the other dependent on Ca^{2+} influx through nifedipine-sensitive Ca^{2+} channels.

Introduction

The contraction of vascular smooth muscle induced by α_1-adrenoceptor activation has been believed to be caused by either Ca^{2+} release from the intracellular stores or Ca^{2+} influx through Ca^{2+} channels. Experimentally, α_1-adrenoceptor-mediated contraction can be separated into phasic and tonic components: the phasic component seems to depend on intracellularly stored Ca^{2+} while the tonic component depends on extracellular Ca^{2+}. The activation of the α_1-adrenoceptor is linked to the hydrolysis of polyphosphoinositide into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (Villalobos-Molina *et al.*, 1982; Hashimoto *et al.*, 1986; Rapoport, 1987). IP_3 can cause a Ca^{2+} release from sarcoplasmic reticulum and a contraction in vascular smooth muscle (Suematsu *et al.*, 1984; Somlyo *et al.*, 1985; Walker *et al.*, 1987). Therefore IP_3 could act as a second messenger responsible for α_1-adrenoceptor-mediated Ca^{2+} release and the phasic component (Hashimoto *et al.*, 1986; Rapoport, 1987). On the other hand, it is still unclear whether the tonic component depends entirely on Ca^{2+} influx, and how the component is related to polyphosphoinositide hydrolysis.

The sustained contraction of rat aorta following α_1-adrenoceptor activation is partially inhibited by Ca^{2+} channel blockers (Godfraind *et al.*, 1982; Beckeringh *et al.*, 1984; Chiu *et al.*, 1986; Bognar & Enero, 1988). The existence of a component in the contraction, which is resistant to Ca^{2+} channel blockers, provides two possibilities: (1) that this component does not require the enhanced Ca^{2+} entry, or (2) that Ca^{2+} entry through 'receptor-operated Ca^{2+} channels' which are insensitive to organic Ca^{2+} channel blockers (Bolton, 1979; Cauvin *et al.*, 1983) contributes to this contraction.

We here report that the tonic contraction due to α_1-adrenoceptor activation of rat aorta is composed of two components, one clearly related to the cascade of PI hydrolysis and the other due to Ca^{2+} influx through nifedipine-sensitive Ca^{2+} channels. Preliminary results of this study have been given elsewhere (Nishimura & Ito, 1989).

[1] Author for correspondence.

Methods

Tension experiments

Rectangular preparations, 1–2 mm wide and 5–6 mm long, were made by cutting open the thoracic aorta isolated from male rats older than 13 weeks of age (300–400 g). The endothelial layer was removed by gently rubbing the intimal surface with a cotton bud. The preparations were suspended in a Magnus bath containing 5 ml physiological saline solution (PSS) aerated with 95% O_2 and 5% CO_2 at 37°C and were equilibrated under a resting tension of 1 g for 1 h before the experiment. The isometric tension was measured by an isometric transducer (Nihon Kohden SB-1T) and recorded on a pen-writing recorder (Rikadenki R-14).

$^{45}Ca^{2+}$ influx experiments

The rate of $^{45}Ca^{2+}$ influx into rat aortic smooth muscle was measured by a cold lanthanum method (Karaki & Weiss, 1979) simultaneously with a tension recording. The thoracic aorta denuded of endothelium was cut into helical strips (4–5 mm in width, 25–30 mm in length and 8–12 mg wet weight) and suspended in a Magnus bath containing 10 ml PSS gassed with 95% O_2 and 5% CO_2 at 37°C. After the equilibration under a resting tension of 1 g for 1 h the isometric tension was recorded as mentioned above. The muscle was exposed to Ca^{2+}-free PSS for 15 min, then challenged by phenylephrine 1×10^{-6} M, and further 10 min later Ca^{2+} 2.5 mM was reintroduced. At an appropriate time after reintroduction of Ca^{2+}, the bath solution was changed to PSS containing $^{45}CaCl_2$ (1 μCi ml^{-1}, cold Ca^{2+} 2.5 mM) and phenylephrine (1×10^{-6} M) within 5 s. The change of solution did not affect the level of tension. As control, the same protocol was performed but without phenylephrine. After the incubation with $^{45}Ca^{2+}$ for 2 min, each preparation was quickly transferred to La^{3+} solution aerated with 100% O_2 at 0.5°C to displace extracellular $^{45}Ca^{2+}$ with La^{3+}. After 30 min washing in cold La^{3+} solution, the preparation was blotted between filter papers (Whatman No. 2) for 10 s under a weight of 3.0 g cm^{-2}, weighed and placed in a scintillation vial. The

muscle was solubilized by 0.5 ml Soluene-350 (Packard) overnight at room temperature. After the addition of 2 ml of scintillation cocktail (ACS-II) the radioactivity was counted in a liquid scintillation counter (Aloka LSC 903).

Solutions and drugs

PSS contained (mM): NaCl 136.8, $MgCl_2$ 0.2, $CaCl_2$ 2.5, KCl 5.4, $NaHCO_3$ 11.9 and glucose 5.5 (pH 7.2–7.4). for the Ca^{2+}-free PSS, $CaCl_2$ was omitted from PSS and 0.5 mM ethylene glycol *bis*(β-aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA) was added. Free Ca^{2+} concentration in the medium-containing EGTA was calculated with the programme of Fabiato & Fabiato (1979). La^{3+} solution for measurement of $^{45}Ca^{2+}$ influx had the following composition (mM): $LaCl_3$ 73.8, glucose 5.5 and Tris-HCl 24, and pH was adjusted to 6.8–6.9 to avoid the precipitation of lanthanum (H. Karaki, personal communication).

The drugs used were phenylephrine (Wako Pure Chemicals), 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate (NCDC, Sigma), cromakalim (Beecham), nifedipine (Sigma), verapamil (Eisai) and 1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride (H-7, Seikagaku Kogyo).

Statistics

All values are expressed as a mean ± s.e.mean (n = number of preparations). To test the effects of drugs on phenylephrine-induced contraction, at first the control response to phenylephrine was obtained, and then the response in the presence of test drug was obtained. The statistical significance was tested for paired data. In the $^{45}Ca^{2+}$ influx study the statistical significance was performed by non-paired t test. For both studies the significance was considered at the level of $P < 0.05$.

Results

Patterns of phenylephrine-induced contraction

In the presence of external Ca^{2+} (2.5 mM), phenylephrine 1×10^{-6} M, induced a contraction in which a phasic component and a tonic component fused but they could be distinguished by the rate of rise. The tonic contraction looked monophasic (Figure 1a). When phenylephrine and 2.5 mM Ca^{2+} were applied simultaneously after preincubation with Ca^{2+}-free PSS (with 0.5 mM EGTA) for more than 15 min, the contraction developed in a stepwise manner. After the rapid upstroke of phasic contraction, the first tonic phase (tonic-I) developed, which lasted 30 s to 4 min, and then the second tonic phase (tonic-II) was superimposed on it (Figure 1b). If the preincubation period with Ca^{2+}-free PSS was less than 15 min, the tonic contraction did not exhibit a biphasic development.

The biphasic tonic contraction was also observed with another protocol (Figure 1c). When phenylephrine was applied during the 15 min exposure to Ca^{2+}-free PSS containing 0.5 mM EGTA, the phasic component was distinctly observed. After the phasic contraction subsided, a small sustained contraction remained as long as the muscle was soaked in Ca^{2+}-free PSS. Subsequent readmission of 2.5 mM Ca^{2+} at 10 min after phenylephrine induced a stepwise development of tension. The initial phase of the tonic contraction (tonic-I) had an amplitude of 45–65% of maximum contraction, and 2–6 min later the second phase of the tonic contraction (tonic-II) superimposed on it. Often a rapid rising phase was distinguished at the beginning of tonic-I. With this protocol the separation of tonic contraction into two phases was observed even when the muscle was preincubated in Ca^{2+}-free PSS for only 1 min before addition of phenylephrine, although in this case tonic-II developed earlier (within 2 min after readmission of Ca^{2+}).

Sensitivity to Ca^{2+} channel blockers and cromakalim

To investigate whether the Ca^{2+} influx through the nifedipine-sensitive Ca^{2+} channel contributes to the tonic contraction, the effect of nifedipine on phenylephrine-induced tonic contraction was examined. As shown in Figure 2a, nifedipine (1×10^{-7} M) applied during the tonic-II phase decreased the tension close to the level of the tonic-I phase. On the other hand, when nifedipine (1×10^{-7} M) was applied before the addition of Ca^{2+}, the tonic-II phase did not appear on the tonic-I phase (Figure 2b). Figure 3a shows the effect of pretreatment with nifedipine, 1×10^{-7} M, on the time course of the tonic contraction. The inhibition by nifedipine was much greater on the later phase than on the early phase. Table 1 shows the dose-effect of nifedipine on phenylephrine-contraction. Since usually at 2 min after the addition of Ca^{2+} the tension stayed in the tonic-I phase and at 10 min the tension reached the steady state, the tension at 2 min may be considered as the tonic-I phase and that at 10 min indicates the sum of the tonic-I and tonic-II phases. Therefore it can be said that nifedipine at above 1×10^{-7} M selectively blocked the tonic-II phase. Another Ca^{2+} channel blocker, verapamil (1×10^{-6} M) also abolished the tonic-II phase while leaving the tonic-I phase unchanged.

The possibility was considered that the incubation of muscles in Ca^{2+}-free PSS containing EGTA for 25 min until the readmission of Ca^{2+} made the membrane abnormally leaky to Ca^{2+} and the Ca^{2+} channel insensitive to Ca^{2+} channel blockers (Ahn *et al.*, 1984; Guan *et al.*, 1988). We checked these points in two experiments. When Ca^{2+} (2.5 mM) was applied to muscles pre-incubated in Ca^{2+}-free PSS containing 0.5 mM EGTA for 30 min with no phenylephrine, the tension did not develop. This suggests that the membrane permeability to Ca^{2+} did not change much by treatment with EGTA. In other experiments, afer 15 min exposure to Ca^{2+}-free PSS containing 0.5 mM EGTA, KCl (20 mM) was added. Subsequent addition of Ca^{2+} (2.5 mM) (10 min later) induced a contraction, which was the same as that induced by 20 mM

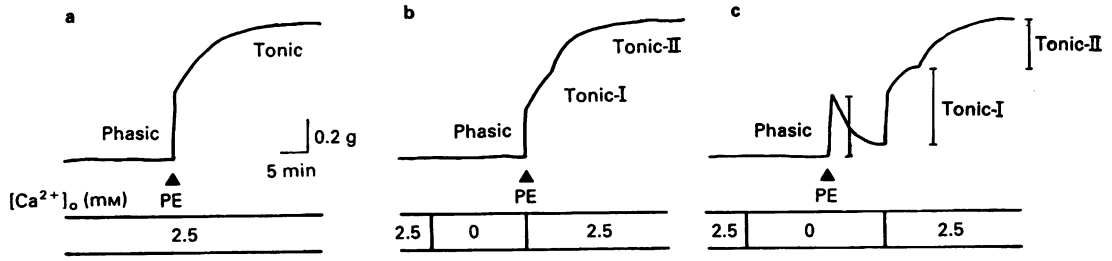

Figure 1 Typical examples of phenylephrine (PE)-induced contraction of rat aorta with different protocols. (a) A contraction induced by 10^{-6} M phenylephrine in normal (2.5 mM Ca^{2+}) PSS. (b) Two-step development of tonic contraction when phenylephrine and 2.5 mM Ca^{2+} were applied simultaneously to the muscle preincubated in Ca^{2+}-free PSS containing 0.5 mM EGTA for 15 min. (c) Separation of phenylephrine-induced contraction into phasic, tonic-I and tonic-II components. In this example, after the muscle was preincubated in Ca^{2+}-free PSS (with 0.5 mM EGTA) for 15 min, phenylephrine (1×10^{-6} M) was applied and 10 min later Ca^{2+} (2.5 mM) was added. This protocol was mainly used for the later experiments.

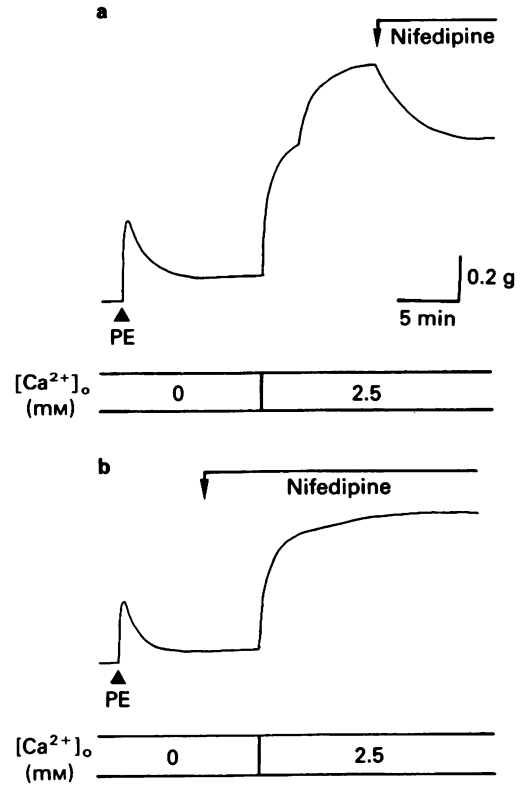

Figure 2 Effects of nifedipine on two components of tonic contraction due to phenylephrine in rat aorta. Phenylephrine (PE)-contraction was elicited by the protocol shown in Figure 1c. Nifedipine (10^{-7} M) was applied during the tonic-II phase (a) or 5 min before the addition of Ca^{2+} (b).

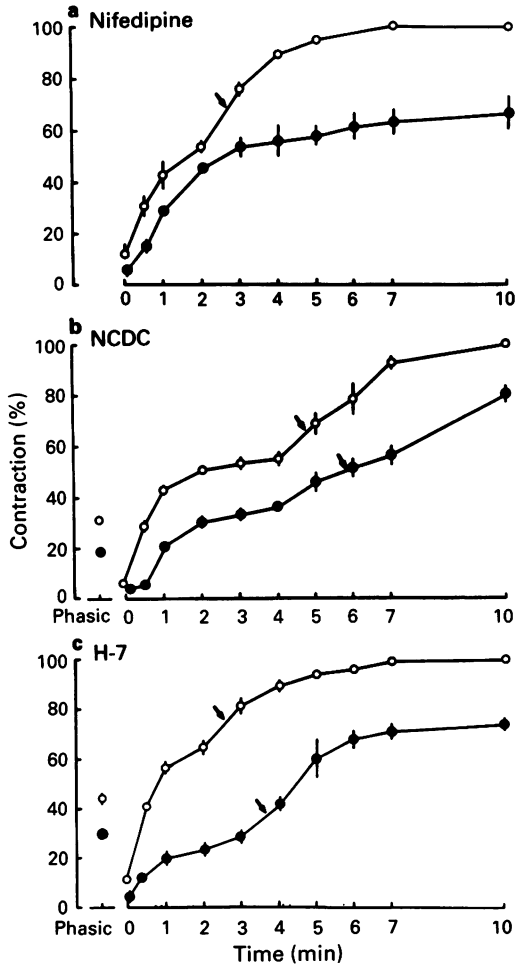

Figure 3 Effects of nifedipine, NCDC and H-7 on the time course of tonic contraction induced by phenylephrine in rat aorta. The protocol shown in Figure 1c was used. (a) Nifedipine 1×10^{-7} M ($n = 8$), (b) NCDC 3×10^{-5} M ($n = 12$), (c) H-7 1×10^{-5} M ($n = 10$). Nifedipine was applied 5 min before the readmission of Ca^{2+} (5 min after phenylephrine) while NCDC and H-7 were applied 15 min before the addition of phenylephrine (1×10^{-6} M). At 0 min Ca^{2+} (2.5 mM) was added. In each graph: (○) control and (●) presence of each drug. Each point represents the mean and vertical lines the s.e.mean. The arrow indicates average time of initiation of the tonic-II phase. With nifedipine, the tonic-II phase did not appear. Statistical significance tested for paired samples, which is not shown in figures, was observed for every point except 0 min for nifedipine and NCDC.

KCl in normal PSS and was abolished by pretreatment with nifedipine (1×10^{-7} M). Therefore it is unlikely that the procedure of incubation in Ca^{2+}-free, EGTA-containing PSS for up to 25 min made the permeability to Ca^{2+} abnormally high or made Ca^{2+} channels resistant to nifedipine.

Nifedipine and verapamil are known to block selectively voltage-dependent Ca^{2+} channels. The greater sensitivity of the tonic-II phase to these substances suggests that the change in membrane potential might be involved in this component. Although here we did not measure the membrane potential of the rat aorta, we tested the effect of cromakalim, which relaxes vascular smooth muscles by a hyperpolarizing action

Table 1 Effects of nifedipine, 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate (NCDC) and 1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride (H-7) on the phenylephrine-induced contraction

	Developed tension (%) 2 min		10 min		*Initiation of tonic-II* (min)		
	Control	*Treated*	*Control*	*Treated*	*Control*	*Treated*	n
Nifedipine							
3×10^{-8} M	56 ± 3	52 ± 6	100 ± 0	84 ± 4*	3.0 ± 0.3	2.7 ± 0.3	8
1×10^{-7} M	57 ± 2	46 ± 2*	100 ± 0	67 ± 7†	2.7 ± 0.2	—	8
3×10^{-7} M	64 ± 2	49 ± 2*	100 ± 0	63 ± 6†	2.5 ± 0.1	—	6
NCDC							
1×10^{-5} M	52 ± 3	39 ± 2†	100 ± 0	92 ± 0*	4.4 ± 0.4	6.0 ± 0.4*	8
3×10^{-5} M	51 ± 2	30 ± 1†	100 ± 0	79 ± 3†	4.7 ± 0.3	5.9 ± 0.3†	12
1×10^{-4} M	45 ± 1	20 ± 2†	100 ± 0	38 ± 4†	3.6 ± 0.3	—	8
H-7							
5×10^{-6} M	57 ± 3	20 ± 3†	100 ± 0	73 ± 4*	2.8 ± 0.2	4.8 ± 0.2†	7
1×10^{-5} M	66 ± 3	23 ± 2†	100 ± 0	74 ± 2*	2.7 ± 0.2	3.8 ± 0.1*	10
2×10^{-5} M	66 ± 3	7 ± 1†	100 ± 0	44 ± 2†	2.7 ± 0.2	4.8 ± 0.1†	6

The contraction was elicited with the protocol shown in Figure 1c. After the observation of control response each drug was applied in Ca^{2+}-free PSS 15 min before the addition of 1×10^{-6} M phenylephrine. The tension is expressed as the relative value of the tension development at 10 min after addition of 2.5 mM Ca^{2+} in control responses. With 1×10^{-7} and 3×10^{-7} M nifedipine, and 1×10^{-4} M NCDC the tonic-II did not appear. The data are mean ± s.e.mean. * $P < 0.05$, † $P < 0.01$ (paired t test).

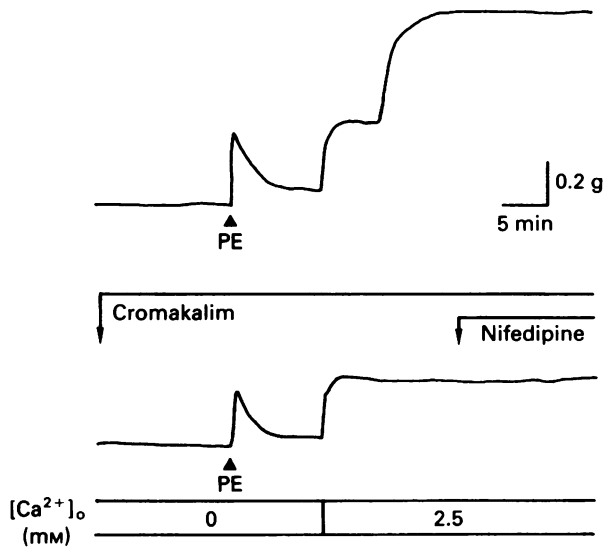

Figure 4 Effect of pretreatment with cromakalim on the development of the phenylephrine (PE)-induced tonic contraction in rat aorta. The protocol shown in Figure 1c was repeated twice for the same muscle. Upper trace, control response in the absence of cromakalim. Lower trace, cromakalim (3×10^{-7} M) was applied 10 min before the addition of phenylephrine. Fifteen min after the addition of Ca^{2+}, nifedipine (1×10^{-7} M) was applied.

(Hamilton *et al.*, 1986; Weir & Weston, 1986; Standen *et al.*, 1989). As shown in Figure 4, cromakalim (3×10^{-7} M) suppressed the tonic-II phase with a small depression of the tonic-I phase. Subsequent addition of nifedipine in the presence of cromakalim did not cause a further inhibition.

Sensitivity to various substances related to phosphoinositide cascade

To determine the dependence of each component of tonic contraction on polyphosphoinositide hydrolysis, the effects of substances which might affect phosphatidylinositol cascade were tested. We used NCDC (Walenga *et al.*, 1980; Nakaki *et al.*, 1985), which has been reported to inhibit phospholipase C, an enzyme that catalyzes phosphatidylinositol 4,5-bisphosphate into IP_3 and diacylglycerol. NCDC was applied 15 min before phenylephrine. Figure 3b shows the effect of NCDC (3×10^{-5} M) on the development of the tonic contraction, and Table 1 summarizes the dose-related effects. Unlike nifedipine, NCDC (1×10^{-5}–1×10^{-4} M) decreased both the early and later phases of the contraction to a similar extent. This means that the decrease of the later phase could result from the inhibition of the tonic-I phase. With 1×10^{-5} and 3×10^{-5} M NCDC, the muscle exhibited a two step development of tonic contraction while the initiation of the tonic-II phase was delayed. With 1×10^{-4} M NCDC, the tonic contraction became monophasic in 6 of the 8 preparations. On the other hand, NCDC at 1×10^{-5} M did not affect the contraction induced by 60 mM KCl in normal PSS, and at 3×10^{-5} M or 1×10^{-4} M it decreased the contraction by $4 \pm 1\%$ ($n = 4$) or $19 \pm 2\%$ ($n = 4$), respectively.

Polyphosphoinositide hydrolysis following α_1-adrenoceptor activation produces diacylglycerol, which activates protein kinase C (C-kinase) (Nishizuka, 1984). Therefore C-kinase can be involved in the phenylephrine-induced tonic contraction. Hidaka *et al.* (1984) reported that H-7 inhibited C-kinase in various tissues, and Khalil & Van Breeman (1988) used this substance to inhibit the enzyme in vascular smooth muscle activated by a phorbol ester or phenylephrine. Here the effect of H-7 on phenylephrine-contraction was tested with the protocol of Figure 1c to see whether C-kinase played a role in the phenylephrine-tonic contraction. Figure 5 shows examples of phenylephrine-contraction in the presence of 1×10^{-5} and 2×10^{-5} M H-7. H-7 decreased the phasic component due to

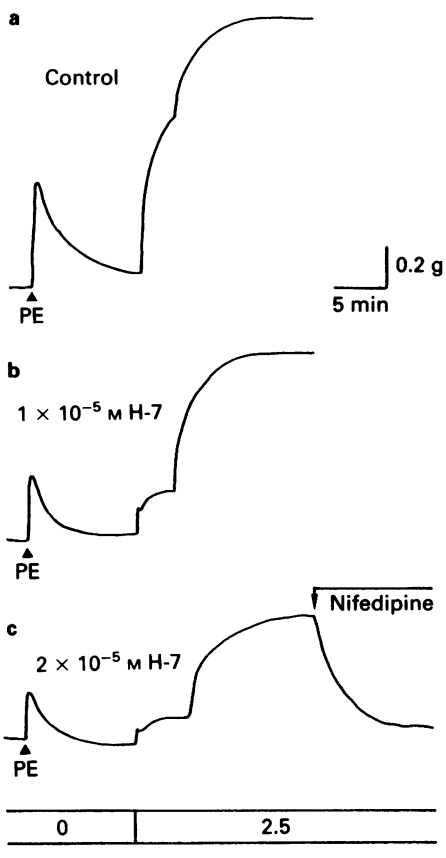

Figure 5 Effect of H-7 on the phenylephrine-induced tonic contraction. The protocol shown in Figure 1c was adapted: (a) the control contraction due to phenylephrine (PE) applied in Ca^{2+}-free PSS and the subsequent addition of Ca^{2+} at 10 min; (b) and (c) H-7 (1×10^{-5}, 2×10^{-5} M) was added 15 min before the application of phenylephrine. In (c), nifedipine 1×10^{-7} M was applied when the tension reached the plateau in the presence of H-7 2×10^{-5} M.

phenylephrine and abolished the residual tonic response in Ca^{2+}-free PSS, consistent with the data on rabbit aorta obtained by Khalil & Van Breemen (1988). In addition, H-7 decreased the amplitude of the tonic-I phase and delayed the onset of the tonic-II phase. The data shown in Figure 3c and Table 1 suggest that the inhibition of the contraction by H-7 mainly resulted from the inhibition of the tonic-I phase rather than the tonic-II phase. On the other hand, the inhibition of the 60 mM KCl-induced contraction in normal PSS by H-7 was $2 \pm 1\%$, $6 \pm 1\%$ and $18 \pm 2\%$ at 5×10^{-6} M, 1×10^{-5} M and 2×10^{-5} M ($n = 4$ for each concentration), respectively.

The effects of nifedipine and H-7 on phenylephrine-induced contraction were additive. The addition of nifedipine (1×10^{-7} M) at the plateau of the tonic-II phase pretreated with H-7 (2×10^{-5} M) decreased the tension close to the original level (Figure 5). When a contraction was elicited by phenylephrine (1×10^{-6} M) in normal PSS, the pretreatment with nifedipine (1×10^{-7} M) inhibited the contraction by $41 \pm 3\%$ ($n = 9$) while H-7 inhibited it by $49 \pm 4\%$ ($n = 7$). The presence of both compounds almost completely inhibited the phenylephrine-contraction (inhibition; $96 \pm 2\%$, $n = 6$). Thus it seems that phenylephrine-contraction involves nifedipine-sensitive and H-7-sensitive components.

$^{45}Ca^{2+}$ influx

To determine the relationship between both phases of phenylephrine-induced tonic contraction and the Ca^{2+} influx from the extracellular fluid we examined the rate of $^{45}Ca^{2+}$ influx. To correlate the $^{45}Ca^{2+}$ influx to contraction, the change of tension was recorded for every preparation. The rate of $^{45}Ca^{2+}$ influx was measured only for the strip which

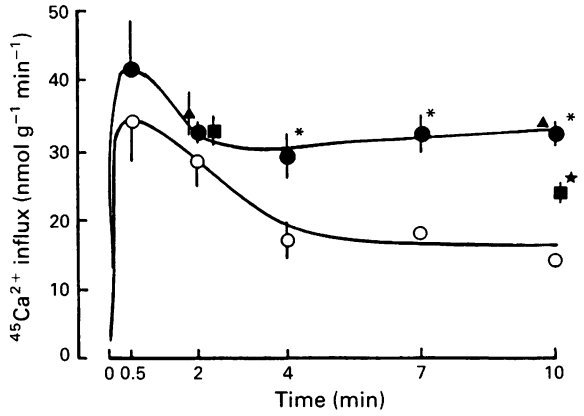

Figure 6 The rate of $^{45}Ca^{2+}$ influx during the tonic-I and tonic-II components of phenylephrine-induced contraction. The same protocol as in Figure 1c was adapted. At 0 min 2.5 mM Ca^{2+} was added to control muscles (○) or phenylephrine-pretreated muscles (●). At the desired time PSS was changed to $^{45}Ca^{2+}$-containing PSS for 2 min and then the muscles were transferred to cold La^{3+} solution. (▲) The $^{45}Ca^{2+}$ influx in the presence of phenylephrine and H-7 1×10^{-5} M; (■) the $^{45}Ca^{2+}$ influx in the presence of phenylephrine and nifedipine 1×10^{-7} M. * Significantly different from control ($P < 0.05$). Each point represents a mean of 8 preparations with s.e.mean shown by vertical lines. * Significantly different from the influx in phenylephrine-treated group.

showed two step development of tonic contraction with the regular protocol (Figure 1c). Here we found that the tonic-I phase lasted 2–3 min in this experimental system and the tonic-II phase reached the steady state within 3 min from the initiation of the phase. After the tension response to phenylephrine was observed without $^{45}Ca^{2+}$, the same protocol was repeated for $^{45}Ca^{2+}$ influx measurement. After 0.5, 2, 4, 7 and 10 min application of 2.5 mM Ca^{2+} to control and phenylephrine-treated muscles the medium was changed to $^{45}Ca^{2+}$-containing medium and 2 min later the muscle was quickly transferred to La^{3+} solution to remove extracellular $^{45}Ca^{2+}$ (Figure 6). The rate of $^{45}Ca^{2+}$ influx was greatest at 0.5 min in both control- and phenylephrine-treated muscles. This suggests that the Ca^{2+} entry is more rapid when Ca^{2+} is entering the cells for replenishment of Ca^{2+} stores than when the cellular Ca^{2+} distribution is close to equilibrium. There was no difference in the rate of $^{45}Ca^{2+}$ influx between control and phenylephrine-treated groups at 0.5 and 2 min, although tension developed in phenylephrine-treated muscles but not in control muscles. On the other hand, the rate of the influx was significantly greater in the phenylephrine-treated group than in the control at 4, 7 and 10 min, which corresponded to the tonic-II phase.

The rate of $^{45}Ca^{2+}$ influx at 2 min of application of Ca^{2+} was the same as that at 10 min in the phenylephrine-treated group. The effects of nifedipine (1×10^{-7} M) and H-7 (1×10^{-5} M) on the rate of $^{45}Ca^{2+}$ influx at 2 and 10 min were examined. Nifedipine did not affect the influx at 2 min but significantly decreased it at 10 min. H-7 did not affect the influx during either phase (Figure 6).

Discussion

By preincubation of the rat aorta in Ca^{2+}-free PSS containing 0.5 mM EGTA, the α_1-adrenoceptor-mediated contraction was divided into at least three components, a phasic component and two components of tonic contraction. In addition, a small residual contraction persisting in the absence of extracellular Ca^{2+} may be another component. The phasic contraction may be due to Ca^{2+} release from sarcoplasmic reticulum and IP_3 may be a second messenger responsible for this (Hashimoto *et al.*, 1986; Rapoport, 1987). Each phase of tonic contraction following the addition of Ca^{2+} showed pharmacologically different responsiveness to various substances, i.e., the tonic-I phase was more sensitive to NCDC and H-7 than the tonic-II phase, whereas the tonic-II phase was more sensitive to nifedipine, verapamil and cromakalim than the other. This suggests that the two phases depend on different mechanisms.

The contraction induced by phenylephrine in normal PSS was partially sensitive to either H-7 or nifedipine, and it was almost completely abolished when both agents were present. Therefore the tonic contraction due to α_1-receptor activation in normal medium consists of two different phases which appear separately by use of the present protocol. Although at present we do not have sufficient data to explain this separation, the protocol used in this study could be useful in investigating the mechanisms involved in the α_1-receptor-mediated contraction.

Before interpreting the data we must consider the specificity of substances used in this study which might affect polyphosphoinositide hydrolysis and C-kinase. Whether NCDC has an action other than an inhibition of phospholipase C is not clear. In this study, NCDC at 1×10^{-4} M considerably decreased the contraction induced by high KCl. Therefore, some non-specific action may be at least partly involved in the inhibition of phenylephrine-contraction by NCDC when the highest concentration was used. H-7 does not selectively inhibit C-kinase (K_i 6.0 μM); it also inhibits cyclic AMP-dependent protein kinase (A-kinase, K_i 3.0 μM), guanosine 3′:5′-cyclic monophosphate (cyclic GMP)-dependent protein kinase (G-kinase, K_i 5.8 μM) and myosin light chain kinase at a high concentration (K_i 97 μM; Hidaka *et al.*, 1984). However, because A-kinase and G-kinase are responsible for the relaxation of vascular smooth muscle rather than its contraction (Rüegg & Paul, 1982; Ignarro & Kadowitz, 1985), it is difficult to suppose that the inhibition of A-kinase or G-kinase by H-7 causes the depression of contraction. At the highest concentration (2×10^{-5} M) H-7 decreased the KCl-induced contraction by 17.6%. This could be ascribed to the inhibition of myosin light chain kinase and this effect might be partly involved in the inhibition of all phases of phenylephrine-contraction at that concentration. Since the inhibition by H-7 of KCl-contraction was much less at lower concentrations, the major cause of inhibition by this agent of tonic-I could be ascribed to inhibition on C-kinase.

Overall, we can say that at the submaximal concentrations used in this study, NCDC or H-7 inhibited the tonic-I phase mainly through an effect on phospholipase C or C-kinase, respectively. Although these effects may have caused the inhibition of the tonic-II phase when the highest concentrations were used, these effects must be less or indirect as compared to the effects on the tonic-I phase.

On the other hand, although the tonic-II phase was inhibited by Ca^{2+} channel blockers the tonic-I phase was not. The resistance of the tonic-I phase to nifedipine was not due to alterations of Ca^{2+} channels following treatment with EGTA. In some studies α_1-adrenoceptor-mediated contraction of rat aorta was partially resistant to Ca^{2+} channel blockers (Beckeringh *et al.*, 1984; Chiu *et al.*, 1986; Bognar & Enero, 1988). The tonic-I and tonic-II phases may correspond to the Ca^{2+} channel blocker-resistant and -sensitive parts of the contraction, respectively.

We measured the Ca^{2+} uptake by the short (2 min) application of $^{45}Ca^{2+}$ at the desired time during the tonic contraction. This procedure would minimize the $^{45}Ca^{2+}$ efflux, hence the increased $^{45}Ca^{2+}$ uptake may represent the unidirectional Ca^{2+} influx (Van Breeman *et al.*, 1985). The rate of $^{45}Ca^{2+}$ influx during the tonic-II phase was significantly greater in phenylephrine-treated muscles than in the control ones. The additional Ca^{2+} influx during this period may contribute to the formation of the tonic-II phase which is sensitive to nifedipine. On the contrary, the rate of $^{45}Ca^{2+}$ influx during the tonic-I phase in phenylephrine-treated muscles was not different from that in the controls, but a contraction appeared

only in phenylephrine-treated muscles. Furthermore, the inhibition of the tonic-I phase by H-7 did not accompany the inhibition of $^{45}Ca^{2+}$ influx. These findings indicate that not only the Ca^{2+} influx but also other mechanisms are involved in the initiation of the tonic-I phase and this mechanism is likely to be associated with C-kinase.

Among the many reports that C-kinase activation induces contraction of vascular smooth muscles, some workers showed that phorbol esters, well-known C-kinase activators, induced a contraction even when a muscle was incubated in Ca^{2+}-free medium (Gleason & Flaim, 1986; Chiu *et al.*, 1987; Sawamura *et al.*, 1987) or when intracellular Ca^{2+} did not increase (Jiang & Morgan, 1987; Litten *et al.*, 1987). Besides, Itoh *et al.* (1986, 1988) reported that a phorbol ester could induce a contraction of skinned vascular smooth muscles by enhancing the Ca^{2+} sensitivity of the contractile protein. Therefore, the activation of C-kinase can cause contraction before the Ca^{2+} channels open.

H-7 suppressed the small residual contraction in Ca^{2+}-free PSS. This small contraction might not be due to Ca^{2+} release (Ito *et al.*, 1986) but result from C-kinase activation (Khalil & Van Breemen, 1988). Since C-kinase activity depends on the concentration of Ca^{2+} (Nishizuka, 1984), the readmission of Ca^{2+} to a muscle pre-exposed to Ca^{2+}-free PSS would enhance C-kinase if diacylglycerol is present, and consequently potentiate the residual contraction to the level of the tonic-I phase. H-7 also decreased the phasic contraction at all concentrations. This inhibition could result from the inhibition of the residual contraction which underlay the phasic contraction.

As well as nifedipine, cromakalim inhibited the tonic-II phase. In the presence of cromakalim the addition of nifedipine did not cause a further inhibition, indicating that the nifedipine-sensitive component equals the cromakalim-sensitive one. Although we did not measure the membrane potential, the data suggest that a depolarization occurred during the tonic-II phase and that it was responsible for the opening of the voltage-dependent Ca^{2+} channel. A question is whether some event(s) in phosphatidylinositol cascade is associated with the change in membrane potential and/or the Ca^{2+} channel opening. Phorbol esters were reported to increase Ca^{2+} influx at concentrations higher than the threshold to induce contraction (Gleason & Flaim, 1986; Litten *et al.*, 1987). Therefore, the relation of C-kinase to nifedipine-sensitive Ca^{2+} channels may be important. Further studies are needed to clarify the role of the phosphatidylinositol cascade in Ca^{2+} channel opening.

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Modulation of the hypothermic and hyperglycaemic effects of 8-OH-DPAT by a_2-adrenoceptor antagonists

[1]Michael J. Durcan, Krystyna M. Wozniak & Markku Linnoila

Laboratory of Clinical Studies, DICBR, National Institute on Alcohol Abuse and Alcoholism, Bldg 10 3C102, 9000 Rockville Pike, Bethesda, MD 20892, U.S.A.

1 The effects of pretreatment with two novel and relatively specific α_2-adrenoceptor antagonists on the hypothermic and hyperglycaemic responses induced by the 5-HT_{1A} receptor agonist 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) were investigated in mice. The α_2-adrenoceptor antagonists used were, atipamezole, which occupies both central and peripheral receptors, and L 659,066, which poorly penetrates the blood brain barrier.

2 Atipamezole (1 and 3 mg kg^{-1}) alone had no effect on body temperature but significantly attenuated the 8-OH-DPAT-induced hypothermic response. The hyperglycaemic effect of 8-OH-DPAT was also attenuated by pretreatment with atipamezole; however, 3 mg kg^{-1} atipamezole did cause some hypoglycaemia when administered alone.

3 Pretreatment with L 659,066 (3–30 mg kg^{-1}) failed to alter the hypothermic effects of 8-OH-DPAT. All doses of L 659,066 tested attenuated 8-OH-DPAT-induced hyperglycaemia, but the highest dose (30 mg kg^{-1}) produced hypoglycaemia when administered alone.

4 The results suggest that the attenuation of 8-OH-DPAT-induced hypothermia by α_2-adrenoceptor antagonists may be centrally mediated whereas the blockade of 8-OH-DPAT-induced hyperglycaemia may involve peripheral mechanisms.

Introduction

8-Hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) is a relatively selective centrally acting 5-hydroxytryptamine (5-HT) agonist (Hjorth *et al.*, 1982), which has been shown to have a high affinity for the 5-HT_{1A} receptor subtype (Middlemiss & Fozard, 1983). In rodents, 8-OH-DPAT produces both hypothermia and hyperglycaemia (Hjorth, 1985; Goodwin *et al.*, 1985; Middlemiss *et al.*, 1985; Gudelsky *et al.*, 1986; Chaouloff & Jeanrenaud, 1987; Wozniak *et al.*, 1988), although the precise mechanism of action of 8-OH-DPAT on body temperature and blood glucose levels is, at present, not fully understood. In addition to its 5-HT agonist properties, 8-OH-DPAT is reported to have α_2-adrenoceptor antagonist properties (Crist & Suprenant, 1987) and it can generalize to the α_2-adrenoceptor antagonist yohimbine in a drug discrimination task (Winter, 1988). Additionally, 8-OH-DPAT has reported activity at dopamine D_2 receptors (Smith & Cutts, 1989; Bull *et al.*, 1990). The relatively selective α_2-adrenoceptor antagonist, idazoxan, can however, block the decrease in extracellular 5-hydroxy-indoleacetic acid (5-HIAA) produced by 8-OH-DPAT and also reverse 8-OH-DPAT-induced hyperglycaemia (Chaouloff & Jeanrenaud, 1987). In this study the effects of pretreatment with the novel and relatively specific α_2-adrenoceptor antagonists atipamezole (Scheinin *et al.*, 1988) and L 659,066 (Clineschmidt *et al.*, 1988) on 8-OH-DPAT-induced responses were investigated in mice. Atipamezole occupies both central and peripheral receptors, whereas L 659,066 poorly penetrates the blood/brain barrier. Therefore, these compounds enabled differentiating central from peripheral effects of α_2-adrenoceptor antagonism on 8-OH-DPAT-induced phenomena.

Methods

Naïve NIH Swiss male mice housed in groups of 5 on a 12 h light:12 h dark cycle with food and water available *ad libitum* were used in all experiments. The mice weighed between 21 and 25 g at the time of testing.

[1] Author for correspondence.

Temperature recording

Core body temperatures were measured with a rectal probe and a digital thermometer (Sensortek Inc.). The probe was inserted 2.5 cm into the rectum of each mouse.

Blood glucose determinations

Blood glucose determinations were made from blood drawn from the tail tip and the measurements made with a Glucometer II reflectance photometer (Ames Division, Miles Laboratories, Inc., Yellow Springs, OH, U.S.A.).

Drug administration

The drugs used in these experiments were 8-OH-DPAT (Research Biochemicals Inc, Natik, MA, U.S.A.), atipamezole (Farmos Group Ltd., Turku, Finland), and L 659,066 ((2R-trans)-N-(2-(1,3,4,6,7,12b-hexahydro-2'-oxospiro(2H-benzofuro(2,3-a)quinolizine-2,3-imidazolin)-3'-yl)ethyl)methanesulphonamide monohydrochloride, MK 912) (Merck, Sharp and Dohme, West Point, PA, U.S.A.).

In Experiment 1 groups of mice (n = 12–14) were given either 1 or 3 mg kg^{-1} atipamezole dissolved in distilled water vehicle (i.p.) or vehicle alone immediately following baseline temperature and blood glucose determination. Twenty minutes later body temperature and blood glucose levels were again measured and subsequently followed by the administration of 0.25 mg kg^{-1} 8-OH-DPAT or saline vehicle (s.c.). Three additional body temperature and blood glucose measurements were performed at 20 min intervals. All injections were administered at a volume of 10 ml kg^{-1}.

In Experiment 2 groups of mice (n = 8–11) were administered 3, 10 or 30 mg kg^{-1} L 659,066 dissolved in distilled water vehicle (i.p.) or vehicle alone immediately following the measurement of a baseline temperature and blood glucose. Twenty minutes later body temperature and blood glucose level were again measured and subsequently followed by the administration of 0.25 mg kg^{-1} 8-OH-DPAT or saline vehicle (s.c.). An additional group of animals received pretreatment with 3 mg kg^{-1} atipamezole (i.p.) followed 20 min later by 0.25 mg kg^{-1} 8-OH-DPAT (s.c.) for the purposes of comparison. Three further body temperature and blood glucose mea-

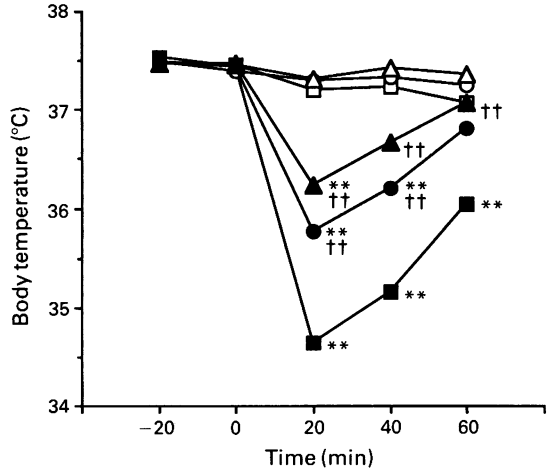

Figure 1 Mean body temperatures of groups of animals pretreated with 1–3 mg kg^{-1} atipamezole 20 min before 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) or vehicle treatment. (□) Vehicle + vehicle; (■) vehicle + 8-OH-DPAT; (○) 1 mg kg^{-1} atipamezole + vehicle; (●) 1 mg kg^{-1} atipamezole + 8-OH-DPAT; (△) 3 mg kg^{-1} atipamezole + vehicle; (▲) 3 mg kg^{-1} atipamezole + 8-OH-DPAT. All s.e.means < 0.3°C. ** $P < 0.01$ vs. vehicle-only treated animals at the same time point; †† $P < 0.01$ vs. vehicle + 8-OH-DPAT treated animals at the same time point.

surements were performed at 20 min intervals. All injections were administered at a volume of 10 ml kg^{-1}.

Statistics

The data were analyzed by a repeated measures analysis of variance with difference between means compared by *post hoc* Tukey tests.

Results

In Experiment 1, 8-OH-DPAT caused a significant reduction in body temperature ($P < 0.01$) when administered alone, whereas atipamezole alone had no effect on body temperature (Figure 1). The 8-OH-DPAT temperature reduction was maximal at 20 min after its administration, decreasing thereafter. This 8-OH-DPAT-induced hypothermia was significantly ($P < 0.01$) attenuated by both doses of atipamezole at each time point post 8-OH-DPAT administration.

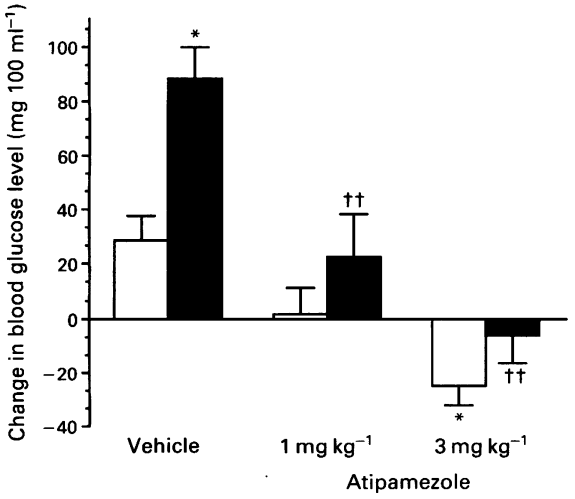

Figure 2 Mean change in blood glucose levels from initial baseline levels of animals pretreated with 1–3 mg kg^{-1} atipamezole before treatment with either vehicle or 8-hydroxy-2(di-n-propylamino)tetralin (8-OH-DPAT); data points shown are for 40 min post 8-OH-DPAT administration (a similar pattern of results is seen at both 20 and 60 min post 8-OH-DPAT, data not shown). Open columns, pretreatment + vehicle; solid columns, pretreatment + 8-OH-DPAT. * $P < 0.05$ vs. vehicle only treated animals; †† $P < 0.01$ vs. vehicle + 8-OH-DPAT-treated animals.

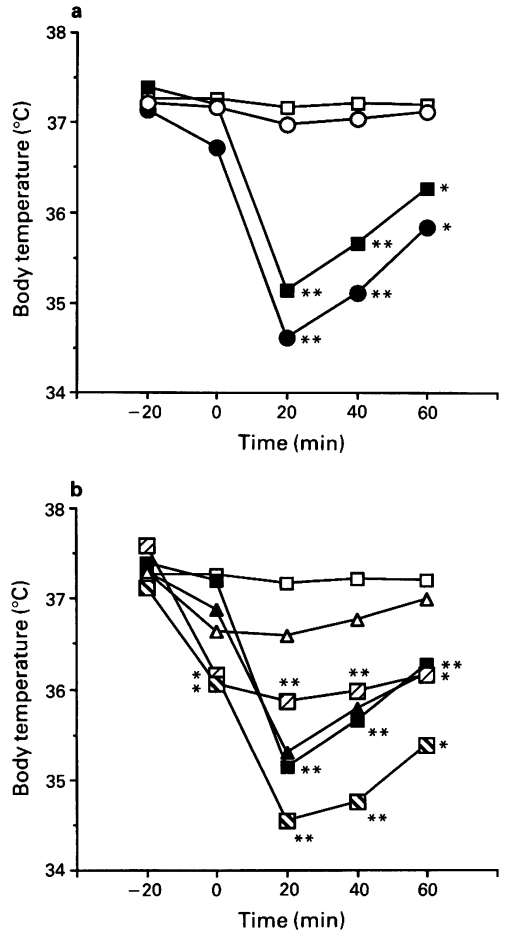

Figure 3 Mean body temperatures of groups of animals pretreated with 3–30 mg kg^{-1} L 659,066 before 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) or vehicle treatment. To enhance clarity the pretreatments with 3 mg kg^{-1} L 659,066 and atipamezole are shown in (a) the pretreatments with 10 and 30 mg kg^{-1} L 659,066 are shown in (b); the vehicle only and vehicle + 8-OH-DPAT groups are the same in both graphs. All s.e.means < 0.3°C. (□) Vehicle + vehicle; (■) vehicle + 8-OH-DPAT; (○) 3 mg kg^{-1} L 659,066 + vehicle; (●) 3 mg kg^{-1} L 659,066 + 8-OH-DPAT; (△) 10 mg kg^{-1} L 659,066 + vehicle; (▲) 10 mg kg^{-1} L 659,066 + vehicle; (▨) 30 mg kg^{-1} L 659,066 + vehicle; (▩) 30 mg kg^{-1} L 659,066 + 8-OH-DPAT. * $P < 0.05$; ** $P < 0.01$ vs. vehicle only treated animals at the same time point.

