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SPECIAL REPORT

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Loss of preconditioning in rabbits with vascular tolerance to nitroglycerin

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A preceding right ventricular overdrive pacing (VOP) of 500 b.p.m. for 5 min, markedly reduced the severity of global myocardial ischaemia produced by a subsequent 5-min VOP in conscious rabbits. This VOP-induced preconditioning developed in parallel with an increase in cardiac cyclic guanosine 3':5'-monophosphate (cyclic GMP) content. VOP-induced preconditioning was abolished when the animals had been made tolerant to the vasodilator effect of nitroglycerin (NG). In the heart of the NG-tolerant rabbits, neither VOP nor preconditioning increased cyclic GMP content. This suggests that changes by NG tolerance of cyclic GMP metabolism may account for the loss of VOP-induced preconditioning.

Keywords: Ischaemic preconditioning; nitroglycerin tolerance; cyclic GMP; ST-segment elevation; LVEDP; rabbit heart

Introduction The intracellular messenger, cyclic guanosine 3':5'-monophosphate (cyclic GMP) has been suggested to play a crucial role in the mechanism of the anti-ischaemic effect of preconditioning induced by ventricular overdrive pacing (VOP) in conscious rabbits (Szilvassy *et al.*, 1994). Results obtained with anaesthetized dogs (Vegh *et al.*, 1992) also suggest that nitric oxide (NO), a stimulator of soluble guanylate cyclase, contributes to the antiarrhythmic effect of preconditioning produced by brief coronary artery occlusions. Moreover, inhibition of the L-arginine-NO pathway has been found to abolish the antiarrhythmic effect of bradykinin, a putative mediator of preconditioning (Vegh *et al.*, 1993). Several studies have shown that the tolerance to organic nitroesters is accompanied by reduced production and increased breakdown of cyclic GMP in vascular and non-vascular tissues (Axelsson & Ahlner, 1987). Therefore, we examined whether nitroglycerin (NG) tolerance influenced VOP-induced preconditioning in conscious rabbits.

Methods Male New Zealand white rabbits (2500–3000 g) were anaesthetized with 15 mg kg^{-1} diazepam (Sigma, St Louis, MO, U.S.A.) and 10 mg kg^{-1} ketamine (EGIS, Budapest, Hungary) given intravenously. A bipolar 'French 4' electrode catheter was introduced into the apex of the right ventricle for pacing and recording the intracavital electrogram (Szilvassy *et al.*, 1993a,b). Polyethylene catheters were inserted into the left ventricular cavity and the central ear artery to measure intraventricular pressure and mean arterial blood pressure (MABP), respectively. One week after surgery, the rabbits were given 150 µl kg^{-1} ethanol, the solvent for NG, subcutaneously four times a day for three days. Next morning global myocardial ischaemia was provoked by VOP of 500 b.p.m. for 5 min. Average intracavital ST-segment elevation during the first five cardiac cycles at sinus rhythm after VOP produced the data for evaluation. Left ventricular end-diastolic pressure (LVEDP) was measured before and immediately after VOP. This VOP was then given for 5 min followed by a second 5-min VOP, and the parameters were determined again. The first VOP served to estimate electrophysiological and haemodynamic changes due to global myocardial ischaemia and to induce preconditioning. The second VOP was to test the preconditioning effect on the parameters measured. The rabbits were then given 50 mg kg^{-1} NG (EGIS, Budapest, Hungary) subcutaneously four times a day for three days to induce NG tolerance and the pacing protocol was repeated. The effect of an intravenous bolus of 30 µg kg^{-1} NG on MABP was tested before and after the three-day NG administration. In the second set of experiments, two groups of electrode catheter-instrumented rabbits (15 of each) underwent the three-day treatment with NG or solvent, respectively. Anaesthetized, open-chest, artificially ventilated rabbits were subjected to VOP of 500 b.p.m. for 5 min as described (Szilvassy *et al.*, 1993a). Normal and NG-tolerant rabbits were divided into three subgroups. One was subjected to a single VOP, then immediately after VOP, samples were taken from the apex of the left ventricle for cyclic GMP determination. Another five rabbits underwent two periods of VOP with an interpacing period of 5 min, and samples were taken immediately after the second VOP. A group was killed for measuring baseline cyclic GMP values. Cardiac cyclic GMP content was measured by means of radioimmunoassay using Amersham kits as described (Szilvassy *et al.*, 1993a). Data expressed as means ± s.d. were statistically analyzed by ANOVA followed by a modified *t* test for multiple comparisons according to Bonferroni.

Results In non tolerant rabbits, 30 µg kg^{-1} intravenous NG decreased MABP from 88 ± 5.8 mmHg to 72 ± 5.1 mmHg ($P < 0.01$), whereas it did not change MABP in tolerant animals (91 ± 4.4 mmHg vs 93 ± 5.2 mmHg). A single VOP induced an intracavital ST-segment elevation of 1.1 ± 0.16 mV in non tolerant rabbits (Figure 1a); nevertheless, when this was preceded by a preconditioning VOP, the ST-segment elevation decreased to 0.6 ± 0.07 mV ($P < 0.001$). In tolerant rabbits, ST-segment elevation induced by a single VOP was more marked than in controls (1.5 ± 0.22 mV vs 1.1 ± 0.16 mV, $P < 0.05$), and it remained unchanged after a preconditioning VOP. The increase in LVEDP produced by a single VOP was markedly decreased by a preconditioning VOP in non tolerant but not in NG tolerant rabbits (Figure 1b). In non tolerant animals, a single VOP significantly increased cardiac cyclic GMP content as compared to resting values (Figure 1c) and preconditioning slightly amplified the VOP-induced cyclic GMP increase. In tolerant animals, however, neither a single VOP nor consecutive periods of VOP changed cardiac cyclic GMP content. Resting LVEDP and cardiac cyclic GMP of NG tolerant rabbits did not significantly differ from that of non tolerant ones (data not shown).

Discussion These results confirm previous findings that the global myocardial ischaemia induced by VOP is markedly

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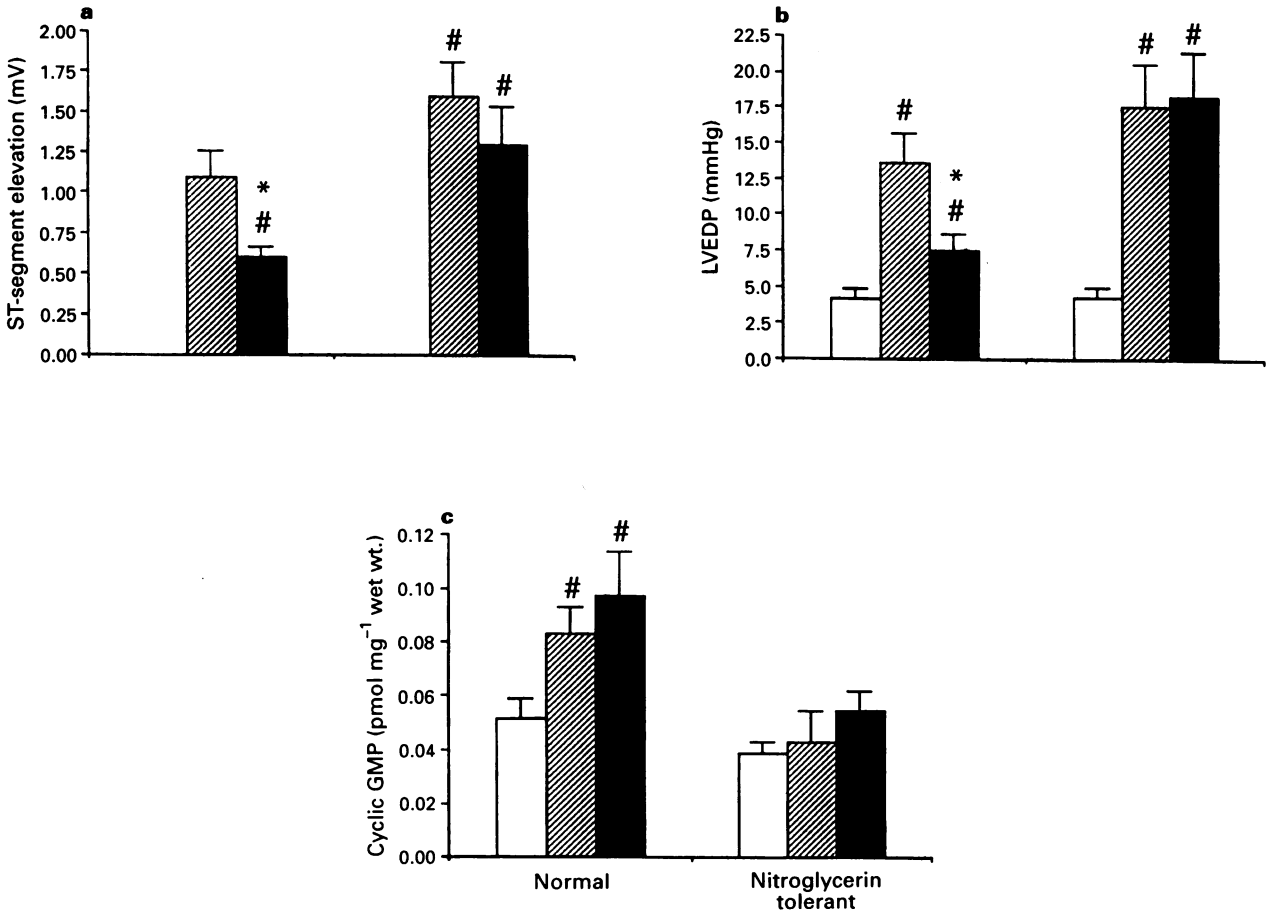


Figure 1 Effect of ventricular overdrive pacing (VOP) on (a) intracavital ST-segment elevation and (b) left ventricular end-diastolic pressure (LVEDP) in conscious rabbits ($n = 6$) and (c) on cardiac cyclic GMP content in anaesthetized rabbits ($n = 5$). Open columns show resting, hatched columns non preconditioned values measured after a single VOP (500 b.p.m. for 5 min), solid columns designate values measured after a 5 min VOP preceded by a preconditioning VOP of the same rate and duration with an interpacing period of 5 min. 'Normal' values are obtained from solvent-treated animals. 'Nitroglycerin tolerant' values derived from rabbits treated with 50 mg kg^{-1} nitroglycerin (NG) four times a day for three days. Data are means ± s.d.; #significant difference between resting and postpacing; *between non preconditioned and preconditioned values, $P < 0.05$.

attenuated by a preceding ischaemic challenge provoked by VOP (Szilvassy *et al.*, 1994). The results also indicate that this protection is lost when tolerance to the vasodilator effect of NG develops. Ischaemic preconditioning is believed to be due to the increased formation and release of endogenous cardioprotective substances. These substances may derive from the vascular endothelium or cardiac myocytes and stimulate soluble guanylate cyclase either directly like NO (Moncada *et al.*, 1991), or indirectly like bradykinin via releasing NO (Vegh *et al.*, 1992; 1993). An increased formation of cyclic GMP may therefore be considered an important step in the signal transduction pathways involved in preconditioning. This concept is in accord with our previous finding that an increase in cardiac cyclic GMP content correlates with the time course of the anti-ischaemic effect of VOP-induced preconditioning (Szilvassy *et al.*, 1994). Pharmacological inhibition of cyclic GMP breakdown has also been found to have an anti-ischaemic effect in conscious rabbits (Szilvassy *et al.*, 1993b). The present data, showing that VOP-induced preconditioning is lost in NG tolerance in association with the inability of VOP to increase myocardial cyclic GMP content, suggest that cyclic GMP signalling contributes to the protection of the ischaemic myocardium.

The mechanism of NG tolerance is unclear, although different hypotheses have been advanced, suggesting (i) the importance of pharmacokinetic factors, (ii) the oxidation of critical sulphydryl groups in the receptor inducing a low affinity state (Axelsson & Ahlner, 1987), and (iii) alterations in the formation and breakdown of cyclic GMP. Cyclic GMP is an important mediator of vasorelaxation induced by NG. Keith *et al.* (1982) found an inhibition of NG-induced cyclic GMP generation in aortic strips of NG-tolerant rats; however, the relaxation induced by 8-bromo-cyclic GMP, a stable, membrane-penetrating cyclic GMP analogue, was unaltered, indicating that, once it is formed, the intracellular action of cyclic GMP is unimpaired. In NG tolerance, Axelsson & Andersson (1983) have shown that guanylate cyclase activity is decreased, while cyclic GMP hydrolysis is enhanced. These findings might explain the impairment of preconditioning in NG-tolerant rabbits. Besides theoretical considerations, our results emphasize the potential risk of nitrate tolerance in clinical patients maintained on prolonged nitrate therapy.

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Differential modulation of κ and μ opioid antinociception by the glycine/NMDA receptor agonist D-serine

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D-Serine, a selective agonist for the strychnine-insensitive glycine allosteric site associated with the NMDA receptor-ion channel complex, was found to modulate differentially the antinociception produced by κ and μ-opioid receptor agonists in the rat formalin test. D-Serine (100 μg, i.c.v.) attenuated the antinociception produced by the selective κ-opioid agonist, enadoline (0.003–0.1 mg kg^{-1}, s.c.) against the tonic, but not acute, phase of the formalin response. Conversely, D-serine potentiated the antinociception produced by morphine (0.3–10 mg kg^{-1}, s.c.) against both the acute and tonic phases. These results demonstrate an important interaction between the opioid and NMDA/glycine systems in the control of nociceptive information possibly at different levels of the neuraxis.

Keywords: Enadoline; morphine; rat formalin test; inflammatory pain

Introduction The excitatory amino acids (EAA) glutamate and aspartate play a major role in the mediation of formalin-induced nociception through an interaction with both non-NMDA (acute phase) and NMDA/glycine (tonic phase) types of receptors (Haley *et al.*, 1990). κ and μ opioid agonists exhibit potent antinociceptive properties against such prolonged tonic/chronic forms of noxious stimulation, particularly when there is an associated inflammatory component (Wheeler-Aceto & Cowan, 1991). κ- and μ-agonists have been shown to inhibit glutaminergic transmission through a predominantly pre- and postsynaptic mechanism of action, respectively, at both a spinal and supraspinal level (Allerton *et al.*, 1988; Lambert *et al.*, 1991; Pinnock, 1992). It is possible that such a modulatory influence may contribute towards the antinociceptive potency of the opioids against formalin-induced nociception. We have previously demonstrated that activation of the glycine-NMDA receptor indirectly modulates the anticonvulsant action of the κ-receptor selective agonist CI-977 (enadoline; Singh *et al.*, 1990). In the present study we have therefore investigated the potential interaction between the opioid system mediating antinociception and the glycine/NMDA receptor complex in the formalin model of acute and prolonged noxious stimulation.

Methods Male Wistar rats (60–90 g) were administered with 50 μl of formalin (5% formaldehyde) into the plantar surface (i.pl.) of the left hindpaw and the time (s) each animal spent licking and biting the injured paw recorded in 5 min bins for a total of 60 min. Cumulative data (mean ± s.e.mean) were then recorded for the early, acute phase (0–10 min) and the late, tonic phase (10–45 min). Enadoline/saline or morphine/saline were administered, respectively, as a 15 and 30 min subcutaneous pretreatment prior to formalin in groups of 6–10 animals. The intracerebroventricular (i.c.v.) injections were performed by exposing the skull of the rats under isoflurane anaesthesia. D-serine (100 μg)/saline was administered, 30 min prior to formalin, by direct injection (5 μl) into the 4th ventricle at lambda with a 5 mm long, 27 guage needle attached to a 10 μl Hamilton syringe and the wound sealed with epoxy resin. Similar injections of Evans Blue dye demonstrated a uniform distribution of injectate throughout the ventricular system within 10–15 min post-administration.

Statistical analyses of the data were carried out using a Mann-whitney U-test. At each dose-level of the opioid agonist, the group that had received D-serine as a pretreatment was compared with the group given the opioid drug plus saline (i.c.v.). A probability level of $P<0.05$ was regarded as significant. ED_{50} (dose producing a 50% reduction in the licking/biting response of control animals) values from graphs of the cumulative data were determined by GRAPHPAD and presented as the mean with 95% confidence limits.

Drugs used were enadoline (CI-977, ((5**R**)-(5α,7α,8β)-N-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4,5]dec-8-yl]-4-benzofuranacetamide monohydrochloride (Parke-Davis Neuroscience Research Centre, Cambridge), morphine sulphate (Savory & Moore, Cambridge), D-serine (Sigma) and isoflurane (Abbot Laboratories, Kent).

Results In control animals receiving saline pretreatments (i.c.v. and s.c.), the formalin-induced acute and tonic phases of licking and biting lasted for 93 ± 10 and 337 ± 22 s ($n = 12$), respectively.

Enadoline (0.003–0.1 mg kg^{-1}, s.c.) caused a dose-dependent inhibition of both the acute and tonic phases of the formalin response with ED_{50} values of 0.009 (0.008–0.010) mg kg^{-1} and 0.007 (0.004–0.013) mg kg^{-1}, respectively (Figure 1a and b). Similarly, morphine caused a dose-dependent inhibition of both acute and tonic phases with ED_{50} values of 2.9 (1.3–6.1) mg kg^{-1} and 3.0 (2.2–4.0) mg kg^{-1}, respectively (Figure 1a and b).

D-Serine (100 μg, i.c.v.), administered 15 min before enadoline (0.01–0.1 mg kg^{-1}, s.c.), caused a significant inhibition of the antinociceptive response to enadoline, at 0.01 and 0.03 mg kg^{-1}, against the tonic phase of the formalin nociceptive response (Figure 1b) with a rightward shift in the control dose-response curve and an increase in the ED_{50} to 0.044 (0.033–0.060) mg kg^{-1}. D-Serine pretreatment had no significant effect on the enadoline-mediated inhibition of the acute phase at any dose (Figure 1a) as shown by the ED_{50} of 0.010 (0.009–0.011) mg kg^{-1}. Conversely, when administered immediately before morphine (0.3–10 mg kg^{-1}, s.c.), D-serine (100 μg, i.c.v.) caused a significant potentiation of the morphine antinociceptive effect, at 1 and 3 mg kg^{-1} against the

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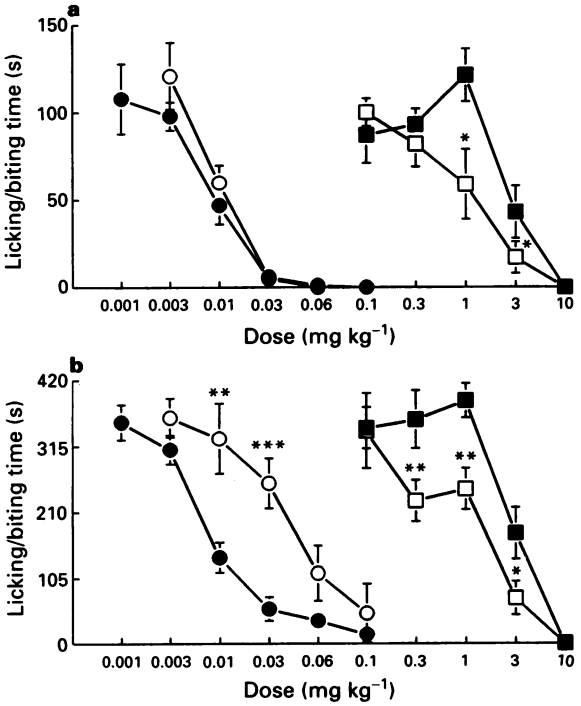


Figure 1 Dose-dependent (mg kg^{-1}, s.c.) effect of enadoline (●, ○) and morphine (■, □) on the (a) acute and (b) tonic phases of the formalin nociceptive response in the absence (solid symbols) and presence (open symbols) of D-serine (100 μg, i.c.v.). Significant differences between D-serine and the respective saline pretreated groups are indicated by asterisks ($^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, Mann-Whitney U-test).

acute phase (Figure 1a) and at 0.3, 1 and 3 mg kg^{-1} against the tonic phase (Figure 1b) of the formalin nociceptive response. A leftward shift was observed in the morphine dose-response curve with a decrease in the ED_{50} to 1.2 (0.5–2.6) and 1.3 (0.4–4.1) mg kg^{-1} for the acute and tonic phases, respectively.

D-Serine (100 μg, i.c.v.) had no significant effect on either the acute or tonic phase of the formalin-induced licking/biting response with values of 62 ± 18 s and 336 ± 53 s ($n = 6$), respectively. In saline (i.c.v.) pretreated control animals the respective values were 83 ± 8 s and 318 ± 22 s ($n = 8$).

Discussion The present study has demonstrated that opioid receptor mediated antinociception in the formalin model can be differentially modulated by the glycine-NMDA agonist D-serine. The most likely site for this interaction is in the spinal cord where κ and μ receptors are widely distributed throughout the superficial layers (laminae I and II) and lamina V of the dorsal horn, regions associated with nociceptive input and transmission (Stevens *et al.*, 1991).

The D-serine-mediated inhibition of tonic but not acute antinociception produced by the κ-selective agonist, enadoline (Hunter *et al.*, 1990), is consistent with the ability of enadoline to cause presynaptic inhibition of EAA release (Lambert *et al.*, 1991; Pinnock, 1992). Thus, since the glycine-NMDA receptor is located on the dorsal horn cells, D-serine would not be able to influence a presynaptic action of enadoline on the terminals of the primary afferents to reduce EAA release and, consequently, the EAA-evoked monosynaptic response (acute phase). In contrast, D-serine was able to reverse the inhibitory effect of enadoline on the subsequent polysynaptic response to formalin which has been associated with the sensitization and plasticity of dorsal horn neurones, particularly transmission neurones located in the deeper laminae (Haley *et al.*, 1990). This could be explained if the κ receptors involved in the modulation of the tonic phase were predominantly located on the terminals of spinal interneurons, *e.g.* immediately presynaptic to the transmission cells, especially those located in the deeper laminae of the dorsal horn. Of course, such an explanation cannot exclude the possibility that the antinociceptive effect of enadoline on the tonic phase may be mediated in part through activation of supraspinal κ receptors.

In contrast to the glycine-NMDA influence on the κ-opioid system, D-serine potentiated morphine-induced attenuation of *both* acute and tonic phases which would suggest a predominantly postsynaptic site of interaction, possibly on the spinal dorsal horn cells. The paradoxical effect of the glycine-NMDA agonist might therefore reflect modulation of distinct interneuronal pathways by the μ and κ opioid systems in the spinal cord. Alternatively, however, it may reflect possible differences in the anatomical localization of the interaction with, for example, a predominant involvement of supraspinal sites in the D-serine modulation of the morphine response. Thus, morphine antinociception has been shown previously to be potentiated by NMDA agonists injected into the periaqueductal grey (Jacquet, 1988) and nucleus raphe magnus (Van Praag & Frenk, 1990).

In conclusion, the present study has shown that an interaction appears to exist between two major systems in the control of nociceptive information, particularly following a prolonged noxious stimulus, that may have important consequences for the treatment of chronic pain.

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Anti-inflammatory and analgesic activity of the bradykinin antagonist, icatibant (Hoe 140), against an extract from *Porphyromonas gingivalis*

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1 *Porphyromonas gingivalis* is one of the bacteria likely to be related to pain in periodontitis. Several enzymes isolated from *P. gingivalis* have been reported to have kininogenase activity. Since kinin release could be held responsible for inflammatory symptoms and pain in periodontitis, we investigated whether the inflammatory and algesic effects of a sonic extract from *P. gingivalis* (PGSE) could be inhibited by the potent bradykinin B_2 receptor antagonist, icatibant (Hoe 140).

2 In anaesthetized rats, the subplantar injection of PGSE (0.1 and 1.0 mg) caused a dose-dependent oedema of the hind paws. The net increase of the paw volume 60 min after the injection was 23 ± 5% and 77 ± 12%, respectively. The oedema was rich in plasma proteins as determined by the Evans blue method. Pretreatment with icatibant (300 nmol kg^{-1}, s.c.) significantly reduced the effect of 1.0 mg of PGSE whereas the effects of 0.1 mg of PGSE remained unaffected.

3 The subplantar injection of 1.0 mg of PGSE in unanaesthetized rats caused nociceptive behavioural responses which started about 5 min after the injection and lasted for about 10–15 min. These responses were completely prevented by pretreatment with icatibant (300 nmol kg^{-1}, s.c.).

4 The present results show that the plasma extravasation induced by non-algesic doses of a sonic extract from *P. gingivalis* are caused by mechanisms other than B_2 kinin receptor activation whereas inflammatory effects of algesic doses are due to the action of kinins. The pain elicited by the extract is solely mediated by kinins and can be prevented by icatibant. The bradykinin antagonist could thus have a potential for a clinical use against pain associated with periodontal inflammation.

Keywords: Bradykinin antagonists; icatibant (Hoe 140); inflammation; pain; *Porphyromonas gingivalis*

Introduction

Porphyromonas gingivalis is a bacterium found in the bacterial flora of infected dental root canals and is significantly related to percussion pain in periodontitis (Hashioka *et al.*, 1992). Several proteinases isolated from *P. gingivalis* have been shown to possess potent kininogenase activity (Hinode *et al.*, 1992; Scott *et al.*, 1993; Sojar *et al.*, 1993). One of these enzymes, lys-gingivain, is apparently the most potent kininogenase reported to date (Scott *et al.*, 1993). In order to elucidate the contribution of kinin release to the inflammatory and algesic effects of the bacterium, we tested the potent and specific bradykinin B_2 receptor antagonist, icatibant (D-Arg-[Hyp^3-Thi^5-D-Tic^7-Oic^8]-bradykinin; formerly named compound I [Lembeck *et al.*, 1991] or Hoe 140 [Hock *et al.*, 1991; Wirth *et al.*, 1991]), against the effects of a sonic extract from *P. gingivalis* (PGSE) following its subplantar injection in rats.

Methods

Experimental procedure

Female Sprague-Dawley rats (200–250 g, Forschungsanstalt für Versuchstierzucht, Himberg, Austria) were anaesthetized with pentobarbitone sodium (40 mg kg^{-1}, i.p.). Icatibant (300 nmol kg^{-1}) was injected s.c.; control animals received a corresponding volume (3 ml kg^{-1}) of a 154 mM solution of NaCl (saline). Twenty min later, a subplantar injection of a sonic extract from *Porphyromonas gingivalis* (PGSE; Scott *et al.*, 1993; 0.1 or 1.0 mg in 50 µl) was given into one hindpaw; the contralateral paw received an equal volume of saline. The paw volumes were measured before, and 5, 10, 20, 25, 30, 45 and 60 min after the injection with a plethysmometer (Ugo Basile, Italy).

For the determination of the extravasation of plasma proteins, Evans blue (20 mg kg^{-1}), an azo dye binding to plasma proteins, was administered i.v. 5 min prior to the subplantar injection of the PGSE. The rats were killed 1 h later by perfusion with 40 ml heparinized saline via the aorta. The hindpaws were cut off, placed in 8 ml formamide and incubated for 36 h at 55°C for the extraction and photometric determination of Evans blue (Gamse *et al.*, 1980).

Nociceptive reactions of unanaesthetized rats were monitored over a period of 30 min following the subplantar injection of PGSE (0.1 and 1.0 mg) under brief nitrous oxide analgesia. The behavioural responses were rated with scores (0 = no reaction; 1 = favouring the uninjected paw; 2 = elevating the injected paw; 3 = licking the injected paw; modifed from Cohen *et al.*, 1984). For each 1 min period a mean score was calculated from the times spent within each score during that period. Pretreatment with icatibant or saline was performed as above.

Substances

The sonic extract from *P. gingivalis* was generously supplied by Cheryl F. Scott (Sol Cherry Thrombosis Research Center, Temple University, Philadelphia, PA, U.S.A.) and Dr Benjamin F. Hammond (Medical College of Pennsylvania, Philadelphia, PA, U.S.A.). Icatibant (D-Arg-[Hyp^3-Thi^5-D-Tic^7-Oic^8]-bradykinin) was a gift from Hoechst AG (Frankfurt am Main, Germany). Pentobarbitone sodium (Nembutal) was from Sanofi (Libourne, France). Evans blue was purchased from Sigma (St. Louis, MO, U.S.A.). Formamide was from E. Merck (Darmstadt, Germany).

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Statistical analysis

All values presented are means ± s.e.mean. Comparisons of the results obtained in icatibant-treated rats with those from control animals were made by the Mann-Whitney U test.

Results

Rat paw oedema

The subplantar injection of PGSE (0.1 and 1.0 mg) into the hindpaws of anaesthetized rats elicited a dose-dependent paw oedema which reached a plateau after 30 min and thereafter remained constant (Figure 1a). The oedema induced by the lower dose of PGSE (23 ± 5% net volume increase at 60 min) was not affected by pretreatment with icatibant (300 nmol kg^{-1}, s.c.), whereas the oedema caused by the higher dose (77 ± 12%) was significantly ($P<0.05$) attenuated (to 24 ± 4%) in rats which had been injected with the bradykinin antagonist.

The extravasation of plasma proteins into the tissue was quantified with the Evans blue method. Similar to the results obtained when the volume of the paw oedema was measured, icatibant did not modify the extravasation of Evans blue caused by 0.1 mg PGSE, whereas the amount of Evans blue determined in the paws following injection of 1.0 mg PGSE was significantly ($P<0.05$) smaller in icatibant-treated rats than in controls.

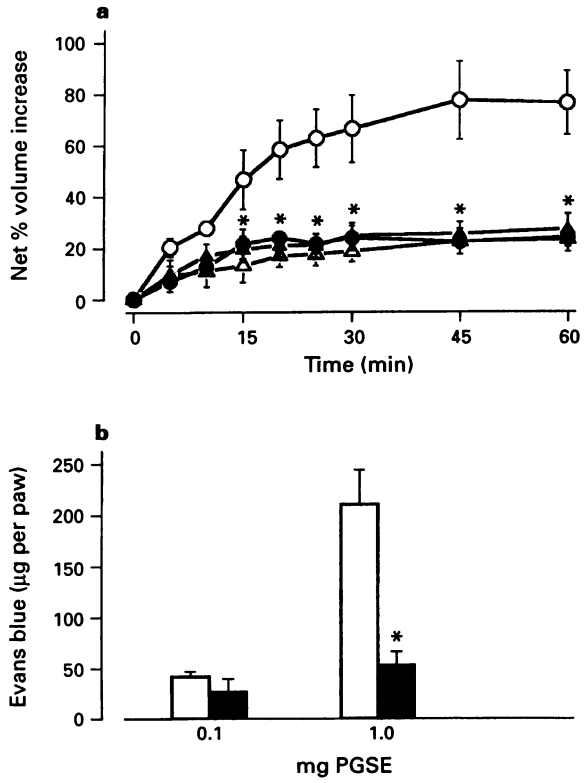


Figure 1 Paw oedema following subplantar injection of a sonic extract from *P. gingivalis* in anaesthetized rats: the increase in paw volume (a) following injection of 0.1 mg (triangles) or 1.0 mg (circles) of the extract was measured repeatedly during a period of 1 h. Extravasation of plasma proteins, quantified by the Evans blue method (b) was determined 60 min after the subplantar injection of the extract. The rats were pretreated s.c. with icatibant (300 nmol kg^{-1}; closed symbols and columns) or with saline (3 ml kg^{-1}; open symbols and columns) 20 min prior to the experiment. Significance of difference of effects of 1.0 mg PGSE in icatibant-treated rats vs control rats: $*P<0.05$. Means ± s.e.mean; $n = 4-6$.

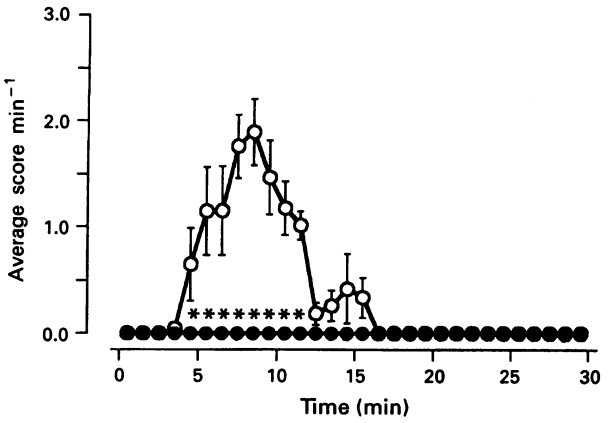


Figure 2 Nociceptive responses to subplantar injections of a sonic extract (1.0 mg) from *P. gingivalis* in unanaesthetized rats: the rats were pretreated s.c. either with icatibant (300 nmol kg^{-1}; ●) or with saline (3 ml kg^{-1}; ○) 20 min prior to the experiment. Behavioural responses were monitored for a period of 30 min after the subplantar injection and rated with scores as described in the Methods section. For each 1 min period an average score value was calculated. Significance of difference to control rats: $*P<0.05$. Means ± s.e.mean; $n = 4-5$.

Nociceptive behavioural responses

Following the subplantar injection of 0.1 mg PGSE into the paws of unanaesthetized rats, no behavioural responses, indicative of nociception, could be observed. However, 1.0 mg PGSE induced such responses (favouring the uninjected paw, elevating or licking the injected paw) starting about 5 min after the injection. For the subsequent 10–15 min, nociceptive behaviour, frequently interrupted by short periods of normal behaviour, was seen. Thereafter, the rats did not show any signs of pain. This pattern of behaviour was completely prevented by pretreatment with icatibant (Figure 2).

Discussion

Porphyromonas gingivalis is an anaerobe bacterium frequently found in severe periodontal inflammatory disease. It is significantly related to subacute clinical symptoms such as percussion pain in apical periodontitis (Hashioka *et al.*, 1992). Several proteinases isolated from *P. gingivalis* were shown *in vitro* to have kininogenase activity (Hinode *et al.*, 1992; Scott *et al.*, 1993; Sojar *et al.*, 1993). One of these enzymes, lys-gingivain, is apparently the most potent kininogenase reported to date (Scott *et al.*, 1993). Unlike gingipain, another proteinase isolated from *P. gingivalis* (Chen *et al.*, 1992), which can be purified from the culture medium and is also capable of initiating kinin production (Travis *et al.*, 1993), lys-gingivain is a membrane-bound enzyme. Lys-gingivain represents about 1% of the total protein of a sonic extract from *P. gingivalis* (PGSE) and seems to be a major component of its proteinase activity (Scott *et al.*, 1993). In order to elucidate the contribution of the kinin release to the inflammatory and algesic effects of *P. gingivalis*, the bradykinin B_2 receptor antagonist, icatibant was tested against effects of PGSE *in vivo*.

The subplantar injection of PGSE caused a dose-dependent increase in vascular permeability as quantified by the increase in paw volume and accumulation of plasma proteins in the tissue. The effects of the lower dose of PGSE (0.1 mg) remained unaffected by icatibant, suggesting that this part of the inflammatory response was due to mechanisms other than kininogenase activity. On the other hand, icatibant

significantly reduced the effects of the higher dose of PGSE (1.0 mg) to approximately the level of responses induced by the lower dose. Since icatibant is a specific bradykinin B_2 receptor antagonist without any inhibitory effects on the actions of other inflammatory mediators (Lembeck *et al.*, 1991; Hock *et al.*, 1991; Wirth *et al.*, 1991) it can be concluded that this part of the inflammatory response is due to activation of B_2 receptors in the tissue. Although we did not use a B_1 receptor antagonist, an involvement of B_1 receptors is not likely since B_1 receptor antagonists have been shown to be ineffective against the oedema-producing actions of bradykinin (Whalley, 1987) or kinin-releasing enzymes (Cirino *et al.*, 1991; Legat *et al.*, 1994) in the rat paw.

The remaining part of the oedema may be due to other components of the extract. Proteinases purified from *P. gingivalis* also have other actions which may subserve inflammatory responses such as disruption of basement membranes of epithelial cells (Shah *et al.*, 1992), cleavage of collagen (Sojar *et al.*, 1993) and fibrinogen (Scott *et al.*, 1993) or activation of complement components (Wingrove *et al.*, 1992).

In contrast to its inflammatory effects, the lower dose of PGSE did not have algesic properties. However, nociceptive behavioural responses could be observed readily when 1.0 mg of PGSE was given as a subplantar injection in unanaesthetized rats. This algesic effect was completely abolished by icatibant which shows that the pain induced by PGSE was entirely due to the action of kinins in the tissue. The nociceptive responses to PGSE (1.0 mg) may appear to be somewhat more short-lived than the effects on plasma extravasation (compare Figures 1 and 2). However, the increase in paw oedema observed at a time when the nociceptive responses have disappeared, i.e. later than 20 min after the subplantar injection of PGSE, was small. This may indicate that tissue kinin concentrations required to induce nociception were higher than those leading to increased permeability.

In summary, the two doses of PGSE (0.1 and 1.0 mg) used in the present investigation represent a non-algesic and an algesic dose of PGSE, respectively. While the inflammatory effects of the non-algesic dose of PGSE are not due to kinin action in the tissue, the increase in vascular permeability caused by the algesic dose of PGSE are caused predominantly by the action of kinins acting on bradykinin B_2 receptors. The pain induced by PGSE is solely mediated by kinins and can be completely prevented by the bradykinin B_2 antagonist, icatibant. Thus, icatibant could be potentially useful for the treatment of pain in severe periodontal disease.

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Adenosine-sensitive α_1-adrenoceptor effects on reperfused ischaemic hearts: comparison with phorbol ester

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1 We have examined the effects of the α_1-adrenoceptor agonists, phenylephrine or methoxamine, on contractility in rat and rabbit isolated hearts as well as their effects on postischaemic ventricular recovery. We compared these effects to those of 12-phorbol 13-myristate acetate (PMA), a direct activator of protein kinase C (PKC).

2 The positive inotropic effect of α_1-receptor agonists was significantly attenuated in the presence of the Na/H exchange inhibitor, methylisobutyl amiloride (MIA, 1 μM), whereas the positive inotropic effect of PMA was unaffected.

3 Reperfusion of rat hearts subjected to either 30 or 60 min of zero-flow ischaemia, resulted in recovery of contractility to $91 \pm 2\%$ and $57 \pm 7\%$ of the preischaemic values, respectively which was unaffected by phenylephrine. In contrast, PMA at a concentration (10 pM) devoid of direct depressant effects, significantly decreased recovery following 60 min of ischaemia to $31 \pm 4\%$ of pre-ischaemic value ($P<0.05$ from control); an effect which was completely prevented by the PKC inhibitor, bisindolylmaleimide. A similar inhibitory effect of PMA and lack of effect of phenylephrine were seen in reperfused rabbit hearts.

4 As α_1-receptor activation has been shown previously to stimulate cardiac adenosine production, we assessed whether blockade of adenosine A_1 receptors with the specific antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, 0.5 μM) would unmask the actions of phenylephrine in hearts subjected to 30 min ischaemia and reperfusion. In the presence of DPCPX, phenylephrine reduced recovery to $44 \pm 9\%$ compared to $82 \pm 10\%$ recovery in the absence of phenylephrine ($P<0.05$). Identical results were observed in rabbit hearts treated with DPCPX in which recovery was reduced from $57.1 \pm 11.2\%$ to $17.8 \pm 6.8\%$ by phenylephrine ($P<0.05$). Another A_1 receptor antagonist, ($\pm$)-N^6-endonorbornan-2-yl-9-methyladenine (N-0861, 0.5 μM) produced virtually identical results to those observed with DPCPX.

5 MIA failed to modulate the inhibition of postischaemic recovery by phenylephrine. Bisindolylmaleimide, on the other hand, partially prevented the effects of phenylephrine on postischaemic contractile dysfunction. The inhibitory effect of either PMA or phenylephrine on postischaemic recovery of both rat and rabbit hearts was generally dissociated from alterations in energy metabolism, although in the case of rat hearts, inhibition by phenylephrine was associated with diminished high energy phosphate content.

6 Our results demonstrate that both α_1-receptor activation as well as direct activation of PKC with phorbol ester can attenuate post-ischaemic ventricular recovery. Moreover, our results strongly suggest that endogenous adenosine protects the heart against the deleterious effects of α_1-receptor activation during ischaemia and reperfusion.

Keywords: Ischaemia; reperfusion; α_1-adrenoceptor activation; phorbol myristate acetate; sodium-hydrogen exchange; protein kinase C; contractility; energy metabolism

Introduction

The existence of α_1-adrenoceptors in the myocardium is well established as is the ability of α_1-receptor activation to produce a positive inotropic effect. While the mechanism(s) for the positive inotropic effect of α_1 stimulation is not known with certainty, stimulation of these receptors has been shown to be linked to enhanced phosphoinositide hydrolysis and the production of various intracellular second messengers, such as inositol phosphates and diacylglycerol. These messengers may be important in mediating the positive inotropic effects of α_1-receptor agonists (for review see Terzic *et al.*, 1993). The enhanced production of diacylglycerol is of particular importance as this effect is sustained and the elevation in diacylglycerol content results in subsequent stimulation of protein kinase C (PKC) and alteration of intracellular processes which are dependent on PKC-mediated phosphorylation (for review see Nishizuka, 1992).

It has recently been proposed that one intracellular process which could mediate the positive inotropic effects of α_1-adrenoceptor agonists is the Na/H exchanger (Otani *et al.*, 1990; Iwakura *et al.*, 1990; Wallert & Frohlich, 1992; Terzic *et al.*, 1992; Gambassi *et al.*, 1992; Puceat *et al.*, 1993). Although one possible mechanism of Na/H exchange activation could be via PKC, in the heart it appears that the enzyme plays little part either in mediation of the positive inotropic effect or the activation of Na/H exchange by α_1-receptor agonists (Puceat *et al.*, 1993). Irrespective of the precise mechanism for Na/H exchange activation, it has been suggested that stimulation of the antiport and the resultant intracellular alkalinization sensitizes myofibrils to calcium which would be expected to produce an increase in contractile force (Terzic *et al.*, 1992; Gambassi *et al.*, 1992; Puceat *et al.*, 1993). Moreover, the activation of Na/H exchange may directly elevate intracellular calcium concentration via Na/Ca exchange (Iwakura *et al.*, 1990). Aside from its potential role in mediation of inotropy, Na/H exchange has been implicated as an important mediator of myocardial reperfusion injury based on numerous observations that pharmacological and non-pharmacological inhibition of the exchanger results in improved postischaemic ventricular recovery (for review see Karmazyn & Moffat, 1993). Therefore, in addition to stimulation of phospholipid catabolism by α_1-agonists, the

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ability of these agents to stimulate Na/H exchange activity suggests that α_1-adrenoceptor activation could exert a deleterious effect on the reperfused myocardium. To examine this potential role of α_1-receptor stimulation, we studied the effects of two α_1-receptor agonists on the response of the rat and rabbit isolated heart to global ischaemia followed by reperfusion. In addition, we compared the effects of these drugs to those of the phorbol ester 12-phorbol 13-myristate acetate, an agent known to activate PKC independent of phosphoinositide hydrolysis (Castagna *et al.*, 1982).

Methods

Animals

Male Sprague-Dawley rats (275–325 g) and male New Zealand white rabbits (2–2.5 kg) were used in this study. Animals were maintained in the Health Sciences Animal Care Facility of the University of Western Ontario in accordance with the guidelines of the Canadian Council on Animal Care (Ottawa, Ontario, Canada).

Heart perfusions

Rats were killed by decapitation whereas rabbits were killed by injection of sodium pentobarbitone (Euthanyl Forte, 108 mg kg^{-1}) into the marginal ear vein. Hearts were rapidly excised and arranged for retrograde perfusion by the standard Langendorff technique. Rat hearts were perfused with Krebs-Henseleit solution consisting of (in mM): NaCl 120, KCl 4.63, KH_2PO_4 1.17, $NaHCO_3$ 20, $CaCl_2$ 1.25, $MgCl_2$ 1.2 and glucose 8. The solution was gassed with a 95% O_2/5% CO_2 mixture and the pH of the solution was 7.4. In initial studies, we observed that function of rabbit hearts perfused with bicarbonate-buffered medium deteriorated with time (approximately 40% loss in contractile force after 2 h perfusion) and hence for these experiments the perfusion medium was changed to a HEPES buffered solution of the following composition (in mM): NaCl 135, HEPES 5, KCl 5.4, NaH_2PO_4 0.33, $CaCl_2$ 2.0, $MgCl_2$ 1.0 and glucose 10.0. This solution reduced the loss in function to less than 10% over a 2 h perfusion duration. The solution was gassed continuously with 100% O_2 and the pH was 7.4. The entire perfusion system was maintained at 37°C by means of a water-jacketed condenser attached to a circulator. Similar techniques were used for the determination of contractile function of both rat and rabbit hearts. Contractile force (apicobasal displacement) was obtained by connecting a force displacement transducer (Grass FT.03) to the apex of the heart at an initial preload stretch of 2 g. The rate of force development ($+dF/dt$) and relaxation ($-dF/dt$) were obtained electronically using a Grass differentiator (Model 7P20). A side-arm off the perfusion cannula was connected to a pressure transducer (Statham Gould P231D) in order to ascertain changes in coronary vascular resistance. However, it should be stated that there were no differences in coronary pressures between any of the experimental groups nor was there any relationship between contractile recovery and coronary pressure under any conditions; therefore coronary pressure data are not reported in this study. Atria were removed and hearts were electrically paced at a rate of 5 Hz and 3 Hz for rat and rabbit hearts, respectively. Flow rate was maintained with a peristaltic pump at 10 ml min^{-1} and 20 ml min^{-1} for rat and rabbit hearts, respectively. All recordings were made using a Grass polygraph.

Following equilibration for 30 min at normal flow rates, total global ischaemia was initiated for either 30 or 60 min (rat hearts) or only for 60 min (rabbit hearts) by turning off the perfusion pump. Following ischaemia, hearts were reperfused by restoring flow to the control level (see above) for a further 30 min.

Drug addition protocols

An initial set of studies was done to determine the concentration-dependent effects of the α_1-agonists, phenylephrine and methoxamine, in normally perfused rat or rabbit (phenylephrine only) hearts. Cumulative increases in drug concentrations were tested either alone or in hearts pretreated with 1 μM of the Na/H exchange inhibitor, methylisobutyl amiloride (MIA). Each concentration was administered for 15 min by which time the effect, if any, stabilized. It is well-established that α_1-receptor agonists exert triphasic contractile effects on cardiac tissue, namely an initial transient positive followed by a negative inotropic effect which then reverses to a sustained increase in contractility (Terzic *et al.*, 1993). It should therefore be noted that the sustained component of the positive inotropic effect of phenylephrine or methoxamine was considered when assessing positive inotropic effects of these compounds.

For ischaemia experiments, 1 μM of either phenylephrine or methoxamine (phenylephrine only for rabbit hearts) was added 15 min prior to initiation of ischaemia. In another set of studies, these agonists were examined in the reperfused heart in the presence of MIA (1 μM). In addition, the effects of the adenosine A_1 receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 0.5 μM) were tested in the presence or absence of MIA or the potent PKC inhibitor, bisindolylmaleimide (20 nM). Further experiments were done to assess the effects of another A_1 receptor antagonist, N-0861, in ischaemic and reperfused rat and rabbit hearts treated with phenylephrine. Drugs were present throughout the complete perfusion period. It should be added that for all experiments involving the α_1-agonists phenylephrine or methoxamine, 1 μM of the β-receptor antagonist, propranolol, was present throughout the perfusion period as well as in the appropriate control experiments for these drugs.

Another set of experiments was performed to study the effects of 10^{-11} M PMA on contractile recovery. For these studies, PMA was added 15 min prior to initiation of ischaemia and allowed to remain for the duration of the perfusion period. The effects of PMA were also examined in the presence of bisindolylmaleimide.

Phenylephrine, methoxamine, and DPCPX were purchased from Research Biochemicals Inc. (Natick, MA, U.S.A.). Bisindolylmaleimide (GF 109203X) was purchased from Calbiochem (La Jolla, CA, U.S.A.). PMA and propranolol were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). N-0861 was a gift from Whitby Research Inc. (Richmond, Virginia, U.S.A.). All chemicals were dissolved in absolute ethanol except phenylephrine, methoxamine and propranolol which are water soluble. Maximum ethanol concentration was 0.005%. N-0861 was dissolved in 0.1 M pH 7.0 phosphate buffer.

Metabolite assays

For determination of tissue metabolites after reperfusion, the heart was clamped with Wollenberger tongs precooled in liquid nitrogen, removed from the cannula and stored in liquid nitrogen until the assays were performed. Assays for adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), creatine phosphate (CP) and lactate were performed on 6% perchloric acid extracts. The assay for glycogen was performed on pulverized tissue. Energy metabolite content was determined enzymatically with a Beckman DU-65 spectrophotometer as previously described (Bergmeyer, 1963).

Data analysis and expression of results

The data were analysed either with Student's t test for unpaired data or analysis of variance followed by Student-Newman-Keuls test to locate differences between groups. Differences were considered significant at the level of

$P<0.05$. Data have been standardized and expressed on a percentage basis. For ischaemia and reperfusion experiments the percentage recovery was calculated by comparing functional parameters during reperfusion to values obtained immediately before initiating ischaemia.

Results

Effects of α_1-adrenoceptor stimulation on rat hearts

Normally perfused hearts To determine direct contractile effects of α_1-agonists, we initially carried out a concentration-response analysis of normally perfused hearts to which increasing concentrations of phenylephrine or methoxamine were added. As summarized in Figure 1, both α_1-agonists produced positive inotropic effects at the lower concentrations. A small decline in contractility, particularly prominent with phenylephrine, was observed with higher agonist concentrations although even with 16 μM phenylephrine contractile force was still significantly higher than values obtained before phenylephrine administration ($P<0.05$). As shown in Figure 1, 1 μM MIA significantly attenuated the positive inotropic effects of both compounds although this was significant at all concentrations only with respect to phenylephrine.

Ischaemic reperfused rat hearts To examine the potential modulatory role of α_1-agonists on postischaemic ventricular recovery, hearts were pretreated with 1 μM of either phenylephrine or methoxamine, a concentration that produced a marked, α_1-mediated positive inotropic effect in the control studies described above. With regard to phenylephrine, rat hearts were subjected to either 30 or 60 min ischaemia, conditions which produce approximately a 90% and 60% recovery in contractile force following reperfusion, respectively. Methoxamine studies were done only on rat hearts which were subjected to 60 min ischaemia. As shown in Figure 2, phenylephrine failed to modify contractile recovery either after 30 or 60 min global ischaemia. In addition, as summarized in Table 1, a similar lack of effect on postischaemic recovery was evident in hearts pretreated with 1 μM methoxamine. No differences in resting tension were observed in reperfused hearts in the presence or absence of α_1-receptor agonists (data not shown).

Although the above results demonstrate a failure of α_1-agonists to attenuate directly postischaemic recovery, as would be predicted for agents which stimulate Na/H exchange, we performed another series of studies to determine whether α_1 agonists can reverse the protective effect of Na/H exchange inhibition with MIA. As is evident from Figure 3, after 60 min ischaemia, reperfusion in the presence of MIA resulted in recovery of contractility which was approximately

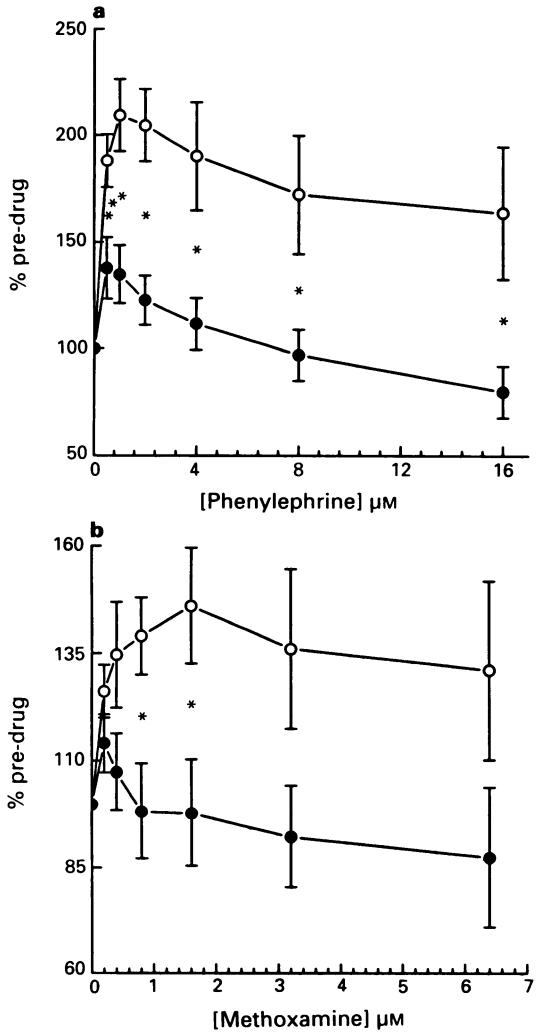


Figure 1 Changes in developed force of rat isolated hearts to increasing concentrations of either phenylephrine (a) or methoxamine (b) in the presence (●) or absence (○) of methylisobutyl amiloride. Values indicate the percentage change for those obtained prior to either phenylephrine or methoxamine addition. *$P<0.05$ between groups of $n = 6$.