The basal blood glucose levels in mice before any treatment were in the range 160–180 mg 100 ml^{-1}. 8-OH-DPAT induced a significant ($P < 0.01$) increase in blood glucose level, although blood glucose levels in the vehicle-treated controls were also moderately elevated; in consequence, the change in glucose level from the initial baseline was analyzed (Figure 2). Pretreatment with atipamezole alone tended to reduce or reverse the increase in blood glucose seen in vehicle-treated controls, with the 3 mg kg^{-1} dose causing significant ($P < 0.01$) hypoglycaemia. Both doses of atipamezole significantly ($P < 0.01$) blocked the 8-OH-DPAT-induced increases in blood glucose levels at each time point.

In Experiment 2, L 659,066 (3 and 10 mg kg^{-1}) had no intrinsic effect on body temperature; however, the highest dose tested (30 mg kg^{-1}) did significantly ($P < 0.05$) reduce core temperature compared to vehicle-treated control values at all time points (Figure 3). The hypothermic effect of 8-OH-DPAT was not significantly affected by pretreatment with any dose of L 659,066, although pretreatment with 3 mg kg^{-1} ati-

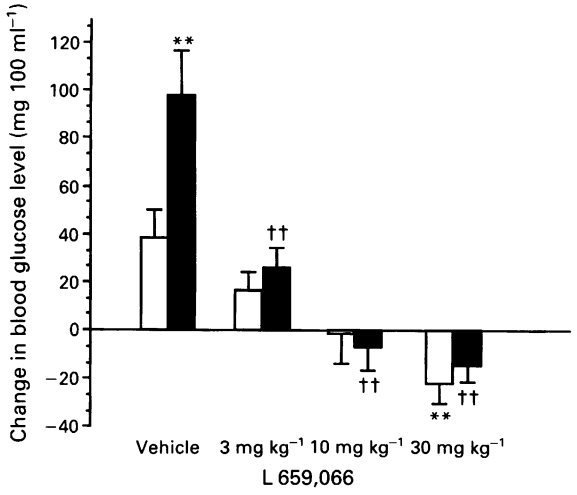

Figure 4 Mean change in blood glucose levels over time from initial baseline levels of animals treated with 3–30 mg kg^{-1} L 659,066 prior to treatment with either vehicle or 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT), data points shown are for 40 min post 8-OH-DPAT administration (a similar pattern of results is seen at both 20 and 60 min post 8-OH-DPAT, data not shown). Open columns, pretreatment + vehicle; solid columns, pretreatment + 8-OH-DPAT. * $P < 0.05$; ** $P < 0.01$ vs. vehicle only treated animals at the same time point; † $P < 0.05$; †† $P < 0.01$ vs. vehicle + 8-OH-DPAT-treated animals at the same time point.

pamezole once again significantly ($P < 0.05$) attenuated the hypothermia at both 20 and 40 min after the administration of 8-OH-DPAT. In this experiment the hypothermic effects of 8-OH-DPAT were no longer significant at the 60 min time point.

L 659,066, 30 mg kg^{-1}, caused a significant ($P < 0.05$) fall in blood glucose levels relative to the vehicle-treated control group, 40 to 80 min after administration, although, once again, an increase in blood glucose level was also seen in the vehicle-treated control group (Figure 4). Significant ($P < 0.01$) 8-OH-DPAT-induced increases in blood glucose levels were detected 40 and 60 min after 8-OH-DPAT administration. This 8-OH-DPAT-induced rise in blood glucose level was significantly ($P < 0.01$) blocked both by 3 mg kg^{-1} of atipamezole and by 3 to 30 mg kg^{-1} of L 659,066.

Discussion

The observed hypothermic and hyperglycaemic effects of 8-OH-DPAT are similar to those described previously (Chaouloff & Jeanrenaud, 1987; Wozniak *et al.*, 1988). The lack of any intrinsic effects of atipamezole on body temperature has also been noted previously (Durcan *et al.*, 1989). The lower doses (3 and 10 mg kg^{-1}) of the mainly peripherally-acting α_2-adrenoceptor antagonist, L 659,066 had no effects on body temperature, although, the highest dose tested (30 mg kg^{-1}) did induce hypothermia. The mechanism of this effect is not clear at the present time.

Although 8-OH-DPAT is reported to have α_2-adrenoceptor antagonist activity (Crist & Surprenant, 1987), its hypothermic effect is thought to be mediated by central 5-hydroxytryptaminergic mechanisms, because it can be blocked by the centrally active 5-HT$_{1A}$ antagonists, methiothepin, spiperone, pizotifen and pindolol (Middlemiss *et al.*, 1985; Gudelsky *et al.*, 1986; Wozniak *et al.*, 1988) whereas xylamidine, which is only active peripherally, is without effect (Gudelsky *et al.*, 1986). Further evidence derives from selective lesions of central 5-hydroxytryptaminergic neurones produced by the neurotoxin 5,7 dihydroxytryptamine, which abolish the hypothermic effects of 8-OH-DPAT, implicating presynaptic 5-HT receptors (presumably 5-HT$_{1A}$). Additionally, depletion of 5-HT with *p*-chlorophenylalanine also abolishes the hypothermic effects of 8-OH-DPAT (Goodwin *et al.*, 1985) and centrally active α_2-adrenoceptor antagonists, unlike 8-OH-DPAT, do not affect body temperature (Durcan *et al.*, 1989; the present study). Furthermore, the blockade of the hypothermic effect of 8-OH-DPAT by α_2-adrenoceptor antagonists appears to be centrally mediated since this effect was not seen following pretreatment with L 659,066 which poorly penetrates into the brain.

The mechanism of the observed attenuation of 8-OH-DPAT-induced hypothermia by atipamezole is unclear. The extent of any direct activity of atipamezole or L 659,066 at 5-HT$_{1A}$ sites is not well established; however, other α_2-adrenoceptor antagonists such as yohimbine, WY 26392 and idazoxan do show significant affinity for 5-HT$_{1A}$ receptor sites (Fozard *et al.*, 1987) and thus direct actions of atipamezole or L 659,066 at 5-HT$_{1A}$ receptor sites cannot be ruled out. α_2-Adrenoceptors have been found on 5-hydroxytryptaminergic neurones (Göthert & Huth, 1980; Frankhuyzen & Mulder, 1980; Göthert *et al.*, 1981), including those in the hypothalamus (Galzin *et al.*, 1982), a brain region associated with thermoregulation (Perkins *et al.*, 1981; Rothwell *et al.*, 1985; Addae *et al.*, 1986). These α_2-adrenoceptors have been reported to modulate 5-HT release (Göthert & Huth, 1980; Ellison & Campbell, 1986), although their precise physiological function remains to be established (Galzin *et al.*, 1984; Blier *et al.*, 1989). Interestingly, the α_2-adrenoceptor antagonists, atipamezole and idazoxan, also attenuate ethanol-induced hypothermia (Durcan *et al.*, 1989), for which central 5-hydroxytryptaminergic mechanisms have been implicated (Ritzmann & Tabakoff, 1976; Tabakoff *et al.*, 1978).

The hyperglycaemic effect of 8-OH-DPAT is thought to be, at least in part, centrally mediated, since it can be produced by central (intracerebroventricular) administration (Chaouloff & Jeanrenaud, 1987). The failure of 8-OH-DPAT to cause an increase in blood glucose in animals pretreated with either atipamezole or L 659,066 indicates that the α_2-adrenoceptor antagonism can influence hyperglycaemia in mice; the α_2-adrenoceptor antagonist, idazoxan, also attenuates 8-OH-DPAT-induced hyperglycaemia in rats (Chaouloff & Jeanrenaud, 1987). The present results are consistent with a recent report demonstrating that L 659,066 prevents the rise in plasma glucose levels seen following administration of the α_2-adrenoceptor agonists clonidine and 3,4-dihydroxyphenylimino-2-imidazoline and also improves glucose tolerance in saline pretreated animals (Goldman *et al.*, 1989). The fact that both L 659,066, which poorly penetrates into the brain, as well as the centrally and peripherally active antagonist, atipamezole, have similar effects implicate peripheral mechanisms in the attenuation of hyperglycaemic effects of 8-OH-DPAT. A number of both *in vivo* (Nakadate *et al.*, 1980a,b) and *in vitro* (Nakaki *et al.*, 1980, 1981; Hillaire-Buys *et al.*, 1985) studies have demonstrated α_2-adrenoceptor involvement in the regulation of insulin secretion. The α_2-adrenoceptor antagonist yohimbine has been shown to increase insulin secretion (Hsu *et al.*, 1987; Ribes *et al.*, 1989). Conversely, the α_2-adrenoceptor agonist clonidine has been shown to decrease insulin levels (Senft *et al.*, 1968; Ismail *et al.*, 1983) and increase blood glucose levels (Rehbinder & Deckers, 1968). 8-OH-DPAT has been reported to cause increases in plasma catecholamines, especially adrenaline and noradrenaline (Bagdy *et al.*, 1989). These may increase blood glucose levels since noradrenaline reduces glucose-induced insulin release from the islets of Langerhans (Morgan & Montague, 1985). The adrenaline enhancing properties of 8-OH-DPAT have been linked to its hyperglycaemic effects and can be blocked by (−)-pindolol, which antagonizes 5-HT$_{1A}$ receptor sites as well as β-adrenoceptors (Chaouloff *et al.*, 1990). 8-OH-DPAT-induced increases in catecholamines may therefore result in the observed increases in blood glucose; an effect which may be attenuated by α_2-adrenoceptor antagonists acting peripherally. A rise in blood glucose is also evident following treatment with vehicle only (possibly as a result of a mild stress resulting from the procedure inducing increased

plasma catecholamine levels), although this is a lesser effect than that seen following 8-OH-DPAT treatment: this effect is also reversed by α_2-adrenoceptor pretreatment.

In summary, 8-OH-DPAT-induced hypothermia is attenuated by a centrally active α_2-adrenoceptor antagonist (atipamezole), whereas it is unaffected by pretreatment with an α_2-adrenoceptor antagonist which poorly penetrates the blood brain barrier (L 659,066). Both antagonists attenuated the hyperglycaemia induced by 8-OH-DPAT. These results suggest that the attenuation of the 8-OH-DPAT-induced hypothermia by atipamezole may be centrally mediated whereas the blockade of hyperglycaemia, evident with both antagonists, may involve peripheral mechanisms.

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Contractile 5-HT_1 receptors in human isolated pial arterioles: correlation with 5-HT_{1D} binding sites

[1]Edith Hamel & Denis Bouchard

Laboratory of Cerebrovascular Research, Montreal Neurological Institute, 3801 University Street, Montréal, Québec, Canada H3A 2B4

1 The 5-hydroxytryptamine (5-HT) receptor responsible for inducing vasoconstriction in human isolated pial arterioles has been pharmacologically characterized.

2 Of several 5-HT agonists tested, 5-carboxamidotryptamine (5-CT) was the most potent and the rank order of agonist potency can be summarized as: 5-CT > 5-HT > RU 24969 = α-methyl-5-HT = methysergide ≫ MDL 72832 = 2-methyl-5-HT ≫ 2-dipropylamino-8-hydroxy-1,2,3,4-tetrahydro-naphthalene (8-OH-DPAT). With few exceptions, the maximal contractile responses of these agonists were comparable to that induced by 5-HT.

3 A correlation analysis performed between the agonists vascular potency (pD_2 values) and their affinities (pK_D values) published at various subtypes of 5-HT binding sites showed a positive significant correlation with rat cortical 5-HT_{1B} ($r = 0.86$; $P < 0.01$) and human caudate 5-HT_{1D} ($r = 0.98$; $P < 0.005$) subtypes.

4 Selective antagonists at 5-HT_2 (ketanserin, mianserin, MDL 11939) and 5-HT_3 (MDL 72222) sites were totally devoid of inhibitory activity on the 5-HT-induced contraction, an observation which agreed with the agonist data and further excluded activation of these receptors. In contrast, the 5-HT_1-like/5-HT_2 antagonist methiothepin and the non-selective 5-HT_{1D} compound metergoline inhibited with high affinity the contraction induced by 5-HT with respective pA_2 values of 8.55 ± 0.16 and 6.88 ± 0.05. This contractile response was, however, insensitive to 5-HT_{1B} (propranolol) and 5-HT_{1C} (mesulergine, mianserin) antagonists.

5 It is concluded that a 5-HT_1-like receptor, which shares strong similarities with the 5-HT_{1D} binding sites identified in human caudate membranes, is mediating the vasocontractile action of 5-HT in human pial arterioles.

Introduction

The importance of 5-hydroxytryptamine (5-HT or serotonin) in the control of the brain circulation has been emphasized recently due to its probable implication in cerebrovascular disorders such as vasospasm and migraine (Anthony & Lance, 1975; MacKenzie *et al.*, 1985; Fozard, 1987). Although its precise role remains unclear and doubts have been formulated as to the origin of the vasoactive 5-HT (platelets and/or specific 5-hydroxytryptaminergic nerves) (Lee, 1989), it is beyond any doubts that various 5-HT-related compounds (primarily 5-HT_2 antagonists) are widely used in the prophylaxis of migraine (Raskin, 1986; Titus *et al.*, 1986). More recently, a 5-HT_1-like agonist (GR-43175; Sumatriptan) has reached Phase III clinical trials and was found beneficial in the treatment of acute migraine headache (Doenicke *et al.*, 1988). On consideration of the multiple subtypes of 5-HT receptors (Bradley *et al.*, 1986; Peroutka, 1988) at which sites the indoleamine can interact and the reported species-related variations in these cerebrovascular receptors (for review, see Young *et al.*, 1987; Lee, 1989), it appears important to define the nature of the contractile 5-HT receptor in human cerebral blood vessels.

Both 5-HT_1 and/or 5-HT_2 receptors have been implicated in the cerebral vasocontractile action of 5-HT depending on the species (for review, see Young *et al.*, 1987; Lee, 1989). In man, most studies were performed before the identification of the various 5-HT receptor subtypes and before the availability of selective drugs at these sites. Several non-selective 5-HT_2 antagonists were originally found to act as non-competitive inhibitors of the 5-HT-induced vasoconstriction in either human basilar (Forster & Whalley, 1982; Muller-Schweinitzer, 1983) or small pial arteries (Hardebo *et al.*, 1978; Edvinsson *et al.*, 1978). Shortly thereafter, Peroutka & Kuhar (1984) using a receptor binding radioautographic approach reported the identification and visualization of 5-HT_1, but not 5-HT_2, receptors in human basilar artery. This contention has received further support from a recent pharmacological study (Parsons *et al.*, 1989) which suggested that the 5-HT receptor in human basilar arteries appears identical to the 5-HT_1-like receptor causing contraction of the dog isolated saphenous vein and cerebral blood vessels from the dog and primate.

In the present study, we have used selective 5-HT agonists and antagonists in an attempt to characterize the 5-HT receptor which mediates contraction of human pial arterioles, a vascular segment of cardinal importance in regulation of cerebral blood flow. The vascular potencies of these agents were compared to their published affinities at various 5-HT receptor subtypes as defined by binding studies. We conclude that a 5-HT_1 receptor, which best correlated with the 5-HT_{1D} binding site identified in human brain, is responsible for the contraction elicited by the indoleamine.

Methods

In vitro *study of human pial arterioles*

Pial arterioles from eleven humans of either sex were obtained post-mortem (delay of 4 to 12 h) from the Montreal Brain Bank at the Douglas Hospital. Patients had died from neurological or non-neurological causes but, in all cases, 5-HT-related medication was not required. The specimens corresponded to a proximal ramification of the middle cerebral artery and were approximately 0.8 mm outside diameter; all vessels were used within 1 h following removal from the cortical surface. The small arteries were cleaned of surrounding tissue under a dissecting microscope and placed in a cold Krebs-Ringer solution of the following composition (in mM): NaCl 118, KCl 4.5, $MgSO_4$-$7H_2O$ 1.0, KH_2PO_4 1.0,

[1] Author for correspondence.

$NaHCO_3$ 25, $CaCl_2$-$2H_2O$ 2.5 and glucose 6.0. Circular segments (3–4 mm in length) were used for recording of the isometric tension developed by the smooth muscle as described previously (Högestätt *et al.*, 1983); changes in tension were measured by a force displacement transducer (GRASS FT 103D) and recorded on a Grass Polygraph Model 7E. All vessel segments were mounted between two L-shaped metal prongs in temperature-controlled (37°C) tissue baths containing the Krebs solution gassed continuously with 5% CO_2 in 95% O_2, in order to maintain the pH at 7.4. Arteriolar segments were then given a mechanical tension of 0.4 g and allowed to stabilize at this level for 60 min. During this period the bathing fluid was replaced regularly by fresh buffer (every 15 min).

Response curves to agonists

Log concentration-response curves were generated for each agonist by cumulative addition of the drug (0.1 nM–1 mM) to vessels equilibrated at the level of the resting tension (mechanical tone of 0.4 g). In every experiment the maximal contractile capacity of the arterial segments was assessed by exposure to 124 mM K^+. For this purpose, NaCl was replaced by KCl in equimolar concentration in the Krebs buffer solution. Relative potencies of the agonists were determined according to their respective pD_2 values (the negative logarithm of the molar concentration of the agonist which produced 50% of the maximal contractile effect; $-\log EC_{50}$) calculated from individual curves with the following equation:

$$pD_2 = -\log[A] - \log\left(\frac{E_{Amax}}{E_A} - 1\right) \quad (1)$$

where E_{Amax} is the maximal contraction induced by agonist A and E_A the contractile effect in response to a given concentration [A] of agonist (Van den Brink, 1977). The E_{Amax} (in g) of the vessel segments to the various agonists were compared with the maximal vasoconstriction elicited by 5-HT in the same preparations, and expressed as percentage of the maximal 5-HT response. For reference, the maximal constriction elicited by 5-HT (10 μM) corresponded to 71 ± 5% ($n = 33$) of the maximal contractile capacity of the cerebrovascular smooth muscle (1.64 ± 0.09 g), as determined by 124 mM K^+.

Determination of antagonist potency

A concentration-response curve to 5-HT was first obtained and when the maximal contraction was reached, the agonist was washed out from the bath and the potency of antagonist determined on the same vessels. Cumulative addition of the agonist was again performed but in the presence of graded concentrations of a given antagonist in the range of 10 nM to 1 μM. In each case, the antagonist was in contact with the vessels for a period of 20 min before reconstruction of the agonist concentration-effect curve. When appropriate the potency of the antagonist was evaluated by the use of pA_2 value for dual antagonist (competitive and metactoid) or the negative logarithm of the molar antagonist concentration, in the presence of which twice the original agonist concentration is needed to cause an effect that is 'relatively' equal to the original effect (Van Den Brink, 1977). The pA_2 was calculated as:

$$pA_2 = -\log[B] + \log\left(\frac{[A]_2}{[A]_1} - 1\right) \quad (2)$$

in which [B] is the antagonist concentration; $[A]_2$ the concentration of agonist needed to reach a vasoconstriction relatively equivalent to the control half-maximal response, in the presence of [B]; $[A]_1$ the concentration of agonist needed to elicit the half-maximal response in the absence of [B] (Van Den Brink, 1977). In addition, the pA_2 values were estimated from the regression lines obtained in the Schild plot analysis (Arunlakshana & Schild, 1959). The competitive nature of the antagonism was also assessed by Schild plot analysis of the agonist dose-ratios obtained for two (metergoline) to three (methiothepin) different antagonist concentrations. The slope of the regression line was determined for each individual arteriolar segment.

Statistical analysis

All results are presented as means ± s.e.mean. Differences in agonists potency (pD_2 values and maximal responses) were evaluated according to one-way analysis of variance (ANOVA) for unequal sample size and Student-Newman-Keuls multiple comparison test. Student's t test was used to evaluate significant antagonist activity. Linear regression lines and correlation coefficients were calculated in order to detect and quantify correlations between vascular and binding potencies of the various agonists. In all cases, statistical significance was assumed when $P \leq 0.05$.

Drugs

The following drugs were purchased: 5-HT creatinine sulphate, (±)-propranolol hydrochloride (Sigma Chemical Co., St. Louis, MO), ketanserin tartrate, mianserin hydrochloride, and 8-OH-DPAT ((±)-2-dipropylamino-8-hydroxy-1,2,3,4-tetrahydro-naphthalene) hydrobromide (RBI, Natick, MA). All other compounds were kindly provided by the following: (±)-α-methyl-5-HT (α-CH_3-5-HT), 2-methyl-5-HT (2-CH_3-5-HT), methysergide hydrogen maleinate and mesulergine (Sandoz, Basel, Switzerland); 5-methoxy-3(1,2,3,6-tetrahydro-4-pyridinyl) 1H indole succinate (RU 24969; Roussel UCLAF, Paris, France); α-phenyl-1-(2-phenylethyl)-4-piperidine methanol (MDL 11939); 1αH,3α,5αH-tropan-3-yl-3,5-dichlorobenzoate (MDL 72222) and 8-[4-(1,4-benzodioxan- 2-ylmethylamino)butyl]- 8-azaspiro[4,5]decane-7,9-dione (MDL 72832) were from the Centre de Recherche Merrell Dow International, Strasbourg, France and methiothepin maleate from Hoffman-Laroche, Basel, Switzerland. Metergoline was a generous gift from Dr Rémi Quirion, Douglas Hospital Research Centre, Verdun, Québec, Canada.

Results

Agonist potency

All 5-HT agonists tested were found to induce concentration-dependent constrictions of human pial arterioles under resting tension (Figure 1, Table 1). Except for methysergide which elicited a contractile response corresponding to only 53% ($P < 0.05$) of that induced by 5-HT itself, all other agonists tested elicited full constriction over the concentration range studied (Figure 1). 5-CT was four fold more potent than 5-HT itself followed by RU 24969, methysergide and α-CH_3-5-HT which were approximately 5 to 12 times less potent than 5-HT in inducing vasoconstriction, as evaluated from their EC_{50} ratios (Table 1). Agonists at 5-HT_{1A} (8-OH-DPAT and MDL 72832) and 5-HT_3 (2-CH_3-5-HT) sites were the least potent compounds and were one hundred to one thousand fold less active than 5-HT on a concentration basis. However, these agents produced maximal responses which either compared well (MDL 72832), were significantly higher (8-OH-DPAT, $P < 0.05$) or slightly less (2-CH_3-5-HT) than that elicited by 5-HT itself (Table 1).

A correlation analysis was performed between the vascular potencies (expressed as pD_2 values taken from Table 1) of the agonists in human pial arterioles and the affinities of these compounds at the various subtypes of 5-HT binding sites as described in the literature (taken as pK_D, pK_i or pIC_{50} values as detailed in legend to Figure 2). No significant correlation could be detected between the affinities of these agents at 5-HT_{1A}, 5-HT_{1C} or 5-HT_2 binding sites characterized in rat

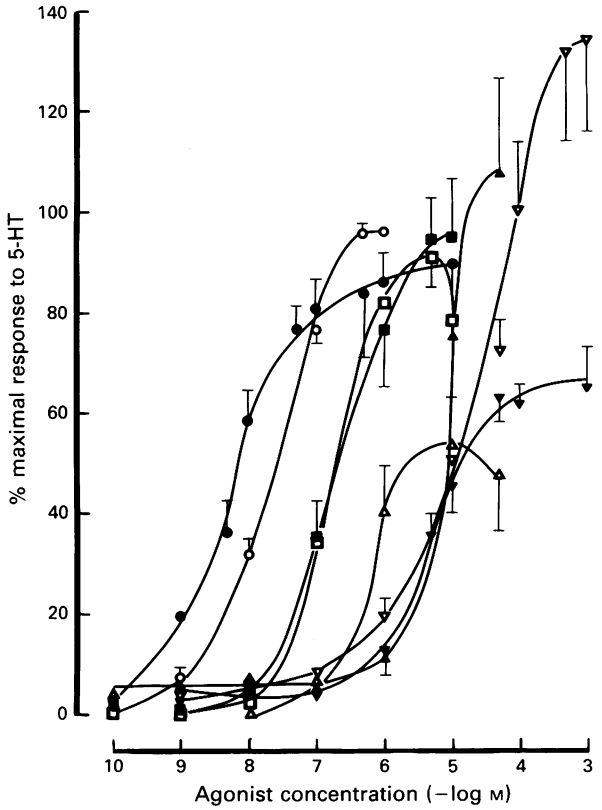

Figure 1 Concentration-response curves to 5-hydroxytryptamine (5-HT) and 5-HT agonists on human pial arterioles under resting tension. The curve to 5-HT (○) represents data obtained with all tissues. Shown are the responses to 5-carboxamidotryptamine (●); RU 24969 (□); α-methyl-5-HT (■); methysergide (△); MDL 72832 (▲); 2-dipropylamino-8-hydroxy-1,2,3,4-tetrahydro-naphthalene (▽) and 2-methyl-5-HT (▼). The complete information for individual potency, maximal response and number of vascular segments is given in Table 1. Vertical bars show s.e.means of $n = 4$ to 33 (see Table 1).

brain or pig choroid plexus membranes and their potencies in inducing vasoconstriction of human pial arterioles (Figure 2). However, significant correlations were found between their affinities at 5-HT$_{1B}$ sites in rat cortex ($r = 0.86$; $P < 0.01$; Figure 2) as well as 5-HT$_{1D}$ sites characterized in human caudate membranes ($r = 0.98$; $P < 0.005$; Figure 2).

Antagonists

Consecutive administration of 5-HT alone to arteriolar segments resulted in a good reproducibility of the vasoconstriction induced by the amine, provided that a 45 to 60 min resting period was allowed before reconstruction of the concentration-response curves. In any instance, a trend towards an increase in maximal response and potency was noted rather than a decrease in vasomotor effects induced by 5-HT.

Antagonists with affinities at 5-HT$_{1B}$ (propranolol), 5-HT$_{1C}$ (mesulergine, mianserin), 5-HT$_2$ (ketanserin, mianserin and MDL 11939) and 5-HT$_3$ (MDL 72222) binding sites were tested as inhibitors of the 5-HT-induced constriction of human pial arterioles. In concentrations which varied between 10 nM to 1 μM, all these compounds were devoid of antagonist activity as neither 5-HT potency (EC_{50}) nor maximal response (E_{Amax}) were significantly modified in the presence of the antagonist (Figure 3 and Table 2). In some cases, the 5-HT response was enhanced rather than depressed as was observed with the 5-HT$_2$ antagonists ketanserin and mianserin (for illustration, see ketanserin Figure 3e). However, such an effect was not noted with the more recently described 5-HT$_2$ antagonist MDL 11939 (Dudley *et al.*, 1988).

In contrast, in the presence of various concentrations of methiothepin, a compound with a broad spectrum of activity which includes all 5-HT$_1$ subtypes as well as the 5-HT$_2$ sites, an apparent parallel rightward shift in the concentration-response curves to 5-HT was observed (Figure 3a; Table 2) with a slight decrease in the maximal effect at higher antagonist concentrations. Similarly, the compound metergoline which exhibits high affinity for various 5-HT receptor subtypes, including 5-HT$_{1D}$ recognition sites in human brain

Table 2 Effects of antagonists on 5-hydroxytryptamine (5-HT)-induced contraction of human pial arterioles

Antagonist	n	*Concentration used*	*pA_2 value* (Schild slope)*
Methiothepin	6	10 nM–1 μM	8.55 ± 0.16 (1.41 ± 0.09)
Metergoline	4	10 nM–1 μM	6.88 ± 0.05 (1.72 ± 0.23)
Mesulergine	3	0.1–1 μM	No effect
Propranol	3	0.1–1 μM	No effect
Ketanserin	5	0.1–1 μM	No effect
Mianserin	5	1 μM	No effect
MDL 11939	3	10 nM–1 μM	No effect
MDL 72222	3	10 nM–1 μM	No effect

The concentrations of antagonist used are listed and given pA_2 values were obtained according to Arunlakshana & Schild as described in Methods. Results are the mean ± s.e.mean from the number n of arteriolar segments indicated. The slopes of the Schild plots are given within parentheses and were significantly different from unity with a $P < 0.05$.

* In the Van Den Brink calculation, the pA_2 values were 8.46 ± 0.24 and 7.24 ± 0.09, respectively, for methiothepin and metergoline.

Table 1 Potencies of various 5-hydroxytryptamine (5-HT) agonists for inducing contraction on human pial arterioles

Agonist	n	E_{Amax} (% of 5-HT E_{Amax})	pD_2 ($-\log EC_{50}$)	$\frac{EC_{50}\ agonist}{EC_{50}\ 5\text{-}HT}$
5-HT	33	100	7.61 ± 0.08[b]	1
5-CT	21	90 ± 5	8.18 ± 0.09[b]	0.25
RU 24969	8	86 ± 7	6.85 ± 0.15[c]	5
α-CH_3-5-HT	10	97 ± 9	6.57 ± 0.16[c]	12
Methysergide	4	53 ± 14[a]	6.30 ± 0.06[c]	13
MDL 72832	5	103 ± 19	5.45 ± 0.08[d]	96
2-CH_3-5-HT	18	68 ± 5	5.20 ± 0.13[d]	450
8-OH-DPAT	10	137 ± 19[b]	4.67 ± 0.21[b]	1160

All values are mean ± s.e.mean from n individual segments as indicated. Those used for 5-HT represent pooled data obtained with all agonist experiments. An EC_{50} of 40.0 ± 6.7 nM was calculated for 5-HT and was used for determination of the agonist relative potency defined as EC_{50} agonist/EC_{50} 5-HT.

[a] $P < 0.05$ when compared with all agonists except 2-CH_3-5HT; [b] $P < 0.05$ with respect to all other agonists; [c] $P < 0.05$ with all agonists except α-CH_3-5-HT, methysergide or RU 24969, respectively; [d] $P < 0.05$ with all compounds but 2-CH_3-5-HT and MDL 72832, respectively, by one way analysis of variance and Student Newman-Keuls multiple range test. For key to abbreviations used see legend of Figure 1.

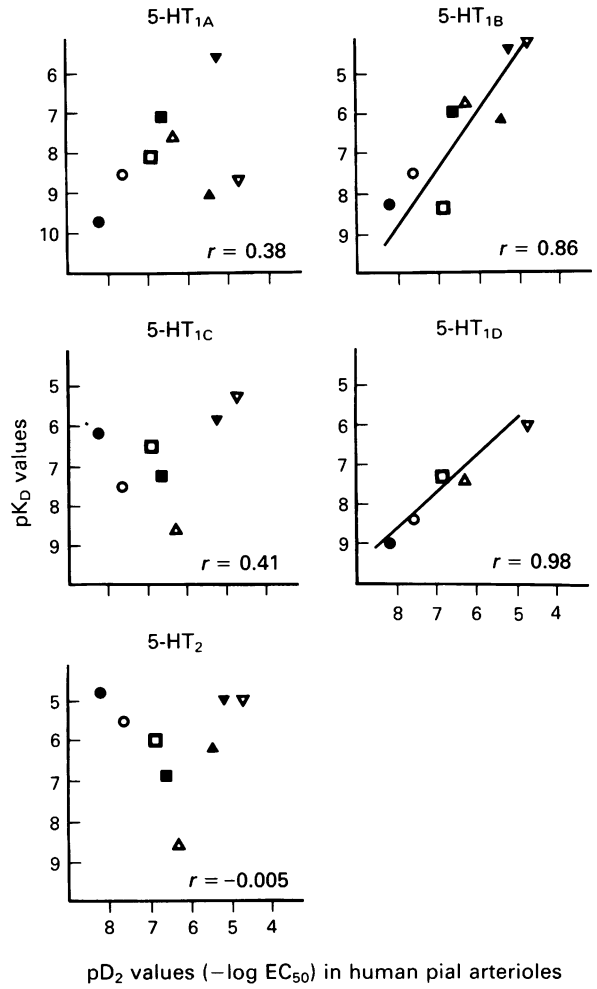

Figure 2 Correlation between the 5-hydroxytryptamine (5-HT) agonists vascular potencies (pD_2 values in human arterioles from Table 1) and their binding affinities (pK_D or pK_i) at various 5-HT receptor subtypes. The agonists used for the correlation analysis are 5-HT (○); 5-CT (●); RU 24969 (□); α-CH_3-5-HT (■); methysergide (△); MDL 72832 (▲); 8-OH-DPAT (▽) and 2-CH_3-5-HT (▼). We have used pK_D values published by Engel *et al.* (1986) in rat brain cortex membranes at 5-HT_{1A}, 5-HT_{1B} and 5-HT_2 binding sites for all agonists except RU 24969 (pK_i values from Glennon, 1987 in rat brain cortex), MDL 72832 (pIC_{50} values obtained in rat brain membranes; Mir *et al.*, 1988) and methysergide (pK_D values from Hoyer, 1988; at 5-HT_{1A} site, pig frontal cortex was used in this study). Binding affinities at 5-HT_{1C} were determined in pig choroid plexus and, as above, pK_D values were used except for RU 24969 and MDL 72832 (Engel *et al.*, 1986; Glennon, 1987; Hoyer, 1988; Mir *et al.*, 1988). At the 5-HT_{1d} subtype, affinities (pK_D or pK_i values) were taken from those obtained in human caudate membranes (Waeber *et al.*, 1988; Herrick-Davis *et al.*, 1988). Correlation coefficients (r) are given in the figure. For the 5-HT_{1B} subtype: $r = 0.86$ ($P < 0.01$) and for the 5-HT_{1D} site, $r = 0.98$ ($P < 0.005$). For key to abbreviations used see legend of Figure 1.

membranes (Waeber *et al.*, 1988), potently inhibited the 5-HT-induced vasoconstriction in human pial arterioles (Figure 3b and Table 2). The pA_2 values estimated in the Schild plot analysis were 8.55 ± 0.16 and 6.88 ± 0.05 for methiothepin and metergoline, respectively. When calculated according to Van Den Brink, pA_2 values of 8.46 ± 0.24 and 7.24 ± 0.09 were obtained for methiothepin and metergoline, respectively. For both antagonists, the slopes of the regression lines in the Schild plots analysis differed significantly from unity (Table 2).

Discussion

The present study on the pharmacological identification of human 5-HT receptors in pial arterioles suggests that 5-HT_{1A}, 5-HT_{1C}, 5-HT_2 and 5-HT_3 receptor subtypes are not involved in the vasoconstriction induced by the indoleamine in this cerebrovascular tissue. More likely, a 5-HT_1-like receptor, which correlated best with the 5-HT_{1D} binding sites identified in human caudate membranes, is responsible for the 5-HT-induced contractile response. This contention is based on both our agonist and antagonist data as well as on the pharmacological properties of these various subtypes of 5-HT receptors (Bradley *et al.*, 1986; Richardson & Engel, 1986; Peroutka, 1988).

We found that two selective 5-HT_{1A} agonists, 8-OH-DPAT (Gozlan *et al.*, 1983; Middlemiss & Fozard, 1983) and MDL 72832 (Mir *et al.*, 1988) had a very low potency in inducing vasoconstriction of human isolated pial arterioles. In fact, their vascular potencies were more than 1000 fold less than their reported affinities at the 5-HT_{1A} subtype (Engel *et al.*, 1986; Glennon, 1987; Hoyer, 1988; Mir *et al.*, 1988), an observation which virtually rules out the stimulation of 5-HT_{1A}

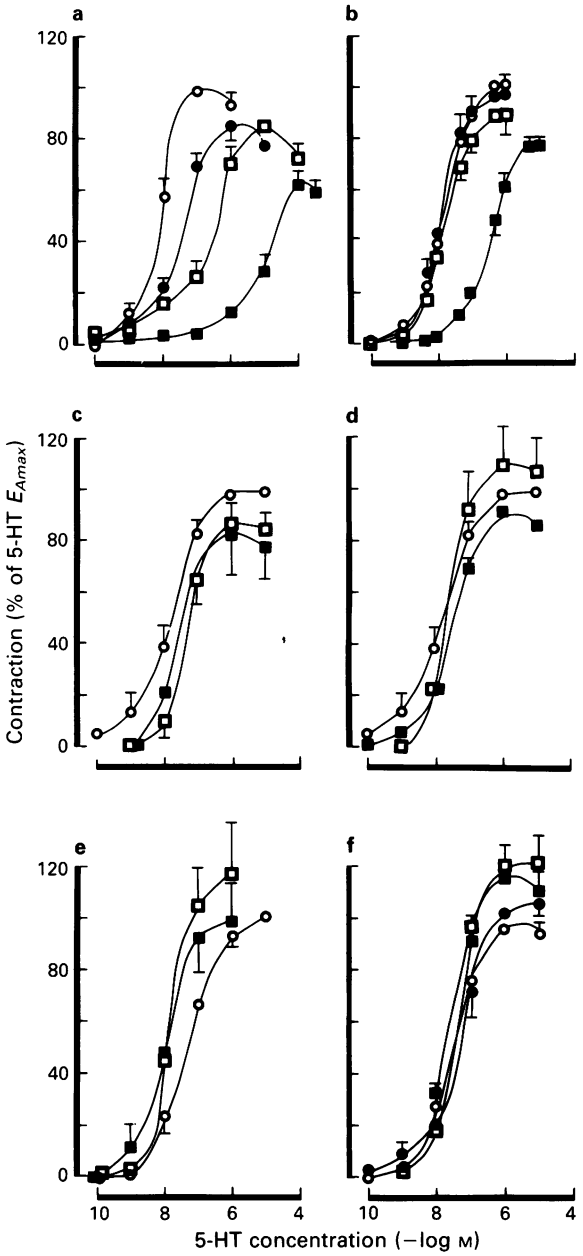

Figure 3 Concentration-response curves for 5-hydroxytryptamine (5-HT) on human pial arterioles in the absence (○, control) and presence of 10 nM (●), 100 nM (□) or 1000 nM (■) methiothepin (a, $n = 6$); metergoline (b, $n = 4$); propranolol (c, $n = 3$); mesulergine (d, $n = 3$); ketanserin (e, $n = 5$); and MDL 72222 (f, $n = 3$). Vertical bars represent s.e.means of $n = 3$–6.

receptors in the 5-HT-induced contraction of pial arterioles. Most probably, at the concentration range at which they were able to elicit potent vasoconstriction (10 μM–1 mM), these agents interacted with other 5-HT receptor subtypes (Engel *et al.*, 1986; Mir *et al.*, 1988) and/or with α-adrenoceptors as suggested previously in other tissues (Timmermans *et al.*, 1984) including cerebrovascular beds (Hamel *et al.*, 1989; Parsons *et al.*, 1989). The exclusion of the 5-HT_{1A} subtype pinpoints to apparent species-related variations, as in the dog basilar artery this subtype of 5-HT receptor appears to mediate contraction and 8-OH-DPAT was found to be as potent as (Taylor *et al.*, 1986) or more potent than (Peroutka *et al.*, 1986) 5-HT. However, more recent studies have suggested that a 5-HT_1-like receptor, different from the 5-HT_{1A}, mediates contraction in this cerebral artery (Connor *et al.*, 1989).

Our experimental data also suggest that 5-HT_{1C} receptors cannot be involved in the 5-HT-mediated vasoconstriction of human pial arterioles. This hypothesis is based primarily on the observation that the potent 5-HT_{1C} antagonists, mesulergine (tested at 0.1 and 1 μM) and mianserin (tested at 1 μM), showed no inhibitory effect in this vascular response. Furthermore, the rank order of agonist potency at this subtype: 5-HT = α-CH_3-5-HT > RU 24969 = 5-CT = 2-CH_3-5-HT > 8-OH-DPAT (Pazos *et al.*, 1984; Engel *et al.*, 1986; Glennon, 1987; Hoyer, 1988) is different from the vascular potency observed in the present study, as clearly evidenced by the correlation analysis. The weak agonist activity of 2-CH_3-5-HT and the lack of antagonism of MDL 72222, two compounds with selective affinity at the 5-HT_3 receptor subtype (Fozard, 1984; Richardson & Engel, 1986), on the 5-HT-induced contraction of human pial arterioles further suggest that this vasomotor effect is not mediated by a 5-HT_3 receptor. Such a conclusion would support the finding that the beneficial effect of MDL 72222 in acute migraine attack (Loisy *et al.*, 1985) is related to nonvascular interactions (Richardson *et al.*, 1985; Orwin & Fozard, 1986).

Earlier studies have demonstrated the non-competitive nature of the weak antagonism exerted by several selective and non-selective 5-HT_2 antagonists on the 5-HT-induced contraction of human cerebral blood vessels (Hardebo *et al.*, 1978; Edvinsson *et al.*, 1978; Foster & Whalley, 1982; Muller-Schweinitzer, 1983). Recently, Parsons and colleagues (1989) showed the absence of antagonist effects of 5-HT-induced constriction of human basilar artery by the 5-HT_2 blocking drug, ketanserin. This observation supports our present findings in human pial arterioles which showed that selective 5-HT_2 compounds including ketanserin, mianserin and MDL 11939 (Engel *et al.*, 1986; Glennon, 1987; Hoyer, 1988; Dudley *et al.*, 1988) failed to exhibit any inhibitory effect on the 5-HT-mediated contraction. In some instances, a slight (non-significant) enhancement in both 5-HT maximal response and potency was noted with 5-HT_2 antagonists such as ketanserin and mianserin. This observation is difficult to explain but it is interesting that such a phenomenon had previously been noted in human cerebral vasculature (Hardebo *et al.*, 1978; Edvinsson *et al.*, 1978) and in other tissues with 5-HT_1-like mediated effects (Newberry & Gilbert, 1989). The exclusion of the 5-HT_2 receptor is further supported by the complete absence of correlation noted between the vascular and binding potencies of the various agonists studied. These results contrast with our previous study in feline cerebral arteries in which we found that the 5-HT_2 antagonists were the most potent inhibitors of the 5-HT vasocontractile response, albeit in an apparently non-competitive manner (Hamel *et al.*, 1989). These discrepancies further emphasize the species-related variations in the identity of contractile 5-HT cerebrovascular receptors.

Several aspects from both our agonist and antagonist data suggest that a 5-HT_1-like receptor is being stimulated by 5-HT to induce contraction of human pial arterioles. On one hand, this hypothesis is based on the higher potency of 5-CT as compared to 5-HT itself (Bradley *et al.*, 1986) and on the overall rank order of agonist potency, in which the agonist property of methysergide is clearly evidenced and which resembles quite closely the order of potency described for 5-HT_1-like-mediated vascular function in the dog sapheneous vein (Engel *et al.*, 1983). On the other hand, the participation of a 5-HT_1-like receptor is further adduced by the fact that the selective 5-HT_2 and 5-HT_3 antagonists were completely inactive whereas methiothepin (which has affinity at 5-$HT_{1A, 1B, 1C, 1D}$ and 5-HT_2 receptors) was able to block potently the 5-HT-induced contraction of human pial arterioles.

Based on our agonist correlation data, two subtypes of 5-HT_1-like receptors, namely 5-HT_{1B} and 5-HT_{1D}, are possible candidates for the receptor mediating constriction of human pial arterioles. However, experiments carried out with propranolol, a potent antagonist at 5-HT_{1B} sites in brain membranes (Middlemiss, 1984; Engel *et al.*, 1986; Glennon, 1987; Hoyer, 1988), showed that this compound was completely devoid of inhibitory activity (up to 1 μM) on the contractile response of human pial arterioles to 5-HT. This observation is supported by the work of Parsons *et al.* (1989) who similarly found that the 5-HT_{1B} antagonist cyanopindolol was inactive in blocking 5-HT vasomotor effects in human basilar artery. Moreover, species differences in the distribution of 5-HT_{1B} and 5-HT_{1D} receptors (Hoyer *et al.*, 1986; Waeber *et al.*, 1988; Herrick-Davis *et al.*, 1988) indicate that the former are absent from human brain, an observation which would argue in favour of the activation of contractile 5-HT_{1D} receptors in human pial arterioles. The strong correlation obtained with the agonist's potency at the 5-HT_{1D} site ($r = 0.98$) would also support such a contention. However, the most convincing evidence to attest for the involvement of a receptor similar to the 5-HT_{1D} subtype was provided by the antagonist activity of metergoline on the 5-HT-induced cerebrovascular response. Though the correlation analysis illustrated in Figure 2 included only the agonists, addition of the metergoline vascular potency (pA_2 of 6.88 or 7.24, see results) and the pK_D value found in human caudate membranes (7.93 $\pm$ 0.23; Waeber *et al.*, 1988) still yielded a highly significant correlation ($r = 0.98$, $P < 0.01$ for either one of the pA_2 values). Methiothepin, on the other hand, was much more potent in inhibiting the 5-HT-induced constriction than predicted from its affinity at human 5-HT_{1D} binding sites (pK_D of 6.77, Waeber *et al.*, 1988). The reason for a higher affinity at cerebrovascular 5-HT receptors is unclear, but it might suggest that the cerebrovascular 5-HT_1 receptor is somewhat different from the caudate 5-HT_{1D} binding sites. Interestingly, such a high potency was also reported for methiothepin at 5-HT_1-like receptors in human basilar artery (Parsons *et al.*, 1989). The apparent lack of antagonism of mianserin, which expectedly should have exerted some blockade effect due to its relative affinity at 5-HT_{1D} sites (Waeber *et al.*, 1988; Hoyer, 1988), most probably results from our experimental conditions (tested at a single dose of 1 μM) which did not allow for any forthcoming effect to be observed. Such an explanation seems likely when considering that 1 μM metergoline potently antagonized the 5-HT response while being only slightly active at 0.1 μM. Had this compound been tested at a unique concentration of 0.1 μM, we would have classified it as being virtually inactive. Such might actually have been the case in the dog saphenous vein where, on the basis of no antagonism of 0.1 μM metergoline, it was concluded that the contractile response to 5-HT was not mediated by 5-HT_{1D} receptors (Humphrey *et al.*, 1988). Indeed, the overall pharmacology of this arterial bed resembles quite closely that described previously in human basilar artery (Parsons *et al.*, 1989) and here in human pial arterioles. Whether or not the 5-HT_1-like contractile cerebrovascular receptor identified in dog, primate and human basilar artery (Connor *et al.*, 1989; Parsons *et al.*, 1989) corresponds to the 5-HT_{1D} subtype remains to be established, but this possibility has not been ruled out (Parsons *et al.*, 1989).

In conclusion, our study suggests that a 5-HT_1-like receptor, which best correlated with the 5-HT_{1D} site identified in

human brain caudate membranes, mediates the vasoconstriction elicited by 5-HT in human pial arterioles. That we have identified a receptor slightly different from the brain caudate nucleus 5-HT_{1D} binding site cannot be excluded; however, our results and the current understanding of 5-HT receptor pharmacology and species distribution strongly emphasize its similarity to the 5-HT_{1D} subtype. When comparing the results of the present study in human pial arterioles with our recent data obtained in cat cerebral arteries, there is a major difference which resides in the participation of 5-HT_2 receptors exclusively in the latter species which, nevertheless, also exhibited the presence of a 5-HT_1-like contractile receptor (Hamel *et al.*, 1989). In the search for anti-migraine drugs, the 5-HT_{1D} site would appear as a good target for controlling cerebrovascular function since agonists and antagonists at this site would, respectively, induce constriction and potently block the contractile effect of endogeneous 5-HT thus resulting in vasodilatation, two vasomotor effects which have been associated with the manifestation of migraine (See Toole, 1984; Fozard, 1987). However, a better understanding of the pharmacology of peripheral 5-HT receptors would appear essential in order to insure the selectivity of 5-HT_{1D}-related drugs for use in cerebrovascular treatment without considerable interaction with non-cerebral vessels (see Angus, 1989).

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Identification of N-iminoethyl-L-ornithine as an irreversible inhibitor of nitric oxide synthase in phagocytic cells

T.B. McCall, [1]M. Feelisch, R.M.J. Palmer & [2]S. Moncada

Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS

1 The synthesis of nitric oxide (NO) from L-arginine by rat peritoneal neutrophils (PMN) and the murine macrophage cell-line J774 and the inhibition of this synthesis by N-iminoethyl-L-ornithine (L-NIO), N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine (L-NNA) and its methyl ester (L-NAME) were investigated.

2 L-NIO was the most potent inhibitor in both types of cells while L-NMMA was less active. L-NNA and L-NAME had no significant effect in PMN and L-NNA produced only approximately 40% inhibition of the generation of NO in the J774 cells at the highest concentration tested (300 μM).

3 The inhibitory effect of L-NIO was rapid in onset, requiring 10 min pre-incubation to achieve its full inhibitory activity, while the other compounds required 20–60 min pre-incubation to achieve their full effect.

4 The inhibitory effect of L-NIO (10 μM) on intact cells could not be reversed by L-arginine (300 μM) but could be prevented by concomitant incubation with this compound (300 μM), while the effect of the other inhibitors could be reversed by a 3–5 fold molar excess of L-arginine.

5 The NO synthase from both PMN and J774 cells was cytosolic and NADPH- but not Ca^{2+}-dependent, with K_m values for L-arginine of 3.3 ± 0.8 and 4.2 ± 1.1 μM respectively.

6 L-NIO was the most potent inhibitor of the neutrophil and J774 enzymes with IC_{50} values of 0.8 ± 0.1 and 3 ± 0.5 μM respectively. Furthermore, the effect of L-NIO was irreversible. The other three compounds were less potent, reversible inhibitors.

7 The inhibitory effects of all these compounds were enantiomerically specific.

8 These data indicate that L-NIO is a novel, potent, rapid in onset and irreversible inhibitor of NO synthase in phagocytic cells. The rapid uptake of L-NIO compared with the other compounds indicates that phagocytic cells have different uptake mechanisms for L-arginine analogues.

Introduction

Neutrophils (PMN; McCall *et al.*, 1989; Salvemini *et al.*, 1989) and macrophages (Hibbs *et al.*, 1988; Marletta *et al.*, 1988; Stuehr *et al.*, 1989) synthesize nitric oxide (NO) from the amino acid L-arginine. This synthesis is inhibited by the L-arginine analogue N^G-monomethyl-L-arginine (L-NMMA; Hibbs *et al.*, 1987; McCall *et al.*, 1989; Salvemini *et al.*, 1989). However, in the PMN this compound only causes partial inhibition of synthesis following a long period of pre-incubation (McCall *et al.*, 1989), suggesting that L-NMMA is a weak inhibitor of the NO synthase in PMN or is only poorly taken up into these cells, or both.

Other L-arginine analogues, such as N-iminoethyl-L-ornithine (L-NIO), N^G-nitro-L-arginine (L-NNA) and its methyl ester (L-NAME) have recently been reported to be potent inhibitors of the NO synthase of adrenal glands (Palacios *et al.*, 1989), brain (Knowles *et al.*, 1990) and vascular endothelial cells (Rees *et al.*, 1990). Present evidence indicates that the NO synthase in endothelial cells and brain differs from that in PMN and macrophages. L-Canavanine, for example, inhibits NO synthesis in phagocytic cells (Hibbs *et al.*, 1987; McCall *et al.*, 1989), but does not affect the generation of NO by brain synaptosomes (Knowles *et al.*, 1989) or endothelial cells (Palmer & Moncada, 1989). Furthermore, L-homoarginine is a weak substrate in phagocytic cells (McCall *et al.*, 1989), but not in endothelial cells (Palmer & Moncada, 1989) or brain synaptosomes (Knowles *et al.*, 1989).

In the present study, we have examined the activity of these inhibitors of NO synthase on the release of NO from rat peritoneal PMN and from the murine macrophage cell line J774, activated with IFN-γ and lipopolysaccharide (LPS), as determined by a bioassay method using inhibition of platelet aggregation (McCall *et al.*, 1989). In addition, we have examined the potency of the inhibitors on the activity of NO synthase from these cells.

Some of these results were presented at the IUPHAR satellite symposium 'EDRF and EDRF-related substances' in Antwerp, Belgium, June 1990.

Methods

Preparation of human platelets and the detection of NO, by use of platelet aggregation as a bioassay, were carried out as previously described (McCall *et al.*, 1989).

Peritoneal PMN were elicited with oyster glycogen (0.2%) and harvested from male Wistar rats (200–250 g). A purified population of PMN (>95% pure) was prepared by Ficoll-Hypaque density gradient centrifugation. The 5% contaminating cells consisted of mast cells (2–3%) and mononuclear cells (2–3%). After hypo-osmotic lysis of erythrocytes, the PMN were resuspended at a final concentration of 1×10^7 cells ml^{-1} in Tyrode solution containing 1 mM Ca^{2+} and 5 μM indomethacin and maintained at 4°C. The PMN preparation was more than 98% viable as assessed by the uptake of Acridine Orange.

J774 cells (American Tissue Culture Catalogue T1B 67, page 231), cultured in stirrer bottles in RPMI 1640 containing 10% foetal calf serum, were stimulated with IFN-γ (150 u ml^{-1}) and LPS (10 μg ml^{-1}) for 18 h. Cells were then harvested by centrifugation, resuspended at a final concentration of 1×10^6 cells ml^{-1} as described for PMN and maintained at room temperature.

Bioassay of NO

Cells were added to indomethacin (5 μM)-treated platelets (1×10^8 platelets in 500 μl) in a Payton aggregometer (37°C,

[1] Present address: Schwarz Pharma 4019, Monheim, F.R.G.
[2] Author for correspondence.

900 r.p.m.) and incubated for 4 min before addition of a submaximal concentration of thrombin (20–40 mu ml^{-1}) to initiate aggregation. The response was monitored for 10 min. The anti-aggregating effect of phagocytic cells was expressed as a percentage (mean ± s.e.mean) of the aggregation induced by thrombin alone.

Preparation of NO synthase

Purified rat peritoneal PMN or activated and non-activated J774 cells, resuspended in HEPES buffer (0.1 M, pH 7.4) containing 100 μM dithiothreitol, were lysed by sonicating twice for 10 s and kept on ice. The lysate was then centrifuged at 105,000 *g* for 30 min at 4°C and the supernatant was incubated with AG50-X8 (Na^+ form; 100 mg ml^{-1} of supernatant) for 5 min at 4°C to deplete endogenous L-arginine.

Spectrophotometric assay for NO synthase activity

The generation of NO by cytosol was measured by difference-spectrophotometry according to the method of Feelisch & Noack (1987). This measurement is based on the quantitative oxidation of oxyhaemoglobin to methaemoglobin in aqueous solution by NO.

The cytosolic preparation was pre-incubated at 37°C in the presence of oxyhaemoglobin (5 μM) and NADPH (100 μM) for 5 min, prior to addition of a submaximally effective concentration of L-arginine (30 μM), to initiate NO generation by the enzyme. The initial rate of production of NO was determined as the difference in absorbance between 401 and 411 nm in a dual beam spectrophotometer (Shimadzu) and the results expressed as pmol NO min^{-1} mg^{-1} protein.

Materials

Oyster glycogen, indomethacin, superoxide dismutase (SOD), N^G-nitro-L-arginine methyl ester (L-NAME), NADPH, L-arginine, dithiothreitol (all Sigma), AG 50-X8 (Bio-Rad), RPMI (Gibco), foetal calf serum (Flow Labs.) human thrombin (Ortha Diagnostic Systems), Ficoll-Hypaque (Pharmacia), *Salmonella typhosa* lipopolysaccharide (Difco), recombinant murine IFN-γ (Genzyme), N-iminoethyl-L-ornithine (L-NIO), N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine (L-NNA), prostacyclin (all Wellcome) were obtained as indicated. Human haemoglobin was prepared as described (Paterson *et al.*, 1976).

Statistics

Student's *t* test (two-tailed) for unpaired data was used to determine statistical significance, and $P < 0.05$ was taken as statistically significant.

Results

Phagocytic cells inhibited platelet aggregation in a cell number-dependent manner with maximal inhibition observed with 1×10^6 PMN and 1×10^5 activated J774 cells ($n = 3$ for each). The inhibitory activity of both cells was dependent on the generation of NO, since it was abolished by haemoglobin and potentiated by superoxide dismutase (SOD; McCall *et al.*, 1989).

Effect of L-arginine analogues on NO release by PMN and J774 cells

The synthesis of NO by PMN (1×10^6) and by J774 cells (1×10^5) was significantly inhibited by L-NIO and L-NMMA in a concentration-dependent and enantiomerically specific manner. In both types of cells L-NIO was more potent than L-NMMA as an inhibitor of NO synthesis (Figure 1). The inhibitory effect of L-NIO (100 μM) was rapid in onset in both

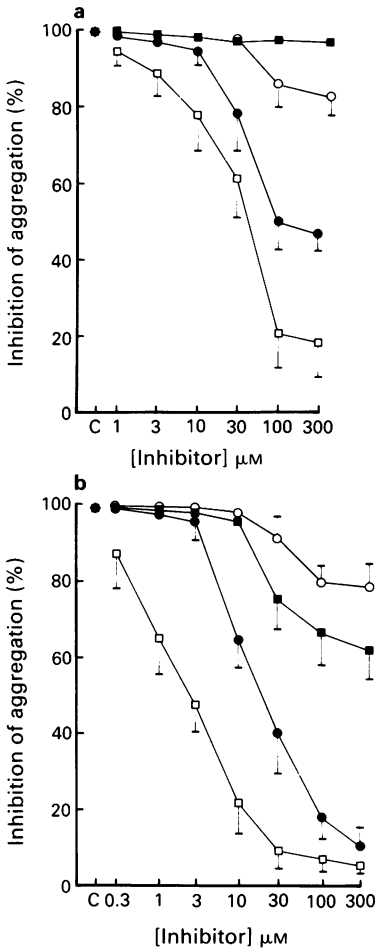

Figure 1 The effect of L-NIO (□), L-NMMA (●), L-NNA (■) and L-NAME (○) on the platelet anti-aggregatory activity of (a) 1×10^6 rat peritoneal PMN and (b) 1×10^5 activated J774 cells. The cells were pre-incubated with L-NIO for 10 min and with the other compounds for 50 min. Each point is the mean of 4–8 separate experiments with s.e.mean shown by vertical bars. C = control.

PMN and J774 cells, being complete within 10 min of pre-incubation (Figure 2). L-NMMA (100 μM), on the other hand, required 50 min of pre-incubation to achieve its full inhibitory activity on both types of cells.

The effect of L-NIO (10 μM) on the synthesis of NO by PMN and J774 cells was irreversible even when a 30 fold molar excess of L-arginine was added 10 min after incubation with the inhibitor ($n = 4$). However, the effect of L-NIO was prevented by concomitant incubation with a 30 fold molar excess of L-arginine ($n = 3$). In contrast, the effect of L-NMMA (300 μM) was fully reversed within 5 min with a 3 fold molar excess of L-arginine ($n = 4$).

The synthesis of NO by J774 cells, but not PMN, was significantly inhibited by L-NNA (Figure 1; $n = 4$). This effect of L-NNA on J774 cells was maximal within 20 min pre-incubation (Figure 2). L-NAME did not cause significant inhibition of NO synthesis by either cell type (Figure 1; $n = 3$–6).

Characteristics of NO synthase from PMN and J774 cells

The synthesis of NO by the cytosolic fraction from PMN and activated J774 cells in the presence of NADPH (100 μM) increased from 10 ± 4 and 12 ± 6 to 152 ± 22 and 181 ± 34 pmol NO min^{-1} mg^{-1} protein respectively ($n = 3$ for each) following addition of L-arginine (30 μM). The K_m for L-arginine of the enzyme from PMN was 3.3 ± 0.8 μM and from J774 cells was 4.2 ± 1.1 μM. The K_m for L-homoarginine of the enzyme from PMN and J774 cells was 15.4 ± 4.4 and

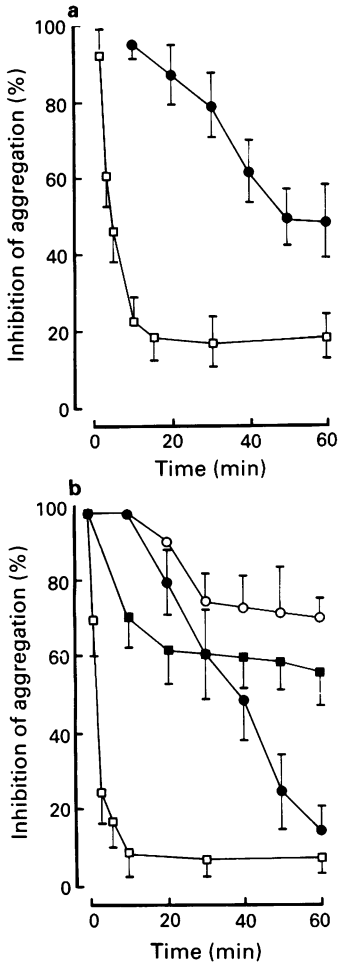

Figure 2 Time course of the effect of (a) L-NIO (□) and L-NMMA (●) on the anti-aggregatory activity of PMN and (b) of L-NIO (□), L-NMMA (●), L-NAME (○) and L-NNA (■) on that of activated J774 cells. All compounds were studied at 100 μM. Each point is the mean of 4–6 separate experiments with s.e.mean shown by vertical bars.

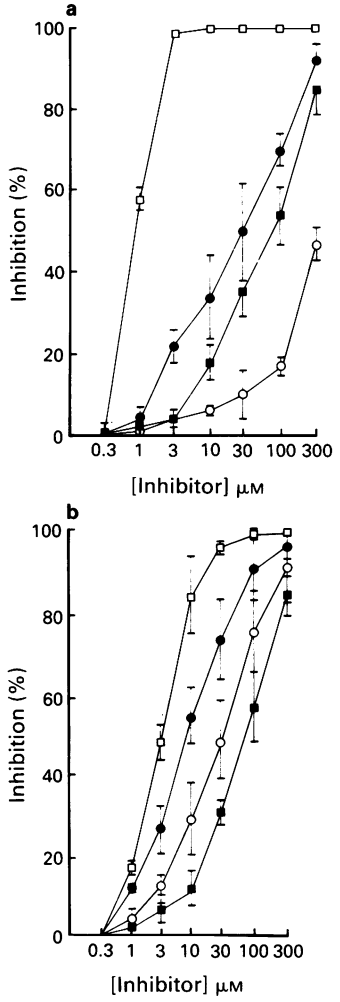

Figure 3 (a) Inhibition by L-NIO (□), L-NMMA (●), L-NNA (■) and L-NAME (○) of the activity of the NO synthase from PMN. Nitric oxide generation by the enzyme was initiated by addition of 30 μM L-arginine. Each point is the mean of 3–5 separate experiments with s.e.mean shown by vertical bars. (b) Inhibition by L-NIO (□), L-NMMA (●), L-NNA (■) and L-NAME (○) of the activity of the NO synthase from J774 cells. Each point is the mean of 3–8 separate experiments with s.e.mean shown by vertical bars.

$13.8 \pm 3.2\,\mu$M respectively. EGTA (100 μM) did not affect the activity of NO synthase from either PMN or J774 cells in the presence of $MgCl_2$ (1 mM; $n = 3$). The NO synthase from both cells was cytosolic, as enzymic activity was not detectable in the pellet following centrifugation at 105,000 g ($n = 3$). The cytosolic preparation from unstimulated J774 cells did not exhibit NO synthase activity ($n = 3$).

Inhibition of NO synthase from PMN and J774 cells by analogues of L-arginine

The NO synthase from PMN and J774 cells in the presence of 30 μM L-arginine was inhibited in a concentration-dependent manner by L-NIO, L-NMMA and L-NNA (Figure 3), with IC_{50} values as indicated in Table 1. The NO synthase from J774 cells was also inhibited in a concentration-dependent manner by L-NAME (Figure 3; Table 1) but this compound caused less than 50% inhibition of NO synthase from PMN at the highest concentration tested (300 μM; $n = 5$). The effects of the compounds were enantiomerically specific since their D-enantiomers did not alter enzyme activity ($n = 3$ for each).

Table 1 Potency of inhibitors of NO synthase from PMN and activated J774 cells in the presence of 30 μM L-arginine ($n = 3$–5)

	Inhibitor IC_{50} (μM)	
	PMN	*J774*
L-NIO	0.8 ± 0.1	3 ± 0.5
L-NMMA	30 ± 4	7.5 ± 1.2
L-NNA	80 ± 8	85 ± 9
L-NAME	<50% inhibition at 300 μM	28 ± 5

L-NIO = N-iminoethyl-L-ornithine; L-NMMA = N^G-monomethyl-L-arginine; L-NNA = N^G-nitro-L-arginine; L-NAME = N^G-nitro-L-arginine methyl ester.

The inhibitory effect of L-NIO (1 μM) on the NO synthase from PMN and from J774 cells (3 μM) was not reversed by L-arginine, even when a 300 fold molar excess was added 3 min after starting the incubation ($n = 4$). In contrast, the inhibitory action of equi-effective concentrations of L-NMMA, L-NAME or L-NNA was reversed by L-arginine (300 μM; Figure 4; $n = 3$–7).

Discussion

Rat peritoneal neutrophils and activated J774 cells inhibited platelet aggregation in a cell number-dependent manner and this activity was potentiated by SOD and inhibited by haemoglobin. Furthermore, the anti-aggregatory activity was inhibited by the L-arginine analogue L-NMMA, an established inhibitor of NO generation in the endothelial cell (Palmer *et*

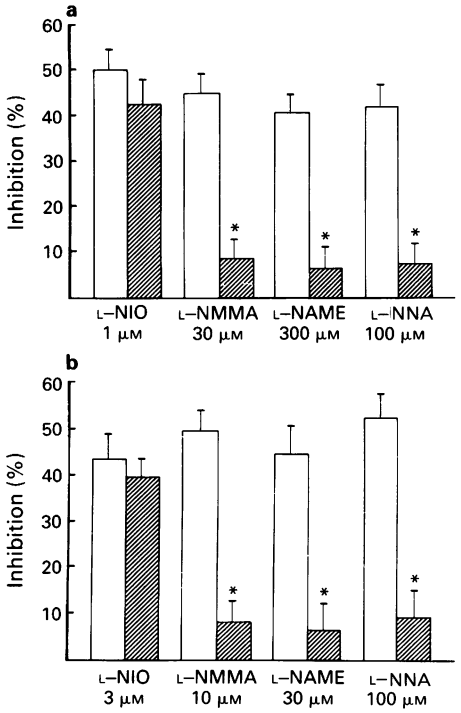

Figure 4 Reversal by L-arginine (300 μM; hatched columns) of the inhibition of NO synthesis in the presence of 30 μM L-arginine (open columns) by equieffective concentrations of L-NIO, L-NMMA, L-NAME and L-NNA in (a) PMN and (b) J774 cells. Each column is the mean from 3–7 separate determinations with s.e.mean shown by vertical bars.

al., 1988) and mouse peritoneal macrophages (Hibbs *et al.*, 1987). These data indicate that both rat peritoneal neutrophils and activated J774 cells synthesize NO.

L-NIO was the most potent inhibitor of the generation of NO in both cell types. In contrast, L-NNA only weakly inhibited NO synthesis by J774 cells and L-NAME had no significant effect on either cell. This profile of inhibition differs from that described in adrenal glands (Palacios *et al.*, 1989), brain (Knowles *et al.*, 1990), vascular endothelial cells (Rees *et al.*, 1990) and human platelets (Radomski *et al.*, 1990).

The effect of L-NIO was rapid in onset in both cell types, whereas L-NMMA required approximately 50 min preincubation before maximal activity was observed. The effect of L-NIO was prevented by concomitant incubation with L-arginine. These observations suggest that there are different mechanisms for uptake of L-arginine analogues in phagocytic cells. Since there is no evidence for substantial differences in the uptake of these compounds into endothelial cells or platelets (Rees *et al.*, 1990; Radomski *et al.*, 1990), further work is required to clarify the mechanisms underlying these variations in different cells.

Two different types of NO synthase have now been recognized. One is cytosolic, Ca^{2+}- and NADPH-dependent and generates NO as a transduction mechanism for stimulation of the soluble guanylate cyclase in vascular endothelium (Palmer & Moncada, 1989), brain (Knowles *et al.*, 1989), adrenal gland (Palacios *et al.*, 1989), platelets (Radomski *et al.*, 1990) and probably other tissues. The second type is present in macrophages, is cytosolic and NADPH- but not Ca^{2+}-dependent (Marletta *et al.*, 1988; Kwon *et al.*, 1989). Furthermore, in macrophages, the enzyme also requires tetrahydrobiopterin (Kwon *et al.*, 1989; Tayeh & Marletta, 1989) and is inducible, releasing NO as part of the cytotoxic functions of these cells (Hibbs *et al.*, 1988). Although circulating human and rabbit neutrophils release small amounts of NO (McCall *et al.*, 1990), it is likely that the enzyme in the cells used in the present study was also induced by the process of migration into the peritoneal cavity.

Our present results support the existence of two different NO synthases since, although both enzymes are inhibited by L-NIO and L-NMMA, the former is a more potent and irreversible inhibitor of the enzyme in phagocytic cells. Furthermore, L-NNA and L-NAME are only weak inhibitors of the NO synthase from phagocytic cells, in contrast to findings in endothelial cells. In addition all these compounds, including L-NIO, are reversible by L-arginine in endothelial cells.

In summary, we have identified L-NIO as a novel, potent and irreversible inhibitor of NO generation in phagocytic cells, which is rapidly taken up by intact cells. If L-NIO exhibits the same characteristics after administration *in vivo*, this compound may be a useful tool to investigate selectively the biological relevance of the production of NO by phagocytic cells.

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Stimulation of neutrophil adherence to vascular endothelial cells by histamine and thrombin and its inhibition by PAF antagonists and dexamethasone

[1]Masako Watanabe, Mikio Yagi, Mitsugu Omata, Noriyasu Hirasawa, *Suetsugu Mue, †Susumu Tsurufuji & Kazuo Ohuchi

Department of Biochemistry, Faculty of Pharmaceutical Sciences, Tohoku University, Aoba Aramaki, Aoba-ku, Sendai, Miyagi 980, Japan; *College of Medical Science, Tohoku University School of Medicine, Seiryo-cho, Aoba-ku, Sendai, Miyagi 980, Japan and †Institute of Cytosignal Research, Inc., Ohmori, Ohta-ku, Tokyo 143, Japan

1 In order to clarify the roles of platelet-activating factor (PAF) in histamine- and thrombin-induced neutrophil adhesion to vascular endothelial cells, the effects of several PAF antagonists were examined. The effects of the glucocorticoid dexamethasone were also examined in order to gain further insight into the anti-inflammatory actions of glucocorticoids.

2 In culture, histamine and thrombin stimulated the adherence of rat peritoneal neutrophils to human endothelial cells from the umbilical vein. They did not stimulate neutrophil adherence in the absence of endothelial cells, suggesting that the target cells for the histamine- and thrombin-induced adherence of neutrophils were endothelial cells, not neutrophils.

3 Several PAF antagonists, such as CV-3988, L-652,731 and Y-24,180 inhibited the histamine- and thrombin-induced neutrophil adherence in a concentration-dependent manner. Indomethacin failed to inhibit it.

4 Dexamethasone, a steroidal anti-inflammatory drug, did not inhibit the histamine- and thrombin-induced adherence of neutrophils to endothelial cells when the drug was present only during the 20 min incubation period for the adherence assay. When the endothelial cells were preincubated for 3 h with dexamethasone, the adherence of neutrophils to endothelial cells induced by histamine or thrombin was not inhibited.

5 When the neutrophils were preincubated for 3 h with dexamethasone, the histamine- and thrombin-induced adherence of neutrophils to endothelial cells was inhibited.

6 Our studies indicate that: (a) adherence of neutrophils to endothelial cells induced by histamine and thrombin is mediated by PAF production since PAF antagonists inhibited the adherence of neutrophils; and (b) neutrophils, not endothelial cells, are the target cells through which dexamethasone acts to inhibit adherence.

Introduction

The adhesion of circulating neutrophils to vascular endothelial cells may play a crucial role in the acute inflammatory responses. The mechanism of the adhesion process has not yet been clarified. Vascular endothelial cells produce various mediators which may regulate neutrophil adhesion, such as endothelial-leukocyte adhesion molecule-1 (ELAM-1) (Pober *et al.*, 1986; Bevilacqua *et al.*, 1989) and intercellular adhesion molecule-1 (ICAM-1) (Dustin *et al.*, 1986; Rothlein *et al.*, 1986). The other strong candidate for neutrophil adhesion is platelet-activating factor (PAF). PAF is reported to be synthesized after stimulation by thrombin (Prescott *et al.*, 1984), histamine, bradykinin, adenosine triphosphate (McIntyre *et al.*, 1985), leukotrienes C_4 and D_4 (McIntyre *et al.*, 1986) and interleukin-1 (Bussolino *et al.*, 1986). Among them, thrombin (Zimmerman *et al.*, 1985a) and leukotrienes C_4 and D_4 (McIntyre *et al.*, 1986) make endothelial cells adherent to neutrophils in a way which is compatible with the time course and dose-dependency of PAF production. Consequently, it is strongly suggested that PAF produced in the endothelial cells by such stimulators plays a significant role in neutrophil adhesion to endothelial cells.

In the present investigations, we found that histamine, like thrombin, also stimulated neutrophil adherence to endothelial cells. In order to clarify further the significance of the role of PAF in neutrophil adherence to endothelial cells, the effects of several PAF antagonists such as CV-3988, L-652,731 and Y-24,180 on histamine- and thrombin-induced neutrophil adherence were examined.

Steroidal anti-inflammatory drugs inhibit neutrophil infiltration into the inflammatory locus (Ohuchi *et al.*, 1982; 1984). Although several reports have suggested that glucocorticoids inhibit neutrophil accumulation at inflammatory sites by inhibiting production of chemoattractants (Kurihara *et al.*, 1984; Tsurufuji *et al.*, 1984; Schleimer *et al.*, 1989), the precise mechanism for the inhibition of neutrophil infiltration by steroidal anti-inflammatory drugs has not yet been clarified. We studied whether histamine- and thrombin-induced neutrophil adherence to endothelial cells is inhibited by the steroidal anti-inflammatory drug dexamethasone, in order to gain further insight into the mechanism of anti-inflammatory activity of dexamethasone.

Methods

Human endothelial cell culture

Human endothelial cells were isolated from the umbilical vein with 0.25% trypsin (Flow Laboratories, McLean, VA, U.S.A.)–0.01% EDTA in phosphate-buffered saline (PBS) solution, and cultured in 75-cm^2 tissue culture flasks (Iwaki Glass, Tokyo, Japan) according to a slight modification of the method described by Jaffe *et al.* (1973b) and Gimbrone (1976). Primary cultures of endothelial cells were grown in medium 199 (M199) (Nissui Seiyaku Co., Tokyo, Japan) containing 20% foetal bovine serum (FBS) (Flow Laboratories) supplemented with amphotericin B solution (1%) (Flow

[1] Author for correspondence.

Laboratories), endothelial mitogen (20 $\mu g\,ml^{-1}$) (Biomedical Technologies, Inc., Stoughton, MA, U.S.A.), porcine intestinal heparin (90 $\mu g\,ml^{-1}$) (Novo Industries A/S, Denmark), penicillin G potassium (18 $\mu g\,ml^{-1}$) and streptomycin sulphate (50 $\mu g\,ml^{-1}$) (Meiji Seika Co., Tokyo, Japan). Culture flasks were incubated at 37°C in an atmosphere of 5% carbon dioxide (CO_2) in air. The culture media were changed every 3 days. When primary cultures were almost confluent, endothelial cells were harvested from flasks by treatment with 0.25% trypsin, and plated into well-plates with a 16-mm diameter (Tissue Culture Cluster 24, Costar, Cambridge, MA, U.S.A.). Subcultures were grown in M199 containing 10% FBS supplemented with the reagents used for primary cultures. The cells were used at the first passage in each experiment. When monolayers of endothelial cells were tightly confluent, they were used for assay of endothelial cell-neutrophil adhesion. Cultures were characterized as endothelial cells based on morphological criteria (Jaffe *et al.*, 1973b) and by indirect immunofluorescence using a specific antiserum to human factor VIII antigen (ICN Biomedicals Inc., Costa Mesa, CA, U.S.A.), a usual marker of endothelial cells (Jaffe *et al.*, 1973a).

Preparation of rat neutrophils

Male rats (350–500 g, Sprague-Dawley strain, specific pathogen-free, Charles River Japan Inc., Kanagawa, Japan) were injected i.p. with Ca^{2+}-free Krebs-Ringer bicarbonate buffer (pH 7.4, 12 ml $100\,g^{-1}$ body weight) containing 1% casein (from milk, vitamin-free, Wako Pure Chemical Ind., Tokyo, Japan) under light diethyl ether anaesthesia. After 15 h, rats were killed under diethyl ether anaesthesia by cutting the carotid artery, and peritoneal fluid was collected. The collected fluid was centrifuged at 250 *g* for 3 min at 4°C, and the cell pellet was washed twice with PBS and once with M199. Cells were finally suspended in M199 containing 0.1% (w/v) essentially fatty acid-free bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO, U.S.A.), at a concentration of 6×10^6 cells ml^{-1}. Under microscopic observation after staining with haematoxylin and eosin, more than 95% of the cells were identified as neutrophils.

Adhesion of neutrophils to gelatin-coated dishes

Neutrophils (3×10^6 cells) in 1.0 ml of the M199-BSA solution containing various concentrations of histamine or thrombin were incubated in each well of gelatin-coated plates with a 16-mm well diameter (Iwaki Glass) at 37°C for 20 min in an atmosphere of 5% CO_2 in air. After the incubation, the wells were washed 3 times with 0.5 ml of M199 to remove non-adherent neutrophils, then 0.4 ml of 0.25% trypsin-EDTA in PBS solution was added, and the samples were incubated for 10 min at 37°C to detach the adherent neutrophils from the wells. After an addition of 0.1 ml of M199 containing 10% calf serum (Flow Laboratories Inc.), the neutrophils were suspended by use of siliconized Pasteur pipettes, and were counted in a haemocytometer.

Endothelial cell-neutrophil adhesion assay

Monolayers of endothelial cells which were tightly confluent were washed twice with 0.5 ml of M199 at 37°C. Then 0.5 ml of neutrophil suspension (3×10^6 cells 0.5 ml^{-1}) was layered over the endothelial cells, 0.5 ml of the medium containing various concentrations of reagents was added, and the samples were incubated at 37°C for 20 min in an atmosphere of 5% CO_2 in air. After the incubation, the wells were washed 3 times with 0.5 ml of M199 to remove non-adherent neutrophils, then 0.4 ml of 0.25% trypsin–0.01% EDTA in PBS solution was added and the samples were incubated for 10 min at 37°C to detach the endothelial cells from the wells and the neutrophils from the endothelial cells. After addition of 0.1 ml of M199 containing 10% calf serum (Flow Laboratories, Inc.), endothelial cells and neutrophils were suspended by use of siliconized Pasteur pipettes. The neutrophils and endothelial cells were counted in a haemocytometer. Neturophil adhesion is expressed as the number of neutrophils that adhered to 100 endothelial cells.

Chemicals

Human plasma thrombin (Sigma Chemical Co.) and histamine (free base, Wako Pure Chemical Ind.) were dissolved in the M199-BSA solution. PAF (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, a mixture of C_{16} and C_{18} forms, Avanti Polar Lipids Inc., Birmingham, AL, U.S.A.) was dissolved in ethanol and an aliquot of the ethanol solution was added to the M199-BSA solution. Synthetic PAF antagonists, CV-3988 ((**RS**)-2-methoxy-3-(octadecylcarbamoyloxy) propyl-2-(3-thiazolo) ethyl phosphate) (Terashita *et al.*, 1983), L-652,731 (*trans* - 2,5 - bis - (3,4,5 - trimethoxyphenyl) tetrahydrofuran) (Hwang *et al.*, 1985) and Y-24,180 ((±)-4-(2-chlorophenyl)-2-[2-(4-isobutylphenyl)ethyl]-6,9-dimethyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepine (Terasawa *et al.*, 1990) were dissolved in ethanol or dimethylsulphoxide (DMSO), and an aliquot of the solution was added to the M199-BSA solution. Dexamethasone (Sigma Chemical Co.) and indomethacin (Sigma Chemical Co.) were dissolved in ethanol, and an aliquot of the solution was added to the M199-BSA solution. The final concentration of ethanol or DMSO in the M199-BSA solution was adjusted to 0.1% (v/v). Control medium contained the same amount of the vehicle.

Statistical analysis

Results were analyzed for statistical significance by Student's *t* test for paired observations.

Results

Effects of thrombin, histamine and PAF on neutrophil adherence to vascular endothelial cells

Figure 1 shows the effects of thrombin, histamine and PAF on the adherence of rat peritoneal neutrophils to human endothelial cells from the umbilical vein. Thrombin (1 unit ml^{-1}) and histamine (10^{-5} M) increased the adherence about two fold. PAF at 10^{-7} M was not as active as thrombin and histamine. These drugs increased the adherence in a concentration-

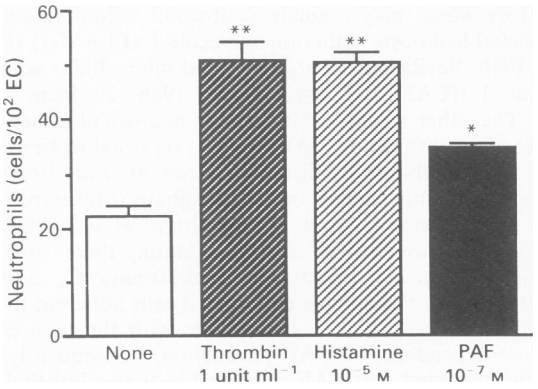

Figure 1 Effects of thrombin, histamine and PAF on neutrophil adhesion to endothelial cells. Neutrophils (3×10^6 cells) in 1 ml of M199 supplemented with 0.1% (w/v) BSA (M199-BSA solution) which contained thrombin (1 unit ml^{-1}), histamine (10^{-5} M) or PAF (10^{-7} M) were incubated with the monolayer of endothelial cells (2×10^5 cells) at 37°C for 20 min. Neutrophil adhesion is expressed as the number of neutrophils that adhered to 100 endothelial cells (EC). Values are the means from 5 to 6 samples with s.e.mean shown by vertical bars. Statistical significance: * $P < 0.01$; ** $P < 0.001$ vs. 'None'. Comparable results were obtained in two additional experiments.

dependent manner. Significant increases in the adherence were observed at concentrations of more than 0.1 units ml^{-1} for thrombin, 10^{-6} M for histamine and 10^{-8} M for PAF (data not shown). Leukotriene B_4 failed to stimulate adherence even at a concentration of 3×10^{-6} M (data not shown).

Effects of thrombin, histamine and PAF on neutrophil adherence to gelatin-coated dishes

To identify the target cells for thrombin, histamine and PAF, neutrophils plus the medium containing one of each of the stimulants were incubated at 37°C for 20 min in gelatin-coated dishes in the absence of endothelial cell monolayers. As shown in Figure 2, thrombin at concentrations of 0.01 to 1 unit ml^{-1} did not affect adherence of neutrophils to gelatin-coated dishes. Histamine at concentrations of 10^{-7} to 10^{-5} M significantly decreased the adherence in a concentration-dependent manner. In contrast, PAF at 10^{-7} M significantly stimulated the adherence. A significant increase in neutrophil adherence was also observed at 10^{-8} M PAF (data not shown).

Effects of PAF antagonists on neutrophil adherence to vascular endothelial cells

The effects of the specific PAF antagonists, CV-3988, L-652,731 and Y-24,180, on thrombin-induced (1 unit ml^{-1}) and histamine-induced (10^{-5} M) neutrophil adherence to endothelial cells are shown in Figures 3, 4, and 5, respectively. These PAF antagonists inhibited both thrombin- and histamine-induced neutrophil adherence in a concentration-dependent manner. Among the three antagonists, Y-24,180 was the most active. Higher concentrations of PAF antagonists tended to inhibit the spontaneous adhesion of neutrophils. The PAF antagonists showed no cytotoxicity to either type of cell as revealed by the dye exclusion test.

Effects of dexamethasone on thrombin- and histamine-induced adherence of neutrophils to vascular endothelial cells

When endothelial cell monolayers were incubated at 37°C for 20 min with neutrophils in the medium containing thrombin (1 unit ml^{-1}) or histamine (10^{-5} M) and various concentrations of dexamethasone (3×10^{-8} to 3×10^{-6} M), neutrophil adherence induced by thrombin or histamine was not affected by the dexamethasone (data not shown). Furthermore, no inhibition of neutrophil adherence was seen when endothelial cell monolayers were preincubated for 3 h in medium containing various concentrations of dexamethasone (3×10^{-8} to 3×10^{-6} M), washed with fresh dexamethasone-free medium, and incubated with neutrophils at 37°C for 20 min in dexamethasone-free medium containing thrombin (1 unit ml^{-1}) or histamine (10^{-5} M) (Figure 6). Neutrophil adherence was not inhibited when the above experiment was repeated with exposure to dexamethasone throughout and with incubation in the conditioned medium derived from the initial incubation with endothelial cells. However, as shown in Figure 7, when neutrophils were preincubated for 3 h in the presence of various concentrations of dexamethasone (3×10^{-8} to 3×10^{-6} M), washed with fresh dexamethasone-free medium, and incubated with endothelial cell monolayers at 37°C for 20 min in the presence of thrombin (1 unit ml^{-1}) or histamine (10^{-5} M) but the absence of dexamethasone, neutrophil adherence to endothelial cells induced by thrombin and histamine was significantly inhibited.

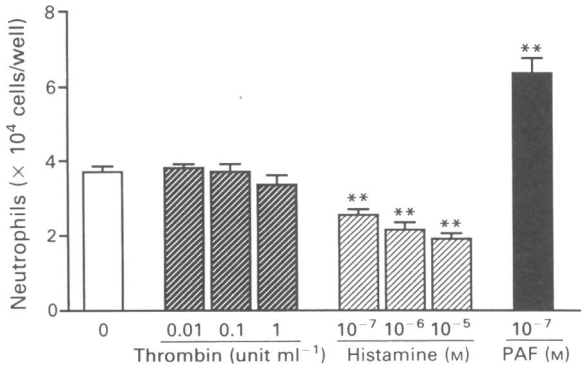

Figure 2 Effects of thrombin, histamine and PAF on neutrophil adhesion to gelatin-coated dishes. Neutrophils (3×10^6 cells) in 1 ml of the M199-BSA solution containing thrombin (1 unit ml^{-1}), histamine (10^{-5} M) or PAF (10^{-7} M) were incubated at 37°C for 20 min in gelatin-coated dishes with a 16 mm diameter, and the number of neutrophils adhering to the dishes was counted. Values are the means from 5 to 6 samples with s.e.mean shown by vertical bars. Statistical significance: ** $P < 0.001$ vs. control. Comparable results were obtained in two additional experiments.

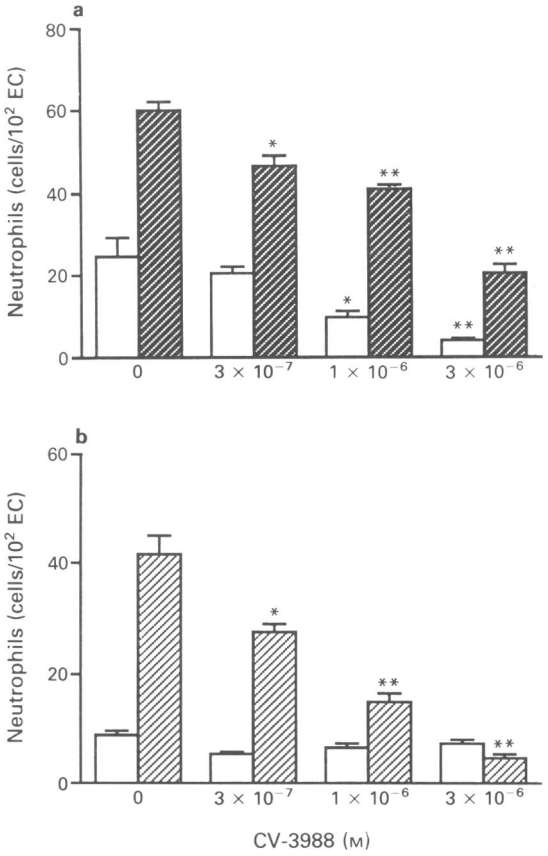

Figure 3 Effects of CV-3988 on thrombin- and histamine-induced adhesion of neutrophils to endothelial cells. Neutrophils (3×10^6 cells) in 1 ml of the M199-BSA solution containing 1 unit ml^{-1} of thrombin (a) or 10^{-5} M of histamine (b) and indicated concentrations of CV-3988 were incubated with a monolayer of endothelial cells (2×10^5 cells) at 37°C for 20 min. Open columns represent the medium in the absence of stimulant. Hatched columns represent the medium which contained thrombin (a) and histamine (b). Values are the means from 5 to 6 samples with s.e.mean shown by vertical bars. Statistical significance: * $P < 0.01$; ** $P < 0.001$ vs. corresponding control. Comparable results were obtained in two additional experiments.

Effects of indomethacin on thrombin- and histamine-induced adherence of neutrophils to vascular endothelial cells

Thrombin-stimulated (1 unit ml^{-1}) neutrophil adherence was not inhibited when endothelial cell monolayers were incubated at 37°C for 20 min with neutrophils in the medium containing various concentrations of indomethacin (3×10^{-8} to

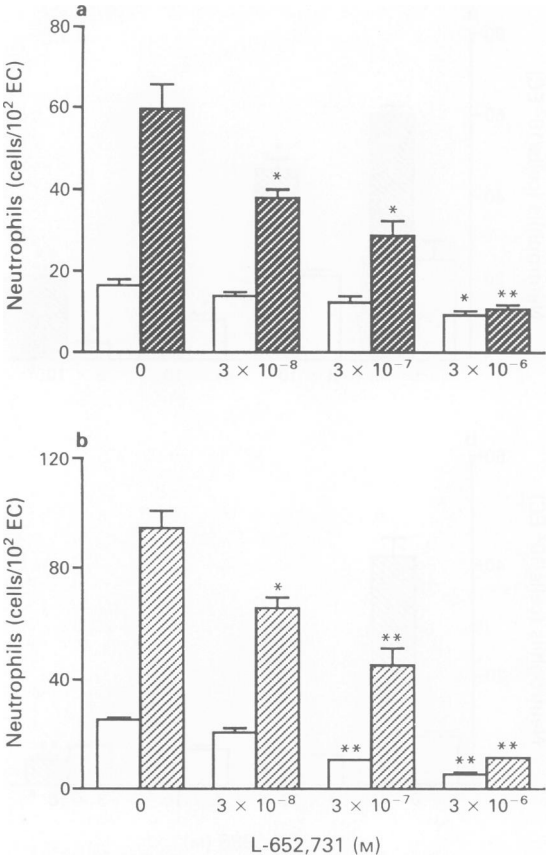

Figure 4 Effects of L-652,731 on thrombin- and histamine-induced adhesion of neutrophils to endothelial cells. Neutrophils (3×10^6 cells) in 1 ml of the M199-BSA solution containing 1 unit ml^{-1} of thrombin (a) or 10^{-5} M of histamine (b) and indicated concentrations of L-652,731 were incubated with a monolayer of endothelial cells (2×10^5 cells) at 37°C for 20 min. Open columns represent the stimulant-free medium. Hatched columns represent the medium which contained thrombin (a) and histamine (b). Values are the means from 5 to 6 samples with s.e.mean shown by vertical bars. Statistical significance: * $P < 0.01$; ** $P < 0.001$ vs. corresponding control. Comparable results were obtained in two additional experiments.

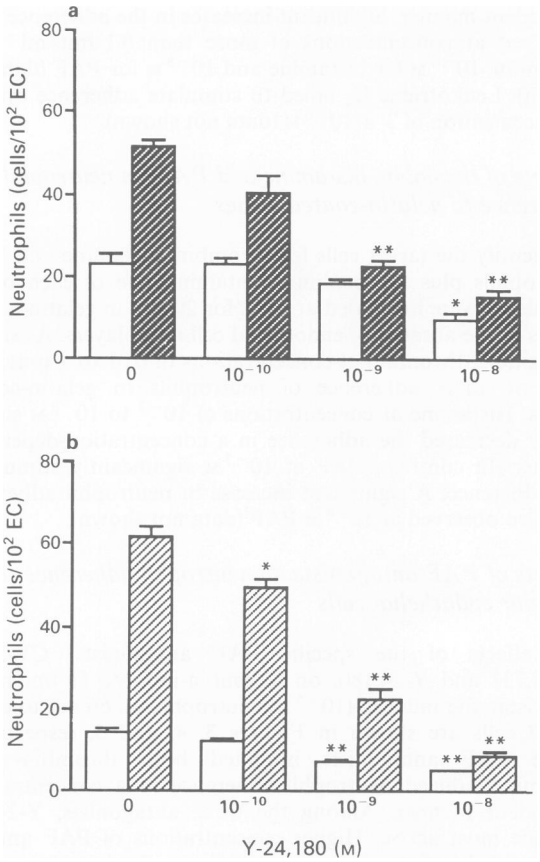

Figure 5 Effects of Y-24,180 on thrombin- and histamine-induced adhesion of neutrophils to endothelial cells. Neutrophils (3×10^6 cells) in 1 ml of the M199-BSA solution containing 1 unit ml^{-1} of thrombin (a) or 10^{-5} M of histamine (b) and indicated concentrations of Y-24,180 were incubated with a monolayer of endothelial cells (2×10^{-5} cells) at 37°C for 20 min. Open columns represent the stimulant-free medium. Hatched columns represent the medium which contained thrombin (a) and histamine (b). Values are the means from 5 to 6 samples with s.e.mean shown by vertical bars. Statistical significance: * $P < 0.01$; ** $P < 0.001$ vs. corresponding control. Comparable results were obtained in two additional experiments.

3×10^{-6} M) (data not shown). Furthermore, when endothelial cell monolayers or neutrophils were preincubated with indomethacin at the same range of concentrations for 1 h, thrombin-induced (1 unit ml^{-1}) or histamine-induced (10^{-5} M) neutrophil adherence was not inhibited by indomethacin at concentrations up to 3×10^{-6} M (data not shown).

Discussion

Preliminary experiments revealed that the adherence rate of peripheral neutrophils from healthy volunteers to vascular endothelial cells derived from one donor differed greatly from volunteer to volunteer. Therefore, in the present investigations, in order to minimize individual differences of neutrophil adherence, rat peritoneal neutrophils were used instead of human peripheral neutrophils. Furthermore, in order to minimize changes in characteristics of vascular endothelial cells caused by a long incubation period with many passages, primary cultured endothelial cells from different human donors were used in each experiment. Consequently, depending upon the differences in the donated umbilical veins, the adherence rate of neutrophils to vascular endothelial cells changed from experiment to experiment. But the inter-experimental difference in the neutrophil adherence rate was much less than that obtained by use of human neutrophils and human vascular endothelial cells.