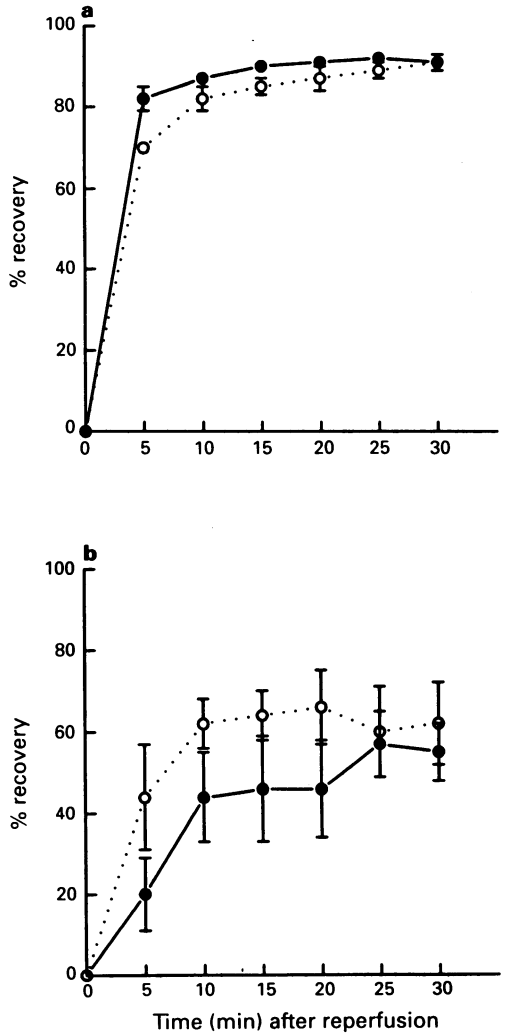


Figure 2 Recovery in developed force of rat isolated hearts subjected to either 30 min (a) or 60 min (b) global zero-flow ischaemia in the presence (○) or absence (●) of phenylephrine 1 μM. Values indicate the percentage recovery in developed force of pre-ischaemic values. $n = 6$.

Table 1 Recovery in contractile force in reperfused rat hearts after 60 min ischaemia in the presence or absence of methoxamine

	Minutes after reperfusion					
Group	5	10	15	20	25	30
Control	20.0 ± 9	44.1 ± 11.2	46.2 ± 13.1	46.3 ± 11.8	56.8 ± 8.3	54.8 ± 7.4
Methoxamine	28.7 ± 6.3	45.5 ± 5.4	48.3 ± 5.2	46.6 ± 3.4	45.5 ± 3.3	46.3 ± 3.8

Values indicate means ± s.e.mean of $n = 5$ for methoxamine and $n = 7$ for control and depict percentage recovery of pre-ischaemic values.

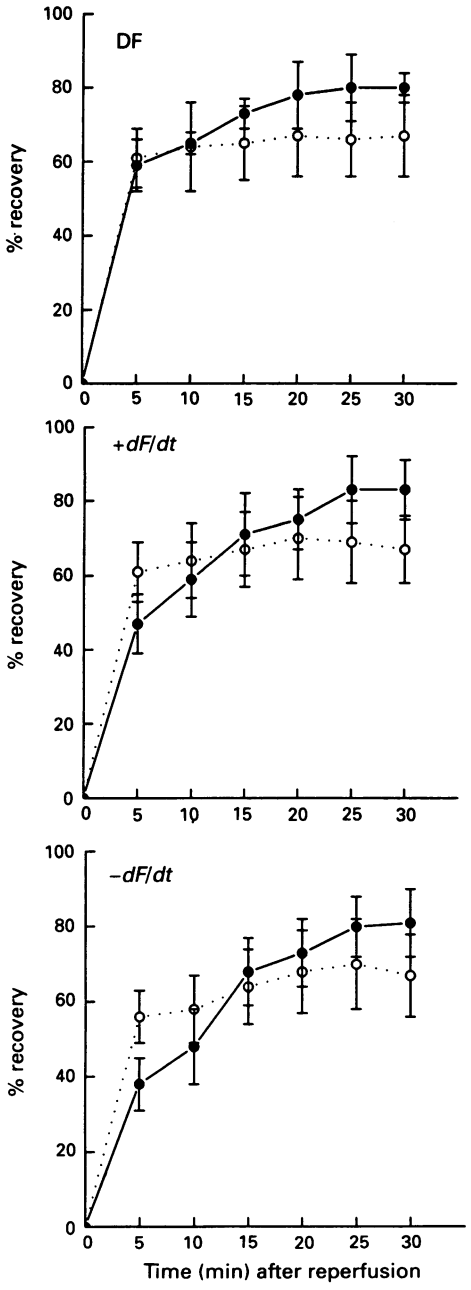


Figure 3 Contractile response of rat isolated hearts treated with methylisobutyl amiloride and subjected to 60 min global zero-flow ischaemia in the presence (○) or absence of phenylephrine 1 μM, (●). Values indicate the percentage recovery in developed force (a), rates of force development (+ dF/dt) (b) or relaxation (− dF/dt) (c) of pre-ischaemic values. $n = 6$.

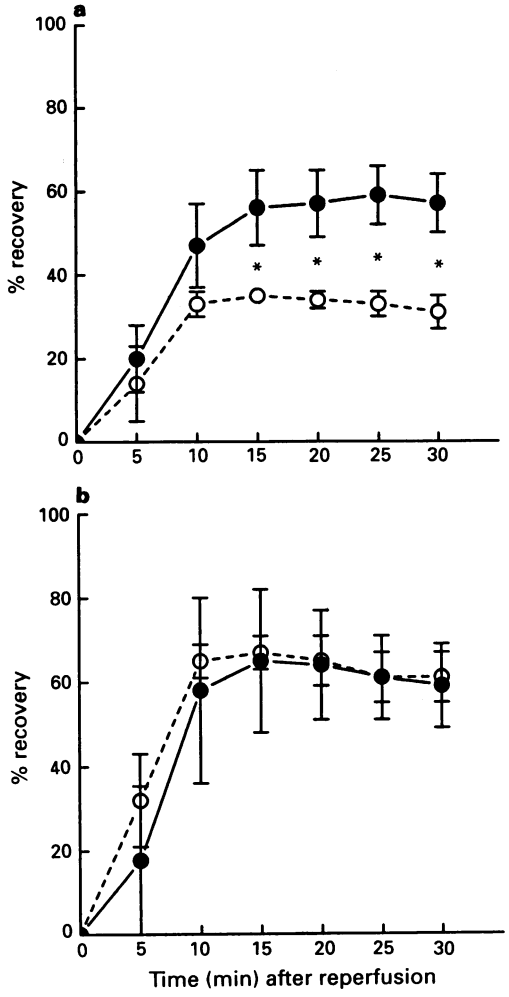


Figure 4 Contractile response of rat isolated hearts subjected to 60 min global zero-flow ischaemia in the presence (○) or absence (●) of 12-phorbol 13-myristate acetate (10 pM) either when the phorbol was present alone (a) or in the presence of the protein kinase C inhibitor, bisindolylmaleimide (b). Values indicate the percentage recovery in developed force of pre-ischaemic values. *$P < 0.05$ from control. $n = 6$.

80% of preischaemic values. Moreover, as shown in Figure 3, in the presence of MIA, recovery was rapid with approximately 60% recovery after 5 min of reperfusion in contrast to 20% recovery at this time seen in the absence of MIA (bottom panel of Figure 2). Phenylephrine failed to exert a significant effect on contractile recovery when added to hearts protected by MIA (Figure 3).

Effects of PMA on the ischaemic reperfused rat heart

To compare the effects of α_1-receptor stimulation with a direct activator of PKC, we initially examined the ability of a very low concentration of PMA (10 pM) to affect postischaemic ventricular recovery. In time-control studies, this concentration of PMA produced a modest (15–20%) increase in contractile force on its own which peaked after 20 min perfusion and which subsequently declined to values identical to those observed prior to PMA administration. This increase in contractility was completely abolished by bisindolylmaleimide.

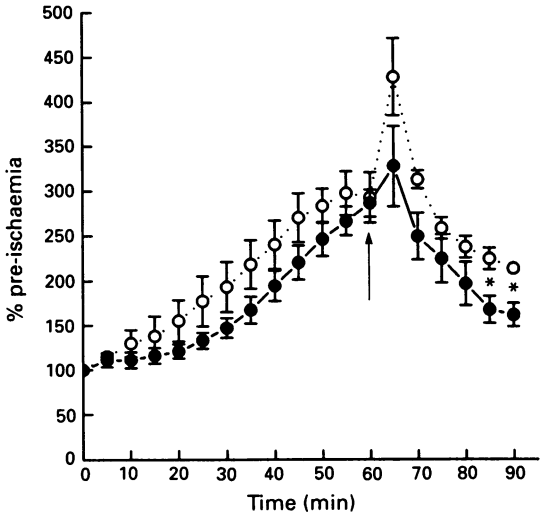


Figure 5 Changes in resting tension during 60 min global zero-flow ischaemia and reperfusion of rat isolated hearts in the presence (○) or absence (●) of 12-phorbol 13-myristate acetate (10 pM). Ischaemia was initiated at 0 min with the time of reperfusion indicated by arrow. Values indicate the percentage change in resting tension from pre-ischaemic values. $*P<0.05$ from control. $n=6$.

As shown in Figure 4a, the presence of 10 pM PMA reduced postischaemic recovery throughout the reperfusion period with significant differences being observed from 15 to 30 min. However, PMA failed to depress contractile recovery when the phorbol was administered in the presence of bisindolylmaleimide (Figure 4b). In addition to a reduction in contractile recovery, the presence of PMA resulted in a trend towards elevated resting tension during ischaemia and a sustained elevation of resting tension during reperfusion which was significantly different from control after 25 and 30 min of reflow (Figure 5).

Effect of adenosine A_1 receptor blockade

Previous reports that α_1-receptors regulate adenosine production (Kitakaze *et al.*, 1987) prompted us to investigate whether endogenous adenosine could antagonize the potential deleterious effects of phenylephrine in reperfused ischaemic hearts. To investigate this possibility, we examined the effects of phenylephrine in the presence of adenosine A_1 receptor blockade. In initial experiments, we found that the A_1 receptor blocker, DPCPX markedly reduced recovery in hearts which were reperfused following 60 min ischaemia. Therefore this series of experiments was performed on hearts subjected to 30 min ischaemia followed by reperfusion. As shown in Figure 6a, with this duration of ischaemia, antagonism of cardiac A_1 receptors with DPCPX produced virtually no effect on postischaemic recovery. However, in the presence of DPCPX, phenylephrine significantly depressed contractile recovery compared to hearts perfused with DPCPX alone ($43.8 \pm 9.0\%$ phenylephrine vs $81.9 \pm 9.9\%$ control, $P<0.05$ after 30 min reperfusion), however this was not associated with elevated resting tension (Table 2). The ability of phenylephrine to inhibit recovery in the presence of DPCPX was not modified by the Na/H exchange inhibitor MIA (Figure 6b) although the profile of postischaemic recovery was somewhat different in the presence of both DPCPX and MIA. In particular, it is noteworthy that in the presence of both agents, phenylephrine depressed recovery throughout most of the perfusion period. In addition, following an initial rapid recovery of contractility, contractility declined with continued reperfusion (Figure 6b), a phenomenon not observed in hearts perfused with DPCPX alone (Figure 6a). We also examined whether an inhibitor of PKC could reverse the deleterious effects of phenylephrine in the presence of

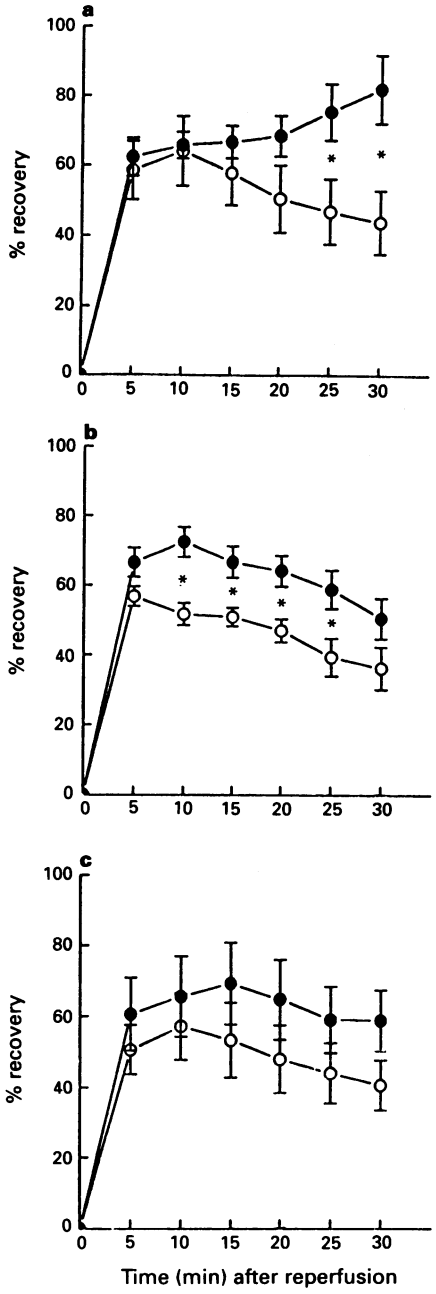


Figure 6 Contractile response of rat isolated hearts treated with 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) and subjected to 30 min global zero-flow ischaemia in the presence or absence of phenylephrine (PE): (a) shows the effect of DPCPX and PE alone whereas (b) and (c) show results of similar experiments carried out in the presence of MIA or bisindolylmaleimide, respectively. (●) DPCPX; (○) DPCPX plus PE. Values indicate the percentage recovery in developed force of pre-ischaemic values. $*P<0.05$ between groups. $n=6$.

DPCPX. As shown in Figure 6c, there were no significant differences between responses observed in the presence or absence of bisindolylmaleimide. However, resting tension was generally higher during reperfusion with phenylephrine in the presence of bisindolylmaleimide and this parameter was significantly higher than in control after 15 min ($115 \pm 7.2\%$ control versus $141.7 \pm 9.5\%$ phenylephrine, $P<0.05$).

Recovery in contractility was further assessed with another antagonist of A_1 receptors, N-0861. As shown in Table 3, phenylephrine significantly depressed contractile recovery in rat hearts subjected to 30 min ischaemia in the presence of N-0861.

Responses of rabbit hearts

Selected experiments were performed on rabbit isolated hearts to confirm the major observations of our study with rat isolated hearts. Table 4 summarizes the contractile response of rabbit isolated hearts to increasing concentrations of phenylephrine. As was observed in rat hearts, MIA significantly attenuated the positive inotropic effect of phenylephrine. The response of rabbit hearts subjected to 60 min ischaemia in the presence or absence of 1 μM phenylephrine was studied under various experimental conditions (Table 5). On its own, phenylephrine failed to alter either postischaemic contractile recovery (Table 5) or the elevation in resting tension (data not shown) observed after restoration of flow. However, in the presence of DPCPX, phenylephrine decreased recovery in rabbit hearts to values significantly different from control after 15 min of reperfusion. Moreover, this effect occurred in the presence of significantly elevated resting tension throughout much of the reperfusion period, an effect which was not observed in rat hearts (Table 2). As for rat hearts, the effect of DPCPX was mimicked by N-0861 (Table 3). In addition, as was observed in rat hearts, PMA significantly depressed recovery of contractility throughout most of the reperfusion period (Table 5); however in contrast to the elevation in resting tension seen with PMA at the end of reperfusion in the rat, no significant differences in this parameter were observed in rabbit hearts (not shown).

Changes in tissue energy metabolite contents in reperfused rat and rabbit hearts

Tables 6 and 7 show levels of various metabolites in reperfused hearts treated either with PMA or with phenylephrine in the presence of DPCPX, conditions which were effective in inhibiting recovery of contractility. As shown in Table 6, PMA failed to alter high energy phosphate or adenine nucleotide content in either rat or rabbit hearts. On the other hand, in the presence of DPCPX, phenylephrine decreased high energy phosphate contents, although this effect was seen only in rat hearts. Moreover, the inhibition of total high energy phosphates was observed in the presence of MIA but was prevented by bisindolylmaleimide (Table 6). Neither lactic acid nor glycogen contents were generally affected by any of the treatments (Table 7). However, in the presence of DPCPX, lactate content was significantly elevated in rabbit hearts treated with phenylephrine (Table 7).

Table 2 Resting tension changes in reperfused rat and rabbit hearts treated with 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) in the presence or absence of phenylephrine

	Minutes after reperfusion					
Group	5	10	15	20	25	30
			Rat			
DPCPX	180.8 ± 21.3	158.3 ± 20.9	138.3 ± 23.9	133.3 ± 22.3	121.7 ± 19.7	120.0 ± 18.1
DPCPX + phenylephrine	204.2 ± 20.0	182.1 ± 18.1	158.0 ± 16.6	144.9 ± 16.4	144.7 ± 18.1	138.8 ± 13.0
			Rabbit			
DPCPX	207.5 ± 7.8	260.0 ± 18.5	250.0 ± 26.1	230.0 ± 28.1	223.0 ± 17.3	205.0 ± 33.2
DPCPX + phenylephrine	217.5 ± 8.1	307.0 ± 28.1	325.0 ± 7.8*	343.0 ± 17.1*	340.0 ± 29.2*	338.0 ± 17.8*

Values indicate means ± s.e.mean of $n = 6$ and indicate percentage of pre-ischaemic values.
*$P < 0.05$ from values obtained with DPCPX alone.

Table 3 Effect of N-0861 on recovery of contractile force of rat and rabbit isolated hearts after 30 and 60 min ischaemia, respectively

	Minutes after reperfusion					
Group	5	10	15	20	25	30
			Rat			
N-0861 alone	67 ± 12	90 ± 11	103 ± 9	105 ± 5	108 ± 6	105 ± 5
N-0861 + phenylephrine	67 ± 5	78 ± 5	83 ± 8	70 ± 6*	75 ± 4*	70 ± 2*
			Rabbit			
N-0861 alone	32 ± 14	53.3 ± 4	96 ± 3	104 ± 7	105 ± 4	107 ± 3
N-0861 + phenylephrine	0	36 ± 8	76 ± 4	74 ± 5*	74 ± 5*	77 ± 4*

Values indicate mean ± s.e.mean of $n = 5$ and indicate percentage recovery of pre-ischaemia values.
*$P < 0.05$ from values obtained with N-0861 alone.

Table 4 Effect of increasing concentrations of phenylephrine on contractile force of rabbit isolated hearts in the presence or absence of methylisobutyl amiloride (MIA)

	Phenylephrine concentration (μM)					
Group	0.1	0.2	0.4	0.8	1.6	3.2
Control	106 ± 3	106 ± 3	106 ± 4	107 ± 5	105 ± 4	107 ± 6
Phenylephrine	116 ± 4	127 ± 6	130 ± 5*	138 ± 7*	145 ± 7*	148 ± 7*
Phenylephrine + MIA	106 ± 2	109 ± 2	115 ± 3	115 ± 4	111 ± 3	125 ± 8

Values indicate means ± s.e.mean of $n = 6$ and indicate percentage of pre-phenylephrine values. Control group depicts hearts perfused over an identical period without phenylephrine.
*$P < 0.05$ from control.

Table 5 Recovery in contractile force of rabbit isolated hearts after 60 min ischaemia under various treatments

	Minutes after reperfusion					
Group	5	10	15	20	25	30
Control	17.2 ± 7.1	33.9 ± 8.6	50.5 ± 7.2	65.1 ± 7.8	73.1 ± 6.8	80.6 ± 11.2
Phenylephrine	15.7 ± 4.2	33.0 ± 9.1	46.5 ± 4.1	58.4 ± 8.1	61.8 ± 5.9	67.9 ± 8.3
PMA	10.2 ± 3.4	13.9 ± 3.1*	31.7 ± 3.9	37.6 ± 4.1*	41.9 ± 7.0*	47.8 ± 11.4
DPCPX	8.3 ± 8.3	16.6 ± 4.2	29.3 ± 3.8	44.9 ± 8.7	53.7 ± 7.9	57.1 ± 11.2
DPCPX + phenylephrine	.02 ± .02	11.8 ± 3.9	14.6 ± 4.1*	15.8 ± 4.2*	16.6 ± 5.4*	17.8 ± 6.8*

Values indicate means ± s.e.mean of $n = 5$ and indicate percentage recovery of pre-ischaemic values. PMA: 12-phorbol 13-myristate acetate; DPCPX: 1,3-dipropyl-8-cyclopentylxanthine.
*$P < 0.05$ from respective control value in the absence of phenylephrine.

Table 6 High energy phosphate and adenine nucleotide content in reperfused rat and rabbit hearts treated with 12-phorbol 13-myristate acetate (PMA) or 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) in the presence or absence of phenylephrine

Group	*ATP*	*CrP*	*Total HEP*	*ADP*	*AMP*	*Total adenine nucleotides*	*Energy charge*
				Rat			
Control	17.1 ± 2.5	21.4 ± 2.9	38.6 ± 5.2	6.5 ± 0.2	3.6 ± 0.7	27.2 ± 2.6	0.74 ± 0.03
PMA	16.3 ± 0.6	21.5 ± 1.0	37.8 ± 1.5	5.6 ± 0.4	2.3 ± 0.1	24.2 ± 0.8	0.79 ± 0.01
DPCPX	17.8 ± 1.3	15.7 ± 1.1	33.5 ± 1.5	4.6 ± 1.2	1.0 ± 0.2	23.4 ± 2.3	0.86 ± 0.02
DPCPX + phenylephrine	10.9 ± 0.8*	12.9 ± 2.1	23.9 ± 2.9*	3.9 ± 1.7	0.9 ± 0.2	15.7 ± 2.6	0.84 ± 0.03
DPCPX + MIA	16.4 ± 0.64	17.3 ± 1.6	33.7 ± 2.1	5.8 ± 0.9	2.2 ± 0.2	24.4 ± 1.7	0.80 ± 0.02
DPCPX + MIA + phenylephrine	13.0 ± 1.6	14.6 ± 1.3	27.7 ± 2.8	4.7 ± 0.6	2.0 ± 0.3	*19.7 ± 1.2	0.78 ± 0.03
DPCPX + Bis	14.8 ± 2.5	18.8 ± 1.5	33.6 ± 3.8	4.9 ± 0.8	2.5 ± 1.2	21.2 ± 3.3	0.81 ± 0.02
DPCPX + Bis + phenylephrine	14.3 ± 0.4	16.9 ± 2.0	31.2 ± 2.9	4.4 ± 0.8	1.8 ± 0.3	20.4 ± 1.8	0.81 ± 0.03
				Rabbit			
Control	14.0 ± 1.6	18.7 ± 1.7	32.7 ± 3.2	4.2 ± 0.2	1.1 ± 0.2	19.3 ± 1.7	0.83 ± 0.01
PMA	15.0 ± 1.5	20.6 ± 1.6	35.5 ± 2.8	4.4 ± 0.6	1.3 ± 0.1	20.6 ± 1.4	0.83 ± 0.02
DPCPX	17.0 ± 1.3	22.1 ± 0.6	39.1 ± 1.7	4.0 ± 0.2	1.0 ± 0.1	22.0 ± 1.2	0.86 ± 0.01
DPCPX + phenylephrine	15.0 ± 1.6	20.8 ± 1.2	35.8 ± 2.5	4.1 ± 0.2	1.2 ± 0.2	20.3 ± 1.5	0.83 ± 0.02

Values indicate means ± s.e.mean of $n = 6$ and are given in $\mu mol\ g^{-1}$ dry wt. Energy charge was calculated from the following equation:

$$\frac{ATP + 0.5\ ADP}{ATP + ADP + AMP}$$

*$P < 0.05$ from DPCPX alone. Bis = bisindolylmaleimide.

Discussion

Potential mechanisms for α_1-adrenoceptor-mediated effects in the heart

The existence of α_1-adrenoceptors in mammalian ventricle has been well documented as has the ability of stimulation of these receptors to produce a positive inotropic effect (for review see Terzic *et al.*, 1993). However, the precise mechanism of action mediating the positive inotropic effect of α_1-receptor stimulation is not known with certainty. Recently, it has been demonstrated that α_1-receptor stimulation with phenylephrine activates Na/H exchange in ventricular myocytes with subsequent intracellular alkalinization (Terzic *et al.*, 1992; Gambassi *et al.*, 1992; Puceat *et al.*, 1993). In this regard, it has been proposed that Na/H exchange induced intracellular alkalinization and a subsequent increase in the myofibrillar sensitivity to calcium represents the primary factor in the production of the positive inotropic effect of α_1-receptor stimulation (Terzic *et al.*, 1992; Gambassi *et al.*, 1992; Puceat *et al.*, 1993). There are a number of lines of evidence which support this hypothesis. First, as noted above it has been reported that increased cell shortening can be dissociated from an elevation in intracellular Ca^{2+} concentration (Terzic *et al.*, 1992; Gambassi *et al.*, 1992; Puceat *et al.*, 1993). Second, in isolated ventricular myocytes, both the positive inotropic effect of α_1-receptor agonists as well as their ability to produce intracellular alkalinization have been shown to be attenuated by Na/H exchange inhibitors (Terzic *et al.*, 1992; Gambassi *et al.*, 1992; Puceat *et al.*, 1993). Lastly, Na/H exchange inhibitors have been shown to attenuate the positive inotropic effect of α_1-receptor agonists in cellular and multicellular preparations (Otani *et al.*, 1990; Terzic *et al.*, 1992; Gambassi *et al.*, 1992; Puceat *et al.*, 1993). The ability of MIA, a potent inhibitor of the sodium-hydrogen exchanger (Talor *et al.*, 1989), to attenuate the positive inotropic effect of both methoxamine and phenylephrine in our study further strengthens the hypothesis promulgated by various investigators that Na/H exchange plays an important role in mediating the positive inotropic effect of α_1-receptor.

Effects of α_1-receptor stimulation on reperfused myocardium: comparison with phorbol ester

Various characteristics of α_1-receptor agonists in the heart are conducive to potential deleterious effects of these compounds on the reperfused, ischaemic myocardium. First, the positive inotropic effect of these compounds could depress postischaemic recovery by increasing myocardial oxygen demand.

Table 7 Lactic acid and glycogen contents in reperfused rat and rabbit hearts treated with 12-phorbol 13-myristate acetate (PMA) or 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) in the presence or absence of phenylephrine

Group	*Lactic acid*	*Glycogen*
	Rat	
Control	2.39 ± 0.72	34.7 ± 8.5
PMA	1.44 ± 0.66	37.1 ± 8.0
DPCPX	1.4 ± 0.4	30.6 ± 3.1
DPCPX + phenylephrine	2.2 ± 0.6	17.9 ± 6.3
DPCPX + MIA	4.3 ± 1.2	47.2 ± 6.9
DPCPX + MIA + phenylephrine	7.2 ± 2.4	30.1 ± 3.4*
DPCPX + Bis	1.7 ± 0.9	37.8 ± 8.1
DPCPX + Bis + phenylephrine	1.9 ± 0.9	38.7 ± 6.4
	Rabbit	
Control	1.1 ± 0.2	55.1 ± 6.1
PMA	1.7 ± 0.7	73.8 ± 4.8
DPCPX	3.3 ± 1.4	47.2 ± 1.4
DPCPX + phenylephrine	7.4 ± 1.0*	43.2 ± 3.6

Values indicate means ± s.e.mean of $n = 6$ and are given in μmol g^{-1} dry wt. Bis:bisindolylmaleimide; MIA: methyl-isobutyl amiloride.
*$P < 0.05$ from DPCPX alone.

Second, a number of investigators have reported that α_1-receptor stimulation is linked to enhanced phospholipid-particularly phosphatidylinositol-hydrolysis which would result in membrane disruption (for review see Terzic *et al.*, 1993). Third, evidence has also been presented that α_1-receptor antagonists protect against reperfusion-induced arrhythmias in a variety of experimental models (Thandroyen *et al.*, 1983; Molina-Viamonte *et al.*, 1991) and reduce Ca^{2+} accumulation (Sharma *et al.*, 1983) in the reperfused myocardium (see Corr *et al.*, 1989 for review and references therein). Fourth, a further potential contributing factor *vis a vis* an α_1-receptor-mediated enhancement of cardiotoxicity is the observation of increased density of α_1-receptor in the ischaemic myocardium of various species (Dillon *et al.*, 1988) although interestingly, this phenomenon was not observed in rat hearts (Dillon *et al.*, 1988). Lastly, in view of the emerging importance of Na/H exchange in mediating myocardial reperfusion injury (see Karmazyn & Moffat, 1993 for review) and, as discussed above, increasing evidence that α_1-receptor agonists stimulate the antiport, it can be hypothesized that activators of the antiport would produce deleterious effects on postischaemic ventricular function.

A major focus of the present study was to examine whether stimulation of α_1-receptor diminishes postischaemic ventricular recovery as well as to compare these effects with a very low concentration of phorbol ester which directly activates PKC (Castagna *et al.*, 1982). It should be emphasized that phorbols produce complex, concentration-dependent effects which may or may not be dependent on PKC activation (Watson & Karmazyn, 1991). While generally considered as negative inotropes, very low concentrations of these compounds have been shown to exert modest positive inotropic effects which are most probably mediated by PKC activation (Ward & Moffat, 1992; Karmazyn & Haist, 1993). Indeed, although not shown in the results, bisindolylmaleimide completely abolished the positive inotropic effect of PMA in normally perfused hearts. Our results demonstrate that in the absence of any direct negative inotropic effect, PMA significantly inhibited ventricular recovery and elevated resting tension during reperfusion. Moreover, this effect was reversed by bisindolylmaleimide, an observation which supports the notion that the PMA-induced depression in recovery was PKC-mediated.

Potential role of endogenous adenosine in modulating effects of α_1-receptor stimulation on ischaemic and reperfused hearts

It was surprising that α_1-receptor activation failed to affect postischaemic ventricular recovery, particularly in view of the preceding discussion suggesting a theoretical basis for α_1-receptor-mediated contractile dysfunction of the reperfused myocardium. Evidence in the literature, albeit discrepant, suggests that α_1-receptors may regulate myocardial adenosine production. For example Kitakaze *et al.* (1987) have shown that in the ischaemic canine myocardium, α_1-receptor blockade results in diminished adenosine release. On the other hand, Hohl *et al.* (1989) have demonstrated in rat ventricular myocytes that α_1-receptor stimulation activates adenylate deaminase, the enzyme which catalyzes the conversion of AMP to IMP, thereby resulting in diminution of adenosine production. It is obviously difficult to compare studies on completely different models. Nonetheless, the *in vivo* study (Kitakaze *et al.*, 1987) utilizing intact canine myocardium suggests that, at least in the ischaemic heart, α_1-receptors represent important regulators of adenosine production through an as yet unknown mechanism. In view of this finding as well as extensive evidence demonstrating a cardioprotective effect of adenosine against various forms of injury, including those accompanying oxidative stress and reperfusion (Karmazyn & Cook, 1992; Lasley & Mentzer, 1992), we hypothesized that endogenous adenosine may serve to counteract the potential deleterious effect of α_1-receptor stimulation. Indeed, it is interesting that in a canine model of myocardial stunning, α_1-receptor activation has been shown to improve recovery and enhance ATP content after reperfusion through a mechanism attributed to elevated adenosine production (Kitakaze *et al.*, 1991). To examine the role of endogenous adenosine, experiments were carried out in the presence of the adenosine A_1 receptor antagonists, DPCPX (von der Leyen *et al.*, 1989; Behnke *et al.*, 1990) or N-0861 (Martin *et al.*, 1993). A_1 antagonists were chosen in view of evidence that it is primarily the activation of these myocardial receptors which mediates the protective effects of the nucleoside and that their antagonism attenuates the protective effects (Karmazyn & Cook, 1992; Lasley & Mentzer, 1992; Ely & Berne, 1992). A_1 receptor antagonists failed on their own to inhibit recovery after reperfusion after 30 and 60 min ischaemia in rat and rabbit hearts, respectively. This finding is in agreement with a previous observation (Angello *et al.*, 1991) and suggests that under these conditions endogenous adenosine may not mediate recovery. However, it is evident from our study that in the presence of DPCPX or N-0861, phenylephrine significantly attenuates postischaemic ventricular recovery in both the rat and rabbit hearts and, at least in the rabbit heart, also elevates resting tension during reperfusion. This finding suggests that endogenous adenosine may function to protect against the deleterious effects of α_1-receptor activation through an as yet unknown mechanism. However, the possibility that DPCPX unmasks the deleterious effects of α_1-receptor agonists through a different mechanism cannot be excluded, particularly in the absence of adenosine measurements. Despite the fact that they demonstrate markedly enhanced potency at the A_1 receptor, it should be noted that both DPCPX and N-0861 can, at higher concentrations, also block the A_2 receptor (Bruns *et al.*, 1987; Martin *et al.*, 1992). Although A_1 receptors are predominant, A_2 receptors have also been found in ventricular cells (Xu *et al.*, 1992; Zucchi *et al.*, 1992; Stein *et al.*, 1993) and it has been suggested that A_2 receptor activation may also result in protection of the ischaemic and reperfused myocardium (Norton *et al.*, 1992). Moreover, in receptor binding studies, K_d values for the A_1 receptor, at least with respect to DPCPX, have been reported to be in low nanomolar concentrations (Bruns *et al.*, 1987; Tawfik-Schlieper *et al.*, 1989), thus substantially lower than those used in the present study. While it is difficult to compare

binding data with functional studies it is unlikely that the effects of either agent was due to A_2 receptor antagonism for a number of reasons. For example, despite the demonstration of A_2 receptors, as noted above, it is well-established that the primary adenosine receptor in the cardiac cell is the A_1 receptor (see Ely & Berne, 1992 for review) and that activation of these receptors is associated with cardioprotective effects in a variety of experimental models (Ely & Berne, 1992). Moreover, in functional studies it has been shown that concentrations of either DPCPX or N-0861 higher than those used by us were required to antagonize competitively the negative inotropic effects of A_1 receptor agonists in ventricular (Endoh *et al.*, 1993) or atrial (Martin *et al.*, 1993) preparations. It should also be added that with respect to N-0861, radioligand binding studies in human and guinea-pig atrial preparations revealed K_i values for the A_1 receptor of 0.7 μM and 0.62 μM, respectively (Shryock *et al.*, 1992), concentrations similar to those used in the present study. Of added importance in excluding A_2-mediated effects in the present paper is the fact that Shryock *et al.* (1992) also demonstrated the inability of 20 μM of N-0861 to prevent adenosine-induced coronary relaxation, an A_2-mediated effect, in guinea-pig isolated hearts.

Potential mechanisms for α_1-adrenoceptor-mediated inhibition of postischaemic recovery in presence of adenosine A_1 receptor blockade

Because of the multitude of effects which have been reported for α_1-adrenoceptor agonists, it is difficult to ascribe a precise mechanism(s) to account for their effects on postischaemic recovery in the presence of an adenosine A_1 receptor antagonist. Our studies however, demonstrate that the activation of Na/H exchange is unlikely to be involved in view of the inability of MIA to modulate the effects of α_1-receptor activation. Thus, it appears that activation of the Na/H antiport is important in mediating the positive inotropic effects of α_1-receptor agonists but not their deleterious effects on postischaemic reperfusion. This may not be particularly surprising as the trans-sarcolemmal pH gradient is the major regulator of Na/H exchange activity (Karmazyn & Moffat, 1993). It is likely that ischaemia-induced intracellular acidosis is of sufficient extent to ensure maximum stimulation of Na/H exchange upon reperfusion, irrespective of α_1-receptor stimulation. In contrast to the complete abolition of PMA-induced inhibition of postischaemic recovery by bisindolylmaleimide, the PKC inhibitor failed to prevent the deleterious effect of phenylephrine in the presence of DPCPX. These results suggest that PKC may not mediate the negative effects of α_1-receptor stimulation on the ischaemic reperfused heart or that the concentration of bisindolylmaleimide used was insufficient to block PKC activity, particularly under conditions of reperfusion in the presence of α_1-receptor activation. While the precise role of PKC in mediating the effects of α_1-receptor activation requires further studies, our results as well as those reported by others nonetheless suggest that different mechanisms mediate the inhibitory effects of PMA and phenylephrine on postischaemic recovery. For example, it has been demonstrated that phorbol esters actually attenuate the positive inotropic effects of α_1-adrenoceptor agonists (Kushida *et al.*, 1988). Furthermore, it has been reported that the effects of α_1-receptor agonists and phorbol esters on changes in the action potential in rat papillary muscle are different and likely occur through different mechanisms (Endou *et al.*, 1991). Moreover, in the present study we have found that bisindolylmaleimide was totally without effect on the positive inotropic influence of α_1-receptor agonists although it totally prevented these effects of PMA. The latter observation is in agreement with other studies which showed that neither the positive inotropic effects of α_1-receptor agonists in rat papillary muscle nor the effects of these agents on Na/H exchange activity in cardiomyocytes are affected by PKC inhibitors (Otani *et al.*, 1990; Puceat *et al.*, 1993). Lastly, as noted below, different profiles of changes in energy metabolites were found in reperfused rat hearts with PMA as compared to phenylephrine.

Whether the deleterious effect of phenylephrine in the presence of adenosine A_1 receptor blockade is mediated via defective energy metabolism is at present inconclusive. A significant inhibition of high energy phosphate content was found after reperfusion of rat hearts in the presence of phenylephrine whereas in rabbit hearts, depressed recovery occurred in the absence of alterations in high energy phosphate contents. Moreover, we observed significantly elevated lactate levels in reperfused rabbit hearts treated with phenylephrine in the presence of DPCPX but not in rat hearts under similar conditions. Lastly, it should be noted that although PMA significantly inhibited recovery in both species, this effect occurred in the absence of any changes in myocardial energy products. Thus, although substantial changes in energy metabolites were seen under various conditions, a dissociation between these changes and contractile recovery is evident, a finding in agreement with other reports utilizing a number of different approaches towards modulation of postischaemic ventricular recovery (Neely & Grotyohann, 1984; van Bilsen *et al.*, 1988; Currie & Karmazyn, 1990).

Conclusions

In conclusion, we have demonstrated that a sub-toxic concentration of the phorbol ester PMA depresses postischaemic ventricular recovery through an apparent PKC-dependent mechanism. On the other hand, activation of α_1-receptors, which results in a cascade of intracellular events including PKC activation, inhibits recovery only in the presence of blockade of adenosine A_1 receptors. As ischaemia results in numerous effects which mimic changes produced by phorbols as well as α_1-receptor agonists including enhanced α_1-receptor density, stimulation of phosphoinositide hydrolysis and PKC activation, our results suggest that these events are important in determining the cardiac response to ischaemia and reperfusion. Lastly, our results strongly suggest that endogenous adenosine plays an important role in minimizing the potential deleterious effects of enhanced α_1-receptor activation in the ischaemic reperfused myocardium.

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The differential effects of felodipine and nitrendipine on cerebral dihydropyridine binding *ex vivo* and the ethanol withdrawal syndrome in mice

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1 The ability of two dihydropyridine calcium channel antagonists, felodipine and nitrendipine both to displace [^{3}H]-isradipine binding in CNS tissue measured *ex vivo* and to protect against the ethanol withdrawal syndrome has been investigated.

2 Mice were injected with various doses of felodipine or nitrendipine and [^{3}H]-isradipine binding measured in brain homogenates prepared 0.5, 3 or 5 h later. Inhibition versus dose curves were sigmoid and the dose required to produce 50% inhibition increased linearly with time after administration. Felodipine was approximately 10 times more potent than nitrendipine.

3 Nitrendipine (50 mg kg^{-1}, i.p.) and felodipine (10 mg kg^{-1}, i.p.) produced around a 75% inhibition of [^{3}H]-isradipine binding 3 h later. Binding of [^{3}H]-nitrendipine to cerebral tissues measured after *in vivo* injection of the ligand was decreased by nitrendipine (50 mg kg^{-1}) and felodipine (10 mg kg^{-1}) to a similar extent.

4 Nitrendipine (50 mg kg^{-1}) prevented the behavioural signs of ethanol withdrawal as measured by handling induced convulsions, but felodipine (10 mg kg^{-1} or 2 mg kg^{-1}) did not provide any protection against this effect of ethanol withdrawal. Felodipine (10 mg kg^{-1}, twice daily) during the course of ethanol treatment also failed to attenuate the withdrawal syndrome.

5 The convulsive response to a mild audiogenic stimulus during ethanol withdrawal was increased following one dose of felodipine (5 mg kg^{-1}, i.p.) but unaffected by nitrendipine.

6 Injection of Bay K 8644 (60 μg, i.c.v.) produced a significant increase in handling-induced convulsive behaviour. Felodipine (10 mg kg^{-1}, i.p.) reduced this behaviour both 60 and 120 min later, while nitrendipine (50 mg kg^{-1}) showed a modest reduction only at 120 min.

7 In contrast, nitrendipine (50 mg kg^{-1}) and felodipine (10 mg kg^{-1}) produced similar effects on the hyperexcitability produced by handling following administration of bicuculline. Hexamethonium (8 mg kg^{-1}) had no effect on this response.

8 No change was found in [^{3}H]-isradipine or [^{125}I]-ω-conotoxin binding to cerebral tissue prepared from ethanol-dependent mice.

9 These results demonstrate that while felodipine and nitrendipine have similar actions on some CNS-mediated effects (raising seizure thresholds to several convulsant drugs), felodipine, in contrast to nitrendipine, has no effect on the ethanol withdrawal syndrome. Suggested explanations for the results include the possibility that nitrendipine may protect against the ethanol withdrawal syndrome via sites other than dihydropyridine receptors: that felodipine has partial agonist actions at dihydropyridine receptors in the CNS or that felodipine has actions which mask its protective effect in ethanol withdrawal.

Keywords: Dihydropyridines; felodipine; nitrendipine; ethanol withdrawal; Bay K 8644; calcium antagonists; seizures; [^{3}H]-isradipine binding

Introduction

Several lines of evidence suggest that dihydropyridine-sensitive calcium channels may be involved in the behavioural and other changes that occur on withdrawal from alcohol administration (for review see Littleton & Little, 1989). The involvement of dihydropyridine-sensitive calcium channels in the ethanol withdrawal behavioural syndrome was indicated by the observation that administration of the calcium channel antagonists, nimodipine, nitrendipine and isradipine significantly decreased the withdrawal convulsive behaviour (Little *et al.*, 1986; Littleton *et al.*, 1990), while the calcium channel activator Bay K 8644 prevented the action of nimodipine in blocking the ethanol withdrawal syndrome (Littleton *et al.*, 1990). The density of dihydropyridine binding was found to be increased after prolonged ethanol treatment, as were the effects of dihydropyridines on phosphatidylinositol turnover and neurotransmitter release (Dolin *et al.*, 1987).

In the current study we have compared the protective action of two dihydropyridine drugs (felodipine and nitrendipine) against the ethanol withdrawal syndrome with their ability to displace binding of radiolabelled isradipine ([^{3}H]-PN 200-110) at the dihydropyridine binding sites in the brain. The protective action of the drugs against the behavioural effects of the calcium channel activator, Bay K 8644, was also examined in control animals, as was the ability of the drugs to inhibit the hyperexcitability produced by handling following administration of bicuculline. The calcium antagonists, nitrendipine and felodipine, like other dihydropyridines, are both highly lipid soluble and we have previously demonstrated that nitrendipine passes the blood brain barrier (Dolin & Little, 1989; Whittington *et al.*, 1991), while felodipine has also been recently reported to have good penetration into the brain (Rigby *et al.*, 1991).

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A preliminary account of some of this work has been presented to the British Pharmacological Society (Watson *et al.*, 1992).

Methods

Animals

Male mice (TO strain, Bantin and Kingman, Hull or Olac, Bicester) weighing 32–38 g were used in all studies. They were made physically dependent on ethanol by a 14 day period of inhalation of ethanol/vapour in an inhalation chamber, at an ethanol concentration of 8 mg l^{-1} (Green *et al.*, 1990b; Whittington *et al.*, 1991). Controls were kept under identical conditions in the absence of ethanol.

Treatment protocol for subsequent measurement ex vivo *of [^{3}H]-isradipine binding in brains of naïve mice*

Control (naïve) mice were injected intraperitoneally with vehicle (Tween 80, 0.5% v/v in distilled water), nitrendipine (0.2, 1, 10 or 50 mg kg^{-1}) or felodipine (0.02, 0.1, 1, 2, or 10 mg kg^{-1}). They were killed either 30 min, 3 h or 5 h later, the brains removed and the whole brain minus cerebellum used for the binding assay (see below).

Treatment protocol for subsequent measurement ex vivo *of [^{3}H]-isradipine binding in brains of ethanol-dependent and control mice*

Following chronic ethanol treatment, mice were withdrawn and injected with felodipine (10 mg kg^{-1}), nitrendipine (50 mg kg^{-1}) or vehicle, 3 h later. At 6 h into withdrawal, the animals were killed and brains minus cerebellum used for measurement of [^{3}H]-isradipine binding. A group of control mice kept in comparable conditions but not made ethanol-dependent (controls) were also given felodipine, nitrendipine or vehicle as above and killed at the same time and [^{3}H]-isradipine binding by brain tissue was measured.

Treatment protocol for subsequent measurement of in vivo *displacement of [^{3}H]-nitrendipine binding in brains of naïve mice*

Control (naïve) mice were injected intraperitoneally with nitrendipine (50 mg kg^{-1}) or felodipine (10 mg kg^{-1}). Thirty minutes later [^{3}H]-nitrendipine (4 μCi in 0.1 ml) was injected via a tail vein. The mice were killed 1 min later, the brains removed and the whole brain used for the binding assay (see below).

Binding of [^{3}H]-isradipine to cerebral tissue

Whole brain (minus cerebellum) was homogenized in 100 volume ice cold Tris-HCl buffer (50 mM, pH 7.4) containing 0.1 mM phenylmethylsulphenyl fluoride. Each assay tube contained [^{3}H]-isradipine (final concentration 1.0 nM) and 400 μl tissue homogenate to a final volume of 500 μl. Nicardipine (10 μM) was used to determine non-specific binding. Tubes were incubated at 37°C for 30 min and the assay terminated by rapid filtration through glass fibre filters using a Skatron Cell Harvester. Filters were washed with polyethylene glycol 8000 (10% w/v) containing $CaCl_2$ (10 mM) and Tris HCl (20 mM, pH 7.4). After drying the filters were immersed in 2.0 ml of Emulsifier Safe scintillant and radioactivity counted.

Measurement of binding of [^{3}H]-nitrendipine to brain tissue after in vivo *injection of ligand*

The method followed that of Supavilai & Karobath (1984) as described in detail in Watson & Little (1994). One min following injection of [^{3}H]-nitrendipine (4 μCi in 0.1 ml) the brain was removed and homogenized at a concentration of 20 mg tissue ml^{-1} in ice cold Tris 4-Cl buffer. One ml of the homogenate was used as a measure of the total activity in each homogenate, to provide a correction for differences in the amount of ligand given to each mouse. Another 1 ml of homogenate was filtered through Whatman GF/B (2.5 cm) filters, and the filters washed quickly by 2 × 5 ml of ice-cold buffer (Tris-Cl, 50 mM, pH 7.4). This provided a measure of the activity bound to the membrane fragments (particulate binding). Each measurement was performed in triplicate. The samples were counted by scintillation spectrometry.

Non-specific binding was determined by incubation of homogenate with nimodipine (10 μM) to measure non-displaceable binding. Specific binding was expressed as a percentage of total activity in the brain homogenate, i.e. the particulate counts minus the nonspecific counts, divided by the total counts in 1 ml of homogenate.

Binding of [^{125}I]-ω-conotoxin to cerebral tissue

Whole brain (minus cerebellum) was homogenized in 1000 volumes (1 mg ml^{-1}) of Tris-HCl buffer (50 mM, pH 7.4) containing EGTA (10 mM). Each assay tube contained [^{125}I]-ω-conotoxin (50 pmol) and 160 μl of tissue preparation. Non-specific binding was determined by addition of ω-conotoxin (0.1 mM). Tubes were incubated at 25°C for 25 min and the incubation terminated by rapid filtration through glass fibre filters using a Skatron Cell Harvester. The buffer was Tris-HCl (100 mM, pH 7.4) containing bovine serum albumin (0.1% w/v) and $CaCl_2$ (92 nM). Filters were presoaked in polyethylenimine. After drying, filters were immersed in Emulsifier Safe scintillant (2 ml) and radioactivity counted.

Measurement of the ethanol withdrawal syndrome

Ethanol-dependent mice were removed from the ethanol chamber and coded between 08 h 00 min and 09 h 00 min in the morning. The dihydropyridines were administered intraperitoneally either at the start of the withdrawal period or 3 h into withdrawal. The severity of the withdrawal syndrome was measured each hour for up to 12 h by rating the handling-induced convulsive behaviour (Goldstein & Pal, 1971; Green *et al.*, 1990b). This method scores both the degree of tremor and convulsive movements, on a scale 0–4 with higher ratings indicating more severe convulsive behaviour. Ratings were made by an observer who was unaware of the prior drug treatment. Each treatment group contained ten mice. Groups were treated with either nitrendipine, felodipine or vehicle, as described in the Results section.

Previous studies have demonstrated that nitrendipine, given concurrently with ethanol in chronic treatment, prevented the ethanol withdrawal syndrome (see Discussion). Separate groups of mice were therefore administered felodipine (10 mg kg^{-1}) or vehicle, twice daily, during the 14 day period of ethanol vapour exposure. Twelve mice were used in each group. The last injection of felodipine was given 24 h before withdrawal of ethanol, and the withdrawal behaviour was examined as described above.

Measurement of the convulsive effect of Bay K 8644

The Bay K 8644 suspension (60 μg in 5 μl) was injected into the left cerebral ventricle of conscious mice and handling-induced convulsive behaviour measured 30, 60, 120 and 240 min later. Groups of mice ($n = 8$) were pretreated with either nitrendipine, felodipine or Tween vehicle 30 min before the injection of Bay K 8644. The convulsive behaviour on handling was rated as described in the section above on the ethanol withdrawal syndrome, by an observer who was unaware of the prior drug treatment.

Measurement of effects of bicuculline on convulsive behaviour

The effects of felodipine and nitrendipine were examined on the behaviour in response to handling after administration of bicuculline. Nitrendipine (50 mg kg^{-1}) or felodipine (10 mg kg^{-1}) were given intraperitoneally, followed 30 min later by bicuculline (2.7 mg kg^{-1}, i.p.). The behaviour in response to handling was then rated every 15 min for 60 min.

Measurement of effects of hexamethonium on convulsive behaviour

To control for possible consequences of the cardiovascular actions of the dihydropyridines on the behaviour measured in the experiments above, the effects of hexamethonium were examined. The dose of this compound was chosen on the basis of an earlier study in which we measured the blood pressure by a noninvasive method (Dolin & Little, 1989). Hexamethonium (8 mg kg^{-1}) lowered the blood pressure to a similar extent as nitrendipine (50 mg kg^{-1}). Hexamethonium (8 mg kg^{-1}) was given intraperitoneally followed 30 min later by bicuculline (2.8 mg kg^{-1}, i.p.). The behaviour in response to handling was then rated every 15 min for 60 min.

Drugs, drug administration and chemicals

The following drugs were used (sources in parentheses). Felodipine (Astra Hässle, Molndal, Sweden) and nitrendipine (Bayer Pharmaceuticals, Newbury) were suspended in Tween (0.5% v/v in distilled water). Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-fluoromethylphenyl)-pyridine-5-carboxylate, Bayer Pharmaceuticals) was suspended in Tween (0.5% v/v in Krebs buffer). Nitrendipine, felodipine and Bay K 8644 were used under red safe-light illumination. Pentylenetetrazol (Sigma Chemical Co., Poole) was dissolved in normal saline (NaCl 0.9% w/v in distilled water). Bicuculline (Sigma Chemical Co., Poole) was dissolved in 1 M HCl, then diluted with saline brought to pH 3 with HCl.

[^{3}H]-isradipine and [^{125}I]-ω-conotoxin were both obtained from N.E.N., Du Pont, Stevenage, Herts. All other reagents were obtained from Merck Ltd, Dagenham, Essex.

Statistics

The ratings of behaviour during withdrawal, after Bay K 8644 and bicuculline were compared by nonparametric analysis of variance, designed for repeat measures on the same animals (Meddis, 1984). In the study on the effects of Bay K 8644, ratings of handling-induced behaviour at each time point were also compared by the Mann-Whitney U test. The incidence of convulsions was analysed by Fisher's exact probability-test.

Results

Assessment of the dissociation of felodipine and nitrendipine from dihydropyridine binding sites in brain

Since a major part of the current investigation relied on measures of [^{3}H]-isradipine binding *ex vivo* following administration of felodipine or nitrendipine, an experiment was first performed to determine whether felodipine or nitrendipine would be likely to dissociate from cerebral binding sites during the preparation of the tissue. The amount of tissue homogenate (80 μl) added to the assay was kept constant, while the total incubation medium containing [^{3}H]-isradipine (1 nM) was increased to 100 μl, 200 μl, 500 μl or 1000 μl.

Mice were injected with felodipine (0.3 mg kg^{-1}), nitrendipine (10 mg kg^{-1}) or vehicle, killed 30 min later and tissues prepared for measurement of [^{3}H]-isradipine binding as described above.