In the present investigations, the effects of several drugs on adherence of rat peritoneal neutrophils to human endothelial cells from the umbilical vein were examined in order to gain further insight into the mechanism of neutrophil adherence and the anti-inflammatory activity of the glucocorticoid, dexamethasone. In this study, we showed that both histamine and thrombin stimulated adherence of rat peritoneal neutrophils to vascular endothelial cells from human umbilical veins. However, when rat peritoneal neutrophils were incubated in gelatin-coated plastic dishes which did not contain vascular endothelial cells, the adherence was not stimulated by thrombin treatment (Figure 2). These results indicate that thrombin-stimulated neutrophil adherence to vascular endothelial cells is dependent on the endothelial cells and does not result from direct activation of the neutrophils by thrombin, as suggested by Zimmerman *et al.* (1985a). In contrast, incubation of neutrophils in the medium containing histamine resulted in a concentration-dependent inhibition of adherence to gelatin-coated plastic dishes (Figure 2). Since histamine is reported to inhibit neutrophil adherence to glass beads by a histamine H_2 receptor-dependent mechanism (Robinson, 1982), the concentration-dependent inhibition to gelatin-coated dishes might be induced by the same mechanism. However, when neutrophils were incubated on a monolayer of endothelial cells in the presence of histamine, neutrophil adhesion to endothelial cells was significantly stimulated (Figure 1). This result indicates that endothelial cells might produce some potent stimulator for adherence which counteracts the H_2

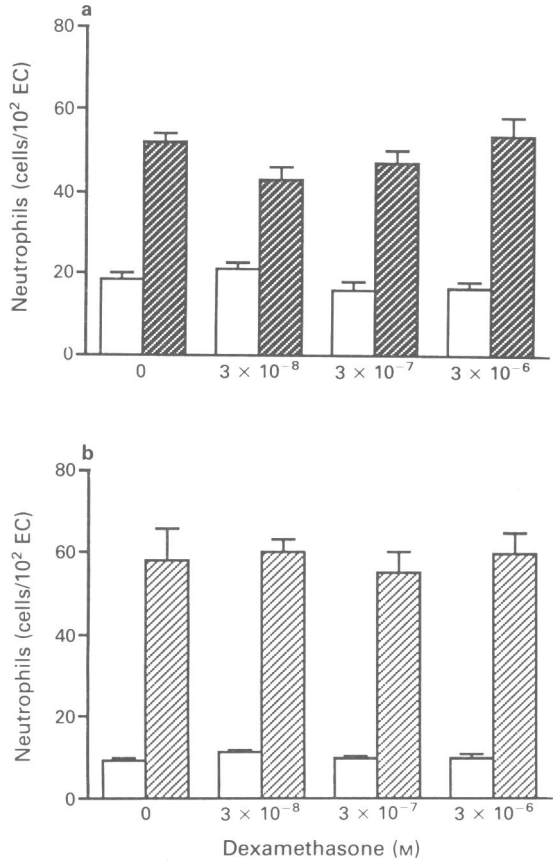

Figure 6 Effects of dexamethasone treatment of endothelial cells on thrombin- and histamine-induced adhesion of neutrophils to endothelial cells. Endothelial cell monolayers (2×10^5 cells) were incubated at 37°C for 3 h in 1 ml of the M199-BSA solution containing indicated concentrations of dexamethasone, washed and further incubated in the medium containing neutrophils (3×10^6 cells) and 1 unit ml^{-1} of thrombin (a) or 10^{-5} M of histamine (b). Open columns represent the stimulant-free medium. Hatched columns represent the medium which contained thrombin (a) and histamine (b). Values are the means from 5 to 6 samples with s.e.mean shown by vertical bars. Comparable results were obtained in three additional experiments.

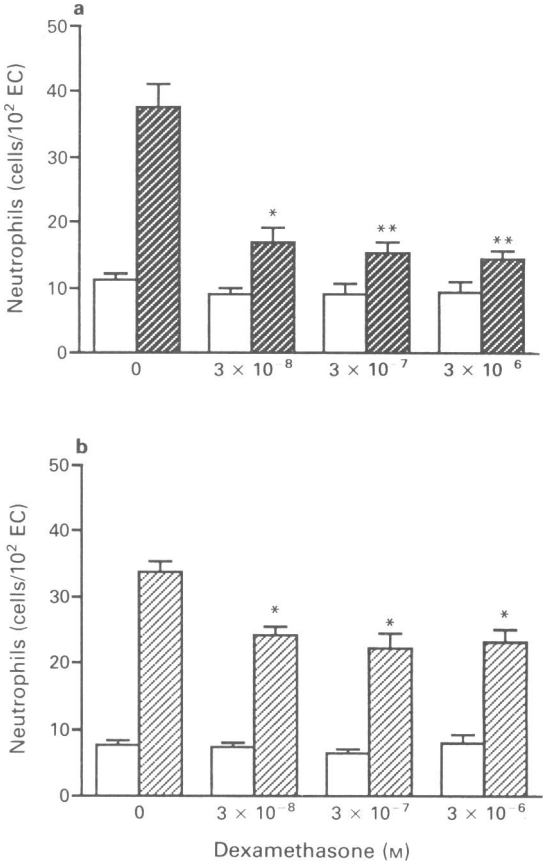

Figure 7 Effects of dexamethasone treatment of neutrophils on thrombin- and histamine-induced neutrophil adhesion to endothelial cells. Neutrophils (3×10^6 cells) were incubated at 37°C for 3 h in 1 ml of the M199-BSA solution containing indicated concentrations of dexamethasone, washed and further incubated with endothelial cell monolayer (2×10^5 cells) in 1 ml of the M199-BSA solution containing 1 unit ml^{-1} of thrombin (a) or 10^{-5} M histamine (b). Open columns represent the stimulant-free medium. Hatched columns represent the medium which contained thrombin (a) and histamine (b). Values are the means from 5 to 6 samples with s.e.mean shown by vertical bars. Statistical significance: * $P < 0.01$; ** $P < 0.001$ vs. corresponding control. Comparable results were obtained in three additional experiments.

receptor-induced inhibition of adherence. In contrast, LTB_4 failed to induce neutrophil adherence to endothelial cells (data not shown). The lack of effect of LTB_4 could be because rat neutrophils are weak responders to this lipid mediator (Kreisle *et al.*, 1985). It is reported that histamine (McIntyre *et al.*, 1985) as well as thrombin (Prescott *et al.*, 1984; Zimmerman *et al.*, 1987) produces PAF in endothelial cells, and exogenous PAF stimulates adhesion of neutrophils to vascular endothelial cells in hamster cheek pouch microcirculation (Dillon *et al.*, 1988). Furthermore, Zimmerman *et al.* (1987) demonstrated that in thrombin-stimulated human endothelium, PAF synthesis and neutrophil adhesion to the endothelial cells were tightly coupled events. Consequently, it is conceivable that histamine as well as thrombin stimulates PAF generation in endothelial cells and promotes neutrophil adherence. In order to investigate this idea, effects of several PAF antagonists or histamine- and thrombin-induced neutrophil adherence to endothelial cells were examined.

Since thrombin- and histamine-induced adherence of neutrophils to vascular endothelial cells was inhibited by several PAF antagonists in a concentration-dependent manner (Figures 3, 4 and 5), PAF produced by vascular endothelial cells or by neutrophils might be responsible for the neutrophil adherence to vascular endothelial cells. However, in human polymorphonuclear leukocytes (PMN), thrombin at 10 units ml^{-1} or histamine at 50 μM failed to induce PAF production (Sisson *et al.*, 1987). Consequently, it is possible that vascular endothelial cells produce PAF, which in turn makes neutrophils adherent to the endothelial cells. This idea is also supported by Zimmerman *et al.* (1990) demonstrating that L-652,731 inhibited the binding of human neutrophils to human endothelial cells activated by thrombin but not to endothelial cells activated by tumor necrosis factor α. Most of the PAF produced by endothelial cells remains cell-associated and it has been suggested that PAF is appropriately located on the endothelial cells to interact with neutrophils (Prescott *et al.*, 1984; McIntyre *et al.*, 1986; Zimmerman *et al.*, 1990). However, in interleukin-1-induced adhesion of PMN to cultured human endothelial cells, Breviario *et al.* (1988) reported that PAF production by endothelial cells did not significantly contribute to PMN adhesion because CV-3988 acts as an inhibitor in paraformaldehyde-fixed endothelial cells. They considered the inhibitory effect of CV-3988 was directed at PAF produced by the PMN themselves in interleukin-1-stimulated adherence. We reported that in an allergic inflammation model of air pouch type in rats (Tsurufuji *et al.*, 1982), neutrophil infiltration into the pouch fluid was significantly inhibited by PAF antagonists (Sugidachi *et al.*, 1988), suggesting that PAF generated in the locus of allergic inflammation plays some significant role in neutrophil infiltration *in vivo*.

As to the effects of indomethacin, Zimmerman *et al.* (1985b) found that PAF production was enhanced by treatment of endothelial cells with indomethacin, indicating that endogenously generated prostacyclin might modulate PAF synthesis.

However, in the present investigations, indomethacin treatment did not affect neutrophil adherence to endothelial cells even at a concentration of 3×10^{-6} M (data not shown).

As to the action of steroidal anti-inflammatory drugs on neutrophil adhesion to vascular endothelial cells, Schleimer *et al.* (1989) observed that 24 h culture of human neutrophils with dexamethasone failed to inhibit the adherence induced by either leukocyte activators, such as formyl-methionyl-leucyl-phenylalanine and PAF, or endothelial activators, such as interleukin-1, lipopolysaccharide or 12-*O*-tetradecanoylphorbol-13-acetate. However, judging from the half-life of neutrophils, the 24 h of culture might be too long to examine the adherence ability of neutrophils. In contrast, the oral administration of prednisone in humans significantly inhibited the adherence of peripheral neutrophils to nylon-wool columns when examined 4 h after prednisone administration (Clark *et al.*, 1979). Furthermore, Rosenbaum *et al.* (1986) reported that spontaneous adherence of rabbit peripheral PMN to rabbit coronary microvascular endothelial cells was reduced when the leukocytes were pretreated with 10^{-7} M dexamethasone for 4 h. However, when the rabbit coronary microvascular endothelial cells were pretreated with dexamethasone, granulocyte adhesion was not inhibited. In the present investigations, we showed that when human endothelial cells were pretreated with dexamethasone for 3 h, neutrophil adhesion induced by thrombin or histamine was not inhibited (Figure 6), but when neutrophils were pretreated with dexamethasone for 3 h, thrombin- and histamine-induced neutrophil adhesion to vascular endothelial cells was significantly inhibited (Figure 7). These results strongly suggest that dexamethasone suppresses histamine- and thrombin-induced neutrophil adherence to vascular endothelial cells and thus may inhibit neutrophil infiltration into the inflammatory locus. Probable target cells for glucocorticoids might be neutrophils, but not vascular endothelial cells. Our preliminary experiments revealed that the inhibitory effect of dexamethasone did not become apparent when endothelial cells were pretreated for 1 h with dexamethasone. The requirement of a time lag before manifesting the effect of dexamethasone might suggest that biosynthesis of certain proteins is necessary for the suppression of neutrophil adherence.

If phospholipase A_2 is exclusively essential for PAF synthesis in vascular endothelial cells, glucocorticoid treatment of vascular endothelial cells might suppress PAF production by inhibiting phospholipase A_2 (Parente & Flower, 1985). However, dexamethasone treatment of vascular endothelial cells failed to inhibit neutrophil adherence (Figure 6), suggesting that dexamethasone could not reduce PAF production by inhibiting phospholipase A_2. It is possible that the alternative physiological pathway involving choline phosphotransferase (Lee *et al.*, 1986) may subserve the generation of intracellular PAF in vascular endothelial cells.

It has been suggested that in thrombin-induced neutrophil adherence to vascular endothelial cells, CDw18 antigen expression on neutrophils plays a significant role in the neutrophil adherence (Bizios *et al.*, 1988). Since PAF is reported to express CDw18 antigen on the outer membrane of neutrophils (Zimmerman & McIntyre, 1988), it is possible that PAF, which is generated in endothelial cells by histamine treatment, might also induce CDw18 complex on neutrophils, which in turn makes neutrophils adherent to endothelial cells. One of the possible mechanisms of steroidal anti-inflammatory drugs on inhibition of neutrophil adherence to vascular endothelial cells might be an inhibition of the expression of CDw18 complex on neutrophils. However, this hypothesis requires further investigation.

In conclusion, histamine as well as thrombin stimulated neutrophil adherence to vascular endothelial cells. The histamine- and thrombin-induced neutrophil adherence was inhibited in a concentration-dependent manner by several PAF antagonists, suggesting that PAF probably produced by endothelial cells might be responsible for neutrophil adherence. The steroidal anti-inflammatory drug dexamethasone inhibited neutrophil adherence when neutrophils were pretreated for 3 h. However, when endothelial cells were pretreated with dexamethasone for 3 h, neutrophil adhesion was not inhibited. Consequently, neutrophils might be target cells for dexamethasone in inhibiting histamine- and thrombin-induced neutrophil adherence to vascular endothelial cells.

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Selectivity profile of the novel muscarinic antagonist UH-AH 37 determined by the use of cloned receptors and isolated tissue preparations

*†[1]J. Wess, †G. Lambrecht, †E. Mutschler, *M.R. Brann & *†F. Dörje

*Laboratory of Molecular Biology, National Institute of Neurological Disorders and Stroke, Building 36, Room 3D-02, National Institutes of Health, Bethesda, MD 20892, U.S.A. and †Department of Pharmacology, University of Frankfurt, Theodor-Stern-Kai 7, Gebäude 75A, D-6000 Frankfurt/M, F.R.G.

1 Functional *in vitro* experiments were carried out to determine the antimuscarinic potencies of the pirenzepine derivative UH-AH 37 (6-chloro-5,10-dihydro-5-[(1-methyl-4-piperidinyl)acetyl]-11H-dibenzo-[b,e] [1,4] diazepine-11-one hydrochloride) at M_1 muscarinic receptors of rabbit vas deferens, M_2 receptors of rat left atria and M_3 receptors of rat ileum. Furthermore, N-[^{3}H]-methylscopolamine competition binding experiments were performed to obtain its affinities for the five cloned human muscarinic receptors (m1–m5) stably expressed in CHO-K1 cells. Pirenzepine served as a reference drug throughout all experiments.

2 In all preparations used, UH-AH 37 interacted with muscarinic receptors in a fashion characteristic of a simple competitive antagonist.

3 In the functional studies, UH-AH 37, like pirenzepine, showed high affinity for M_1 (pA_2 8.49) and low affinity for M_2 muscarinic receptors (pA_2 6.63). In contrast to pirenzepine, UH-AH 37 also displayed high affinity for M_3 receptors (pA_2 8.04).

4 In agreement with its functional profile, UH-AH 37 bound with highest affinity to m1 (pK_i 8.74) and with lowest affinity to m2 receptors (pK_i 7.35). Moreover, it showed a 7 fold higher affinity for m3 (pK_i 8.19) than for m2 receptors, whereas pirenzepine bound to both receptors with low affinities.

5 The binding affinity of UH-AH 37 for m4 and m5 receptors (pK_i 8.32 for both receptors) was only ca. 2.5 fold lower than that for m1 receptors, while the corresponding affinity differences were 6 and 13 fold in case of pirenzepine.

6 In conclusion, the receptor selectivity profile of UH-AH 37 differs clearly from that of its parent compound, pirenzepine, in both functional and radioligand binding studies, the major characteristics being its pronounced M_2 (m2)/M_3 (m3) selectivity. UH-AH 37 thus represents a useful tool for the further pharmacological characterization of muscarinic receptor subtypes.

Introduction

Radioligand binding and functional studies suggest that there are at least three pharmacologically distinct muscarinic receptor subtypes (M_1–M_3) (Birdsall *et al.*, 1987; Mitchelson, 1988). While M_1 receptors are mainly present in neuronal tissues, M_2 and M_3 receptors are found predominantly in peripheral effector organs and lower brain areas. The individual receptor subtypes can be distinguished pharmacologically by their affinities for various selective antagonists. While pirenzepine preferentially binds to M_1 receptors (Hammer *et al.*, 1980; Doods *et al.*, 1987), AF-DX 116 (Hammer *et al.*, 1986; Giachetti *et al.*, 1986) and methoctramine (Melchiorre *et al.*, 1987; Wess *et al.*, 1988) selectively interact with the M_2 subtype. Hexahydro-sila-diphenidol (Lambrecht *et al.*, 1989; Waelbroeck *et al.*, 1989) and 4-diphenyl-acetoxy-N-methyl- piperidine methiodide (4-DAMP, Barlow & Shepherd, 1985; Waelbroeck *et al.*, 1988), on the other hand, show high affinities for both M_1 and M_3 receptors.

Recently, molecular cloning studies have revealed the existence of five molecularly distinct muscarinic receptor proteins (m1–m5) (Kubo *et al.*, 1986a,b; Peralta *et al.*, 1987; Bonner *et al.*, 1987; 1988). The antagonist binding properties of the expressed m1, m2 and m3 receptors generally correlate well with those of the pharmacologically defined M_1, M_2 and M_3 subtypes (Peralta *et al.*, 1987; Akiba *et al.*, 1988; Buckley *et al.*, 1989). By contrast, little is known about the pharmacological equivalents of the m4 and m5 gene products.

Preliminary results have indicated that a recently synthesized pirenzepine derivative, UH-AH 37 (6-chloro-5,10-dihydro-5-[(1-methyl-4-piperidinyl)acetyl]-11H-dibenzo-[b,e][1,4]diazepine-11-one hydrochloride) (Figure 1), may represent an interesting and novel tool for the pharmacological characterization of muscarinic receptor subtypes (Doods & Mayer, 1989; Eberlein *et al.*, 1989). In functional experiments, this antagonist, in contrast to pirenzepine, blocked M_3 receptors mediating contraction of the guinea-pig ileum with ca. 10 fold higher affinity than M_2 receptors in guinea-pig atria mediating negative chronotropic effects (Doods & Mayer, 1989). In radioligand binding studies, however, UH-AH 37 displayed similarly low affinities for cardiac M_2 and glandular M_3 receptors (Doods & Mayer, 1989). It has therefore been suggested that ileal and glandular M_3 receptors may be different (Doods & Mayer, 1989; Eberlein *et al.*, 1989).

Based on these findings, we have evaluated the pharmacological profile of UH-AH 37 in more detail. First, *in vitro* functional studies were carried out to reassess its affinity towards atrial M_2 and ileal M_3 receptors in a different species (rat) and

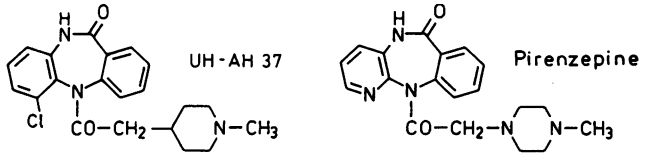

Figure 1 Chemical structure of pirenzepine and UH-AH 37.

[1] Author for correspondence at present address: Laboratory of Molecular Biology, National Institute of Neurological Disorders and Stroke, Building 36, Room 3D-02, National Institutes of Health, Bethesda, MD 20892, U.S.A.

to study, in addition, its antagonistic potency at M_1 receptors of the rabbit vas deferens (Eltze, 1988; Eltze *et al.*, 1988). Second, radioligand binding studies were performed to determine the affinities of UH-AH 37 for all five cloned human muscarinic receptors (m1–m5) stably expressed in CHO-K1 cells. Pirezepine served as a reference drug throughout all experiments. Our data show that UH-AH 37 possesses a selectivity profile which, in contrast to a previous study (Doods & Mayer, 1989), differs from that of pirenzepine in both functional and radioligand binding studies.

Methods

Functional studies

Rabbit isolated vas deferens Experiments on rabbit isolated vas deferens were performed according to Eltze (1988) and Eltze *et al.* (1988). Male New Zealand white rabbits (2.5–3.0 kg) were killed by i.v. injection of pentobarbitone sodium (120 mg kg^{-1}). Vasa deferentia were removed and divided into four segments of approximately 1.5 cm length. The preparations were set up in 7 ml organ baths containing modified Krebs buffer of the following composition (mM): NaCl 118.0, KCl 4.7, $CaCl_2$ 1.8, $MgSO_4$ 0.6, KH_2PO_4 1.2, $NaHCO_3$ 25.0, and (+)-glucose 11.1; 1 μM yohimbine was included to block α_2-adrenoceptors. The bath fluid was gassed continuously with 95% O_2 + 5% CO_2 and maintained at 31°C. A basal tension of 750 mg was applied, and after a 30 min equilibration period, twitch contractions were elicited by field stimulation with platinum electrodes (0.05 Hz, 0.5 ms, 30 V). Contractions were measured isometrically by a force-displacement transducer connected to a Hellige amplifier and a Rikadenki recorder. The M_1 receptor agonist 4-Cl-McN-A-343 (Eltze *et al.*, 1988) inhibited the neurogenic twitch contractions in a concentration-dependent fashion.

Rat isolated left atria and ileum Wistar rats (150–200 g) of either sex were killed by cervical dislocation. Left atria and ileal longitudinal muscle strips of ca. 1.5 cm length (Paton & Zar, 1968) were set up, under a tension of 500 mg, in 6 ml organ baths in oxygenated (95% O_2 + 5% CO_2) Tyrode solution (pH 7.4) of the following composition (mM): NaCl 137.0, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.05, $NaHCO_3$ 11.9, NaH_2PO_4 0.42, and (+)-glucose 5.6. The temperature of the bath fluid was maintained at 32°C (atria) and 37°C (ileum), respectively. Atria were paced electrically (2 Hz, 3 ms, 4–10 V) by means of two platinum electrodes. Negative inotropic effects to the agonist arecaidine propargyl ester (Moser *et al.*, 1989) were measured as isometric contractions. Responses of ileal longitudinal muscle to the same agonist were measured as isotonic contractions. Atrial and ileal responses were recorded as described for the rabbit vas deferens.

Determination of antagonist affinities After an equilibration time of 30–60 min, concentration-response curves were constructed by cumulative addition of the agonist (van Rossum, 1963). When these responses were constant, concentration-response curves were repeatedly obtained in the presence of antagonists. Four to five different concentrations of each antagonist were used (log concn. interval 0.5; $n = 4$–6 for each concn.). The antagonists were allowed to equilibrate for 30–60 min (vas deferens and ileum) or 1 h (atria), respectively. Preliminary experiments showed that these time intervals were sufficient for equilibration of the different antagonist concentrations. Agonist EC_{50} values in the absence and presence of antagonists were determined graphically from plots of log agonist concentration vs. % response in order to calculate dose ratios. The slopes of Arunlakshana-Schild plots (Arunlakshana & Schild, 1959) were determined by linear regression by the method of least squares. pA_2 values were estimated by fitting to the data the best straight line with a slope of unity, as required by the theory (Tallarida *et al.*, 1979).

Radioligand binding studies

Transfections and tissue culture The CHO-K1 (Chinese hamster ovary) cell lines stably expressing the human m2–m5 receptors have been described previously (Buckley *et al.*, 1989). By using a strategy which involves the cotransfection of the muscarinic receptor gene with a neomycin resistance gene as a selectable marker (Buckley *et al.*, 1989), we also obtained a CHO-K1 cell line stably expressing the human m1 receptor (Dörje *et al.*, 1991). Receptor levels, as determined by N-[^{3}H]-methylscopolamine ([^{3}H]-NMS) binding, were (fmol per mg membrane protein): m1 (2520), m2 (750), m3 (1830), m4 (1780), and m5 (950).

Cells were grown as monolayers in 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum, 0.1 mM non-essential amino acids, and 100 u ml^{-1} penicillin and streptomycin.

Membrane preparation and binding assays Transfected cells were grown to about 70–80% confluency, washed, scraped into ice-cold binding buffer, and homogenized for 30 s in a Tekmar Tissumizer (setting 50). Membranes were pelleted at 15,000 *g*, rehomogenized and stored at −80°C prior to use.

Binding assays were carried out essentially as described by Buckley *et al.* (1989). Binding buffer consisted of 25 mM sodium phosphate (pH 7.4) containing 5 mM $MgCl_2$. Assays were conducted in 1 ml final volume. Final membrane protein concentrations (in μg ml^{-1}) were: m1 (6), m2 (10), m3 (5), m4 (3), and m5 (4). The [^{3}H]-NMS concentration used was 150–300 pM. Ten different concentrations of the cold inhibitors were employed. Specific binding was defined as the difference in [^{3}H]-NMS binding in the absence and presence of 1 μM atropine. Incubations were carried out at 22°C for 3 h. Binding was terminated by filtration through a Brandel cell harvester onto Whatman GF/C filters. Membranes were washed three times with ice-cold binding buffer, transferred to 10 ml of scintillation fluid (NEN Aquasol), and counted in an LKB Beta counter. Data were fitted to the equation %[^{3}H]-NMS bound = $100 - (100\ x^n/IC_{50})/(1 + x^n/IC_{50})$ to obtain the Hill number n and to %[^{3}H]-NMS bound = $100 - (100\ x/IC_{50})/(1 + x/IC_{50})$ to derive the IC_{50} value (x = concentration of the cold inhibitor). IC_{50} values were converted to K_i values according to the method of Cheng & Prusoff (1973). Data were analyzed by a nonlinear least squares curve fitting procedure using the programme DATAPLOT (distributed by the National Technical Information Services) and run on a VAX II computer.

Statistical evaluation

Data are presented as means ± s.e.mean of *n* experiments. Differences between mean values were tested for statistical significance by Student's *t* test; the level of significance was chosen at $P < 0.05$.

Drugs

The following drugs were used: N-[^{3}H]-methylscopolamine ([^{3}H]-NMS 71 Ci $mmol^{-1}$; New England Nuclear, Boston, Ma., U.S.A.); atropine sulphate and yohimbine hydrochloride (Sigma, München, F.R.G.); UH-AH 37 (6-chloro-5,10-dihydro-5-[(1-methyl-4-piperidinyl)acetyl]-11H-dibenzo-[b,e][1,4] diazepine-11-one hydrochloride) and pirenzepine dihydrochloride (kindly provided by Thomae, Biberach, F.R.G.). 4-Cl-McN-A-343 (4-(4-chlorophenylcarbamoyloxy)-2-butynyl- trimethylammonium iodide) (Nelson *et al.*, 1976) and arecaidine propargyl ester (Mutschler & Hultzsch, 1973) were synthesized in one of our laboratories. All other chemicals were of reagent grade and were used as purchased.

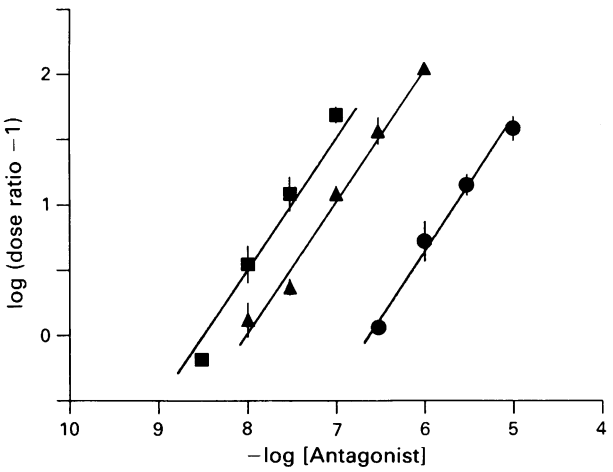

Figure 2 Arunlakshana-Schild plots for the antagonistic effects of UH-AH 37 on M_1 (■, rabbit vas deferens), M_2 (●, rat atria), and M_3 (▲, rat ileum) receptors. 4-Cl-McN-A-343 (vas deferens) (Eltze *et al.*, 1988) and arecaidine propargyl ester (atria and ileum) (Moser *et al.*, 1989) were used as agonists. Each point represents the mean of 4–6 determinations with s.e.mean shown by vertical lines.

Results

Functional studies

UH-AH 37, like pirenzepine, concentration-dependently blocked the 4-Cl-McN-A-343-induced inhibition of twitch contractions of the rabbit vas deferens mediated by M_1 receptors, as well as the negative inotropic and contractile responses to arecaidine propargyl ester in rat atria and ileum mediated by M_2 and M_3 receptors, respectively (Figure 2). Increasing concentrations of the antagonists produced parallel shifts of the agonist concentration-response curves progressively to the right without appreciable changes in basal tension or maximum agonist responses. In all cases, Arunlakshana-Schild plots were linear over the tested antagonist concentration-range (Figure 2), and the slopes of the regression lines were not significantly different from unity ($P > 0.05$) (Table 1). In agreement with its reported selectivity profile (Lambrecht *et al.*, 1989), pirenzepine showed high affinity for M_1 receptors in rabbit vas deferens (pA_2 8.24), but low affinities for atrial M_2 (pA_2 6.31) and ileal M_3 receptors (pA_2 6.89) (Table 1). Like pirenzepine, UH-AH 37 displayed high affinity for M_1 (pA_2 8.49) and low affinity for M_2 receptors (pA_2 6.63). However, in contrast to pirenzepine, UH-AH 37 retained relatively high affinity for M_3 receptors (pA_2 8.04). UH-AH 37 was thus able to discriminate clearly between M_2 and M_3 receptors, displaying a 26 fold selectivity for the latter receptor subtype (Table 1, Figure 2).

Table 1 Antimuscarinic potencies (pA_2 values) and slopes of Arunlakshana-Schild plots (given in parentheses) for pirenzepine and UH-AH 37 at M_1, M_2 and M_3 receptors

Compound	*Rabbit vas deferens* (M_1)	*Rat atria* (M_2)	*Rat ileum* (M_3)
Pirenzepine	8.24 ± 0.06[a]	6.31 ± 0.07	6.89 ± 0.03
	(1.19 ± 0.10)	(1.05 ± 0.06)	(0.96 ± 0.05)
UH-AH 37	8.49 ± 0.06	6.63 ± 0.05	8.04 ± 0.04
	(1.23 ± 0.09)	(0.99 ± 0.09)	(1.01 ± 0.06)

Mean values ± s.e.mean are given (n = 16–21). As the slopes of the Arunlakshana-Schild regression lines were not significantly different from unity ($P > 0.05$), pA_2 values were obtained by imposing the unity constraint (Tallarida *et al.*, 1979).

[a] Data taken from Lambrecht *et al.* (1988).

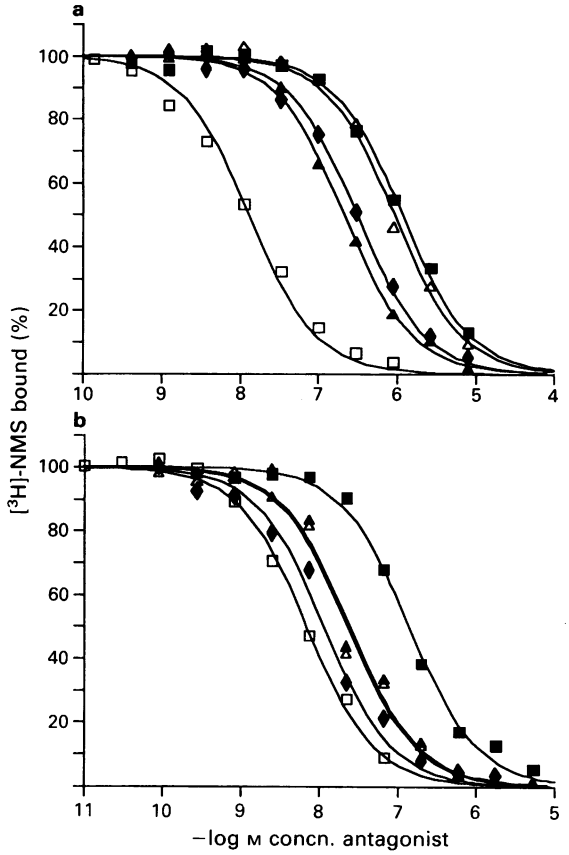

Figure 3 Displacement of specific N-[^{3}H]-methylscopolamine ([^{3}H]-NMS) binding to the human m1 (□), m2 (■), m3 (△), m4 (▲), and m5 (◆) receptors stably expressed in CHO-K1 cells by pirenzepine (a) and UH-AH 37 (b). Binding studies were carried out as described under Methods. Curves are representative of 3–6 independent experiments carried out in duplicate. Curves were generated by computer fit according to a one binding site model.

Radioligand binding studies

UH-AH 37 and pirenzepine competitively displaced [^{3}H]-NMS binding to the five cloned human muscarinic receptors

Table 2 Binding affinities (pK_i values[a]) of pirenzepine and UH-AH 37 for the five cloned human muscarinic receptors (m1–m5) stably expressed in CHO-K1 cells (Hill coefficients in parentheses)

Compound	*m1*	*m2*	*m3*	*m4*	*m5*
Pirenzepine	8.20 ± 0.13	6.65 ± 0.05	6.86 ± 0.06	7.43 ± 0.05	7.05 ± 0.04
	(0.94 ± 0.07)	(1.00 ± 0.04)	(1.02 ± 0.05)	(0.99 ± 0.07)	(0.92 ± 0.08)
UH-AH 37	8.74 ± 0.02	7.35 ± 0.04	8.19 ± 0.08	8.32 ± 0.05	8.32 ± 0.01
	(0.96 ± 0.04)	(0.98 ± 0.06)	(1.00 ± 0.05)	(0.94 ± 0.05)	(0.96 ± 0.10)

Data are presented as means ± s.e.mean of 3–6 independent experiments each peformed in duplicate. N-[^{3}H]-methylscopolamine ([^{3}H]-NMS) competition binding studies were carried out as described under Methods. Hill coefficients were not significantly different from unity ($P > 0.05$). The affinities of [^{3}H]-NMS for the individual receptor subtypes were (in pM): m1, 54 ± 1; m2, 83 ± 4; m3, 52 ± 2; m4, 26 ± 5; m5, 106 ± 11 (Dörje *et al.*, 1991).

[a] $pK_i = -\log K_i$.

(m1–m5) individually expressed in CHO-K1 cells (Figure 3). All inhibition curves were characterized by Hill coefficients not significantly different from unity ($P > 0.05$; Table 2). In agreement with its reported selectivity profile (Buckley *et al.*, 1989), pirenzepine showed highest affinity for m1 receptors (pK_i 8.20), its affinities towards m2, m3, m4, and m5 receptors being 35, 25, 6, and 13 fold lower, respectively (Table 2). Analogously, UH-AH 37 showed highest affinity for m1 (pK_i 8.74) and lowest affinity to m2 receptors (pK_i 7.35). In contrast to pirenzepine, UH-AH 37 displayed high affinities for m3, m4, and m5 receptors (Table 2, Figure 3). The affinities of UH-AH 37 for these three receptor subtypes were only about 3 fold lower than its affinity for m1 receptors (Table 2).

Discussion

The receptor selectivity profile of the pirenzepine derivative UH-AH 37 has been evaluated in functional and radioligand binding studies and compared to that of its parent compound. In all preparations used, UH-AH 37, like pirenzepine, interacted with muscarinic receptors in a fashion characteristic of a simple competitive antagonist. In the functional studies, agonist concentration-response curves were progressively displaced to the right without depression of maximum responses. Moreover, the slopes of the Schild regression lines obtained in the functional studies and the Hill coefficients of the inhibition binding curves were not significantly different from unity.

In the functional studies, UH-AH 37, like pirenzepine, showed high affinity for M_1 receptors in rabbit vas deferens (pA_2 8.49) and low affinity for M_2 receptors in rat atria (pA_2 6.63). However, in contrast to pirenzepine, which also exhibited low affinity to M_3 receptors in rat ileum, UH-AH 37 displayed relatively high affinity for this receptor subtype (pA_2 8.04), thus clearly discriminating between M_2 and M_3 receptors. A similar selectivity has been reported in functional studies on atrial and ileal tissues of the guinea-pig (Doods & Mayer, 1989). Furthermore, preliminary experiments have shown that UH-AH 37 also blocks M_3 receptors in other smooth muscle preparations such as rat bladder and guinea-pig trachea with high affinity (pA_2 ca. 8; Mutschler & Riotte, unpublished results). The ability of UH-AH 37 to bind to M_3 receptors with higher affinity than to M_2 receptors is shared by other selective antagonists such as 4-DAMP (Barlow & Shepherd, 1985) and hexahydro-sila-diphenidol (Lambrecht *et al.*, 1989; Waelbroeck *et al.*, 1989). However, while these two antagonists show equally high affinities for M_1 and M_3 receptors (Lambrecht *et al.*, 1989; Waelbroeck *et al.*, 1988; 1989), UH-AH 37 exhibits an approximately 3 fold preference for M_1 receptors.

As observed in the functional studies, UH-AH 37 and pirenzepine also yielded different affinity profiles in the radioligand binding studies. In general, the affinitites of these two antagonists for the cloned human m1, m2 and m3 receptors correlated well with those obtained for the M_1, M_2 and M_3 receptor subtypes. This finding further supports the notion that the M_1, M_2 and M_3 subtypes may represent the pharmacological equivalents of the structurally defined m1, m2 and m3 receptors (Akiba *et al.*, 1988; Buckley *et al.*, 1989). In agreement with the functional data, UH-AH 37 and pirenzepine bound with highest affinity to m1 (pK_i 8.74 and 8.20, respectively) and with lowest affinity to m2 receptors (pK_i 7.35 and 6.65, respectively). Pirenzepine bound to m3 receptors with similarly low affinity (pK_i 6.86) as to m2 receptors. UH-AH 37, by contrast, retained relatively high affinity for m3 receptors (pK_i 8.19). Thus, UH-AH 37 showed a similar affinity profile in the functional and radioligand binding studies which is characterized by high affinity for M_1 (m1), somewhat lower affinity for M_3 (m3), and low affinity for M_2 (m2) receptors.

A comparison of the binding affinities of UH-AH 37 and pirenzepine for m4 and m5 receptors reveals another striking difference between the selectivity profiles of the two antagonists. Pirenzepine showed relatively low affinities for these two receptor subtypes (pK_i 7.43 and 7.05, respectively) as compared to its affinity for m1 receptors (pK_i 8.20), thus displaying a clear overall preference for m1 receptors. UH-AH 37, on the other hand, bound to m4 and m5 receptors with affinities (pK_i 8.32 in both cases) which were only 2.5 fold smaller than the corresponding m1 value (pK_i 8.74).

In contrast to our findings, it has been reported that UH-AH 37, despite being a potent antagonist of ileal M_3 receptors in functional studies, exhibited low affinity for M_3 receptors in rat submandibular gland in radioligand binding studies (Doods & Mayer, 1989). However, UH-AH 37 binding to glandular tissue was characterized by a Hill coefficient significantly smaller than one, indicating either the lack of equilibrium binding conditions or the presence of a mixture of different muscarinic receptor subtypes. In agreement with the latter notion, it has been found in mRNA mapping studies that at least two different muscarinic receptor subtypes (m1 and m3) are expressed in exocrine glands (Maeda *et al.*, 1988). Based on this finding, binding studies using glandular tissue as a source of M_3 receptors have to be interpreted with caution. As shown in this study, this limitation can be overcome by the use of clonal cell lines individually expressing the different muscarinic receptor subtypes, thus allowing the unambiguous determination of drug affinities to clearly defined receptor species. This approach will not only be useful in clarifying existing discrepancies between functional and conventional radioligand binding studies, but will also play a major role in the future development of subtype-selective drugs.

In conclusion, we have shown that UH-AH 37 displays a selectivity profile which is different from that of its parent compound, pirenzepine. The major difference between the affinity patterns of the two antagonists is that UH-AH 37 is able to discriminate clearly between M_2 (m2) and M_3 (m3) receptors. It thus represents a useful tool for the further pharmacological characterization of muscarinic receptor subtypes.

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Octimibate, a potent non-prostanoid inhibitor of platelet aggregation, acts via the prostacyclin receptor

[1]Janet E. Merritt, [2]Trevor J. Hallam, Anthony M. Brown, Isobel Boyfield, David G. Cooper, Deirdre M.B. Hickey, [2]Albert A. Jaxa-Chamiec, Alberto J. Kaumann, *Mary Keen, #Eamonn Kelly, [3]Ursula Kozlowski, James A. Lynham, Kitty E. Moores, Kenneth J. Murray, †John MacDermot & [4]Timothy J. Rink

SmithKline Beecham Pharmaceuticals, The Frythe, Welwyn, Herts AL6 9AR; *Department of Pharmacology, University of Birmingham, The Medical School, Birmingham B15 2TJ; #Department of Pharmacology, The Medical School, University Walk, Bristol BS8 1TD and †Department of Clinical Pharmacology, Royal Postgraduate Medical School, Du Cane Road, London W12 0NN

1 Octimibate, 8-[(1,4,5-triphenyl-1H-imidazol-2-yl)oxy]octanoic acid, is reported to have antithrombotic properties. This is in addition to its antihyperlipidaemic effects which are due to inhibition of acyl-CoA:cholesterol acyltransferase (ACAT). The aim of this study was to investigate the mechanism of the antithrombotic effect of octimibate, and to determine whether the effects of octimibate are mediated through prostacyclin receptors.

2 In suspensions of washed (plasma-free) human platelets, octimibate is a potent inhibitor of aggregation; its IC_{50} is approx. 10 nM for inhibition of aggregation stimulated by several different agonists, including U46619 and ADP. The inhibitory effects of octimibate on aggregation are not competitive with the stimulatory agonist; the maximal response is suppressed but there is no obvious shift in potency of the agonist. In platelet-rich plasma, octimibate inhibits agonist-stimulated aggregation with an IC_{50} of approx. 200 nM.

3 Octimibate also inhibits agonist-stimulated rises in the cytosolic free calcium concentration, $[Ca^{2+}]_i$, in platelets. Both Ca^{2+} influx and release from intracellular stores are inhibited. The effects of octimibate on aggregation and $[Ca^{2+}]_i$ are typical of agents that act via elevation of adenosine 3':5'-cyclic monophosphate (cyclic AMP). Similar effects are seen with forskolin, prostacyclin (PGI_2) and iloprost (a stable PGI_2 mimetic).

4 Octimibate increases cyclic AMP concentrations in platelets and increases the cyclic AMP-dependent protein kinase activity ratio. Octimibate stimulates adenylyl cyclase activity in human platelet membranes, with an EC_{50} of 200 nM. The maximal achievable activation of adenylyl cyclase by octimibate is 60% of that obtainable with iloprost. Octimibate has no effect on the cyclic GMP-inhibited phosphodiesterase (phosphodiesterase-III), which is the major cyclic AMP-degrading enzyme in human platelets.

5 Octimibate inhibits, apparently competitively, the binding of [^{3}H]-iloprost (a stable PGI_2 mimetic) to platelet membranes; the estimated K_i is 150 nM.

6 The platelets of different species show considerable differences in the apparent potency of their inhibition of aggregation by octimibate; platelets from cynomolgus monkeys are 3 fold more sensitive than those from humans, while rat, cat and cow platelets are 50, 100, and 250 fold less sensitive than human platelets. The sensitivity of these different species to iloprost, however, varies over a range of only 10 fold with no obvious difference between primates and non-primates.

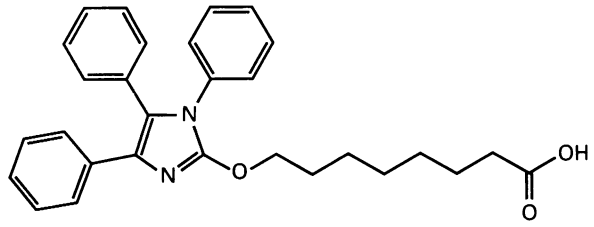
7 Octimibate appears to be a potent agonist (aggregation), or partial agonist (adenylyl cyclase), at prostacyclin receptors and is the first non-prostanoid agent of this type to be identified. The species differences in relative potency of octimibate and iloprost may reflect the existence of receptor subtypes.

Introduction

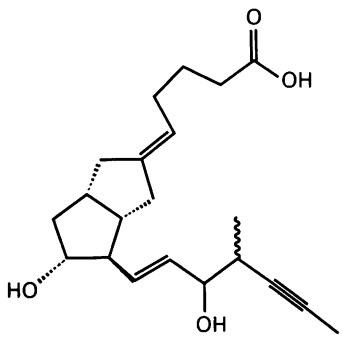
Octimibate, 8-[(1,4,5-triphenyl-1H-imidazol-2-yl)oxy]octanoic acid, has been described as having antihyperlipidaemic, antiatherosclerotic and antithrombotic properties (European Patent Specification, EP-B-130526). Octimibate (10–100 mg kg^{-1}, daily) has been shown to reduce both plasma cholesterol and atherosclerotic lesions in fat-fed rabbits and minipigs (Lautenschlager *et al.*, 1986; Rucker *et al.*, 1988), and it causes a shift from esterified to free cholesterol in cholesterol-loaded macrophages (Schmitz *et al.*, 1985). These antihyperlipidaemic and antiatherosclerotic effects of octimibate have been ascribed to its inhibition of the cholesterol esterifying enzyme, acyl-CoA:cholesterol acyltransferase (ACAT). However, the antithrombotic activity of octimibate cannot be explained by its inhibition of ACAT. *In vitro*, aggregation of human platelets induced by adenosine diphosphate (ADP), arachidonic acid, collagen, platelet-activating factor and thrombin was inhibited by octimibate with IC_{50} values in the range of 1–9 μM. *In vivo*, ADP-induced thrombus formation in the microcirculation of the hamster cheek pouch was reduced after octimibate doses of 10–100 mg kg^{-1} (Lautenschlager *et al.*, 1986). Octimibate thus appears to be a functional antagonist of platelet aggregation in that it inhibits aggregation stimulated by a variety of agonists, rather than acting as a specific receptor antagonist. However, the way in which octimibate inhibits platelet aggregation has not been described to date.

Here, we have explored the mechanism of action of octimibate, and provide evidence that it acts (at least in part) via the prostacyclin (PGI_2) receptor to inhibit platelet aggregation.

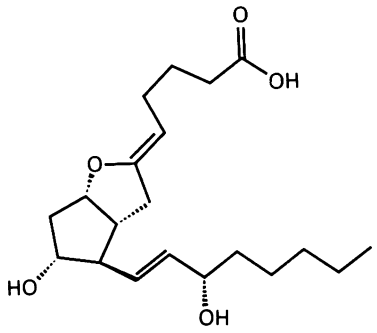
[1] Author for correspondence.
Present addresses:
[2] Glaxo Group Research Ltd., Greenford Road, Greenford, Middlesex UB6 0HE.
[3] Roche Products Ltd., Welwyn Garden City, Herts. AL7 3AY.
[4] Amylin Corporation, 9373 Towne Centre Drive, Suite 250, San Diego, California 92121, USA.



Octimibate



Iloprost



Prostacyclin
(PGI_2)

Figure 1 The structures of octimibate (8-[(1,4,5-triphenyl-1H-imidazol-2-yl)oxy]octanoic acid), iloprost and prostacyclin (PGI_2).

The effects of octimibate on human platelets have been compared to PGI_2 and iloprost (a stable PGI_2 mimetic); the structures of these three compounds are shown in Figure 1. We have investigated the effects of octimibate on platelet aggregation, cytosolic free calcium concentration ($[Ca^{2+}]_i$), adenosine 3': 5'-cyclic monophosphate (cyclic AMP) concentrations and cyclic AMP-dependent protein kinase activity, adenylyl cyclase activity, and binding of [^{3}H]-iloprost to platelet membranes. Since the potency of iloprost is reported to vary in different species (Sturzebecher & Losert, 1987; Armstrong *et al.*, 1989), the effects of octimibate and iloprost have been compared on inhibition of aggregation of platelets from several different species. Many prostanoid agonists at the PGI_2 receptor have been described (Oliva & Nicosia, 1987). However, octimibate is the first non-prostanoid agonist to be described for the PGI_2 receptor on any cell-type or tissue, including platelets.

Methods

Preparation of human platelets

Blood was freshly collected from healthy volunteers, who gave informed consent, and platelets were prepared as previously described (Hallam *et al.*, 1984). For experiments with platelet-rich plasma (PRP), blood was collected into 1/10th volume citrate dextrose (7 mM citric acid, 139 mM trisodium citrate, 93 mM glucose), and was centrifuged for 5 min at 500 *g*. The upper layer of PRP was removed, and was diluted 1/2 with buffer containing (mM): NaCl 145, KCl 5, $MgCl_2$ 1, HEPES 10 (pH 7.4 at 37°C) and glucose 10. The final platelet density was approx. 1.5×10^8 cells ml^{-1}. The resulting 50% PRP was incubated with aspirin (100 μM) for 15 min at 37°C before use in the aggregation experiments.

For experiments with washed platelets, blood was collected into 1/7th volume acid citrate dextrose (citric acid 65 mM, trisodium citrate 85 mM, glucose 110 mM), and was centrifuged for 5 min at 500 *g*. The upper layer of PRP was removed, and aspirin (100 μM) was added. The PRP was then centrifuged for 15 min at 200 *g*, and the resultant platelet pellet was resuspended (at approx. 1.5×10^8 cell ml^{-1}) in medium containing (mM): NaCl 145, KCl 5, $MgCl_2$ 1, $CaCl_2$ 0.2, HEPES 10 (pH 7.4 at 37°C), glucose 10 and apyrase 10 μg ml^{-1}. For experiments to measure $[Ca^{2+}]_i$, the PRP was incubated with quin2 AM (20 μM) for 30 min at 37°C before centrifugation.

Essentially similar methods were used for preparation of platelets from different animal species.

Measurement of aggregation

Aggregation was measured at 37°C in a 4 channel aggregometer (PAP-4 from Biodata Corp.). For the experiments with washed platelets, $CaCl_2$ (1 mM) and fibrinogen (1 mg ml^{-1}) were added to aliquots of cells just before use. Aliquots (0.7 ml) of PRP or washed platelets were continuously stirred, and the test compound was added 2 min before the aggregatory stimulus. The final concentration of dimethylsulphoxide (DMSO) was 0.1%. The extent of aggregation was assessed 4 min after addition of the stimulus, and the effects of the compounds were measured as % inhibition of the control response in the absence of inhibitor (vehicle present). Aggregation was stimulated by U46619 (1 μM) or ADP (20 μM), except where dose-response curves for U46619 and ADP are shown.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured as previously described (Rink *et al.*, 1982; Hallam *et al.*, 1984). The PRP was incubated with quin2 AM, as described above, and the washed quin2-loaded platelets were used for these experiments. Quin2 fluorescence was measured at 340 nm excitation, 500 nm emission in a Perkin-Elmer LS-5 fluorimeter at 37°C. $[Ca^{2+}]_i$ was calculated from the quin2 fluorescence as previously described (Tsien *et al.*, 1982). $CaCl_2$ (1 mM) or EGTA (1 mM) were added to cells (0.7 ml) as required. The test compound was added 2 min before the stimulatory agonist (ADP 20 μM). The final concentration of DMSO was 0.1%. The effects of the compounds were measured as % inhibition of the initial peak in fluorescence evoked by the stimulus.

Measurement of the cyclic AMP-dependent protein kinase activity ratio and of platelet cyclic AMP content

Suspensions of washed human platelets (prepared as above) were incubated with the test compounds for 2 min at 37°C. The incubations were terminated with 9 volumes of homogenizing buffer containing (mM): KH_2PO_4 10, EDTA 10, isobutyl methylxanthine 0.5, Triton X-100 (0.2% w/v), pH 6.8 (Seiler *et al.*, 1987). The resulting homogenates were used directly in the cyclic AMP-dependent protein kinase activity ratio assay (Murray *et al.*, 1990a). Phosphotransferase activity was measured using malantide (a synthetic dodecapeptide) as substrate in the absence and presence of exogenous cyclic AMP. Activity in the absence of exogenous cyclic AMP is a measure of the amount of active enzyme in the platelets following incubation with the test compound, and activity in the presence of exogenous cyclic AMP is a measure of the total

amount of enzyme present in the platelets. The cyclic AMP-dependent protein kinase activity ratio therefore measures the amount of cyclic AMP-dependent protein kinase that is active in the platelets after the 2 min incubation with the test compound as a fraction of the total activity.

For measurement of platelet cyclic AMP content, parallel aliquots of washed platelets were incubated with the test compounds for 2 min at 37°C. These incubations were terminated by addition of 0.1 volumes of $HClO_4$ (3 M). After 30 min at 4°C, these extracts were centrifuged for 1 min at 12,000 *g*. Perchloric acid was removed from aliquots (0.2 ml) of the supernatant by addition of 0.25 ml of a 1:1 mixture of 1,1,2,2-dichlorodifluoroethane and tri-n-octylamine, followed by thorough mixing then centrifugation (Sharps & McCarl, 1982). The upper aqueous layer was taken for measurement of cyclic AMP. Cyclic AMP was assayed with the kit detailed in the Materials section, and platelet cyclic AMP content is expressed as pmol/10^8 platelets.

Preparation of human platelet membranes

Platelet-rich plasma concentrates were obtained from the Blood Transfusion Service, Cambridge, and membranes were prepared by a modification of the method of Edwards *et al.* (1987). The PRP was centrifuged for 15 min at 800 *g*, and the supernatant was discarded. The rest of the procedure was carried out at 4°C. The platelet pellets were resuspended in buffer containing 0.25 mM EDTA and 5 mM Tris-Cl, homogenized with a Dounce homogenizer (10 strokes with a loose pestle), and then centrifuged at 26,000 *g* for 20 min. The pellet was washed twice by resuspension in buffer containing 0.25 mM EDTA and 50 mM Tris-Cl, followed by homogenization, then centrifugation at 26,000 *g* for 10 min. The final membrane pellet was homogenized in buffer containing 0.25 mM EDTA and 50 mM Tris-Cl, and stored at −70°C. This membrane suspension contained approximately 12 mg protein ml^{-1}. All Tris buffers were adjusted to pH 7.4 at 20°C. Protein determinations were according to the method of Bradford (1976).

Measurement of platelet membrane adenylyl cyclase activity

Membranes were prepared as described above, and adenylyl cyclase activity was measured by a modification of the method of Salomon *et al.* (1974). The incubation (total volume of 50 μl) contained ATP 100 μM, [α-^{32}P]-ATP 1–2 μCi, phosphocreatine 20 mM, creatine kinase 40 u ml^{-1}, myokinase 40 u ml^{-1}, GTP 10 μM, cyclic AMP 1 mM, EGTA 100 μM, $MgCl_2$ 5 mM, EDTA 10 μM, Tris-Cl 50 mM, pH 7.4 at 20°C, approximately 30 μg platelet membrane protein, 0.4% dimethylsulphoxide (DMSO) and the test compounds. The incubations, for 10 min at 37°C, were initiated by addition of platelet membranes and terminated by addition of 100 μl of a solution containing cyclic AMP 40 mM, [^{3}H]-cyclic AMP (approx. 10,000 c.p.m.), ATP 10 mM and 1% SDS, followed by addition of 1 ml of water. [α-^{32}P]-ATP and [^{32}P]-cyclic AMP were separated by a two step chromatography method, Dowex, followed by alumina (Salomon *et al.*, 1974). [^{3}H]-cyclic AMP was used to monitor recovery of cyclic AMP from the columns, and cyclic AMP formation was expressed as pmol cyclic AMP min^{-1} mg^{-1} protein.

Inhibition of [^{3}H]-iloprost binding to platelet membranes

Membranes were prepared as described above, and inhibition of [^{3}H]-iloprost binding was measured as described by Hall & Strange (1984). The incubation (total volume of 270 μl) contained Tris-Cl 50 mM (pH 7.4 at 20°C), $MgCl_2$ 4 mM, EDTA 40 μM, [^{3}H]-iloprost 10 nM, membrane protein 0.4–0.8 mg, 1.85% DMSO, and various concentrations of unlabelled iloprost or octimibate. For determination of non-specific binding, 20 μM unlabelled iloprost was included. (Specific binding was 85–90% of total binding). The tubes (triplicates for each condition) were set up on ice, and then incubated for 30 min at 37°C. The incubations were terminated by rapid filtration on Whatman GF/B filters using a Brandel harvester. The filters were washed three times with Tris-Cl 50 mM, $MgCl_2$ 5 mM, pH 7.4 at 20°C, and then counted for radioactivity.

From two preliminary experiments measuring binding of [^{3}H]-iloprost as a function of [^{3}H]-iloprost concentration, K_D values of 16.7 nM and 22.6 nM were obtained. A mean value of 19.6 nM was then used in the Cheng and Prusoff equation (Cheng & Prussoff, 1973) to estimate the K_i values for iloprost and octimibate from the IC_{50} values obtained in binding inhibition experiments. Data were fitted to a one site model using Grafit (Erithacus Software Ltd., Staines, Middx).

Analysis of data

Concentration-inhibition curves were fitted to the logistic equation by computer using the programme ALLFIT (DeLean *et al.*, 1978). This analysis yields values for the IC_{50} and the Hill coefficient.

Materials

Octimibate was synthesized, according to the method described by Lautenschlager *et al.* (1986), in the Department of Medicinal Chemistry, SmithKline Beecham Pharmaceuticals, Welwyn, and was dissolved in DMSO. Iloprost was obtained from Schering AG, Postfach 650311, D-1000 Berlin 65, Germany, and was dissolved in ethanol (0.5 mg 10 μl^{-1}) then diluted to 0.5 mg ml^{-1} in a solution containing 0.9% NaCl and 10 mM Tris-Cl, pH 8.3. PGI_2 was from Sigma, and was dissolved at 1 mg ml^{-1} in a solution containing 90% ethanol and 10 mM NaOH, then diluted in a solution containing 0.9% NaCl and 10 mM Tris, pH 8.5. U46619 (dideoxy-11α, 9α-epoxymethano-prostaglandin $F_{2\alpha}$) was from The Upjohn Company, Kalamazoo, MI, U.S.A. ADP, GTP and EDTA were from Boehringer. Quin2 acetoxymethyl ester (quin2 AM) was from Calbiochem, EGTA was from Fluka, and the Dowex cation exchange resin (AG50W-X4, H^+ form) was from Bio-Rad. [α-^{32}P]-ATP (10–50 Ci $mmol^{-1}$), [^{3}H]-cyclic AMP (30 Ci $mmol^{-1}$), the cyclic AMP assay kit (RPA 508) and [^{3}H]-iloprost (TRK 839) were from Amersham International plc. All other materials were from Sigma or BDH.

Results

Effect of octimibate on platelet aggregation

Figure 2 shows the effect of octimibate, iloprost, PGI_2 and forskolin on aggregation of washed human platelets stimulated with the thromboxane mimetic, U46619 (1 μM). Figure 2a shows typical aggregation traces for platelets stimulated with U46619, following a 2 min preincubation with various concentrations of octimibate. This clearly shows that octimibate is a potent inhibitor of U46619-stimulated aggregation, with maximal inhibition at 25 nM octimibate. From the dose-response curve shown in Figure 2b, the IC_{50} for inhibition of U46619-stimulated platelet aggregation by octimibate is 10.5 ± 0.4 nM (n = 9), and the Hill coefficient of the inhibition curve is 3.5 ± 0.4. Figure 2b also shows inhibition of aggregation by PGI_2 (IC_{50} = 0.36 ± 0.01 nM, Hill coefficient = 3.4 ± 0.3, n = 1), iloprost (IC_{50} = 0.89 ± 0.03 nM, Hill coefficient = 3.5 ± 0.5, n = 8) and forskolin (IC_{50} = 106 ± 10 nM, Hill coefficient = 2.7 ± 0.6, n = 1).

Octimibate inhibited platelet aggregation stimulated by a variety of agonists in addition to U46619. ADP-stimulated aggregation was potently inhibited by octimibate with an IC_{50} of 8.4 ± 0.1 nM, n = 4 (see Table 1 and Figure 3). Octimibate also inhibited aggregation stimulated by vasopressin (0.5 μM), 5-hydroxytryptamine (2.5 μM with 10 μM adrenaline) or thrombin (0.1 u ml^{-1}) with IC_{50} values of 2 nM, 18 nM and 50 nM

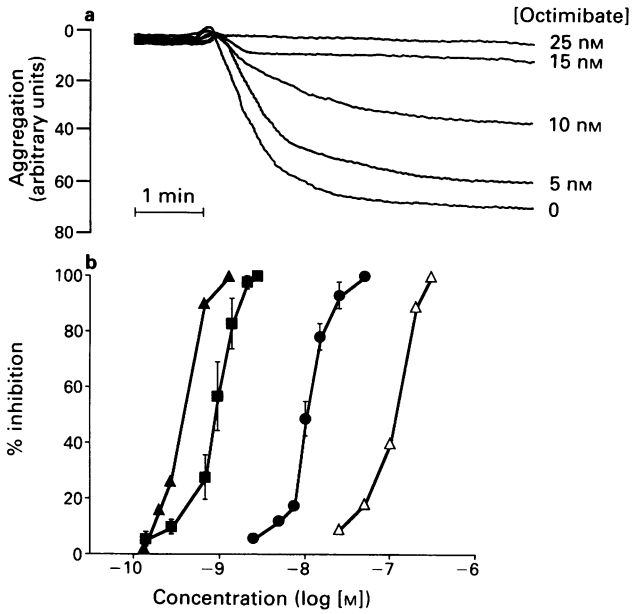


Figure 2 The effects of octimibate, iloprost, PGI_2 and forskolin on aggregation of washed human platelets stimulated with U46619. (a) Typical aggregation traces showing U46619 (1 μM)-stimulated aggregation of washed platelets in the presence of varying concentrations of octimibate (0–25 nM) added 2 min before U46619. (b) Dose-response curves for inhibition of aggregation by PGI_2 (▲), iloprost (■), octimibate (●) and forskolin (△). The compounds were added 2 min before U46619 (1 μM). Percentage inhibition is calculated as inhibition of the maximal extent of aggregation measured 4 min after addition of U46619. Data are means of 8 experiments with iloprost, 9 experiments with octimibate, and are single experiments with PGI_2 and forskolin; vertical bars show s.e.mean; where not shown, the error bars are within the symbol size.

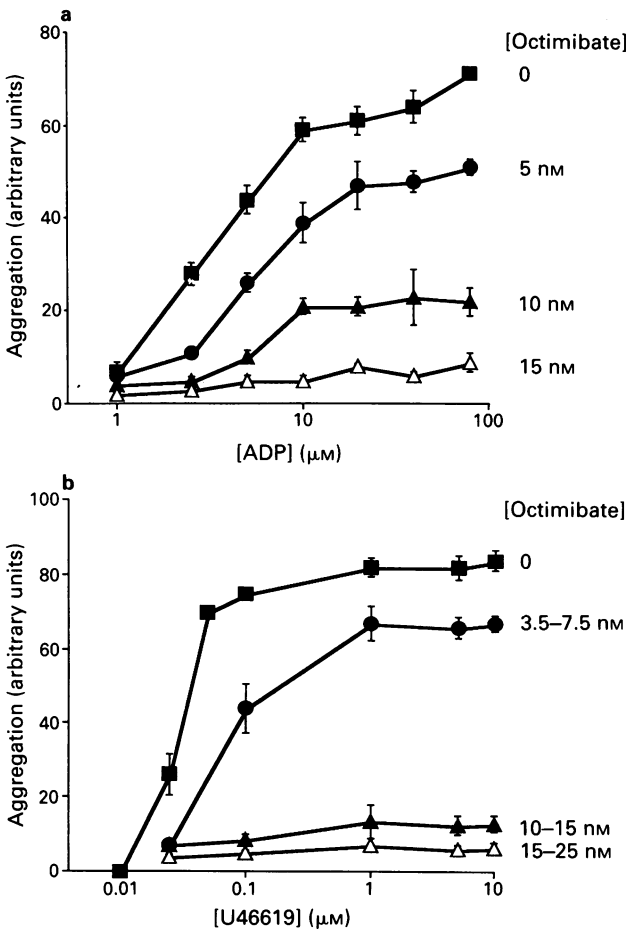


Figure 3 Effect of octimibate on aggregation of washed human platelets stimulated by various concentrations of ADP (a) or U46619 (b). Washed platelets were incubated for 2 min with the concentrations of octimibate indicated, and then challenged with varying concentrations of ADP or U46619. (Where a range of octimibate concentrations are shown, this indicates that different concentrations were needed on different platelet preparations to obtain the same % inhibition.) Aggregation is shown as the extent of aggregation (arbitrary units) 4 min after addition of the stimulus. Data are means of 3 experiments, s.e.mean shown by vertical bars; where not shown, the error bars are within the symbol size.

respectively. Since octimibate inhibited aggregation stimulated by several different agonists, it is unlikely to be acting as a receptor antagonist. Such functional inhibition would therefore be expected to be 'non-competitive' with the agonist. Figure 3 demonstrates clearly that the inhibitory effect of octimibate on ADP- and U46619-evoked aggregation of washed platelets was not competitive. The dose-response curves for ADP (Figure 3a) and U46619 (Figure 3b) showed a decrease in maximum effect with increasing concentrations of octimibate, while there was no detectable shift in EC_{50} for the agonist. The inhibitory effect of iloprost showed similar characteristics to that of octimibate (results not shown).

Octimibate also inhibited aggregation of platelets in plasma, although its potency was considerably reduced compared to that with washed platelets. The IC_{50} for inhibition of U46619-stimulated aggregation of platelet-rich plasma by octimibate was 207 ± 4 nM ($n = 10$), compared to 10.5 ± 0.4 nM ($n = 9$) for inhibition of U46619-stimulated

Table 1 Effects of octimibate and iloprost on human platelets

	Octimibate	*Iloprost*	*Ratio* octimibate/iloprost
K_i (nM) for binding	153 ± 28 (7)	31 ± 4 (14)	4.9
Stimulation of adenylyl cyclase			
EC_{50} (nM)	204 ± 8 (7)	6.8 ± 0.7 (15)	30
Relative maximum effect	0.57 ± 0.02 (7)	1.0	
Inhibition of rise in $[Ca^{2+}]_i$			
IC_{50} (nM) for washed platelets stimulated by ADP	9.8 ± 0.4 (3)	1.7 ± 0.1 (3)	5.8
Inhibition of aggregation			
IC_{50} (nM) for washed platelets			
stimulated by U46619	10.5 ± 0.4 (9)	0.9 ± 0.03 (8)	11.7
stimulated by ADP	8.4 ± 0.1 (4)	0.7 ± 0.05 (4)	12.0
Inhibition of aggregation			
IC_{50} (nM) for platelet-rich plasma			
stimulated by U46619	207 ± 4 (10)	1.6 ± 0.09 (5)	129
stimulated by ADP	226 ± 4 (3)	1.4 ± 0.03 (3)	161

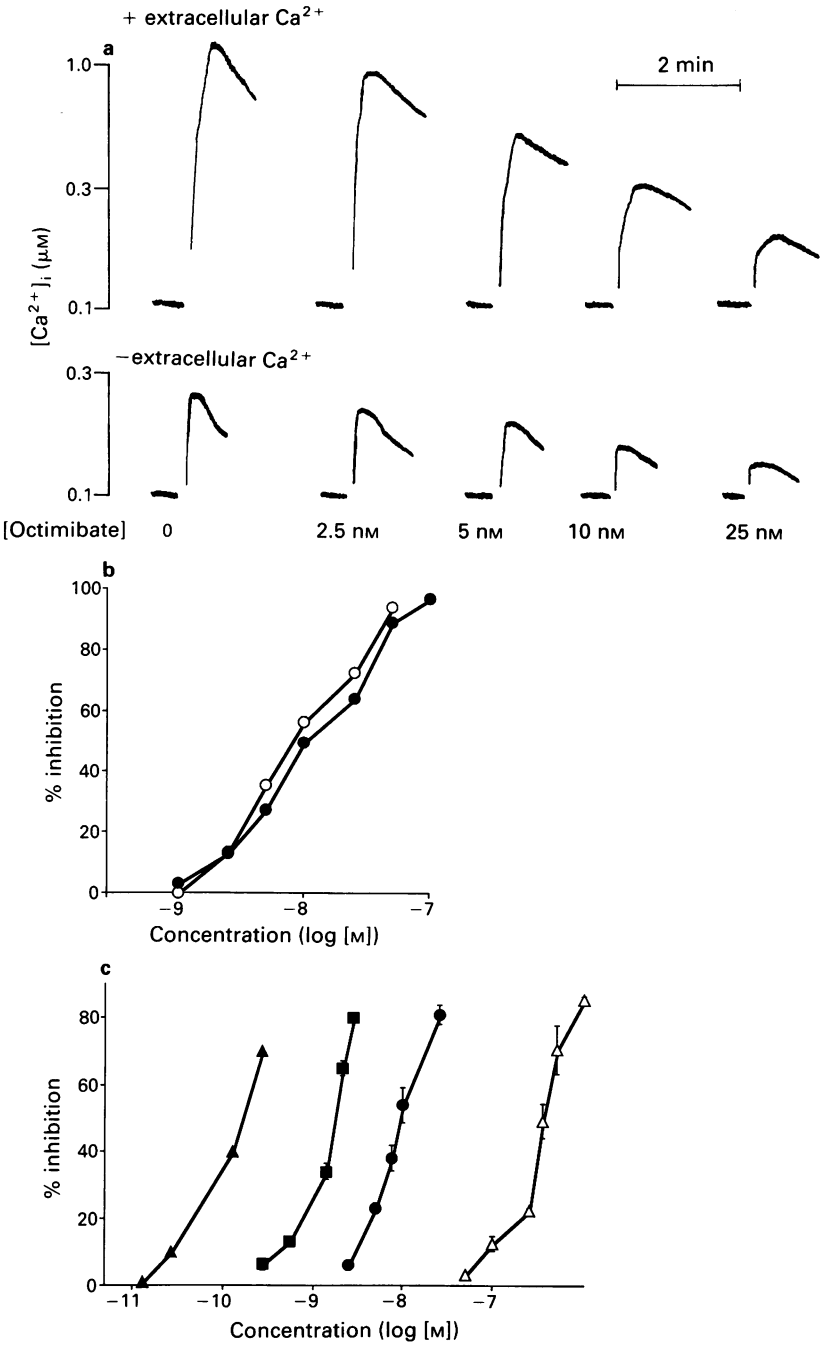


Figure 4 The effects of octimibate, iloprost, PGI_2 and forskolin on ADP-stimulated increases in $[Ca^{2+}]_i$ in quin2-loaded washed human platelets. (a) Typical quin2 fluorescence traces (calibrated to $[Ca^{2+}]_i$) showing ADP (20 μM)-stimulated increases in $[Ca^{2+}]_i$ in washed platelets in the presence of various concentrations of octimibate (0–25 nM) added 2 min before ADP. Experiments were carried out in the presence of extracellular Ca^{2+} (1 mM $CaCl_2$ added) or in the absence of extracellular Ca^{2+} (1 mM EGTA added). (b) Dose-response curves for inhibition of the ADP-stimulated increase in quin2 fluorescence by octimibate in the presence of extracellular Ca^{2+} (total rise in $[Ca^{2+}]_i$; ○) or in the absence of extracellular Ca^{2+} (Ca^{2+} release from intracellular stores; ●). Experimental details are as described in (a). Percentage inhibition is calculated as inhibition of the maximum peak in fluoresence measured after addition of ADP. Data are means ± ranges of duplicate determinations in one experiment. (Where not shown, the error bars are within the symbol size.) (c) Dose-response curves for inhibition of the ADP-stimulated increase in quin2 fluorescence in the presence of extracellular Ca^{2+} by PGI_2 (▲), iloprost (■), octimibate (●) and forskolin (△). The compounds were added 2 min before ADP (20 μM). Percentage inhibition is calculated as inhibition of the maximum peak in fluorescence measured after addition of ADP. Data are means of 5 experiments with octimibate, 3 experiments with iloprost and forskolin, and a single experiment with PGI_2; vertical bars show s.e.mean; where not shown, the error bars are within the symbol size.

aggregation of washed platelets. Similar results were obtained for ADP-stimulated platelets, IC_{50} values of 226 ± 4 nM (n = 3) and 8.4 ± 0.1 nM (n = 4) in the presence and absence of plasma respectively. The presence of plasma had little effect on the inhibitory activity of iloprost; the IC_{50} values were 1.6 ± 0.09 nM (n = 5) in plasma, compared to 0.9 ± 0.03 nM (n = 8) on washed platelets stimulated with U46619. These results are summarised in Table 1.

The inhibitory effects of both octimibate and iloprost were reversible. Platelet-rich plasma was incubated with concentrations of compound that caused 100% inhibition of aggregation, then centrifuged, and the platelets were resuspended in buffer (as described in the method section for preparation of washed platelets). When these platelets were then challenged with an aggregatory stimulus (U46619), there was no inhibition of aggregation.

Effect of octimibate on agonist-stimulated $[Ca^{2+}]_i$ rises in platelets

Figure 4 shows the effect of octimibate, iloprost, PGI_2 and forskolin on $[Ca^{2+}]_i$ responses of washed human platelets stimulated with ADP (20 μM). Figure 4a shows typical fluorescence traces, calibrated to $[Ca^{2+}]_i$, for quin2-loaded platelets stimulated with ADP in the presence or absence of extracellular Ca^{2+} (1 mM $CaCl_2$ or 1 mM EGTA added respectively). The platelets were preincubated for 2 min with the concentrations of octimibate shown. The figure clearly shows that octimibate is a potent inhibitor of ADP-stimulated rises in $[Ca^{2+}]_i$, both in the presence or absence of extracellular Ca^{2+}. In the presence of extracellular Ca^{2+}, the ADP-stimulated rise in $[Ca^{2+}]_i$ is due to a combination of Ca^{2+} release from intracellular stores and influx of extracellular Ca^{2+} (Rink & Sage, 1990). Under these experimental conditions, where the cells are heavily loaded with a $[Ca^{2+}]_i$ indicator dye (quin2) to buffer changes in $[Ca^{2+}]_i$ (Merritt *et al.*, 1990), 25–30% of the rise in fluorescence is due to Ca^{2+} release from intracellular stores and the rest is due to influx of extracellular Ca^{2+} (compare control traces plus and minus extracellular Ca^{2+} in Figure 4a). The inhibitory effects of octimibate in the presence of extracellular Ca^{2+} are therefore predominantly due to inhibition of Ca^{2+} influx. When the platelets are incubated in the absence of extracellular Ca^{2+} (1 mM EGTA added), the ADP-stimulated rise in $[Ca^{2+}]_i$ is due to release from intracellular stores. This is also shown in Figure 4a, which shows inhibition of this response by octimibate. Both Ca^{2+} release from intracellular stores and influx of extracellular Ca^{2+} show similar sensitivities to inhibition by octimibate, as shown in Figures 4a and 4b. Figure 4b shows dose-response curves from a single experiment for inhibition of total $[Ca^{2+}]_i$ rises and Ca^{2+} release from intracellular stores by octimibate. The IC_{50} values for octimibate were 9.0 ± 0.6 nM (Hill coefficient 1.3 ± 0.1) and 11.6 ± 0.8 nM (Hill coefficient 1.2 ± 0.1) respectively.

Figure 4c shows dose-response curves for inhibition of the total ADP-stimulated increase in $[Ca^{2+}]_i$ by octimibate (IC_{50} = 9.8 ± 0.4 nM, Hill coefficient = 1.8 ± 0.1, n = 5), PGI_2 (IC_{50} = 0.16 ± 0.01 nM, Hill coefficient = 1.5 ± 0.2, n = 1), iloprost (IC_{50} = 1.7 ± 0.11 nM, Hill coefficient = 2.4 ± 0.5, n = 3), and forskolin (IC_{50} = 370 ± 26 nM, Hill coefficient = 2.2 ± 0.4, n = 3). For each of these compounds, similar concentrations were required to inhibit both Ca^{2+} release from intracellular stores and influx of extracellular Ca^{2+}: 67 ± 4% (n = 3) and 81 ± 3% (n = 3) inhibition respectively by 25 nM octimibate; 62 ± 6% (n = 3) and 65 ± 2% (n = 3) inhibition respectively by 2.1 nM iloprost; 74% (n = 1) and 70% (n = 1) inhibition respectively by 0.27 nM PGI_2; 61 ± 3% (n = 3) and 71 ± 7% (n = 3) inhibition respectively by 500 nM forskolin.

Effect of octimibate and iloprost on cyclic AMP metabolism in platelets

Figure 5 shows the effect of octimibate and iloprost on the cyclic AMP content (Figure 5a) and the cyclic AMP-dependent protein kinase activity ratio (Figure 5b) of human platelets following parallel 2 min incubations at 37°C. It is clear that both iloprost and (to a lesser extent) octimibate have stimulated increases in cyclic AMP content and activation of cyclic AMP-dependent protein kinase (an increase in the activity ratio). It should be noted that, at concentrations of octimibate and iloprost that are required to inhibit platelet aggregation (Figure 2 and Table 1), the stimulated increases in cyclic AMP content and cyclic AMP-dependent protein kinase activity are very small. This stimulated increase in platelet cyclic AMP content could be due to either stimulation of adenylyl cyclase or inhibition of cyclic nucleotide phosphodiesterase.

Figure 6 shows that both octimibate and iloprost activate adenylyl cyclase in human platelet membranes. The EC_{50} for activation of adenylyl cyclase by octimibate is 204 ± 8 nM

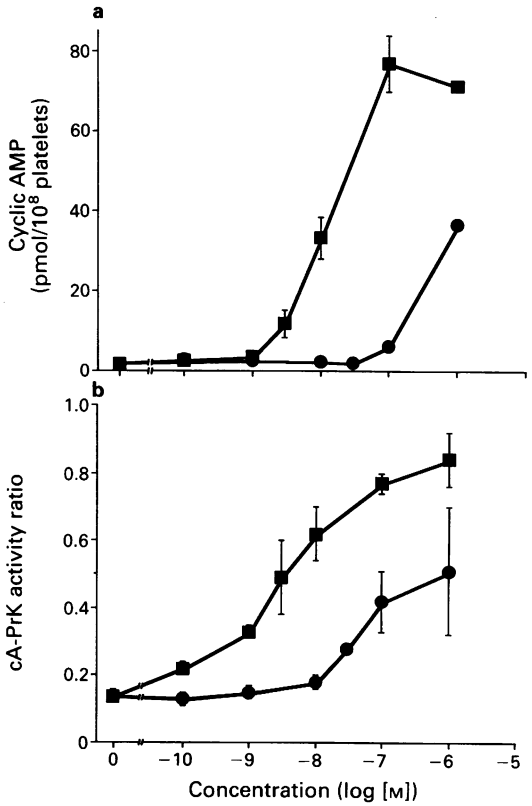


Figure 5 The effects of octimibate and iloprost on cyclic AMP levels (a) and the cyclic AMP-dependent protein kinase activity ratio (b) in washed human platelets. Platelet suspensions were incubated for 2 min in parallel incubations with various concentrations of octimibate (●) or iloprost (■), and following termination of the incubations, cyclic AMP levels (a) or cyclic AMP-dependent protein kinase activity ratios (cA-PrK) (b) were measured. Data are means of 1–5 (mostly 4) experiments; vertical bars show s.e.mean; where not shown, the error bars are within the symbol size.

(n = 7), Hill coefficient is 1.4 ± 0.1. The EC_{50} for activation by iloprost is 6.8 ± 0.7 nM (n = 15), Hill coefficient is 1.2 ± 0.1. The maximum effect of octimibate relative to that of iloprost is 57 ± 2% (n = 7). The maximum effect of PGI_2 was similar to that of iloprost (results not shown). High concentrations of octimibate (> 10 μM) appear to inhibit adenylyl cyclase activ-

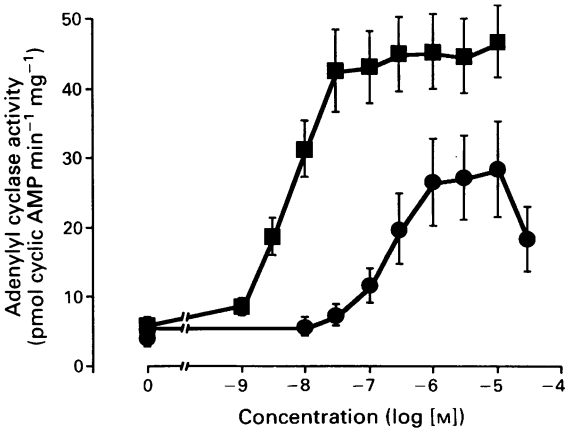


Figure 6 The effects of octimibate and iloprost on adenylyl cyclase activity in human platelet membranes. Adenylyl cyclase activity is shown as pmol cyclic AMP formed min^{-1} mg^{-1} protein during a 10 min incubation at 37°C. Dose-response curves are shown as means of 7 experiments for octimibate (●) and 15 experiments for iloprost (■); vertical bars show s.e.mean, where not shown, the error bars are within the symbol size.

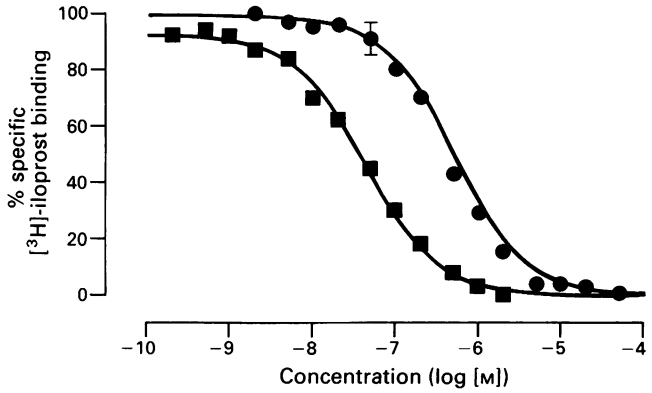


Figure 7 Inhibition of [^{3}H]-iloprost binding to platelet membranes by iloprost or octimibate. [^{3}H]-iloprost binding is shown as % specific binding, after subtraction of nonspecific binding, during a 30 min incubation at 37°C. Results are shown as means of triplicates from a single representative experiment with iloprost (■) and octimibate (●). The curves were obtained by fitting the data to a single site model. Vertical bars show s.e.mean; where not shown, the error bars are within the symbol size.

ity. This is most probably due to a nonspecific effect, since concentrations of octimibate $> 10\,\mu$M cause red blood cell haemolysis.

An alternative or additional means for elevation of cellular cyclic AMP could be inhibition of phosphodiesterase. The major cyclic AMP-metabolising phosphodiesterase in platelets is the cyclic GMP-inhibited phosphodiesterase, phosphodiesterase III, (Murray *et al.*, 1990b). Octimibate, at concentrations up to 1 μM, had no effect on the activity of this enzyme.

Inhibition of [^{3}H]-iloprost binding by octimibate

Figure 7 shows curves for inhibition of specific binding of [^{3}H]-iloprost to human platelet membranes by unlabelled iloprost or octimibate; the results are from a single typical experiment carried out in triplicate. For both ligands, the data appear to fit well to a single site analysis. Octimibate inhibited completely the specific binding of [^{3}H]-iloprost. The IC_{50} values for iloprost and octimibate were 46.5 ± 5.2 nM ($n = 14$) and 231 ± 42 nM ($n = 7$) respectively. These IC_{50} values correspond to K_i values of 30.9 ± 3.5 nM ($n = 14$) for iloprost and 153 ± 28 nM ($n = 7$) for octimibate. Maximal binding of [^{3}H]-iloprost was 0.96 ± 0.22 pmol mg^{-1} protein ($n = 5$). Both this value and the K_d of 19.6 ± 3.0 nM ($n = 2$, with range) for [^{3}H]-iloprost are consistent with values reported by others (e.g. Hall & Strange, 1984).

Effects of octimibate on aggregation of platelets from different species

Table 1 summarises all the results obtained with octimibate and iloprost on human platelets, and Table 2 shows the effects of octimibate and iloprost on aggregation of washed platelets from several different species. From Table 2, it is evident that octimibate is a potent inhibitor of aggregation of human and cynomolgus monkey platelets, but is 20–200 fold less potent on all other species tested (similar results were obtained using platelet-rich plasma). The potency of iloprost varied by no more than a factor of 10 between species, with no obvious differences between primates and non-primates. Table 2 also shows the ratio of potency of octimibate/iloprost for each species. It is evident that octimibate is, relatively, at least 20 fold more potent on primates that non-primates, when considering the species variation of both octimibate and iloprost.

Discussion

In addition to its antihyperlipidaemic and antiatherosclerotic actions, octimibate has been reported to have antithrombotic activity with inhibition of platelet aggregation at concentrations of 1–9 μM (Lautenschlager *et al.*, 1986). Here, we have shown that octimibate is considerably more potent for inhibition of human platelet aggregation than was previously reported. We have shown that octimibate inhibits platelet aggregation with an IC_{50} of about 10 nM for washed platelets and about 200 nM for platelets in plasma. The reduction in potency of octimibate in plasma is presumably due to binding to plasma proteins. As the inhibitory effect was not competitive with the stimulatory agonist (U46619 or ADP), octimibate was not acting as a receptor antagonist.

Many agonists that stimulate platelet aggregation also evoke rises in $[Ca^{2+}]_i$ (Rink *et al.*, 1982; Hallam *et al.*, 1984; Rink & Sage, 1990), and inhibition of agonist-stimulated rises in $[Ca^{2+}]_i$ can result in inhibition of agonist-stimulated aggregation (Sage & Rink, 1985; Merritt *et al.*, 1989). We have shown that octimibate inhibits agonist-stimulated $[Ca^{2+}]_i$ rises in platelets with an IC_{50} value of about 10 nM, a similar potency to that for inhibition of aggregation. Agonist-stimulated rises in $[Ca^{2+}]_i$ are due to both release of Ca^{2+} from intracellular stores and influx of extracellular Ca^{2+} (Rink & Sage, 1990). Octimibate inhibited both Ca^{2+} release from stores and influx over the same dose-range, thus it cannot be acting as a plasma membrane Ca^{2+} channel blocker since a Ca^{2+} channel blocker would be expected to inhibit selectively influx of Ca^{2+} (Merritt *et al.*, 1989). Agents, such as forskolin, PGI_2 and PGD_2, that elevate cyclic AMP are known to inhibit agonist-stimulated $[Ca^{2+}]_i$ rises in platelets (Feinstein *et al.*, 1983; Rink & Sanchez, 1984). Forskolin has been found to inhibit both Ca^{2+} release from internal stores and influx with similar potency, and also to inhibit aggregation (Sage & Rink, 1985). Here, we have confirmed that both forskolin and PGI_2 (and iloprost, a stable PGI_2 mimetic), inhibit agonist-stimulated platelet aggregation and rises in $[Ca^{2+}]_i$ due to both store release and influx, with similar effects to those of octimibate. Experiments were therefore carried out to see if octimibate evoked increases in cyclic AMP in platelets.

We have shown that octimibate, as well as iloprost, does evoke increases in platelet cyclic AMP content and increases the platelet cyclic AMP-dependent protein kinase activity ratio (i.e. the proportion of the enzyme that is active following incubation of platelets with the test compound). At concentrations of octimibate or iloprost that inhibit platelet aggregation, the stimulated increases in cyclic AMP or cyclic AMP-dependent protein kinase activity ratio are almost undetectable. Such a result was not unexpected, since a similar

Table 2 Inhibition of washed platelet aggregation from different species

Species	*Stimulus*	*IC_{50} (nM)* Octimibate	Iloprost	*Ratio* (octimibate/iloprost)
Human	U46619	10.5	0.9	12
Cynomolgus monkey	U46619	3.8	1.0	4
Guinea-pig	U46619	204	0.7	296
Dog	PAF	310	1.9	163
Rat	ADP	520	1.6	325
Cat	U46619	1100	4.0	275
Cow	PAF	2700	8.5	318

relation between elevation of cyclic AMP and inhibition of platelet aggregation has already been demonstrated for the phosphodiesterase inhibitor, siguazodan, whereby very small increases in cyclic AMP are associated with inhibition of platelet aggregation (Murray et al., 1990b). The octimibate-stimulated rise in cyclic AMP could be due to either inhibition of phosphodiesterase or stimulation of adenylyl cyclase. The main phosphodiesterase isozyme found in platelets is the cyclic GMP-inhibited phosphodiesterase (Murray et al., 1990b), and we have shown that the activity of this enzyme is not affected by octimibate, but octimibate does stimulate adenylyl cyclase activity in platelet membranes.

Platelets are known to have several receptors, including PGI_2, prostaglandin D_2 (PGD_2) and adenosine (A_2), that are coupled to stimulation of adenylyl cyclase (Miller & Gorman, 1979; Huttemann et al., 1984; Ashby, 1989; Keen et al., 1989a) and inhibit aggregation (Moncada et al., 1976; Rink & Sage, 1990). Iloprost is a stable PGI_2 mimetic (Gryglewski & Stock, 1987), and inhibition of [^{3}H]-iloprost binding can be used to assay the interaction of ligands with the PGI_2 receptor (Hall & Strange, 1984). We have shown that octimibate inhibits completely the binding of [^{3}H]-iloprost to platelet membranes, with an estimated K_i of 150 nM. This result suggests that octimibate binds to the same site as iloprost, the PGI_2 receptor, although additional binding sites for octimibate to other receptor-types cannot be excluded. The K_i value for inhibition of binding of [^{3}H]-iloprost by octimibate (150 nM) is similar to the EC_{50} value for activation of adenylyl cyclase (200 nM). These results suggest the major (or only) site of action of octimibate in stimulating adenylyl cyclase in platelets is at the PGI_2 receptor. The K_i value for inhibition of binding of [^{3}H]-iloprost by iloprost (30 nM) is greater than the EC_{50} value for activation of adenylyl cyclase (7 nM), which suggests that there could be spare receptors for activation of adenylyl cyclase by iloprost.

Octimibate also inhibited [^{3}H]-iloprost binding and activated adenylyl cyclase in membranes of NCB-20 cells (Kelly, Keen & MacDermot, unpublished observations), a cell line reported to have receptors for PGI_2 (Blair et al., 1980; Blair & MacDermot, 1981). Desensitization of PGI_2 receptors is reported to be homologous in NCB-20 cells (Kelly et al., 1990), and iloprost-pretreatment of these cells reduced considerably their subsequent response to octimibate as well as iloprost (Kelly, Keen & MacDermot, unpublished observations). This result provides further support for the hypothesis that the effects of octimibate are mediated (at least in part) through the same receptor as that for iloprost (i.e. the PGI_2 receptor).

Interaction of octimibate with the PGI_2 receptor could explain some of the antiatherosclerotic effects of octimibate. Octimibate has been reported to increase cholesterol efflux from cholesterol-loaded macrophages by causing a shift from esterified to free cholesterol (Schmitz et al., 1985; Lautenschlager et al., 1986). These effects of octimibate have been assumed to be due to inhibition of cholesterol esterification by ACAT. However, PGI_2 is known to decrease the lipid content of cholesterol-loaded macrophages (Willis et al., 1986) and vascular smooth muscle cells where it elevates cyclic AMP and activates neutral cholesterol ester hydrolase through cyclic AMP-dependent phosphorylation (Hajjar, 1986). Some of the antiatherosclerotic effects of octimibate could therefore be due to PGI_2 receptor-mediated activation of neutral cholesterol ester hydrolase in addition to the previously reported inhibition of ACAT.

Although octimibate appears similar to iloprost in its spectrum of activities, some differences are also apparent. The maximum activation of adenylyl cyclase obtained with octimibate is considerably less than that obtained with iloprost or PGI_2; the maximum activation seen with octimibate was 60% of that seen with iloprost. This result might suggest that octimibate is acting as a partial agonist at the PGI_2 receptor. An alternative or additional explanation is that there is a nonspecific inhibitory effect superimposed. The decrease in adenylyl cyclase activity at very high concentrations of octimibate appears to be due to nonspecific effects, since high concentrations of octimibate cause red blood cell haemolysis. From these results it is therefore not possible to determine whether octimibate is a full or a partial agonist at the PGI_2 receptor, and further experiments were not possible due to the problems of insolubility and inhibition of adenylyl cyclase activity at high concentrations of octimibate.

Octimibate showed considerably more species variation than either iloprost (Sturzebecher et al., 1987; Armstrong et al., 1989), PGI_2 (Whittle & Moncada, 1984; Oliva & Nicosia, 1987) or other PGI_2 mimetics including carbacyclin (Oliva & Nicosia, 1987). Octimibate was highly selective for inhibition of primate platelet aggregation compared to other species. This differential species sensitivity of octimibate might indicate the presence of different receptor subtypes in primates compared to other species. Obviously such results highlight the need for caution when interpreting results obtained on tissue from other species and from animal models.

To date, all known PGI_2 mimetics are prostanoid-type molecules (Oliva & Nicosia, 1987). Octimibate is structurally very different, and is a novel and potent inhibitor of platelet aggregation. The species variation of octimibate points to differences between the biological effects of octimibate and iloprost. It was therefore of interest to see whether octimibate could distinguish between platelet and vascular PGI_2 receptors, since such a compound could have significant therapeutic potential (Keen et al., 1989b). The results of these studies are reported in the following paper (Merritt et al., 1991).

We would like to thank Schering AG (Berlin) for supplying us with iloprost, the Blood Transfusion Service (Cambridge) for providing us with outdated concentrated platelet-rich plasma, the Royal Veterinary College (South Mimms, London) and the Huntingdon Research Centre (Huntingdon) for providing us with blood from different animals. Thanks also to Mr V.P. Shah (Department of Medicinal Chemistry, SmithKline Beecham Pharmaceuticals, Welwyn, Herts) for synthesis of octimibate. M.K. and E.K. were supported by a programme grant from the Wellcome Trust.

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Primate vascular responses to octimibate, a non-prostanoid agonist at the prostacyclin receptor

[1]Janet E. Merritt, Anthony M. Brown, **Stuart Bund, David G. Cooper, †John W. Egan, [2]Trevor J. Hallam, **Anthony M. Heagerty, Deirdre M.B. Hickey, Alberto J. Kaumann, *Mary Keen, #Eamonn Kelly, †Cheryl A. Kenney, †Andrew J. Nichols, †Edward F. Smith III, [3]George T.G. Swayne, §John MacDermot & [4]Timothy J. Rink

SmithKline Beecham Pharmaceuticals, The Frythe, Welwyn, Herts. AL6 9AR; †SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania 19406, U.S.A.; **Department of Medicine, Clinical Sciences Building, Leicester Royal Infirmary, Leicester LE2 7LX; *Department of Pharmacology, University of Birmingham, The Medical School, Birmingham B15 2TJ; #Department of Pharmacology, The Medical School, University Walk, Bristol BS8 1TD and §Department of Clinical Pharmacology, Royal Postgraduate Medical School, Du Cane Road, London W12 0NN

1 Octimibate is a potent inhibitor of human platelet aggregation, and appears to act (at least in part) through the prostacyclin receptor, as described in the preceding paper. Here, the vascular effects, both *in vitro* and *in vivo*, of octimibate have been compared to those of the stable prostacyclin (PGI_2) mimetic, iloprost. Since octimibate shows extensive species variation and is potent at inhibiting platelet aggregation in primates, all of the experiments reported here have been carried out with primate tissue or *in vivo* in cynomolgus monkeys.

2 Activation of adenylyl cyclase in human lung membranes appears to involve stimulation of the vascular PGI_2 receptor. Octimibate, as well as iloprost, stimulates adenylyl cyclase in this preparation. The EC_{50} values for iloprost and octimibate are 50 nM and 340 nM respectively. These values are similar to those seen with human platelet membranes. As with platelets, the maximal activation achievable with octimibate is 60% of that seen with iloprost. This result suggests that octimibate is a partial agonist for stimulation of adenylyl cyclase.

3 Iloprost (10–100 nM) relaxes human coronary and mesenteric artery precontracted with KCl, and also relaxes cynomolgus monkey aorta precontracted with phenylephrine. Octimibate appears to be a partial agonist for relaxation of human coronary artery precontracted with KCl; the intrinsic activity of octimibate (10 μM) is 0.15 compared to iloprost, and octimibate surmountably antagonizes the relaxant effects of iloprost with a K_p of 200 nM. Octimibate (up to 10 μM) evokes only weak relaxation of human mesenteric artery (precontracted with KCl) and cynomolgus monkey aorta (precontracted with phenylephrine).

4 The effects of iloprost and octimibate were compared *in vivo* in cynomolgus monkeys. In addition to inhibiting *ex vivo* platelet aggregation, both compounds cause hypotension with little effect on heart rate. The dose-response curves for inhibition of *ex vivo* platelet aggregation and a fall in mean arterial blood pressure were compared. The dose-separation (i.e., the relative differences in effective concentrations) for the two responses is similar with both iloprost and octimibate.

5 Since the peripheral resistance vessels are intimately involved in regulation of systemic arterial blood pressure, the effects of both agents were tested on human peripheral resistance vessels (150–400 μm diameter) *in vitro*. These vessels are relaxed by both iloprost and octimibate following precontraction with KCl. The IC_{50} value for iloprost is 44 nM, and 1.7 μM octimibate evokes 50% of the maximal relaxation obtained with iloprost. Thus, the relative potencies of the two compounds in relaxing human subcutaneous resistance vessels are similar to their relative potencies in inhibiting platelet responses. This result correlates with the lack of platelet versus vascular selectivity seen with the *in vivo* monkey studies.

6 These results suggest that octimibate, a partial agonist at the prostacyclin receptor, is unable to discriminate between platelet and vascular prostacyclin receptors in primates.