Administration of either compound resulted in a substantial inhibition of *ex vivo* [^{3}H]-isradipine binding, the value of which decreased only slightly with increasing assay volume for both drugs (Figure 1). The data suggest therefore that *ex vivo* binding gives a reasonable estimate of *in vivo* receptor occupancy of both drugs, justifying its use in this investigation.

[^{3}H]-isradipine binding in the brain, measured ex vivo *at various times after injection of felodipine or nitrendipine*

The degree of inhibition of [^{3}H]-isradipine binding by various doses of felodipine and nitrendipine, was measured *ex vivo* either 30 min, 3 h or 5 h after injection of felodipine (Figure 2a) or nitrendipine (Figure 2b). The inhibition versus dose curves were sigmoid and the dose required to produce a 50% inhibition of binding increased linearly with time after administration (Figure 3). These data also indicate that over 10 times the dose of nitrendipine was required at any time to achieve the same degree of inhibition as that achieved by injection of felodipine.

The binding of [^{3}H]-nitrendipine measured in vivo *in whole brain following felodipine or nitrendipine injections*

Administration of nitrendipine (50 mg kg^{-1}) or felodipine (10 mg kg^{-1}) caused displacement of [^{3}H]-nitrendipine measured *in vivo* (Figure 4). The extent of displacement was similar for these doses of the two compounds.

[^{3}H]-isradipine binding in the brains of ethanol-dependent mice injected with felodipine or nitrendipine

Mice dependent on ethanol were injected with either felodipine (10 mg kg^{-1}) or nitrendipine (50 mg kg^{-1}) 3 h into withdrawal. These doses were chosen from the above study on displacement of [^{3}H]-isradipine binding by these compounds, as being necessary to produce around a 75% occupation of dihydropyridine binding sites.

There was a similar degree of inhibition of [^{3}H]-isradipine binding following felodipine or nitrendipine in both the control and ethanol withdrawn mice (Table 1). The degree of inhibition of [^{3}H]-isradipine binding was also similar to that seen in naïve mice (Table 1).

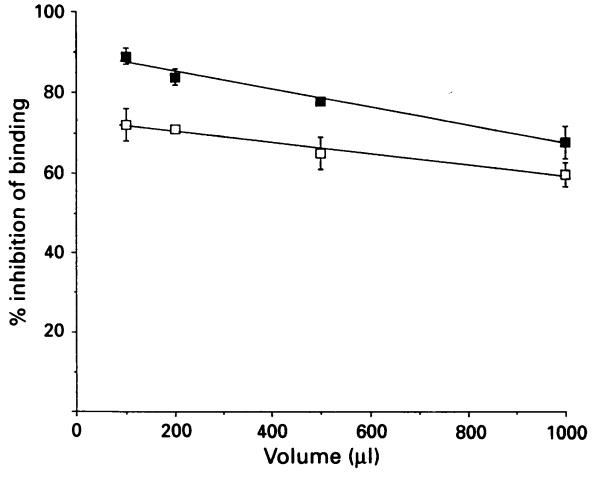


Figure 1 The relationship between the incubation volume and percentage inhibition of binding of [^{3}H]-isradipine in cerebral tissue *in vivo* following injection of nitrendipine, 10 mg kg^{-1} (■) or felodipine, 0.3 mg kg^{-1} (□). Results expressed as mean with s.e.mean ($n = 4$).

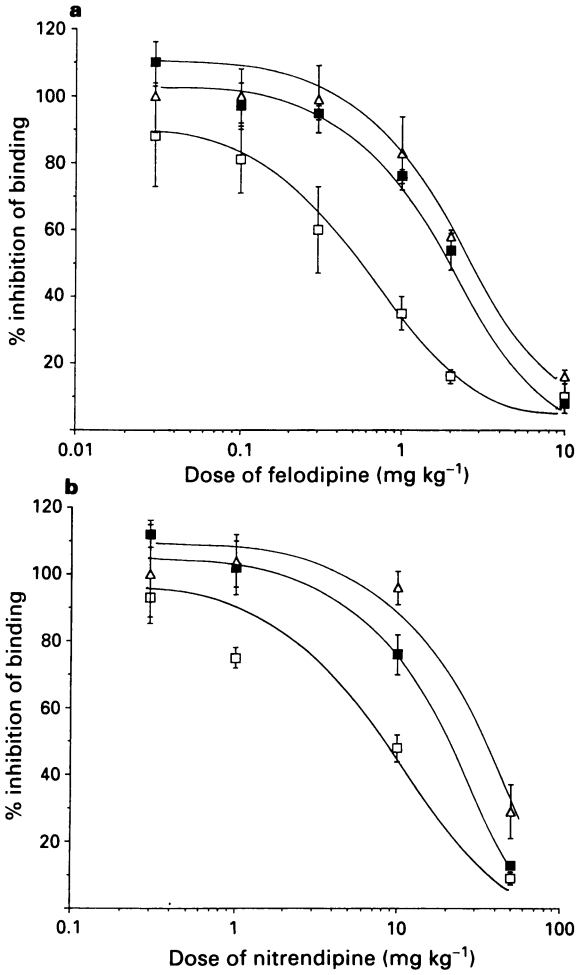


Figure 2 Percentage inhibition of [^{3}H]-isradipine binding in cerebral tissues *ex vivo* at various times following injection of dihydropyridines. (a) The inhibition of binding 30 min (□), 3 h (■) or 5 h (△) following various doses of felodipine. (b) The inhibition of binding 30 min (□), 3 h (■) or 5 h (△) following various doses of nitrendipine. Results shown mean ± s.e.mean of 4 separate determinations.

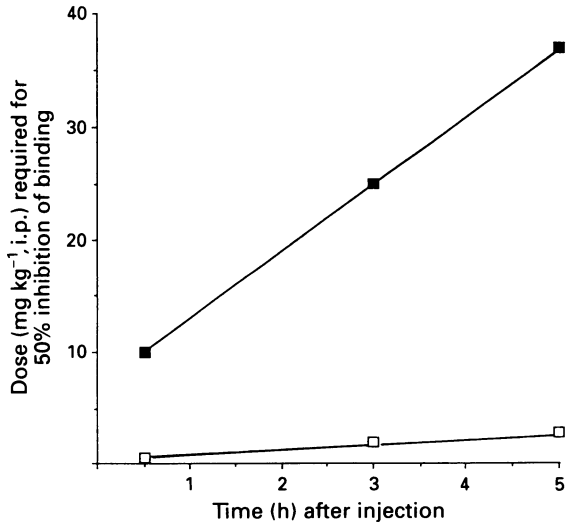


Figure 3 The dose of nitrendipine (■) or felodipine (□) required to produce a 50% inhibition of cerebral [^{3}H]-isradipine binding at various times following dihydropyridine administration i.p. Data recalculated from results given in Figure 2a and b.

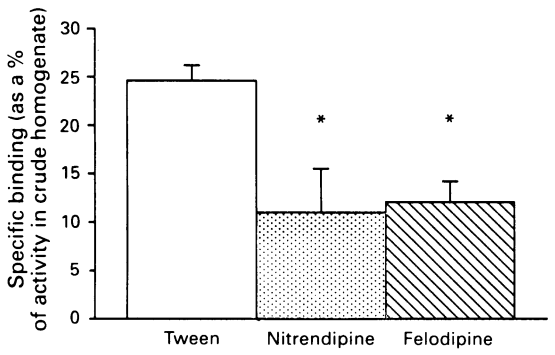


Figure 4 Displacement of [^{3}H]-nitrendipine in whole brain following intravenous injection of the ligand and intraperitoneal injections of felodipine (10 mg kg^{-1}) or nitrendipine (50 mg kg^{-1}). Both compounds caused significant displacement of the ligand $^*P<0.05$, compared with the results after injection of vehicle.

[^{125}I]-ω-conotoxin binding in the brain of ethanol-dependent mice injected with felodipine or nitrendipine

Specific [^{125}I]-ω-conotoxin binding was similar in control and ethanol-dependent mice at 3 h into withdrawal (Table 1). Administration of nitrendipine (50 mg kg^{-1}) or felodipine (10 mg kg^{-1}) did not alter the degree of [^{125}I]-ω-conotoxin binding in the control or ethanol withdrawn mice after a further 3 h (Table 1).

Effects of felodipine and nitrendipine on the ethanol withdrawal syndrome

Acute administration of felodipine (10 mg kg^{-1}) immediately on withdrawal or 3 h after withdrawal from ethanol did not produce any significant alteration of the withdrawal behaviour, as measured by handling-induced behaviour (Figure 5a and b). In contrast, confirming previous studies (see Discussion), nitrendipine (50 mg kg^{-1}) administration, given at 3 h after withdrawal produced a complete attenuation of withdrawal hyperexcitability (Figure 5b). When a mild audiogenic stimulus was given at 9 h into the withdrawal period, a syndrome of clonic seizures accompanied by wild running was produced in some animals (Figure 6a); this syndrome was not seen in control animals which had not received ethanol. There was a significant increase ($P<0.05$) in the incidence of this response in animals given felodipine (5 mg kg^{-1}) on withdrawal, compared with those given vehicle injections. The increased incidence in mice given felodipine (2 mg kg^{-1}) did not reach statistical significance, while the incidence following felodipine (10 mg kg^{-1}) did not differ from vehicle injected animals. Nitrendipine (25 or 50 mg kg^{-1}) did not significantly alter the incidence of this response (Figure 6a).

Effects of felodipine given concurrently with ethanol in chronic treatment

The administration of felodipine (10 mg kg^{-1}) twice daily throughout the ethanol treatment did not prevent the withdrawal hyperexcitability, as measured by ratings of convulsive behaviour (Figure 6b).

Effects of pretreatment with felodipine or nitrendipine on handling-induced convulsive behaviour induced by Bay K 8644 injection

Injection of Bay K 8644 (60 μg, i.c.v.) produced a significant increase in handling-induced convulsive behaviour ($P<0.01$) for at least 2 h after injection; this declined to control values

Table 1 *Ex vivo* binding of [^{3}H]-isradipine and [^{125}I]-ω-conotoxin in control and ethanol-treated mice 3 h following vehicle, nitrendipine (50 mg kg^{-1}) or felodipine (10 mg kg^{-1})

	[^{3}H]-isradipine		*[^{125}I]-ω-conotoxin binding*	
Injected	*Control*	*Ethanol-treated*	*Control*	*Ethanol-treated*
Vehicle	16.00 ± 0.42	16.50 ± 0.43	12.39 ± 0.43	11.33 ± 0.55
Nitrendipine	2.63 ± 0.32 [84%]	2.52 ± 0.24 [85%]	12.75 ± 0.56	12.59 ± 0.35
Felodipine	3.69 ± 0.37 [77%]	4.93 ± 0.61 [71%]	12.75 ± 0.29	11.94 ± 0.39

Experiment performed 3 h into ethanol withdrawal. Results expressed in fmol mg^{-1} tissue and shown as mean ± s.e.mean (n = 10). Figures in square brackets are percentage inhibition of binding in dihydropyridine-treated mice compared to relevant vehicle treated animals.

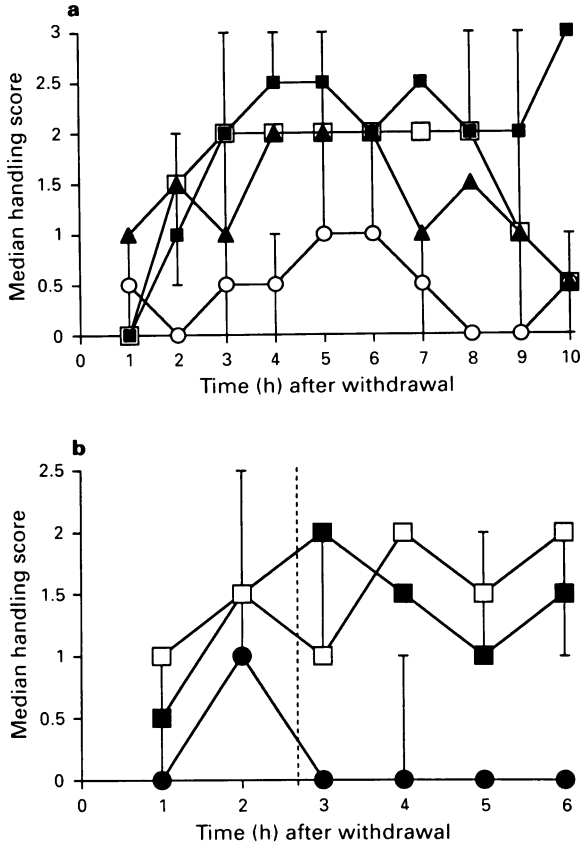


Figure 5 Handling-induced convulsive behaviour following ethanol withdrawal in mice. Values are medians with interquartile ranges on every third value. (a) Drugs or vehicle given immediately on withdrawal; (○) control animals, not treated with ethanol, given Tween vehicle at 0 h; (□) Tween vehicle on withdrawal from ethanol; (▲) felodipine (2 mg kg^{-1}) on withdrawal from ethanol; (■) felodipine (10 mg kg^{-1}) on withdrawal from ethanol. (b) (□) Tween vehicle given 3 h after withdrawal from ethanol; (■) felodipine (10 mg kg^{-1}) given 3 h after withdrawal from ethanol; (●) nitrendipine (50 mg kg^{-1}) given 3 h after withdrawal from ethanol. Dashed line indicates time of drug injection.

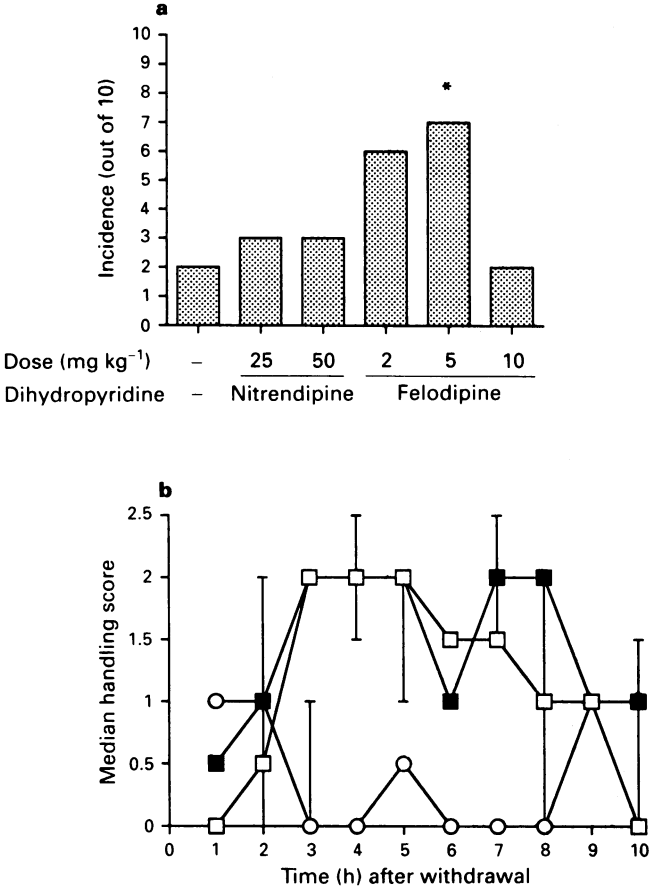


Figure 6 (a) Number of mice showing clonic seizures and wild running in response to a mild audiogenic stimulus, 9 h after ethanol withdrawal. Drugs were given immediately on withdrawal from the chronic ethanol treatment. (b) Handling-induced convulsive behaviour following ethanol withdrawal in mice. Values are medians with interquartile ranges on every third value. (○) Control animals, not treated with ethanol; (□) Tween vehicle given chronically with the ethanol; (■) felodipine (10 mg kg^{-1}) given chronically with the ethanol.

by 4 h. Felodipine (10 mg kg^{-1}) given 30 min before the Bay K 8644, significantly ($P<0.01$) reduced the convulsive behaviour measured at 60 min or 120 min after the Bay K 8644 injection, when comparisons were made with concurrent results obtained from animals given the vehicle (Table 2). In contrast, injection of nitrendipine (50 mg kg^{-1}) 30 min before the Bay K 8644, was without effect on the convulsive behaviour at 60 min after the Bay K 8644 injection, and had a marginal but statistically significant ($P<0.05$) effect at 120 min after the Bay K 8644 (Table 2). Neither felodipine nor nitrendipine reduced the handling scores measured at 30 min after the administration of Bay K 8644.

Effects of pretreatment with felodipine or nitrendipine on handling-induced convulsive behaviour following bicuculline injection

Injection of bicuculline produced a significant increase in handling-induced convulsive behaviour ($P<0.01$) for 60 min after injection. Nitrendipine (50 mg kg^{-1}) or felodipine (10 mg kg^{-1}, i.p.) given 30 min before the bicuculline, reduced the convulsive behaviour (Table 3). The effects of both dihydropyridines were significant ($P<0.05$, compared over the whole experimental period, with results after injection of vehicle).

Table 2 Effect of Bay K 8644 on handling-induced convulsive behaviour and the effect of pretreatment with felodipine or nitrendipine

Injected i.p.	*Bay K 8644 (i.c.v.)*	*Time* (min) *30*	*60*	*120*	*240*
Vehicle	– –	0 (0–1.5)	1 (0.5–2)	0 (0–1.5)	0.5 (0–1)
Vehicle	+	3 (3–3.5)†	2.5 (2.5–3)†	2 (1–2.5)†	1 (1–2)
Felodipine	+	3 (2.5–3)	1 (1–2)**	1 (0–1)**	0 (0–0.5)
Nitrendipine	+	3 (3–4)	2 (2–2)	1.5 (1–1.5)*	1 (0–1)

The median convulsive scores (with interquartile ranges) following injection of Bay K 8644 (60 μg, i.c.v.) and the effect of pretreatment with felodipine (10 mg kg^{-1}) or nitrendipine (50 mg kg^{-1}). Different from vehicle-treated mice not given Bay K 8644: †$P<0.01$. Different from vehicle-treated mice given Bay K 8644: **$P<0.01$; *$P<0.05$.

Table 3 Effect of pretreatment with felodipine, nitrendipine or hexamethonium on the action of bicuculline (2.8 mg kg^{-1}) on handling-induced convulsive behaviour

	Time (min) *after bicuculline injection* 15	30	45	60
Tween	–	–	2 (1–2.5)	2 (1.5–2)
Felodipine 10 mg kg^{-1}	–	–	0 (0–0)**	0 (0–0)**
Nitrendipine 50 mg kg^{-1}	–	–	0.5 (0–1)*	0 (0–0)**
Saline	3 (2–3)	2 (2–3)	2 (1–2)	1 (1–2)
Hexamethonium 8 mg kg^{-1}	2 (2–3)	2 (1.5–3)	2 (1–2)	1 (0–1.5)

Values are median convulsive scores (with interquartile ranges).
**$P<0.01$ compared with vehicle-treated mice; *$P<0.05$ compared with vehicle-treated mice.

Effects of treatment with hexamethonium on handling-induced convulsive behaviour following bicuculline injection

Hexamethonium (8 mg kg^{-1}) had no effect on the hyperexcitability produced by bicuculline (2.8 mg kg^{-1}) (Table 3).

Discussion

Both felodipine and nitrendipine are essentially insoluble in aqueous solution, and non-specifically bound dihydropyridine would therefore not be expected to pass into the aqueous phase during the *ex vivo* assay and interfere with the binding measures. Furthermore the initial experiments on the dissociation of felodipine and nitrendipine from cerebral dihydropyridine binding sites showed that this would be very small during the course of the assay (Figure 1). Overall therefore it was felt that one could have confidence in using *ex vivo* binding as a reflection of receptor occupancy *in vivo*. This was confirmed by the similarity of the displacement *in vivo* of [^{3}H]-nitrendipine measured at a single time point.

Our data thus enabled us to determine the degree of receptor occupancy following both different doses of the dihydropyridine drugs and at various times following drug administration (Figures 2 and 3). Using this information we then selected doses of felodipine and nitrendipine to produce a similar degree of receptor occupancy *in vivo* and examined the consequences of the drugs on the ethanol withdrawal syndrome. Nitrendipine, as has been shown previously (Littleton *et al.*, 1990), prevented the appearance of the ethanol withdrawal syndrome. Felodipine, in contrast, had no effect on the behavioural excitability caused by withdrawal as measured by handling-induced convulsive behaviour. The degree of receptor occupancy following felodipine in the ethanol-dependent mice appeared slightly lower than after nitrendipine but the small size of this difference made it unlikely that lower receptor occupancy following felodipine was the reason for the lack of effect of this compound on the syndrome. This view is supported by the effect of nitrendipine on withdrawal which lasted for several hours after administration, since examination of Figures 2 and 3 shows that by 5 h after injection the degree of receptor occupancy by nitrendipine would be substantially lower than that 30 min following felodipine. Even concomitant felodipine administration during the period when ethanol was being administered failed to alter the withdrawal syndrome, again in contrast to nitrendipine (Whittington *et al.*, 1991).

Previous studies have provided considerable evidence to suggest that dihydropyridine-sensitive binding sites are involved in ethanol dependence. This evidence has been obtained not only from behavioural studies showing prevention of the withdrawal syndrome by a variety of calcium antagonists (Little *et al.*, 1986; Whittington *et al.*, 1991), but also from neurochemical studies (Dolin *et al.*, 1987; Whittington *et al.*, 1991) and electrophysiological analysis of withdrawal hyperexcitability. The latter experiments have demonstrated selective and stereospecific prevention by isradipine of epileptiform activity produced by ethanol withdrawal in isolated hippocampal slices (Whittington & Little, 1991) and increased dihydropyridine-sensitive calcium spikes during the withdrawal phase (Whittington *et al.*, 1992). However, the present results suggest that occupation of dihydropyridine binding sites may not necessarily prevent the ethanol withdrawal syndrome. Although felodipine showed competition for these binding sites, it did not protect against the convulsive behaviour which can be produced by handling during ethanol withdrawal. The increased incidence of audiogenic seizures seen in ethanol-dependent animals given felodipine, further illustrated the real difference between the actions of nitrendipine and those of felodipine on ethanol withdrawal hyperexcitability. It should be emphasised that these results probably have little relevance to any action of dihydropyridines when they are given clinically for hypertension since the human daily therapeutic dose is 20–50 times lower than those used in this study.

One explanation for these results might be that felodipine has a partial agonist action at dihydropyridine receptors. This would explain the actions on the binding and on the withdrawal signs. The state of the receptor may be altered by the chronic ethanol treatment. It has been found in cultured cells that the relative proportions of agonist and antagonist properties of dihydropyridines can be altered by changes in G-proteins (Scott & Dolphin, 1987). It is also possible that felodipine may have an additional action at a different site from nitrendipine on the dihydropyridine complex. This com-

plex contains at least five different binding sites and we recently showed that diltiazem, which increases dihydropyridine binding through an allosteric action at another site on the complex, increased both ethanol withdrawal hyperexcitability and the behavioural effects of Bay K 8644 (Watson & Little, 1992a,b), even though in the periphery, diltiazem blocks calcium channels.

There is also evidence that the membrane potential affects the binding of dihydropyridines and that depolarization increases the affinity for the receptor (Bean, 1989). Nevertheless the behavioural effects of the calcium channel agonist Bay K 8644, were more effectively antagonized by felodipine than nitrendipine. These data, at least, suggest that felodipine is acting on the CNS opposing the action of a drug that is acting at calcium channels.

It is also clear that felodipine and nitrendipine can produce some similar actions in the brain since they had similar anticonvulsant actions against bicuculline. The mechanism by which dihydropyridines produce their weak anticonvulsant action however is not known, but the present results suggest that it differs from that by which nitrendipine prevents the ethanol withdrawal syndrome. Previous experiments using nitrendipine and nimodipine against convulsion incidence after other convulsant drugs showed no protective effect (Dolin *et al.*, 1988).

Of course, it cannot be assumed that dihydropyridines have effects in the brain that are solely related to their actions at the dihydropyridine binding site. Previous studies have found verapamil and nicardipine to be potent 5-HT_2 receptor antagonists (Affolter *et al.*, 1985; Green *et al.*, 1990a) and, while felodipine bound poorly to the site, it nevertheless at a high dose antagonized the 5-HT_2 receptor-mediated head twitch behaviour of mice, in common with several other Ca^{2+} antagonists (Green *et al.*, 1990a). Such data serve to demonstrate the problems in trying to correlate *in vivo* functional changes with either *in vitro* or *in vivo* biochemical analysis. In this regard the lack of change in [^{3}H]-isradipine binding in the ethanol-dependent mice contrasts with several reports of increases in dihydropyridine binding measured *in vitro* (Dolin *et al.*, 1987; Dolin & Little, 1989; Whittington *et al.*, 1991) but is in agreement with another report on the failure to find a significant increase in [^{3}H]-nitrendipine binding to synaptosomes prepared from ethanol-dependent rats (Woodward *et al.*, 1990). It is also in contrast to the study on *in vivo* [^{3}H]-nitrendipine binding, measured during ethanol withdrawal (Watson & Little, 1994). With regard to the *in vitro* studies it is possible that this difference was due to the different ligand used, as the other studies have used either [^{3}H]-nitrendipine or [^{3}H]-nimodipine. The actions of nitrendipine and other dihydropyridines which protect against the withdrawal syndrome are not necessarily mediated via the 'L' subtype of channel. These compounds are known to affect other sites, including actions at low concentrations on the adenosine transporter (Deckert *et al.*, 1993). Felodipine, and isradipine, may not share these actions.

It should also be noted that we found no change *ex vivo* in [^{125}I]-ω-conotoxin binding in the ethanol-dependent mice even though it has been reported that the response of synaptosomes to the addition of ω-conotoxin can change in ethanol-dependent rats (Woodward *et al.*, 1990).

In conclusion therefore, we have demonstrated that felodipine will bind to dihydropyridine sites in the brain and does so when given at much lower doses than nitrendipine. However, nitrendipine administration decreased the ethanol withdrawal syndrome while felodipine administration was without effect on the handling-induced convulsions, and, at one dose, increased the incidence of audiogenic seizures. Given the considerable evidence for the involvement of dihydropyridine sites in the ethanol withdrawal syndrome, five possibilities exist to explain the data. (1) The drugs do not act at identical sites in the brain; (2) felodipine may act as a partial agonist at some sites in the CNS; (3) the two enantiomers of felodipine may have differential relative effects at certain sites; (4) felodipine may have actions which mask or prevent the protective effect on withdrawal; (5) the action of dihydropyridines in protecting against the withdrawal syndrome is an effect of the drugs which is distinct from their action at dihydropyridine binding sites.

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Effects of GABA and various allosteric ligands on TBPS binding to cloned rat $GABA_A$ receptor subtypes

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1 [^{35}S]*t*-butylbicyclophosphorothionate (TBPS) is a high affinity ligand for the picrotoxin site of $GABA_A$ receptors. Here we examined TBPS binding to the cloned receptors made of α1, α3 or α6 in combination with β2 or β2 and γ2 subunits, in the presence of GABA and several allosteric ligands (diazepam, methyl 6,7-dimethoxy-4-methyl-β-carboline-3-carboxylate (DMCM), 3α,21-dihydroxy-5α-pregnan-20-one (5α-THDOC), pentobarbitone and Zn). The cloned receptors were transiently expressed in SF-9 insect cells by infecting with recombinant baculoviruses.

2 In αβ subtypes, GABA at nanomolar concentrations enhanced TBPS binding but inhibited binding at micromolar concentrations. Half maximal GABA concentrations for enhancement or inhibition of TBPS binding were correlated with high and low affinity GABA binding sites, respectively, in individual subtypes. The maximal enhancement of binding also varied according to the α isoform (α3β2>>α1β2>α6β2). In αβγ subtypes, TBPS binding was unaffected by GABA at nanomolar concentrations, but was inhibited by GABA at micromolar concentrations. Addition of γ2 thus appeared to abolish conformational coupling between high affinity GABA sites and TBPS sites, and also altered low affinity GABA sites; in particular, the half maximal GABA concentration for inhibition of TBPS binding changed from >100 (α6β2) to 1 μM (α6β2γ2).

3 Allosteric ligands also altered TBPS binding to sensitive $GABA_A$ receptor subtypes. For instance, diazepam only in the α1β2γ2 and α3β2γ2 subtypes, and 5α-THDOC in all the subtypes enhanced TBPS binding in the absence of GABA, and intensified the inhibitory effect of GABA. Pentobarbitone exhibited only the latter effect in all the subtypes we examined.

4 DMCM and Zn, inhibitors of GABA-induced Cl currents in αβγ and αβ subtypes, respectively, produced opposite effects to agonists, decreasing TBPS binding in the absence of GABA and attenuating (or eliminating in the case of Zn) the inhibitory effect of GABA on TBPS binding.

5 These results show that GABA binding sites and their conformational coupling with TBPS sites are differentially affected by the α isoform (particularly α6 as compared to α1 or α3) and by quaternary interactions involving the γ2 subunit. Moreover, changes in TBPS binding by allosteric ligands include not only direct (allosteric) effects on TBPS sites but also indirect effects via GABA sites, and are consistent with their known subtype selectivity and functionality from previous studies.

Keywords: GABA; $GABA_A$ receptors; $GABA_A$ receptor subunits; GABA ligands; allosteric [^{3}H]TBPS binding; cloned receptors

Introduction

[^{35}S]*t*-butylbicyclophosphorothionate (TBPS) is a high affinity ligand to the picrotoxin site on $GABA_A$ receptors (Squires *et al.*, 1983), which is presumably located near the mouth of the chloride channel of the receptor complex. TBPS binding has received much attention because of its sensitivity to GABA and various allosteric ligands (Trifiletti *et al.*, 1984; Gee *et al.*, 1986; Lloyd *et al.*, 1990; Im & Blakeman, 1991; Sieghart, 1992; Squires & Seaderap, 1993). It has been shown in rat brain membranes that GABA inhibits TBPS binding, and its action is potentiated by agonists, such as benzodiazepines, neurosteroids and barbiturates, and partially reversed by inverse agonists, such as β-carbolines (Trifiletti *et al.*, 1984; Gee *et al.*, 1986; Lloyd *et al.*, 1990; Im & Blakeman, 1991; Sieghart, 1992; Squires & Seaderap, 1993). These results appear to be consistent with TBPS being a probe sensitive to the conformational changes in the chloride channel of the receptor complex. However, recent cloning studies have revealed that $GABA_A$ receptors are made of various subunits which exist in several isoforms in mammalian brains, and that there is functional diversity of cloned receptors depending on their subunit compositions (Barnard *et al.*, 1987; Schofield, 1989; Olsen & Tobin, 1990; Sigel *et al.*, 1990; Verdoorn *et al.*, 1990). This physical and functional multiplicity of $GABA_A$ receptors in brain membranes makes it difficult to study mechanisms of actions for GABA and allosteric ligands on TBPS binding. It would be useful to study TBPS binding in individual cloned receptors. Earlier we carried out an initial study on TBPS binding to α1 containing receptors (Pregenzer *et al.*, 1993), expressed in SF-9 insect cells infected with baculovirus carrying cDNAs for $GABA_A$ subunits (Carter *et al.*, 1992). This current study represents an expanded investigation on the effects of GABA and several allosteric ligands on TBPS binding to three major classes of $GABA_A$ receptor subtypes, Type 1 (α1β2γ2), Type 2 (α3β2γ2) and the third receptor type unique to cerebellar granule cells (α6β2γ2), and their counterparts without γ2. We found that GABA-sensitivity of TBPS binding varied widely among the subtypes, and that changes in TBPS binding by allosteric ligands correlate with their functional characteristics deduced from electrophysiological studies of the various $GABA_A$ receptor subtypes.

Methods

The growth of SF-9 cells as well as that of the baculoviruses for cloning of GABA receptors have been described previously (Carter *et al.*, 1992). Briefly, SF-9 cells were grown in serum-free Grace medium (Gibco) to a cell density of 1×10^6 cells ml^{-1} and then infected with baculovirus constructs (AcNPV) carrying rat cDNAs for $GABA_A$ receptor subunits (Levitan *et al.*, 1988; Khrestchatisky *et al.*, 1989; Shivers *et al.*, 1989; Ymer *et al.*, 1989; Luddens *et al.*, 1990). The original titer of each virus was 1×10^8 plaque fermenting units (PFU) ml^{-1}. Baculoviruses carrying desired cDNAs

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were mixed in equal ratio (1×10^6 virus particles for each) and co-infected into SF-9 cells. The cells were grown in 2 l batches, harvested 60 h after infection, and homogenized in a solution containing NaCl 118 mM, KCl 5 mM and HEPES/Tris 20 mM pH 7.3, with Polytron PT 3000 (Brinkman) for 4 min. Unbroken cells and large nuclei aggregates were removed by centrifugation at 1000 g for 10 min. The membranes were recovered with a second centrifugation of the supernatant at 40,000 g for 50 min, were resuspended to a final concentration of 5 mg ml^{-1} in a solution containing sucrose 300 mM, Tris/HCl 5 mM, pH 7.5, and glycerol (a final concentration of 20%), and stored at −80°C.

Binding of radioactive ligands was measured by filtration techniques as described elsewhere (Pregenzer *et al.*, 1993). Briefly, for binding of [^{3}H]-flunitrazepam and [^{3}H]-muscimol we used a medium containing mM: NaCl 118, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2, HEPES/Tris (pH 7.3) 20, varying concentrations of the radioactive ligand (0.5 to 60 nM for flunitrazepam, and 2 to 160 nM for muscimol), and 30 μg membrane proteins in a total volume of 500 μl. The reaction was carried out at 4°C for 60 min. For TBPS binding, we used a medium containing NaCl 1 M, and Tris/HCl (pH 7.4) 10 mM, [^{35}S]-TBPS ranging from 2 to 160 nM, and 50 μg membrane proteins in a total volume of 500 μl. For experiments to examine the effects of GABA or allosteric ligands, the level of [^{35}S]-TBPS was fixed at 3 nM. The mixtures were incubated for 120 min at 24°C. At the end of the reaction, the membranes were separated by filtration over a Whatman GF/B filter under vacuum, and used to determine membrane bound radioactivity. Non-specific binding was estimated in the presence of 200 μM diazepam for [^{3}H]-flunitrazepam, 100 μM muscimol for [^{3}H]-muscimol, or 1 mM picrotoxin for [^{35}S]-TBPS, and was subtracted to compute specific binding.

Dissociation constants (K_d) and maximal binding sites (B_{max}) for [^{3}H]-muscimol, [^{3}H]-flunitrazepam, and [^{35}S]-TBPS were obtained from Scatchard analysis of the binding data and are presented as the mean ± standard errors (s.e.) from three experiments, each consisting of triplicate measurements. In the range of ligand concentrations used here, Scatchard plots for muscimol, TBPS and flunitrazepam were linear and fit one site models. Dose-response profiles for GABA action on TBPS binding were analysed by the following logistic equations (Black & Leff, 1983):

$$E = Emax[GABA]^n/([K_{0.5}]^n + [GABA]^n) \quad \text{Equation 1}$$

$$E = ((Emax1[GABA]^n/([K1_{0.5}]^n + [GABA]^n + 100) - ((Emax1 + 100)[GABA]^n/([K2_{0.5}]^n + [GABA]^n) \quad \text{Equation 2}$$

where E is GABA-induced response (% of control), Emax is the maximal response, $K_{0.5}$ (equivalent to EC_{50} or IC_{50}) is GABA concentration for the half maximal response, and n is the degree of cooperativity. Equation 1 was used for a monophasic response and Equation 2 for a biphasic response for GABA (see the α1β2 profile Figure 1). The analysis was carried out using least-squares fitting methods (Sigma plot).

GABA-mediated Cl^- currents in SF-9 cells were measured by the whole cell configuration of the patch clamp technique (Hamill *et al.*, 1981; Draguhn *et al.*, 1990). Briefly, patch pipettes prepared from borosilicate glass tubes were filled with a solution containing (mM): CsCl 140, EGTA 11, $MgCl_2$ 4, ATP 2 and HEPES 10, pH 7.3. The external solution contained (mM): NaCl 135, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1.8 and HEPES 5 pH 7.2. GABA and drugs were dissolved in the external solution to a final concentration of 5 μM, unless indicated otherwise, and were applied through a U-tube placed within 100 μm of the target cell. The current was recorded with an Axopatch 1D amplifier and a CV-4 headstage (Axon Instrument Co.). GABA currents were measured at the holding potential of −60 mV at room temperature (21–24°C).

[^{3}H]-flunitrazepam, [^{3}H]-Ro 15-4513, [^{3}H]-muscimol and [^{35}S]-TBPS were obtained from NEN Research Products, Du Pont Company. GABA, DMCM and TBPS were purchased from Research Biochemical Incorporated. BaculoGold transfection kit and SF-9 cells were obtained from PharMingen. Pentobarbitone and 5α-THDOC were from the Upjohn Company.

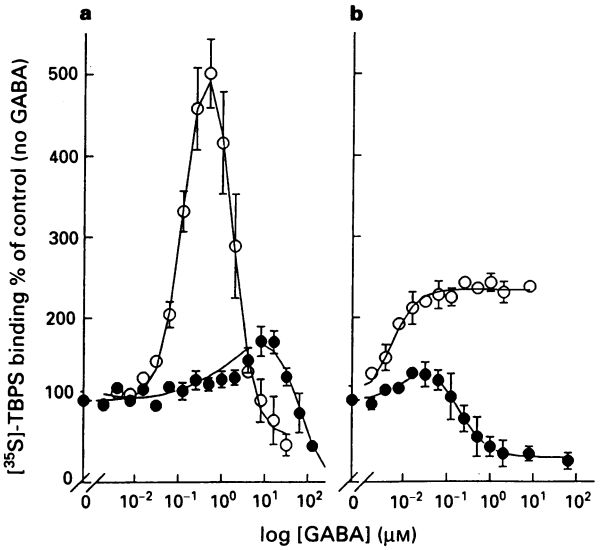


Figure 1 Sensitivity of TBPS binding to GABA concentrations in subtypes of cloned $GABA_A$ receptors. Binding of [^{35}S]-TBPS (3 nM) was measured in the presence of various GABA concentrations, using filtration techniques, with the membranes of SF-9 cells infected for 60 h with baculovirus carrying indicated cDNAs for $GABA_A$ receptors. Non-specific binding of the ligand was estimated in the presence of 1 mM picrotoxin and used to compute the specific binding. In (a) (○) α3β2; (●) α3β2γ2; (b) (○) α6β2; (●) α6β2γ2. The binding data were normalized to that observed without GABA, and represent the mean ± s.e. from three experiments. The other experimental details were as described in Methods.

Results

Ligand binding parameters in cloned $GABA_A$ receptor subtypes

We compared equilibrium binding parameters for [^{3}H]-muscimol, [^{3}H]-flunitrazepam (or [^{3}H]-Ro 15-4513 for the α6β2γ2 subtype) and [^{35}S]-TBPS in the α3β2, α3β2γ2, α6β2 and α6β2γ2 subtypes of cloned $GABA_A$ receptors in membranes of SF-9 cells which had been infected with corresponding recombinant baculoviruses (Table 1). The data confirmed several trends we had already observed with the α1β2 and α1β2γ2 subtypes in an earlier study (Pregenzer *et al.*, 1993). (1) Addition of γ2 to the α3β2 or α6β2 subtype had no appreciable effect on the dissociation constants (K_d) for muscimol and TBPS. (2) Benzodiazepine binding (Ro 15-4513 or flunitrazepam) was observed only in the receptors containing γ2. (3) The maximal binding site (B_{max}) for muscimol which represents the high affinity GABA site closely matched with that for TBPS, and may represent the total number of $GABA_A$ receptors. Besides these trends, we noted that the K_d values for muscimol and TBPS in the receptors containing α6 (an average value of 4 nM for muscimol and 47 nM for TBPS) were considerably lower than those observed in the receptors containing α3 (an average value of 13 nM for muscimol and 128 nM for TBPS). Furthermore, the B_{max} for Ro 15-4513 in the α3β2γ2 subtype was nearly equal to those for muscimol and TBPS, but about 30% greater than that for flunitrazepam. Both ligands were expected to interact with the same site (benzodiazepine site). Further study may be needed to clarify this discrepancy.

Table 1 Binding parameters for [^{3}H]-muscimol, [^{3}H]-Ro 15-4513, [^{3}H]-flunitrazepam and [^{35}S]-TBPS at equilibrium

	Muscimol		*Ro 15-4513*		*Flunitrazepam*		*TBPS*	
	K_d	B_{max}	K_d	B_{max}	K_d	B_{max}	K_d	B_{max}
α3β2	14.2 ± 4.8	5.1 ± 0.4	ND	ND	ND	ND	122 ± 17	4.4 ± 3
α3β2γ2	9.1 ± 2.2	4.2 ± 0.4	8.7 ± 2	4.5 ± 0.3	4.7 ± 0.4	3.2 ± 0.4	135 ± 47	4.5 ± 0.6
α6β2	3.1 ± 0.5	5.2 ± 0.5	ND	ND	ND	ND	48 ± 7	5.8 ± 0.6
α6β2γ2	4.8 ± 0.3	9.5 ± 1.3	11.6 ± 2.1	11.4 ± 1.2	ND	ND	45 ± 7	8.8 ± 0.5

Binding studies were carried out in the cell membranes of SF-9 cells infected for 60 h with baculoviruses carrying indicated cDNAs for rat $GABA_A$ receptor subunits. Experimental conditions were described in detail in Methods. The parameters were obtained from Scatchard analysis of binding data, and represent the mean ± standard errors from three experiments, each consisting of triplicate measurements. The unit for K_d values is nM, and that for B_{max} values is pmol mg^{-1} protein. ND: No detectable binding with the radioactive ligands at 10 nM.

GABA-sensitivity of TBPS binding

We examined the effects of GABA at various concentrations on TBPS (3 nM) binding to the α3β2, α3β2γ2, α6β2 and α6β2γ2 subtypes (Figure 1). In the α3β2 subtype, GABA at low concentrations (<0.5 μM) enhanced TBPS binding by more than 400%, but at high concentrations (>1 μM) inhibited TBPS binding (nearly abolished it at 50 μM). The data were analysed with Equation 2. For the enhancement phase, we found $EC_{50} = 110 \pm 10$ nM for GABA, $n = 1.4 \pm 0.2$, and $E_{max} = 438 \pm 16\%$, and for the inhibition phase, $IC_{50} = 2$ μM, and $n = 1.7 \pm 0.4$. The increased TBPS binding in the presence of low GABA (0.25 μM) was due to a decrease in the K_d for TBPS, from 111 ± 6 to 45 ± 1 nM, with little change in B_{max} (3 pmol mg^{-1} protein for this particular preparation). In preparations for the α3β2γ2 subtype, on the other hand, GABA at low concentrations (<1 μM) had no effect, but at high concentrations (>10 μM) inhibited TBPS binding, except for a minor enhancement (about 60% above the control) which peaked at 10 μM GABA. The receptor subtype responsible for the peak is not certain. The peak could not be due to traces of the α3β2 subtype because of the difference in GABA sensitivity (10 μM vs 0.5 μM GABA for α3β2), and also could not be attributed to the α3β2γ2 subtype, because of its disappearance in the presence of Zn (50 μM), which has been shown to interact with the αβ subtypes, but not with the αβγ subtypes (Draguhn *et al.*, 1990) (see below).

In the α6β2 subtype, TBPS binding profile was quite different from that in the α3β2 subtype. GABA monophasically enhanced TBPS binding with no sign of inhibition even at 100 μM. Analysis of the data with Equation 1 yielded an $EC_{50} = 5.2 \pm 0.5$ nM, $n = 1.3 \pm 0.2$ and $E_{max} = 134 \pm 29\%$. In the α6β2γ2 subtype, again GABA at low concentrations had no effect, but at high concentrations inhibited TBPS binding with a minor Zn-sensitive enhancement of about 25% at 50 nM GABA, probably representing a minor receptor subtype of unknown subunit composition.

Differential modes of modulation of TBPS binding by allosteric ligands

We examined the effects of several allosteric ligands (diazepam, methyl 6,7-dimethoxy-4-methyl-β-carboline-3-carboxylate (DMCM), 3α,21-dihydroxy-5α-pregnan-20-one (5α-THDOC), pentobarbitone and Zn) on TBPS binding to the various subtypes of cloned $GABA_A$ receptors. Two parameters were used to characterize the drug effect on TBPS binding: (1) the drug-induced change in TBPS binding in the absence of GABA (%) and (2) the change in the GABA concentration to reduce TBPS binding to 50% of the control level (IC_{50}, without GABA and drugs) (Table 2).

Diazepam, 5α-THDOC and pentobarbitone are agonists for $GABA_A$ receptors, and interact with three major modulatory sites on $GABA_A$ receptors, namely the benzodiazepine, neurosteroid and barbiturate sites, respectively. In the absence of GABA, diazepam and 5α-THDOC enhanced TBPS binding, but pentobarbitone did not (Figure 2). Diazepam (0.2 μM) increased TBPS binding by 29 ± 6 and 54 ± 7% in the α1β2γ2 and α3β2γ2 subtypes, respectively, but not in the α6β2γ2 and the subtypes without γ2. 5α-THDOC (0.8 μM) increased TBPS binding by 42 ± 5, 44 ± 11, 10 ± 22, 51 ± 45, 228 ± 15 and 14 ± 8% in the α1β2γ2, α3β2γ2, α6β2γ2, α1β2, α3β2 and α6β2 subtype, respectively. Pentobarbitone (20 μM) failed to enhance TBPS binding in all these subtypes. In the presence of GABA, however, all these agonists potentiated the inhibitory effect of GABA on TBPS binding, as shown by changes in the IC_{50} value for GABA (Figure 2 and Table 2). Diazepam reduced the IC_{50} value from 8.2 ± 0.6 to 4.5 ± 0.8 μM in the α1β2γ2 subtype, and from 116 ± 12 to 54 ± 8 μM in the α3β2γ2 subtype, but produced no changes in the other subtypes. 5α-THDOC reduced

Table 2 Differential effects of allosteric ligands on TBPS binding in the subtypes of cloned $GABA_A$ receptors

	Control		*Diazepam*		*DMCM*		*Pentobarbitone*		*5α-THDOC*	
	% change (no GABA)	IC_{50} (μM)	% change (no GABA)	IC_{50} (μM)	% change (no GABA)	IC_{50} (μM)	% change (no GABA)	IC_{50} (μM)	% change (no GABA)	IC_{50} (μM)
α1β2γ2	100	8.2 ± 0.6	129 ± 6	4.5 ± 0.8	47 ± 8	20 ± 3	100 ± 3	4.7 ± 0.5	142 ± 5	1.4 ± 0.4
α3β2γ2	100	116 ± 12	154 ± 7	54 ± 8.3	86 ± 8	265 ± 60	98 ± 12	47 ± 22	144 ± 11	42 ± 12
α6β2γ2	100	1 ± 0.4	–	–	–	–	97 ± 3	0.1 ± 0.03	110 ± 22	0.01 ± 0.005
α1β2	100	1.3 ± 0.3	–	–	–	–	105 ± 5	0.4 ± 0.1	151 ± 45	0.17 ± 0.05
α3β2	100	9.0 ± 1.1	–	–	–	–	97 ± 3	3.9 ± 0.2	328 ± 15	1.3 ± 0.3
α6β2	100	>100	–	–	–	–	87 ± 5	3.7 ± 2	114 ± 8	0.1 ± 0.05

Binding of TBPS (6 nM) in the subtypes of $GABA_A$ receptors was examined as a function of GABA concentration (0.01 to 100 μM) with or without diazepam (0.2 μM), DMCM (0.1 μM), 5α-THDOC (0.8 μM) or pentobarbitone (20 μM). We monitored drug effects with two parameters: (1) % change (no GABA) in the level of TBPS binding by the ligand in the absence of GABA as normalized to the value observed without the ligand, and (2) the value of IC_{50}, representing the GABA concentration with or without the drug, which reduces TBPS binding to the 50% level of that observed in the absence of GABA and drugs. The values represent the means ± standard errors from at least three experiments, each consisting of triplicate measurements.

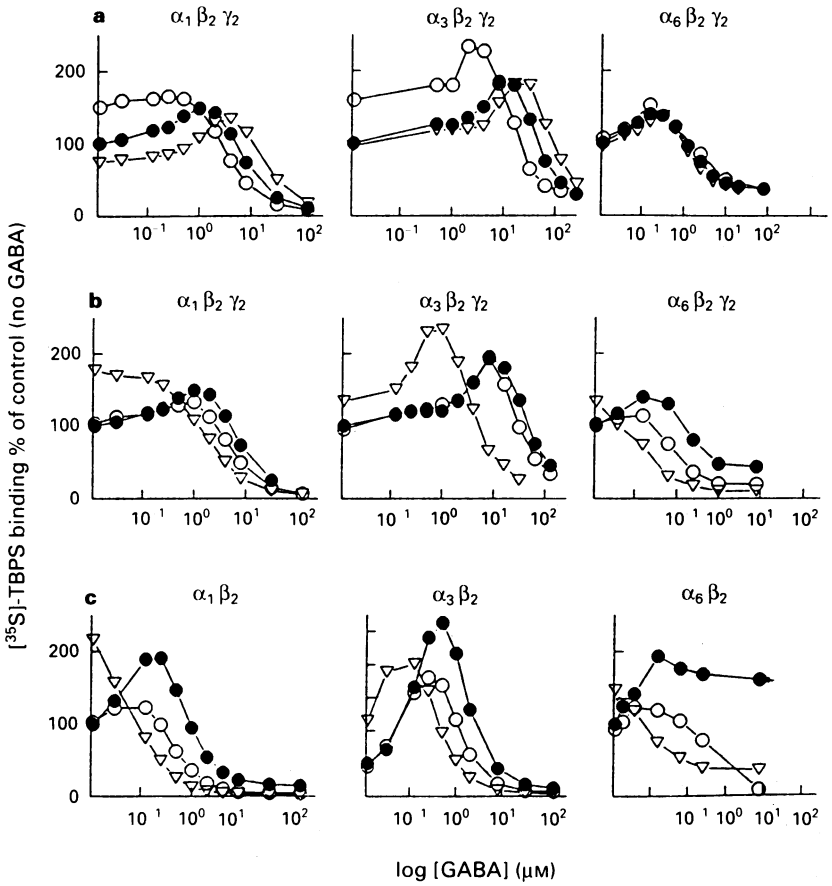


Figure 2 Effects of diazepam, DMCM, 5α-THDOC and pentobarbitone on GABA-sensitive TBPS binding in various subtypes of cloned $GABA_A$ receptors. TBPS binding (in the presence of GABA from 0 to 100 μM) was measured without or with diazepam (0.2 μM), DMCM (0.1 μM), 5α-THDOC (0.8 μM) or pentobarbitone (20 μM) in membranes of SF-9 cells infected with baculovirus carrying indicated cDNAs for $GABA_A$ receptor subunits. In (a) (●) control; (○) diazepam; (▽) DMCM. In (b) (●) control; (○) pentobarbitone; (▽) 5α-THDOC. In (c), (●) control; (○) pentobarbitone; (▽) 5α-THDOC. The data were normalized to the level of TBPS binding observed without GABA, and represent the mean from triplicate measurements. Two parameters were obtained from the data: the % changes in TBPS binding without GABA (control), and the changes in the GABA concentration to reduce the level of TBPS binding to 50% of the control (IC_{50}) by the drugs. The results are listed in Table 2. For abbreviations, see text.

the IC_{50} value from the control (shown in parentheses for each subtype) to 1.4 ± 0.4 (8.2 ± 0.6, α1β2γ2), 42 ± 12 (116 ± 12, α3β2γ2), 0.01 ± 0.005 (1 ± 0.4, α6β2γ2), 0.17 ± 0.05 (1.3 ± 0.3, α1β2), 1.3 ± 0.3 (9 ± 1.1, α3β2), and 0.1 ± 0.05 μM (<100, α6β2). Pentobarbitone also reduced the IC_{50} value in all the subtypes, to 4.7 ± 0.5 (8.2 ± 0.6, α1β2γ2), 47 ± 22 (116 ± 12, α3β2γ2), 0.1 ± 0.03 (1 ± 0.4, α6β2γ2), 0.4 ± 0.1 (1.3 ± 0.3, α1β2), 3.9 ± 0.2 (9 ± 1.1, α3β2), and 3.7 ± 2 μM (<100, α6β2). These results reflect two types of drug actions, allosteric modulation of GABA sites (changes in the IC_{50} value) and direct (allosteric) action on TBPS site (enhancement of TBPS binding in the absence of GABA). Pentobarbitone was unique among agonists in its inability to influence TBPS sites in the absence of GABA.

DMCM, a benzodiazepine inverse agonist, produced effects that were the opposite to those of agonists. In the absence of GABA, DMCM (0.1 μM) inhibited TBPS binding by −53 ± 8 and −14 ± 8% in the α1β2γ2 and α3β2γ2 subtype, respectively, and increased the IC_{50} value to 20 ± 3 (8.2 ± 0.6, α1β2γ2) and 265 ± 60 μM (116 ± 12, α3β2γ2), respectively. DMCM produced no effect in the other subtypes, displaying the same subtype selectivity as diazepam (Pritchett *et al.*, 1989a,b; Luddens *et al.*, 1990).