Introduction

Octimibate, 8-[(1,4,5-triphenyl-1H-imidazol-2-yl)oxy]octanoic acid, was previously described as having antihyperlipidaemic, antiatherosclerotic and antithrombotic properties (European Patent Specification, EP-B-130526; Lautenschlager *et al.*, 1986; Rucker *et al.*, 1988). In the preceding paper (Merritt *et al.*, 1991), we showed octimibate to be a potent non-prostanoid inhibitor of platelet aggregation (IC_{50} of 10 nM) and provided evidence that the effects of octimibate are mediated, at least in part, through activation of the prostacyclin (PGI_2) receptor. Iloprost is a stable PGI_2 mimetic (Gryglewski & Stock, 1987) but there appeared to be some differences between the effects of iloprost and octimibate on platelets (Merritt *et al.*, 1991). The maximal activation of human platelet adenylyl cyclase by octimibate is considerably lower than that seen with iloprost or PGI_2, suggesting that octimibate is a partial agonist at the platelet PGI_2 receptor. There is extensive species variation in responsiveness of platelets to octimibate compared to iloprost; octimibate was potent only in primates. The species variation in platelet responsiveness seen with octimibate suggested that octimibate might be distinguishing a PGI_2 receptor subtype unique to primates. It was therefore of interest to determine whether octimibate might also distinguish tissue variations between PGI_2 receptors within a given species. The biological importance of PGI_2 and the potential for tissue selectivity have been extensively reviewed (Moncada, 1982; Keen *et al.*, 1989a).

PGI_2 causes relaxation of many different arterial strips or rings from various species although venous strips are not

[1] Author for correspondence.
[2] Glaxo Group Research Ltd., Greenford Road, Greenford, Middlesex UB6 0HE.
[3] Department of Discovery Biology, Pfizer Central Research, Sandwich, Kent CT13 9NJ.
[4] Amylin Corporation, 9373 Towne Centre Drive, Suite 250, San Diego, California 92121, U.S.A.

relaxed (Gryglewski, 1987; Oliva & Nicosia, 1987). In several species, PGI_2 or its stable analogues cause a fall in blood pressure (Oliva & Nicosia, 1987). A major effect of prostacyclin in man, as well as other species, is dilatation of arterioles but not postcapillary venules (Gryglewski, 1987). This results in a reduction in peripheral vascular resistance and causes peripheral vasodilatation evidenced as facial flushing, and headache (Pickles & O'Grady, 1982; Keen *et al.*, 1989a). These vascular effects of PGI_2 have limited the clinical utility of PGI_2 and its analogues as antithrombotics. The most widespread use of PGI_2 and its stable analogues is in extracorporeal haemoperfusion systems to prevent aggregation and deposition of platelets (Moncada, 1982; Keen *et al.*, 1989a). Infusions of PGI_2 or iloprost have also been used in the treatment of peripheral vascular disease; their effectiveness here is probably due to both their effects on platelets and vascular effects (Belch *et al.*, 1983; Keen *et al.*, 1989a). If a compound were to distinguish platelet and vascular PGI_2 receptors, it could have great therapeutic potential as a highly effective antithrombotic. [It should be noted that all the anti-aggregatory effects we observed with octimibate and iloprost were additional to that of aspirin; Merritt *et al.*, 1991]. The idea of looking for a PGI_2 analogue selective for platelets has been discussed extensively (e.g. Whittle & Moncada, 1984; Oliva & Nicosia, 1987; Armstrong *et al.*, 1989; Keen *et al.*, 1989a). To date, the only compound reported to discriminate between PGI_2 receptors in platelets and vascular smooth muscle is (5Z)-carbacyclin (Corsini *et al.*, 1987). However, these authors used human platelets and rabbit mesenteric artery, so it is not clear whether their result reflects a true tissue variation or merely a species variation in the PGI_2 receptor.

Since octimibate shows selectivity for primate platelets, all the studies we describe here with vascular responses have been carried out on primate (human or cynomolgus monkey) tissue. Human lung provides a source of tissue for biochemical experiments on the vasculature, so we compared the effects of iloprost and octimibate on activation of human lung membrane adenylyl cyclase. We then looked at the effects of iloprost and octimibate on the large primate vessels that were available, human coronary and mesentery artery, and cynomolgus monkey aorta. Experiments were then carried out *in vivo* with cynomolgus monkeys to see if iloprost and octimibate differed in their relative effects on platelet and vascular responses. Since many of the vascular effects of iloprost *in vivo* are due to effects on peripheral resistance vessels rather than large vessels, the effects of iloprost and octimibate were compared on human peripheral resistance vessels.

Methods

Measurement of human lung membrane adenylyl cyclase activity

Human lung tissue was obtained post-operatively and stored at −70°C. (Note, it is important that the tissue should be free of charcoal, which might absorb the test compounds. For this reason, the tissue was taken from a young non-smoker.) For the preparation of membranes, the tissue was homogenized in buffer containing 0.29 M sucrose and 25 mM Tris-Cl, pH 7.4 at 4°C, then centrifuged for 10 min at 500 *g*. This low-speed supernatant was used as a source of membranes as the response was unstable on further processing of the tissue. Adenylyl cyclase activity was measured as described by Keen *et al.* (1989b) and Edwards *et al.* (1987), which is a modification of the method of Salomon *et al.* (1974). Reaction mixtures of 100 μl contained Tris-Cl 50 mM (pH 7.4 at 37°C), $MgCl_2$ 5 mM, creatine phosphate 20 mM, creatine kinase 10 u, adenosine 3′:5′-cyclic monophosphate (cyclic AMP) 1 mM, GTP 4 μM, Ro20-1724 0.25 mM (a phosphodiesterase inhibitor), ATP 1 mM with 2–6 μCi [α-^{32}P]-ATP, drugs or vehicle as appropriate, and 0.2–0.4 mg of homogenate protein. Reactions were prepared on ice and then incubated at 37°C for 15 min. Reactions were terminated by addition of 800 μl of 6.25% (w/v) trichloroacetic acid. [^{3}H]-cyclic AMP (about 15,000 c.p.m.) was added to each tube, and the tubes were centrifuged for 15 min at 200 *g*. A two step chromatographic procedure was used to separate [^{32}P]-cyclic AMP from [α-^{32}P]-ATP (Salomon *et al.*, 1974). [^{3}H]-cyclic AMP was used to monitor recovery of cyclic AMP from the columns, and adenylyl cyclase activity is expressed as pmol cyclic AMP formed min^{-1} mg^{-1} protein.

Relaxation of large primate vessels

Aortae from cynomolgus monkeys were provided by Huntingdon Research Centre Ltd., PO Box 2, Huntingdon, Cambs PE18 6ES. Human coronary arteries were obtained from Papworth-Everett Hospital, Cambridge, and mesenteric arteries were obtained from Addenbrooke's Hospital, Cambridge. Helicoidal strips of human tissue (Kaumann, 1983) and strips or rings of monkey aorta were mounted in organ baths as described by Kaumann & Frenken (1988). The medium, (composition mM: NaCl 89, KCl 5, $CaCl_2$ 2.25, $MgSO_4$ 0.5, $NaHCO_3$ 29, Na_2HPO_4 1, Na fumarate 10, Na pyruvate 5, Na L-glutamate 5, glucose 10, ascorbate 0.2, EDTA 40 μM and cocaine 6 μM) was maintained at 37°C and continuously gassed with 95% O_2/5% CO_2. The tissues were attached to strain gauge transducers connected via amplifiers to polygraphs, and tissue responses were measured as changes in isometric force. The contractile stimulus was phenylephrine (10 μM) for the monkey aortae and 45 mM KCl for the human vessels. When 45 mM KCl was included in the medium, the concentration of NaCl was correspondingly reduced. The tissues were precontracted to achieve a stable tone and then iloprost or octimibate was added cumulatively in order to establish a dose-response curve for relaxation. Data were analysed and equilibrium dissociation constants (K_p) for a partial agonist were calculated as described by Marano & Kaumann (1976).

In vivo *studies with cynomolgus monkeys*

All procedures were approved by the Institutional Animal Care and Use Committee of SmithKline Beecham Pharmaceuticals, and are in accordance with NIH guidelines for the use of experimental animals. Male cynomolgus monkeys (*Macaca fascicularis*) were fasted for 12 h prior to surgery, but were allowed water *ad libitum*. Animals were sedated with ketamine (15 mg) and atropine (40 μg kg^{-1}), and then anaesthetized with a mixture of oxygen, nitrous oxide and isofluorane (60%:20% 10%). A catheter was inserted through the skin into the saphenous vein and was used to administer saline and either drug or vehicle. One of the femoral arteries was exposed surgically, and a catheter was inserted, which was used for collection of arterial blood samples. Arterial blood pressure was continuously monitored (Statham P23 DC pressure transducer) on a multichannel dynograph (Electronics for Medicine recorder).

Blood samples were collected into sodium citrate (final concentration 0.38%), then centrifuged for 10 min at 200 *g* to obtain platelet-rich plasma (PRP). The PRP was removed and the remaining blood was centrifuged for 2 min at 1000 *g* to obtain platelet-poor plasma (PPP). The PRP was diluted with the PPP to produce a platelet count of 3×10^8 platelets ml^{-1}. Aggregation of PRP in aliquots (300 μl), stimulated with either ADP (10 μM) or U46619 (10 μM), was monitored in an aggregometer (Chrono-Log 400-VS, Chrono-Log Corp., Havertown, PA, U.S.A.). The mean platelet aggregation value was expressed as a percentage of the corresponding control sample

(taken before administration of drug). Blood samples were taken immediately after surgery (30 min before infusion of the test compound) and at the end of the infusion of the test compound.

Treatment with either octimibate (1–100 nmol kg^{-1} min^{-1} i.v.). iloprost (0.03–1 nmol kg^{-1} min^{-1}, i.v.) or vehicle (20% PEG 40 plus 80% isotonic saline) was initiated approximately 30 min after the first blood sample was taken. All solutions were sterile filtered (0.2 μm filter), and were prepared fresh daily. Drug or vehicle was administered at a rate of 0.2 ml min^{-1} for 60 min. The drug infusion was terminated immediately if the mean arterial blood pressure dropped below 35 mmHg. The animals were allowed to recover at the end of the experiment. The mean arterial blood pressure and heart rate responses to drug infusion were determined by averaging the readings between 30 and 60 min, and expressing this average response as a percentage of the initial predrug value. Results are expressed as ED_{20} for the drug dose resulting in a 20% fall in mean arterial blood pressure and ID_{50} for the drug dose resulting in 50% inhibition of platelet aggregation. These values were determined by linear regression analysis of the dose-response curves.

Relaxation of human peripheral resistance vessels

Resistance artery segments, approximately 2 mm long, were dissected from biopsies of skin and subcutaneous tissue (approx. 0.5 × 0.5 × 1.0 cm) taken from the gluteal region of healthy volunteers under local anaesthetic (3.5 ml 2% lignocaine hydrochloride) (Aalkjaer *et al.*, 1986; 1987). Local ethical committee approval had been granted, and the volunteers gave informed consent. The mean age of the 6 subjects (5 females and one male) was 41.5 ± 4.1 years. Arterial segments were mounted as ring preparations in a myograph (Mulvany & Halpern, 1977) modified to permit the study of two vessels (Mulvany & Nyborg, 1980). Arteries were mounted on two 40 μm diameter stainless steel wires, and were held in physiological saline containing (mM): NaCl 119, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.18, $MgCl_2$ 1.17, $CaCl_2$ 2.5, glucose 5.5 and EDTA 26 μM, pH 7.4 when gassed with 5% CO_2/95% O_2. After warming to 37°C for 30 min, the passive tension-internal circumference relation was determined (Mulvany & Halpern, 1977). Arteries were set to normalised internal circumferences, L_0, where $L_0 = 0.9L_{100}$ and L_{100} is the internal circumference the artery would have when relaxed and under a transmural pressure of 100 mmHg (Mulvany & Halpern, 1977). Effective normalised lumen diameters, I_0, are calculated as L_0/π. Contractile responses at these settings are near maximal (Aalkjaer *et al.*, 1987). The normalised lumen diameter for these experiments was 238 ± 21 μm (12 vessels).

A standard start 'wake-up' protocol was applied which consisted of 4 activations. Each activation was for 2 min, with complete relaxation between. The complete bath solution was changed for each addition, as well as the washes. The activations consisted of 3 additions of high KCl, where the NaCl in the basic medium is replaced with KCl, and one addition of the high KCl medium containing 5 μM noradrenaline. For the experiments with iloprost and octimibate, the vessels were contracted with 45 mM KCl (the basic medium contained 45 mM KCl, with a corresponding reduction in NaCl), which gave stable near maximal responses. Once a stable response had been obtained, dose-responses for relaxation by iloprost were carried out. Each addition of test compound, in medium containing 45 mM KCl, was accompanied by a complete change of solution in the bath. At the end of the dose-response, the tissue was washed by several complete changes of solution in the bath. The vessel was then left for 45 min in normal medium to allow recovery. After the recovery period, the vessel was again contracted with 45 mM KCl, and a dose-response for relaxation by octimibate or iloprost was carried out. The Results section (Figure 5) provides further details and validation of this protocol.

Materials

Octimibate and iloprost were obtained and solubilised as described in the preceding paper (Merritt *et al.*, 1991).

Results

Effects of octimibate and iloprost on primate vascular responses in vitro

Figure 1 shows that both iloprost and octimibate activate adenylyl cyclase in human lung membranes. Although this preparation contains a mixture of both vascular and airways smooth muscle, the response to PGI_2 receptor-stimulation is known to be predominantly due to stimulation of the vascular smooth muscle (Haye-Legrand *et al.*, 1987). Figure 1 shows results from a single experiment carried out in duplicate, and similar results were obtained in a second experiment. The EC_{50} for stimulation of adenylyl cyclase was 52 nM for iloprost and 342 nM for octimibate (the EC_{50} was 367 nM for octimibate in a second experiment). The maximal stimulation seen with octimibate was 57% of the response seen with iloprost.

Figure 2 shows the effects of octimibate and iloprost on human arterial vessels. Human coronary (Figure 2a) or mesenteric arteries (Figure 2b) were precontracted with 45 mM KCl, and then dose-response curves for relaxation were carried out for iloprost, octimibate, or iloprost in the presence of octimibate. Relaxation was measured as a percentage of the maximal relaxation obtained for each vessel with sodium nitroprusside (0.2 mM), administered to terminate the experiment. The two tissue preparations were from different donors, and each represents single experiments containing 2–4 replicates. It is clear that iloprost relaxes both coronary and mesenteric arteries, with maximal relaxation at 100 nM iloprost. Octimibate (1–10 μM) causes only a small relaxation of these vessels. Octimibate (1–3 μM) reduces the responsiveness of the vessels to iloprost such that higher concentrations of iloprost are needed in the presence of octimibate to achieve maximal relaxation. Due to limited tissue availability it was not possible to obtain full dose-response curves for the mesenteric

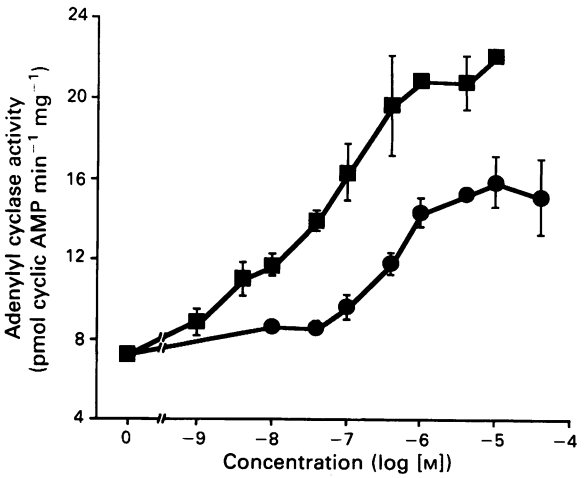

Figure 1 Activation of adenylyl cyclase in human lung membranes by octimibate and iloprost. Adenylyl cyclase activity is shown as pmol cyclic AMP formed min^{-1} mg^{-1} protein during a 15 min incubation at 37°C. The data are from a single experiment carried out in duplicate, and are shown as means with ranges (vertical bars). Dose-response curves are shown for octimibate (●) and iloprost (■). (Similar results were obtained with octimibate in a second experiment.)

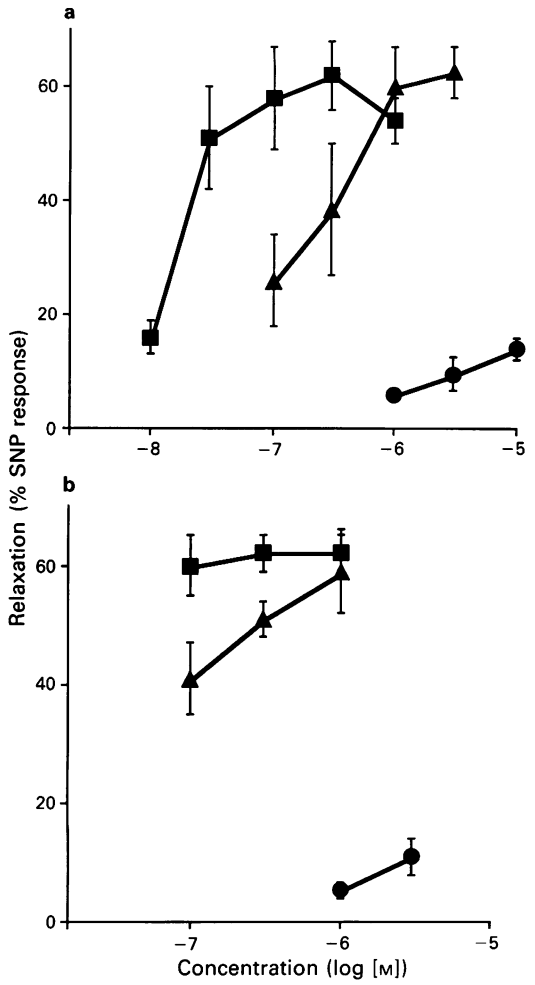

Figure 2 Dose-response curves for relaxation of human coronary (a) and mesenteric (b) arteries by iloprost (■) and octimibate (●). Dose-response curves were also carried out for iloprost in the presence of 1 μM octimibate (▲) for the coronary artery, and for iloprost in the presence of 3 μM octimibate (▲) for the mesenteric artery. In each case, relaxation is expressed as a percentage of the maximal relaxation obtained in that tissue with sodium nitroprusside (SNP, 0.2 mM). The vessels were precontracted with 45 mM KCl. Results are means of 2–4 strips (vertical bars show s.e.mean but where $n = 2$, the error bar shows the range). The donor of the coronary artery was a 23 year old male, and for the mesenteric artery was a 67 year old male.

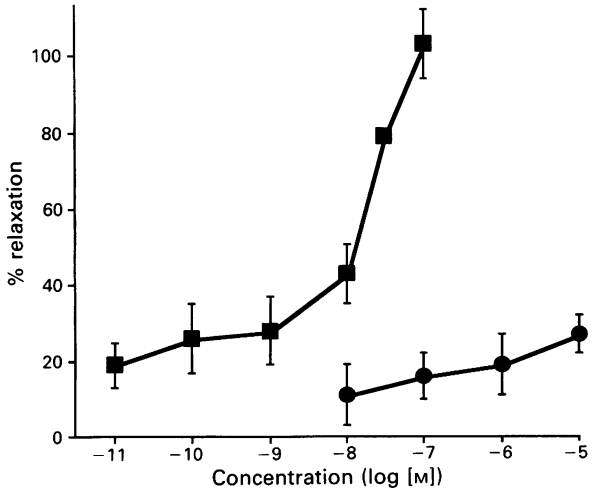

Figure 3 Cynomolgus monkey aortic rings and strips were precontracted with phenylephrine (10 μM), and dose-response curves for relaxation were carried out with iloprost (■) and octimibate (●); 100% relaxation represents basal tone. Results are means of 4–5 strips or rings obtained from 3 animals; vertical bars show s.e.mean.

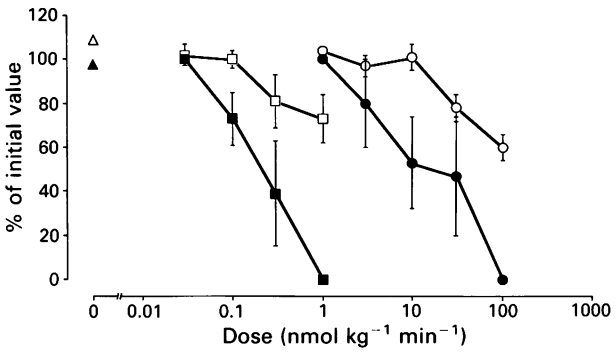

Figure 4 *In vivo* studies of the effects of octimibate and iloprost in cynomolgus monkeys. Anaesthetized cynomolgus monkeys were infused for 60 min with iloprost (■, □) or octimibate (●, ○) at the doses shown. The vehicle (▲, △) was 20% PEG400 in 0.9% NaCl. At the end of the infusion, mean arterial blood pressure (open symbols) and *ex vivo* platelet aggregation, stimulated with 10 μM U46619 (closed symbols), were monitored. The results are expressed as percentages of the initial preinfusion value. Results are means of 4 animals in each group; vertical bars show s.e.mean.

artery; however, the results with the coronary artery (Figure 2a) clearly show that 1 μM octimibate causes a rightward shift of almost one log unit in the iloprost dose-response curve. Octimibate therefore appears to be acting as a surmountable antagonist of iloprost-stimulated relaxation of the coronary artery, with an equilibrium dissociation constant (K_p) of around 200 nM.

Figure 3 shows the effect of octimibate and iloprost on cynomolgus monkey aorta precontracted with phenylephrine. It is clear that iloprost causes dose-dependent relaxation of this vessel, with complete relaxation evoked by 100 nM iloprost. Octimibate causes marginal relaxation at concentrations up to 10 μM. These results are the means from 3 animals.

Effects of octimibate and iloprost in vivo *in cynomolgus monkeys*

Figure 4 and Table 1 show the effects of i.v. administration of octimibate or iloprost in cynomolgus monkeys. It is clear that both octimibate and iloprost caused dose-dependent decreases in blood pressure and *ex vivo* platelet aggregation, as shown in Figure 4. The effects of both octimibate and iloprost on heart rate were minimal (results not shown). Table 1 shows ED_{20} values for the fall in mean arterial blood pressure (drug dose needed for a 20% decrease) and ID_{50} values for inhibition of *ex vivo* platelet aggregation (drug dose needed for a 50% decrease). Platelet aggregation was stimulated with either U46619 (dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$) or ADP. The ED_{20} values were chosen for assessment of effects on blood pressure since a 20% fall in blood pressure would be clinically significant. Table 1 also shows the ED_{20}/ID_{50} dose-ratio as an indication of platelet versus vascular selectivity. Figure 4 and Table 1 show that octimibate is approximately 90 fold less potent than iloprost *in vivo* in its effect on blood pressure and inhibition of platelet aggregation. Table 1 shows that the ED_{20}/ID_{50} ratio is the same for both octimibate and iloprost, which shows that octimibate exhibits no more platelet versus vascular selectivity than iloprost.

Effects of octimibate and iloprost on human peripheral resistance vessels in vitro

Since the peripheral resistance vessels are intimately involved in regulation of systemic arterial blood pressure and octimibate had small effects on large primate vessels *in vitro* but produced major haemodynamic effects *in vivo*, the *in vitro* effects of octimibate on peripheral resistance vessels were assessed. Figure 5 shows that both octimibate and iloprost

Table 1 Summary of the effects of iloprost and octimibate in cynomolgus monkeys

	Blood pressure (mean arterial)	*Platelet aggregation (ex vivo)*			
		ADP-stimulated		*U46619-stimulated*	
	ED_{20} (nmol kg^{-1} min^{-1})	ID_{50} (nmol kg^{-1} min^{-1})	ED_{20}/ID_{50}	ID_{50} (nmol kg^{-1} min^{-1})	ED_{20}/ID_{50}
Iloprost	0.35	0.082	4.3	0.21	1.7
Octimibate	30.7	7.7	4.0	19.6	1.6
Ratio (oct/ilo)	88	94		93	

evoke relaxation of human peripheral resistance vessels (150–400 μm diameter) precontracted with 45 mM KCl. Figure 5a shows a representative tracing from a single vessel. Once a stable baseline had been obtained, the vessel was contracted by addition of medium containing 45 mM KCl, and then iloprost was added to cause relaxation. Following several washes and a 45 min recovery period, the same vessel was again contracted with 45 mM KCl and then octimibate was added to cause relaxation. Since this protocol required successive dose-response curves to be carried out on the same vessels to overcome possible variability between preparations, it was important to demonstrate that the vessels did not become desensitized to iloprost. Three successive dose-responses to iloprost were therefore carried out with washes and a 45 min recovery period between each; Figure 5b (mean of 4 vessels from 2 donors) shows that the three successive iloprost dose-response curves are virtually identical, which confirms that the tissue does not desensitise under these conditions. Figure 5c shows dose-response curves for relaxation of the same vessels (mean of 8 vessels from 4 donors) by both iloprost and octimibate. It is clear that octimibate, as well as iloprost, evokes significant dose-dependent relaxation of these vessels. The EC_{50} for relaxation by iloprost is 44 ± 3 nM (Hill coefficient 1.1 ± 0.07). Due to lack of solubility, it was not possible to

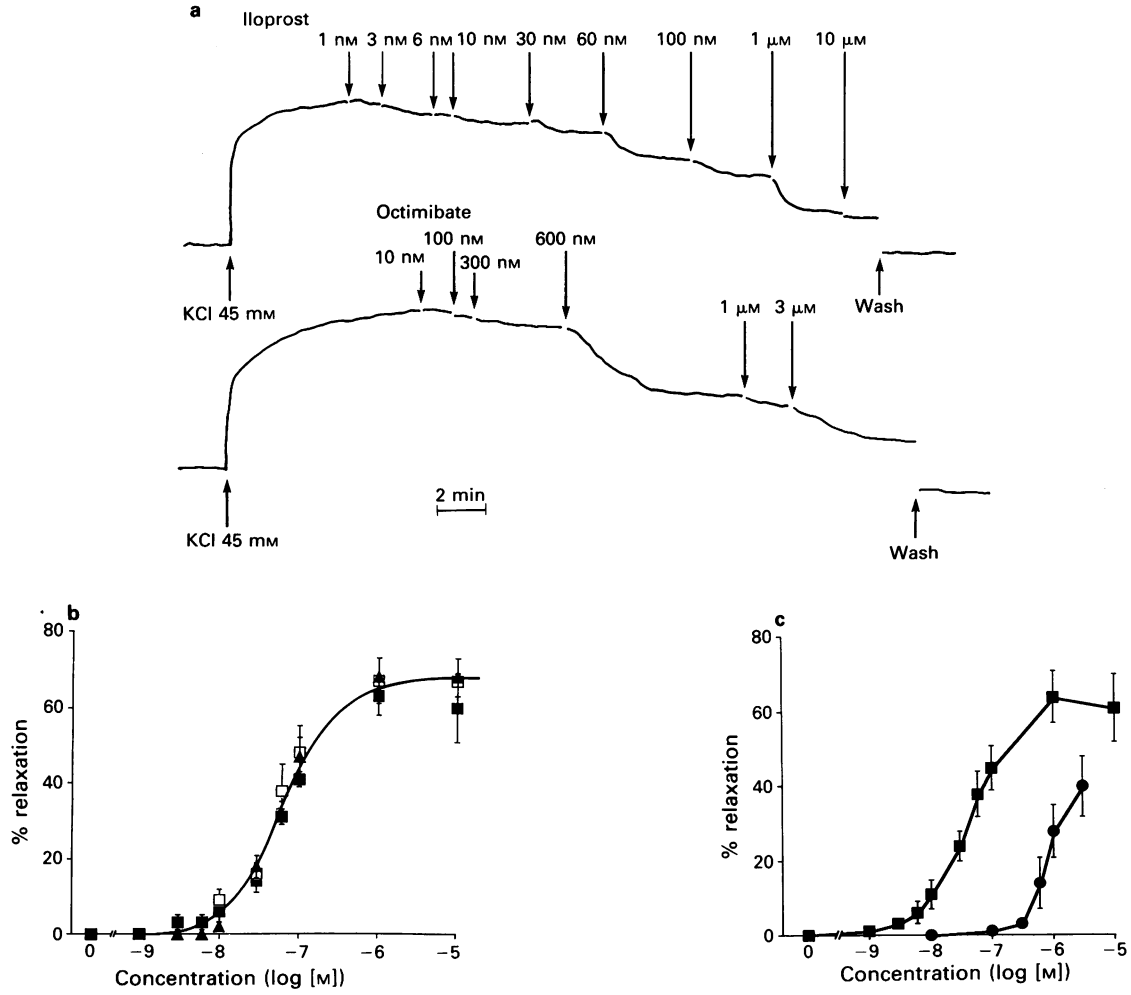

Figure 5 Relaxation of human peripheral resistance vessels by iloprost and octimibate. The mean diameter of the vessels was 238 ± 21 μm (n = 12), and the mean age of the donors (5 females and one male) was 41.5 ± 4.1 years. (a) Typical traces from a single vessel. KCl (45 mM) and iloprost or octimibate, at the concentrations indicated, were added at the times shown. The break between each dose represents a complete change of medium in the bath. There is a wash and 45 min recovery period between the iloprost and octimibate experiments. (b) Dose-response curves for relaxation by iloprost of vessels precontracted with KCl (45 mM). Three successive dose-responses to iloprost were carried out on the same vessels, with washes and a 45 min recovery period between each. The graph shows the first (■), second (□) and third (▲) iloprost dose-curves, with data represented as means of 4 vessels from 2 donors; vertical bars show s.e.mean. (c) Dose-response curves for relaxation by iloprost (■) and octimibate (●) of vessels precontracted with KCl (45 mM). The same vessels were used to construct both dose-curves, as explained and illustrated in Figure 5a. Results are means of 8 vessels from 4 donors; vertical bars show s.e.mean.

obtain a maximal response with octimibate; however, 1.7 μM octimibate evokes 50% of the maximal relaxation obtained with iloprost, and the Hill coefficient for the octimibate dose-response curve is 1.2.

Discussion

These results show that both octimibate and iloprost activate adenylyl cyclase in human lung membranes with EC_{50} values of 350 nM and 50 nM respectively. The EC_{50} value for octimibate is similar to the value of 200 nM that we observed for human platelet membranes. With both lung and platelets the maximal activation of adenylyl cyclase seen with octimibate was 60% of that seen with iloprost; it is not clear whether this reflects true partial agonism or nonspecific inhibitory effects of high concentrations of octimibate. Assuming that responses of the lung to iloprost reflect behaviour of the vasculature (Haye-Legrand *et al.*, 1987), these biochemical results show that human vascular and platelet PGI_2 receptors are responding similarly to octimibate. Octimibate is therefore unable to distinguish tissue specific PGI_2 receptors, as we had originally hoped, based upon its species selectivity.

From these biochemical results, octimibate and iloprost might be expected to have similar functional effects on vascular smooth muscle. Octimibate had a weak relaxant effect on large primate arteries (human coronary and mesenteric artery and monkey aorta) in comparison to the relaxant effect of iloprost; this result suggests that octimibate is acting as a partial agonist. The adenylyl cyclase results also suggested that octimibate might be a partial agonist, so it is possible that octimibate cannot activate adenylyl cyclase sufficiently to relax these large vessels. The results with human coronary artery provide further support for the hypothesis that octimibate is functioning as a partial agonist at the PGI_2/iloprost receptor. In the presence of octimibate, the iloprost dose-response curve was shifted to the right. The K_p value for octimibate was calculated to be around 200 nM, which is similar to the K_i of 150 nM for binding of octimibate to the platelet iloprost receptor described in the preceding paper (Merritt *et al.*, 1991). This result provides further support to the hypothesis that the binding site for octimibate is no different in platelets and vasculature.

Even if octimibate does not distinguish 'tissue-specific' receptors, its partial agonism might provide functional selectivity for inhibition of platelet aggregation over vascular relaxation. The results from the large primate vessels *in vitro* suggest that this may be so. The next experiment was to investigate the effects of octimibate *in vivo* to see whether the apparent functional selectivity observed *in vitro* was still evident. At doses needed for antiplatelet effects, iloprost and PGI_2 are well known to cause vascular side effects *in vivo* (Pickles & O'Grady, 1982; Gryglewski, 1987; Keen *et al.*, 1989a). It was hoped that octimibate would show a better platelet versus vascular selectivity than iloprost. The results with cynomolgus monkeys, however, showed that octimibate was no different from iloprost in platelet versus vascular selectivity.

The lack of platelet selectivity *in vivo* compared to the *in vitro* results obtained so far suggested that the *in vitro* vascular assays with large vessels were less relevant to the *in vivo* vascular effects of octimibate. Since small resistance vessels rather than the large conductance vessels have a major role in the regulation of systemic arterial blood pressure, the effects of octimibate and iloprost were examined *in vitro* on human peripheral resistance vessels. These vessels were relaxed by octimibate as well as iloprost, which probably explains the *in vivo* effects of octimibate.

In conclusion, octimibate and iloprost appear to have similar effects on platelets and small resistance vessels *in vitro*, and to have similar effects *in vivo*. However, octimibate is less effective in relaxing large vessels *in vitro*; this might be because octimibate is a partial agonist at the PGI_2 receptor and the larger vessels have a lower receptor density. Although octimibate is a novel type of compound acting at the PGI_2 receptor and exhibits species selectivity, we have been unable to demonstrate the hoped for selectivity between platelets and vascular resistance vessels in primates. These results emphasise the importance of examining species differences in exploring the pharmacology of new compounds, and of using primate, preferably human, tissue if possible. A comparison of, for example, human platelets and dog artery relaxation or blood pressure responses could well give rise to seriously flawed predictions of likely behaviour in terms of selectivity of effect in clinical studies in man.

We would like to thank Schering AG (Berlin) for supplying us with iloprost, Huntingdon Research Centre (Huntingdon) for providing us with cynomolgus monkey aortas, Addenbrooke's Hospital (Cambridge) for the human mesenteric arteries, and the surgical staff of Papworth-Everett Hospital (Cambridge) for the human coronary arteries. We are grateful to the staff of the Laboratory Animal Sciences Department, SmithKline Beecham Pharmaceuticals (Philadelphia, USA) for their help with the *in vivo* experiments with cynomolgus monkeys; particular thanks to Ms S. Campbell, Mr E. Jenkins and Mr J. Kissinger. We would also like to thank the following for their expert technical assistance: Mr G. Dane, Ms M. Greener, Ms J. Vasko and Ms A. Wright. M.K. and E.K. were supported by a programme grant from the Wellcome Trust.

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Parainfluenza virus infection damages inhibitory M_2 muscarinic receptors on pulmonary parasympathetic nerves in the guinea-pig

[1]Allison D. Fryer & *David B. Jacoby

Department of Environmental Health Sciences, School of Hygiene and Public Health, Johns Hopkins University, Baltimore, Maryland, 21205 U.S.A. and *Johns Hopkins Asthma and Allergy Center, 301 Bayview Blvd., Baltimore, Maryland, 21224, U.S.A.

1 The effect of viral infection on the function of neuronal M_2 muscarinic autoreceptors in the lungs was studied in anaesthetized guinea-pigs.

2 Guinea-pigs were inoculated intranasally with either parainfluenza type 3 or with a vehicle control. Four days later the animals were anaesthetized, paralysed and artificially ventilated. Pulmonary inflation pressure, tidal volume, blood pressure, and heart rate were recorded. Both vagus nerves were cut and electrical stimulation of the distal portions caused bronchoconstriction (measured as an increase in pulmonary inflation pressure) and bradycardia.

3 In control animals, pilocarpine (1–100 $\mu g\,kg^{-1}$, i.v.) attenuated vagally-induced bronchoconstriction by stimulating inhibitory M_2 muscarinic receptors on parasympathetic nerves in the lungs. Conversely, blockade of these receptors with the antagonist gallamine (0.1–10 $mg\,kg^{-1}$, i.v.) produced a marked potentiation of vagally-induced bronchoconstriction. These results confirm previous findings.

4 In guinea-pigs infected with parainfluenza virus, pilocarpine did not inhibit vagally-induced bronchoconstriction. Furthermore, gallamine did not potentiate vagally-induced bronchoconstriction to the same degree as in uninfected controls.

5 There was no increase in baseline pulmonary inflation pressure in the infected animals over the controls. Receptors on airway smooth muscle were unchanged by viral infection since large doses of pilocarpine caused equivalent bronchoconstriction in both groups of animals. Gallamine inhibited the vagally-induced fall in heart rate equally in both groups of animals indicating that virus-induced changes in M_2 receptor function on pulmonary parasympathetic nerves are not part of a generalized decrease in M_2 receptor function.

6 These results demonstrate that the M_2 muscarinic receptor-mediated inhibition of acetylcholine release from parasympathetic nerves in the lungs is decreased in animals infected with parainfluenza virus. Loss of this inhibition would result in increased release of acetylcholine from the parasympathetic nerves and may explain virus-induced airway hyperresponsiveness.

Introduction

Viral infections of the lung exacerbate asthma in children and in adults (Frick *et al.*, 1979; Henderson *et al.*, 1979; Welliver, 1983; Little *et al.*, 1978). In normal subjects viral infection of the lung produces temporary increases in baseline airways resistance (Johanson *et al.*, 1969; Picken *et al.*, 1972; Blair *et al.*, 1976; Hall *et al.*, 1976) and increases bronchial reactivity to a variety of stimuli (Aquilina *et al.*, 1980; Empey *et al.*, 1976; Little *et al.*, 1978). These changes often persist for weeks beyond the period of clinical illness.

The mechanisms by which viruses induce airway hyperresponsiveness are poorly understood. There is no evidence that viral infection causes abnormalities in airway smooth muscle function. Contraction of airway smooth muscle *in vitro* to muscarinic agonists and histamine has been reported to be unaltered by viral infection (Buckner *et al.*, 1981; 1985; Jacoby *et al.*, 1988). *In vivo*, the bronchoconstrictor response to aerosolized acetylcholine (ACh) is the same in guinea-pigs infected with parainfluenza virus as in sham-infected animals (Dusser *et al.*, 1989).

Virus-induced hyperresponsiveness may be the result of a defect in the parasympathetic nervous system. In normal subjects, bronchoconstriction induced by exercise or by inhalation of histamine or cold air was temporarily potentiated during and immediately after respiratory viral infections (Empey *et al.*, 1976; Aquilina *et al.*, 1980). Both of these responses were blocked by atropine indicating potentiation of a vagal reflex. A defect in the efferent limb of the parasympathetic nervous system was suggested by Buckner *et al.* (1985) who demonstrated that bronchoconstriction induced by electrical stimulation of the vagus nerves was potentiated in virus-infected guinea-pigs.

In the airways, release of ACh is under the local control of muscarinic receptors on postganglionic, parasympathetic nerves (Fryer & Maclagan, 1984; 1987a,b; Blaber *et al.*, 1985; Faulkner *et al.*, 1986). Under physiological conditions these autoreceptors inhibit ACh release, thereby limiting vagally-induced bronchoconstriction. Blockade of these receptors with selective antagonists such as gallamine potentiates vagally-mediated bronchoconstriction as much as 10 fold. Therefore, it is possible that loss of these neuronal receptors may contribute to virus-induced hyperresponsiveness. These experiments were carried out to determine whether virus infection alters the function of inhibitory muscarinic receptors on the pulmonary parasympathetic nerves.

Methods

Virus infection

Parainfluenza type 3 (ATCC VR-93) was grown in Rhesus monkey kidney cell monolayers in L-15 medium for one week at 34°C. Cells and medium were frozen and thawed, cleared by low-speed centrifugation, and stored in aliquots at −70°C.

Specific antigen-free guinea-pigs were anaesthetized with

[1] Author for correspondence.

methohexitone (20 mg kg^{-1}, i.p.). Animals in the infected group were inoculated intranasally with 1 ml virus solution that contained 10^5 $TCID_{50}$ ml^{-1} (10^5 times the concentration required to produce infection in 50% of Rhesus monkey kidney monolayers), obtained by diluting the viral stock in Dulbecco's phosphate-buffered saline. Animals in the uninfected (control) group were inoculated intranasally with fluids obtained from virus-free Rhesus monkey kidney cells that were prepared and diluted in phosphate-buffered saline in the same way as the viral solutions. Control and infected animals were housed in separate laminar flow rooms.

Virus isolation and titration

After physiological studies were completed, the guinea-pig lungs were removed and stored at −70°C. Frozen samples were thawed, weighed, and homogenized in 2 ml phosphate-buffered saline (Polytron, Brinkman, Lucerne, Switzerland). Virus was eluted from the tissue homogenate by incubation at 34°C for 1 h. The suspensions were centrifuged at 400 *g* for 30 min, and the supernatants were inoculated in serial 10 fold dilutions into fresh Rhesus monkey kidney cell monolayers. After one week's incubation at 34°C, the monolayers were washed and the medium was replaced with a 0.5% suspension of guinea-pig erythrocytes. After 1 h, the erythrocytes were washed off, and the monolayers were examined under an inverted phase-contrast microscope (Olympus) for evidence of haemadsorption (sticking of erythrocytes to the surface of cells expressing the viral hemagglutinin on their surfaces) (Shelokov *et al.*, 1958). Only data from virus-exposed guinea-pigs with proven parainfluenza infections are reported.

Anaesthesia

Guinea-pigs (Dunkin Hartley; 250–350 g) were used four days after inoculation with virus or with control media. They were anaesthetized with urethane (1.5 g kg^{-1}) injected intraperitoneally. This dose of urethane produces a deep anaesthesia lasting 8–10 h (Green, 1982). None of the experiments described lasted for longer than 3 h and depth of anaesthesia was monitored by observing for fluctuations in heart rate and blood pressure. Guinea-pigs were handled in accordance with the standards established by the U.S.A. Animal Welfare Acts set forth in National Institute of Health guidelines and the Policy and Procedures Manual published by the Johns Hopkins University School of Hygiene and Public Health Animal Care and Use Committee.

Measurement of pulmonary inflation pressure (Ppi)

Once the guinea-pigs were anaesthetized, a carotid artery was cannulated for measurement of blood pressure and heart rate. Cannulae were placed into both jugular veins for the administration of drugs. Both vagi were cut and the distal portions placed on shielded platinum electrodes immersed in a pool of liquid paraffin. The animal's body temperature was maintained at 37°C with a heating blanket.

The animals were paralysed with suxamethonium (infused at 10 μg kg^{-1} min^{-1}) and ventilated with a positive pressure, constant volume animal ventilator (Harvard). Airflow was recorded as the pressure drop across a Fleisch pneumotachograph (3/0) measured with a Grass differential pressure transducer (PT5B). The airflow signal was integrated to give tidal volume. Pulmonary inflation pressure (Ppi) was measured with a Spectromed pressure transducer (DTX). All signals were displayed on a Grass polygraph. Po_2 and Pco_2 were measured in arterial blood samples at the beginning and end of each experiment (Corning 170 pH/blood gas analyser).

A positive pressure of 85–100 mmH_2O was needed for adequate ventilation of the animals. Bronchoconstriction was measured as the increase in Ppi over the basal inflation pressure produced by the ventilator (Dixon & Brodie, 1903). The sensitivity of the method was increased by a baseline subtractor device (University of Maryland, Dept. of Biophysics) which cut off the basal inflation pressure allowing the increase in Ppi to be recorded at a greater amplification on a separate channel of the polygraph (Burden & Parkes, 1971). With this method, increases in pressure as small as 2–3 mmH_2O could be recorded accurately. Changes in pulmonary inflation pressure reflect changes in resistance and compliance of the lungs.

Simultaneous stimulation of both vagus nerves (2–15 Hz, 0.2 ms, 5–30 V), produced a bronchoconstriction and a fall in heart rate. The nerves were stimulated regularly at 2 min intervals and the number of pulses per train were kept constant for each set of experiments. All animals were pretreated with guanethidine 5 mg kg^{-1}, i.v. This dose of guanethidine has been demonstrated to deplete noradrenaline and it produced a temporary reduction in the magnitude of the vagally-induced bronchoconstriction and bradycardia. Thirty minutes after guanethidine, when the responses to stimulation of the vagus nerves were back to pre-guanethidine values and were reproducible, cumulative dose-response curves measuring the effect of pilocarpine or gallamine on vagally-induced bronchoconstriction were performed. Doses of pilocarpine greater than 30 μg kg^{-1} produced a transient bronchoconstriction. Therefore, the effect of these doses of pilocarpine on vagally-induced bronchoconstriction was measured after the Ppi had returned to baseline. At the end of each experiment vagally-induced bronchoconstriction and bradycardia were abolished by atropine (1 mg kg^{-1}, i.v.) indicating that both of these responses were mediated via release of ACh onto muscarinic receptors.

Drugs

Gallamine, pilocarpine, suxamethonium, atropine, and urethane were purchased from Sigma, St. Louis, MO, U.S.A.; guanethidine was supplied by CIBA, Summit, NJ, U.S.A.; and methohexitone was purchased from Eli Lilly, Indianapolis, IN, U.S.A. All drugs were dissolved and diluted in 0.9% NaCl solution. Rhesus monkey kidney cells were purchased from Viromed, Minnetonka, MN, U.S.A.

Statistics

The effects of viral infection on dose-response curves to pilocarpine and gallamine were compared by two-way analysis of variance. The initial responses to stimulation of the vagus nerves were compared between control and infected guinea-pigs by unpaired Student's *t* tests. A *P* value less than 0.05 was considered significant.

Results

All virus-exposed animals became infected with the virus. Homogenates of lungs from virus-exposed guinea-pigs contained $10^{4.7} \pm 10^{0.22}$ $TCID_{50}$/100 mg tissue weight (geometric mean ± s.e.mean). Lungs from control guinea-pigs contained no titratable virus.

There was no difference in baseline Ppi (85–100 mmH_2O) or baseline heart rate (280–320 beats min^{-1}) between control and virus-infected guinea-pigs. Electrical stimulation of the vagus nerves caused bronchoconstriction (measured as an increase in Ppi) and bradycardia in both groups. There were no differences between control and infected guinea-pigs in these responses (see histograms in Figures 1, 3 and 4).

In control guinea-pigs, stimulation of M_2 muscarinic receptors on the parasympathetic nerves by pilocarpine inhibited vagally-induced bronchoconstriction in dose-dependent fashion. The degree of inhibition was also dependent on the frequency of electrical stimulation. Pilocarpine was somewhat more effective in inhibiting bronchoconstriction elicited at 2 Hz compared with 15 Hz (Figure 1a–c, open squares).