Zn represents another class of inhibitors of GABA-induced Cl^- currents, selective for the α1β2 subtype (not effective in α1β2γ2) (Draguhn *et al.*, 1990). Here we also confirmed similar Zn-sensitivity for the α3β2 subtype (Figure 3). Zn at 25 μM nearly abolished GABA-induced Cl^- currents in the α3β2, but reduced Cl^- currents by only 30 ± 9% in the α3β2γ2 subtype. As expected, Zn affected TBPS binding only in αβ subtypes in the manner opposite to the agonists. In the absence of GABA, Zn (50 μM) decreased TBPS binding (−37 ± 11%) to the α3β2 subtype, but produced no appreciable effect in the α3β2γ2 subtype. In the presence of GABA, Zn abolished GABA-mediated inhibition of TBPS binding to the α3β2 subtype; for instance, GABA (50 μM) alone reduced TBPS binding by 80%, but with Zn (50 μM) enhanced it to over 200% as normalized to the control level (no GABA). In the membrane preparations for the α3β2γ2 subtype, however, Zn (50 μM) shifted the GABA-dose response profile for TBPS binding to the left, largely because of disappearance of a minor Zn-sensitive enhancement in the preparations, as mentioned above; for instance, in the presence of Zn (50 μM), GABA monophasically blocked TBPS binding with an IC_{50} = 10 μM (compare with IC_{50} = 116 ± 12 μM without Zn) and n = 1.8. The profile for Zn-sensitive TBPS binding in the preparations for the α3β2γ2 subtype was obtained by the differences between the profiles with and without Zn (Figure 4, inset). An earlier study reported similar selective effects of Zn on TBPS binding to the α1β2 subtype (Pregenzer *et al.*, 1993).

Discussion

In this study we examined the effects of GABA and several allosteric ligands on TBPS binding to three major classes of $GABA_A$ receptor subtypes, Type 1 (α1β2γ2), Type 2 (α3β2γ2),

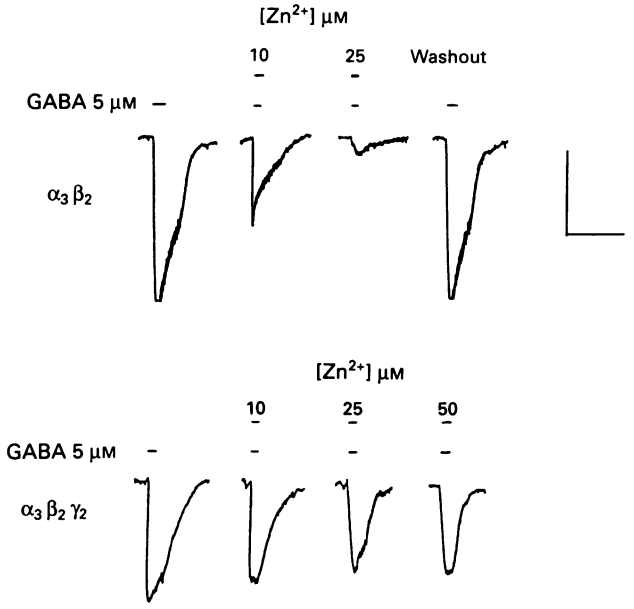


Figure 3 Representative current traces showing inhibition of GABA-induced Cl^- currents by Zn in the $\alpha3\beta2$, but not in the $\alpha3\beta2\gamma2$ subtype. GABA-induced Cl^- currents were measured in the SF-9 cells transiently expressing the $\alpha3\beta2$ or $\alpha3\beta2\gamma2$ subtypes using the whole cell configuration of patch clamp techniques. The currents were induced at the holding potential of -60 mV (under a symmetrical Cl^- gradient) by application of 5 μM GABA without or with Zn (10, 25 or 50 μM). Zn at 25 μM nearly abolished GABA-induced Cl^- in the $\alpha3\beta2$ subtype in a reversible manner, but was largely ineffective in blocking the Cl^- currents in the $\alpha3\beta2\gamma2$ subtype. Only $30 \pm 9\%$ ($n = 3$) current reduction was observed in the presence of Zn at 50 μM. The horizontal calibration bar represents 30 s, and the vertical bar 200 pA for the upper panel, and 500 pA for the lower panel.

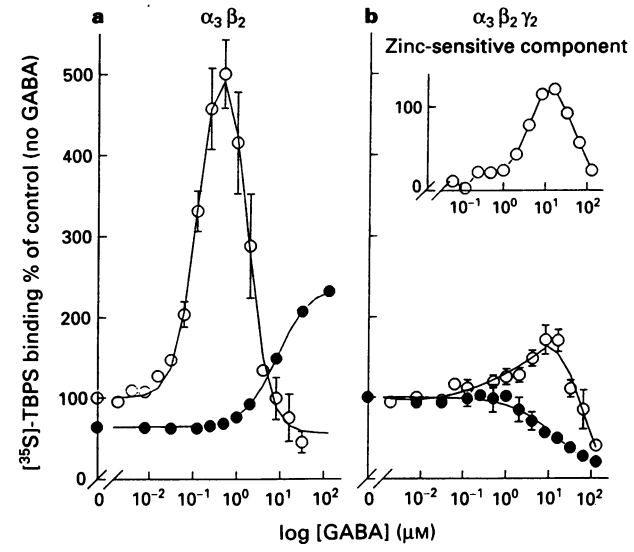


Figure 4 Effect of Zn on TBPS binding in (a) the $\alpha3\beta2$ and (b) $\alpha3\beta2\gamma2$ subtypes of cloned GABA$_A$ receptors. TBPS binding in the presence of GABA (0 to 100 μM) was measured with (●) or without Zn (○) at 50 μM. The binding data were normalized to that observed without GABA, and represent the mean ± s.e. from three experiments. In the preparations for the $\alpha3\beta2\gamma2$ subtype, we noted a Zn-sensitive component, shown in the inset.

the receptor subtype unique to cerebellar granule cells ($\alpha6\beta2\gamma2$), and their counterparts without γ2. Several findings are noteworthy. In αβ subtypes, the biphasic effect of GABA on TBPS binding (Figure 1) was differentially affected by the α isoform. Various α isoforms induced changes in the half maximal GABA concentration for enhancement of TBPS binding with EC_{50} values of 94 ± 10 nM, 110 ± 10 and 5.2 ± 0.5 nM for $\alpha1\beta2$, $\alpha3\beta2$ and $\alpha6\beta2$, respectively, and for inhibition of TBPS binding with IC_{50} values for GABA of 2, 2.7 and >100 μM for $\alpha1\beta2$, $\alpha3\beta2$ and $\alpha6\beta2$, respectively. The maximal enhancement of TBPS binding by GABA also varied with the α isoform (153 ± 10, 438 ± 16 and $139 \pm 29\%$ of TBPS binding for $\alpha1\beta2$, $\alpha3\beta2$ and $\alpha6\beta2$, respectively). All the values for α1 containing receptors were from an earlier study (Pregenzer *et al.*, 1993). Interestingly, the EC_{50} value in each subtype matched well with its K_d value for the high affinity GABA site (83, 71 and 12 nM for $\alpha1\beta2$, $\alpha3\beta2$ and $\alpha6\beta2$, respectively). Stimulation of TBPS binding thus seems to result from interaction of GABA with its high affinity site. Inhibition of TBPS binding, on the other hand, may reflect interaction of GABA with its low affinity site, and could be related to channel openings, which were observed only in the presence of GABA at micromolar concentrations in patch-clamp studies with various subtypes of cloned GABA$_A$ receptors (Puia *et al.*, 1991). We propose that the IC_{50} values approximate the K_d values for low affinity GABA sites. According to this proposal, the $\alpha6\beta2$ subtype possesses low affinity GABA sites ($IC_{50} > 100$ μM) much different from those on the $\alpha1\beta2$ (2 μM) and $\alpha3\beta2$ subtype (2.7 μM), suggesting the α isoform as the major determinant for low affinity GABA sites. Also the α isoform influences the degree of coupling between GABA (high affinity) and TBPS sites, as reflected by the α isoform-dependent change in the maximal enhancement of TBPS binding by GABA.

Addition of γ2 to αβ subtypes brought about two changes; the disappearance of enhancement of TBPS binding by GABA at nanomolar concentrations and changes in the IC_{50} values for inhibition of TBPS binding by GABA (8.2, 10 and 1 μM for the $\alpha1\beta2\gamma2$, $\alpha3\beta2\gamma2$ and $\alpha6\beta2\gamma2$ subtype, respectively). Note that the IC_{50} for the $\alpha3\beta2\gamma2$ subtype was obtained in the presence of Zn to eliminate the minor Zn-sensitive component as discussed in the Results section. It appears that the quaternary interactions involving the γ2 subunit abolished conformational coupling between high affinity GABA sites and TBPS sites, and altered low affinity GABA sites, particularly for the $\alpha6\beta2\gamma2$ (IC_{50} change from >100 to 1 μM). It should be noted that addition of γ2 produced no appreciable change in high affinity GABA site as monitored with [^{3}H]-muscimol binding (see Table 1 and Pregenzer *et al.*, 1993). These results emphasize functional and physical distinctions between high and low affinity GABA sites in various GABA$_A$ receptors.

Our current study with various allosteric ligands generally confirmed earlier observations in rat brain membranes that agonists potentiate, and inverse agonists attenuate the inhibitory effect of GABA on TBPS binding (Gee *et al.*, 1986; Trifiletti *et al.*, 1986; Lloyd *et al.*, 1990; Im & Blakeman, 1991; Sieghart, 1992; Squires & Seaderap, 1993). Subtype selectivity for individual ligands observed with TBPS binding also matched well with that observed with ligand binding studies. For instance, diazepam and DMCM (0.1 μM) affected TBPS binding in the $\alpha1\beta2\gamma2$ and $\alpha3\beta2\gamma2$ subtypes to which the drugs bind with high affinity, but produced no effect on TBPS binding in the $\alpha6\beta2\gamma2$ and αβ subtypes where the drugs at the concentrations we used here showed no binding activity (Pritchett *et al.*, 1989a,b; Luddens *et al.*, 1990). The $\alpha6\beta2\gamma2$ subtype appears to represent the diazepam-insensitive population of GABA$_A$ receptors in bovine cerebellar membranes (Turner *et al.*, 1991). Several other interesting points are: (1) in the $\alpha6\beta2$ subtype 5α-THDOC (0.8 μM) and pentobarbitone (20 μM) reduced IC_{50} value from <100 μM to 0.1 and 3.9 μM, respectively. This suggests a marked allosteric modulation of low affinity GABA sites by the drugs. (2) Diazepam and 5α-THDOC enhanced TBPS binding in the absence of GABA, but pentobarbitone did not. This differential property among agonists would be useful for classifying novel ligands for GABA$_A$ receptors. (3) We detected a minor Zn-sensitive

subtype in the preparations of the $\alpha 3\beta 2\gamma 2$ subtype. This subtype shares the same Zn-sensitivity with the $\alpha 3\beta 2$, but differs in GABA concentrations required to induce a maximal enhancement of TBPS binding (10 μM as compared to 0.5 μM for $\alpha 3\beta 2$). The subunit composition of this subtype would be of future research interest.

In summary, differential modulation of TBPS binding by GABA at various concentrations in cloned $GABA_A$ receptors (particularly $\alpha\beta$ subtypes) provided important information on both high and low affinity GABA binding sites, and was useful for understanding how a subunit or isoform contributes to GABA binding sites and their coupling with TBPS sites. The α isoform seems to contribute much to GABA binding sites, since the presence of $\alpha 6$ produced drastic changes in high and low affinity GABA sites as compared to those observed with $\alpha 1$ or $\alpha 3$. Addition of $\gamma 2$ to $\alpha\beta$ subtypes or presence of allosteric ligands, on the other hand, seems to induce marked changes in low affinity GABA sites. In addition, the α isoform and the $\gamma 2$ subunit influence conformational coupling between GABA sites and TBPS sites.

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Antiarrhythmic effects of BN-063, a newly synthesized adenosine A_1 agonist, on myocardial ischaemia in rats

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1 It has been shown that adenosine is able to reduce the severity of arrhythmias induced by myocardial ischaemia. In isolated preparations, the antiarrhythmic effect of adenosine on ventricular myocardium is known to antagonize the catecholamine-induced stimulation of intracellular cyclic AMP production, an effect mediated via adenosine A_1 receptors.

2 The aim of this study was to evaluate the antiarrhythmic effect of BN-063 (1-cyclopropylisoguanosine), a newly synthesized selective adenosine A_1 agonist, on ventricular arrhythmias in rats.

3 Arrhythmias were induced by left coronary artery ligation or by administration of isoprenaline (7 mg kg^{-1}) subcutaneously. Pretreatment with BN-063 (0.25, 0.5 and 1.0 mg kg^{-1}) 10 min prior to occlusion significantly delayed the onset of ventricular arrhythmias, reduced the total number of ventricular premature contraction (VPC) and ventricular tachycardia (VT), decreased the incidence of VT and ventricular fibrillation (VF) and mortality during the first 30 min following left coronary artery ligation. In contrast, pretreatment with 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), an adenosine A_1 antagonist, was arrhythmogenic during the ischaemic period. The rate-pressure product, an index for indirect measurement of myocardial oxygen consumption, was also significantly reduced by BN-063 during ligation time.

4 The incidence of VT, VF and mortality was also significantly reduced when BN-063 was administered after left coronary artery ligation.

5 BN-063 converted the VF induced by isoprenaline to normal sinus rhythm and improved the survival rate.

6 It is concluded that, through activation of adenosine A_1 receptors, BN-063 can suppress ventricular arrhythmias induced by myocardial ischaemia and catecholamines. The antiarrhythmic actions of BN-063 may be mediated by reducing heart rate and antagonizing the stimulatory effects of catecholamine in myocardial ischaemia.

Keywords: Adenosine; myocardial ischaemia; catecholamine; arrhythmias; ventricular fibrillation; A_1 agonist

Introduction

In acute myocardial ischaemia, the arrhythmogenic effects of stimulated sympathetic neural activity or increased catecholamine concentrations are well documented. High local noradrenaline (NA) levels may be achieved by either local nonexocytotic release (Abrahamsson *et al.*, 1985; Schomig *et al.*, 1987) or exocytotic NA release due to activation of cardiac sympathetic nerves (Lombardi *et al.*, 1983; Zaza & Schwartz, 1985). However, catecholamine-induced increases in the inward calcium current (I_{Ca}) and transient inward current (I_{Ti}) (Belardinelli & Isenberg, 1983; Fedida *et al.*, 1987) which have been implicated in the genesis of afterdepolarization and triggered activity, are potentially arrhythmogenic in the ischaemic heart. Myocardial ischaemia facilitates adenosine release which may reduce the consequences of impaired myocardial tissue perfusion. Activation of adenosine receptors, which regulate adenylate cyclase activity through guanosine triphosphate binding proteins in the heart, influences the regulation of coronary blood flow, atrioventricular conduction and attenuates the effects of catecholamine stimulation and myocardial oxygen supply/demand imbalance associated with ischaemia. Endogenous adenosine has been proposed to play the role of 'endogenous myocardial antiarrhythmic substance' (Fagbemi & Parratt, 1984). In addition, evidence that exogenous adenosine reduces the severity of ventricular arrhythmias induced by ischaemia has also been shown *in vivo* (Fagbemi & Parratt, 1984; Wainwright & Parratt, 1988; Wainwright *et al.*, 1993). The precise mechanisms underlying this antiarrhythmic effect are not clear. In view of the evidence that adenosine receptor blockade with 8-phenyltheophylline is arrhythmogenic (Culling *et al.*, 1984), it has been suggested that the antiarrhythmic effect of adenosine may be mediated via adenosine receptors.

Numerous studies show that elevation of myocardial adenosine 3':5'-cyclic monophosphate (cyclic AMP) is implicated in the generation of early ventricular ischaemic arrhythmias (Lerman & Belardinelli, 1990). It is likely that the antiarrhythmic effect of adenosine is achieved by antagonism of catecholamine stimulation resulting from suppressing catecholamine-induced increases in adenylyl cyclase activity and intracellular cyclic AMP. These actions of adenosine are likely to be mediated through the A_1 adenosine receptor coupled to the catalytic subunit of adenylyl cyclase through a pertussis toxin-sensitive guanine nucleotide binding regulatory (G_i) protein (Belardinelli & Pelleg, 1990).

BN-063 (1-cyclopropylisoguanosine) is a newly synthesized analogue of doridosine (1-methylisoguanosine) (Chern *et al.*, 1991), a naturally occurring nucleoside isolated early in 1980 from an aqueous ethanolic extract of the sponge *Tedania digitata* (Cook *et al.*, 1980). Doridorsine could produce a reduction in arterial pressure and slowing of heart rate in mammals via adenosine receptor stimulation (Jacobson *et al.*, 1992). On the basis of our *in vivo* and receptor-binding studies (Tao *et al.*, 1993), BN-063 was shown to be a selective adenosine A_1 receptor agonist. Therefore, the purpose of the present study was to assess the effects of BN-063 on ventricular arrhythmias resulting from left coronary artery ligation or isoprenaline administered subcutaneously.

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Methods

Arrhythmias induced by left coronary artery ligation

Experimental preparation Adult Sprague-Dawley rats of either sex weighing 250–300 g were anaesthetized with intraperitoneal urethane (1.2 g kg^{-1}). Tracheostomy was performed and an intubating cannula was connected to a rodent ventilator and the animals were ventilated artificially with room air. Respiratory rate was synchronised with the rat's spontaneous rate (60–80 strokes min^{-1}, 1 ml 100 g^{-1}). Arterial blood pH and blood gases were maintained within normal physiological limits (pH: 7.35–7.45; P_{CO_2}: 30–35 mmHg; P_{O_2}: 85–100 mmHg) by adjusting the respiratory rate and tidal volume. The left femoral artery and vein were cannulated for the measurement of arterial blood pressure and heart rate via a Statham pressure transducer and a Biotechnometer (Gould, U.S.A.) and for the administration of drugs, respectively. Electrocardiograms were recorded from standard lead II limb leads, with a positive electrode connected to the left hind leg, a negative electrode to the right foreleg and a ground electrode to the left foreleg. An oscilloscope ECG monitor (DSO 420, Gould, USA) was used to display the ECG continuously throughout the experiments. All signals, including the ECG and haemodynamic data, were recorded on the chart paper.

After a left-side thoracotomy was performed at the fifth intercostal space, the pericardium was incised and the heart exteriorised. A loose ligature (6/0 silk suture) was placed around the left coronary artery close to its origin. The rat was then allowed to stabilize for 30 min. During this period, if the rat showed functional instability such as hypotension (systolic blood pressure value less than 100 mmHg) or occurrence of cardiac arrhythmias, it was discarded. About 1–2 rats were discarded in each treatment group.

Experimental protocol After the 30 min stabilization, animals were randomly assigned to one of three main treatment groups: (1) saline injection only (control group; $n = 10$); (2) treated with drugs 10 min before ligation, including 4 subgroups ($n = 10$); (i) low-dose BN-063 (1-cyclopropylisoguanosine) (0.25 mg kg^{-1}), (ii) intermediate-dose BN-063 (0.5 mg kg^{-1}), (iii) high-dose BN-063 (1.0 mg kg^{-1}) and (iv) 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 0.5 mg kg^{-1}), an adenosine A_1 antagonist; (3) treated with BN-063 after ligation when arrhythmias occur, including 3 subgroups ($n = 7$); (i) low-dose BN-063 (0.25 mg kg^{-1}), (ii) intermediate-dose BN-063 (0.5 mg kg^{-1}) and (iii) high-dose BN-063 (1.0 mg kg^{-1}). After the ligature was tied, blood pressure, heart rate and electrocardiogram were then continously monitored throughout the 30 min of postligation period. Early ventricular arrhythmias which occur within 30 min of the onset of myocardial ischaemia were assessed by the onset, incidence and total number of ventricular premature contraction (VPC), ventricular tachycardia (VT) and ventricular fibrillation (VF).

Arrhythmias induced by isoprenaline administered subcutaneously

Male Sprague-Dawley rats (380–420 g) were anaesthetized with pentobarbitone sodium (50 mg kg^{-1}) given intraperitoneally. The electrocardiograms were measured from lead II limb leads. Blood pressure and heart rate were monitored by a Statham pressure transducer from a catheter placed in the left femoral artery and the left femoral vein was cannulated for the administration of drugs. When they were haemodynamically stable, (±)-isoprenaline (7 mg kg^{-1}) was injected subcutaneously. The onset of the effect of isoprenaline was indicated by an increase in the heart rate to around 500 beats min^{-1} and a significant decrease in blood pressure. In addition, the J point of the ST segment on the ECG became clearly depressed. As the VF induced by isoprenaline was occurring, saline or BN-063 (0.5 mg kg^{-1}) was administered immediately. The conversion of VF to normal sinus rhythm and prevention of mortality were used as protective indices.

Data analysis

All data are expressed as mean ± standard error of the mean (s.e.mean). The Yates corrected chi-square test was used to analyse the difference in the incidence of arrhythmias and survival rate between control and treated groups. Student's t test was used to test the difference in the onset of arrhythmias between control and treated groups. The other parameters were compared by a one-factor analysis of variance. If this analysis indicated significant differences among the group means, the control group was compared with each of the treatment groups by means of the Newman-Keuls method. A P value of less than 0.05 was considered as statistically significant.

Drugs

BN-063 solution was prepared according to the method described previously by Tao *et al.* (1993). (±)-Isoprenaline was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) was purchased from Research Biochemicals Incorporated Co. (Natick, MA, U.S.A.).

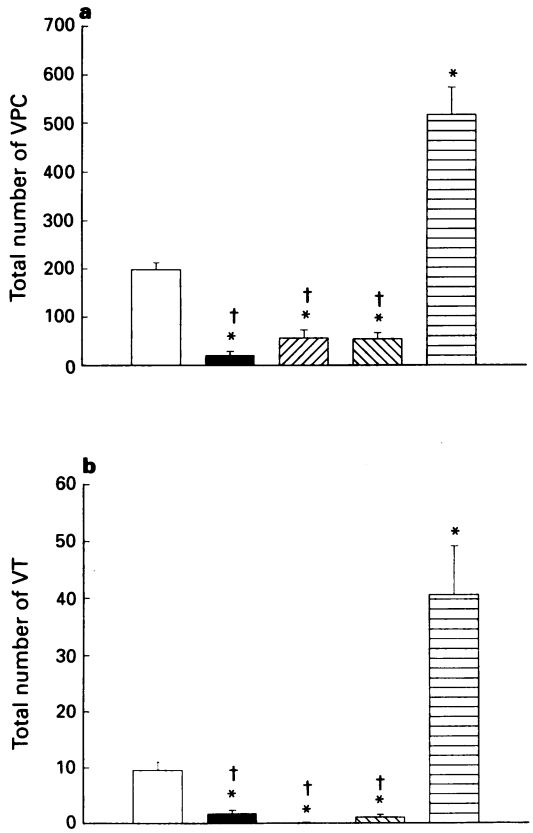


Figure 1 Effects of pretreatment with BN-063 on the number of (a) ventricular premature contraction (VPC) and (b) ventricular tachycardia (VT) which occurred during 30 min coronary artery occlusion period in anaesthetized rats. (□) Saline; (■) BN-063 0.25 mg kg^{-1}; (▨) BN-063 0.5 mg kg^{-1}; (▧) BN-063 1.0 mg kg^{-1}; (▤) DPCPX 0.5 mg kg^{-1}. Values are expressed as means ± s.e.mean ($n = 10$). *$P < 0.05$ vs control value; †$P < 0.05$ vs DPCPX-treated values.

Results

Left coronary artery ligation

Haemodynamic parameters Before left coronary artery ligation, the three doses of BN-063 (0.25, 0.5 and 1.0 mg kg^{-1}) significantly reduced heart rate and the rate-pressure product, an index of myocardial oxygen consumption, but did not significantly affect systolic, diastolic and mean arterial blood pressures. After left coronary artery ligation, the heart rate and rate-pressure product were depressed significantly in the three BN-063-treated groups (Table 1). However, at the high dose (1.0 mg kg^{-1}) of BN-063, a significant reduction in mean blood pressure during the first 15 min of occlusion was observed.

The effects of BN-063 on ischaemic arrhythmias Ligation of left coronary artery invariably resulted in ventricular arrhythmias, which commenced within 4–5 min of occlusion, manifesting as ventricular premature contractions (VPC), ventricular tachycardia (VT) and ventricular fibrillation (VF). Table 2 shows the effects of pretreatment with BN-063 on the onset and incidence of arrhythmias following coronary artery occlusion in the rat. After left coronary artery ligation, all of the rats in the control group developed arrhythmias during the 30 min post-ligation period. Pretreatment with BN-063 before coronary artery ligation abolished the occurrence of VF, markedly delayed the onset of arrhythmias and significantly reduced the incidence of VT at intermediate and high doses (0.5 and 1.0 mg kg^{-1}), as compared to control and DPCPX-treatment. BN-063 also resulted in a significant reduction in total number of VPC and VT when compared with control and DPCPX treated group (Figure 1). Pretreatment with DPCPX, a selective antagonist of adenosine A_1 receptors, before coronary artery ligation resulted in an augmented arrhythmia count, with no effect on the incidence and onset of arrhythmias. The survival rate of BN-063 treated groups was 100% which was significantly higher than that of control and DPCPX-treated groups (Figure 2). After left coronary ligation, VF was terminated by BN-063 administration and the incidence of VT was significantly reduced at intermediate dose (0.5 mg kg^{-1}) (Figure 3). The survival rates of BN-063-treated groups were 100%, which were significantly higher than that of control group (Figure 4).

The effect of BN-063 on isoprenaline-induced arrhythmia

The times to onset of VF induced by isoprenaline in the control and BN-063-treated groups were 49.56 ± 7.84 and 54.96 ± 11.68 min respectively, which were not statistically different. Table 3 shows that the mortality rate of the control group was 70%, indicating the VF induced by isoprenaline could occasionally revert to sinus nodal rhythm spontaneously. Treatment with BN-063 (0.5 mg kg^{-1}) at the onset of ventricular fibrillation reduced mortality to zero. It is evidence that BN-063 could convert ventricular fibrillation of all rats to normal sinus rhythm and preserve survival.

Table 1 Summary of haemodynamic parameters during experimental protocol where BN-063 was given 10 min before coronary artery ligation

Group	*Baseline*	*OCC*	*O–15*	*O–30*
Heart rate (beats min^{-1})				
Control	303 ± 14	303 ± 14	312 ± 12	305 ± 13
BN-063 0.25 mg kg^{-1}	291 ± 6	225 ± 4*	264 ± 4	279 ± 8
BN-063 0.5 mg kg^{-1}	307 ± 13	225 ± 12*	241 ± 10*	269 ± 18
BN-063 1.0 mg kg^{-1}	283 ± 22	182 ± 13*	198 ± 14*	220 ± 15*
Mean blood pressure (mmHg)				
Control	79 ± 3	79 ± 3	74 ± 2	74 ± 4
BN-063 0.25 mg kg^{-1}	80 ± 4	75 ± 4	72 ± 4	72 ± 3
BN-063 0.5 mg kg^{-1}	76 ± 6	69 ± 5	67 ± 4	68 ± 4
BN-063 1.0 mg kg^{-1}	77 ± 3	67 ± 3*	64 ± 3*	65 ± 3
Rate-pressure product (× 1000)				
Control	37 ± 2.2	37 ± 2.2	32.5 ± 2.6	32 ± 2.4
BN-063 0.25 mg kg^{-1}	37.6 ± 2.8	26 ± 1.2*	27 ± 1.6	28 ± 1.5
BN-063 0.5 mg kg^{-1}	38.3 ± 2.8	26.3 ± 1.5*	24.6 ± 1.2*	25.5 ± 1.3
BN-063 1.0 mg kg^{-1}	35.3 ± 1.4	21.8 ± 1.1*	20.1 ± 2.4*	21.7 ± 1.2*

OCC = immediately after occlusion; O–15 = 15 min after occlusion; O–30 = 30 min after occlusion; values are expressed as means ± s.e.mean; *$P<0.05$ vs control values. BN-063 = 1-cyclopropylisoguanosine.

Table 2 Effects of pretreatment with BN-063 on the onset and incidence of ventricular arrhythmias following coronary artery occlusion in the rat

		VPC		*VT*		*VF*	
Group	*n*	onset (min)	incidence (%)	onset (min)	incidence (%)	onset (min)	incidence (%)
Control	10	5.69 ± 0.22	100	6.30 ± 0.32	100	9.47 ± 0.75	70
BN-063 (0.25 mg kg^{-1})	10	9.96 ± 0.90*+	100	12.84 ± 0.59*+	70	–	0*+
BN-063 (0.5 mg kg^{-1})	10	9.83 ± 1.71*+	100	–	0*+	–	0*+
BN-063 (1.0 mg kg^{-1})	10	11.36 ± 0.70*+	100	12.02 ± 1.00*+	50*+	–	0*+
DPCPX (0.5 mg kg^{-1})	10	4.63 ± 0.24	100	4.75 ± 0.40	100	8.19 ± 1.15	100

n is number of rats. VPC = ventricular premature contraction; VT = ventricular tachycardia; VF = ventricular fibrillation. *$P<0.05$ compared to control group; +$P<0.05$ compared to DPCPX-treated group.

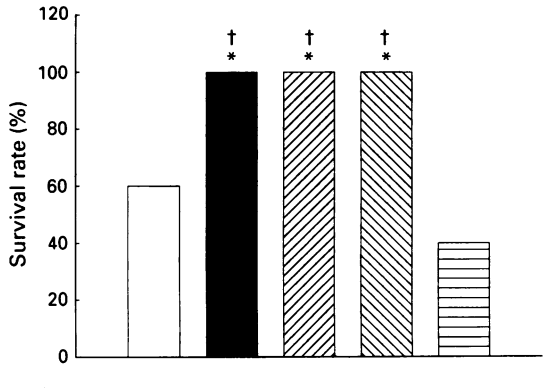


Figure 2 Effects of pretreatment with BN-063 on the survival rate following 30 min coronary artery occlusion period in anaesthetized rats. (□) Saline; (■) BN-063 0.25 mg kg^{-1}; (▨) BN-063 0.5 mg kg^{-1}; (▩) BN-063 1.0 mg kg^{-1}; (▤) DPCPX 0.5 mg kg^{-1}. *$P<0.05$ vs control value; †$P<0.05$ vs DPCPX-treated values ($n = 10$).

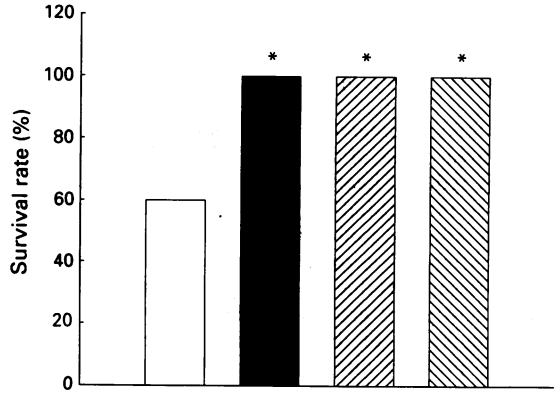


Figure 4 Effects of post-treatment with BN-063 on the survival rate following 30 min coronary artery occlusion period in anaesthetized rats. (□) Saline, $n = 10$; (■) BN-063 0.25 mg kg^{-1}, $n = 7$; (▨) BN-063 0.5 mg kg^{-1}, $n = 7$; (▩) BN-063 1.0 mg kg^{-1}, $n = 7$; *$P<0.05$ vs control value.

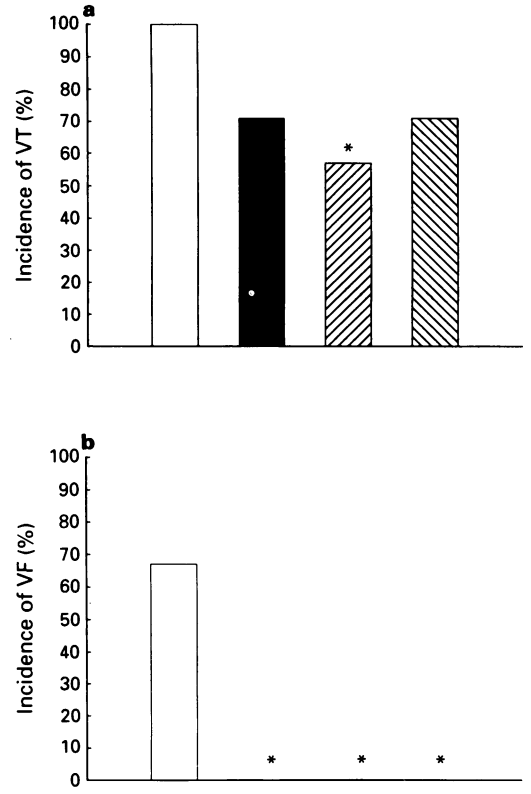


Figure 3 Effects of post-treatment with BN-063 on the incidence of (a) ventricular tachycardia (VT) and (b) ventricular fibrillation (VF) which occurred during 30 min coronary artery occlusion period in anaesthetized rats. (□) Saline, $n = 10$; (■) BN-063 0.25 mg kg^{-1}, $n = 7$; (▨) BN-063 0.5 mg kg^{-1}, $n = 7$; (▩) BN-063 1.0 mg kg^{-1}, $n = 7$; *$P<0.05$ vs control value.

Discussion and conclusions

In the coronary artery ligation model, a highly reproducible experimental animal model, pretreatment with BN-063 markedly delayed the onset of arrhythmias, attenuated the severity of arrhythmias and reduced the incidence of arrhythmias significantly. This demonstrated that the early ventricular arrhythmias resulting from acute myocardial ischaemia were efficiently improved by this selective adenosine A_1 agonist. In addition, blockade of adenosine A_1 receptors with DPCPX enhanced arrhythmogenicity. These results further support the hypothesis that adenosine A_1 receptors in the heart may play an important role against the malignant ventricular arrhythmias induced by acute myocardial ischaemia. However, the paradoxical failure of a high dose of BN-063 (1.0 mg kg^{-1}) to abolish VT may result from severe bradycardia and subsequent hypotension leading to accelerated myocardial ischaemia during the early ligation period. Furthermore, the findings of this study clearly showed that the occurrence of ventricular fibrillation was abolished in all BN-063-treated groups. One very interesting observation, however, is that the adenosine A_1 agonist – BN-063 has a specific effect on certain types of arrhythmia, i.e. ventricular fibrillation. This finding is consistent with the reports by Wainwright & Parratt in an adenosine study (Wainwright & Parratt, 1988). Certainly ventricular fibrillation is the most important arrhythmia to suppress under conditions of acute myocardial ischaemia. The antiarrhythmic effect of BN-063 administered after ligation was also evident, but the potency and efficacy was less than that of pretreatment with BN-063. Previous studies showed that the rat heart is deficient in functional collaterals (Johns & Olson, 1954). In accordance with this, we suggest that perhaps the protective effect could be achieved even in the absence of BN-063 in the ischaemic region of heart. It is now well established that adenosine exerts pronounced negative chronotropic and dromotropic actions on sinus node

Table 3 The effect of BN-063 on the mortality caused by isoprenaline administered subcutaneously

Groups	*n*	*Body weight* (g)	*Onset of VF* (min)	*Survival* (*n*)	*Mortality* (%)
Control	10	414 ± 16	49.56 ± 7.84	3	70
BN-063 (0.5 mg kg^{-1})	10	408 ± 14	54.96 ± 11.68	10	0*

n is the number of rats. VF = ventricular fibrillation. *$P<0.05$ compared to control group.

automaticity and AV nodal conduction, respectively, to reduce heart rate (Pelleg & Belardinelli, 1993). This action of adenosine is mediated by an A_1 adenosine subtype. This mechanism may be responsible for the effects of the adenosine A_1 agonist BN-063 which produced a marked and sustained reduction in heart rate in these studies. Previous studies have shown that there was a positive correlation between heart rate and the severity of ischaemia induced arrhythmias (Coker & Parratt, 1985; Wainwright & Parratt, 1993). The slowing of cardiac rate by BN-063 could reduce cardiac work and oxygen consumption of ischaemic myocardium, thus contributing to protective effects of BN-063 under conditions of ischaemia and to maintaining cellular electrophysiological stability. This concept is consistent with the cardioprotection produced by a selective adenosine A_1 receptor agonist **R**-PIA (**R**-N^6-2-phenylisopropyladenosine) (Wainwright & Parratt, 1993) and a new bradycardic agent FR76830 described by Ishibashi *et al.* (1990). Furthermore, the presence of BN-063 in the ischaemic region may produce further protective effects on ventricular myocytes, for example, by antagonizing the effects of catecholamines. It is well known that enhanced cardiac sympathetic activation, with an increased local release of noradrenaline accompanies acute ischaemia and is a major contributory factor to arrhythmogenesis (Lombardi *et al.*, 1983; Abrahamsson *et al.*, 1985; Zaza & Schwartz, 1985; Schomig *et al.*, 1987). Catecholamine induced increases in the inward calcium current (I_{Ca}) would be potentially arrhythmogenic in the ischaemic heart (Boachie-Ansah *et al.*, 1993). There was a highly significant correlation between adenosine and the stimulation of catecholamines in *in vitro* studies. Adenosine not only antagonizes isoprenaline-induced increases in I_{Ca} but also inhibits catecholamine induced I_{Ti} and triggered activity (Belardinelli & Isenberg, 1983; Fedida *et al.*, 1987). It is likely that adenosine antagonism of catecholamine stimulated increases in I_{Ca} resulted from effects on intracellular cyclic AMP levels (Lerman & Belardinelli, 1990). These actions of adenosine are mediated through adenosine A_1 receptors coupled to the catalytic subunit of adenylyl cyclase through a pertussis toxin-sensitive guanine nucleotide binding regulatory (G_i) protein (Belardinelli & Pelleg, 1990). Thus, adenosine A_1 agonist BN-063 may also mediate the antiadrenergic actions of adenosine to produce further protective effects in ischaemic ventricular tissue.

Catecholamine-induced increases in I_{Ca} and inward background current, which are associated with an increasing intracellular cyclic AMP, are potentially arrhythmogenic in ventricular myocytes (Egan *et al.*, 1988; Boachie-Ansah *et al.*, 1993). It has been suggested that activation of catecholamine induced currents could generate an ectopic ventricular focus leading to tachycardia. If more than one focus were activated asynchronously then the rhythm could become chaotic and might precipitate ventricular fibrillation (Egan *et al.*, 1988). Accordingly, subcutaneous administration of isoprenaline was used to facilitate ventricular fibrillation *in vivo*. Results of our isoprenaline-induced arrhythmia studies revealed that BN-063 is effective in termination of catecholamine-induced ventricular fibrillation. This antiarrhythmic action of BN-063 may result from inhibiting the formation of cyclic AMP through inhibition of adenylate cyclase. Further studies are needed to investigate this suggestion.

How the activation of adenosine A_1 receptor enhanced myocardial resistance to ischaemic injury remains unclear. However, the therapeutic effect of an adenosine A_1 receptor agonist may be related to: (1) both negative chronotropic and dromotropic effects of the adenosine A_1 receptor stimulation on the conducting system resulting in a decrease in myocardial oxygen consumption (Pelleg & Belardinelli, 1993); (2) suppression of the action of catecholamines including inhibition of catecholamine-induced increases in myocardial cyclic AMP (Boachie-Ansah *et al.*, 1993); (3) reduced noradrenaline release from sympathetic nerve endings, an effect mediated through presynaptic adenosine A_1 receptors (Richardt *et al.*, 1987). Recently, a series of studies indicated that injections of adenosine can mimic the protection against myocardial ischaemia induced by preconditioning, including reduced severity of life threatening ventricular arrhythmias that occur during prolonged occlusion and reduced myocardial infarct size (Vegh *et al.*, 1992; Downey *et al.*, 1993). However, the mechanism by which preconditioning protects the heart remains unknown. Gross & Auchampach proposed that opening of ATP-sensitive potassium channels mediates preconditioning (Auchampach *et al.*, 1991). In ventricular myocytes, activation of adenosine A_1 receptors can open ATP-sensitive potassium channels by a mechanism involving Gi protein (Kirsch *et al.*, 1990). The increased K^+ flux may result in hyperpolarization to counteract the depolarizing effect of ischaemia leading to a reduction in the influx of Ca^{2+}. This may contribute to the antiarrhythmic mechanism of adenosine in preconditioning. Therefore, the antiarrhythmic action of BN-063 in myocardial ischaemia may be partially achieved via opening ATP-sensitive potassium channels.

In conclusion, ventricular arrhythmias induced by myocardial ischaemia and catecholamines could be suppressed by BN-063 through the activation of adenosine A_1 receptors. The beneficial effects of BN-063 could be of considerable clinical use in the prevention and treatment of ischaemia-induced arrhythmias.

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Down-regulation of bombesin binding to guinea-pig pancreatic acinar cells during homologous desensitization

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1 [^{125}I]-Tyr^4-bombesin exhibited saturable binding to pancreatic acinar cells.

2 Preincubation of cells at 37°C with 0.03 nM–1 μM-bombesin for 10 min followed by acid or neutral washes reduced subsequent binding of [^{125}I]-Tyr^4-bombesin in a concentration-dependent manner by up to 90%.

3 In cell suspensions, bombesin raised the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) and the $[Ca^{2+}]_i$ response was reduced by pre-exposure to the agonist.

4 In individual superfused cells, bombesin at 1 nM normally caused a large $[Ca^{2+}]_i$ transient followed by sustained $[Ca^{2+}]_i$ oscillations. The cells exhibited a variable degree of desensitization when subsequently exposed to higher agonist concentrations.

5 Preincubation with bombesin for 10 min caused a concentration-related reduction of subsequent amylase responses to bombesin.

6 Down-regulation of receptor binding is a rapid phenomenon during bombesin exposure explaining, at least partially, tachyphylaxis of $[Ca^{2+}]_i$ and amylase responses.

Keywords: Acinar cell; amylase; bombesin; calcium; cholecystokinin; pancreas; receptor

Introduction

Amylase release from pancreatic acinar cells is stimulated by a number of agonists including bombesin (Jensen *et al.*, 1978; Uhleman *et al.*, 1979). The signal generated by interaction with the acinar cell receptor for bombesin (Jensen *et al.*, 1978; Zhu *et al.*, 1991) is at least partially transduced by lysis of polyphosphoinositides leading to formation of Ca^{2+} mobilizing inositol 1,4,5-tris-phosphate and protein kinase C activating diacylglycerol (Pandol *et al.*, 1985; Sjödin *et al.*, 1990; Matozaki *et al.*, 1991). Interaction with the receptor induces amylase secretion which exhibits a relatively rapid tachyphylaxis both *in vitro* (Shinozaki & Funakoshi, 1988) and *in vivo* (Coy *et al.*, 1992).

In individual superfused rat acinar cells, low concentrations of bombesin ($\leqslant$ 0.1 nM) have been reported to induce sustained oscillations of the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) whereas concentrations at or above 1 nM evoke a large $[Ca^{2+}]_i$ transient followed by a small sustained elevation (Matozaki *et al.*, 1991). The aim of the present study was to investigate whether exposure of guinea-pig pancreatic acinar cells to bombesin decreases their subsequent capacity to bind the peptide and to determine whether such an effect is related to reduced $[Ca^{2+}]_i$ and amylase responses to bombesin.

Methods

Preparation of pancreatic acinar cells

Male guinea-pigs of the Hartley strain (150–200 g), obtained from Sahlins försöksdjursfarm, Malmö, Sweden, were deprived of food overnight and killed by CO_2. The pancreas was dissected free from fat and mesentery. Dispersed acinar cells were prepared by the technique of Amsterdam and Jamieson (1972). The method included digestion of the pancreas with crude collagenase and hyaluronidase and a Ca^{2+} chelation step with addition of ethylenediamine-tetraacetic acid. Cells were suspended in Krebs-Ringer bicarbonate (KRB) buffer (pH 7.4) equilibrated with 95% O_2 and 5% CO_2 and containing amino acid and vitamin supplement (Eagle, 1959) with 0.01% (w/v) soybean trypsin inhibitor, 15 mM glucose, 1% (w/v) bovine serum albumin and 0.5 mM Ca^{2+}. Suspensions of isolated cells were used for binding experiments and for $[Ca^{2+}]_i$ determinations. For measurements of amylase release and of $[Ca^{2+}]_i$ in individual cells, pancreatic acini together with single cells were prepared by a modification (Barlas *et al.*, 1982) of the method of Peikin *et al.* (1978). The method included digestion with purified collagenase. For $[Ca^{2+}]_i$ and amylase experiments, cells and acini were normally suspended in a medium equilibrated with O_2 and containing (mM): HEPES 24.5 (pH 7.4), NaCl 98, KCl 6, Na_2HPO_4 2.5, $CaCl_2$ 0.5, $MgCl_2$ 1.0, sodium pyruvate 5, sodium fumarate 5, sodium glutamate 5, glucose 13.5, glutamine 2, 1% (w/v) albumin, 0.01% (w/v) trypsin inhibitor, 2% (v/v) amino acid and 1% (v/v) vitamin mixture.

Binding experiments

Suspensions of 16.4 ± 0.6 × 10^6 cells ml^{-1} (mean ± s.e. mean; $n = 12$) in KRB-buffer were incubated at indicated temperatures with (6–17) × 10^{-11} M [^{125}I]-Tyr^4-bombesin, with and without unlabelled peptide. At specified times after addition of tracer, duplicate or triplicate 100 μl samples of cell suspensions were layered over 300 μl ice-cold KRB buffer in microfuge tubes of polypropylene. The cells were centrifuged for 15 s at 9,000 *g* in a Beckman Model B microcentrifuge (Beckman Instruments, Palo Alto, CA, U.S.A.). The pellets were washed four times in KRB-buffer. The tubes containing washed pellets were placed in a Packard 800 C or 5780 gamma counter (United Technologies-Packard, Downer's Grove, IL. U.S.A.). Duplicate samples were also taken from the incubation mixture containing both cells and medium and counted. Binding of labelled peptide was expressed as a percentage of added radioactivity. Cell-associated radioactivity in the presence of 10^{-6} M unlabelled peptide (non-saturable binding) was 5.4 ± 1.4% of total binding (mean ± s.e.mean; $n = 9$). Saturable binding was calculated by subtraction of non-saturable binding from total binding. Inhibition of [^{125}I]-Tyr^4-bombesin binding by increasing concentrations of unlabelled bombesin was analyzed by a computerized

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non-linear least-squares algorithm (Munson & Rodbard, 1980).

Surface bound bombesin or [^{125}I]-Tyr^4-bombesin was released by washes of cells at pH 7.4 or 3.1. At specified times, cells were centrifuged at 100 *g* for 2 min. The pellets were suspended in KRB buffer (pH 7.4) or in 0.02 M acetic acid containing 0.15 M NaCl (pH 3.1) for 4 min. This procedure was repeated once. In experiments with pre-exposure to bombesin, cells were suspended once in KRB buffer after acid washes before resuspension in incubation medium.

[Ca^{2+}]$_i$-experiments

Cells ($1-3 \times 10^6$) were suspended in 5 ml of the HEPES-buffered medium described above and 5 µl 1 mM-fura-2 acetoxymethylester in dimethylsulphoxide added. After incubation for 40 min at 37°C, the cells were washed with medium lacking the indicator. The cells were then suspended in 1 ml medium and transferred to a 1 cm quartz cuvette placed in a thermostatically controlled (37°C) cuvette holder of a time-sharing multichannel spectrophotofluorometer allowing continuous recording of the 340/380 nm fluorescence excitation ratio. [Ca^{2+}]$_i$ was then calculated as previously described (Gylfe, 1988).

For microfluorometric measurements, acini and single cells were allowed to attach for 1–2 h to cover glasses coated with poly-L-lysine or Matrigel. The cells were loaded with Fura-2, by incubation for 40 min with 1 µM-Fura-2-acetoxymethylester. The cover glasses with cells were rinsed and used as the bottom of an open chamber designed for microscopic work (Gylfe, 1990). The chamber was connected to a two-channel peristaltic pump, allowing steady superfusion at a rate of 1.0 ml min^{-1}. The chamber was placed on the stage of an inverted microscope (Nikon Diaphot) within a climate box maintained at 37°C. The microscope was equipped for epifluorescence microscopy continuously recording the 340/380 nm fluorescence excitation ratio allowing calculation of [Ca^{2+}]$_i$ (Gylfe, 1990; Sjödin *et al.*, 1991) of individual cells.

Amylase release experiments

In experiments defining the dose-response-relationship for bombesin-stimulated amylase secretion, acini were incubated at 37°C for 30 min with different concentrations of bombesin. In other experiments elucidating the effect of pre-exposure to bombesin, acini were incubated with different concentrations of bombesin for 10 min followed by centrifugation at 50 *g* for 1 min. Acini were resuspended in HEPES-buffered medium for 4 min and pelleted. This washing procedure was repeated once. The acini were then incubated with agonist for 20 min. At the beginning and end of the incubation, amylase activity was recorded in the extracellular medium. Total amylase was determined in the mixture of acini and incubation medium after lysis of cells by a ten fold dilution with a solution containing 0.01 M sodium phosphate (pH 7.8), 0.1% (w/v) albumin and 0.1% (w/v) sodium dodecyl sulphate. Amylase activity was measured using the Phadebas reagent (Ceska *et al.*, 1969) and release was calculated as the percentage of the amylase content of the acini.

Substances

Crude (Sigma type I, 125 u mg^{-1}) and purified (Sigma type VII 1000–3000 u mg^{-1}) collagenase, hyaluronidase (Sigma type III, 600 u mg^{-1}), poly-L-lysine, purified soybean trypsin inhibitor and 2-hydroxy-ethyl-piperazine-*N*-sulphonic acid (HEPES) were from Sigma Chemical Co., St. Louis, MO, U.S.A.; Molecular Probes, Inc., Eugene, OR, U.S.A., supplied the acetoxymethylester of Fura-2. Bovine serum albumin, fraction V, was obtained from ICN Life Sciences, Cleveland, OH, U.S.A. and Phadebas amylase test tablets were from Pharmacia-LKB Biotechnology, Uppsala, Sweden. Gibco Bio-Cult, Ltd, Paisley, U.K. provided modified Eagle's medium amino acid solution (50 times concentrated) and vitamin solution (100 times concentrated). Matrigel was obtained from Collaborative Research, Bedford, MA, U.S.A. [^{125}I]-Tyr^4-bombesin with specific activity of 2200 Ci mmol^{-1} was from New England Nuclear, Dreieich, Germany. Bombesin and the sulphated form of cholecystokinin octapeptide (CCK-8) were from Peninsula Lab, Inc., Belmont, CA, U.S.A. All other chemicals were of analytical grade.

Results

Ligand binding

[^{125}I]-Tyr^4-bombesin bound rapidly to acinar cells at 37°C with saturation after 15 min (Figure 1). Maximal saturable binding was about 30% of added radioactivity. Cell-associated radioactivity resistant to acid washes (pH 3.1) increased from 20% after 15 min to about 30% of maximal binding following 60 min incubation.

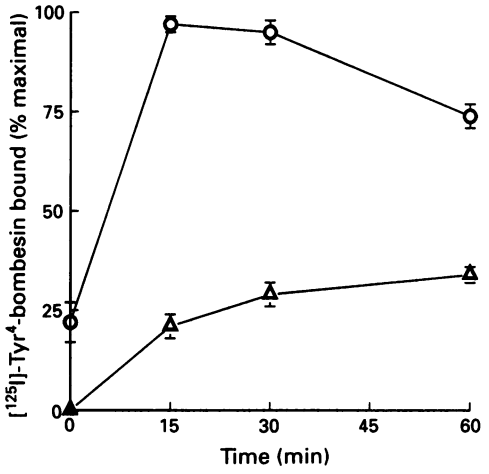


Figure 1 Time-course for saturable [^{125}I]-Tyr^4-bombesin binding to pancreatic acinar cells. Cells were incubated with ~0.1 nM [^{125}I]-Tyr^4-bombesin at 37°C for indicated times and washed at pH 7.4 (○) or suspended and washed at pH 3.1 (△). Saturable binding is expressed as % of maximal saturable binding in each experiment. Means ± s.e.mean of 4 experiments.

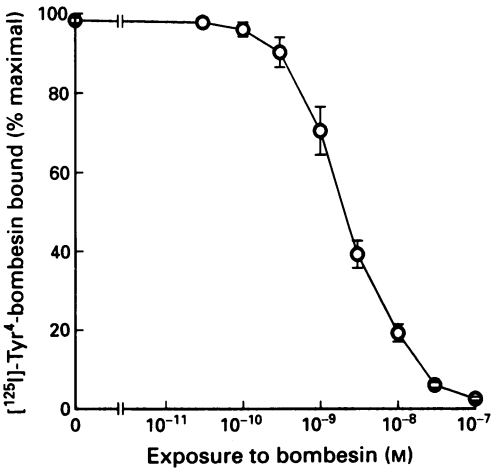


Figure 2 Ability of bombesin to inhibit saturable binding of [^{125}I]-Tyr^4-bombesin (~0.1 nM) to pancreatic acinar cells incubated at 37°C for 30 min. Saturable binding is expressed as % of maximal saturable binding in each incubation series. Mean ± s.e.mean of 4 experiments.

Saturable binding of [^{125}I]-Tyr^4-bombesin was inhibited in a concentration-dependent way by bombesin (Figure 2). A computerized analysis of two combined experiments (Munson & Rodbard, 1980) indicated that the data were compatible with both one- and two-site models. The one-site model gave a K_D of 1.4×10^{-9} M and the number of binding-sites per cell was 9100. A two-site model did not give a significantly better fit. However, if cells were preincubated for 10 min with 3×10^{-9} M bombesin, a two-site model was preferred ($P < 0.01$) with dissociation constants of 8.9×10^{-10} and 9.4×10^{-9} M. The number of sites per cell was 2000 and 3900 of the high- and low-affinity type, respectively, indicating a reduction of the total number of binding-sites following pre-exposure to agonist.

Preincubation of cells for 10 min with different concentrations of bombesin followed by washes at pH 7.4, dose-dependently reduced subsequent tracer binding (Figure 3a). Also after washes at pH 3.1, which *per se* reduced the capacity of the cells to bind tracer, a corresponding concentration-dependent inhibition of [^{125}I]-Tyr^4-bombesin binding was seen, irrespective of whether the preincubation period was 10 min (Figure 3b) or 5 min (not shown).

The ability of neutral (pH 7.4) and acid washes (pH 3.1) to release surface-bound [^{125}I]-Tyr^4-bombesin was studied after 30 min incubation at pH 7.4 and 4°C. At this temperature there is little internalization of bombesin. Washing of cells for 8 min at pH 7.4 reduced saturable binding from 15.6 to 4.7% whereas similar washing at pH 3 eliminated such binding (Table 1).

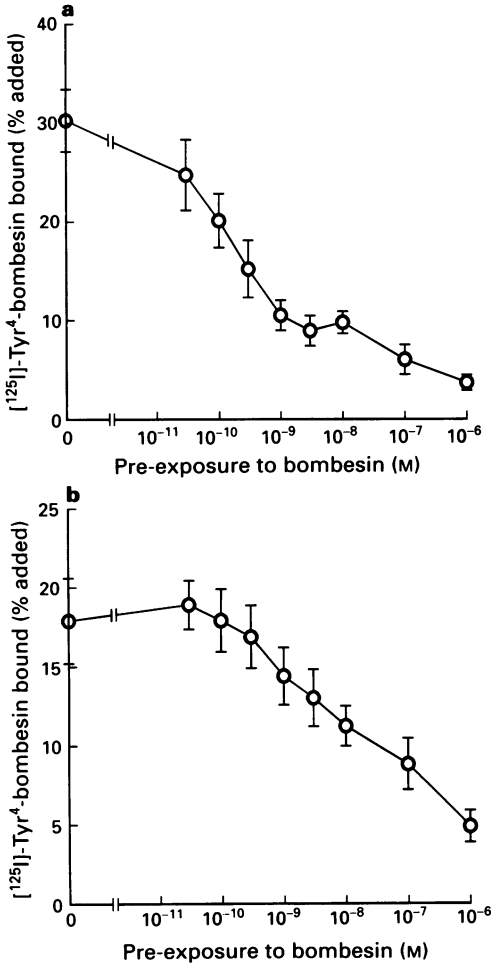


Figure 3 Effects of preincubation of pancreatic acinar cells with indicated concentrations of bombesin on subsequent saturable binding of [^{125}I]-Tyr^4-bombesin. After preincubation for 10 min at 37°C, cells were washed in neutral medium (a) or acid (pH 3.1, b) for two periods of 4 min and finally incubated for 30 min with ~0.1 nM [^{125}I]-Tyr^4-bombesin in the presence and absence of 1 μM bombesin. Means ± s.e.mean of 5 (a) or 6 (b) experiments.

Cytoplasmic calcium concentrations

In suspensions of acinar cells, 10^{-10} M bombesin induced a moderate relatively sustained rise of $[Ca^{2+}]_i$. Subsequent addition of 10^{-8} M bombesin evoked a further transient elevation corresponding to 35% of the peak response of naive cells. After a final addition of 10^{-9} M CCK-8, $[Ca^{2+}]_i$ reached about 20% of levels attained in previously unstimulated cells (Figure 4). An initial addition of 10^{-9} M bombesin caused a greater, partly sustained elevation of $[Ca^{2+}]_i$

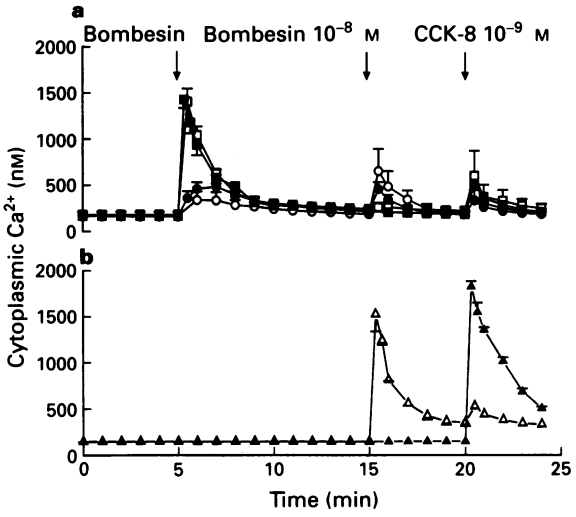


Figure 4 Effects of different concentrations of bombesin on $[Ca^{2+}]_i$ of acinar cells in suspensions. Fura-2-loaded cells ($1-3 \times 10^6$) were incubated in 1 ml medium at 37°C and stimulated initially with bombesin at 10^{-10} M (○), 10^{-9} M (●), 10^{-8} M (□) or 10^{-7} M (■) followed by additions of 10^{-8} M bombesin and 10^{-9} M cholecystokinin octapeptide (CCK-8) (a). In control experiments, cells were not stimulated initially but only by the later additions of 10^{-8} M bombesin and 10^{-9} M CCK-8 (△) or by 10^{-9} M CCK-8 alone (▲, b). The data are from 3–4 experiments. Means ± s.e.mean.

Table 1 Efficiency of various washing procedures in releasing surface-bound [^{125}I]-Tyr^4-bombesin

Direct centrifugation		*Washing at pH 7.4*		*Washing at pH 3.1*	
Total (%)	*Saturable* (%)	*Total* (%)	*Saturable* (%)	*Total* (%)	*Saturable* (%)
16.8 ± 1.7	15.6 ± 1.9	4.8 ± 1.8	4.7 ± 1.8	0.2 ± 0.1	0

Cell-associated radioactivity (% bound) after 30 min incubation with ~0.1 nM [^{125}I]-Tyr^4-bombesin at 4°C and pH 7.4 followed by direct centrifugation (9,000 *g*) of acinar cells and subsequent washing of pellets in buffer or by centrifugation (100 *g*) and resuspension of cells for 2 periods of 4 min with buffer at pH 7.4 or with 0.02 M acetic acid containing 0.15 M NaCl at pH 3.1 with subsequent centrifugation (9,000 *g*) and washing of pellets. Values are means ± s.e.mean; $n = 4$.

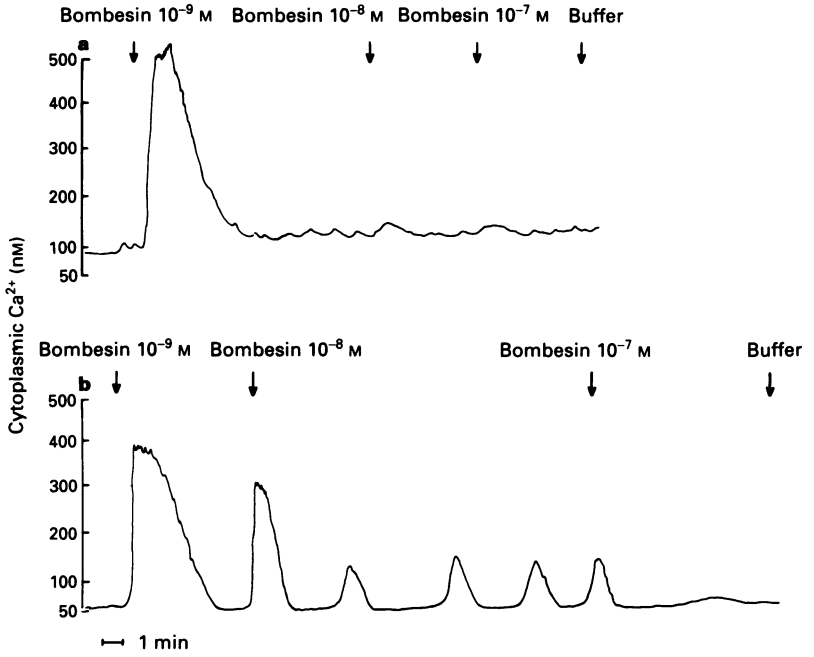


Figure 5 Effects of different concentrations of bombesin on $[Ca^{2+}]_i$ of individual acinar cells. Fura-2 loaded cells were superfused at 37°C with medium containing indicated concentrations of bombesin.

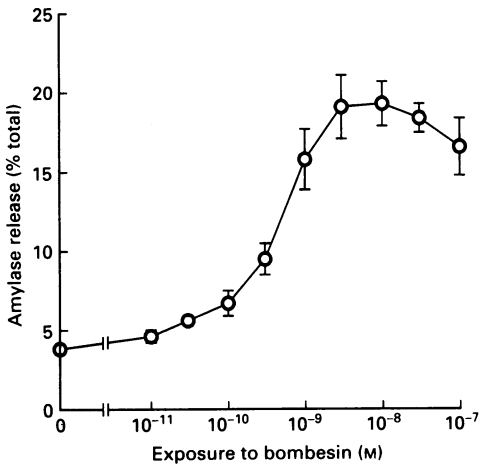


Figure 6 Ability of bombesin to evoke release of amylase (% of total) from dispersed pancreatic acini incubated at 37°C for 30 min with indicated concentrations of bombesin. Means ± s.e.mean of 10–13 incubation series.

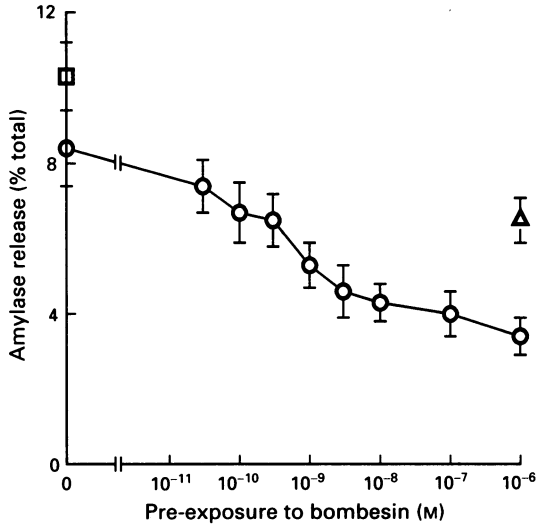


Figure 7 Effects of preincubation of pancreatic acini for 10 min at 37°C with indicated concentrations of bombesin on amylase release from acini after washing for two periods of 4 min with neutral medium and subsequent incubation with 2×10^{-8} M bombesin or 10^{-9} M cholecystokinin octapeptide (CCK-8) (□; △) for 20 min during which amylase release was determined and expressed as % of total amylase after subtraction of basal release. Means ± s.e.mean of 12–14 incubation series.

followed by limited subsequent responses to 10^{-8} M bombesin and 10^{-9} M CCK-8. Naive cells reacted to 10^{-8} and 10^{-7} M bombesin with high initial $[Ca^{2+}]_i$-peaks followed by sustained elevated levels identical with those following stimulation with 10^{-9} M bombesin. Subsequent addition of 10^{-8} M bombesin caused little or no $[Ca^{2+}]_i$-elevation.

Figure 5 shows recordings of $[Ca^{2+}]_i$ of two individual cells, responding to 10^{-9}–10^{-7} M bombesin. Each cell reacted to 10^{-9} M bombesin with an initial large transient followed by smaller oscillations. One cell (Figure 5a) was almost totally desensitized to higher agonist concentrations, whereas another exhibited a partially reduced responsiveness (Figure 5b). Of 18 examined cells, 16 exhibited desensitization with response patterns similar to the cells illustrated in Figures 5a and b. Particularly, the initial $[Ca^{2+}]_i$ transients were reduced when cells had been pre-exposed to lower agonist concentrations.

Amylase release

Amylase responses of suspended acini to various concentrations of bombesin for 30 min at 37°C increased concentration-dependently (Figure 6). Maximal effects were obtained at 3×10^{-9}–10^{-8} M, whereas 10^{-7} M evoked less secretion. The amylase response to 2×10^{-8} M was reduced in a

concentration-related way by up to about 60% following preincubation for 10 min with increasing concentrations of the peptide (Figure 7). Preincubation of cells with 10^{-6} M bombesin also reduced the amylase response to 10^{-9} M CCK-8 by about 35%.

Discussion

The guinea-pig pancreatic acinar cells reacted with concentration-dependent increases of $[Ca^{2+}]_i$ and amylase release on stimulation with bombesin. The kinetics of the $[Ca^{2+}]_i$-responses in suspensions of cells revealed an initial peak phase followed by a second partially sustained elevation in agreement with previous studies (Sjödin *et al.*, 1990; Matozaki *et al.*, 1991). In individual cells, the normal response pattern consisted in large initial $[Ca^{2+}]_i$ transients followed by oscillations of moderate amplitudes. The $[Ca^{2+}]_i$-response consequently faded during continuous bombesin stimulation and disappeared completely in some cells when exposed to high agonist concentrations.

Loss of responsiveness to an agonist may be due to a variety of mechanisms. We have recently reported that the specific desensitization of pancreatic acinar cells to substance P (SP) is related to a rapid and profound down-regulation of receptor binding (Sjödin *et al.*, 1994) whereas the inhibition of enzyme secretion during stimulation of acinar cells with supramaximal concentrations of CCK-8 involves desensitization to cytoplasmic Ca^{2+} (Nilsson *et al.*, 1994).

The present experiments demonstrate that pre-exposure to bombesin for only 5–10 min reduces subsequent agonist binding, extending previous findings of reduced bombesin binding following 60 min pre-exposure to agonist in rat pancreas (Zhu *et al.*, 1991). The efficiency of the washing procedures indicates that the reduction cannot be explained by prolonged receptor occupancy. It is also clear that exposure to high bombesin concentrations not only inhibits subsequent [^{125}I]-Tyr^4-bombesin binding and reduces the $[Ca^{2+}]_i$ and amylase responses to this agonist, but also inhibits the responses to CCK-8. The tachyphylaxis of the $[Ca^{2+}]_i$ and amylase responses to bombesin thus appears to involve both a specific mechanism related to homologous agonist receptor binding and a more general phenomenon also affecting the response to CCK-8. In this respect, the present findings differ from those of Lee *et al.* (1980) which indicated that desensitization induced by bombesin did not affect amylase responses to CCK-8.

Previous studies have indicated that bombesin receptors belong to a single affinity class with a K_D of around 2 nM (Jensen *et al.*, 1978; Zhu *et al.*, 1991). However, two bombesin receptor subtypes have recently been detected in the digestive tract (Jensen & Coy, 1991) and in brain (Ladenheim *et al.*, 1992). The present study suggests that preincubation with bombesin reveals two different affinity states for agonist binding and that the number of sites is reduced by pre-exposure to the agonist. However, it should be noted that the results were obtained under steady-state and not true equilibrium conditions.

Both SP (Sjödin *et al.*, 1994) and CCK-8 (Menozzi *et al.*, 1991) appear to stimulate secretion by interaction with low-affinity receptors and the desensitizing effects of SP and CCK-8 also take place at higher agonist concentrations (Abdelmoumene & Gardner, 1980; Menozzi *et al.*, 1989; Sjödin *et al.*, 1994). For bombesin, 3×10^{-11}–10^{-10} M evoked threshold $[Ca^{2+}]_i$ and amylase responses whereas 3×10^{-9}–10^{-8} M induced maximal effects. At concentrations above 10^{-8} M, secretion was slightly submaximal in naive cells. Down-regulation of bombesin binding and desensitization of $[Ca^{2+}]_i$ and amylase responses after previous exposure to the agonist took place over a wide range of concentrations from 3×10^{-11} to 10^{-7} M. It is possible that the broad concentration-range reflects involvement of more than one mechanism.

Pre-exposure of acini to bombesin reduced subsequent agonist-stimulated amylase release by up to 60%. The $[Ca^{2+}]_i$ experiments demonstrated that desensitization mainly affected the initial response, whereas the sustained later phase was less affected. This observation may explain the remaining enzyme secretion after pre-exposure to high agonist concentrations. Since the inhibitory mechanism mentioned involving desensitization to cytoplasmic Ca^{2+} was restricted to the second phase of enzyme secretion (Nilsson *et al.*, 1994), it may not be of major importance for the observed desensitization elicited by bombesin.

The present results indicate that down-regulation of binding of bombesin to pancreatic acinar cells is one mechanism of importance for tachyphylaxis of the $[Ca^{2+}]_i$ and amylase responses. At high bombesin concentrations, the cells also exhibit reduced responsiveness to other agonists.

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Novel histamine H_3 receptor antagonists: affinities in an H_3 receptor binding assay and potencies in two functional H_3 receptor models

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1 We determined the affinities of ten novel H_3 receptor antagonists in an H_3 receptor binding assay and their potencies in two functional H_3 receptor models. The novel compounds differ from histamine in that the aminoethyl side chain is replaced by a propyl or butyl chain linked to a polar group (amide, thioamide, ester, guanidine, guanidine ester or urea) which, in turn, is connected to a hexocyclic ring or to an alicyclic ring containing an alkyl residue.

2 The specific binding of [^{3}H]-N$^\alpha$-methylhistamine to rat brain cortex membranes was monophasically displaced by each of the ten compounds at pK_i values ranging from 7.56 to 8.68.

3 Inhibition by histamine of the electrically evoked tritium overflow from mouse brain cortex slices preincubated with [^{3}H]-noradrenaline was antagonized by the ten compounds and the concentration-response curve was shifted to the right with apparent pA_2 values ranging from 7.07 to 9.20.

4 The electrically induced contraction in guinea-pig ileum strips (which was abolished by atropine) was inhibited by the H_3 receptor agonists **R**-(−)-α-methylhistamine (pEC_{50} 7.76), N$^\alpha$-methylhistamine (7.90) and imetit (8.18). The concentration-response curve of **R**-(−)-α-methylhistamine was shifted to the right by thioperamide (apparent pA_2 8.79) and by the ten novel compounds (range of pA_2 values 6.64–8.81).

5 The affinities and potencies were compared by linear regression analysis. This analysis was extended to thioperamide, the standard H_3 receptor antagonist, which is also capable of differentiating between H_{3A} and H_{3B} sites. Comparison of the apparent pA_2 values in the two functional H_3 receptor models yielded a regression coefficient of 0.77 ($P<0.02$). When the pA_2 of the drugs in the mouse brain cortex were compared to the pK_i for H_3 sites (ten novel compounds) and for H_{3A} sites (thioperamide), a significant correlation ($r = 0.87$; $P<0.001$) was obtained. There was, however, no significant correlation when the pK_i of thioperamide for H_{3B} sites was used instead ($r = 0.52$). In a similar manner, comparison of the pA_2 in the guinea-pig ileum with the pK_i in the binding assay yielded a significant correlation ($r = 0.70$, $P<0.05$) only when the pK_i of thioperamide for H_{3A} sites was used but not when its pK_i for H_{3B} sites was considered ($r = 0.17$, NS).

6 On the basis of these results, structure-activity relationships for the novel H_3 receptor antagonists, and the nature of the H_3 receptors in the guinea-pig ileum and mouse brain, are considered.

Keywords: [^{3}H]-N$^\alpha$-methylhistamine binding; rat brain cortex membranes; noradrenaline release; mouse brain cortex slices; guinea-pig ileum strips; H_3 receptor subtypes; presynaptic receptors; histamine; **R**-(−)-α-methylhistamine; thioperamide

Introduction

A new type of histamine receptor, termed H_3, has been characterized by Arrang *et al.* (1983), at which impromidine (a partial H_2 receptor agonist) and burimamide (an H_2 receptor antagonist) proved to be potent antagonists. Subsequently, Arrang *et al.* (1987) showed that thioperamide is superior to impromidine and burimamide with respect to its antagonistic potency and, more importantly, its selectivity towards H_3 receptors. Subsequently, a variety of H_3 receptor antagonists have been synthesized (for review, see Leurs & Timmerman, 1992; Lipp *et al.*, 1992) and the structural requirements necessary for an H_3 receptor antagonist have been defined (Lipp *et al.*, 1992). Thus, an antagonistic effect at H_3 receptors can be expected for a molecule which consists of a nitrogen-containing heterocyclic ring (most favourably an imidazole) connected to a polar group via an alkyl chain. The affinity is increased by a lipophilic residue linked to the polar group by a spacer. Numerous H_3 receptor antagonists have recently been synthesized by some of us according to this construction pattern (Schwartz *et al.*, 1992) and ten of these compounds will be considered in the present study.

We determined the affinities of these compounds for H_3 receptors in a radioligand binding study and their antagonistic potencies in two functional H_3 receptor models. [^{3}H]-N$^\alpha$-methylhistamine was used for the binding studies. The specific binding of this ligand to rat brain membranes is displaced biphasically by thioperamide and burimamide; this finding led West *et al.* (1990) to propose the existence of H_3 receptor subtypes, termed H_{3A} and H_{3B}. According to this classification, the H_3 receptor involved in the inhibitory effect of histamine on noradrenaline release in the mouse brain cortex can be classified as H_{3A} (Schlicker *et al.*, 1992). Combining both experimental approaches with the H_3 receptor model in the guinea-pig ileum, the functional H_3 receptor model used most frequently (for review, see Vollinga *et al.*, 1992), we tried to obtain further evidence in favour of the occurrence of H_3 receptor subtypes. Another aim of the present study was to demonstrate structure-activity relationships for the novel H_3 receptor antagonists.

Methods

Binding studies

Binding experiments were carried out according to West *et al.* (1990) with the modifications described by Kathmann *et*

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al. (1993). Cerebral cortices obtained from male Wistar rats were homogenized (Potter-Elvehjem; 10 up and down strokes, in a period of 1 min) in 25 volumes of ice-cold Tris-HCl buffer (Tris 50 mmol l^{-1}, pH 7.5; EDTA 5 mmol l^{-1}; sucrose 10.27%) and centrifuged at 1000 g for 10 min (4°C). The supernatant was centrifuged at 35000 g for 10 min and the pellet was resuspended in 10 volumes of Tris-HCl buffer and frozen at − 80°C.

The binding assay was performed in Tris-HCl buffer (Tris 50 mmol l^{-1}, pH 7.5; EDTA 5 mmol l^{-1}) in a final volume of 0.5 ml containing 0.2–0.3 mg protein. [^{3}H]-N$^\alpha$-methylhistamine (specific activity 80 Ci $mmol^{-1}$) was used at a concentration of 0.2 nmol l^{-1}. The incubation (30°C) was terminated after 40 min by filtration through polyethyleneimine (0.3%)-pretreated Whatman GF/C filters. Each filter was placed in a counting vial and 6 ml of the commercially available scintillant Ready Gel (Beckman, Fullerton, CA, U.S.A.) was added. The radioactivity of the filters was determined in a Beckman LS 6000TA counter.

Non-specific binding (7% of total binding) was determined in the presence of **R**-(−)-α-methylhistamine (**R**-αMH) 2 μmol l^{-1}. Protein was assayed according to the method described by Bradford (1976). Data were analysed using the programmes LIGAND (McPherson et al., 1983) and GraphPadInPlot (GPIP; GraphPad Software, San Diego, CA, U.S.A.).

Superfusion studies

Superfusion experiments were performed according to Schlicker et al. (1992). Briefly, cerebrocortical slices from male NMRI mice were incubated for 60 min at 37°C with physiological salt solution (PSS; for composition, see Kathmann et al., 1993) containing [^{3}H]-noradrenaline 25 nmol l^{-1} (specific activity 52.3 Ci $mmol^{-1}$) and subsequently superfused with PSS (37°C; 110 min) containing desipramine 1 μmol l^{-1} plus rauwolscine 1 μmol l^{-1}. The superfusate was collected in 5-min samples. Tritium overflow was evoked by two 2-min periods of electrical field stimulation (36 rectangular pulses of 50 mA and 2 ms; 0.3 Hz) 40 (S_1) and 90 min (S_2) after onset of superfusion. Histamine was added to the PSS from 62 min of superfusion onward and the histamine receptor antagonist under study was present in the PSS throughout superfusion. At the end of superfusion, the slices were solubilized with 0.5 ml Soluene (Packard, Meriden, CT, U.S.A.) and 7 ml scintillant (containing 6 g PPO and 0.1 g POPOP in 1 l toluene) was added. The vials containing the superfusate samples were supplemented with 6 ml Ready Gel. The radioactivity of the solubilized slices and superfusate samples was determined in a Beckman LS 6000TA counter.

Stimulation-evoked tritium overflow was calculated by subtraction of the basal from the total tritium efflux during the period of stimulation and the subsequent 13 min and was expressed as percentage of the tritium in the slices at the onset of stimulation; basal efflux was assumed to decline linearly from the 5-min period before to that 15–20 min after onset of stimulation. To determine the effect of histamine (in the presence or absence of antagonists) on the evoked overflow, the ratio of the overflow evoked by S_2 over that evoked by S_1 was calculated (S_2/S_1). For quantification of the effects of histamine receptor antagonists on the evoked overflow, the tritium overflow evoked by S_1 was compared to the corresponding value obtained in the absence of the antagonist.

Organ bath studies

Longitudinal muscle strips (ca. 2 cm) were prepared from the small intestine, 20–50 cm proximal to the ileocaecal valve, of female guinea-pigs (350–450 g). The muscle strips were mounted between two platinum electrodes (4 mm apart) in 20 ml Krebs buffer (composition in mmol l^{-1}: NaCl 118, KCl 5.6, $MgSO_4$ 1.18, $CaCl_2$ 2.5, NaH_2PO_4 1.28, $NaHCO_3$ 25 and glucose 5.5), connected to an isometric transducer, continuously gassed with carbogen (95% O_2:5% CO_2) and maintained at 37°C. After equilibration of the muscle segments for 1 h with washings every 10 min, they were stimulated continuously with rectangular pulses of 15 V and 0.5 ms at a frequency of 0.1 Hz. After 30 min of stimulation, cumulative concentration-response curves with half-logarithmic increments of **R**-αMH were recorded until no change in response was found. Subsequently, the preparations were washed 3 times every 10 min without any stimulation. The antagonist under study was allowed to equilibrate with the tissue for 20–30 min before the re-determination of the concentration-response curve of **R**-αMH. Up to three concentration-response curves could be constructed in one preparation. Mepyramine 1 μmol l^{-1} was routinely present in the medium in order to block H_1 receptors.

Calculations and statistics

The apparent pA_2 value of the test compounds against histamine (mouse brain cortex slices) or **R**-αMH (guinea-pig ileum strips) was calculated according to formula 4 of Furchgott (1972): $pA_2 = \log([E']/[E] - 1) - \log[B]$, where [E'] and [E] are the concentrations of the agonist producing the half-maximum effect in the presence and absence of the antagonist, respectively, and [B] is the concentration of the antagonist. In mouse brain cortex slices, the maximum inhibitory effect obtainable with histamine was consistently about 70%; thus, the concentration producing an inhibition of 35% was used for determination of the pEC_{50} and apparent pA_2. In guinea-pig ileum strips, the maximum effect obtainable with agonists is highly variable ranging from less than 30% to nearly 100%; only tissues in which the maximum effect of the first curve was more than 30% were considered. For determination of the pEC_{50} and apparent pA_2, the concentrations producing the half-maximum effect in the individual tissues were used.

Results are given as means ± s.e.mean of n experiments (functional experiments) or of n experiments in triplicate (binding experiments). In order to evaluate whether the displacement of [^{3}H]-N$^\alpha$-methylhistamine binding by the drugs under study is better fitted by a one- or two-site model, the F-test was used (partial F-test; de Lean et al., 1982). The pK_i and the pA_2 values of the test compounds were compared by linear regression analysis; the Bonferroni correction (Holm, 1979) was used since the same data were subjected repeatedly to the regression analysis.

Drugs

The following were used: N$^\alpha$-[methyl-^{3}H]-methylhistamine dihydrochloride, (−)-[ring-2,5,6-^{3}H]-noradrenaline base (NEN, Dreieich, Germany); atropine sulphate, histamine dihydrochloride (Merck, Darmstadt, Germany); desipramine hydrochloride (CIBA-Geigy, Wehr, Germany); 2,5-diphenyloxazole (PPO), 1,4-di-[2-(5-phenyloxazolyl)]-benzene (POPOP; Zinsser, Frankfurt, Germany); imetit, mepyramine, **R**-(−)-α-methylhistamine dihydrogen maleate, N$^\alpha$-methylhistamine dihydrochloride and the test compounds (for chemical structures, see Table 1) numbers **3**, **4**, **8** and **9** (free bases), number **1** (dihydrochloride) and numbers **2**, **5**, **6**, **7** and **10** (maleates) were synthesized at the Institut für Pharmazie (Freie Universität, Berlin, Germany; the synthesis of the novel H_3 receptor antagonists will be described elsewhere); rauwolscine hydrochloride (Roth, Karlsruhe, Germany).

Results

Affinities of the test compounds for H_3 receptor binding sites

We have recently shown that [^{3}H]-N$^\alpha$-methylhistamine binds saturably to an apparently homogeneous class of receptors

Table 1 Chemical structures, affinities for H_3 binding sites and antagonistic potencies in two functional H_3 receptor models of ten newly synthesized compounds and of thioperamide

Compound	*Chemical structure*				*Binding experiments*	*Functional experiments*	
					pK_i	Apparent pA_2	
	n	R^1	R^2	R^3	R^4	Rat brain cortex membranes[a]	Mouse brain cortex slices[b]	Guinea-pig ileum strips[c]
1	2	H	NH	NH	$NH\text{-}CH_2-$	8.68	9.20	8.81
2	2	H	0	0	$(CH_2)_2-$	8.52	8.28	7.23
3	2	H	NH	N-C-OC(CH₃)₃ (O)	$NH\text{-}CH_2-$	7.98	8.67	7.38
4	3	H	NH	N-C-OC(CH₃)₃ (O)	NH-	7.86	7.28	6.93
5	2	CH_3	NH	0	$(CH_2)_2-$	7.76	7.98	7.27
6	2	H	NH	0	$(CH_2)_2-$	7.66	7.47	6.81
7	2	H	NH	0	$(CH_2)_2-$	7.63	7.18	7.21
8	2	H	NH	0	NH-	7.58	7.07	7.26
9	2	H	NH	S	$(CH_2)_2-$	7.57	7.40	7.51
10	2	H	NH	0	$(CH_2)_2-$	7.56	7.27	6.64
Thioperamide						8.31, 7.19[d]	8.67[e]	8.79

[a]pK_i values were determined from the concentration-response curves (shown in Figure 1) of the novel H_3 receptor antagonists for their inhibitory effect on [^{3}H]-N$^\alpha$-methylhistamine binding to rat brain cortex membranes. Means of 4–8 experiments; the s.e.mean values (not shown) ranged from 0.02 to 0.10.

[b]The apparent pA_2 value of 1 was obtained from the concentration-response curves (shown in Figure 2) of histamine for its inhibitory effect on the electrically-evoked tritium overflow from superfused mouse brain cortex slices (preincubated with [^{3}H]-noradrenaline). The apparent pA_2 values of the other newly synthesized compounds were obtained from corresponding concentration-response curves (not shown). Means of 4–11 experiments; the s.e.mean values (not shown) ranged from 0.06 to 0.26.

[c]The apparent pA_2 value of 1 was obtained from the concentration-response curves (shown in Figure 3) of **R**-(−)-α-methylhistamine for its inhibitory effect on the electrically induced contraction in guinea-pig ileum strips. The apparent pA_2 values of the other newly synthesized compounds and of thioperamide were obtained from corresponding concentration-response curves (not shown). Means of 8–20 experiments; the s.e.mean values (not shown) ranged from 0.04 to 0.16.

[d]The values, which represent the pK_i for H_{3A} and H_{3B} binding sites, respectively, are taken from West *et al.* (1990; almost identical binding assay as in the present study).

[e]From Schlicker *et al.* (1992).

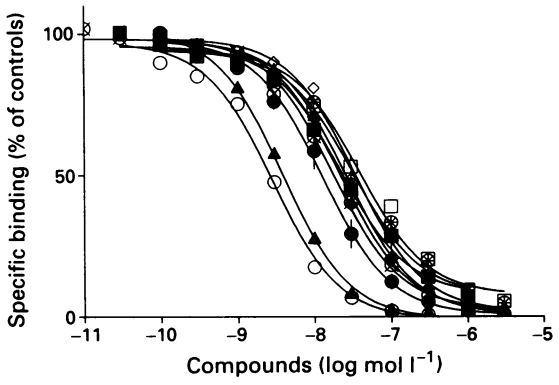


Figure 1 Inhibition of specific [^{3}H]-N$^\alpha$-methylhistamine binding to membranes from rat brain cortex by ten newly synthesized H_3 receptor antagonists. Means ± s.e.mean from 4–8 experiments (in triplicate) are shown (for some data points, s.e.mean is contained within the symbol): (□) **10**; (■) **7**; (△) **6**; (▲) **2**; (◇) **9**; (◆) **5**; (○) **1**; (●) **3**; (⊗) **4** and (⊛) **8**. For details of compounds, see Table 1.

($n_H \sim 1$) in rat brain cortex membranes, with a B_{max} of 98 ± 6 fmol mg^{-1} protein and a K_D of 0.70 ± 0.03 nmol l^{-1} (Kathmann *et al.*, 1993). The displacement of the specific binding of [^{3}H]-N$^\alpha$-methylhistamine 0.2 nmol l^{-1} in this preparation by the ten newly synthesized compounds is shown in Figure 1. Analysis of the competition curves by computer programmes and subsequent statistical evaluation by the *F*-test showed that for 7 of the 10 compounds a one-site fit is better than a two-site fit in all individual experiments. With respect to **10**, a two-site fit was better in 2 of 6 experiments, and with respect to **4** and **6** a two-site fit was better in one of 5 and 6 experiments, respectively. For the established H_3 receptor antagonist thioperamide, a two-site fit is consistently superior to a one-site fit (West *et al.*, 1990). The pK_i values are listed in Table 1.

Antagonistic potencies of the test compounds at H_3 receptors in the mouse brain cortex

Tritium overflow, evoked by the first period of electrical stimulation (S_1), was 6.89 ± 0.06% of tissue tritium in 33 controls; the S_2/S_1 value amounted to 1.09 ± 0.02. Histamine, which was present in the medium during S_2 only, inhibited the electrically evoked tritium overflow (S_2/S_1) in a concentration-dependent manner. The maximum inhibitory effect was about 70%; the concentration producing an inhibition of 35% (EC_{50}) was 0.13 μmol l^{-1} (Figure 2). Compound **1**, which was present in the medium throughout superfusion, shifted to the right the concentration-response curve of histamine for its inhibitory effect on the electrically evoked tritium overflow (Figure 2). Similar rightward shifts of the concentration-response curve of histamine were also obtained for the other test drugs (not shown). The apparent pA_2 values determined for the ten compounds are given in Table 1. The compounds by themselves did not affect the electrically evoked tritium overflow (S_1) (not shown).

Antagonistic potencies of the test compounds at H_3 receptors in the guinea-pig ileum

Electrical stimulation increased the tension in the guinea-pig ileum strips by 6.0 ± 0.09 mN ($n = 125$). The electrically induced contraction was abolished by atropine 1 μmol l^{-1} ($n = 8$, not shown). **R**-αMH inhibited the electrically induced contraction in a concentration-dependent manner. The maximum effect was variable; the EC_{50} (i.e. the concentration producing

the half-maximum inhibitory effect in the individual preparations) was 17 nmol l^{-1} (Figure 3). The effect of **R**-αMH was mimicked by imetit (EC_{50} 7 nmol l^{-1}) and by N^{α}-methylhistamine (EC_{50} 13 nmol l^{-1}; $n = 8$ each; results not shown).

The concentration-response curve of **R**-αMH was shifted to the right by **1** (Figure 3), by the other test compounds and by thioperamide (not shown). The apparent pA_2 values of the ten newly synthesized compounds and of thioperamide are given in Table 1. The novel H_3 receptor antagonists and thioperamide did not affect the electrically induced contraction by themselves (not shown).

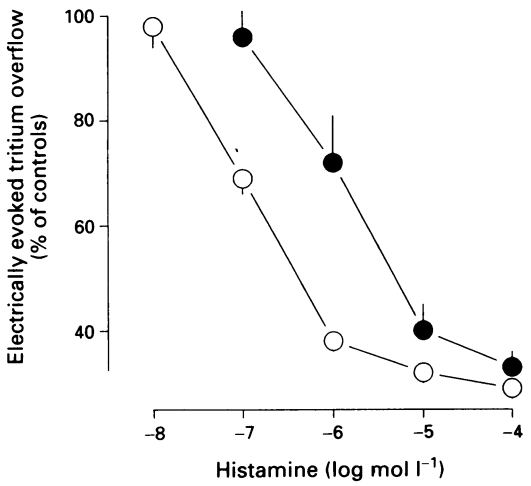


Figure 2 Antagonism by **1** of the inhibitory effect of histamine on the electrically evoked tritium overflow from superfused mouse brain cortex slices preincubated with [^{3}H]-noradrenaline. (○) Histamine; (●) plus compound **1** 0.01 μmol l^{-1}. The medium contained desipramine 1 μmol l^{-1} plus rauwolscine 1 μmol l^{-1} and, in some slices, **1** throughout superfusion and histamine from 62 min of superfusion onward. Tritium overflow was evoked twice after 40 and 90 min (S_1, S_2), and the ratio of the overflow evoked by S_2 over that evoked by S_1 was determined (S_2/S_1). Tritium overflow is given as a percentage of the S_2/S_1 value in the corresponding controls (no histamine; not shown). Means ± s.e.mean of 4–9 experiments.

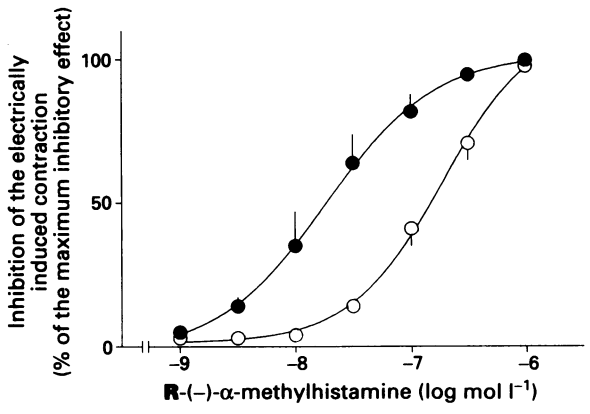


Figure 3 Antagonism by **1** of the inhibitory effect of **R**-(−)-α-methylhistamine (**R**-αMH) on the electrically induced contraction of guinea-pig ileum strips: (●) **R**-αMH; (○) plus compound **1** 0.01 μmol l^{-1}. The preparations were exposed to medium containing mepyramine 1 μmol l^{-1} and were stimulated continuously. After construction of the first concentration-response curve to **R**-αMH, the strips were washed and then equilibrated with **1** for 20–30 min and a second concentration-response curve to **R**-αMH was generated. Values are expressed as percentages of the effect obtained with **R**-αMH 1 μmol l^{-1} (in the first concentration-response curve), which produces the maximum inhibitory effect (75 ± 8%). Means ± s.e. mean of 4 experiments. In each of the 4 tissues, a third concentration-response curve to **R**-αMH was constructed in the presence of **1** 0.1 μmol l^{-1} (not shown).

Correlations of the affinities and potencies of the test compounds and of thioperamide

The affinities (pK_i) of the newly synthesized H_3 receptor antagonists and of thioperamide for [^{3}H]-N^{α}-methylhistamine binding and their potencies (apparent pA_2) in the two functional H_3 receptor models (mouse brain cortex; guinea-pig ileum) were compared by linear regression analysis (Figure 4 and 5). Thioperamide was included in the regression analysis since it is the reference H_3 receptor antagonist and, in addition, is capable of differentiating between H_{3A} and H_{3B} binding sites.

When the apparent pA_2 values of the eleven H_3 receptor antagonists in the mouse brain cortex model were compared to their pK_i values for H_3 binding sites and, in the case of thioperamide, to its pK_i for H_{3A} binding sites, a highly significant correlation was obtained. When, however, the pK_i of thioperamide for H_{3B} binding sites was used instead, the correlation was no longer significant (Figure 4a). Comparison of the apparent pA_2 values of the H_3 receptor antagonists in

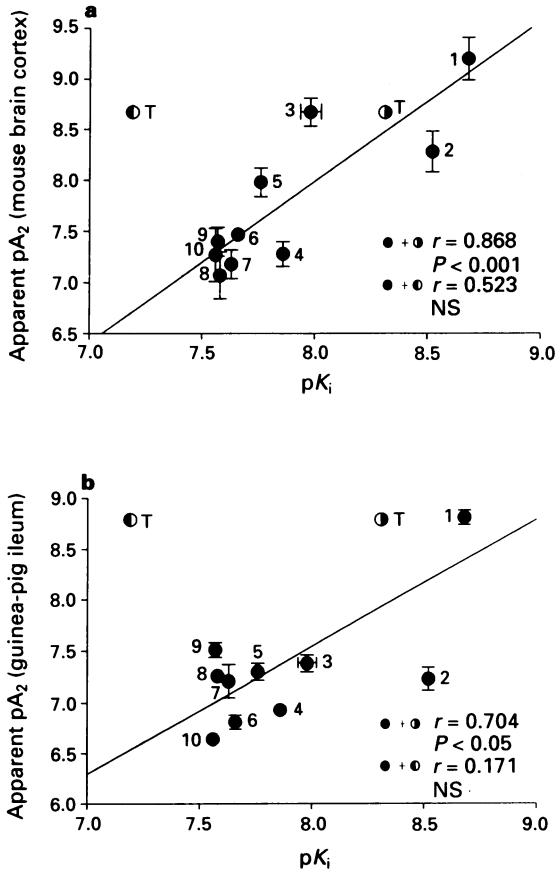


Figure 4 Correlation of the affinities (pK_i) of ten newly synthesized compounds and of thioperamide (T), as measured by displacement of specific [^{3}H]-N^{α}-methylhistamine binding, with their potencies (apparent pA_2), as determined by their abilities to antagonize (a) the effect of histamine on the electrically evoked tritium overflow from mouse brain cortex slices and (b) the effect of **R**-(−)-α-methylhistamine on the electrically induced contraction in guinea-pig ileum strips. S.e.means are shown; in some instances, s.e.mean is smaller than the symbol. The numbers of the compounds correspond to those given in Table 1. Data (taken from Table 1) were statistically analyzed by linear regression analysis. Two regression lines were calculated for the data points shown in either panel. Thus, the apparent pA_2 values of the 11 drugs were compared to their pK_i values for H_3 binding sites and to the pK_i value of thioperamide for H_{3A} sites (◑) or its pK_i for H_{3B} sites (◐). Only the regression lines for the first type of analysis are shown. In (a), the equations of the regression lines are: $y = 1.58 \times - 4.64$ (H_{3A}) and $y = 0.88 \times + 0.95$ (H_{3B}). In (b), the equations of the regression lines are: $y = 1.25 \times - 2.43$ (H_{3A}) and $y = 0.28 \times + 5.24$ (H_{3B}).

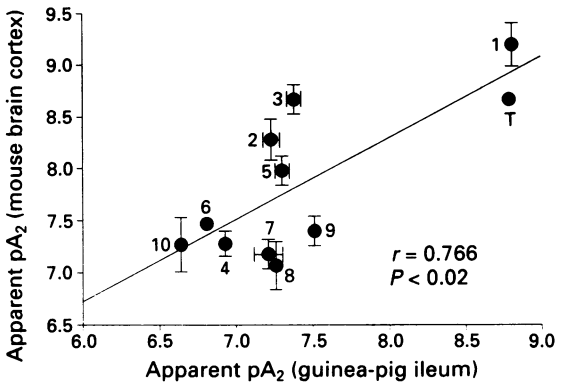


Figure 5 Correlation of the potencies (apparent pA_2) of ten newly synthesized compounds and of thioperamide (T), as determined by their ability to antagonize the effect of histamine on the electrically evoked tritium overflow from mouse brain cortex slices, with their potencies as determined by their ability to antagonize the effect of R-(−)-α-methylhistamine on the electrically induced contraction of guinea-pig ileum strips. Data (taken from Table 1) were statistically analyzed by linear regression analysis. S.e.means are shown; in some instances, s.e.mean is smaller than the symbol. The numbers of the compounds correspond to those given in Table 1. The equation of the regression line is: $y = 0.79 \times + 2.00$.

the guinea-pig ileum model with their pK_i for H_3 sites and the pK_i of thioperamide for H_{3A} sites also yielded a significant correlation. Again, replacement of the pK_i for H_{3A} sites by the pK_i for H_{3B} sites resulted in the loss of a significant correlation (Figure 4b). When the apparent pA_2 values in the two functional H_3 receptor models were compared, a significant correlation occurred (Figure 5).

Discussion

It was the aim of the present study, based on ten recently synthesized H_3 receptor ligands, to examine further the problem of H_3 receptor heterogeneity and to demonstrate structure-activity relationships for the novel compounds. We determined the affinities of the compounds for H_3 binding sites labelled by [^{3}H]-N$^\alpha$-methylhistamine in rat brain cortex membranes (according to West *et al.*, 1990, modified by Kathmann *et al.*, 1993). Furthermore, the potencies of the compounds for the H_3 receptor modulating noradrenaline release (an H_{3A} receptor according to the H_3 receptor subclassification postulated by West *et al.*, 1990) were determined in mouse brain cortex slices (according to Schlicker *et al.*, 1992). Finally, the potencies of the compounds were also determined in another functional H_3 receptor model, i.e. in the guinea-pig ileum (see Vollinga *et al.*, 1992, for references). Under the experimental conditions used in the present study (which were similar to those in the study of Vollinga *et al.*, 1992), the electrically induced contraction can be ascribed to the release of acetylcholine (since it was abolished by atropine). The inhibitory effect of **R-αMH** on the electrically induced contraction is mediated via H_3 receptors; thus, the H_3 receptor antagonist thioperamide (Arrang *et al.*, 1987) antagonized the effect of **R-αMH** with a pA_2 value of about 8.5. In addition, the recently described H_3 receptor agonist, imetit (Garbarg *et al.*, 1992; Howson *et al.*, 1992; van der Goot *et al.*, 1992; Kathmann *et al.*, 1993) potently mimicked the effect of **R-αMH**.

Two approaches were chosen to obtain further evidence in favour of the heterogeneity of H_3 receptors. First, the effects of the novel H_3 receptor antagonists on the specific binding of [^{3}H]-N$^\alpha$-methylhistamine to rat brain cortex membranes were studied. The compounds, unlike thioperamide (West *et al.*, 1990) and burimamide (West *et al.*, 1990; Kathmann *et al.*, 1993), exhibited monophasic displacement curves although three compounds (**4**, **6**, **10**) produced a biphasic displacement in a minority of experiments. This might suggest that these compounds exhibit slightly different affinities for the H_{3A} and H_{3B} sites, which cannot be detected easily. The chemical structures of these compounds might represent a starting point for the synthesis of H_3 receptor antagonists differentiating more markedly between H_{3A} and H_{3B} sites.

Second, we determined the antagonistic potencies (expressed as apparent pA_2 values) of the novel compounds and of thioperamide in two functional H_3 receptor models which differ with respect to (i) the species (mouse vs. guinea-pig), (ii) the tissue (cerebral cortex vs. ileum) and (iii) the transmitter (noradrenaline vs. acetylcholine). It has been shown for other types of presynaptic receptors, including α_2-adrenoceptors (see Trendelenburg *et al.*, 1993, for references) and 5-hydroxytryptamine receptors (see Hoyer & Middlemiss, 1989, for review), that their pharmacological properties strongly depend on these three parameters. However, the two functional H_3 receptors examined in the present study appear to be very similar, if not identical, as shown by the significant correlation between the antagonistic potencies of the eleven compounds. In addition, comparison of the apparent pA_2 values obtained in both models shows that the difference is lower than 0.9 log units for each compound if one takes into account that in the guinea-pig ileum the average level of pA_2 values was lower by 0.42 log units than in the mouse brain. Comparison of the potencies of the compounds with their affinities (expressed as pK_i) for H_3 binding sites yielded a significant correlation for both functional H_3 receptor models, if the pK_i value of thioperamide for H_{3A} sites was considered but not if its pK_i value for H_{3B} sites was used instead. Our data are compatible with the view that the H_3 receptor in the guinea-pig ileum, like that in the mouse brain cortex (Schlicker *et al.*, 1992), belongs to the H_{3A} subclass.

Considering the affinities and potencies of the compounds at the H_3 binding sites, the following structure-activity relationships may be deduced. (1) The amide **7**, the corresponding thioamide **9** as well as the comparable urea derivative **8** do not differ in their affinity for H_3 receptors. (2) The guanidine **1** possesses the highest affinity of all of the test compounds, possibly due to the high basic capacity of this functional group and its ability to form hydrogen bonds. The guanidine moiety, which is protonated under physiological conditions, might interact with a carboxylate moiety of the H_3 receptor. An interaction between the $-NH_3^+$ of histamine and the carboxylate group of an aspartic acid residue has been postulated for H_1 and H_2 receptors (see Ganellin *et al.*, 1992, for references). Compared to **1**, the affinity of the corresponding guanidine ester **3** is lower. The reason may be that the basic capacity of the guanidine moiety as well as its ability to form hydrogen bonds is attenuated by substitution with the *t*-butyloxy-carbonyl residue, which exhibits an inductive effect (-I) although steric effects cannot be totally excluded. (3) Comparison of the different cyclic alkyl residues of compounds **6**, **7** and **10** reveals almost the same affinities. Thus, steric effects do not appear to play a major role for the affinity for H_3 receptors. One might assume that, with respect to this part of the molecule, hydrophobic interactions are more important for the binding to the receptor than steric effects.

In conclusion, ten novel compounds, including amides, guanidine esters as well as a guanidine, an ester, a thioamide and an urea derivative, which have been synthesized according to the general construction pattern for H_3 receptor antagonists described by Lipp *et al.* (1992), exhibit intermediate to high affinities and potencies at H_3 receptors. The novel compounds, for which some structure-activity relationships could be derived, do not differentiate between H_{3A} and H_{3B} sites. The H_3 receptors in the two functional models, i.e. in the mouse brain cortex and in the guinea-pig ileum, closely resemble each other. The H_3 receptor in the guinea-pig ileum

(like that in the mouse brain cortex; Schlicker *et al.*, 1992) may belong to the H_{3A} subclass according to West *et al.* (1990).

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Permeation properties and modulation of volume-activated Cl^--currents in human endothelial cells

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1 We have studied the permeation and pharmacological properties of a recently described volume-activated, calcium-insensitive, small-conductance Cl^--channel in endothelial cells from human umbilical vein.

2 The relative permeability for various anions was $I^- > Cl^- \sim Br^- > F^- >$ gluconate$^-$ (1.63 ± 0.36: $1 : 0.95 \pm 0.16 : 0.46 \pm 0.04 : 0.19 \pm 0.07$, $n = 10$).

3 5-Nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) induced a fast and reversible block of the current ($K_I = 29\ \mu mol\ l^{-1}$).

4 Extracellular ATP induced a low-affinity block of the current, that showed a small voltage-dependence ($K_I = 4.9\ mmol\ l^{-1}$ at $+80$ mV and $K_I = 8.2\ mmol\ l^{-1}$ at -80 mV).

5 Extracellularly applied arachidonic acid ($10\ \mu mol\ l^{-1}$) irreversibly blocked the current in 5 out of 9 cells. This block seems to be non-specific, because other ionic currents, e.g. inwardly rectifying K^+ currents, were blocked as well.

6 Tamoxifen induced a high affinity block of the current ($K_I = 2.9\ \mu mol\ l^{-1}$). Block and reversal of block were however much slower than with NPPB.

7 Cytotoxic compounds, which are substrates of the P-glycoprotein multidrug transporter, loaded into endothelial cells via the patch pipette, exerted only minor effects on the volume-activated current. Vinblastine and colcemid did not affect the volume-activated current, whereas daunomycin and vincristine induced a slow 'run-down' of the current.

8 The similarity between permeation and pharmacological properties of volume-activated Cl^--currents in endothelial cells and those in many other cell types may suggest that they all belong to the same family of volume-activated small-conductance Cl^--channels. Evidence that they belong to the class of P-glycoprotein associated Cl^--channels is however only marginal, whereas their biophysical characteristics differ significantly from those of the ClC-2 volume-activated Cl^--channels.

Keywords: Endothelial cells; volume regulation; patch clamp; chloride currents

Introduction

Mechanical stimuli, such as cell swelling, induce several cellular responses in endothelial cells, among which is the activation of a chloride conductance channel (Nilius *et al.*, 1994; Oike *et al.*, 1994). This channel has a small conductance, is insensitive to intracellular Ca^{2+} and is not modulated by the adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent protein kinase A (Nilius *et al.*, 1994). It resembles the Cl^--channel observed in secretory chromaffin cells (Doroshenko *et al.*, 1991), human colon carcinoma cells HT_{29} (Kunzelmann *et al.*, 1992) and Jurkat T lymphocytes (Lewis *et al.*, 1993). The biological importance of this channel in endothelial cells is not restricted to a possible function in volume regulation, but it also plays a role as a mechanotransducer in these cells (Oike *et al.*, 1994).