In guinea-pigs infected with parainfluenza virus, pilocarpine was not an effective inhibitor of vagally-induced broncho-

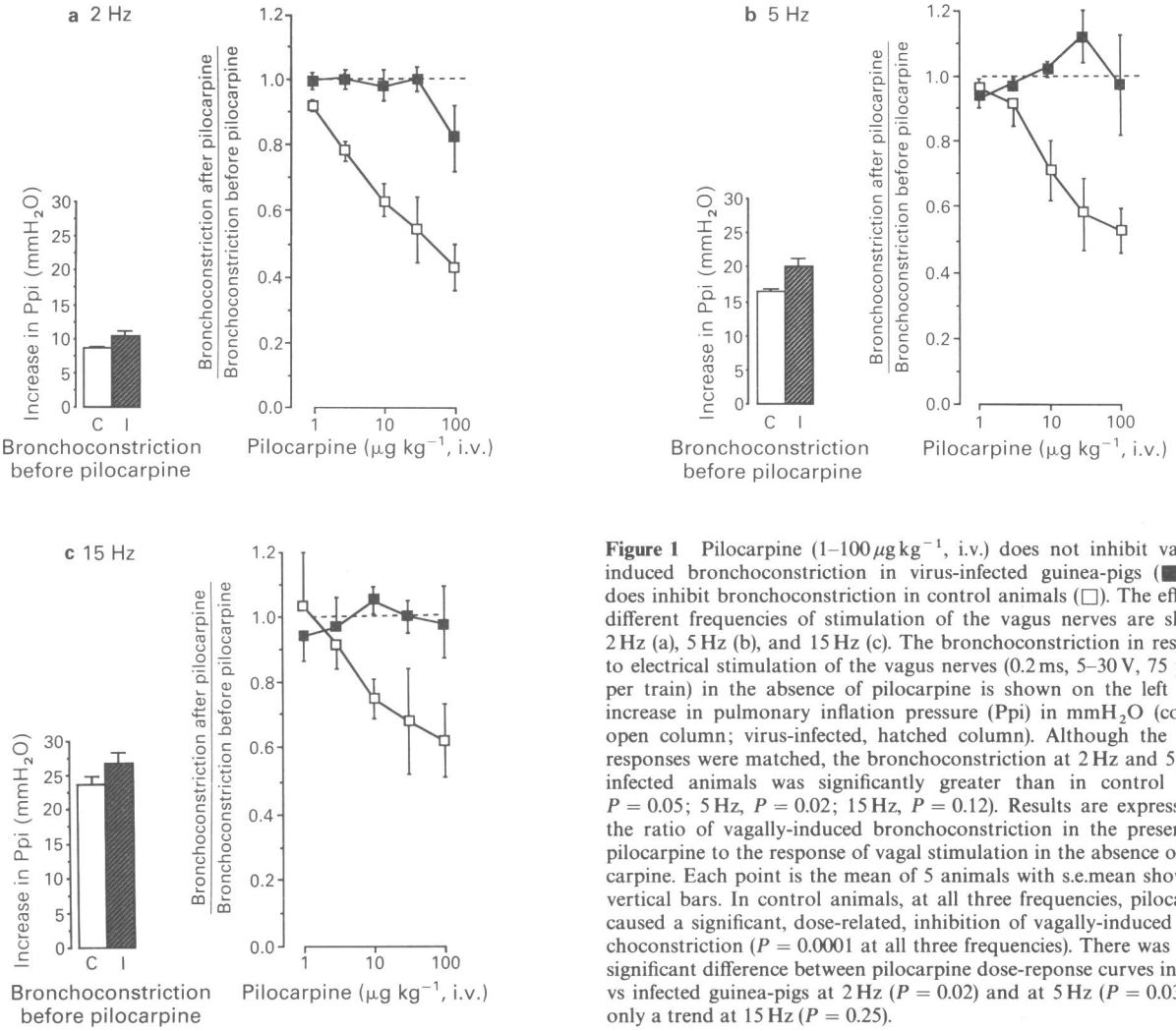

Figure 1 Pilocarpine (1–100 $\mu g\,kg^{-1}$, i.v.) does not inhibit vagally-induced bronchoconstriction in virus-infected guinea-pigs (■) but does inhibit bronchoconstriction in control animals (□). The effect at different frequencies of stimulation of the vagus nerves are shown: 2 Hz (a), 5 Hz (b), and 15 Hz (c). The bronchoconstriction in response to electrical stimulation of the vagus nerves (0.2 ms, 5–30 V, 75 pulses per train) in the absence of pilocarpine is shown on the left as an increase in pulmonary inflation pressure (Ppi) in mmH_2O (control, open column; virus-infected, hatched column). Although the initial responses were matched, the bronchoconstriction at 2 Hz and 5 Hz in infected animals was significantly greater than in control (2 Hz, $P = 0.05$; 5 Hz, $P = 0.02$; 15 Hz, $P = 0.12$). Results are expressed as the ratio of vagally-induced bronchoconstriction in the presence of pilocarpine to the response of vagal stimulation in the absence of pilocarpine. Each point is the mean of 5 animals with s.e.mean shown by vertical bars. In control animals, at all three frequencies, pilocarpine caused a significant, dose-related, inhibition of vagally-induced bronchoconstriction ($P = 0.0001$ at all three frequencies). There was also a significant difference between pilocarpine dose-reponse curves in sham vs infected guinea-pigs at 2 Hz ($P = 0.02$) and at 5 Hz ($P = 0.03$), but only a trend at 15 Hz ($P = 0.25$).

constriction. At 2 Hz, only the highest dose of pilocarpine (100 $\mu g\,kg^{-1}$) inhibited vagally-induced bronchoconstriction (Figure 1a), while at 5 and 15 Hz no dose of pilocarpine was able to inhibit vagally-induced bronchoconstriction in virus-infected guinea-pigs (Figure 1b,c). Doses greater than 100 $\mu g\,kg^{-1}$ pilocarpine were not used because they caused sustained bronchoconstriction via stimulation of muscarinic receptors on the airway smooth muscle.

In both control and infected guinea-pigs, pilocarpine caused a transient bronchoconstriction (at 30–100 $\mu g\,kg^{-1}$) and fall in heart rate (at 1–100 $\mu g\,kg^{-1}$) by stimulating muscarinic receptors on airway smooth muscle and cardiac muscle. There were no differences in these responses between the control and infected animals (Figure 2).

In control animals gallamine (0.1–10 $mg\,kg^{-1}$) potentiated vagally-induced bronchoconstriction in a dose-dependent fashion. In guinea-pigs infected with parainfluenza virus this potentiation was attenuated, and the dose-response curve shifted approximately one log unit to the right (Figure 3). In the heart, gallamine inhibited vagally-induced bradycardia to the same extent in both control and virus-infected animals (Figure 4).

Discussion

In control guinea-pigs, pilocarpine inhibited and gallamine potentiated bronchoconstriction elicited by electrical stimulation of the vagus nerves. These effects are due to stimulation (pilocarpine) and blockade (gallamine) of inhibitory M_2 muscarinic receptors on the pulmonary parasympathetic nerves (Fryer & Maclagan, 1984; 1987a,b; Blaber *et al.*, 1985; Faulkner *et al.*, 1986).

Both pilocarpine-induced inhibition and gallamine-induced potentiation of vagally-mediated bronchoconstriction were markedly decreased in guinea-pigs infected with parainfluenza virus. Thus the neuronal M_2 receptors cannot be stimulated by exogenous agonists since pilocarpine did not inhibit

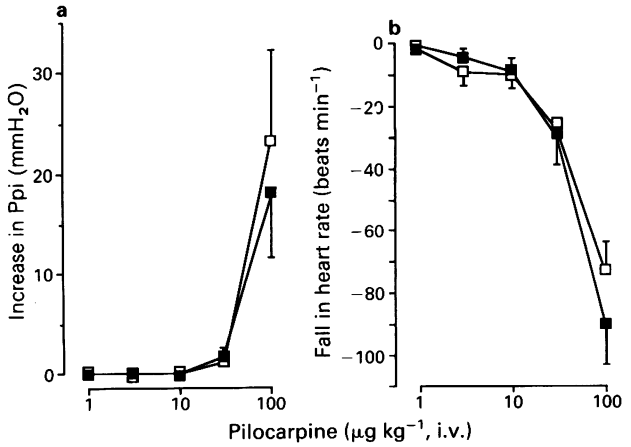

Figure 2 Pilocarpine (1–100 $\mu g\,kg^{-1}$, i.v.) causes a similar degree of bronchoconstriction (a) measured as an increase in pulmonary inflation pressure (Ppi) and bradycardia (b) measured as a fall in heart rate in control (□) and virus-infected (■) guinea-pigs. Each point is the mean of 5 animals with s.e.mean shown by vertical bars.

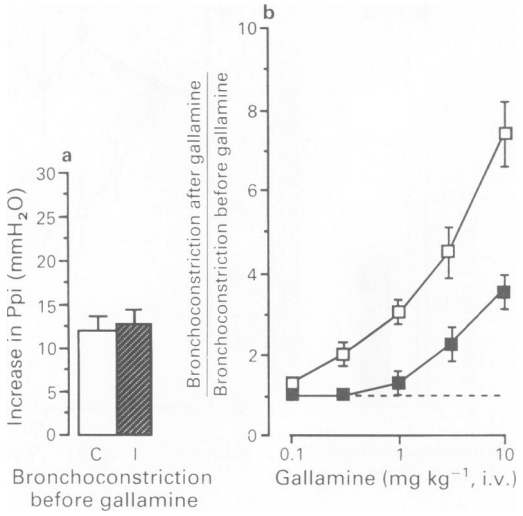

Figure 3 Potentiation of vagally-induced bronchoconstriction by gallamine (0.1–10 mg kg^{-1}, i.v.) is attenuated in guinea-pigs which are infected with parainfluenza virus (■) compared to control guinea-pigs (□) (b). The bronchoconstriction in response to electrical stimulation of the vagus nerves (0.2 ms, 15 Hz, 5–30 V, 75 pulses per train) in the absence of gallamine is shown in (a) as an increase in pulmonary inflation pressure (Ppi) in mmH_2O (control, open column; virus-infected, hatched column; there was no significant difference between these responses, $P = 0.29$). Results are expressed as the ratio of the response to vagal stimulation in the presence of gallamine to the response to vagal stimulation in the absence of gallamine. Each point is the mean of 5 animals with s.e.mean shown by vertical bars. In control animals, gallamine caused a significant, dose-related, potentiation of vagally-induced bronchoconstriction ($P = 0.0001$). There was also a significant difference between gallamine dose-response curves in sham vs infected guinea-pigs ($P = 0.013$).

vagally-induced bronchoconstriction. Neither can these receptors be stimulated by endogenous ACh, since if ACh were stimulating these autoreceptors blockade by gallamine would have potentiated vagally-induced bronchoconstriction.

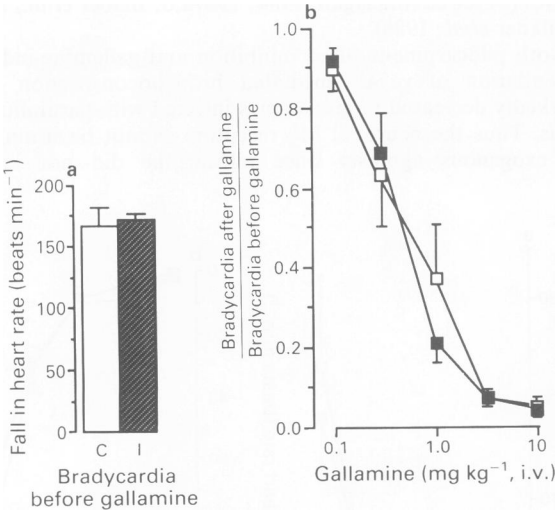

Figure 4 Gallamine (0.1–10 mg kg^{-1}, i.v.) causes a similar degree of inhibition of vagally-induced bradycardia in control (□) and virus-infected (■) guinea-pigs (b). Bradycardia elicited by electrical stimulation of the vagus nerves (0.2 ms, 15 Hz, 5–30 V, 75 pulses per train) was measured. The bradycardia in response to vagal stimulation in the absence of gallamine is shown in (a) as a fall in heart rate in beats min^{-1} (control, open column; virus-infected, hatched column; there was no statistical difference between these responses, $P = 0.34$). Results are expressed as the ratio of the response to vagal stimulation in the presence of gallamine to the response to vagal stimulation in the absence of gallamine. Each point is the mean of 5 animals with s.e.mean shown by vertical bars.

Damage to neuronal M_2 receptors would be expected to increase the baseline bronchoconstrictor response to vagal stimulation in virus-infected animals. Buckner *et al.* (1985) demonstrated such an increase in guinea-pigs infected with parainfluenza virus type 3. In our study, we found a great deal of variability in the response to electrical stimulation of the vagus nerves, which we attribute to variation in the isolation and preparation of the vagus nerves. A different voltage (range 5–30 V) was selected, and kept constant throughout each experiment in order that the initial bronchoconstrictor responses were similar between sham-infected and virus infected guinea-pigs. Because the voltages were varied between experiments the results of Buckner *et al.* (1985) were not directly confirmed. However, there was a trend (although not statistically significant) indicating that a given bronchoconstriction was reached with less voltage in virus-infected animals (11.0 ± 2.8 V) than in sham-infected animals (18.2 ± 5.0 V).

The function of neuronal muscarinic receptors is dependent upon the frequency at which the vagus nerve is stimulated (Fryer & Maclagan, 1984). These receptors function best, and the effects of antagonists at these receptors are most apparent, when the nerves are stimulated at higher frequencies (5–15 Hz). Conversely it is easier to demonstrate the effect of exogenous agonists when the nerve is firing at lower frequencies. We have confirmed that pilocarpine is a more effective agonist at 2 Hz in control guinea-pigs (Figure 2). In virus-infected animals we tested whether the effect of pilocarpine was inhibited at a range of frequencies because the vagus nerves in the lung fire normally at 12–15 Hz (Mitchell *et al.*, 1987).

The changes in the effects of gallamine and pilocarpine on vagally-induced bronchoconstriction cannot be accounted for by alterations in resistance since baseline Ppi was the same in both infected and control animals. Neither of these effects is related to changes in muscarinic receptors on airway smooth muscle since the pilocarpine-induced bronchoconstriction was identical in control and virus-infected animals (see Figure 2a).

Parainfluenza virus primarily infects the airway epithelium and does not usually spread to infect tissues outside the lungs. Therefore, M_2 receptors in other organs, such as the heart, should be unchanged by viral infection of the lungs. That virus-induced changes in M_2 receptors in the lung are not part of a generalized decrease in M_2 receptor function was demonstrated since bradycardia (mediated by M_2 muscarinic receptors, Hammer *et al.*, 1986) in response to both vagal stimulation (Figure 4a) and pilocarpine (Figure 2b) was not altered from control. Furthermore, gallamine was equally potent in inhibiting vagally-induced bradycardia in control and virus-infected animals (Figure 4).

Viral infection increases vagally-mediated reflex bronchoconstriction in guinea-pigs (Buckner *et al.*, 1985) and humans (Empey *et al.*, 1986; Aquilina *et al.*, 1980). The mechanism for this potentiation is unclear. Since bronchoconstriction in response to stimulation of the vagus nerves was potentiated in guinea-pigs infected with parainfluenza virus (Buckner *et al.*, 1985) it appears that at least part of this potentiation results from a defect in the efferent limb of the reflex. Damage to the inhibitory M_2 muscarinic receptors on the pulmonary vagus nerves, as we have demonstrated, may explain this increase in vagally-induced bronchoconstriction.

The mechanism of these changes in M_2 muscarinic receptor function in the airways of virus-infected guinea-pigs is unknown. We have recently shown that exposure of membrane preparations of guinea-pig lungs to parainfluenza virus *in vitro* decreases the affinity of agonists for a portion of the muscarinic receptors (Fryer *et al.*, 1990). This effect is due to viral neuraminidase, as it can be mimicked by an equivalent concentration of purified neuramidinase and blocked by a neuraminidase inhibitor. Because exposure to parainfluenza virus *in vitro* caused a similar decrease in agonist affinity for all of the muscarinic receptors in a membrane preparation of guinea-pig heart, which contains only M_2 receptors, it is pos-

sible that viral neuraminidase is decreasing agonist affinity selectively for M_2 receptors in the lung. This is consistent with the fact that M_2 muscarinic receptors contain a large number of sialic acid residues (Peterson *et al.*, 1986) which would be susceptible to cleavage by neuraminidase and that the sialic acid residues are involved in agonist binding to M_2 receptors (Gies & Landry, 1988).

M_2 muscarinic receptors on parasympathetic nerves in the lungs normally function to inhibit release of ACh from parasympathetic nerves in the lungs. Blockade of these autoreceptors removes the negative feedback control they provide, resulting in a potentiation of vagally-induced bronchoconstriction. The data presented here demonstrate that in guinea-pigs infected with parainfluenza virus, inhibition of ACh release by neuronal M_2 muscarinic receptors in the lungs is decreased. Loss of this inhibitory control would result in potentiation of any vagally-mediated bronchoconstriction, including reflex bronchoconstriction. It is possible that virus-induced airway hyperresponsiveness is the result of viral damage to neural M_2 muscarinic receptors in the lungs.

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Properties of 5-hydroxytryptamine$_3$ receptor-gated currents in adult rat dorsal root ganglion neurones

[1]Brian Robertson & Stuart Bevan

Cellular Electrophysiology, Sandoz Institute for Medical Research, Gower Place, London WC1E 6BN

1 Responses to 5-hydroxytryptamine (5-HT) were examined on rat dorsal root ganglion (DRG) neurones maintained in tissue cultures, by use of whole cell recording techniques.

2 5-HT (usually 10 μM) evoked a depolarization associated with an increase in membrane conductance in 40% of DRG neurones. There was a considerable variation in the size and persistence of this response between different batches of cells.

3 The 5-HT response was mimicked by applying the agonists 2-methyl-5-HT (10 μM) and phenylbiguanide (10 μM). Responses were blocked by ICS 205–930 (100 nM), but not by methysergide (0.1–1.0 μM).

4 5-HT currents could be carried by sodium and caesium ions, but not by choline ions. The amplitude and duration of the 5-HT responses were dependent on the concentration of divalent cations in the extracellular solution: both became greater when calcium and magnesium concentrations were decreased.

5 Staurosporine, a putative antagonist of protein kinases, inhibited responses to 5-HT.

Introduction

5-Hydroxytryptamine (5-HT) is known to depolarize and excite mammalian sensory neurones (Wallis *et al.*, 1982; Christian *et al.*, 1989) and is also able to excite peripheral nociceptive terminals (Richardson *et al.*, 1985). Several subclasses of 5-HT receptors have been described based on the actions of various agonists and antagonists (see Fozard, 1989, for review). While some of the 5-HT receptor subtypes are coupled to second messenger systems such as adenylate cyclase and phospholipase C (see e.g. Peroutka, 1988), other 5-HT receptors are believed to activate ion channels directly (Derkach *et al.*, 1989). Pharmacological experiments on human blister bases have shown that the 5-HT receptor associated with nociceptive neurones is of the 5-HT$_3$ subclass (Richardson *et al.*, 1985). However, the mechanisms underlying this activation are poorly understood.

Here we have investigated the actions of 5-HT on dorsal root ganglion neurones from adult rats in order to elucidate the pharmacology of the receptor and characteristics of the evoked current.

Methods

Experiments were carried out on isolated single cells from adult rats. Dorsal root ganglia (cervical, thoracic and lumbar) were excised aseptically from decapitated adult rats. Following removal of most of the nerve roots and capsular connective tissue, ganglia were prepared according to the methods described by Lindsay (1988), being digested sequentially with collagenase (0.125%) and trypsin (0.25%) and then mechanically dissociated to a suspension of single cells. The neurones were normally plated either directly, or following neuronal enrichment, on polyornithine-laminin coated glass cover slips, although other substrates were tested in some experiments (see Results). Cultures were maintained in Hank's F-15 medium supplemented with nerve growth factor (NGF) 1–5 μg ml^{-1}. Usually recordings were made as soon as possible after plating (1–24 h), whilst neurones possessed few neurites, since these would compromise the fidelity of voltage clamp and dialysis of the cell interior. In some cases 5-HT sensitivity of the cells was examined after longer times in culture. Electrophysiological recordings were performed at 20–22°C.

The normal extracellular solution had the following composition (in mM): NaCl 140, KCl 5, $CaCl_2$ 1, $MgCl_2$ 1, glucose 25, and HEPES 10, adjusted to pH 7.4 with NaOH. In some experiments choline chloride replaced NaCl. The concentration of divalent ions was varied as described in the text.

Patch recording pipettes were pulled from 1.5 mm o.d. electrode glass (GC150TF, Clark Electromedical Instruments) in two stages on a Brown-Flaming (Sutter Instrument Company) puller, heat-polished and filled with a filtered solution containing (in mM): CsCl (or KCl) 140, HEPES 10, MgATP 2, EGTA 5 and $CaCl_2$ 1 at pH 7.4 with CsOH (or KOH).

Recordings were made with a List Electronics EPC7 voltage clamp amplifier, and whole-cell currents were recorded on both magnetic tape and chart recorder. In some experiments recordings were also made from outside-out membrane patches obtained from responsive cells.

Drugs were applied to cells in the bath perfusate, or, for more rapid application by puffer-pipette with a pressure-ejection system. Care was taken to exclude 'puffer artefacts' with this method of application. In later experiments, solutions were applied to individual neurones by a 'U' tube fast perfusion system (Krishtal & Pidoplichko 1980). There was no difference in results observed between these two rapid methods of application.

5-Hydroxytryptamine creatine sulphate (5-HT) was purchased from Sigma, 2-methyl 5-HT (2 Me 5-HT), phenylbiguanide (PBG), methysergide and ICS 205-930 ((3α-tropanyl)-1H-indole-3-carboxylic acid ester) were obtained from Sandoz Ltd. Staurosporine was purchased from Fluka.

Results

All of the experiments described here were performed on DRG neurones isolated from adult rats. Figure 1a illustrates typical inward currents and the corresponding increase in membrane conductance produced by brief applications of 10 μM 5-HT. The agonist-induced currents rapidly rise to a peak, and decay slowly over many seconds. In 118 different preparations, 164 out of 401 (41%) cells responded in this way to 10 μM 5-HT. However, in the majority of these responsive preparations the evoked currents were very small (less than

[1]Present address and correspondence: Electrophysiology Laboratory, Wyeth Research, Huntercombe Lane South, Taplow, Maidenhead SL6 0PH.

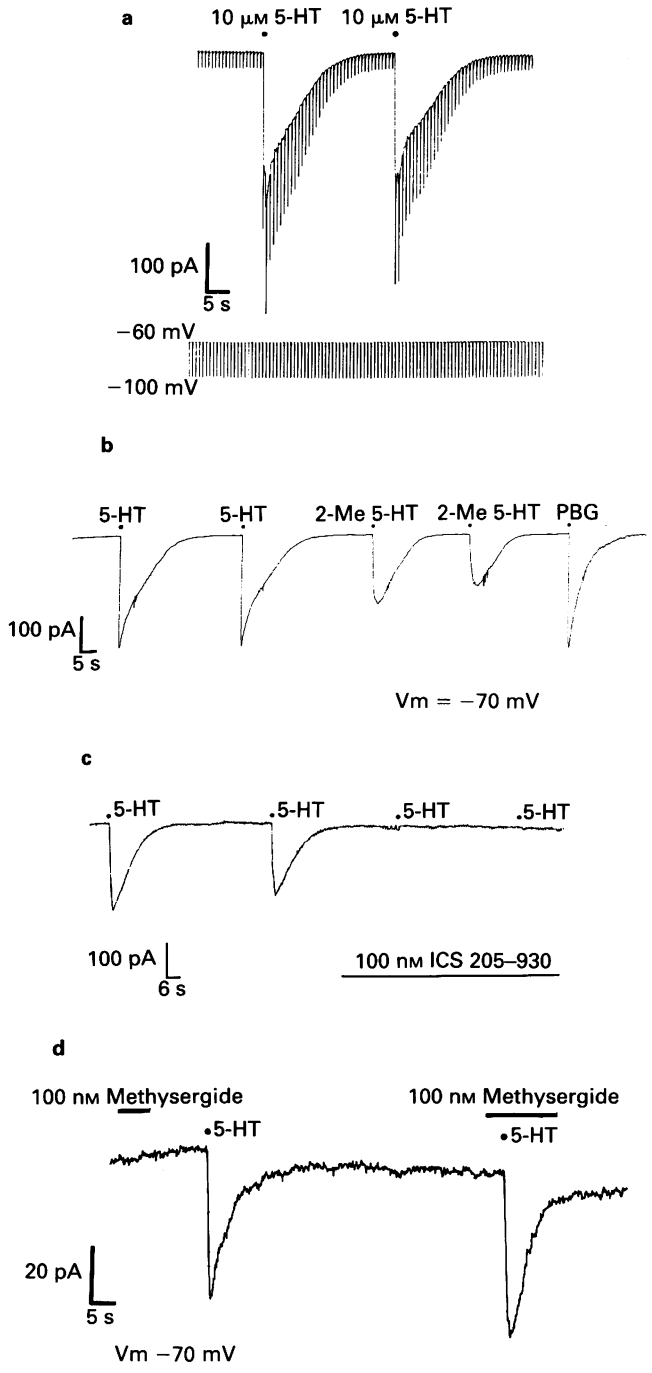

Figure 1 (a) Inward currents in response to two 2 s applications of 10 μM 5-hydroxytryptamine (5-HT) from nearby puffer pipettes. Cell voltage clamped at −60 mV with hyperpolarizing voltage steps to −100 mV, revealing the increase in cell conductance on application of the agonist. Responses recorded in normal extracellular solution without added calcium. Caesium-filled recording electrodes. Cell 180.14. (b) Responses to 10 μM 5-HT, 10 μM 2-methyl 5-HT (2-Me 5-HT) and 10 μM phenylbiguanide (PBG) at −70 mV. Cell 180.14. (c) Bath applied ICS 205-930 (100 nM) blocks the inward current produced by 10 μM 5-HT. Block by the antagonist is fully reversible. Cell 148.10. (d) Methysergide (100 nM) does not affect the 5-HT (10 μM)-induced inward current. Potassium internal solution. Cell 120.2.

20 pA, holding potential −80 mV) and were not analysed further. The remaining responsive cells showed large (>200 pA), robust currents that were more suitable for experimentation. Since the frequency and size of the response varied considerably between cultures, experiments were done to modify the culture conditions. This included varying the time in culture (one to seven days), omitting NGF from the culture medium, and varying the cell substrate by plating the cells on either tissue culture plastic, glass, laminin or polyornithine coated coverslips, or a monolayer of rat cortical astrocytes. Also cultures were treated with dibutyryl cyclic AMP (1–100 μM). Cultures made from selected spinal ganglia were also tested. However, no obvious correlation was noted between these various treatments and the presence or magnitude of the 5-HT response.

The 5-HT-induced currents were believed to be mediated by the 5-HT$_3$ receptor subtype since the responses could also be elicited by applying the structurally related agonist 2-methyl 5-HT (2-Me 5-HT) (Figure 1b), which is considered to be selective for the 5-HT$_3$ subtype (Richardson *et al.*, 1985; Neijt *et al.*, 1988). Out of 26 cells tested that responded to 10 μM 5-HT, all responded to 10 μM 2-Me 5-HT. The agonist phenylbiguanide (PBG, 10 μM), which depolarizes rat isolated vagus nerves (Ireland & Tyers 1987), was also effective in gating a transient inward current, identical to that evoked by 10 μM 5-HT on the same cells (n = 12) (see Figure 1b and Figure 4b).

Further evidence that the 5-HT$_3$ receptor subtype is involved in these responses was obtained by use of 5-HT receptor antagonists. Figure 1c shows complete block of the 5-HT-induced inward current by including the selective 5-HT$_3$ antagonist ICS 205-930 (100 nM) in the perfusate. The antagonism produced by this compound was fully reversible (n = 11). Methysergide is a less selective antagonist with actions against 5-HT$_1$ and 5-HT$_2$ receptor subtypes (Bradley *et al.*, 1986). We found that methysergide at concentrations of 0.1 μM (n = 4, see Figure 1d) and 1.0 μM (n = 3, not shown) had no effect on 5-HT-induced inward currents; at these concentrations both 5-HT$_1$ and 5-HT$_2$ receptors will be blocked (e.g. Peroutka, 1988).

We were next interested in determining the ionic basis of the 5-HT$_3$ current. When the amplitude of successive 5-HT responses remained stable over a period of several minutes, it was possible to determine the current reversal potential by interpolation. Figures 2a and b illustrate the results from one such experiment. With caesium as the principal intracellular cation and with sodium ions externally, the mean reversal potential (E_{rev}) determined from measurements on 8 separate cells was +6.9 ± 1.76 mV (mean ± s.e.mean). Calculation of the sodium permeability to caesium permeability gives a ratio of 1.32. (P_{Na}/P_{Cs} = 1.32). However, if the current was carried by chloride ions, then the reversal potential would also have been around 0 mV under these recording conditions, i.e. symmetrical chloride on both sides of the cell membrane. In order to prove that this current is predominantly a cation conductance, it was necessary to substitute the external cation with a large, and presumably impermeant cation. We found that replacing external sodium ions with the large cation choline completely reversibly abolished the inward current (n = 8; Figure 3). Together, these data suggest that 5-HT activates a cation channel which is permeable to sodium and caesium, but not choline (or chloride) ions.

No clear single channel currents were evoked by 5-HT in outside-out membrane patches. The responses appeared to be smaller versions (~10–20 pA) of the whole cell currents with no discernible single channel openings or closures. Also little or no increase in current noise was associated with 5-HT-induced responses in whole cells (see for example Figure 1).

Other experiments showed that 5-HT responses in these peripheral neurones are modulated by the concentration of divalent cations in the extracellular solution. Figure 4a shows the effect of reducing extracellular calcium concentration from 1 mM to zero (approximately 15 μM). Here the response to a one second application of 5-HT was increased in amplitude and duration as calcium concentration was decreased. Figure 4b shows a similar effect when the agonist PBG was applied. A more dramatic effect was observed when calcium concentration was rapidly increased from 0 to 5 mM (Figure 4c). In this example the charge transferred for a given application of 5-HT is markedly reduced. The inhibitory action of calcium was fully and rapidly reversible. Changes in the level of external magnesium ions similarly altered the size and duration of

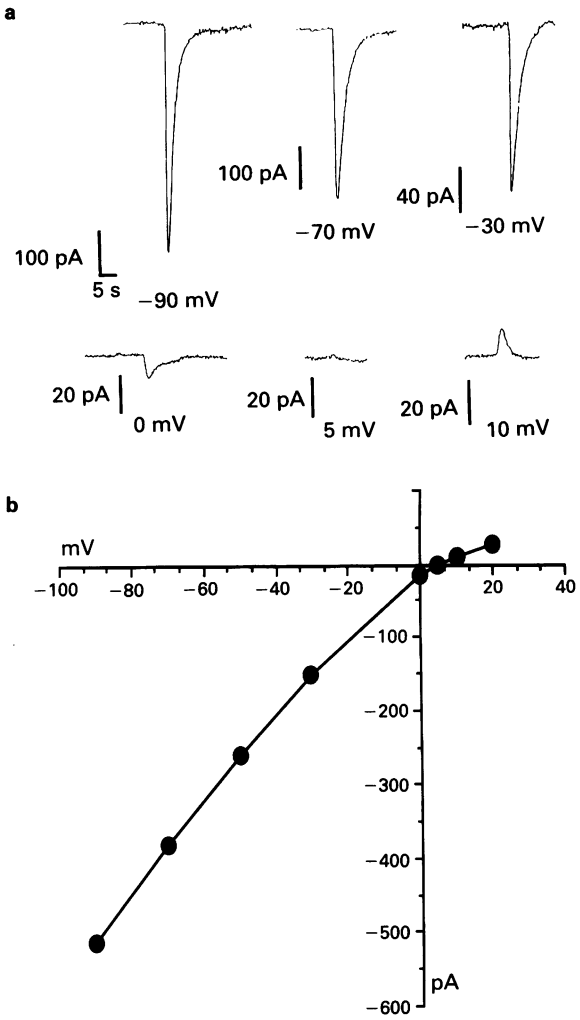

Figure 2 (a) Currents elicited by 1 s application of 10 μM 5-hydroxytryptamine (5-HT) at different membrane potentials (shown below). Note difference in amplitude calibration for current traces (b) Current-voltage relation for currents shown in 2a. E_{rev} + 5 mV is sufficiently different from E_{Cl} to suggest a cation permeability. P_{Na}/P_{Cs} = 1.21. Na external soln., Cs internally, cell 123.29.

responses to 5-HT (not shown). The effects of various external divalent cations are summarized in Table 1.

Recent experiments (e.g. Yakel & Jackson, 1988) have suggested that intracellular second messengers may be involved in the genesis and maintenance of 5-HT_3 responses. In order to investigate the possibility that protein kinases can influence

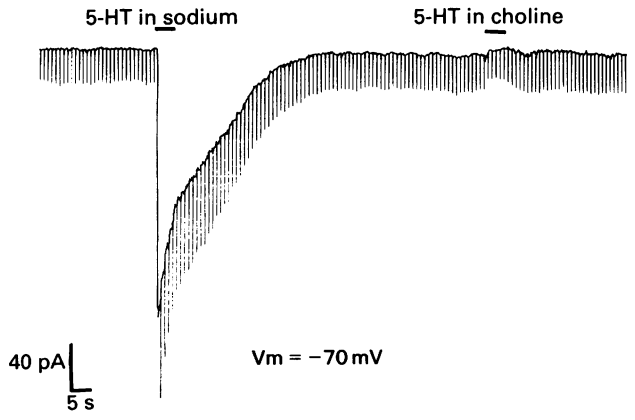

Figure 3 Complete replacement of extracellular sodium chloride with choline chloride abolishes the inward current response to 5-hydroxytryptamine (5-HT). Current deflections are produced by voltage steps from −70 to −100 mV. K^+ internal solution. Cell 117.9.

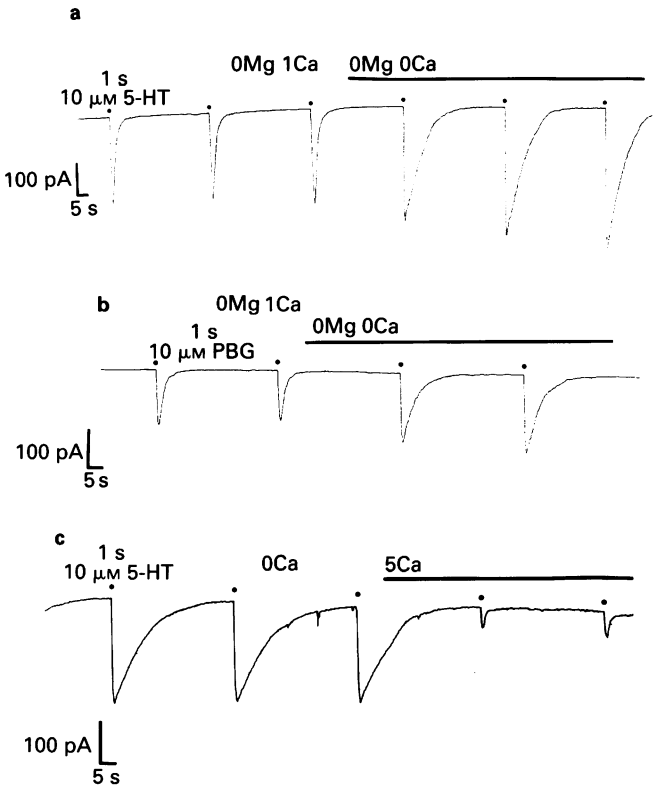

Figure 4 Reduction of extracellular calcium ion concentration from 1 mM to 0 mM potentiates the inward current produced by 10 μM 5-hydroxytryptamine (5-HT) (a) and 10 μM phenylbiguanide (PBG) (b). 0 external Mg in external soln. Cell 172.1. (c) Increase in external Ca_o from 0 (added) to 5 mM markedly attenuates the 5-HT-induced current. Cell 163.5.

5-HT sensitivity, experiments were conducted with the microbial alkaloid staurosporine (isolated from culture broths of *Streptomyces*), which is a potent, but probably nonselective, antagonist of various protein kinases presently available (Tamaoki *et al.*, 1986; Ruegg & Burgess, 1989). Figure 5a shows that a brief application of 100 nM staurosporine reduces the subsequent response to 10 μM 5-HT. This transient inhibition was completely reversible (n = 8). Inclusion of staurosporine (20 nM) in the bathing medium also resulted in an inhibition of 5-HT responses (n = 3), although some recovery was observed even in the continued presence of staurosporine (Figure 5b).

Discussion and conclusions

The results described here show that 5-HT elicits an inward current in adult rat DRG neurones, leading to a membrane depolarization. Depolarization of visceral primary afferent

Table 1 Effects of divalent cations on the amplitude and half-time of decay of 5-hydroxytryptamine-induced currents in dorsal root ganglion (DRG) neurones

Divalent ion concentration	*Amplitude* (%)	n	*Half time of decay* (%)	n
0 Ca	100	21	100	21
1 Ca	76	10	50	13
5 Ca	49	4	32	4
5 Ba	44	3	22	2
1 Mg	65	2	52	2
5 Mg	68	3	59	3
1 Cd	70	1	40	1

n = number of cells, with values determined from at least 3 measurements from each cell.

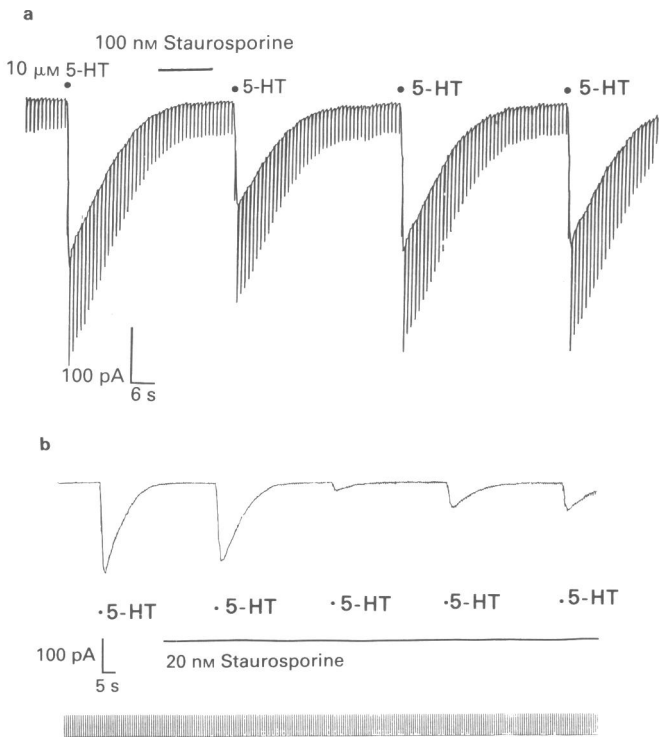

Figure 5 (a) A brief application (15 s) of 100 nM staurosporine from an adjacent puffer pipette before a 1 s pulse of 10 μM 5-hydroxytryptamine (5-HT) reduces the agonist-induced current. This antagonism is fully reversible. Current pulses produced by voltage steps from −70 to −120 mV. Cell 138.5. (b) Inward current produced by 1 s application of 10 μM 5-HT is diminished by bath-applied staurosporine (20 nM), although some recovery is observed. Cell 143.12.

neurones by 5-HT has been described previously by a number of authors and has been ascribed to an influx of sodium ions (Wallis *et al.*, 1982; Higashi & Nishi, 1982). 5-HT is also known to excite nociceptive somatosensory neurones and application of 5-HT to a blister base gives rise to the sensation of pain (Keele & Armstrong, 1964; Richardson *et al.*, 1985). This activation of nociceptive neurones *in situ* is mimicked by 2-Me 5-HT and PBG, which are thought to be selective agonists for 5-HT$_3$ receptors, while the response to 5-HT is inhibited by the 5-HT$_3$ antagonist, ICS 205–930.

Our results on isolated DRG neurones showed that 5-HT$_3$ receptors are coupled to a cation channel in the neuronal membrane. This channel was slightly more selective for sodium ions over caesium, and was impermeable to choline ions. The cation selectivity of this channel is similar to that recently shown by Peters *et al.* (1988) for 5-HT$_3$-activated responses in neuroblastoma cells. However, a sodium/potassium permeability ratio of 2.3 was obtained by Higashi & Nishi (1982) in rabbit nodose ganglion cells. Their higher value is probably due to technical differences, i.e. whole cell clamp with intracellular dialysis and known ion concentrations on both sides of the cell membrane, versus clamp with a high resistance microelectrode of cells in intact ganglia. Yakel & Jackson (1988) and Yang & Hille (1990) suggest that the 5-HT$_3$ channel may be similar to the nicotinic acetylcholine (ACh) channel at the vertebrate endplate, since the permeability ratios for monovalent cations are similar.

Recent evidence (Lambert *et al.*, 1989) suggests that the 5-HT$_3$ channel in neuroblastoma cells has a low single channel conductance, of the order of a few hundred fS. This is unlike the ACh channel which supports much greater ion transport rates (~40 pS). In contrast, Derkach *et al.* (1989) have shown that 5-HT$_3$ receptors in guinea-pig submucous plexus neurones are linked to cation channels with conductances of 15 and 9 pS. Peters *et al.* (1990) have recently reported a conductance value of 17 pS in rabbit nodose neurones. On the basis of our own restricted data, we believe that the 5-HT$_3$ channel in rat DRG neurones has a low single channel conductance for the following reasons. Firstly, we could find no clear single channel events produced by 5-HT in outside-out patches obtained from cells which gave a robust response to the agonist. Small responses could sometimes be obtained with 5-HT, but these currents were miniature versions of the whole cell current response, with a rapid increase to a peak followed by an exponential decay. Secondly, there was very little current variance associated with the agonist-induced whole cell currents. The most economical explanation for these results is that the macroscopic current is produced by many ion channels with a very small conductance which, singly, are almost undetectable in isolated patches. The presence of a few channels in a patch combine to give a very small response whose profile mirrors that of the whole cell. A similar phenomenon has been encountered by Cull-Candy *et al.* (1988) for kainate-gated channels in cerebellar granule cells. A low single channel conductance implies that the neurone has to possess many more ion channels to ensure that a significant current is generated in response to the agonist.

Smith (1966) was the first to report that raised extracellular calcium antagonized 5-HT responses in cat superior cervical ganglion neurones *in vivo*. Later, Nash & Wallis (1981) showed that divalent cations modulated 5-HT responses, but not nicotinic responses, on rabbit superior cervical ganglion (SCG) neurones. In the present voltage clamp study, changing the concentration of divalent cations in the perfusate appeared to have dramatic effects on the magnitude of the membrane currents activated by 5-HT. When the calcium concentration was increased from 0 mM to 1 mM a significant decrease in both peak amplitude and half time of decay resulted and the combination of these two effects resulted in an ~60% reduction of total charge moved across the membrane for each 5-HT response. These effects are quantitatively very similar (judging by their dose-response curve to Ca^{2+}) to those recently obtained by Peters *et al.* (1988) for N1E-115 neuroblastoma cells.

We do not yet know how divalent ions are able to modulate 5-HT$_3$ responses so dramatically, or what role this may play physiologically. It might be suggested that the 5-HT$_3$ channel has a high affinity binding site for divalent ions which, when occupied, considerably reduces the chances of other cations (like Na^+) entering and passing through the channel. This binding site may be 'blind' to the membrane electric field, since the antagonistic action of Ca^{2+} is voltage-insensitive (Peters *et al.*, 1988). Further experiments will be necessary to determine the mechanism of calcium action on these channels. Presumably, any artificial compound that binds at the divalent site could be a useful 5-HT$_3$ antagonist.

In the course of this study, we found that 5-HT responsiveness varied considerably between batches of cells. We are still uncertain as to the cause of this variability. One possibility that we considered was that the status of the intracellular second messenger systems influences 5-HT$_3$ responses (Peroutka, 1988). Yakel & Jackson (1988) have proposed that desensitization in this system is modulated by adenylate cyclase. All of our attempts at increasing the proportion of responsive cells by including dibutyryl cyclic AMP in the cell culture medium proved unsuccessful.

However, the protein kinase antagonist staurosporine inhibited the 5-HT response. Similar results have been obtained with rat isolated spinal cord tail preparation (A. Dray, personal communication). We have also noted that brief exposures to staurosporine antagonize 5-HT responses in the PC12 phaeochromocytoma cell line (unpublished observations). The possible interaction between staurosporine and 5-HT$_3$ responses warrants further investigation to determine if the inhibition is related to an action on PKC or to some other mechanism.

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Inhibition of inositol 1,4,5-trisphosphate formation by cyclic GMP in cultured aortic endothelial cells of the pig

Derek Lang & [1]Malcolm J. Lewis

Department of Pharmacology & Therapeutics and Cardiology, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN

1 In cultured endothelial cells of the pig the endothelium-derived relaxing factor (EDRF) releasing agent thrombin ($2\,u\,ml^{-1}$) caused a significant increase in basal levels of both guanosine 3':5'-cyclic monophosphate (cyclic GMP) and inositol 1,4,5-trisphosphate (IP_3). This increase was time dependent, with peak levels occurring at 2 min and returning towards basal values after 5 min.

2 Pretreatment of the cells with the EDRF inhibitors haemoglobin (1 μM) or L-N^G-nitro arginine (50 μM) significantly reduced the cyclic GMP response to thrombin. Both agents also resulted in significant elevations in basal levels of IP_3. The IP_3 response to thrombin was significantly enhanced at all time points by haemoglobin and at 5 min for L-N^G-nitro arginine, when compared with the response to thrombin alone.

3 Pretreatment of the cells with either sodium nitroprusside (10 μM) or atrial natriuretic peptide (1 μM) caused a significant elevation of basal cyclic GMP levels. Although subsequent exposure to thrombin caused a further increase in cyclic GMP, which together with the rise induced by the previous two agents was significantly greater than the increase caused by thrombin alone, the incremental increase induced by thrombin was markedly less in the presence of nitroprusside or atrial natriuretic peptide. Both these agents, as well as 8-bromo cyclic GMP, resulted in a significant suppression of the IP_3 response to thrombin.

4 These findings show that one mechanism for the inhibitory effect of cyclic GMP on EDRF release from endothelium may be through the inhibition of IP_3 formation in response to EDRF releasing agents.

Introduction

Inositol 1,4,5-trisphosphate (IP_3) is the second messenger which mobilizes intracellular stores of calcium in a number of cell types (Berridge & Irvine, 1984; Hashimoto *et al.*, 1986; Berridge, 1987). It is produced when phosphatidyl-inositol 4,5-bisphosphate (PIP_2) is hydrolysed by phospholipase C in response to cell-surface receptor activation (Berridge & Irvine, 1984; Berridge, 1984; Hokin, 1985; Downes & Michell, 1985). The other product of this hydrolysis is sn 1,2-diacylglycerol which activates protein kinase C (Nishizuka, 1984). In many cell types a guanyl nucleotide transducing-protein (G protein) couples the receptor to phospholipase C and in some cases this step is sensitive to inhibition by pertussis toxin (Berridge, 1987).

A rapid formation of IP_3 in endothelial cells occurs in response to several agonists including thrombin (Moscat *et al.*, 1987; Pollock *et al.*, 1988), bradykinin (Derian & Moskowitz, 1986; Lambert *et al.*, 1986), ADP and ATP (Forsberg *et al.*, 1987; Pirotton *et al.*, 1987) and mellitin, a direct activator of phospholipase C (Loeb *et al.*, 1988). These agonists also stimulate the release of endothelium-derived relaxing factor (EDRF) (for review see Angus & Cocks, 1989), the critical signal for EDRF release being elevation of intracellular Ca^{2+} levels (for review see Newby & Henderson, 1990).

EDRF, recently discovered to be nitric oxide (Palmer *et al.*, 1987) acts like the nitrovasodilator drugs through stimulation of soluble guanylate cyclase and the elevation of intracellular levels of guanosine 3':5'-cyclic monophosphate (cyclic GMP) (Katsuki *et al.*, 1977; Ignarro *et al.*, 1981; Rapoport *et al.*, 1983a,b; Griffith *et al.*, 1985; Forstermann *et al.*, 1986). Atrial natriuretic peptide (ANP) similarly causes intracellular cyclic GMP levels to be elevated in vascular smooth muscle (Winquist *et al.*, 1989), doing so by specific activation of particulate guanylate cyclase (Waldman *et al.*, 1984).

The stimulated rise in cyclic GMP produced by EDRF and other cyclic GMP-elevating agents which results in vascular smooth muscle relaxation is associated with both the inhibition of influx of extracellular calcium as well as a decrease in the release of intracellular calcium (Karaki *et al.*, 1984; Collins *et al.*, 1986). The cause of the inhibition of contraction and of intracellular Ca^{2+} release, was thought to result from the inhibitory effect of cyclic GMP on phosphatidylinositol hydrolysis (Rapoport, 1986) in rat aorta, and inhibition of IP_3 formation by cyclic GMP has now been shown also in rabbit aorta (Lang & Lewis, 1989).

Stimulation of EDRF release in cultured endothelium results in elevation of cyclic GMP levels in the endothelium itself (Martin *et al.*, 1988; Smith & Lang, 1990). Furthermore, elevation of endothelial cell levels of cyclic GMP with either 8-bromo cyclic GMP (Evans *et al.*, 1988) or ANP (Hogan *et al.*, 1989) inhibits EDRF release. The mechanism of this effect remains unknown however, but might be through inhibition of the rise in IP_3 levels in endothelium induced by EDRF releasing agents. To investigate this possibility in the present study, we have examined the effects of alteration of endothelial cell levels of cyclic GMP on IP_3 formation in cultured cells stimulated with thrombin.

Methods

Preparation of cultured endothelial cells

Aortae, from approximately 16 week old pigs, were removed immediately after slaughter at the local abattoir, and flushed with 0.9% (w/v) sterile NaCl containing benzylpenicillin $200\,u\,ml^{-1}$ with streptomycin $200\,\mu g\,ml^{-1}$. The proximal end of the vessel was tied off and the distal end cannulated with a 50 ml syringe containing the same saline. The lumen of the vessel was then filled with the saline for transportation back to the laboratory.