Pharmacological properties and modulation of these chloride channels differ substantially between different cell types. More specifically, they show a different sensitivity to blockers of the adenosine 5'-triphosphate (ATP)-dependent transporter of hydrophobic substrates, a P-glycoprotein encoded by the MDR-1 gene that is responsible for multidrug resistance. Blockers of this transporter, such as 1,9-dideoxyforskolin (DDFSK) and verapamil, inhibit the volume-activated Cl^- current in some cell types (Diaz *et al.*, 1993; Nilius *et al.*, 1994), but have no effect in Jurkat T-lymphocytes (Lewis *et al.*, 1993). This different sensitivity may be of special interest, because it has been proposed (Valverde *et al.*, 1992; Gill *et al.*, 1993) that this P-glycoprotein is associated with volume-activated Cl^--currents. It has also been shown that activation of this Cl^- current is inhibited by intracellular loading of MDR-1-transfected NIH3T3 fibroblasts with substrates of the P-glycoprotein transporter (Gill *et al.*, 1992).

In this study we describe the anion selectivity and the effects of different blockers on the volume-activated Cl^- channel in endothelial cell. These data may be important not only for a pharmacological characterization of this channel, but may provide possible tools that could be used to interfere with mechanotransducing properties of endothelial cells.

Methods

Isolation and culture of endothelial cells

Endothelial cells were obtained from human umbilical cord veins by a collagenase digestion procedure, as described previously (Jaffe *et al.*, 1973). They were grown in Medium 199 containing 10% human serum, $2\ mmol\ l^{-1}$ L-glutamine, $100\ u\ ml^{-1}$ penicillin and $100\ g\ l^{-1}$ streptomycin. Cultures were maintained at 37°C in a fully humidified atmosphere of 10% CO_2 in air. The culture medium was exchanged every 48 h. Cells were detached by exposure to trypsin ($0.5\ g\ l^{-1}$) in a Ca^{2+}- and Mg^{2+}-free solution for approximately 180 s and reseeded on gelatin coated coverslips. We kept the cells in culture two to four days before use. We used in our experiments only non confluent single endothelial cells from primary culture up to the second passage.

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Current measurements

Whole-cell membrane currents were measured using ruptured patches or patches perforated with nystatin. Ruptured patches were used only in those experiments in which we wanted to apply a compound to the cell interior via the pipette. Otherwise we always used nystatin perforated patches to avoid disturbance of the intracellular Ca^{2+}-buffering mechanisms (Horn & Marty, 1988). Currents were monitored with an EPC-7 (List Electronic, Germany) patch clamp amplifier and sampled at 4 ms intervals (2048 points per record, filtered at 100 Hz). We used the following voltage protocol: from a holding potential of 0 mV we applied a step to −80 mV for 1.2 s. This was followed by a step to −150 mV for 0.4 s and a linear voltage ramp to +100 mV. This protocol was repeated every 10 s. The average current during successive voltage steps to −80 mV was used to reconstruct the time course of the volume-activated current. The voltage-dependence and reversal potential of the current were derived from the current recorded during the voltage ramp.

Solutions

The standard bath solution was a modified Krebs solution of the following composition (in mmol l^{-1}): NaCl 132, $CaCl_2$ 1.5, KCl 5.9, $MgCl_2$ 1.2, HEPES-NaOH 11.5, glucose 10, titrated to pH 7.3 with NaOH. Osmolarity was 290 mOsm l^{-1}. The experimental isotonic solution contained (in mmol l^{-1}): NaCl 105, $MgCl_2$ 0.5, $CaCl_2$ 1.3, HEPES-NaOH 10 and D-mannitol 70. Cell swelling was induced by omitting mannitol from this solution. All solutions were adjusted with TrisOH to a pH of 7.4. For anion substitution experiments, NaCl was replaced by either NaI, NaBr, NaF or Na-gluconate. For the experiments with gluconate $[Ca^{2+}]_e$ was corrected for the Ca^{2+} chelating effect of this anion. Osmolarity of all solutions was measured with a vapour pressure osmometer, Wescor 5500 (Schlag, Meßinstrumente, Gladbach, Germany).

For nystatin perforated patches we used a nystatin stock solution of 50 mg l^{-1} dissolved in dimethylsulphoxide (DMSO) that was stored at −20°C. Immediately prior to use this stock solution was diluted 1000 times in the pipette solution and ultrasonicated. The tip of the pipette was filled by capillarity with nystatin-free pipette solution, and the pipette was backfilled with nystatin-containing solution. Access occurred within 5 to 10 min. The standard pipette (internal) solution was made up in Milli-Q water (Millipore) and contained (in mmol l^{-1}): K-aspartate 100, KCl 40, Na_2-ATP 4, HEPES 10, EGTA 0.1 buffered at pH 7.2 with KOH. The pipette solution for the permeation experiments contained (in mmol l^{-1}): KCl 105, $MgCl_2$ 1.2, HEPES-KOH 10, Na_2ATP 4, EGTA-KOH 1, D-mannitol 70. Both pipette solutions had an osmolarity of 290 mOsm l^{-1}.

Experiments were performed at room temperature between 20 and 22°C.

The following substances were used to modulate volume-activated currents: NPPB (5-nitro-2-(3-phenylpropylamino)-benzoic acid, kindly provided by Dr Lang, Hoechst AG, Germany), tamoxifen ((Z)-1-(*p*-dimethylaminoethoxyphenyl)-1,2-diphenyl-1-butene, Sigma), cylosporin (Sandoz, Basel), daunomycin, vincristine, vinblastine, colcemid (Sigma) and arachidonic acid (Sigma). Arachidonic acid was dissolved in DMSO and stored after sonication in vials under pure N_2 at −20°C until use. Stimulation of purinoceptors by extracellular ATP was prevented by suramin 25 μmol l^{-1} (courtesy of Dr Kazda, Bayer AG).

Statistics

Pooled data are given as mean ± standard error of the mean. For significance, Student's *t* test was used (level of significance, $P<0.05$). Fitting routines were used from the software package Origin (MicroCal Software, Northampton, MA, U.S.A.).

Results

Permeation properties of the endothelial volume-induced Cl^--current

Volume-activated Cl^- currents in endothelial cells have been analyzed in detail with respect to possible modulation mechanisms (Nilius *et al.*, 1994). Ion selectivity has not yet been investigated and was examined in the following anion-substitution experiments. Stationary current-voltage relationships were obtained from voltage ramps applied before and during superfusing the cells with hypotonic solution. In the absence of extracellular potassium, these $I-V$ curves lack the inwardly-rectifying potassium current component at negative potentials (Nilius *et al.*, 1993). The $I-V$ curve of the volume-activated current and its reversal potential are obtained from the difference between these curves. Because of the symmetrical Cl^--concentrations in bath and pipette solution, the reversal potential of the volume-induced current was close to 0 mV (Figure 1a). Replacement of extracellular Cl^- by I^-, Br^-, F^- or gluconate displaced the reversal potential of the volume-induced current. The permeability of the various

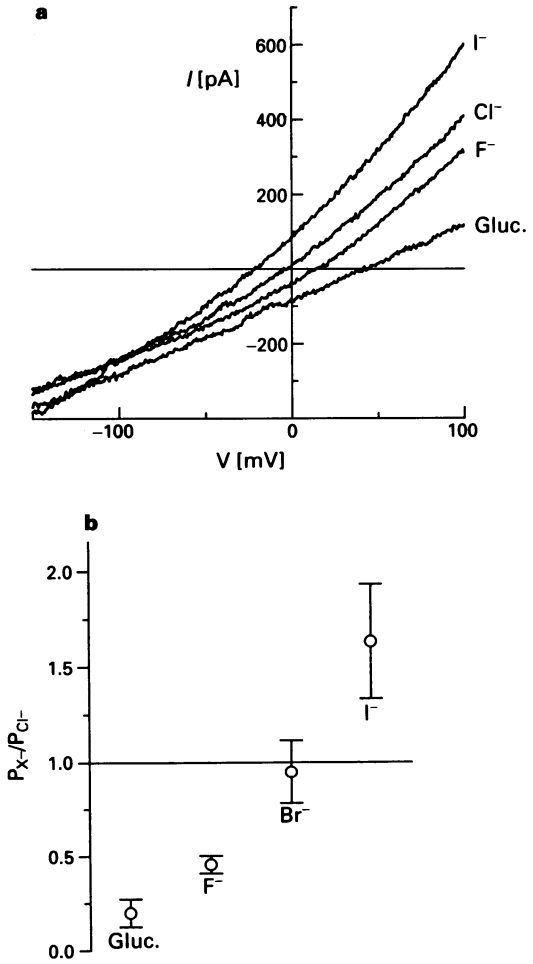


Figure 1 Anion selectivity of the volume-activated current. (a) Current-voltage relationships of the volume-activated current in the presence of various extracellular anions. These curves were obtained from ramp protocols, as described in the methods. (b) Permeability of various anions relative to that of Cl^-, calculated from the reversal potential of the volume-activated current using equation [1]. Each data point represents mean ± s.e.mean from 10 cells.

anions relative to that of Cl^- was calculated from the Hodgkin–Huxley–Katz equation:

$$V_{rev} = \frac{RT}{F} \times \ln \frac{[Cl]_i}{[Cl]_e + (P_X/P_{Cl}) \times [X]_e} \quad [1]$$

were R, T, F have their usual meaning, P_{Cl} and P_X represent the permeability for Cl^- and either of the above anions. $[Cl]_i$ and $[Cl]_e$ correspond to the intra- and extracellular Cl^--concentrations, and $[X]_e$ to the extracellular concentration of the test anion. From the measured reversal potentials (V_{rev}) we calculated the following anion selectivity sequence (Figure 1b, data from 10 cells): $P_I > P_{Cl} \approx P_{Br} > P_F \gg P_{Gluc} = 1.63 \pm 0.36:1: 0.95 \pm 0.16:0.46 \pm 0.04:0.19 \pm 0.07$. This sequence has been observed for many volume-activated Cl^--currents.

Effect of Cl^--channel blockers on the volume-induced current

In a previous report, we have already shown that the classical Cl^- channel blocker DIDS . blocked the volume-activated current, while another Cl^- channel blocker, DCDPC, was ineffective (Nilius *et al.*, 1994). Figure 2 shows the effects of yet another channel blocker NPPB (5-nitro-2-(3-phenylpropylamino)-benzoic acid) on the volume-activated current. The simultaneous application of NPPB (100 μmol l^{-1}) and hypotonic solution prevented activation of the Cl^- current (Figure 2a). Washout of NPPB in isotonic Krebs solution induced a pronounced overshoot of the inward current at −80 mV, indicating that unblock of the channel by NPPB precedes deactivation of the channel in isotonic solutions (Figure 2a). Current-voltage relationships obtained from ramp protocols applied under these various experimental conditions are shown in Figure 2b. It is obvious that NPPB blocked the volume-activated current, but it also affected the background holding current measured at negative potentials (see Figure 2a and b). Figure 3a shows an experiment in which NPPB was applied via a fast perfusion system at four different concentrations during a maintained

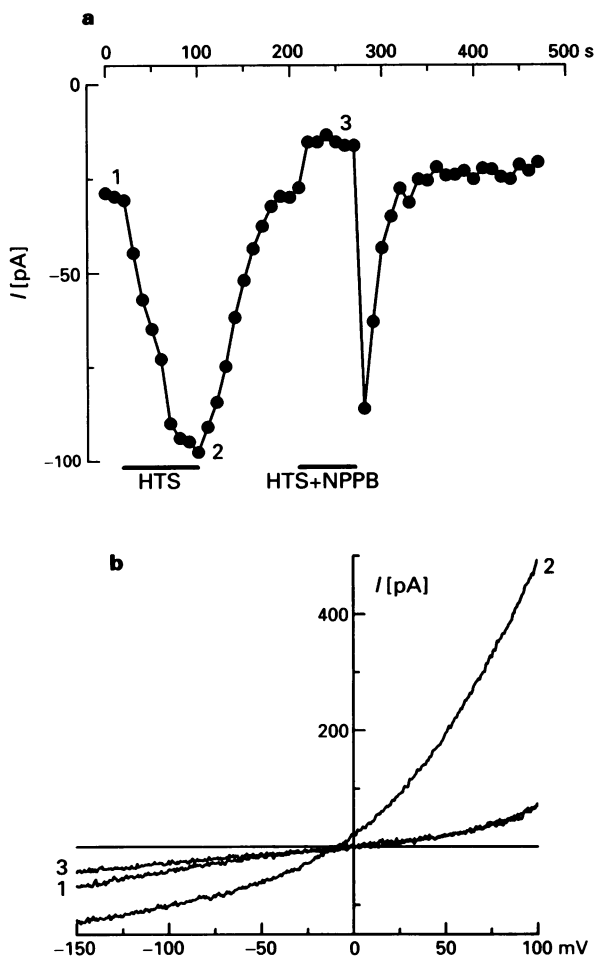


Figure 2 Block of the volume-activated current by 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB). (a) Averaged currents calculated from the initial voltage step to −80 mV during consecutive voltage protocols. Challenge with hypotonic solution (HTS) activated an inward Cl^- current. Applying hypotonic solution together with 100 μmol l^{-1} NPPB (HTS + NPPB) not only prevented activation of the swelling induced current, but also significantly reduced the basal current. Also note the current overshoot when NPPB is washed out in isotonic solution, suggesting that reversal of block of the current is faster than deactivation. (b) Current-voltage relationships measured at the times indicated in (a). The volume-activated current reversed at −8 mV indicating that Cl^- is the main charge carrier (Cl^- equilibrium potential is −18 mV). Note the suppression of the current below its level in control solution at negative voltages in the presence of NPPB.

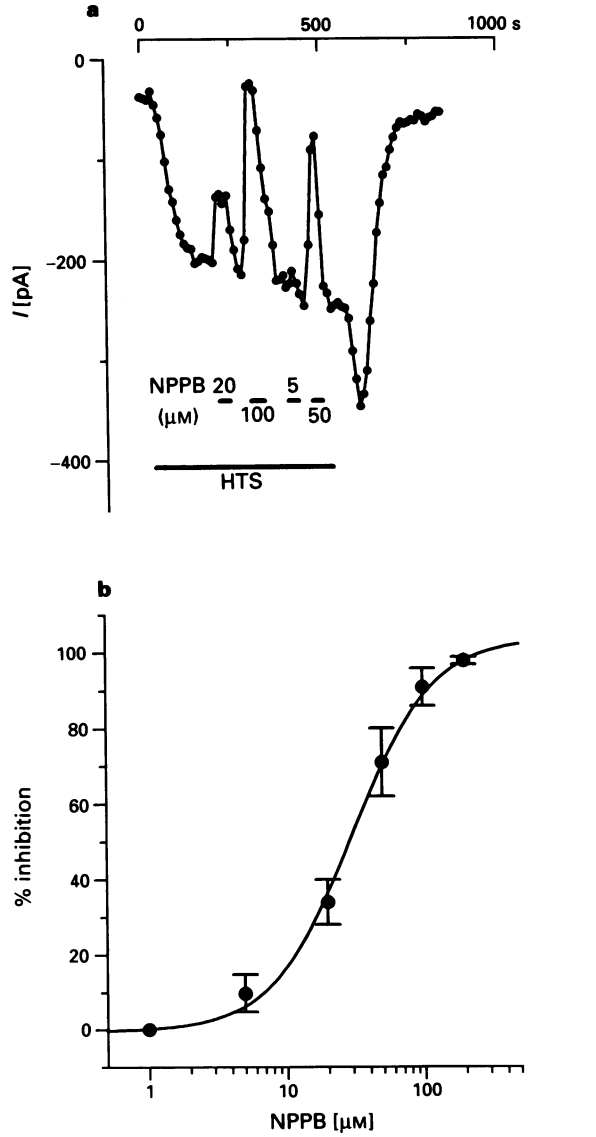


Figure 3 Concentration-dependence of the inhibition of the volume-activated current by 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB). (a) Repetitive applications of NPPB at different concentrations during a prolonged challenge with hypotonic solution (HTS) caused a fast and reversible block of the volume-activated current. Data points represent averaged currents at −80 mV during repetitive applications of the voltage protocol. (b) Concentration-dependence of the inhibition by NPPB. The data points, which were obtained from 4 cells, were fitted to equation [2] with a K_I concentration for half-maximal inhibition of 29 μmol l^{-1}.

period of hypotonic challenge. Each of these concentrations induced a rapid and reversible concentration-dependent block of the volume-activated current. The concentration-dependence of this inhibition, expressed as a percentage of the control current, is represented in Figure 3b. It could be fitted to the equation

$$\% \text{ inhibition} = \frac{100}{1 + K_I/[\text{NPPB}]} \quad [2]$$

in which [NPPB] represents the NPPB concentration. The concentration for half-maximal block K_I was 29 μmol l^{-1} (data from 4 cells).

Modulation of the hypotonic solution (HTS)-current by ATP

It is well-known that volume-activated Cl^--channel depend on intracellular ATP (MDR-1 transfected NIH3T3 fibroblasts: Gill *et al.*, 1992; Jurkat cells: Lewis *et al.*, 1993). We have confirmed this finding in our endothelial cells (Nilius *et al.*, 1994; Oike *et al.*, 1994). It has also been shown that extracellular nucleotides partially impair volume-regulatory responses (Paulmichl *et al.*, 1993; Gschwentner *et al.*, 1993) and more recently that they weakly block volume-activated Cl^--currents (Ackerman *et al.*, 1994). We have therefore investigated whether the endothelial volume-activated current is also sensitive to extracellular ATP. Figure 4a shows an experiment in which various ATP-concentrations (in the presence of 25 μmol l^{-1} suramin to block purinoceptors) were applied to the extracellular bath solution. The amplitude of the Cl^- current was reduced and its time course of activation was much slower in the presence of ATP. Figure 4b shows the effect of ATP (10 mmol l^{-1}) on the HTS-induced current at two different potentials. It is obvious that the inhibition is much faster at positive potentials. The current at −80 mV reached half maximal inhibition after 73 ± 15 s. At +80 mV, it occurred after 35 ± 9 s (n = 3 cells). The block by ATP is reversible and is dependent on the ATP-concentration, as shown in Figure 4c at these same two potentials. The concentration for half maximal block, calculated according to equation [2] was 8.2 ± 2.1 mmol l^{-1} at −80 mV and 4.9 ± 0.4 mmol l^{-1} at +80 mV, suggesting a possible weak voltage-dependence of the ATP block.

Modulation of the hypotonic solution (HTS)-induced-current by arachidonic acid (AA)

Mechanical stimulation, including cell swelling, induces in endothelial cells a release of calcium from intracellular pools, that can be mimicked by arachidonic acid (Oike *et al.*, 1994). The effects of AA on the Cl^- current were however quite variable (Nilius *et al.*, 1994) and apparently not very specific. We have therefore compared the effects of extracellularly applied AA (10 μmol l^{-1}) on the HTS-activated Cl^- current with those on the inwardly rectifying K^+-current, which is

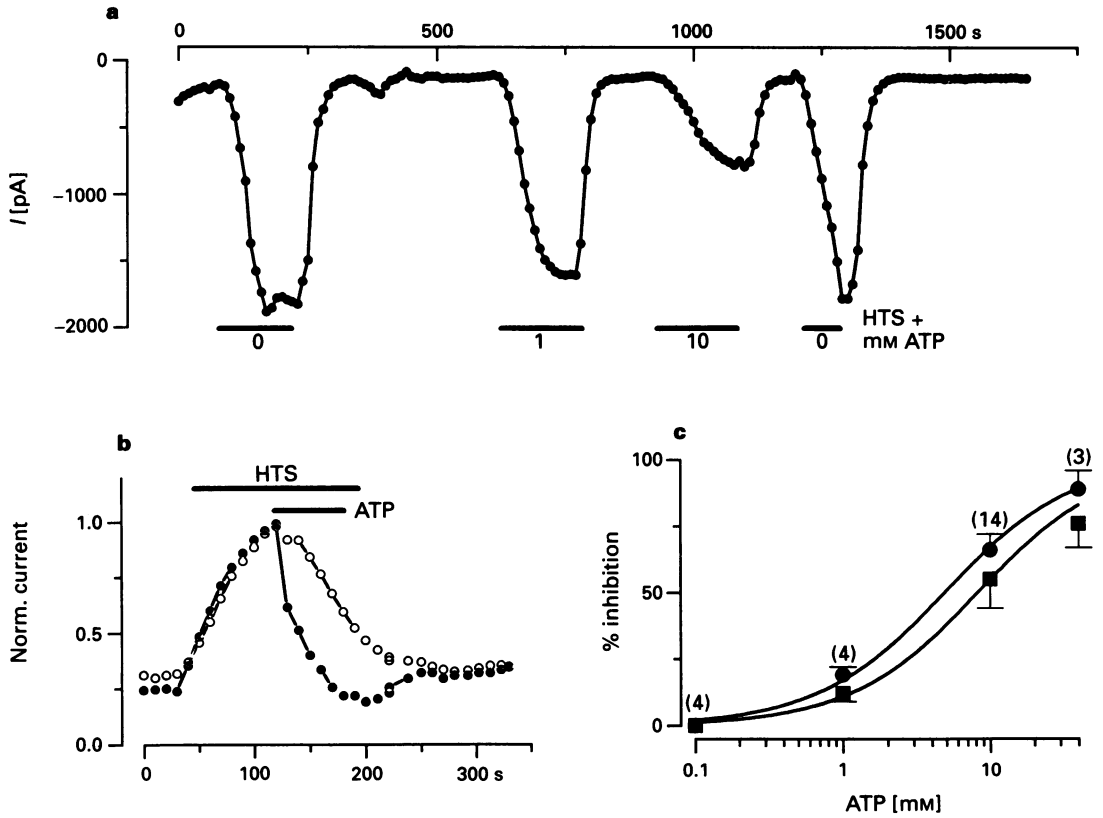


Figure 4 The endothelial volume-activated Cl^- channel is sensitive to extracellular ATP. (a) Amplitude of the volume-activated current at −80 mV in the presence of different concentrations of extracellular ATP. These amplitudes represent average values during the initial voltage step to −80 mV, and are obtained from consecutive voltage protocols applied every 10 s. Hypotonic challenge (HTS) and ATP were applied simultaneously. Activation of purinoceptors was suppressed by suramin (25 μmol l^{-1}). (b) Effect of membrane potential on the rate of current inhibition by extracellular ATP (10 mmol l^{-1}). Averaged currents in a window around −80 mV (○) and +80 mV (●) were calculated during subsequent voltage protocols, and normalized to the maximal current activated by cell swelling at each potential. Block of the current is much faster at positive membrane potentials. (c) Concentration-dependence of the blocking effect of extracellular ATP at −80 (■) and +80 (●) mV. Average currents in the presence and absence of ATP were obtained from a small voltage window around each potential. Inhibition of the current was expressed as a percentage of the control current at each potential. Concentrations for half maximal block shifted from 4.9 mM at +80 mV to 8.2 mM at −80 mV, indicating a more sensitive block of the volume-activated Cl^- channel at positive membrane potentials. The number in parenthesis is the number of cells at both voltages. Data points represent mean ± s.e.mean.

present in the majority of the endothelial cells. AA cause a clear block of the HTS-induced current in 5 out of 9 cells. Figure 5a shows a typical example of this inhibition. Application of AA after activation of the volume-induced current caused a prompt inhibition of the current to a level below that in isotonic solutions. A subsequent stimulation with hypotonic solution in the presence of AA did not evoke any effect. Current voltage-relationships of this cell, obtained from voltage ramps in isotonic and hypotonic solutions before application of AA and in the presence of AA are shown in Figure 5b. It is obvious that the membrane current stimulated by HTS is inhibited at all voltages to a level below that in isotonic solutions in the absence of AA. The effect of AA on the $I-V$ curve of another cell in isotonic solution is shown in Figure 5c(i). The AA-blocked current, shown in Figure 5c(ii) fits well with the pattern of the K^+-inward rectifier current. The inhibitory effects of AA therefore seem to be non-specific.

Effects of modulators of P-glycoprotein transporters on HTS-induced currents

It has recently been proposed that expression of P-glycoprotein is associated with a volume-activated chloride conductance (Gill *et al.*, 1992; Valverde *et al.*, 1992). The observation that compounds, such as DDFSK and verapamil which inhibit the multi-drug resistance transporter, also block the HTS-activated Cl^--current in these cells and in our endothelial cells (Nilius *et al.*, 1994) is consistent with this contention. In addition, there is evidence both at the RNA messenger level (RT-PCR) and at the protein level (immunofluorescent studies with monoclonal antibodies against P-glycoprotein) that our endothelial cells express MDR-1 and P-glycoprotein (De Greef *et al.*, 1994; see also Hegemann *et al.*, 1992). These findings prompted us to investigate in more detail this presumed association between P-glycoprotein and volume-activated Cl^- current in our endothelial cells.

Cyclosporin is a very efficient blocker of the P-glycoprotein transporter. At a concentration of 10 μmol l^{-1}, cyclosporin caused in 5 out of 7 cells a 34 ± 6% inhibition of the HTS-activated current. In the other 2 cells it had no effect. At lower concentrations that are used for transporter block, cyclosporin exerted only marginal effects on the current, as has also been described by Mintenig *et al.* (1993).

The anti-oestrogen, tamoxifen, is another tool that blocks the presumed P-glycoprotein associated Cl^- current (cited as reference 7 in Valverde *et al.*, 1993) and is supposed to be a high affinity inhibitor of this current. We have also tested the effect of tamoxifen on the endothelial HTS-activated Cl^- conductance. Figure 6a shows that tamoxifen (10 μmol l^{-1}) added to the HTS-external solution was able to prevent activation of the current. If however, tamoxifen was added after activation of the current by HTS, the inhibition and the recovery or reversal of blockade were rather slow, as can be seen in Figure 6b. From 4 cells we obtained a time to half maximal block of 44 ± 7 s and a time for half maximal reversal of block of 97 ± 12 s. Thus, block and its reversal are much slower than for any of the other extracellular blockers, e.g. NPPB which block the currents already complete during the interval between two voltage ramps. This differential pattern of block hints at a different blocking mechanism of the HTS current by tamoxifen. The concentration dependence of the block by tamoxifen is shown in Figure 6c. The concentration K_I for half maximal block from the fit of the data to equation [2] was 2.9 ± 0.18 μmol l^{-1}.

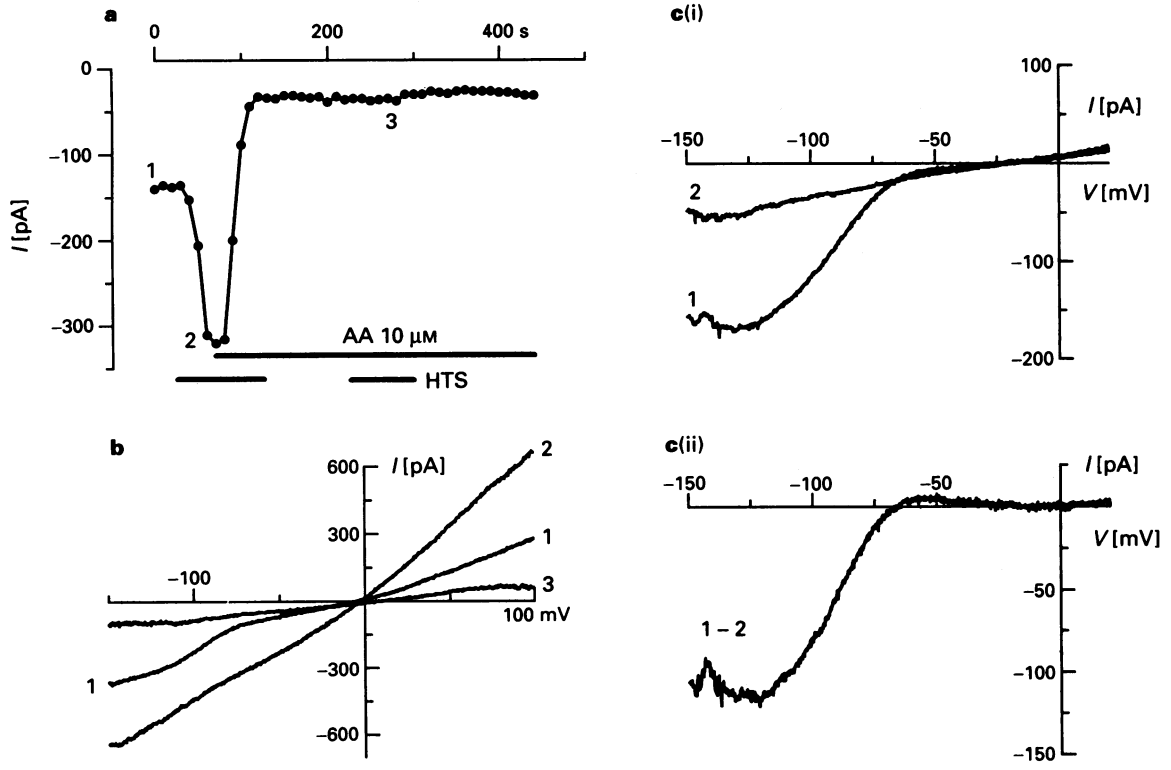


Figure 5 Arachidonic acid (AA) blocks volume-activated Cl^- and inward rectifying K^+ currents. (a) AA (10 μmol l^{-1}), applied extracellularly, induces a fast and irreversible block of the hypotonic solution (HTS)-activated current to a level below that in isotonic solutions. A subsequent HTS-challenge does not activate any current. Data points represent average current values at −80 mV during repeated voltage protocols. (b) Current-voltage relationships obtained at the times indicated in (a). Note that the HTS-induced current is reduced at all voltages to a level below that in isotonic solutions in the absence of AA. (c) Current-voltage relationships in isotonic solution in the absence (1) and in the presence (2) of 10 μmol l^{-1} AA (c(i)). The $I-V$ curve of the AA-blocked current component (C2), obtained as the difference of traces (1) and (2), shows the characteristics of an inwardly rectifying K^+-current, which has been described in detail elsewhere (Nilius *et al.*, 1993).

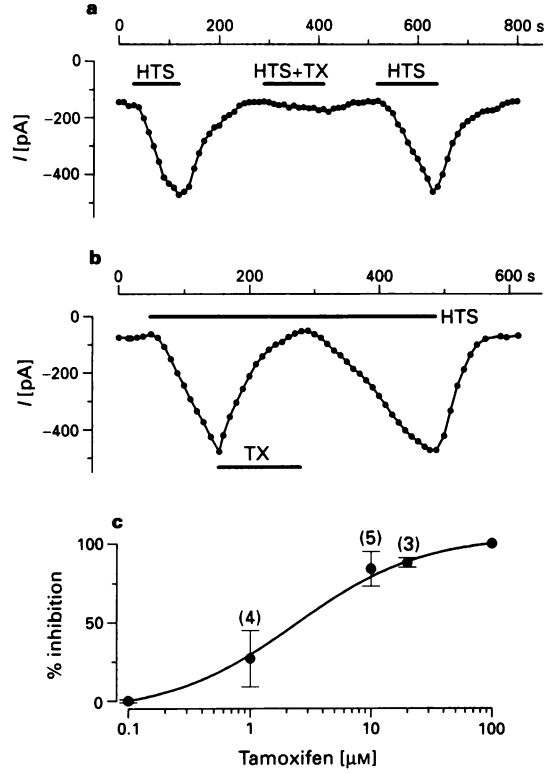


Figure 6 Block of volume-induced Cl^- current by the anti-estrogen, tamoxifen (TX). (a) TX (10 μmol l^{-1}) applied together with hypotonic solution (HTS), prevents activation of the volume-activated membrane current. Data points represent average currents at the −80 mV voltage step during voltage protocols repeated every 10 s. (b) Application of TX (10 μmol l^{-1}) during the HTS-induced current causes a slow inhibition of the current. Recovery during the subsequent washout is also slow. Data points represent average currents at −80 mV. (c) Concentration-dependence of the current inhibition, expressed as a percentage of the control current, by tamoxifen. The number of observations is given in parentheses. The solid line shows the fit of equation [2] to the data points with a K_I value of 2.9 μmol l^{-1}.

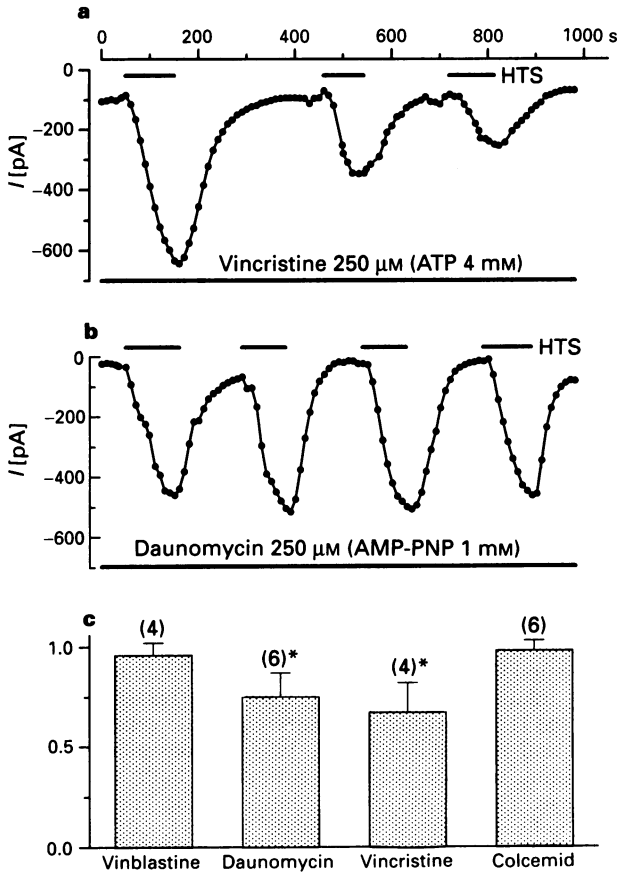


Figure 7 Modulation of volume-activated Cl^- current by substrates of the P-glycoprotein multidrug-resistance transporter. (a) Repetitive stimulation of an endothelial cell loaded via the patch pipette with vincristine (250 μmol l^{-1}) together with ATP (4 mmol l^{-1}) resulted in a slow run-down of the hypotensive solution (HTS)-activated current. Data points represent average current values at −80 mV. The first challenge to HTS was applied 5 min after breaking into the cell. This behaviour could be observed in 50% of the tested cells. Similar data were obtained if the cells were loaded with daunomycin, whereas vinblastine and colcemid did not have any effect. (b) Loading the cell with daunomycin together with AMP-PNP (1 mmol l^{-1}) did not result in a significant run-down of the HTS-induced current. Similar results were obtained with vincristine. (c) Synopsis of all experimental data. The current amplitude during the third application of HTS in the presence of the various cytotoxic drugs is expressed as a percentage of that during the first hypotonic challenge. Vinblastine and colcemid did not have any significant effect, whereas daunomycin and vincristine caused a gradual decline of the HTS-induced current during repetitive stimulations. However in neither of the experiments was it possible to prevent current activation by HTS.

Stimulation of the P-glycoprotein-transporter by loading cells with various of its hydrophobic substrates interferes with activation of the volume-activated Cl^- channel (Gill *et al.*, 1992). Activation of the transporter by these substrates prevents activation of the current induced by cell swelling, but these substrates are without effect on pre-activated Cl^--currents. This has led to the idea of a bifunctional P-glycoprotein that can function either as a transporter or as a channel. Both modes require intracellular ATP, but the channel mode does not require ATP-hydrolysis. Non-hydrolyzable ATP-analogues, such as AMP-PNP, will therefore prevent activation of the transporter but will not interfere with the channel mode. We have loaded endothelial cells via the patch pipette with 250 μmol l^{-1} of various cytotoxic substrates of the P-glycoprotein transporter, i.e. vincristine, daunomycin, vinblastine or colcemid, in the presence of either 4 mmol l^{-1} ATP or 1 mmol l^{-1} AMP-PNP. After an equilibration period of 5 to 8 min, cells were stimulated by HTS. The first stimulation with HTS induced a large current in each of the 27 cells that were investigated. Currents of similar amplitude were evoked during subsequent HTS-applications if vinblastine ($n = 4$) or colcemid ($n = 6$) were used as the substrate. In the presence of vincristine we observed in 2 out of 4 cells a run-down of the current during repeated HTS-stimulations (Figure 7a). A similar run-down was observed in 3 out of 6 cells loaded with daunomycin. This run-down did not occur if the cells were loaded with 1 mmol l^{-1} AMP-PNP instead of ATP ($n = 3$ cells), as shown in Figure 7b. To quantify our data, we have normalized the amplitude of the current observed during the third application of HTS to that during the first application (Figure 7c). It is evident from this figure that daunomycin and vincristine caused a small but significant decrease of the volume-activated current, but that neither vinblastine nor colcemid had any effect. These effects are much less pronounced than those reported for MDR-1 transfected NIH3T3 fibroblasts (Gill *et al.*, 1992) and for S1/1.1 cells permanently transfected with human MDR1 (Mintenig *et al.*, 1993).

Discussion

Volume-activated Cl^- channels, that were first described in chromaffin cells (Doroshenko & Neher, 1992), have now been observed in a huge number of cell types, e.g. epithelial

cell, tumour cells, and even in excitable cells, such as myocardial cells (Sorota, 1992; Zhang *et al.*, 1993). These channels are not only involved in volume regulation (for a review see Hoffmann & Simonson, 1989; Grinstein & Forskett, 1990; McCarty & O'Neill, 1992), but may play a role in other cell functions. In endothelial cells these channels can also be activated by other mechanical stimuli, such as shear stress and mechanical deformation (Oike *et al.*, 1994). Their activation may contribute to a stabilization of the inwardly driving force for calcium ions during mechanical stimuli.

The endothelial channels have a tiny conductance and their activation does not depend on intracellular calcium. They are not modulated via the PKA pathway, and require intracellular ATP for maintained activation. These characteristics seem to be common to most volume-activated Cl^--channels. In this paper, we have further investigated the macroscopic properties of the volume-activated Cl^--current in endothelial cells. The main purpose was to establish a possible relation with volume-activated Cl^--channels that have been described in terms of their molecular structure, such as ClC-2 and P-glycoprotein.

The anion selectivity sequence for the endothelial Cl^- channel is $P_I > P_{Br} \approx P_{Cl} > P_F >> P_{Gluc}$, which is similar to that of most volume-activated Cl^--currents (Kubo & Okada, 1992; Lewis *et al.*, 1993; Diaz *et al.*, 1993). The ClC-2 channel, that can also be activated by hypotonic solutions, however has a much higher permeability for Cl^- than for Br^- and I^- (Thiemann *et al.*, 1992). It also shows inward rectification, while the volume-activated currents in endothelial cells are outwardly rectifying (Nilius *et al.*, 1994). The volume-activated Cl^--current in endothelial cells is efficiently blocked by the Cl^--channel blocker NPPB. The ClC-2 channel on the other hand is only moderately affected by the classical Cl^--channel blockers (Thiemann *et al.*, 1992; Gründer *et al.*, 1992). Permeation, blocking and kinetic properties of volume-activated Cl^- currents in endothelial cells are therefore clearly distinct from those of the ClC-2 channel.

We have reported previously that the volume-activated Cl^--current of endothelial cells can be blocked by DDFSK and verapamil (Nilius *et al.*, 1994), which are supposed to inhibit volume-activated Cl^--currents associated with P-glycoprotein (Pgp, Valverde *et al.*, 1992). Tamoxifen has been proposed as a higher affinity inhibitor of Pgp-associated, cell volume regulated Cl^--currents (cited as reference 7 in Valverde *et al.*, 1993). We show here that 10 μmol l^{-1} tamoxifen completely inhibits volume-activated currents in endothelial cells. Cytotoxic drugs, that are substrates of the P-glycoprotein transporter have previously been shown to prevent activation of the volume-activated Cl^- channel (Gill *et al.*, 1992) and have been used to define a class of Cl^- channel blockers (class I of specific Pgp-associated Cl^- channel blockers, Mintenig *et al.*, 1993). The effects of these cytotoxic drugs were less convincing in our endothelial cells, although we could demonstrate the presence of Pgp. Some of them were completely ineffective, while others induced a slow run down of the current.

A third channel that might be involved in volume regulation is the recently proposed I_{cln}-channel, which is blocked by extracellular nucleotides (Paulmichl *et al.*, 1992). Extracellular ATP also blocked volume-activated Cl^--currents in endothelial cells. This observation would be consistent with such a channel, but it appears that the I_{cln} protein is mainly cytosolic and therefore an unlikely candidate for a channel (Krapivinsky *et al.*, 1994).

In conclusion, volume-activated Cl^- channels in endothelial cells share many properties with those in other cells. They are certainly different from the ClC-2 channel, whereas the evidence that they are associated with P-glycoproteins is still rather weak.

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Regional haemodynamic responses to acetylcholine, methoxamine, salbutamol and bradykinin during lipopolysaccharide infusion in conscious rats

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1 The aim of the study was to assess the regional haemodynamic responsiveness to vasoconstrictor and vasodilator challenges during continuous 24 h infusion of lipopolysaccharide (LPS) in conscious Long Evans rats.

2 Rats were chronically instrumented for the measurement of regional haemodynamics (either internal and common carotid or renal, superior mesenteric and hindquarters) and received 3 min of infusions of acetylcholine (22 nmol min^{-1}), methoxamine (120 nmol min^{-1}), salbutamol (0.83 nmol min^{-1}) and bradykinin (14.4 nmol min^{-1}) at 2, 6 and 24 h after the start of saline or LPS (150 $\mu g\ kg^{-1}\ h^{-1}$) infusion (rats with carotid probes received only acetylcholine and methoxamine).

3 During infusion of LPS there was a changing haemodynamic profile. After 2 h, there was a modest hypotension and vasodilatation in the internal carotid, renal and hindquarters vascular beds. After 6 h, arterial blood pressure had returned to baseline, there was still vasodilatation in the renal vascular bed but vasoconstriction in the internal and common carotids and the hindquarters. After 24 h, there was hypotension, tachycardia and generalized vasodilatation.

4 Acetylcholine caused a fall in blood pressure, tachycardia and hyperaemic vasodilatation in the carotid and renal vascular beds. Throughout the infusion of LPS, the carotid vasodilator response was enhanced after 2 h, reduced after 6 h and enhanced again after 24 h, whereas the renal vasodilator response to acetylcholine was either reduced (6 h) or absent (2 and 24 h); at this juncture the hypotensive response to acetylcholine was also enhanced and the tachycardia was reduced.

5 Methoxamine caused a rise in blood pressure, a fall in heart rate, and vasoconstriction in all the vascular beds monitored. During infusion of LPS, the pressor response to methoxamine was consistently reduced as were the vasoconstrictor responses in the carotid and mesenteric vascular beds, whereas the renal and hindquarters vasoconstrictor responses to methoxamine were only significantly reduced at some time points (renal 6 h, hindquarters 2 and 6 h).

6 Salbutamol caused hypotension, tachycardia and hyperaemic vasodilatation, particularly in the hindquarters vascular bed. Throughout the infusion of LPS, the cardiovascular responses to salbutamol were substantially attenuated.

7 Bradykinin caused hypotension, tachycardia and hyperaemic vasodilatation in the renal, mesenteric and hindquarters vascular beds. During the infusion of LPS, the hypotensive response to bradykinin was consistently augmented, and the tachycardia was consistently reduced, but the regional haemodynamic profile changed with time. Thus, after 2 h, the mesenteric vasodilator response was augmented and the hindquarters vasodilator response was reduced; after 6 h, the mesenteric vasodilator response appeared normal, but the renal and hindquarters vasodilator responses were reduced; after 24 h, the hindquarters vasodilator response was markedly augmented and the renal response had changed to a vasoconstriction.

8 The present findings indicate marked regional variations in response to acetylcholine, methoxamine, salbutamol and bradykinin with time during LPS infusion. The changes observed are likely to reflect the interplay of a number of endogenous vasodilator and vasoconstrictor systems; further investigations will be required to clarify the mechanisms involved.

Keywords: Lipopolysaccharide; acetylcholine; methoxamine; salbutamol; bradykinin; regional haemodynamics

Introduction

We have recently described the profile of regional and cardiac haemodynamic changes during a continuous low dose infusion of lipopolysaccharide (LPS) in conscious rats (Gardiner *et al.*, 1992a; 1994). Some of the changes described in this model are similar to those seen in clinical septicaemia, i.e., a raised cardiac output with marked peripheral vasodilatation (see Parrillo, 1993). In man, this hyperdynamic state and the subsequent development of septic shock is often associated with reduced pressor responsiveness to vasoconstrictors (Chernow *et al.*, 1982), which may be related to induction of the calcium-independent form of nitric oxide synthase (iNOS) (see Moncada *et al.*, 1991).

Several animal models of septic shock, induced by LPS administration, show reduced responsiveness to vasoconstrictors *in vivo* (Parratt, 1973; Schaller *et al.*, 1985; Guc *et al.*, 1990; Szabó *et al.*, 1993), and *ex vivo* (Wakabayashi *et al.*, 1987; Julou-Schaeffer *et al.*, 1990). Additionally, there are reports of reduced responses to vasodilators such as acetylcholine (Altura *et al.*, 1985; Young *et al.*, 1991; Guc *et al.*, 1991; Parker & Adams, 1993), salbutamol (Guc *et al.*, 1991) and bradykinin (Altura *et al.*, 1985; Guc *et al.*, 1991) in different animal models of septic shock, although the mechanisms underlying these abnormalities are not entirely clear.

The aim of the present work was to assess the regional vascular responses to vasodilator and vasoconstrictor chal-

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lenges during continuous low dose infusion of LPS in conscious rats. We chose to examine responses to infusions of acetylcholine, salbutamol and bradykinin, since we have previously shown a component of each of these to be inhibited by nitric oxide synthase inhibitors (Gardiner *et al.*, 1990e; 1991); the vasoconstrictor we used was methoxamine. Preliminary accounts of some of these results have been presented to the British Pharmacological Society (Waller *et al.*, 1993; 1994).

Methods

Two groups of male, Long Evans rats (350–450 g) were used in the studies. The rats were anaesthetized (sodium methohexitone, 60 mg kg^{-1}, i.p., supplemented as required) and the first group had pulsed Doppler probes (Haywood *et al.*, 1981) sutured around both common carotid arteries after the external carotid artery had been ligated on the left side to provide a measurement of internal carotid blood flow only on this side, and common carotid blood flow on the right side (Gardiner *et al.*, 1990b). The second group of rats had probes sutured around the left renal, and superior mesenteric arteries and the distal abdominal aorta, below the level of the ileocaecal artery, to monitor blood flow to the hindquarters as described previously (Gardiner *et al.*, 1990c,d,e). At least 7 days later, the rats were anaesthetized (sodium methohexitone, 40 mg kg^{-1}, i.p., supplemented as required), and had a catheter implanted in the abdominal aorta (via the caudal artery) for recording mean arterial pressure (MAP), and 3 catheters implanted in the right jugular vein for the separate administration of LPS and vasoactive drugs. Rats were returned to their home cages for at least 24 h before experiments were begun, and all subsequent procedures were performed without disturbing the animals. Double channel swivels (Blair *et al.*, 1980) were used to allow overnight intravenous infusion of saline or LPS, and also intra-arterial infusion of heparinised saline (15 u ml^{-1}) to maintain catheter patency. During the experiments, continuous recordings were made of heart rate, mean and phasic blood pressures and either internal and common carotid Doppler shift signals or renal, mesenteric and hindquarters Doppler shift signals (using a modified (Gardiner *et al.*, 1990a) pulsed Doppler system (Crystal Biotech, Holliston, U.S.A.)). Changes in regional vascular conductances were calculated from mean Doppler shift and MAP (Gardiner *et al.*, 1990c,d,e).

Following a 30 min period of recording baseline cardiovascular variables, rats with carotid probes received 3 min infusions (0.15 ml min^{-1}) of acetylcholine (22 nmol min^{-1}) or methoxamine (120 nmol min^{-1}) in a random order, with at least 10 min between each infusion. The animals then received a continuous, 24 h infusion (0.3 ml h^{-1}) of isotonic saline (154 mmol l^{-1} NaCl) or LPS (150 $\mu g\ kg^{-1}\ h^{-1}$) in saline. The animals were then re-challenged with infusions of acetylcholine and methoxamine at 2, 6 and 24 h during LPS infusion. The rats with renal, mesenteric and hindquarters probes underwent a similar protocol to that described above, but in addition to receiving acetylcholine and methoxamine, each animal also received 3 min infusions of salbutamol (0.83 nmol min^{-1}) and bradykinin (14.4 nmol min^{-1}). The experimental design meant that it was not possible to administer more than one dose of each of the vasoactive substances. The single doses were therefore chosen on the basis of our previous work (Gardiner *et al.*, 1991; 1992b).

Data analysis

Intra-group changes relative to the baseline, and changes in responses to vasoactive substances based on the areas under or over the curves (AUC or AOC during 3 min drug infusions), were analysed by Friedman's test (Theodorsson-Norheim, 1987) or by Wilcoxon's signed rank test as appropriate. Inter-group changes were analysed using the Mann Whitney U test; a P value <0.05 was taken to indicate a significant difference.

Drugs

Acetylcholine chloride (Sigma), methoxamine hydrochloride B.P. (Wellcome Foundation), and lipopolysaccharide (*E. coli* serotype 0127:B8)(Sigma) were dissolved in isotonic saline. Bradykinin (Bachem, UK), was dissolved in saline containing 1% bovine serum albumin, and salbutamol hemisulphate salt (Sigma) was dissolved in saline containing ascorbic acid (1 mmol l^{-1}).

Results

Carotid haemodynamics

Resting cardiovascular variables before and during infusion of saline or LPS (Table 1) There were no significant differences between the initial resting cardiovascular variables of the two groups prior to infusion of saline or LPS. Saline infusions caused no significant changes in baseline cardiovascular status over 24 h. However, 2 h after the start of infusion of LPS, there was a modest hypotension associated with slight vasodilatation in the internal carotid vascular bed. After 6 h of LPS infusion, MAP had returned to baseline, but there was reduced flow and conductance in the internal and common carotid vascular beds. Twenty four hours after the onset of LPS infusion, there was hypotension and tachycardia, associated with increased flow and conductance in the internal and common carotid vascular beds (Table 1).

Cardiovascular responses to acetylcholine before and during infusion of saline or LPS (Figure 1) Before the start of saline infusion, acetylcholine caused a depressor response and tachycardia, associated with increases in internal and common carotid flows and vascular conductances. Saline infusion had no significant effect on the responses to acetylcholine over 24 h.

Prior to infusion of LPS, responses to acetylcholine were not significantly different from control responses in the saline group. After 2 h of LPS infusion, the tachycardic response to acetylcholine was reduced but the depressor response was unchanged, although the increases in internal and common carotid flows and vascular conductances were enhanced. Six hours after the onset of LPS infusion, the tachycardic response to acetylcholine was still reduced but the depressor response and the increases in internal carotid flow and conductance were not different from control. However, the common carotid flow and conductance responses to acetylcholine were reduced. After 24 h of LPS infusion, the tachycardic response to acetylcholine was reduced but the depressor response was now enhanced. The acetylcholine-induced increase in flow in the common carotid vascular bed was reduced, but the conductance responses in both vascular beds were enhanced.

Cardiovascular response to methoxamine before and during infusion of saline or LPS (Figure 2) Before the onset of saline infusion, methoxamine caused pressor and bradycardic responses, associated with reductions in internal and common carotid flows and vascular conductances. Saline infusion had no significant effect on responses to methoxamine over 24 h.

Prior to infusion of LPS, responses to methoxamine were not significantly different from control responses in the saline group. Two hours after the onset of LPS infusion, the bradycardic response to methoxamine was unchanged, but the pressor response was reduced; this was associated with abolition of the reduction in common carotid flow, and reduced internal and common carotid conductance changes. After 6 h of LPS infusion, and also after 24 h, the bradycar-

Table 1 Resting cardiovascular variables and changes (Δ) in response to saline (0.3 ml h^{-1}) and lipopolysaccharide (LPS, 150 μg kg^{-1} h^{-1}) infusion in conscious Long Evans rats instrumented with carotid probes

		Resting	Δ 2 h	Δ 6 h	Δ 24 h
Heart rate	Saline	362 ± 8	− 2 ± 5	− 3 ± 5	− 11 ± 4
(beats min^{-1})	LPS	351 ± 17	25 ± 19	18 ± 12	69 ± 31*
Mean arterial pressure	Saline	107 ± 2	− 1 ± 2	0 ± 3	5 ± 2
(mmHg)	LPS	105 ± 2	− 14 ± 3*	3 ± 4	− 17 ± 2*
Internal carotid flow	Saline	2.5 ± 0.3	0.0 ± 0.1	0.1 ± 0.2	0.0 ± 0.2
(kHz)	LPS	2.4 ± 0.2	0.0 ± 0.1	− 0.6 ± 0.1*	0.5 ± 0.2*
Common carotid flow	Saline	3.7 ± 0.4	− 0.2 ± 0.2	0.6 ± 0.2	0.4 ± 0.3
(kHz)	LPS	4.3 ± 0.5	− 0.2 ± 0.2	− 1.4 ± 0.2*	1.0 ± 0.2*
Internal carotid conductance	Saline	23 ± 2	0 ± 1	1 ± 2	− 2 ± 2
([kHz $mmHg^{-1}$] 10^3)	LPS	23 ± 2	4 ± 2*	− 6 ± 2*	10 ± 3*
Common carotid conductance	Saline	35 ± 4	− 1 ± 2	6 ± 3	2 ± 3
([kHz $mmHg^{-1}$] 10^3)	LPS	41 ± 5	5 ± 2	− 14 ± 1*	19 ± 4*

Values means ± s.e.mean. *$P < 0.05$ versus resting value. ($n = 9$).

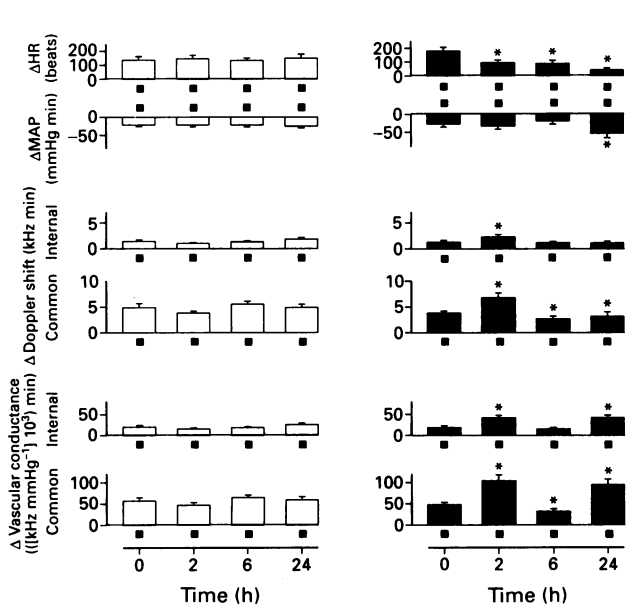


Figure 1 Changes (areas under or over curves AUC, $AOC_{0-3\,min}$) in heart rate (HR), mean arterial pressure (MAP) and internal carotid and common carotid blood flow (Doppler shift) and vascular conductance, in response to acetylcholine infusion (22 nmol min^{-1}) before and during 24 h saline (0.3 ml h^{-1}) (open columns) or lipopolysaccharide (LPS, 150 μg kg^{-1} h^{-1}) (solid columns) infusion in conscious Long Evans rats ($n = 9$ in each group). Values are mean ± s.e.mean, *$P < 0.05$ versus time 0 (Friedman's test); ■ represents a significant response ($P < 0.05$, Wilcoxon's signed rank test).

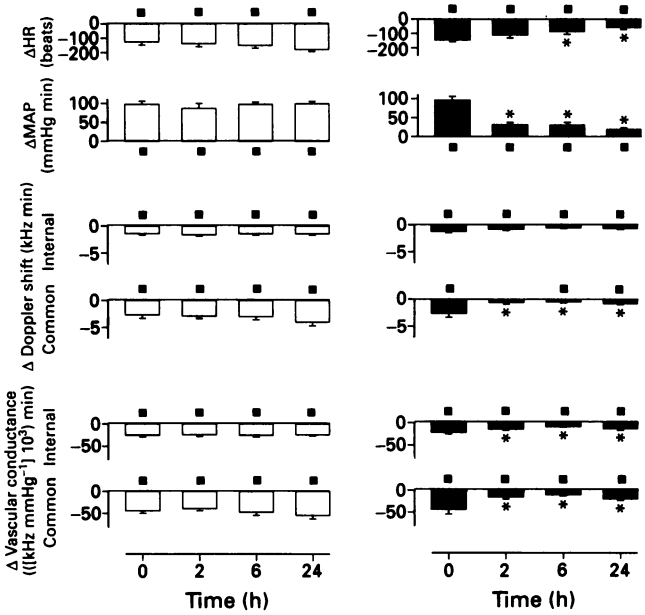


Figure 2 Changes (areas under or over curves AUC, $AOC_{0-3\,min}$) in heart rate (HR), mean arterial pressure (MAP) and internal carotid and common carotid blood flow (Doppler shift) and vascular conductance, in response to methoxamine infusion (120 nmol min^{-1}) before and during 24 h saline (0.3 ml h^{-1}) (open columns) or lipopolysaccharide (LPS, 150 μg kg^{-1} h^{-1}) (solid columns) infusion in conscious Long Evans rats ($n = 9$ in each group). Values are mean ± s.e.mean, *$P < 0.05$ versus time 0 (Friedman's test); ■ represents a significant response ($P < 0.05$, Wilcoxon's signed rank test).

dic response to methoxamine was reduced, and the pressor response was still attenuated, as were the reductions in internal and common carotid flows and vascular conductances.

Renal, mesenteric and hindquarters haemodynamics

Resting cardiovascular variables before and during infusion of saline or LPS (Table 2) There were no significant differences between the initial, resting cardiovascular variables of the 2 groups prior to infusion of saline or LPS. During the 24 h infusion of saline, there were significant reductions in mesenteric blood flow at 2 and 6 h and a small reduction in heart rate at 24 h.

Two hours after the onset of LPS infusion, there was a slight hypotension and a tachycardia, associated with substantial increases in renal flow and conductance, and a smaller increase in hindquarters conductance. Mesenteric flow was reduced to a degree similar to that in the saline controls. After 6 h of LPS infusion, MAP was normal, but there was still a tachycardia. Renal flow and conductance were still elevated, but mesenteric flow was normal, whereas hindquarters blood flow and conductance were reduced. After 24 h of LPS infusion, there was hypotension and tachycardia, associated with marked increases in renal and hindquarters flows and conductances. Although there was vasodilatation in the mesenteric vascular bed, flow was not increased.

Cardiovascular response to acetylcholine before and during infusion of saline or LPS (Figure 3) Before the start of saline infusion, acetylcholine caused hypotension and tachycardia, associated with substantial increases in renal flow and conductance, and modest reductions in mesenteric flow and conductance. During saline infusion, there was a significant

Table 2 Resting cardiovascular variables and changes (Δ) in response to saline (0.3 ml h^{-1}) and lipopolysaccharide (LPS, 150 μg kg^{-1} h^{-1}) infusion in conscious Long Evans rats instrumented with renal, mesenteric and hindquarters probes

		Resting	Δ 2 h	Δ 6 h	Δ 24 h
Heart rate	Saline	331 ± 5	1 ± 7	5 ± 6	− 13 ± 7*
(beats min^{-1})	LPS	333 ± 9	44 ± 9*	32 ± 6*	116 ± 11*
Mean arterial pressure	Saline	102 ± 2	2 ± 2	1 ± 3	0 ± 2
(mmHg)	LPS	103 ± 1	− 13 ± 3*	− 4 ± 2	− 20 ± 2*
Renal flow	Saline	8.3 ± 1.0	− 0.7 ± 0.4	− 1.0 ± 0.4	− 1.3 ± 0.6
(kHz)	LPS	6.4 ± 0.7	3.3 ± 0.5*	2.3 ± 0.3*	5.0 ± 0.7*
Mesenteric flow	Saline	7.6 ± 0.6	− 1.3 ± 0.4*	− 0.5 ± 0.2*	− 0.1 ± 0.5
(kHz)	LPS	6.7 ± 0.7	− 1.5 ± 0.5*	− 0.1 ± 0.7	0.1 ± 0.4
Hindquarters flow	Saline	5.5 ± 0.7	− 0.5 ± 0.2	− 0.6 ± 0.3	− 0.9 ± 0.5
(kHz)	LPS	5.4 ± 0.5	0.3 ± 0.5	− 1.3 ± 0.3*	2.7 ± 0.5*
Renal conductance	Saline	81 ± 9	− 8 ± 4	− 10 ± 3	− 12 ± 5
([kHz $mmHg^{-1}$] 10^3)	LPS	61 ± 6	45 ± 6*	26 ± 4*	75 ± 8*
Mesenteric conductance	Saline	75 ± 7	− 15 ± 5	− 5 ± 3	− 2 ± 4
([kHz $mmHg^{-1}$] 10^3)	LPS	66 ± 7	− 7 ± 5	2 ± 7	16 ± 4*
Hindquarters conductance	Saline	55 ± 8	− 6 ± 2	− 6 ± 3	− 9 ± 6
([kHz $mmHg^{-1}$] 10^3)	LPS	53 ± 5	12 ± 7*	− 10 ± 4*	45 ± 6*

Values means ± s.e.mean. *$P<0.05$ versus resting value. ($n = 8$).

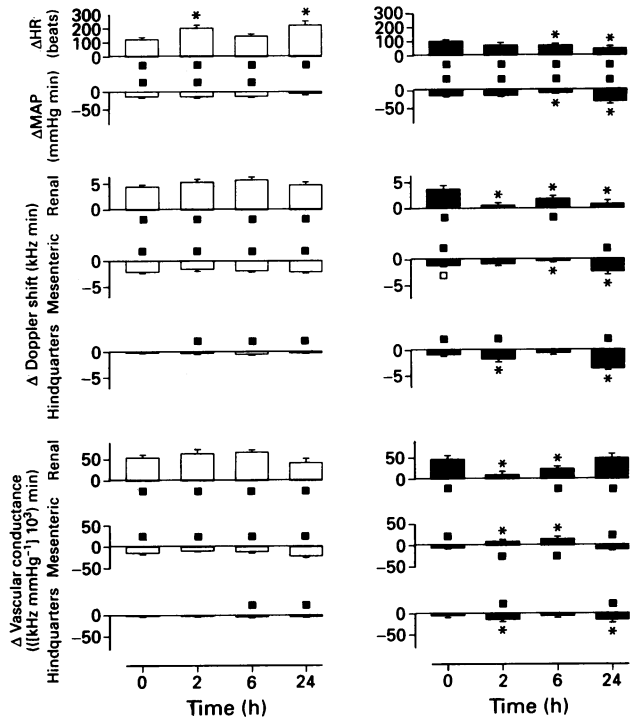


Figure 3 Changes (areas under or over curves AUC, $AOC_{0-3\,min}$) in heart rate (HR), mean arterial pressure (MAP) and renal, mesenteric and hindquarters blood flow (Doppler shift) and vascular conductance in response to acetylcholine infusion (22 nmol min^{-1}) before and during 24 h saline (0.3 ml^{-1} h^{-1}) (open columns) or lipopolysaccharide (LPS, 150 μg kg^{-1} h^{-1}) (solid columns) infusion in conscious Long Evans rats ($n = 8$ in each group). Values are mean ± s.e.mean, *$P<0.05$ versus time 0 (Friedman's test); □$P<0.05$ versus control group at time 0 (Mann Whitney U test); ■ represents a significant response ($P<0.05$, Wilcoxon's signed rank test).

increase in the tachycardic response to acetylcholine, at both 2 and 24 h, but all other responses were unchanged.

Prior to infusion of LPS, responses to acetylcholine were not significantly different from control responses in the saline group, except for a less marked reduction in mesenteric flow in the former group. Two hours after the onset of infusion of LPS, the acetylcholine-induced depressor and tachycardic responses were unchanged. However, the increases in renal flow and vascular conductance were abolished, the mesenteric vasoconstrictor response was reversed to a modest vasodilator response, and there was a greater reduction in hindquarters flow, which was accompanied by significant vasoconstriction. After 6 h of LPS infusion, the depressor and tachycardic responses to acetylcholine were reduced. The acetylcholine-induced increases in renal flow and conductance were, once again, present, albeit attenuated. The mesenteric vasodilator response seen at 2 h was still present, but there were no acetylcholine-induced changes in hindquarters flow or vascular conductance. After 24 h of LPS infusion, the tachycardic response to acetylcholine was still reduced, but the depressor response was now enhanced. The increase in renal vascular conductance was not different from the control response although there was no associated increase in flow. The mesenteric vasoconstrictor response to acetylcholine was back to control levels, although there was a greater reduction in mesenteric flow, and greater reductions in hindquarters flow and conductance, than prior to LPS infusion.

Cardiovascular response to methoxamine before and during infusion of saline or LPS (Figure 4) Before the start of saline infusion, methoxamine caused pressor and bradycardic responses, associated with reductions in mesenteric and hindquarters flows and renal, mesenteric and hindquarters vascular conductances. During the infusion of saline there was a significant, but modest, reduction in the mesenteric flow and vasoconstrictor response to methoxamine at 2 h.

Prior to infusion of LPS, responses to methoxamine were not significantly different from control responses in the saline group. Two hours after the onset of infusion of LPS, the bradycardic response to methoxamine was unchanged, but the pressor response was reduced; this was associated with reductions in mesenteric and hindquarters flow and conductance responses. After 6 h of LPS infusion, the bradycardic response to methoxamine was again unchanged, and the pressor response was still reduced. In the renal vascular bed, the vasoconstrictor response to methoxamine was reduced and the change in flow was no longer significant. In the mesenteric vascular bed, there was a reduced vasoconstrictor response, associated with a reduction in flow that was not different from the control response; in the hindquarters vascular bed, the flow response to methoxamine was abolished and the vasoconstrictor response was reduced. After 24 h of LPS infusion, the bradycardic and pressor responses to methoxamine were reduced, and the renal haemodynamic changes were not different from control, whereas the mesenteric flow and conductance changes were reduced; the hindquarters flow response was reduced, but the conductance change was normal.

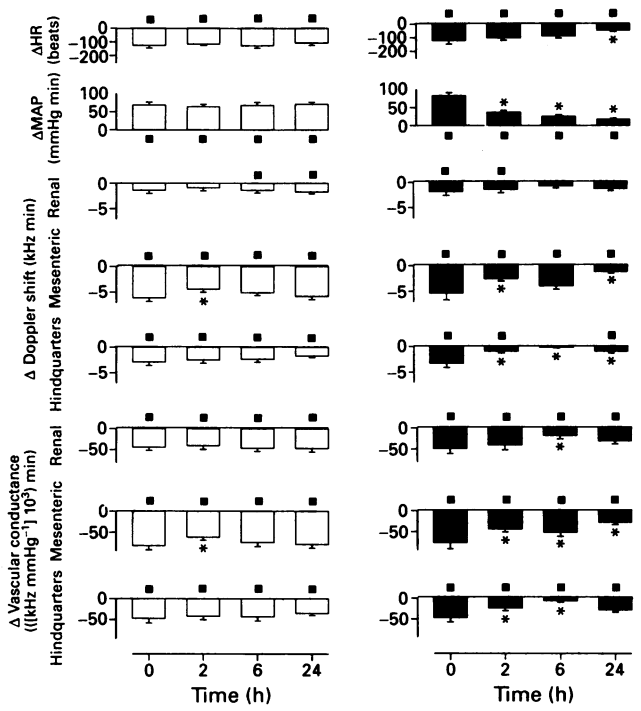


Figure 4 Changes (areas under or over curves AUC, $AOC_{0-3\,min}$) in heart rate (HR), mean arterial pressure (MAP), renal, mesenteric and hindquarters blood flow (Doppler shift) and vascular conductance, in response to methoxamine infusion (120 nmol min^{-1}) before and during 24 h saline (0.3 ml^{-1} h^{-1}) (open columns) or lipopolysaccharide (LPS, 150 μg kg^{-1} h^{-1}) (solid columns) infusion in conscious Long Evans rats ($n = 8$ in each group). Values are mean ± s.e.mean, $^*P < 0.05$ versus time 0 (Friedman's test); ■ represents a significant response ($P < 0.05$, Wilcoxon's signed rank test).

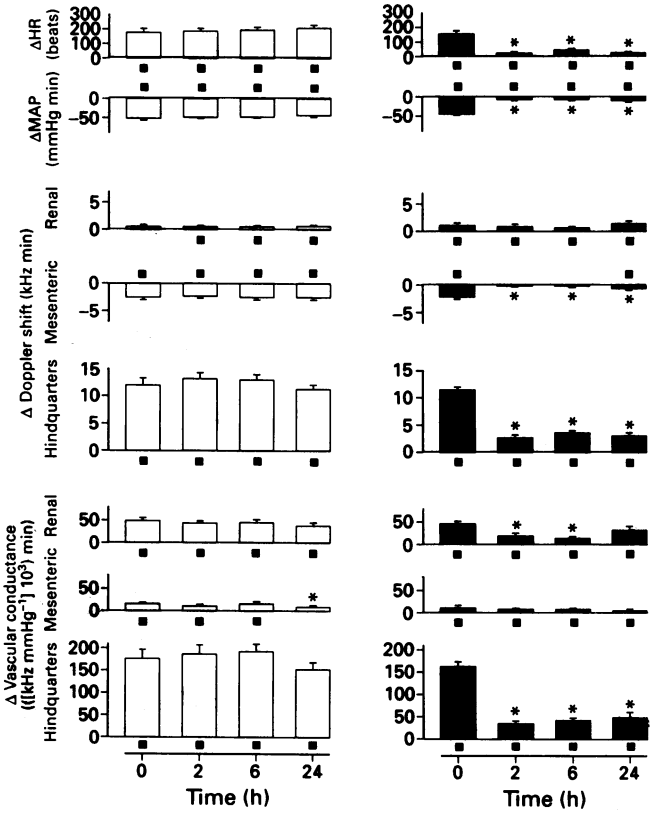


Figure 5 Changes (areas under or over curves AUC, $AOC_{0-3\,min}$) in heart rate (HR), mean arterial pressure (MAP), renal, mesenteric and hindquarters blood flow (Doppler shift) and vascular conductance in response to salbutamol infusion (0.83 nmol min^{-1}) before and during 24 h saline (0.3 ml^{-1} h^{-1}) (open columns) or lipopolysaccharide (LPS, 150 μg kg^{-1} h^{-1}) (solid columns) infusion in conscious Long Evans rats ($n = 8$ in each group). Values are mean ± s.e.mean, $^*P < 0.05$ versus time 0 (Friedman's test); ■ represents a significant response ($P < 0.05$, Wilcoxon's signed rank test).

Cardiovascular response to salbutamol before and during infusion of saline or LPS (Figure 5) Before the onset of saline infusion, salbutamol caused depressor and tachycardic responses, associated with a substantial increase in hindquarters flow and conductance, a smaller increase in renal conductance, and a modest reduction in mesenteric flow associated with a small vasodilatation. During saline infusion there was a significant reduction in the mesenteric vasodilator response to salbutamol at 24 h, but all other responses were unchanged.

Prior to infusion of LPS, responses to salbutamol were not significantly different from control responses in the saline group. Two hours after the start of infusion of LPS, the tachycardic and depressor responses to salbutamol were reduced, the increases in hindquarters flow and conductance were attenuated, the reduction in mesenteric flow was abolished, and the increase in renal conductance was reduced. After 6 h of LPS infusion, responses to salbutamol were not different to those observed at 2 h. After 24 h of LPS infusion, the tachycardic and depressor responses to salbutamol were still reduced, as were changes in hindquarters flow and conductance and mesenteric flow, whereas the increase in renal conductance was back to control values.

Cardiovascular response to bradykinin before and during infusion of saline or LPS (Figure 6) Before the start of saline infusion, bradykinin caused a tachycardic and depressor response, associated with substantial increases in mesenteric and hindquarters flows and conductances, and smaller increases in renal flow and conductance. During saline infusion, the increase in mesenteric flow in response to bradykinin was reduced at 6 and 24 h, and there was a reduction in the mesenteric vasodilator response and an increase in the tachycardic response at 24 h.

Prior to infusion of LPS, responses to bradykinin were not different from the control responses in the saline group, except for a less marked increase in mesenteric flow in the former group. Two hours after the onset of infusion of LPS, the tachycardic response to bradykinin was reduced and the depressor response was enhanced. Under these conditions, the bradykinin-induced increase in mesenteric flow was not different from control, but the vasodilatation was enhanced and the renal vasodilator response to bradykinin was unchanged, although it was associated with a decrease, rather than an increase, in flow. Likewise, the bradykinin-induced increase in hindquarters flow seen under control conditions was replaced by a decrease in flow, and the increase in conductance was reduced. After 6 h of LPS infusion, the tachycardic response to bradykinin was reduced and the depressor response was enhanced. Mesenteric flow and conductance responses were normal, the renal vasodilator response was reduced, with the decrease in renal flow seen at 2 h still present; the hindquarters flow and conductance responses were reduced. After 24 h of LPS infusion, the tachycardic and depressor responses to bradykinin were still reduced or enhanced, respectively. The mesenteric vasodilator response was not different from control, although there was no increase in flow. The decrease in renal flow seen at 2 and 6 h was still present, but was now associated with vasoconstriction rather than vasodilatation. The hindquarters vasodilatation was enhanced, although the increase in flow was reduced.

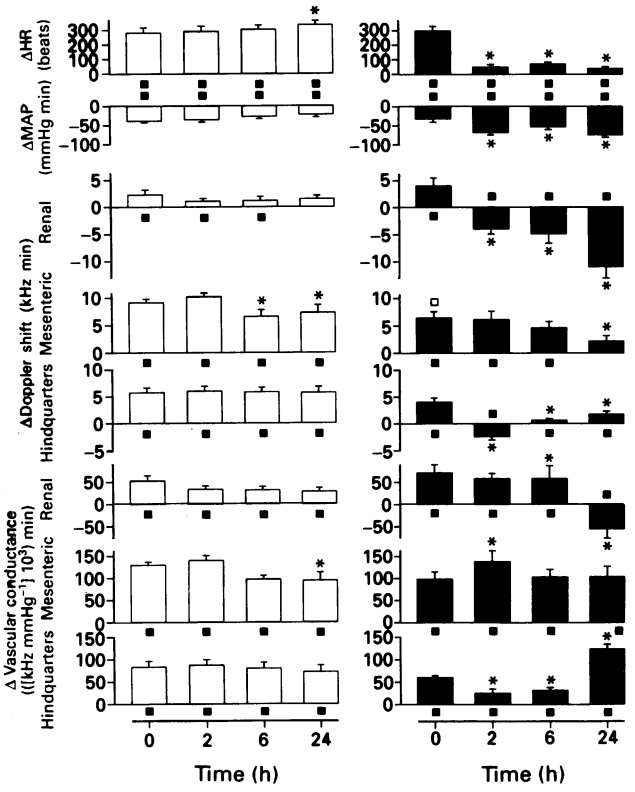


Figure 6 Changes (areas under or over curves AUC, $AOC_{0-3\,min}$) in heart rate (HR), mean arterial pressure (MAP), renal, mesenteric and hindquarters blood flow (Doppler shift) and vascular conductance in response to bradykinin infusion (14.4 nmol min^{-1}) before and during 24 h saline (0.3 $ml^{-1}\,h^{-1}$) (open columns) or lipopolysaccharide (LPS, 150 $\mu g\,kg^{-1}\,h^{-1}$) (solid columns) infusion in conscious Long Evans rats ($n=8$ in each group). Values are mean ± s.e.mean, $^{*}P<0.05$ versus time 0 (Friedman's test); $^{\square}P<0.05$ versus control group at time 0 (Mann Whitney U test); ■ represents a significant response ($P<0.05$, Wilcoxon's signed rank test).

Discussion

Previous work (Gardiner *et al.*, 1992a) has shown that LPS infusion at a dose of 150 $\mu g\,kg^{-1}\,h^{-1}$ for 24 h elicited differential and changing haemodynamic patterns between 0–3, 4–8, and 8–24 h after the start of LPS infusion. Therefore, in the present work, changes in regional haemodynamics and responses to vasodilator and vasoconstrictor challenges were assessed before and during the 3 phases of response to LPS i.e., around 2, 6 and 24 h after the onset of administration.

After 2 h of LPS infusion, there was a modest hypotension, but the most striking change in haemodynamic status was a renal hyperaemic vasodilatation. It is possible that the latter was due to a loss of vasoconstrictor tone and/or activation of vasodilator mechanisms. However, since the renal vasoconstrictor response to methoxamine was not diminished at this stage, and infusion of LPS causes sympatho-excitation independent of baroreflex mechanisms (Burnier *et al.*, 1988) it is likely that the increases in renal flow and conductance were due to activation of one or more vasodilator systems. The two most obvious vasodilator candidates are nitric oxide (NO) and prostanoids. Whilst we have no direct evidence regarding the activity of cyclo-oxygenase under our conditions, it is known that prostanoids are involved in endotoxaemia (Parratt & Sturgess, 1974; Herman & Vane, 1976; Wise *et al.*, 1980; Myers *et al.*, 1993). However, since prostaglandin E_2 (PGE_2) causes renal vasoconstriction in the rat (Gerber & Nies, 1979; Quilley *et al.*, 1989), and PGI_2 causes mesenteric vasodilatation with little effect on renal blood flow in conscious rats (Gardiner *et al.*, unpublished observations), the haemodynamic changes noted must have involved other prostanoids, if the latter were involved at all.

Expression of iNOS can begin to occur within 3 h after administration of endotoxin as a large bolus dose (Salter *et al.*, 1991; Szabó *et al.*, 1993), but it is unlikely that there would have been marked expression of iNOS within 2 h of the onset of the low dose LPS infusion used here. Thus, if NO was involved in the early renal haemodynamic changes it is probable it was due to LPS-induced activation of constitutive NO synthase (Salvemini *et al.*, 1990; Thiemermann & Vane, 1990; Szabó *et al.*, 1993). However, it would be expected that this event would be associated with hyporesponsiveness to α-adrenoceptor agonists (Szabó *et al.*, 1993) except, possibly, in the mesenteric vascular bed (Mitchell *et al.*, 1993), and yet of all the beds studied, the kidney was the only one that did not show a diminution in responsiveness to methoxamine 2 h after the onset of LPS infusion. That finding, together with the relative lack of change in resting haemodynamics, except in the kidney, indicates that the LPS-induced renal hyperaemic vasodilatation is not likely to have been attributable to NO alone.

Systemic infusion of acetylcholine causes selective hyperaemic vasodilatation in the renal and carotid vascular beds and these changes are sensitive to inhibition of NO synthase (Gardiner *et al.*, 1991). However, in the present study, 2 h after the beginning of LPS infusion, the renal vasodilator response to acetylcholine was abolished, but the carotid vasodilatation was markedly enhanced. So, whatever the explanation, it is apparent that the factor(s) affecting basal haemodynamic status are not straightforwardly linked to the factor(s) affecting responsiveness to acetylcholine. Others have reported diminished responsiveness to acetylcholine in pithed rats (Guc *et al.*, 1991), in rat mesenteric metarterioles (Altura *et al.*, 1985) and guinea-pig aortic ring preparations (Young *et al.*, 1991) following LPS administration. Both Altura *et al.* (1985) and Young *et al.* (1991) suggested their findings may have been due to endothelial damage, but, clearly, such an effect could not explain an enhanced vasodilator responsiveness to acetylcholine, as seen in the carotid vascular beds. Considering the differences in the changes in resting haemodynamics and responsiveness to acetylcholine in renal and carotid vascular beds, it is feasible that the resting hyperaemia in the former explained the apparent loss of hyperaemic effect of acetylcholine, i.e. renal flow was near maximal even before challenge with acetylcholine. If changes in resting renal haemodynamic status 2 h after the onset of LPS prevented any assessment of responsiveness to acetylcholine in that vascular bed, then it could be argued that the increases in responsiveness to acetylcholine in the carotid vascular beds were due to the absence of resting hyperaemia, and possibly represented an increased acetylcholine-mediated release of NO attributable to LPS-induced activation of constitutive NO synthase (Salvemini *et al.*, 1990; Thiemermann & Vane, 1990; Szabó *et al.*, 1993).

Two hours after the beginning of LPS infusion, the diminished pressor and carotid, mesenteric and hindquarters vasoconstrictor effects of methoxamine are consistent with reports of vascular hyporeactivity to a variety of vasoconstrictor agents in other rat models (Fink *et al.*, 1985; Schaller *et al.*, 1985; Guc *et al.*, 1990; Hollenberg *et al.*, 1993) and in man (Chernow *et al.*, 1982). It is generally agreed that NO-mediated mechanisms are involved (see Moncada *et al.*, 1991) and, if so, it is interesting that not all vascular beds responded uniformly and that the pattern of hyporesponsiveness changed with time during LPS infusion (see below). Regional differences in the effects of NO-mediated hyporesponsiveness to phenylephrine and other vasoconstrictors following administration of LPS have recently been reported by Mitchell and co-workers (1993). However, they observed that the mesenteric vascular bed showed maintained vasoconstrictor responsiveness *ex vivo*, whereas the present study indicates that finding may not pertain *in vivo*. Interestingly, Kovách *et*

al. (1991) demonstrated regional heterogeneity in the changes in vasoconstrictor and vasodilator responsiveness in isolated blood vessels taken from animals subjected to haemorrhagic shock.

There was a marked reduction in the depressor, tachycardic and regional haemodynamic (particularly hindquarters vasodilator) effects of salbutamol 2 h after the onset of LPS infusion. In contrast to the other vasodilator challenges, the changes in responsiveness to salbutamol seen at 2 h were generally maintained throughout the 24 h infusion of LPS. Thus, the most likely explanation of this effect is reduced β_2-adrenoceptor sensitivity (Auclair *et al.*, 1983; Van Heuven-Nolsen *et al.*, 1986), possibly due to β-adrenoceptor down-regulation secondary to elevated circulatory catecholamines (Chernow *et al.*, 1982; Schaller *et al.*, 1985). Such a proposal is consistent with the finding that there was also a reduced hyperaemic hindquarters vasodilator response to bradykinin 2 h after the start of LPS infusion, since elsewhere we have shown that this effect of bradykinin probably involves β_2-adrenoceptor activation (Gardiner *et al.*, 1992b).

The changes in haemodynamic status and cardiovascular responsiveness which occurred between 2 and 6 h after LPS infusion were: (1) normalization of the mean arterial blood pressure, (2) conversion of the small carotid and hindquarters vasodilations to vasoconstrictions, (3) reversal of the enhanced carotid vasodilator response to acetylcholine to a normal (internal) or slightly diminished (common) response, (4) development of reduced renal vasoconstrictor effect of methoxamine.

The vasoconstrictions seen in the carotid and hindquarters vascular beds could represent an amalgam of a waning vasodilator influence, together with developing activation of vasoconstrictor systems. Interestingly, the responsiveness to methoxamine was still markedly reduced in those vascular beds at that stage, so either sympathetic activation (Chernow *et al.*, 1982; Schaller *et al.*, 1985; Burnier *et al.*, 1988) was sufficiently large to evoke some degree of vasoconstriction, or the hyporesponsiveness was not a generalized phenomenon and other vasoconstrictor substances were able to exert an effect.

The change in basal carotid haemodynamics was associated with a change in the responsiveness of that vascular bed to acetylcholine, i.e. there was if anything a diminished, rather than augmented vasodilatation. The increase in basal tone would be expected to enhance vasodilator effects. If the carotid vasodilator response to acetylcholine was NO-mediated, then a reduced response could be attributable to a defect at the level of the muscarinic receptors, NO production, or post-receptor events.

The changes in haemodynamic status and cardiovascular responsiveness which occurred between 6 and 24 h after LPS infusion were: (1) the development of a tachycardia and hypotension, (2) conversion of the carotid and hindquarters vasoconstrictions to hyperaemic vasodilatations together with enhancement of the renal hyperaemic vasodilatation and the development of mesenteric vasodilatation, (3) enhancement of the depressor response to acetylcholine together with enhancement of the carotid and normalization of the renal vasodilatations, (4) apparent normalization of the vasoconstrictor responses to methoxamine in the renal and hindquarters vascular beds and, (5) development of a renal vasoconstrictor and enhanced hindquarters vasodilator response to bradykinin.

The vasodilatation seen in all vascular beds after 24 h of LPS infusion are likely to represent the enhanced activation of vasodilator systems such as those influenced by iNOS and cyclo-oxygenase-2 (COX-2). Although we have no direct evidence of the induction of these systems, other studies have reported the induction of iNOS by LPS *in vitro* (Radomski *et al.*, 1990) and *in vivo* (Salter *et al.*, 1991; Szabó *et al.*, 1993). Similarly LPS is known to stimulate the induction of COX-2 *in vitro* in human monocytes (Fu *et al.*, 1990) and bovine endothelial cells (Akarasereenont *et al.*, 1994), and *in vivo* in murine macrophages (Masferrer *et al.*, 1990), resulting in enhanced release of prostanoids.

The potentiated hypotensive effect of acetylcholine after 24 h of LPS infusion was not related to enhanced hyperaemia in any of the vascular beds monitored, although the carotid vasodilatation was augmented and the tachycardia was markedly suppressed. It is feasible that under control conditions, the infusion of acetylcholine elicited an increase in cardiac output which offset the hypotension. After 24 h of LPS infusion, when resting cardiac output is markedly elevated (Gardiner *et al.*, 1994) any further cardiac stimulation may have been impaired.

Responsiveness to methoxamine after 24 h of LPS infusion was still markedly reduced in the carotid and mesenteric vascular beds, whereas in the renal and hindquarters vascular beds, the vasoconstrictions were no longer significantly reduced. This apparent normalization was against the background of little or no reduction in flow and hence the conductance changes could have been largely autoregulatory.

The reversal from a renal vasodilatation at 6 h to a renal vasoconstriction at 24 h in response to bradykinin suggests the unmasking of a vasoconstrictor component. We know that systemic administration of bradykinin causes β_2-adrenoceptor mediated vasodilatation in the renal and hindquarters vascular beds (Gardiner *et al.*, 1992b). It is therefore feasible that reduced β_2-adrenoceptor sensitivity could reduce the bradykinin-induced renal vasodilator component, unmasking a vasoconstrictor component. But the renal vasodilator response to salbutamol, the β_2-adrenoceptor agonist, was less attenuated at 24 h than at earlier stages during the LPS infusion. Also, the hindquarters vasodilator response to bradykinin was enhanced, yet the response to salbutamol in that vascular bed was still markedly diminished. We, therefore, have no ready explanation for the changes in the regional haemodynamic profile of bradykinin.

Overall, the results of the present study demonstrate time-dependent changes in the cardiovascular response to a number of agonists during LPS infusion. The results also show differential changes in response to the various agonists within different vascular beds. The specific mechanisms causing these time-dependent regional changes will require further investigation.

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The imidazoline site involved in control of insulin secretion: characteristics that distinguish it from I_1- and I_2-sites

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1 The nature of the binding site mediating the insulin secretagogue activity of certain imidazoline compounds remains unclear and the pharmacology of the I_1- and I_2-imidazoline sites, described in many tissues, does not correlate with the observed responses to imidazolines in islets. In the present paper, we describe further results which support the concept that the islet imidazoline site may represent a novel subtype of imidazoline receptor.

2 Culture of rat isolated islets in the presence of imidazoline secretagogues (either efaroxan or phentolamine) resulted in loss of responsiveness on subsequent re-exposure to these agents. However, culture of islets with either idazoxan or UK14,304 (imidazoline ligands that do not stimulate insulin secretion) did not lead to any loss of response when the islets were subsequently exposed to efaroxan. By contrast, islets cultured with UK14,304 (a potent α_2-adrenoceptor agonist), displayed loss of sensitivity to noradrenaline, consistent with down-regulation of α_2-adrenoceptors.

3 In order to characterize the imidazoline site further, radioligand binding studies were performed in membranes from RINm5F insulinoma cells using [^{3}H]-RX821002, an imidazoline insulin secretagogue that does not interact significantly with imidazoline sites in other tissues. [^{3}H]-RX821002 labelled α_2-adrenoceptors with high affinity (2.01 ± 0.7 nM) but also labelled a second, non-adrenoceptor site with much lower affinity.

4 Under conditions of α_2-adrenoceptor blockade (in the presence of adrenaline), efaroxan displaced [^{3}H]-RX821002 binding to the low affinity site, in a dose-dependent manner. Competition studies employing additional imidazoline compounds of varying secretagogue activity revealed that the pharmacological profile of the low affinity site correlates well with that observed in secretion experiments.

5 The results obtained from the down-regulation experiments with isolated islets and from the radioligand binding studies suggest that the low affinity [^{3}H]-RX821002 binding site may represent the functional receptor responsible for the secretagogue activity of imidazoline compounds in the endocrine pancreas and that it has a pharmacological profile distinct from those of I_1- and I_2-sites.

Keywords: Islets of Langerhans; insulin secretion; imidazoline; I-site; RX821002; efaroxan; RINm5F cells; down-regulation

Introduction

Non-adrenoceptor imidazoline binding sites represent a new class of receptors which are thought to mediate the central anti-hypertensive actions of clonidine and related drugs (Michel & Ernsberger, 1992; Ernsberger *et al.*, 1992). In addition, they may also mediate contraction of peripheral smooth muscle, stimulation of insulin release and alterations in renal metabolism induced by imidazoline compounds (Atlas, 1991). Two principal imidazoline receptor subtypes have been defined, based on their affinity for clonidine or idazoxan. I_1-imidazoline sites are labelled preferentially by [^{3}H]-clonidine, or its derivative [^{3}H]-*p*-aminoclonidine, and are pharmacologically distinct from imidazoline recognition sites labelled with [^{3}H]-idazoxan (I_2-sites), in that the latter show 100 fold lower affinity for clonidine and do not recognize imidazoles (Michel & Ernsberger, 1992).

Recent studies have shown that certain compounds which possess an imidazoline moeity within their structure, are able to enhance the rate of insulin secretion, both *in vivo* and *in vitro* (Ahrén & Lundquist, 1985; Schulz & Hasselblatt, 1988; 1989a; Chan *et al.*, 1988; 1991a). $^{86}Rb^+$ efflux experiments and electrophysiological studies have provided evidence that this response is due to a reduction in potassium efflux through ATP-sensitive K^+ channels in the islet β-cell plasma membrane, resulting in membrane depolarization (Chan & Morgan, 1990; Chan *et al.*, 1991a,b; Dunne, 1991; Jonas *et al.*, 1992). In functional secretion experiments, we have shown that, in the endocrine pancreas, the secretory response induced by the imidazoline efaroxan shows stereoselectivity and is subject to down-regulation in the presence of agonist (Chan *et al.*, 1993). These data suggest that an 'imidazoline receptor' is responsible for mediating the effects of imidazoline compounds in islets, although the nature of the binding site is still unclear. I_2-receptors (also known as non-adrenoceptor idazoxan binding sites (NAIBS)) have been described on pancreatic islets (Brown *et al.*, 1993; Lacombe *et al.*, 1993) and on RINm5F insulinoma cells (Remaury & Paris, 1992). However, the emerging pharmacology of the defined I_1- and I_2-sites does not correlate well with the observed responses to imidazolines in islets. Thus, the possibility remains that the islet imidazoline receptor may represent a novel, third type of imidazoline binding site.

To date, it has not proved possible to identify the islet imidazoline receptor using radioligand binding techniques as the imidazoline compounds which are the most effective insulin secretagogues are not readily available in labelled form. The major exception to this, clonidine, labels multiple binding sites in many tissues, making it an unsatisfactory ligand. Therefore, in the present work, we have exploited the finding that the imidazoline α_2-antagonist, RX821002, can stimulate insulin secretion (Chan, 1990; Gross *et al.*, 1991) but does not interact significantly with imidazoline sites in other tissues (Langin *et al.*, 1989). We show that [^{3}H]-RX821002 can label a low affinity site on RINm5F cell membranes, the pharmacology of which is consistent with the possibility that it may represent the putative imidazoline site responsible for regulation of β-cell K^+_{ATP} channel activity. In addition, we present further data from desensitization experiments which support the concept that the imidazoline

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site mediating the secretagogue activity of imidazolines, such as efaroxan and phentolamine, is distinct from the designated I_1- and I_2-sites.

Methods

Insulin secretion experiments

Islets of Langerhans were isolated from the pancreata of male Wistar rats by collagenase digestion (Montague & Taylor, 1968). The isolation medium was a bicarbonate-buffered physiological saline solution (Gey & Gey, 1936), pH 7.4, containing 4 mM glucose and 2 mM $CaCl_2$. For islet incubations, this medium was supplemented with 1 mg ml^{-1} bovine serum albumin. In static incubation experiments, groups of three isolated islets were incubated for 60 min at 37°C with test reagents. After this time, samples of the medium were removed for measurement of their insulin content by radioimmunoassay (RIA).

For desensitization experiments, groups of isolated islets were cultured in RPMI-1640 containing glutamine (2 mM), foetal calf serum (10% v/v), penicillin (400 iu ml^{-1}), streptomycin (200 $\mu g\ ml^{-1}$) and test reagents, in a humidified atmosphere of air:CO_2 (95:5) for 18 h. After this time, islets were washed and selected by hand under a dissecting microscope for use in static incubation experiments.

Radioligand binding experiments

Membranes were prepared from RINm5F cells, cultured in RPMI-1640 supplemented with 10% (v:v) foetal calf serum. Briefly, cell monolayers were washed with phosphate buffered saline and the cells scraped in ice-cold TEM buffer (50 mM Tris-HCl, pH 7.5; 1 mM EDTA; 10 mM $MgCl_2$) supplemented with 50 μM phenylmethylsulphonyfluoride. Following homogenization and centrifugation (40,000 *g*, 20 min, 4°C), the resulting pellet was resuspended in TEM buffer and re-centrifuged. The final crude membrane fraction was resuspended in TEM buffer and aliquots were quickly frozen and stored at − 70°C. Protein content was measured by the bicinchoninic acid method (Smith *et al.*, 1985).

Membranes (50–100 μg protein), [^{3}H]-RX821002 and competing drugs were incubated in a final volume of 100 μl TEM buffer for 60 min at 25°C. The reaction was stopped by adding 3 ml of ice-cold TEM buffer. Bound and free radioligand were separated by filtration through Whatman GF/B filters under vacuum. Filters were washed and their radioactivity measured after addition of scintillant.

For saturation studies, [^{3}H]-RX821002 was used over the range 1–100 nM and non-specific binding was defined in the presence of 100 μM phentolamine. In experiments designed to study specifically binding at the 'imidazoline' site, incubations were performed in the presence of 10 μM adrenaline to block α_2-adrenoceptors. The radioligand binding data were analyzed by iterative non-linear curve fitting using the Fig P Curve Fitter Programme (Biosoft, Cambridge, UK).

Materials

[^{3}H]-RX821002 (specific activity 59 Ci $mmol^{-1}$) was purchased from Amersham International. RX821002 ((2-2-methoxy-1,4-benzodioxan-2-yl)-2-imidazoline) and efaroxan were gifts from Reckitt & Colman Products. Diazoxide was obtained from Glaxo Pharmaceuticals. UK14,304 (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline) and prazosin were gifts from Pfizer Research. Phentolamine mesylate was donated by CIBA Labs. Adrenaline and clonidine were from Sigma Chemical Co.

Statistics

Statistical analysis was performed using Student's *t* test for unpaired data and results were considered significant when $P < 0.05$.

Results

Down-regulation of imidazoline-mediated potentiation of insulin-release

It has been shown previously that imidazoline compounds which can stimulate insulin secretion directly, are also able to overcome the inhibitory effects of diazoxide on insulin release (Plant & Henquin, 1990; Chan & Morgan, 1990; Chan *et al.*, 1991a,b). Therefore, the use of diazoxide provides a convenient assay system for determining the effectiveness of imidazolines to interact functionally with islet cells. Figure 1 shows the effects of culturing islets in 100 μM efaroxan (a), phentolamine (b), idazoxan (c) and UK14,304 (d) on the ability of the imidazoine secretagogues efaroxan and phentolamine to antagonize diazoxide-induced inhibition of insulin release. Islets which had been cultured in the presence of either efaroxan (a) or phentolamine (b) were found to be refractory to these compounds upon subsequent re-exposure. Thus, in efaroxan-cultured islets, efaroxan failed to elicit any reversal of the inhibition of glucose-induced insulin release mediated by diazoxide (Figure 1a). Similarly, the capacity of phentolamine to antagonize the inhibitory effects of diazoxide was also impaired in these islets (Figure 1b). Conversely, culture of islets in the presence of either idazoxan (Figure 1c) or UK14,304 (Figure 1d), imidazoline ligands (I_2-site) which do not stimulate insulin release (Chan & Morgan, 1990; Chan *et al.*, 1991b; Brown *et al.*, 1993), did not lead to any loss of response when the islets were subsequently exposed to efaroxan or phentolamine.

In contrast, after islets were cultured in the presence of UK14,304 (a potent α_2-agonist), noradrenaline was unable to inhibit glucose (20 mM)-induced insulin secretion (Figure 1d). This loss of sensitivity to noradrenaline is consistent with down-regulation of α_2-adrenoceptors during exposure to the α_2-agonist. Culture in the absence of UK14,304 did not alter the response of islets to noradrenaline (data not shown; see Hurst & Morgan, 1990). Furthermore, since the ability of efaroxan and phentolamine to antagonize the actions of diazoxide was still evident after exposure to UK14,304, this confirms that the insulin secretagogue activity of these compounds does not involve interaction with α_2-adrenoceptors.

Effects of RX821002 on insulin secretion from rat isolated islets

In insulin secretion experiments, RX821002 (100 μM) significantly stimulated basal insulin release from isolated rat islets (Table 1). Moreover, RX821002 was also able to antagonize the inhibitory effect of diazoxide on glucose-induced insulin secretion (Table 1). These data confirm previous studies suggesting that the mechanism of action of imidazoline compounds involves interaction with a regulatory component associated with ATP-sensitive potassium channels (Plant & Henquin, 1990; Chan & Morgan, 1990; Chan, 1993).

Identification of RX821002 binding sites in RINm5F cell membranes

The binding of [^{3}H]-RX821002 to RINm5F membranes was rapid, with the steady state being reached within 20 min at 25°C (data not shown). When employed at concentrations between 1–10 nM, [^{3}H]-RX821002 labelled a high affinity site which was saturable (Figure 2). However, raising the concentration of [^{3}H]-RX821002 further, revealed the presence of a second binding site of much lower affinity, which remained less than fully saturated even at a radioligand concentration of 100 nM. Analysis of the data by iterative non-linear curve fitting confirmed the presence of at least two sites. The high affinity site bound [^{3}H]-RX821002 with a K_D of 2.01 ± 0.7 nM ($n = 4$) and B_{max} of 34 ± 10.4 fmol mg^{-1} protein, consistent with binding to α_2-adrenoceptors. By contrast, the second site exhibited a much lower affinity with an estimated K_D of

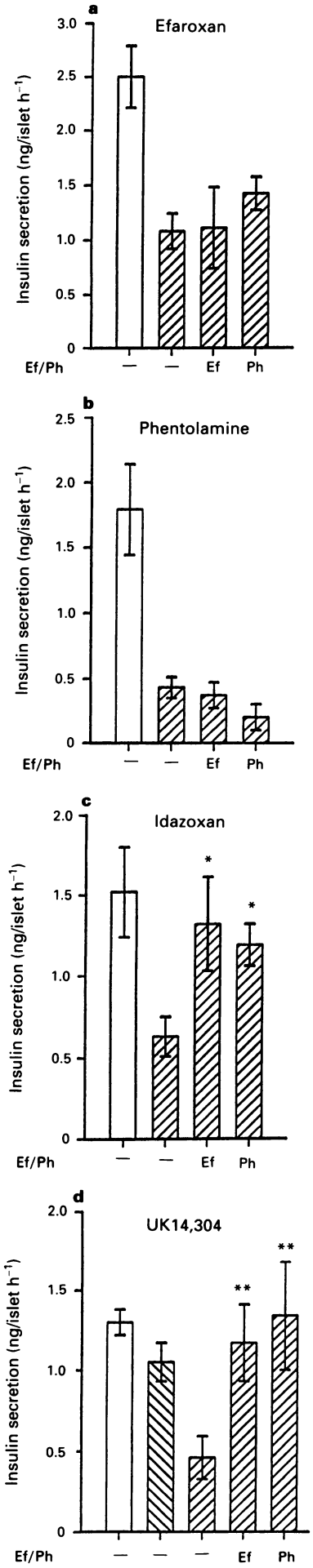


Figure 1 Effects of culture conditions on the ability of imidazoline insulin secretagogues to antagonize the inhibition of insulin secretion induced by diazoxide. Groups of rat isolated islets were cultured for 18 h in medium supplemented with 100 μM efaroxan (a), phentolamine (b), idazoxan (c) or UK14,304 (d). Islets were washed and incubated in the presence of 20 mM glucose. Open columns represent secretion in response to 20 mM glucose alone. Diazoxide (250 μM; [▨]), noradrenaline (10 μM; [▩]), efaroxan (100 μM; Ef) and phentolamine (100 μM; Ph) were included as shown. Insulin secretion was measured after 60 min of incubation at 37°C. Results represent mean values ± s.e.mean for 7–8 observations. $*P<0.01$, $**P<0.001$; relative to incubation with diazoxide in the absence of imidazoline.

Table 1 Effects of RX821002 on basal insulin release and on the inhibitory response to diazoxide in rat islets

Glucose (mM)	*RX821002* (100 μM)	*Diazoxide* (125 μM)	*Insulin secretion* (ng/islet h^{-1})
4	–	–	0.55 ± 0.04
4	+	–	0.81 ± 0.10*
20	–	–	2.11 ± 0.08
20	–	+	0.61 ± 0.07
20	+	+	1.52 ± 0.11**

Groups of three isolated islets were incubated for 60 min at 37°C, in the presence of test reagents as shown. After this time, samples of medium were removed for measurement of insulin content by radioimmunoassay. Data are presented as mean values ± s.e.mean for 12–18 observations. $*P<0.0125$; $**P<0.001$; relative to incubation in the absence of RX821002.

145 ± 22 nM ($n = 4$) and B_{max} of 364 ± 22 fmol mg^{-1} protein. It was difficult to determine the K_D of the low affinity site with any degree of accuracy since the site remained less than fully saturated under all conditions tested and the value obtained from non-linear curve fitting represents a minimum estimate.

Pharmacology of the low affinity [^{3}H]-RX821002 binding site

Further characterization of the low affinity [^{3}H]-RX821002 binding site in competition binding assays employing imidazoline compounds of varying secretagogue activity was difficult, since the K_D of the site is very low and the proportion of non-specific binding increased significantly at high concentrations of radioligand. However, experiments utilizing 50 nM [^{3}H]-RX821002 and performed in the presence of 10 μM adrenaline, revealed a dose-dependent displacement of [^{3}H]-RX821002 binding to the low affinity site by efaroxan (Figure 3). Under these conditions, approximately 45% of total non-adrenoceptor [^{3}H]-RX821002 binding was displaced by 50 μM efaroxan. Figure 4 shows the displacement of [^{3}H]-RX821002 binding by a variety of compounds acting at α-adrenoceptors. The imidazoline ligands efaroxan, RX821-002, phentolamine, and clonidine (10 μM) significantly displaced the binding of the radioligand, whereas idazoxan and UK14,304 (10 μM) had no significant effect on the binding of [^{3}H]-RX821002 at this low affinity site. This excludes the possibility that displacement was from residual α_2-adrenoceptors since all of these compounds are potent α_2-ligands in the endocrine pancreas. Prazosin also failed to alter RX821-002 binding indicating that α_1-adrenoceptors were not responsible for the low affinity binding of the radioligand. Significantly, this pharmacological profile correlates well with the capacity of imidazoline compounds to stimulate insulin release since efaroxan, RX821002, phentolamine and clonidine (under conditions of α_2-blockade) are all secretagogues

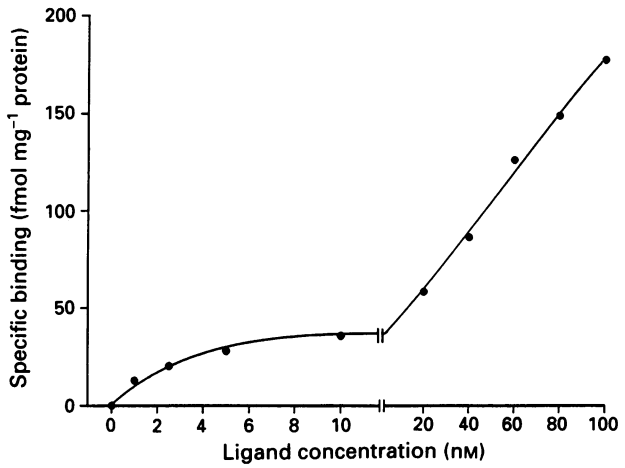


Figure 2 RINm5F membranes were incubated with [^{3}H]-RX821002, over the concentration range 1.0–100 nM, for 60 min at 25°C. Non-specific binding was determined in the presence of 100 μM phentolamine. Shown is a representative experiment with each point determined in triplicate. Binding data were analyzed by iterative non-linear curve fitting using the Fig P Curve Fitter Programme (Biosoft, Cambridge, U.K.).