Endothelial cells were isolated essentially as described by Gordon & Martin (1983). Briefly, the intercostal arteries were ligated, the lumen emptied of the saline and filled with 0.2% collagenase (type II, Sigma) in Medium E199 and incubated at 37°C for 20 min. The cells were then harvested into 40 ml of Medium E199 supplemented with 10% foetal calf serum, 10%

[1] Author for correspondence.

newborn serum, glutamine 6 mM, benzylpenicillin 200 u ml^{-1}, streptomycin 200 $\mu g\,ml^{-1}$ and kanamycin 100 $\mu g\,ml^{-1}$. The cells were subsequently seeded into three six-well plates (well area = 9.62 cm^2) The culture medium was replaced the next day and then every other day until the cells became confluent, usually within 5–7 days.

Experimental protocol

The culture medium was removed and the cells washed with 2 × 2 ml of Krebs-Ringer bicarbonate (KRB) solution of the following composition (mM): NaCl 95.5, KCl 4.8, $MgSO_4$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11. The cells were then incubated in 2 ml of KRB containing lithium chloride 10 mM at 37°C under an atmosphere of 5% CO_2 in air for at least 90 min. Drugs were added at the concentrations and times indicated in the Results.

At the appropriate time the KRB was rapidly removed and the reaction terminated by the addition of 0.5 ml of ice cold 5% (v/v) perchloric acid (PCA). The cells were scraped from the well and together with a further 0.5 ml of PCA were placed in plastic tubes. This combined 1 ml volume of PCA was then centrifuged at 13000*g* for 2 min. The resulting supernatant was aspirated into separate plastic tubes, previously cooled on dry ice and, along with the cell debris pellet stored at −20°C until assay within 1 month. Supernatants were frozen immediately in this way to prevent breakdown of the inositol phosphates.

Measurement of IP_3 cyclic GMP and DNA

The supernatants were thawed and 400 μl of each transferred to separate tubes containing 100 μl of 10 mM EDTA (pH 7.0). Samples were then neutralized by adding 300 μl of 1:1 (v/v) mixture of 1,1,2-trichloro-trifluoroethane and tri-n-octylamine followed by vigorous vortexing for 90 s. The IP_3 and cyclic GMP content of the aqueous upper layer was measured with commercially available kits (Amersham International, U.K. and New England Nuclear Research Products, F.R.G., respectively).

The DNA content of the pellet was measured by the fluorimetric method of Kissane & Robins (1958). The IP_3 content of each well was expressed as pmol μg^{-1} DNA and the cyclic GMP as fmol μg^{-1} DNA.

Drugs

Atrial natriuretic peptide (human sequence), sodium nitroprusside, thrombin (human), L-N^G-nitro arginine and haemoglobin (bovine) were obtained from Sigma Chemical Company, UK. All were dissolved in distilled water immediately prior to use except in the case of L-N^G-nitro arginine where the water was acidified with HCl.

Haemoglobin solutions were reduced to the ferrous form with dithionite as described by Martin *et al.* (1986). Sera, culture medium, glutamine and kanamycin were obtained from Flow Laboratories, U.K., benzyl penicillin (crystopen) from Glaxo, U.K., and streptomycin sulphate from Evans Medicals Limited, U.K.

Statistics

Both IP_3 and cyclic GMP values are expressed as the means ± standard error of the mean (s.e.mean). For analysis of within-group data, a one-way analysis of variance was used followed by Dunnett's multiple range test to identify significant differences at the 0.05 level. For between-group data Tukey's test was used; comparisons were considered significantly different when $P < 0.05$.

Results

Thrombin

Figure 1 shows the levels of IP_3 and cyclic GMP following incubation of the cultured endothelial cells with thrombin

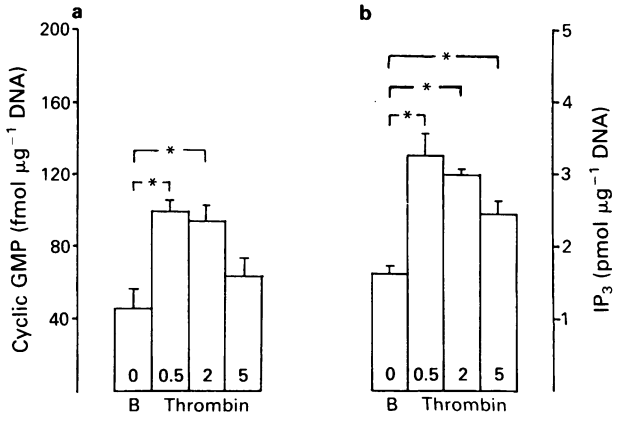

Figure 1 Histogram showing basal (B) concentrations (mean with s.e.mean shown by vertical bars) of guanosine 3′: 5′-cyclic monophosphate (cyclic GMP) (a) and inositol 1,4,5-trisphosphate (IP_3) (b) in cultured aortic endothelial cells of the pig and following stimulation with thrombin (2 u ml^{-1}) for 0.5, 2 and 5 min. (* $P < 0.05$ cf basal values; $n \geqslant 5$).

(2 u ml^{-1}) for 30 s, 2 min and 5 min. The data show a significant rise in IP_3 levels after 30 s declining thereafter towards basal values but remaining significantly elevated at 5 min. The pattern of change in cyclic GMP levels is similar to that of IP_3 with a significant increase at 30 s remaining elevated at 2 min and declining back to basal levels at 5 min.

Haemoglobin

Figure 2 shows the results in thrombin-stimulated cells but following preincubation of the cells for 15 min with haemoglobin (1 μM). The data again shows a significant rise in IP_3 levels at each time point following thrombin stimulation when compared with basal levels in the absence of haemoglobin ($P < 0.05$ at all time points). The rise in IP_3 observed at 30 s, 2 min and 5 min after thrombin addition was significantly greater than that observed in the absence of haemoglobin ($P < 0.05$ at all time points). Haemoglobin alone also caused a significant increase in IP_3 compared with basal levels ($P < 0.05$). There were no significant changes in cyclic GMP levels either in the presence of haemoglobin alone or after the addition of thrombin.

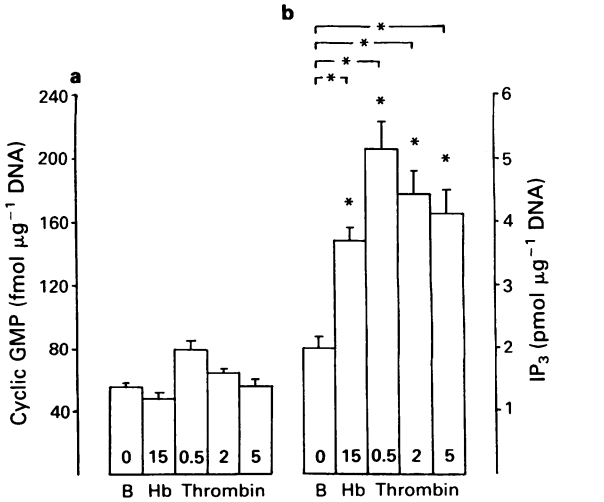

Figure 2 Histogram showing basal (B) concentrations (mean with s.e.mean shown by vertical bar) of guanosine 3′:5′-cyclic monophosphate (cyclic GMP) (a) and inositol 1,4,5-trisphosphate (IP_3) (b) in cultured aortic endothelial cells of the pig and after incubation with haemoglobin (Hb; 1 μM) for 15 min followed by stimulation with thrombin as for Figure 1. (*(in brackets) $P < 0.05$ cf. basal values; * $P < 0.05$ cf. values for thrombin in the absence of haemoglobin; $n \geqslant 5$).

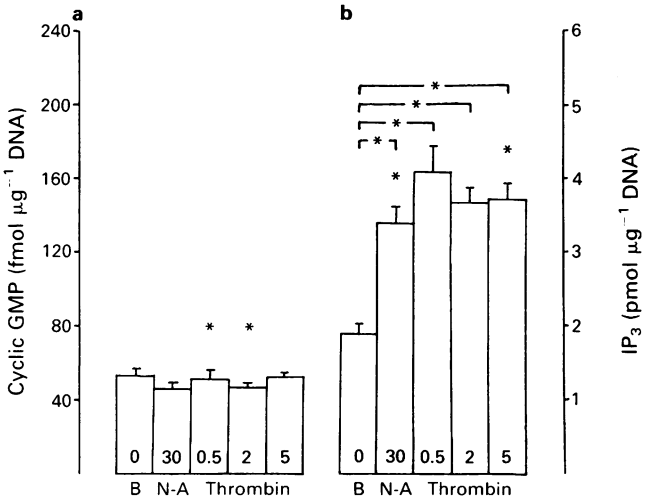

Figure 3 Histogram showing basal (B) concentrations (mean with s.e.mean shown by vertical bars) of guanosine 3':5'-cyclic monophosphate (cyclic GMP) (a) and inositol 1,4,5-trisphosphate (IP_3) (b) in cultured aortic endothelial cells of the pig and after incubation with L-N^G-nitro arginine (N-A; 50 μM) for 30 min followed by incubation with thrombin as for Figure 1. (*(in brackets) $P < 0.05$ cf. basal values; * $P < 0.05$ cf. values for thrombin in the absence of L-N^G-nitro arginine; $n \geqslant 5$).

L-N^G-nitro arginine

Figure 3 shows the changes in IP_3 and cyclic GMP levels following pre-incubation of the cells for 30 min with L-N^G-nitro arginine (50 μM). Like haemoglobin, this inhibitor of EDRF production resulted in a significant increase in IP_3 levels, compared with basal values in the absence of thrombin ($P < 0.05$). In the presence of thrombin, IP_3 increased significantly at each time point ($P < 0.05$) and remained significantly higher at 5 min when compared with the cells stimulated with thrombin alone ($P < 0.05$). Cyclic GMP levels did not alter following thrombin stimulation in the presence of this agent.

Sodium nitroprusside

Figure 4 shows the effect of pre-incubation of the cells for 30 s with sodium nitroprusside (10 μM). The data show no significant rise in IP_3 levels above basal levels at any of the time points studied, following thrombin stimulation. However, incubation with nitroprusside alone caused a significant increase in cyclic GMP over basal levels ($P < 0.05$), which

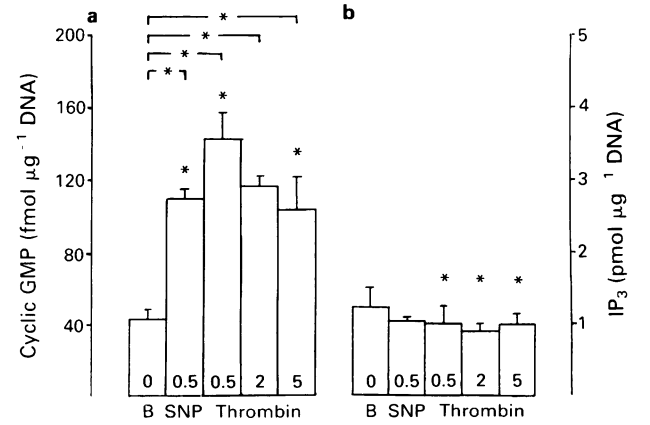

Figure 4 Histogram showing basal (B) concentrations (mean with s.e.mean shown by vertical bars) of guanosine 3':5'-cyclic monophosphate (cyclic GMP) (a) and inositol 1,4,5-trisphosphate (IP_3) (b) in cultured aortic endothelial cells of the pig and after incubation with sodium nitroprusside (SNP; 10 μM) and for 0.5 min followed by incubation with thrombin as for Figure 1. (*(in brackets) $P < 0.05$ cf. basal values; * $P < 0.05$ cf. values for thrombin in the absence of sodium nitroprusside; $n \geqslant 5$).

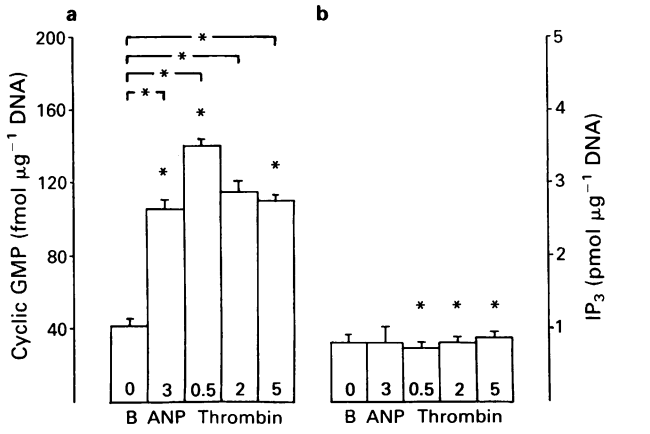

Figure 5 Histogram showing basal (B) concentrations (mean with s.e.mean shown by vertical bars) of guanosine 3':5'-cyclic monophosphate (cyclic GMP) (a) and inositol 1,4,5-trisphosphate (IP_3) (b) in cultured aortic endothelial cells of the pig and after incubation with atrial natriuretic peptide (ANP; 1 μM) for 3 min followed by incubation with thrombin as for Figure 1. (*(in brackets) $P < 0.05$ cf basal values; * $P < 0.05$ cf. values for thrombin in the absence of atrial natriuretic peptide; $n \geqslant 5$).

were significantly enhanced by subsequent incubation with thrombin when compared with basal values ($P < 0.05$ at all time points) or when compared to levels obtained following incubation with thrombin alone ($P < 0.05$ at 30 s and 5 min).

Atrial natriuretic peptide

Figure 5 again shows a thrombin time course but following preincubation of the cells for 3 min with ANP (1 μM). As for nitroprusside, the presence of ANP resulted in complete inhibition of the IP_3 response to thrombin. ANP alone significantly elevated cyclic GMP levels above basal values ($P < 0.05$) and significantly enhanced the thrombin-stimulated rise when compared to basal ($P < 0.05$ at all time points) or when compared with values in the absence of ANP ($P < 0.05$ at 30 s and 5 min).

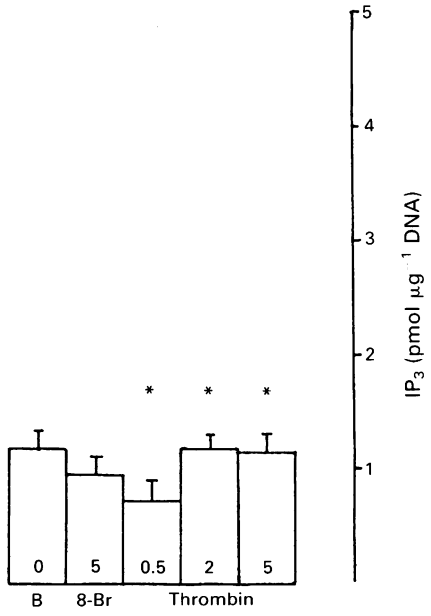

Figure 6 Histogram showing basal (B) concentrations (mean with s.e.mean shown by vertical bars) of inositol 1,4,5-trisphosphate (IP_3) in cultured aortic endothelial cells of the pig and after incubation with 8-bromo cyclic GMP (8-Br; 100 μM) for 5 min followed by incubation with thrombin as for Figure 1. (* $P < 0.05$ cf. values for thrombin in the absence of 8-bromo cyclic GMP; $n \geqslant 5$).

8-bromo cyclic GMP

In the experiments with this lipid-soluble analogue of cyclic GMP, IP_3 levels only were measured since this agent directly elevates cyclic GMP levels in cells. As shown in Figure 6 incubation with 100 μM for 5 min produced complete inhibition of IP_3 formation in response to thrombin.

Discussion

The data show that there is a rapid rise in both IP_3 and cyclic GMP levels in cultured endothelial cells of the pig following exposure to the EDRF-releasing agent thrombin. When the cells were preincubated with the two cyclic GMP elevating agents sodium nitroprusside and ANP, not only were basal cyclic GMP levels increased but, in the presence of thrombin the level remaining elevated for longer. Furthermore, in the presence of elevated basal cyclic GMP levels, the subsequent response of the cells to thrombin was blunted i.e. the incremental increase in cyclic GMP was markedly reduced. Under these conditions, the IP_3 response to thrombin was completely inhibited. Conversely when the cells were preincubated with the EDRF inhibitor haemoglobin (Martin *et al.*, 1985) or the inhibitor of EDRF formation L-N^G-nitro arginine (Moore *et al.*, 1990), the increase in cyclic GMP following thrombin, was significantly less when compared to thrombin alone, and the IP_3 response was significantly enhanced.

These findings therefore confirm earlier work which showed that elevated levels of cyclic GMP inhibit phosphatidylinositol turnover in platelets (Takai *et al.*, 1981) and also in vascular smooth muscle (Rapoport, 1986). They also confirm our own studies showing an inhibitory effect of cyclic GMP on stimulated IP_3 levels in vascular smooth muscle (Lang & Lewis, 1989). The observation that the thrombin-induced incremental increase in cyclic GMP in the presence of nitroprusside or atrial natriuretic factor was reduced, also suggests that EDRF release from the cells was inhibited by these agents. A finding which again confirms our earlier studies that elevation of endothelial cell levels of cyclic GMP inhibits EDRF release (Evans *et al.*, 1988; Hogan *et al.*, 1989).

The mechanism responsible for the cyclic GMP-induced inhibition in endothelial cells is unknown at present. It is possible that, as suggested for the action of cyclic GMP in vascular smooth muscle and platelets (Takai *et al.*, 1981; Rapoport, 1986; Lang & Lewis, 1989), there is inhibition of the transduction mechanisms between the cell surface receptors and IP_3 formation. This is likely to be at the level of either a G protein or possibly phospholipase C. Evidence in favour of these sites of action of cyclic GMP in vascular smooth muscle has recently been provided by Hirata and colleagues (1990). These workers showed that the inhibitory effect of cyclic GMP on phosphoinositide hydrolysis and GTPase activity in homogenates and membrane preparations of cultured bovine aortic smooth muscle cells, resulted from an inhibition of guanine nucleotide regulatory protein activation and the interaction between guanine nucleotide regulatory protein and phospholipase C. Thrombin-induced EDRF release is thought to involve a G protein in its transduction mechanism since pertussis toxin blocks EDRF release by this agent (Flavahan *et al.*, 1989).

Although we have previously shown an inhibitory effect of cyclic GMP on acetylcholine- and substance P-induced EDRF release from rabbit blood vessels (Evans *et al.*, 1988; Hogan *et al.*, 1989), it is not known whether cyclic GMP will inhibit EDRF release, and possibly IP_3 formation, in endothelial cells from all species, with all agonists. If the mechanism of action of cyclic GMP is by inhibition of the transduction mechanisms between receptor and IP_3 information, it is possible that only those agonists coupled to a specific G protein would be inhibited by cyclic GMP. We have previously shown that cyclic GMP has no effect on EDRF release induced by ATP (Evans *et al.*, 1988). By use of intracellular calcium measurements as an indication of endothelial cell activation, it has also been demonstrated that 8-bromo cyclic GMP did not inhibit increases in intracellular calcium induced by thrombin in human umbilical vein endothelial cells (Jaffe *et al.*, 1987), or by histamine in human aortic endothelial cells (Ryan *et al.*, 1988). It has been shown that ADP utilizes a different G protein from thrombin in the transduction mechanism for EDRF release (Flavahan *et al.*, 1989). The nature of the G proteins involved in the activation of human endothelium by thrombin and histamine is not known but if they resemble those for ADP, and maybe the related purine ATP, this could explain the previous negative findings of the effect of 8-bromo cyclic GMP on EDRF release induced by these agents in human cells.

It is likely therefore that the inhibitory effects of cyclic GMP and of EDRF itself on its own release, is important only for those agonists utilizing a specific G protein coupled to IP_3 formation.

In conclusion, the present study provides a possible explanation for the inhibitory effects of cyclic GMP on EDRF release observed by us previously (Evans *et al.*, 1988; Hogan *et al.*, 1989). However, it is unlikely that endothelial cell activation and EDRF release induced by all agonists from endothelial cells of all species will be affected in this way.

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Ageing is associated with increased 5-HT_2-receptor affinity and decreased receptor reserve in rat isolated coronary arteries

Niels C. Berg Nyborg

Department of Pharmacology, Aarhus University, DK-8000 Aarhus C, Denmark

1 The 5-hydroxytryptamine (5-HT)-receptor subtype and affinity for 5-HT was determined in large and small coronary arteries isolated as ring segments from the proximal and distal part of the left coronary artery of 3 month (young) and 2-year old (old) rats.

2 Ketanserin induced a rightward shift of the 5-HT concentration-response curve in both proximal and distal coronary arteries from young rats. The slopes of the Schild-plots were indistinguishable from unity and the estimated pA_2 values were 9.11 and 9.27 for proximal and distal coronary arteries, respectively. These data indicate a homogeneous population of 5-HT_2-like receptors in the coronary arteries.

3 The contractile effect of 5-HT as well as the sensitivity to 5-HT was greater in proximal and distal coronary arteries from old than from young rats.

4 The apparent 5-HT_2-receptor affinity, $-\log(K_A[M])$, and fractional receptor-occupancy for relative responses between 10% and 90% of maximum was determined by partial irreversible inhibition of the 5-HT_2-receptors with phenoxybenzamine.

5 Ageing was associated with an increase in 5-HT_2-receptor affinity for 5-HT in both proximal and distal coronary arteries, whereas the fractional receptor occupancy for half-maximal response to 5-HT decreased with age.

6 5-HT_2-receptor affinity for 5-HT could account for the 5-HT sensitivity of distal coronary arteries in both young and old rats but not in proximal coronary arteries as the slope of the regression line of plots of 5-HT_2-receptor affinity vs. sensitivity was indistinguishable from unity in only the distal vessels.

7 The 5-HT_2-receptor affinity for 5-HT was linearly correlated to the fractional receptor occupancy for half maximal response, suggesting that the 5-HT_2-receptor reserve or density down-regulates the receptor affinity for 5-HT.

8 The results indicate that the increase in 5-HT sensitivity and contractile effect in rat coronary arteries rely upon an increase in both 5-HT_2-receptor agonist affinity and efficiency of the excitation-contraction coupling process in the vascular smooth muscle.

Introduction

5-Hydroxytryptamine (5-HT, serotonin) is a powerful constrictor of large epicardial coronary arteries in a number of species including man and is considered to be involved in the pathophysiological mechanisms of coronary artery spasm (Kalsner, 1982; Ganz & Alexander, 1985).

The action of 5-HT in the coronary circulation is complex, involving stimulation of contractile 5-HT receptors of different subtypes on the smooth muscle cells (Angus, 1989), and stimulation of 5-HT_1-type receptors on the endothelial cells causing release of endothelial-derived relaxing factor(s) (EDRF) in some species (Cocks & Angus, 1983). 5-HT can also potentiate noradrenaline-induced contractions in rat caudal arteries (Van Neuten *et al.*, 1981). The contractile effect of 5-HT involves only receptors of the 5-HT_2-type in isolated coronary arteries of the rat (this paper) and is not antagonized by concomitant release of EDRF (Nyborg & Mikkelsen, 1990). The rat coronary artery therefore offers a simple system with which to study 5-HT-receptor characteristics.

We have previously shown that the maximal 5-HT-induced contraction is related to the size of the coronary artery (Nyborg & Mikkelsen, 1988a; 1990), and sensitivity to 5-HT is slightly greater in proximal than distal arteries (Nyborg & Mikkelsen, 1988a; 1990). A similar size-dependent effect of 5-HT has been observed in dog coronary artery strips (Pourquett *et al.*, 1982). Furthermore, the sensitivity as well as maximal response to 5-HT increases with age in small intramyocardial flow-regulating coronary arteries in rats (Nyborg & Mikkelsen, 1988b).

The mechanical response of vascular smooth muscle is an integrated function in which receptor-agonist dissociation constant or affinity, receptor density, and intracellular second messengers play an important role as modulators determining the agonist concentration-response relationship (Kenakin, 1984). The present experiments were designed to analyze the effect of ageing on the 5-HT_2-receptor dissociation constant, K_A, and the relationship between relative 5-HT_2-receptor occupancy and vessel response in large (proximal epicardial) and small (distal intramural) coronary arteries from 3 month old, and 2 year old, male Wistar rats.

Methods

Arterial ring segments were isolated from the same anatomical location in the proximal, epicardial, and distal, intramural, part of the left coronary artery in hearts from 3 month and 2 year old rats (Nyborg, 1985; 1990; Nyborg & Mikkelsen, 1988a) and mounted on an isometric myograph (Mulvany & Halpern, 1977; Mulvany & Nyborg, 1980).

Experimental procedure

The arteries were equilibrated at 37°C for 30 min in oxygenated (5% CO_2 in O_2) physiological saline solution (PSS) with the following composition (mM): NaCl 119, $NaHCO_3$ 25, KCl 4.7, $CaCl_2$ 1.5, K_2HPO_4 1.18, $MgSO_4$ 1.17, EDTA 0.026 and glucose 11, pH 7.4, before the vessel internal circumference, L_0, was set to 90% the circumference, L_{100}, the vessels would have if relaxed and exposed to a passive transmural pressure of 100 mmHg (13.3 kPa) (Nyborg *et al.*, 1988). The effective vessel lumen diameter, l_0, was calculated as $L_0 \pi^{-1}$.

The vessels were repetitively contracted with 125 mM K-PSS (similar to PSS except that NaCl was exchanged for KCl on an equimolar basis) until reproducible contractions were

obtained. The endothelial function was checked by estimating 10^{-5} M acetylcholine-induced relaxation of the arteries contracted with 10^{-5} M prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$).

The maximal active contractile capacity of the vessels, E_{max}, was determined by measuring the difference in wall tension of vessels when maximally contracted with K-PSS to which 10^{-5} M 5-HT and $PGF_{2\alpha}$ were added and when maximally relaxed in Ca^{2+}-free PSS (similar to PSS except that $CaCl_2$ was omitted and replaced with 0.1 mM EGTA).

Neuronal and extraneuronal 5-HT uptake was blocked throughout all 5-HT concentration-response experiments with cocaine 3×10^{-6} M (Fukuda *et al.*, 1986).

The vessel responses are presented in terms of either effective active wall tension, δT_0, calculated as increase in vessel wall force above resting level divided by twice the vessel segment length, or in terms of relative responses.

Characterization of 5-HT-receptor subtype with ketanserin

The subtype of the 5-HT-receptor present on the proximal and distal coronary vascular smooth muscle cells was determined only in young rats by use of the competitive 5-HT$_2$-receptor antagonist, ketanserin, by the method of Arunlakshana & Schild (1959).

Characterization of 5-HT-receptor dissociation constant

The 5-HT-receptor dissociation constant, K_A(M), was determined by use of the approach proposed by Furchgott (1966). Two cumulative 5-HT concentration-response curves were obtained. The second curve was obtained after the vessels were treated with phenoxybenzamine (Pbz) 3×10^{-8} M for 15 min, which irreversibly blocks 5-HT receptors (Weiner, 1985), and thoroughly washed in drug-free PSS for 45 min. Previous experiments have shown the reproducibility of rat coronary 5-HT concentration-response curves (Nyborg & Mikkelsen, 1988a,b)

Reciprocals of equieffective concentrations of 5-HT before (A(M)) and after treatment with Pbz (A'(M)) were determined. The slope and y-axis intercept of the regression line (least squares method) of plots of 1/A(M) vs. 1/A'(M) were used to calculate K_A: (slope − 1)/y-axis intercept.

The relative receptor occupancy (R_a/R_t) was calculated for each vessel from the equation derived by Furchgott & Bursztyn (1967), where $R_a/R_t = A(M)/(A(M) + K_A(M))$. A(M) and K_A(M) are 5-HT concentration and receptor dissociation constant, respectively. Occupancy-response curves were constructed by plotting the calculated occupancy against the corresponding response from the control concentration-response curve.

The concentration-response curves to 5-HT were fitted to the classical 'Hill-equation': $R/R_{max} = A(M)^n/(A(M)^n + EC_{50}(M)^n)$, where R/R_{max} is relative vessel response to the agonist, A(M), and EC_{50}(M) is concentration of agonist required to give half maximal vessel response (R_{max}), when A(M) and EC_{50}(M) are given in molar concentrations. n is a curve fitting parameter or 'Hill-coefficient'.

Vessel sensitivity, EC_{50}(M), to 5-HT and the 5-HT-receptor dissociation constant, K_A(M), are presented in the text as pD_2 and pK_A values, respectively, where $pD_2 = -\log(EC_{50}(M))$ and $pK_A = -\log(K_A(M))$.

Drugs

Drugs used were 5-hydroxytryptamine creatinine sulphate complex (Sigma), cocaine hydrochloride (DAK), $PGF_{2\alpha}$ (Dinoprost, UpJohn), ketanserin (Janssen Pharmaceutical) and phenoxybenzamine HCl (a gift from Smith Kline and French). The phenoxybenzamine stock solution was made in ethanol. Stock solutions of the other drugs were made in distilled water.

Statistics

The results were analyzed by two-way analysis of variance (2-way ANOVA) (Sokal & Rohlf, 1969), which allows comparison of differences between vessel size (proximal vs. distal) and age (young vs. old), respectively, providing the interaction term is not significant. The statistical program SPSS/PC+ was used for data handling and statistical analysis. P_{size} and P_{age} gives the probability that the differences observed between vessel size (proximal vs. distal) and age (young vs. old), respectively, could have arisen by chance. Where the probability for interaction, P_x, was significant, indicating that the parameters tested in the two vessel sizes were differently affected by ageing, conclusions about the effect of ageing on the parameter could not be drawn. In these cases a t rest was applied on the data comparing young with old rats within each type of vessel, either proximal or distal. The level of significance was for all tests set at $P < 0.05$.

Results

Vessel characteristics

The effective lumen diameter of the proximal and distal vessels was differently affected by ageing ($P_x = 0.003$). The lumen diameters of proximal arteries increased from $351 \pm 26\,\mu$m in young rats ($n = 10$) to $503 \pm 19\,\mu$m ($n = 10$) ($P < 0.001$, t test) in old rats whereas the diameter of distal arteries increased from $230 \pm 19\,\mu$m ($n = 8$) to $313 \pm 19\,\mu$m ($n = 6$) ($P < 0.01$, t test), in young and old rats, respectively.

E_{max} (see Methods) was not significantly different between proximal and distal vessels and it was not affected by ageing, being $3.04 \pm 0.40\,Nm^{-1}$ ($n = 10$) and 3.01 ± 0.39 ($n = 10$) in proximal arteries, and $2.51 \pm 0.38\,Nm^{-1}$ ($n = 8$) and $3.14 \pm 0.51\,Nm^{-1}$ ($n = 6$) in distal arteries from young and old rats, respectively.

The spontaneous myogenic tone of the arteries was greater in distal arteries: $0.28 \pm 0.14\,Nm^{-1}$ ($n = 8$) and $0.55 \pm 0.17\,Nm^{-1}$ ($n = 6$) for young and old rats, respectively, than in proximal arteries: $0.02 \pm 0.01\,Nm^{-1}$ ($n = 10$) and $0.19 \pm 0.09\,Nm^{-1}$ ($n = 10$) young and old rats, respectively ($P_{size} = 0.007$; $P_{age} = 0.050$).

The relaxation induced by acetylcholine 10^{-5} M of the $PGF_{2\alpha}$ (10^{-5} M)-precontracted vessels was not significantly different between the proximal and distal arteries nor between the young and old rats.

Effect of 5-hydroxytryptamine

5-HT induced a concentration-dependent contraction in proximal and distal coronary arteries from young and old rats (Figure 1). The maximal active tension induced by 5-HT, expressed as a percentage of E_{max}, was significantly greater ($P_{size} = 0.028$) in proximal vessels: $76 \pm 5\%$ ($n = 10$) and $85 \pm 3\%$ ($n = 10$), young and old rats, respectively, than in distal vessels: $62 \pm 9\%$ ($n = 8$) and $75 \pm 10\%$ ($n = 6$), young and old rats, respectively. The maximal response to 5-HT was slightly but significantly greater ($P_{age} = 0.049$) in vessels from old rats compared to vessels from young rats.

5-HT-receptor classification with ketanserin

Ketanserin, 10^{-9}–10^{-8} M, induced a rightward shift of the 5-HT concentration-response curve of both proximal and distal coronary arteries from young rats. The slope of the regression lines was not significantly different than unity: 0.97 ± 0.05 ($n = 5$) and 0.99 ± 0.10 ($n = 5$) in proximal and distal arteries, respectively. The estimated pA_2 values were 9.11 ± 0.03 ($n = 5$) and 9.27 ± 0.09 ($n = 5$) in the proximal and distal arteries, respectively.

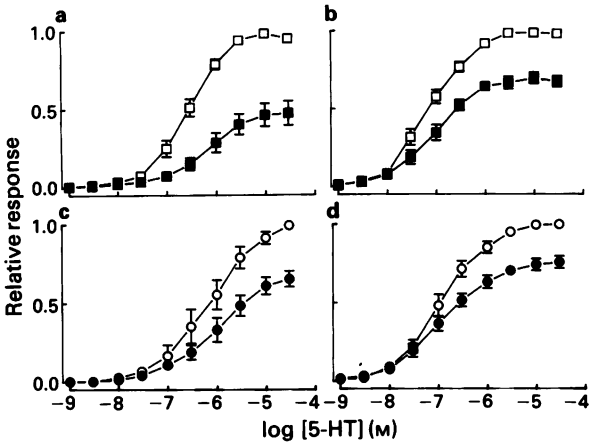

Figure 1 5-Hydroxytryptamine (5-HT) concentration-response relation of proximal (a,b) and distal (c,d) coronary arteries from 3 month old (a,c) and 2 year old (b,d) rats before (open symbols) and after treatment with phenoxybenzamine (closed symbols). Vessel responses were normalized with respect to the maximal response to 5-HT of each individual vessel before treatment with phenoxybenzamine. Points show mean of 6–10 vessels and vertical bars show s.e.mean.

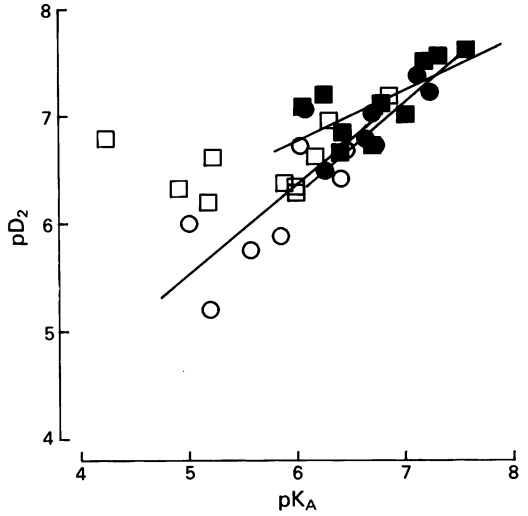

Figure 2 Plots of 5-HT_2-receptor affinity, pK_A(M), versus 5-HT sensitivity, pD_2, in isolated proximal (squares) and distal (circles) coronary arteries from 3 month old (open symbols) and 2 year old (closed symbols) rats.

5-HT-receptor affinity

Treatment of vessels with 3×10^{-8} M Pbz for 15 min depressed the maximal response and displaced the 5-HT concentration-response curve to the right (Figure 1). The 5-HT concentration-response curve of vessels from the young rats was affected by treatment with Pbz more than vessels from old rats ($P_{age} = 0.012$). The maximal response of proximal arteries to 5-HT was more depressed than in that of distal arteries ($P_{size} = 0.029$). The maximal response after Pbz treatment was 49 ± 8% ($n = 10$) and 70 ± 5% ($n = 8$) in the proximal and distal arteries from young rats, respectively, and 72 ± 4% ($n = 10$) and 74 ± 3% ($n = 6$), respectively, in those from old rats.

The sensitivity to 5-HT was significantly ($P_{age} \ll 0.001$) greater in vessels from old than from young rats. The pD_2-values increased from 6.55 ± 0.10 ($n = 10$) to 7.13 ± 0.11 ($n = 10$) in proximal arteries, and from 6.18 ± 0.23 ($n = 8$) to 6.94 ± 0.14 ($n = 6$) in distal arteries, respectively. The difference in pD_2 values between proximal and distal arteries was close to becoming significant ($P_{size} = 0.059$).

The 5-HT_2-receptor affinity, pK_A(M), for 5-HT was significantly greater in older than younger rats ($P_{age} \ll 0.001$). The pK_A(M) values for proximal and distal segments from young rats were 5.63 ± 0.25 ($n = 10$) and 5.83 ± 0.19 ($n = 8$), respectively. The corresponding pK_A(M) values for proximal and distal arteries from old rats were 6.78 ± 0.16 ($n = 10$) and 6.79 ± 0.14 ($n = 6$), respectively. The affinity for 5-HT was not influenced by vessel size ($P_{size} = 0.582$).

The relationship between vessel 5-HT_2-receptor affinity, pK_A(M), and sensitivity, pD_2, to 5-HT was linear in distal arteries from both young and old rats (Figure 2) and the slopes of the regression lines were not significantly different from unity. Slopes were 0.83 ± 0.32 ($r = 0.72$, $P = 0.0412$) and 0.87 ± 0.19 ($r = 0.92$, $P = 0.0102$) in young and old rats, respectively. No relationship between affinity and sensitivity in proximal coronary arteries from young rats (Figure 2) could be found as the slope of the regression line was 0.15 ± 0.14 ($r = 0.37$, $P = 0.2963$). The slope of the regression line for proximal arteries from old rats was significant: 0.48 ± 0.18 ($r = 0.68$, $P = 0.0277$), but less than unity ($P < 0.05$).

The affinity for 5-HT was correlated with the relative 5-HT_2-receptor occupancy for half-maximal response in proximal arteries from both young and old rats. Slopes were 4.92 ± 0.96 ($r = 0.87$, $P = 0.0009$) and 2.41 ± 0.84 ($r = 0.71$, $P = 0.0212$), respectively (Figure 3).

5-HT_2-receptor occupancy and response

An estimate of the relative receptor-occupancy (R_a/R_t) at half-maximal response was made with the equation derived by Furchgott & Bursztyn (1967): 18 ± 4% ($n = 10$) and 35 ± 7% ($n = 8$) of the 5-HT_2-receptors had to be occupied to give half maximal response in proximal and distal coronary arteries of young rats, respectively. The corresponding values were 33 ± 5% ($n = 10$) and 42 ± 3% ($n = 6$), respectively, in old rats ($P_{size} = 0.017$; $P_{age} = 0.027$). An alternative estimate of the 5-HT_2-receptor reserve ($pD_2 - pK_A$(M)) also indicated a lower receptor reserve in distal arteries than in proximal arteries and lower receptor reserve in older than younger rats. The differences in pD_2 and pK_A(M) values in proximal and distal coronary arteries from young rats were 0.92 ± 0.23 ($n = 10$) and 0.35 ± 0.16 ($n = 8$) and in old rats 0.36 ± 0.11 ($n = 10$) and 0.15 ± 0.06 ($n = 6$), respectively. The 5-HT receptor reserve was not limited since the difference between pD_2 and pK_A was significantly different from zero in each of the vessel groups.

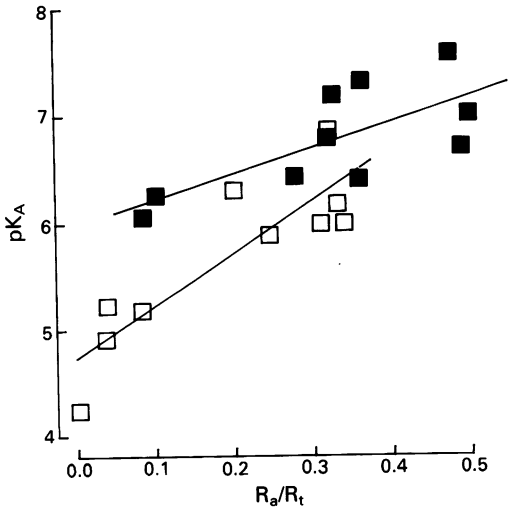

Figure 3 Plots of relative 5-HT_2-receptor occupancy (R_a/R_t) at 5-HT for half-maximal response versus 5-HT_2-receptor affinity, pK_A(M) in proximal coronary arteries from 3 month old (□) and 2 year old (■) rats. The slopes of the regression lines were 4.92 ± 0.96 and 2.41 ± 0.84, young and old rats, respectively.

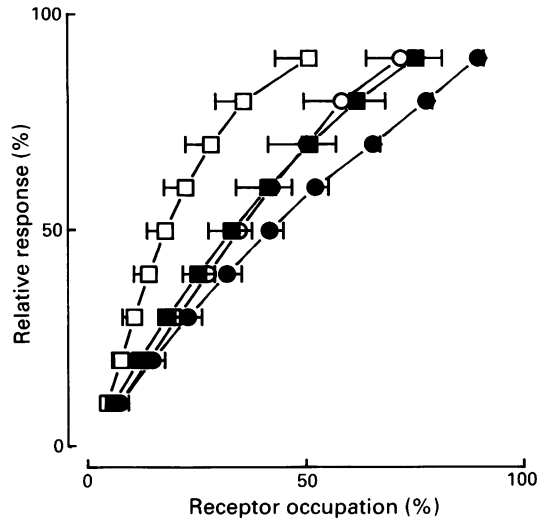

Figure 4 Relationship between relative 5-HT$_2$-receptor occupancy and response to 5-HT in proximal (squares) and distal (circles) coronary arteries from 3 month old (open symbols) and 2 year old (closed symbols) rats.

Figure 4 shows the relationship between relative 5-HT$_2$-receptor occupancy and vessel response in the range 10%–90% of the maximal response. The occupancy-response curves for distal arteries were positioned to the right of age-matched proximal arteries and those for arteries from old rats likewise lay to the right of the corresponding curve from young rats.

Discussion

Receptors may be characterized either by competitive antagonists (Arunlakshana & Schild, 1959), or by their receptor-agonist dissociation constant (Furchgott, 1966; Furchgott & Bursztyn, 1967). The present experiments indicate that 5-HT interacts with a homogeneous population of 5-HT$_2$-like receptors in proximal and distal segments of the left coronary artery in the rat. This is indicated by the slopes of the Schild-plots and the estimated pA_2 values for ketanserin which were indistinguishable from unity and close to the previously reported pA_2 value in bovine coronary arteries, respectively (Kaumann, 1983). The 5-HT$_2$-receptor affinity for ketanserin seems, however, to vary considerably among tissues (Leff & Martin, 1988). The effect of ketanserin was not tested in vessels from old rats so we cannot exclude the possibility that the receptor population may become nonhomogeneous with age.

The possible interference from agonist-released endothelium-derived relaxing factor (EDRF) during construction of the concentration-response curve is a complicating factor in receptor characterization (Furchgott, 1983). Although 5-HT is a potent stimulator of coronary endothelial cells in certain species (Cocks & Angus, 1983; Cohen *et al.*, 1983; Angus, 1989), 5-HT does not induce EDRF release in rat coronary arteries (Nyborg & Mikkelsen, 1990). Consequently 5-HT-receptor affinity can be estimated without removal of endothelium.

The method used for determination of 5-HT$_2$-receptor affinity (Furchgott, 1966; Furchgott & Bursztyn, 1967) requires thermodynamic equilibrium between free and receptor-bound agonist as well as a slope factor, or Hill-coefficient, equal to unity. The slope factor was indistinguishable from unity in all groups of vessels although there was a tendency towards a higher slope factor in proximal arteries (results not shown). Another method for determination of receptor affinities, the so-called 'operational model' (Black *et al.*, 1985) has not gained general acceptance (Mackay, 1988a). Black *et al.* (1985) also estimated 5-HT$_2$-receptor affinity in rat aorta using the 'Furchgott-method' (null method). The average dissociation constant for 5-HT was about 5–10 times greater than that estimated in the present work. These findings indicate that there may be some variation in 5-HT$_2$-receptor affinity among vascular beds. The ratio between the highest and lowest value of the dissociation constant in the rat aorta was not more than 1.7 (Black *et al.*, 1985) which is much lower than in the coronary arteries where this ratio was 90 in proximal and 29 in distal segments from young rats. The variability in 5-HT$_2$-receptor affinity was about 3 times lower in vessels from old rats. Receptors with similar affinities should, according to receptor theory, belong to the same sub-type (Fuder *et al.*, 1981). Thus the coronary 5-HT$_2$-receptors appear to be rather nonhomogeneous despite their affinity for ketanserin varying only by a factor of 3.

The estimated 5-HT$_2$-receptor affinity for 5-HT was considerably increased in vessels from old rats. The present method estimates the apparent affinity and not the 'true' receptor affinity (Mackay, 1989b) because 5-HT-induced contractions involve activation of second messengers, for example phosphoinositols and calcium ions (Nakaki *et al.*, 1985). It is not possible to determine whether the increase in the apparent 5-HT$_2$-receptor affinity is related to changes in the receptor itself or to alterations in functions of intracellular second-messengers.

The vessel sensitivity and 5-HT$_2$-receptor affinity for 5-HT was linearly correlated in distal vessels from both young and old rats. The slope of the regression lines were not distinguishable from unity. This suggests that variation in 5-HT$_2$-receptor affinity can explain the variation in sensitivity to 5-HT of these arteries as it can for the action of noradrenaline on α_1-adrenoceptors in rabbit arteries (Bevan *et al.*, 1986). A similar relationship between affinity and sensitivity to 5-HT could not, however, be demonstrated in proximal coronary arteries indicating that some factor(s) must interact with these parameters.

The relative 5-HT$_2$-receptor occupancy for a given response is not a direct measurement of receptor density but it is likely that a high 5-HT$_2$-receptor reserve is associated with a high receptor density. The linear relation between the relative 5-HT$_2$-receptor occupancy and 5-HT$_2$-receptor affinity for 5-HT indicates that the 5-HT$_2$-receptor affinity may be down-regulated by the receptor density. This may explain why the relationship between affinity and sensitivity in proximal arteries deviates from unity and why affinity increases as the receptor reserve decreases with age. It may be noted that the regression lines for affinity vs. sensitivity of proximal vessels from young and old rats have slopes equal to unity when the vessels with the highest receptor reserve (relative receptor occupancy low) are removed.

Down-regulation of 5-HT$_2$-receptor affinity by its density may actually be desirable from a physiological point of view, because a high receptor density without a lowering of receptor affinity would move the 5-HT concentration-response curve to the left and perhaps allow contraction of the coronary artery at very low 5-HT concentrations. One study (Laher & Bevan, 1985) indicates a similar relation between receptor reserve and α_1-adrenoceptor affinity: A 4 fold greater relative receptor occupancy for half-maximal response was accompanied by a 4 fold increase in receptor affinity for noradrenaline when small intrapulmonary arteries of the rabbit were compared with large intrapulmonary arteries. The relation was not apparent, however, when other vascular beds were compared (Laher & Bevan, 1985).

The 5-HT$_2$-receptor reserve (antilog($pD_2 - pK_A(M)$)) decreased with age and was lower also in distal coronary arteries compared to proximal segments in both young and old rats. The lower receptor reserve in distal arteries was accompanied by smaller maximal contractile responses in these vessels to 5-HT, as one might expect if the excitation-contraction coupling process is similar in both segments. The receptor reserve was not limited in any of the vessel groups. Thus, the low response to 5-HT in distal segments indicates

that the 5-HT_2-receptor excitation-contraction coupling process is relatively weak in rat coronary artery smooth muscle. The increased response to 5-HT in both proximal and distal coronary arteries from old rats, despite a reduced 5-HT_2-receptor reserve, is the opposite of what one would expect. The increased effectiveness of 5-HT in the arteries from the older rats may suggest that the excitation-contraction coupling process is up-regulated but the mechanism of this is obscure.

Regulation of receptor affinities and density by antagonists and agonists is well known for α_1-adrenoceptors in the mesenteric circulation of rats (Colluci *et al.*, 1981). Administration of 5-HT-receptor agonists to rats has also been shown to down-regulate the number of brain 5-HT_2-receptor binding sites within a few hours, without affecting receptor affinity (Buckholtz *et al.*, 1988). 5-HT released in the coronary circulation during preparation could potentially interact with the 5-HT_2-receptors causing changes in their density, but this cannot explain the variability in receptor affinity in the vessels.

The results confirm that the sensitivity to and effect of 5-HT in rat coronary arteries increases with age (Nyborg & Mikkelsen, 1988b). The underlying mechanisms seem to involve increased 5-HT_2-receptor affinity and greater efficiency of the excitation-contraction coupling process in the vascular smooth muscle cells as the 5-HT_2-receptor reserve decreases with age. The data suggest the presence of an intrinsic process by which down-regulation of 5-HT_2-receptor affinity is controlled by the 5-HT_2-receptor reserve or density. The probability of suffering a fatal 5-HT-induced contraction of the coronary circulation is likely to increase with age, at least in rats.

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