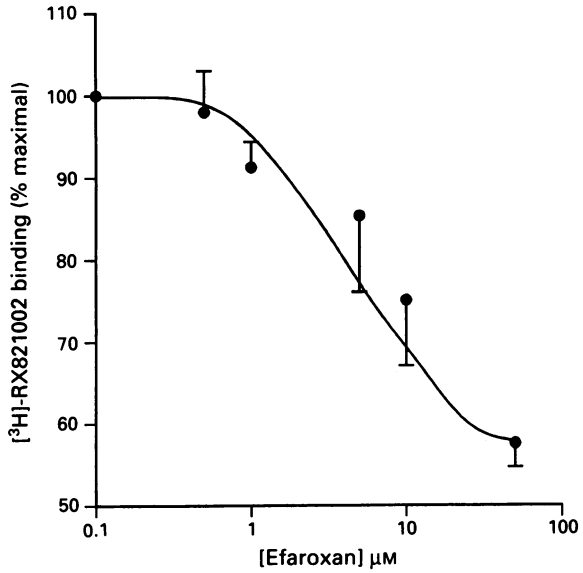


Figure 3 Displacement of [^{3}H]-RX821002 binding to the low affinity binding site in RINm5F membranes by efaroxan. RINm5F membranes were incubated with [^{3}H]-RX821002 (50 nM) in the presence and absence of efaroxan (0.1–100 μM), for 60 min at 25°C. Adrenaline (10 μM) was included to block α_2-adrenoceptors. Each point represents the mean ± s.e.mean for 12 observations.

whereas idazoxan and UK14,304 do not augment insulin secretion (Chan *et al.*, 1988; Schulz & Hasselblatt, 1989b; Chan & Morgan, 1990; Brown *et al.*, 1993). Use of a higher concentration of idazoxan (50 μM) in competition experiments did result in some displacement (20%) of [^{3}H]-RX821002 binding (not shown) suggesting that idazoxan may interact with the site when present at very high concentrations.

Discussion

The ability of several imidazoine compounds to increase insulin release both *in vitro* and *in vivo* has been ascribed to their blockade of ATP-sensitive potassium channels in islet

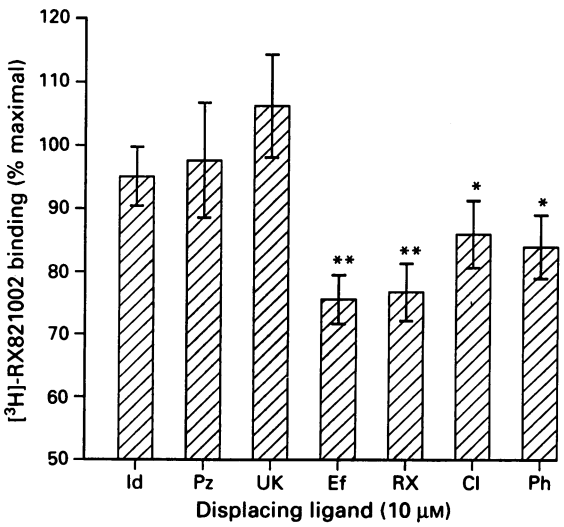


Figure 4 Effects of compounds acting on α-adrenoceptors on the binding of [^{3}H]-RX821002 to the low affinity binding site in RINm5F membranes. Competition binding studies were performed on RINm5F membranes with a single concentration (50 nM) of [^{3}H]-RX821002. Competing agents were present at a concentration of 10 μM; idazoxan (Id); prazosin (Pz); UK14,304 (UK); efaroxan (Ef); RX821002 (RX); clonidine (Cl); and phentolamine (Ph). Adrenaline (10 μM) was included in all tubes to block α_2-adrenoceptors. The data are expressed as percentages of the control binding measured in the absence of competitor. Each point represents the mean ± s.e.mean for 12–18 observations. *$P<0.01$; **$P<0.001$, versus the control.

β-cells (Plant & Henquin, 1990; Chan, 1993). As yet, the nature of the binding site mediating this effect has not been disclosed. In this study, we have sought to identify a radioligand that maybe suitable for labelling of the novel 'imidazoline' binding site responsible for mediating the secretagogue activity of certain imidazoline compounds.

It might be anticipated that clonidine (or a derivative) would be a suitable ligand for labelling of islet imidazoline receptors since clonidine stimulates insulin secretion under conditions of α_2-adrenoceptor blockade (Schulz & Hasselblatt, 1989b; Lacombe *et al.*, 1993). To investigate this possibility, preliminary studies were performed in which the high specific activity ligand [^{125}I]-iodoclonidine was used to label clonidine binding sites in RINm5F cells. RINm5F is a rat islet derived cell line which expresses a wide range of receptors and has been used extensively to investigate the mechanisms of signal transduction by cell surface receptors that control insulin secretion (Praz *et al.*, 1983; Aminaroff *et al.*, 1988). These cells express imidazoline receptors that regulate K^+_{ATP} channel activity (Chan *et al.*, 1991a; Dunne, 1991) and thus, we have used crude membrane preparations from RINm5F cells in the radioligand binding studies. [^{125}I]-iodoclonidine interacted with both α_2-adrenoceptors and imidazoline sites in RINm5F membranes but in the latter case binding was displaced more potently by idazoxan than by efaroxan (unpublished observations), suggesting that it represented mainly interaction with I_2-sites (Ernsberger *et al.*, 1992). Thus, it was inappropriate to employ [^{125}I]-iodoclonidine in further studies due to its multiple sites of interaction.

Therefore, we next considered the possibility that [^{3}H]-RX821002 might be an appropriate ligand. [^{3}H]-RX821002 is an alpha-2 antagonist of the imidazoline family which has been shown to be highly specific for α_2-adrenoceptors with no significant interaction with I_2-sites (NAIBS), over the range of concentrations used in binding studies (Langin *et al.*, 1989). However, we have shown in a previous study that RX821002 can potentiate insulin secretion from rat isolated islets (Chan, 1990) and similar results have also been reported in perfused rat pancreas (Gross *et al.*, 1991). This

response does not reflect α_2-receptor blockade since it is not reproduced by other equally effective α_2-antagonists that are not imidazolines (Chan, 1993). It seems likely, therefore, that the response to RX821002 is mediated by the putative islet imidazoline receptor site implicated in regulation of β-cell potassium permeability.

Binding experiments revealed that [^{3}H]-RX821002 labelled a low affinity site on RINm5F cell membranes, the pharmacology of which is consistent with the possibility that it may represent the previously elusive 'imidazoline' receptor responsible for regulation of β-cell K^+_{ATP} channel activity. The site is clearly distinct from either α_2-adrenoceptors or I_2-sites since the residual binding of [^{3}H]-RX821002, measured in the presence of adrenaline, was not displaced by idazoxan or UK14,304, which interact with both types of receptor with high affinity (Ernsberger *et al.*, 1992; Brown *et al.*, 1993). Furthermore, the site is unlikely to represent an I_1-imidazoline site since efaroxan is reported to be an I_1-antagonist (Ernsberger *et al.*, 1992) but acts agonistically on insulin secretion and K^+_{ATP} channel blockade (Chan & Morgan, 1990; Chan *et al.*, 1991b). Furthermore, the low potency with which efaroxan stimulates insulin secretion (Chan & Morgan, 1990) and displaces [^{3}H]-RX821002 binding (Figure 3) are not consistent with interaction at an I_1 site (Ernsberger *et al.*, 1992).

A number of previous studies have reported the labelling of pancreatic β-cell α_2-adrenoceptors with [^{3}H]-RX821002 (Remaury & Paris, 1992; Lacombe *et al.*, 1993), although none of these has described an additional lower affinity site. Indeed, we also conducted an earlier study in rat isolated islets which revealed only a single [^{3}H]-RX821002 binding site (Morgan *et al.*, 1989). The important difference in the present work is that the range of concentrations of [^{3}H]-RX821002 employed has been extended considerably to facilitate labelling of any low affinity sites. At concentrations below 10 nM, [^{3}H]-RX821002 labels α_2-adrenoceptors exclusively in RINm5F cells and pancreatic islets, and the parameters for α_2-adrenoceptor binding determined in the present work are in excellent agreement with those reported in RINm5F cells by Remaury & Paris (1992).

In view of the very low affinty of the [^{3}H]-RX821002 site identified in this work, it is surprising that the site could be detected by use of vacuum filtration methods to separate bound from free ligand. It might be anticipated that the rate of dissociation of ligand from receptor would be sufficiently rapid that the ligand would dissociate during filtration and washing. Despite these considerations, however, we were consistently able to detect displaceable binding of [^{3}H]-RX821-002 to RINm5F cell membranes over a large number of experiments. It is noteworthy that MacKinnon *et al.* (1993) have also recently characterized a low affinity imidazoline receptor (K_D; 128 nM) in rat kidney membranes using a tritiated ligand and vacuum filtration methods.

Down-regulation of receptors in response to chronic agonist exposure has been widely reported in pathological conditions and during chronic drug treatment. A number of observations have indicated that imidazoline sites are also regulated in this manner (Hamilton *et al.*, 1992; 1993; Olmos *et al.*, 1992; 1993). Indeed, alterations in imidazoline receptor site number and/or regulation have been implicated in the pathogenesis of endogenous depression and hypertension (Olmos *et al.*, 1992; 1993). In the present study, we have confirmed that the imidazoline site responsible for mediating the insulin secretagogue activity in islets can be down-regulated on long-term exposure (18 h) to agonists. However, imidazolines which are unable to stimulate insulin release directly (idazoxan and UK14,304) did not affect the capacity of efaroxan and phentolamine to antagonize the inhibitory actions of diazoxide on secretion in these cultured islets. Thus, these data suggest that efaroxan and phentolamine act as agonists at the islet imidazoline site. Efaroxan has been classified as a potent antagonist at the I_1-site since it can inhibit the rise in arterial blood pressure induced by monoxidine, a highly selective and potent agonist at I_1-sites (Ernsberger *et al.*, 1992). Thus, it would appear that the islet imidazoline site displays pharmacological characteristics that differ from those of I_1-sites. Moreover, idazoxan and UK14,304 have been documented to act at I_2-sites in brain and kidney (Bidet *et al.*, 1990; Hamilton *et al.*, 1992; 1993). Since neither of these compounds is an insulin secretagogue, nor acts as antagonist to the activity of efaroxan (Brown *et al.*, 1993), and was unable to down-regulate the actions of efaroxan and phentolamine in the present study, it can be concluded that the islet imidazoline site represents an imidazoline-preferring site that is also distinct from the I_2-site.

In conclusion, in isolated islets RX821002 can stimulate insulin release by a mechanism which involves interaction with a regulatory component involved in control of ATP-sensitive potassium channel activity. Besides labelling the α_2-adrenoceptors with high affinity, [^{3}H]-RX821002 also labelled with much lower affinity, a class of binding sites with a pharmacological profile similar to that expected of the site involved in stimulation of insulin release by imidazoline receptors. This site displays characteristics which are distinct from those of the designated I_1- and I_2-sites. The radioligand [^{3}H]-RX821002 may be of value for assay of the islet imidazoline receptor during purification.

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Comparative pharmacology of analogues of S-nitroso-*N*-acetyl-DL-penicillamine on human platelets

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1 The effects of two new analogues of S-nitroso-*N*-acetyl-DL-penicillamine (SNAP), S-nitroso-*N*-formyl-DL-penicillamine (SNFP) and S-nitroso-DL-penicillamine (SNPL), on platelet function were examined *in vitro*.

2 SNAP and its analogues were potent inhibitors of platelet aggregation and inducers of disaggregation.

3 All compounds inhibited fibrinogen binding to platelets.

4 They also decreased the release of P-selectin from platelets.

5 Both inhibition of fibrinogen binding and release of P-selectin correlated with an increase in intraplatelet cyclic GMP concentrations.

6 At concentrations sufficient to inhibit platelet function and induce cyclic GMP formation (0.01–3 μM), the release of NO could be detected from SNPL but not from SNAP and SNFP.

7 Release of NO from all compounds was detected at concentrations $\geqslant 10\,\mu M$.

8 Thus, the spontaneous release of NO from SNPL explains the actions of this compound on platelet function; however, platelet-mediated mechanisms may be involved in the release of NO from SNAP and SNFP.

Keywords: Human platelets; S-nitroso-*N*-acetyl-DL-penicillamine analogues; aggregation; fibrinogen binding; P-selectin; cyclic GMP; nitric oxide

Introduction

In vitro, S-nitrosylation of molecules containing functional thiol groups leads to the formation of S-nitrosothiols. Although the biological significance of S-nitrosylation *in vivo* is still uncertain, this reaction may result in stabilization of the chemical and pharmacological properties of NO and generation of compounds with NO-donating properties (Stamler *et al.*, 1992; Venturini *et al.*, 1993). S-nitroso-*N*-acetyl-DL-penicillamine (SNAP) is a stable inhibitor of platelet aggregation (Radomski *et al.*, 1992). We have synthesized two novel analogues of SNAP, S-nitroso-*N*-formyl-DL-penicillamine (SNFP) and S-nitroso-DL-penicillamine (SNPL), and have examined their pharmacological behaviour on human platelets *in vitro*.

Methods

Platelet-rich plasma (PRP) and washed platelet suspensions (WP)

Human blood was collected and PRP and prostacyclin (PGI_2)-washed platelet suspensions were prepared as described by Radomski & Moncada (1983).

Platelet aggregation and release of ATP

These were measured in a platelet-ionized calcium lumi-aggregometer. S-nitrosothiols were incubated with WP (2.5×10^8 platelets ml^{-1}) for 1 min prior to the addition of collagen (1 $\mu g\,ml^{-1}$) and their effects on platelet aggregation studied for 3 min. In some experiments haemoglobin (5 μM) was added 1 min before S-nitrosothiols. In another set of experiments S-nitrosothiols (all at 1 μM) were incubated for 60 min at 37°C.

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Platelet disaggregation

Platelet disaggregation was measured in PRP following initiation of aggregation by ADP (3–5 μM). S-nitrosothiols were added 1 min after ADP and their effects studied for 5 min.

Intraplatelet cyclic GMP and cyclic AMP concentrations

These were measured in WP by enzyme immunoassay as described previously (Radomski *et al.*, 1992). Briefly, WP were incubated in the aggregometer for 10 min at 37°C in the presence or absence of S-nitrosothiols. Following incubation, EGTA (5 mM) was added and platelets lysed by two cycles of freezing in liquid nitrogen and thawing at 37°C. The lysate was centrifuged (10,000 *g* for 5 min) and the supernatant assayed for cyclic nucleotides by the dual range acetylation enzyme immunoassay (Amersham).

[^{125}I]-fibrinogen binding to platelets

Binding to platelets of [^{125}I]-fibrinogen was measured as described by Marguerie *et al.* (1979) with some modifications. Washed platelets (10^8 ml^{-1}) were incubated in the presence or absence of S-nitrosothiols for 15 min. Binding was initiated by adding [^{125}I]-fibrinogen (50 $\mu g\,ml^{-1}$) and ADP (50 μM) in a final volume of 500 μl, conditions in which B_{max} is approx. 4000 c.p.m./10^6 platelets and $K_D = 13.3 \pm 2.0\,\mu g\,ml^{-1}$ fibrinogen ($n = 3$). After 15 min, samples (50 μl) of the reaction mixture were layered on 200 μl of 15% sucrose solution and centrifuged for 1 min at 10,000 *g*. The samples were immediately frozen in dry ice and the radioactivity associated with the platelet pellet was counted in a γ counter.

P-selectin (CD62, GMP-140, PADGEM)

P-selectin release was measured by enzyme immunoassay in the supernatants of WP (2.5×10^8 platelets ml^{-1}) stimulated with collagen (0.3–10 $\mu g\,ml^{-1}$) in the presence or absence of

S-nitrosothiols or PGI_2. The concentrations of S-nitrosothiols or PGI_2 (3 or 10 μM and 3 or 10 nM, respectively) were selected to achieve a similar inhibitory effect (approx. 95%) on the aggregation induced by 3 μg ml^{-1} of collagen. The inhibitors were added 1 min before collagen and their effects studied for 5 min. At the end of incubation EDTA (5 mM) was added, then the samples were centrifuged at 10,000 g for 1 min and the supernatant assayed for the presence of P-selectin (British Biotechnology).

The release of NO from S-nitrosothiols

NO release from S-nitrosothiols was measured by the method described by Feelisch & Noack (1987). S-nitrosothiols were incubated for 5 min at 37°C with or without intact platelets, platelet lysate, platelet cytosol (100,000 g for 30 min) all prepared from 2.5×10^8 platelets, reduced glutathione (30 μM), ascorbic acid (30 μM) or superoxide dismutase (20 u ml^{-1}) and the rate of NO release was measured in a dual wave spectrophotometer (Shimadzu).

S-nitrosothiols

SNAP was synthesized by the method of Field *et al.* (1978). SNPL and SNFP were synthesized according to the method of Hart (1985). For structures, see Figure 1.

Reagents

Human haemoglobin was prepared by the method of Paterson *et al.* (1976). Adenosine 5'-diphosphate (ADP), adenosine 3':5'-triphosphate (ATP), ethylenediaminetetraacetic acid (EDTA), luciferin-luciferase reagent, sucrose, superoxide dismutase, glutathione, ethyleneglycol-*bis*(β-aminoethylether) *N,N,N',N'*-tetraacetic acid (EGTA), Arg-Gly-Asp-Ser (Sigma), prostacyclin sodium salt (Wellcome), L-ascorbic acid (BDH), collagen (Hormon-Chemie), [^{125}I]-fibrinogen (Amersham), guanosine 3':5'-cyclic monophosphate (cyclic GMP) and adenosine 3':5'-cyclic monophosphate (cyclic AMP) enzyme immunoassay (Amersham), Tyrode salt solution (Gibco, BRL) and P-selectin enzyme immunoassay (British Biotechnology) were obtained from the sources indicated.

Statistics

Results are mean ± s.e.mean of at least three separate experiments. They were compared by analysis of variance. The comparisons between all pairs of groups were made using the Bonferroni P value (Computer programme GraphPad 1990), and $P<0.05$ was considered as statistically significant.

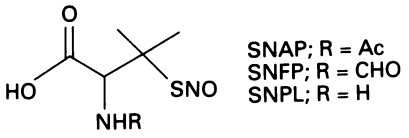


Figure 1 The chemical structures of S-nitroso-*N*-acetyl-DL-penicillamine (SNAP) and its two novel analogues: S-nitroso-DL-penicillamine (SNPL) and S-nitroso-*N*-formyl-DL-penicillamine (SNFP).

Results

Effects of S-nitrosothiols on platelet aggregation, ATP release and disaggregation

Incubation of S-nitrosothiols (0.01–10 μM) with WP resulted in a concentration-dependent inhibition of collagen-induced aggregation and of ATP release from platelets (Figure 2a and Table 1), SNAP and SNFP being significantly more potent ($P<0.05$, $n=3-7$) than SNPL. The inhibitory activity of these S-nitrosothiols was reversed by haemoglobin (5 μM) (Figure 2b). SNAP and SNFP were stable as inhibitors of

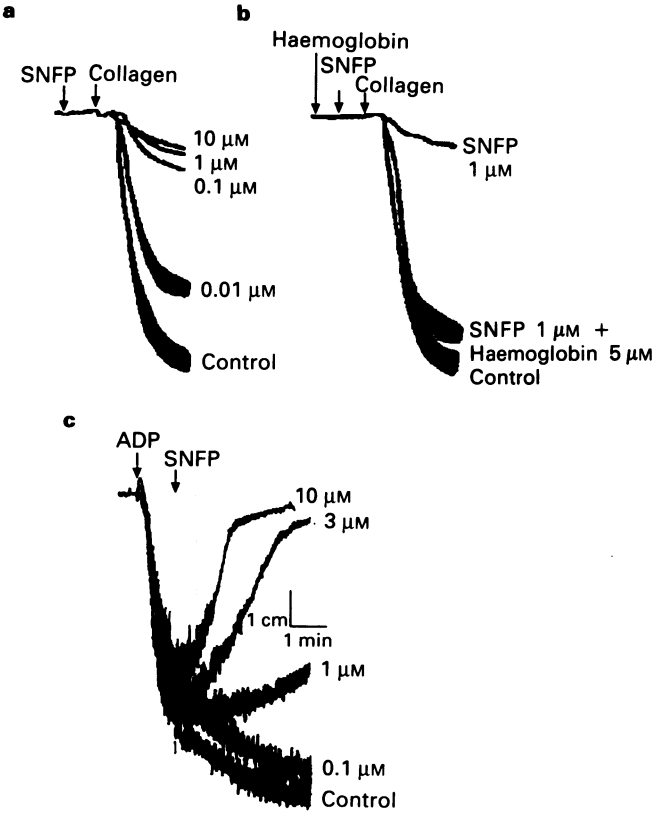


Figure 2 Effect of SNFP on platelet aggregation in washed platelets (WP) (a, b) and platelet-rich plasma (PRP) (c). (a) Inhibition by SNFP (0.01–10 μM) of collagen (1 μg ml^{-1})-induced aggregation. (b) Reversal by haemoglobin (5 μM) of the effect of SNFP (1 μM) on collagen (1 μg ml^{-1})-induced aggregation. (c) Stimulation by SNFP (0.1–10 μM) of disaggregation of platelets aggregated with ADP (5 μM). Representative tracings of five separate experiments.

Table 1 Inhibition of aggregation and ATP release (washed platelets, WP) and stimulation of disaggregation (platelet-rich plasma, PRP) by S-nitrosothiols (R-SNO)

R-SNO ($n=3-7$)	*Inhibition of collagen-induced aggregation* (IC_{50}, μM)	*Inhibition of collagen-induced release of ATP* (IC_{50}, μM)	*Stimulation of disaggregation of platelets aggregated by ADP* (EC_{50}, μM)
SNFP	0.05 ± 0.01	0.15 ± 0.07	0.63 ± 0.21
SNPL	0.38 ± 0.05	0.59 ± 0.13	0.25 ± 0.01
SNAP	0.12 ± 0.02	0.18 ± 0.11	1.09 ± 0.32

SNFP was the most potent inhibitor of collagen-induced aggregation whereas SNPL was the most potent stimulator of disaggregation ($P<0.05$, $n=3-7$). The aggregation was induced in WP by collagen (1 μg ml^{-1}) and in PRP by ADP (3–5 μM). The concentration of aggregating agent was selected in each experiment to produce 75–90% of maximal aggregation.

platelet aggregation. Indeed, the inhibitory activities of these S-nitrosothiols at 1 μM were 94.0 ± 1.2 and 86.8 ± 1.7% before and 92.7 ± 2.3 and 87.9 ± 0.7%, respectively (n = 3–4, $P > 0.05$), after 60 min incubation at 37°C. In contrast, the inhibitory activity of SNPL (1 μM) decayed when stored at 37°C for 60 min from 86.9 ± 6.1 to 56.9 ± 17.5% ($n = 4$, $P < 0.05$).

The addition of S-nitrosothiols (0.01–10 μM) 1 min after induction of platelet aggregation with ADP (3–5 μM) in PRP resulted in a concentration-dependent induction of platelet disaggregation (Figure 2c and Table 1). SNPL was more potent ($P < 0.05$, $n = 3–7$) than SNAP and SNFP as an inducer of disaggregation. With the exception of SNPL, higher concentrations of these compounds were needed for induction of disaggregation than for inhibition of aggregation (Table 1).

Cyclic GMP and cyclic AMP

The basal concentrations of cyclic GMP and cyclic AMP in platelets were 131 ± 33 and 84 ± 27 fmol/10^8 platelets, respectively ($n = 4$). Incubation of platelets with SNAP, SNFP and SNPL (0.01–10 μM) caused a concentration-dependent increase in intraplatelet cyclic GMP (Figure 3 and Table 2). These compounds were equipotent as stimulators of cyclic GMP in platelets. The concentrations of cyclic AMP were not significantly affected by 10 μM of SNAP, SNFP and SNPL (102 ± 38, 54 ± 28, 69 ± 37 fmol/10^8 platelets respectively, $P > 0.05$, $n = 4$).

[^{125}I]-fibrinogen binding assay

SNAP, SNFP and SNPL caused a concentration-dependent inhibition of [^{125}I]-fibrinogen binding to the platelets (Figure 4 and Table 2). These S-nitrosothiols were equipotent in inhibiting fibrinogen binding to platelets. There was a significant correlation between the concentrations of S-nitrosothiols needed for half-maximal stimulation of intraplatelet cyclic GMP and those required for inhibition of fibrinogen binding (Table 2).

Release of P-selectin

Collagen (0.3–10 μg ml^{-1}) resulted in aggregation and the release of P-selectin from platelets (Figure 5). Both the aggregation and release induced by collagen were inhibited by SNPL, SNFP and SNAP (3–10 μM) (Figure 5). No significant differences were found in the inhibitory activities of these S-nitrosothiols ($n = 3$, $P > 0.05$).

To compare the effects of PGI_2 and SNPL as inhibitors of P-selectin release, the concentrations of PGI_2 (3 or 10 nM) and SNPL (3 or 10 μM) were selected to achieve maximal (approx. 95%) inhibition of collagen (3 μg ml^{-1})-induced aggregation. These concentrations were used to examine their effects on collagen (0.3–10 μg ml^{-1})-induced release of P-selectin. PGI_2 also inhibited both aggregation and P-selectin release induced by collagen. There was no significant difference between the two compounds when tested against 0.3–3 μg ml^{-1} of collagen. However, against the highest concentration of collagen (10 μg ml^{-1}), SNPL (52 ± 3% of maximal release) proved to be significantly ($P < 0.05$, $n = 3$) more effective than PGI_2 (70 ± 4% of maximal release) in inhibiting P-selectin release from platelets.

Release of NO

SNPL when dissolved in Tyrode solution released NO in a concentration-dependent manner (Table 3). The release of

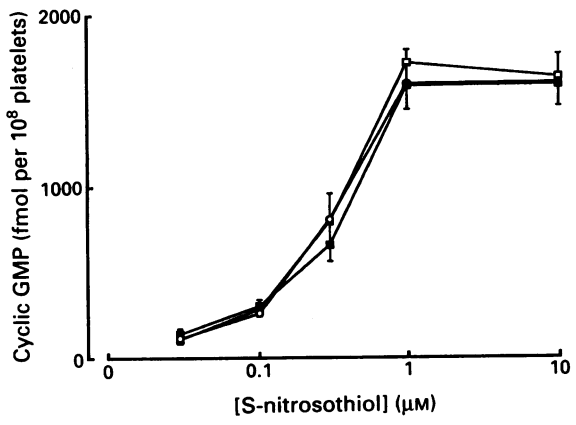


Figure 3 Increase in intraplatelet cyclic GMP concentrations in washed platelets (WP) by S-nitrosothiols. SNAP (□), SNPL (○) and SNFP (■) at concentrations ranging from 0.03 to 3 μM were equipotent as stimulators of the soluble guanylate cyclase. Data are mean ± s.e.mean, $n = 3–4$.

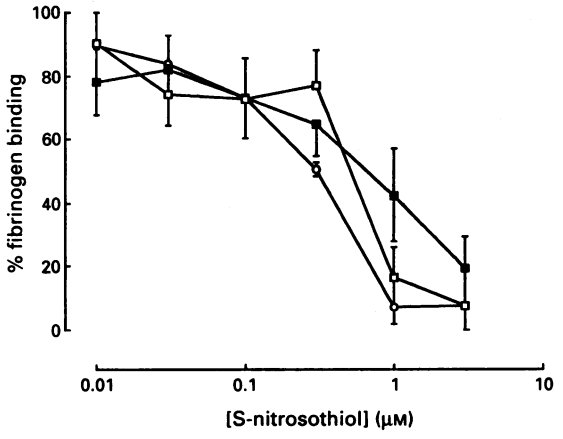


Figure 4 Inhibition by S-nitrosothiols of ADP (50 μM)-induced [^{125}I]-fibrinogen binding to washed platelets, for which B_{max} is approx. 4000 c.p.m./10^6 platelets and K_D = 13.3 ± 2.0 μg ml^{-1} fibrinogen ($n = 3$). SNAP (□), SNPL (○) and SNFP (■) were equipotent in inhibiting fibrinogen binding to platelets. Data are mean ± s.e.mean, $n = 3$.

Table 2 Correlation between intraplatelet cyclic GMP (cGMP) concentrations and inhibition of [^{125}I]-fibrinogen binding to washed platelets (WP)

R-SNO ($n = 3–7$)	*Half maximal stimulation of intraplatelet cGMP* (EC_{50}, μM)	*Inhibition of [^{125}I]-fibrinogen binding* (IC_{50}, μM)	*Correlation between cGMP and fibrinogen binding* (r)
SNFP	0.35	0.64 ± 0.13	0.88
SNPL	0.29	0.34 ± 0.07	0.95
SNAP	0.40	0.34 ± 0.07	0.97

There was a significant correlation between inhibition of fibrinogen binding and increases in cyclic GMP concentrations induced by each of the S-nitrosothiols (R-SNO).

NO from this compound was detected at concentrations as low as 0.1 μM. However, the release of NO from SNAP and SNFP was not detected at concentrations lower than 10 μM.

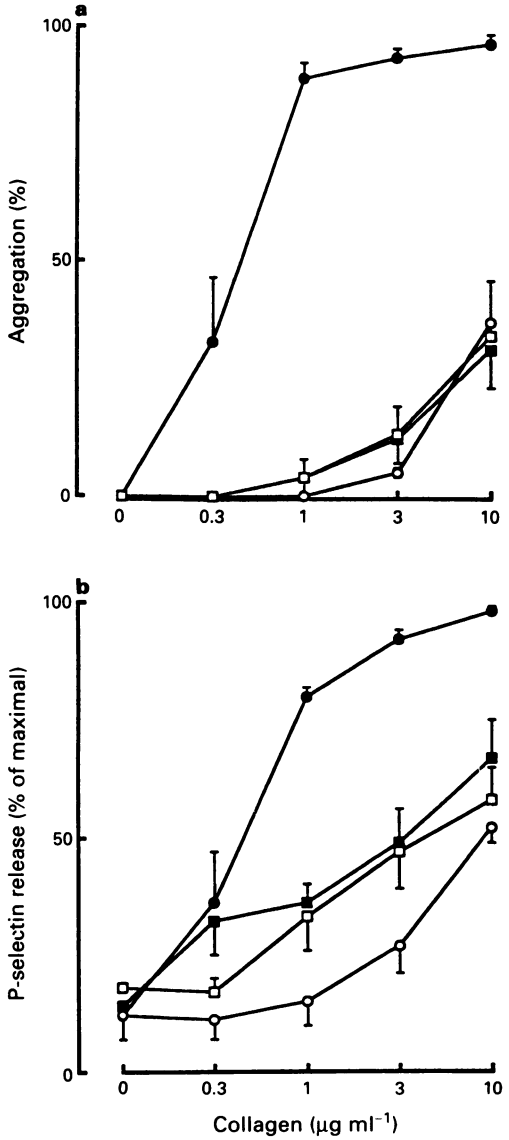


Figure 5 Inhibition by SNAP and its analogues of collagen (0.3–10 μg ml^{-1})-induced platelet aggregation (a) and P-selectin release (b) from washed platelets. The concentration (3 or 10 μM) of SNAP (□), SNPL (○) and SNFP (■) was selected in each experiment in order to achieve maximal (90–95%) inhibitory effect on the aggregation induced by 3 μg ml^{-1} of collagen (●). Data are mean ± s.e.mean, $n = 3$–4.

Table 3 Formation of NO from S-nitrosothiols (R-SNO)

R-SNO	*NO release* (pmol min^{-1})		
(μM, $n = 4$)	*SNPL*	*SNFP*	*SNAP*
0.01	ND	ND	ND
0.1	1.29 ± 0.02	ND	ND
1.0	52.34 ± 1.23	ND	ND
3.0	253.70 ± 2.73	ND	ND
10.0	743.00 ± 5.60	2.15 ± 0.02	5.15 ± 0.64

ND: not detectable (<0.1 pmol min^{-1}).
SNPL (0.1–10 μM) resulted in the release of NO.
The release of NO from SNAP and SNFP was detected only at concentrations higher than 3 μM. Nitric oxide was detected spectrophotometrically by monitoring the rate of NO-induced conversion of oxyhaemoglobin to methaemoglobin.

The addition of platelet lysate, intact platelets, platelet cytosol, reduced glutathione, ascorbic acid or superoxide dismutase did not increase the rate of NO release from these compounds at concentrations up to 10 μM ($n = 3$, data not shown).

Discussion

We have shown that the analogues of SNAP, SNFP and SNPL, are inhibitors of platelet aggregation and the release reaction induced by collagen. Thus, modification of the chemical structure of SNAP resulted in S-nitrosothiols capable of inhibiting platelet function *in vitro*. The potency of these S-nitrosothiols was comparable to that of NO, since their anti-aggregating and disaggregating effects were detected at submicromolar concentrations. However, in contrast to NO (Radomski *et al.*, 1987), their anti-aggregating activity was stable for at least 60 min when incubated at 37°C. The order of stability was SNAP = SNFP > SNPL, indicating that the presence of formyl or acetyl groups results in stabilization of the pharmacological properties of these molecules. Platelet aggregation induced by ADP in plasma depends on the exposure of the fibrinogen receptor (GP IIb/IIIa) and formation of fibrinogen bridges between platelets (Bennet & Vilaire, 1979; Marguerie *et al.*, 1979; Nachman *et al.*, 1984). Since S-nitrosothiols proved to be effective inducers of disaggregation of ADP-aggregated platelets, we examined the effect of these compounds on fibrinogen binding to platelets. SNAP, SNFP and SNPL inhibited fibrinogen binding to platelets at concentrations that also inhibited platelet aggregation and caused disaggregation. Moreover, these actions correlated well with the formation of cyclic GMP elicited by these compounds. Similar observations have been published for S-nitrosoacetylcysteine by Mendelsohn and colleagues (1990). Thus, it is likely that the platelet-inhibitory activity of S-nitrosothiols depends on cyclic GMP-mediated inhibition of fibrinogen binding to platelets. Since S-nitrosothiols (≤ 10 μM) did not increase intraplatelet cyclic AMP concentrations, it is unlikely that the pharmacological effects of SNAP and its analogues were mediated via inhibition by cyclic GMP of low K_M cyclic AMP phosphodiesterase and subsequent increase in cyclic AMP concentrations (Maurice & Haslam, 1990). The mechanism by which cyclic GMP inhibits fibrinogen binding is unclear. It has been suggested that increases in cyclic GMP lead to the phosphorylation of a number of platelet proteins including a 46-kDa vasodilator-stimulated phosphoprotein (VASP). This may result in inhibition of phosphatidylinositol turnover, protein kinase C activity and the exposure of the fibrinogen receptor (Takai *et al.*, 1981; Mellion *et al.*, 1983; Halbrugge *et al.*, 1990).

We have also shown that these S-nitrosothiols are effective inhibitors of the release of P-selectin from platelets. P-selectin is a glycoprotein first identified in the platelet α-granules (Stenberg *et al.*, 1985) that mediates the interactions between platelets, leukocytes and endothelium (for review, see Bevilacqua & Nelson, 1993). When platelets are stimulated by aggregating agents such as collagen, P-selectin is rapidly selected to the plasma membrane to promote adhesion of cells to vascular endothelium or to neutrophils and monocytes at the site of tissue injury (Johnston *et al.*, 1989; Larsen *et al.*, 1989; Hamburger & McEver, 1990; Geng *et al.*, 1990; Palabrica *et al.*, 1992). Thus, if endogenous NO is a regulator of P-selectin expression, our results provide an explanation for the finding that inhibitors of NO synthase *in vivo* promote the formation of platelet-leukocyte aggregates (Kurose *et al.*, 1993). As was the case with fibrinogen binding, the inhibition of P-selectin release correlated with the increase in platelet cyclic GMP concentrations. Although SNPL appeared more effective than PGI_2 as an inhibitor of P-selectin release, full concentration-response curves will be required to substantiate this point. This is specially interesting in view of the suggestion that elevations of cyclic GMP may be more

significant than increases in cyclic AMP as a mechanism for the regulation of interaction between cells (Radomski *et al.*, 1987).

The release of NO, with the subsequent NO-mediated increase in cyclic GMP, is considered necessary for the pharmacological activity of S-nitrosothiols (Feelisch & Noack, 1987). This release can be spontaneous, stimulated by some thiols, or may require enzymatic activity, as is the case with S-nitrosoglutathione (Feelisch & Noack, 1987; Kowaluk & Fung, 1990; Radomski *et al.*, 1992). In our experiments there was a good correlation between NO release from SNPL and the pharmacological and biochemical effects of this S-nitrosothiol. However, using the oxyhaemoglobin assay, we failed to detect NO release from SNAP and SNFP at concentrations ($<10\,\mu$M) that produced both an anti-platelet effect and stimulation of the soluble guanylate cyclase. The release could not be detected even in the presence of intact platelets, platelet fractions or reducing agents known to enhance the rate of NO release from other NO donors (Feelisch & Noack, 1987; Radomski *et al.*, 1992). It is important to note that higher concentrations of these S-nitrosothiols ($\geqslant 10\,\mu$M) did result in the release of detectable amounts of NO.

Thus, SNPL differs from SNAP and SNFP because it: (a) releases NO at lower concentrations, (b) is less stable, (c) is more potent as an inducer of platelet disaggregation, and (d) is less potent as an inhibitor of collagen-induced aggregation and ATP release from platelets. In other respects, i.e. stimulation of the soluble guanylate cyclase, inhibition of fibrinogen binding to platelets and inhibition of P-selectin release, all three S-nitrosothiols are equipotent. These results suggest that the release of NO from SNAP and SNFP occurs inside the platelet while SNPL releases NO spontaneously. Spontaneous release of NO from SNPL may explain its increased potency as an inducer of disaggregation, since a freely diffusible molecule such as NO will probably reach and exert its action on most of the aggregated platelets, whereas SNFP and SNAP would have a more restricted access. This might indeed be the case, since it has been suggested that SNAP requires activation of glyceraldehyde-3-phosphate dehydrogenase for the transport of NO in platelets (McDonald *et al.*, 1993). Alternatively, if SNAP and SNFP stimulate the soluble guanylate cyclase directly, the release of NO would not be a necessary prerequisite for their pharmacological action.

In summary, the analogues of SNAP, SNPL and SNFP, although different from each other in their chemical stability and rate of NO release, are potent stimulators of the soluble guanylate cyclase and inhibitors of platelet function.

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Electrophysiological evidence for different release mechanism of ATP and NO as inhibitory NANC transmitters in guinea-pig colon

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1 The effect of the P_2-purinoceptor antagonist, suramin, the specific N-type voltage-dependent calcium channel blocker, ω-conotoxin GVIA (ω-CgTx) and the δ-opioid receptor agonist [D-Pen^2,D-Pen^5] enkephalin (DPDPE) on the apamin-sensitive and apamin-resistant inhibitory junction potentials (i.j.ps) produced by electrical field stimulation (EFS) were investigated by means of a sucrose-gap technique in the circular muscle of the guinea-pig colon.

2 After incubation of muscle strips in either atropine (1 μM), guanethidine (3 μM) and N^G-nitro-L-arginine (L-NOARG, 30 μM) or atropine, guanethidine and apamin (0.3 μM), the addition of the NK_1 receptor antagonist, SR 140,333 (1 μM) abolished the non-adrenergic, non-cholinergic (NANC) excitatory junction potential (e.j.p.) and unmasked a pure apamin-sensitive i.j.p. (in the presence of L-NOARG) or a pure apamin-resistant i.j.p. (in the presence of apamin). Both types of i.j.p. were abolished by tetrodotoxin.

3 Suramin (30–300 μM) concentration-dependently inhibited the apamin-sensitive i.j.p., while the apamin-resistant i.j.p. was not significantly affected by suramin (up to 300 μM). L-NOARG (30 μM) markedly reduced the apamin-resistant i.j.p.

4 The δ-opioid receptor agonist, DPDPE (0.03–3 μM) concentration-dependently reduced the apamin-sensitive i.j.p., while leaving the apamin-resistant i.j.p. unaffected. Naloxone (1 μM) prevented the i.j.p. inhibition evoked by DPDPE (0.3 μM).

5 ω-CgTx (0.3 μM) markedly reduced the apamin-sensitive but not the apamin-resistant i.j.p. The application of DPDPE (3 μM), after development of a steady state inhibitory effect by ω-CgTx, evoked further inhibition of the apamin-sensitive i.j.p., similar to the effect produced by DPDPE alone. The L-type calcium channel blocker, nifedipine (1 μM) did not significantly affect either the apamin-sensitive or the apamin-resistant i.j.ps.

6 These findings support the purinergic origin of the fast, apamin-sensitive i.j.p. produced by EFS in the circular muscle of the guinea-pig colon and strongly suggest that the apamin-sensitive and the apamin-resistant components of the evoked i.j.p. utilize different mechanisms for the secretion of the NANC transmitters, ATP and NO, respectively.

Keywords: Non-adrenergic, non-cholinergic transmission; apamin; inhibitory junction potentials; suramin; nitric oxide (NO); ω-conotoxin; δ-opioid receptor agonist; [D-Pen^2,D-Pen^5] enkephalin

Introduction

At present, it appears established that more than one transmitter is responsible for non-adrenergic, non-cholinergic (NANC) inhibition in the gut. There is considerable evidence that adenosine triphosphate (ATP; Burnstock, 1981; Vladimirova & Shuba, 1984; Zagorodnyuk & Shuba, 1986; Crist *et al.*, 1992), vasoactive intestinal polypeptide (VIP; Grider & Rivier, 1990; Crist *et al.*, 1992) and nitric oxide (NO, Boeckxstaens *et al.*, 1990; for review see Sanders & Ward, 1992) are involved as transmitters in NANC inhibitory neural responses to the intestinal smooth muscle from different animal species and man. Previously, we have shown that at least three inhibitory mechanisms are involved in the NANC inhibitory junction potential (i.j.p.) and relaxation in the circular muscle of the guinea-pig colon: (i) apamin-sensitive, (ii) N^G-nitro-L-arginine (L-NOARG)-sensitive and (iii) apamin- and L-NOARG-resistant (Maggi & Giuliani, 1993; Zagorodnyuk *et al.*, 1993). For the apamin-sensitive phase of i.j.p., ATP was suggested as a likely mediator in the guinea-pig and human colon (Vladimirova & Shuba, 1984; Zagorodnyuk & Shuba, 1986; Zagorodnyuk *et al.*, 1989). The L-NOARG-sensitive phase of i.j.p. and relaxation of the smooth muscle of the guinea-pig colon implies a possible role of NO or NO-generating substance(s) as inhibitory transmitter (Maggi & Giuliani, 1993; Zagorodnyuk *et al.*, 1993).

The aims of this study were two fold: first, we investigated the effect of suramin, a selective P_2-purinoceptor antagonist (Den Hertog *et al.*, 1989; Hoyle *et al.*, 1990) on the apamin-sensitive and apamin-resistant i.j.p. in order to verify the 'purinergic hypothesis' for the apamin-sensitive part of NANC inhibitory innervation of the circular muscle of guinea-pig colon. Second, we aimed to assess whether two agents known to interfere with transmitter release at autonomic neuroeffector junctions, the peptide blocker of N-type calcium channels ω-conotoxin (ω-CgTx, Miller, 1987; Maggi *et al.*, 1988) and the δ-opioid receptor-selective agonist [D-Pen^2,D-Pen^5] enkephalin (DPDPE, Kosterlitz, 1982; Hoyle *et al.*, 1990a) might exert a differential inhibition on the apamin-sensitive and apamin-resistant i.j.ps in the circular muscle of the guinea-pig colon.

Methods

A single sucrose-gap, modified as described by Artemenko *et al.* (1982) and Hoyle (1987) was used to investigate simultaneously changes in the membrane potential and contractile activity (load 1–2 mN) of the smooth muscle of guinea-pig

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proximal colon to electrical field stimulation (EFS). The sucrose-gap method was the same as described in detail previously (Zagorodnyuk *et al.*, 1993). Briefly, circular muscle strips approximately 0.5–0.8 mm wide and 10 mm long were superfused with oxygenated (95% O_2 and 5% CO_2) Krebs solution (35 ± 0.5°C) at a rate of 1 ml min^{-1}. The composition of Krebs solution (mM) was as follows: NaCl 119, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.5, KCl 4.7, $CaCl_2$ 2.5 and glucose 11.

Junction potentials were evoked by submaximal electrical field stimulation (EFS, 10 Hz for 0.5 s, i.e. six stimuli) with 40 V stimulus strength and 0.2 ms pulse width.

When studying the effect of suramin, ω-CgTx and DPDPE, the smooth muscle strips were incubated for at least 2 h in Krebs solution containing either atropine (1 μM), guanethidine (3 μM), L-NOARG (30 μM) and SR 140,333 (1 μM), or atropine (1 μM), guanethidine (3 μM), apamin (0.3 μM) and SR 140,333 (1 μM), respectively, for studying their effect on the apamin-sensitive or the apamin-resistant i.j.p., respectively. The tachykinin NK_1 receptor antagonist, SR 140,333 (Emonds-Alt *et al.*, 1993) was present in most experiments to abolish the NANC excitatory junction potential (e.j.p.) which interfered with NANC i.j.p. (see results).

For both types of i.j.ps (apamin-sensitive and apamin-resistant), the following parameters were evaluated: latency, amplitude, duration, time to peak (t_1) and time of recovery from peak to baseline (t_2).

Statistical analysis

All data in the text are mean ± standard error of the mean (s.e.mean). Statistical analysis was performed by means of Student's *t* test for paired or unpaired data, or by means of analysis of variance, when applicable. A P level <0.05 was considered statistically significant.

Drugs

Drugs used were: atropine HCl (Serva, Heidelberg, Germany); guanethidine sulphate (ICF); N^G-nitro-L-arginine (L-NOARG), apamin, [D-Pen^2,D-Pen^5] enkephalin (DPDPE) and naloxone (Sigma); ω-conotoxin GVIA (ω-CgTx, Peninsula). SR 140,333 or (S)-1-{2-[3-(3,4-dichlorophenyl)-1-3(3-isopropoxyphenylacetyl) piperidin-n-3-yl] ethyl)-4phenyl-1-azoniabicyclo[2,2,2]octane chloride was a kind gift from Dr X. Emonds-Alt, Sanofi Research, Montpellier, France. Suramin was a kind gift of Prof. M. Costa, Dept. of Human Physiology, Flinders University, Bedford Pk, SA, Australia.

Results

General

In the presence of atropine (1 μM) and guanethidine (3 μM), EFS (10 Hz, 40 V, 0.2 ms for 0.5 s) evoked a composite i.j.p. and relaxation of the circular muscle of the guinea-pig colon (Figure 1). In most experiments (e.g. Figure 1), the two components of the i.j.p. are not easily resolved from each other but, after addition of apamin or L-NOARG, the observed changes in latency, amplitude, duration, time to peak (t_1) and time of recovery to baseline (t_2) (Table 1; Zagorodnyuk *et al.*, 1993) clearly indicate that apamin (0.3 μM) blocked the first fast phase of i.j.p. while leaving the slow phase unaffected. On the contrary, L-NOARG (30 μM) inhibited the second, slow phase, of i.j.p. leaving the early, apamin-sensitive, phase unaffected (Figure 1). Under the present experimental conditions, apamin had little effect on the amplitude of the evoked NANC relaxation which was largely inhibited or suppressed by L-NOARG (Figure 1).

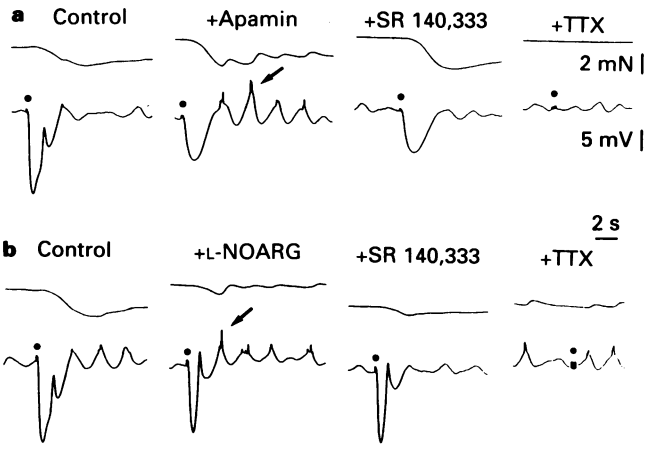


Figure 1 Typical tracings showing the effects of apamin (0.3 μM), N^G-nitro-L-arginine (L-NOARG, 30 μM), SR 140,333 (1 μM) and tetrodotoxin (TTX, 0.3 μM) on the junction potentials and changes in tension evoked by electrical field stimulation (EFS, 10 Hz, 0.5 s, 40 V, 0.2 ms, applied at dots) in the circular muscle from guinea-pig proximal colon. The effect of apamin and L-NOARG is shown after 20 min of action; the effect of SR 140,333 (1 μM) applied in apamin- or L-NOARG-containing Krebs solution is shown at 40 min of action; TTX (0.3 μM for 10 min) blocked i.j.p.s evoked by EFS in apamin- or L-NOARG-containing Krebs solution. Upper tracing is mechanical activity, lower tracing membrane potential. Arrows indicate e.j.p.s.

In the presence of either apamin or L-NOARG a NANC e.j.p. followed by spikes was evident in the response to EFS (arrows in Figure 1) which interferred with the evaluation of drug action on the apamin-sensitive or apamin-resistant i.j.p. Since we showed previously that activation of tachykinin NK_1 receptors mediates the NANC e.j.p. in this preparation, we used the NK_1 receptor antagonist, SR 140,333 (Emonds-Alt *et al.*, 1993) to eliminate this component of the response to EFS. The addition of SR 140,333 (1 μM) to the Krebs solution containing apamin or L-NOARG abolished the NANC e.j.p. and enabled study of a pure NANC apamin-resistant or apamin-sensitive i.j.p. (Figure 1). The electrophysiological parameters of these i.j.ps are summarized in Table 1. Both the apamin-sensitive and apamin-resistant i.j.ps

Table 1 Electrophysiological parameters of the apamin-sensitive and apamin-resistant inhibitory junction potential (i.j.p.) evoked by electrical field stimulation (10 Hz, 40 V, 0.2 ms pulse width for 0.5 s) in the circular muscle of the guinea-pig colon

	Latency (ms)	*Amplitude* (mV)	*Duration* (ms)	t_1 (ms)	t_2 (ms)
Apamin-sensitive i.j.p.	192 ± 3	11.8 ± 0.6	1161 ± 56	497 ± 6	664 ± 54
Apamin-resistant i.j.p.	292 ± 10	8.0 ± 0.7	2660 ± 201	1153 ± 53	1529 ± 166

Each value is mean ± s.e.mean of 26–27 experiments for the apamin-sensitive i.j.p. and 14–15 experiments for the apamin-resistant i.j.p.
All values of the apamin-sensitive i.j.p. are significantly different from the corresponding values of the apamin-resistant i.j.p., $P<0.05$.
t_1 = time to peak; t_2 = time of recovery to baseline.

were abolished by 10 min application of tetrodotoxin (TTX, 0.3 μM, $n = 4$) (Figure 1).

Effects of suramin on the apamin-sensitive and apamin-resistant i.j.ps

Application of suramin (30–300 μM) in L-NOARG- or apamin-containing Krebs solution produced a decrease in tone of the muscle strips with no significant changes in membrane potential. Suramin (30–300 μM) concentration-dependently inhibited the apamin-sensitive i.j.p. (Figure 2 and Figure 4). The inhibition developed slowly and 30–40 min superfusion with suramin was required to reach a steady state. The maximal effect of suramin (300 μM) was 67.7 ± 4.3% ($n = 3$) inhibition of the control response. The latency of the i.j.p. was slightly but significantly increased by suramin being 205 ± 3.4 ms ($n = 6$) and 247 ± 12 ms ($n = 6$, $P < 0.05$) before and after suramin (100–300 μM). The effects produced by the two concentrations of suramin on this parameter were comparable and data were pooled for statistical analysis.

Suramin, at the maximal concentration tested (300 μM) did not affect significantly the amplitude of the apamin-resistant i.j.p. during 40 min of application (Figure 2). At the same time, the apamin-resistant i.j.p. was inhibited by L-NOARG (30 μM) by 62 ± 3% ($n = 3$).

Effects of DPDPE and ω-CgTx on the apamin-sensitive and apamin-resistant i.j.ps

The δ-opioid receptor agonist, DPDPE (3 μM) did not produce a significant change in potential of the smooth muscle membrane. At the same time, DPDPE (0.03–3 μM) concentration-dependently inhibited the apamin-sensitive i.j.p. (Figure 3 and Figure 4). The maximum inhibitory effect (60 ± 5%, $n = 6$) was observed with 3 μM DPDPE at 3–7 min of application. The i.j.p. inhibition by DPDPE was not complete, leaving about 40% of the i.j.p. amplitude unaffected. Naloxone (1 μM) prevented the i.j.p. inhibition caused by DPDPE (0.3 μM): DPDPE produced 45 ± 0.9% and 9 ± 0.9% inhibition of i.j.p. before and after naloxone, respectively ($n = 4$, $P < 0.05$).

At the highest concentration tested (3 μM), DPDPE did not significantly affect the amplitude of the apamin-resistant i.j.p. (Figure 3).

The application of ω-CgTx (0.3 μM) in apamin- or L-NOARG-containing Krebs solution did not evoke any significant change in membrane potential of the circular smooth muscle. ω-CgTx (0.3 μM) inhibited the amplitude of the apamin-sensitive i.j.p. by 64.1 ± 3.9% ($n = 7$) (Figure 5). The inhibitory effect developed slowly and reached a steady state within 20 min. The application of DPDPE (3 μM) after development of a steady-state inhibitory effect of ω-CgTx,

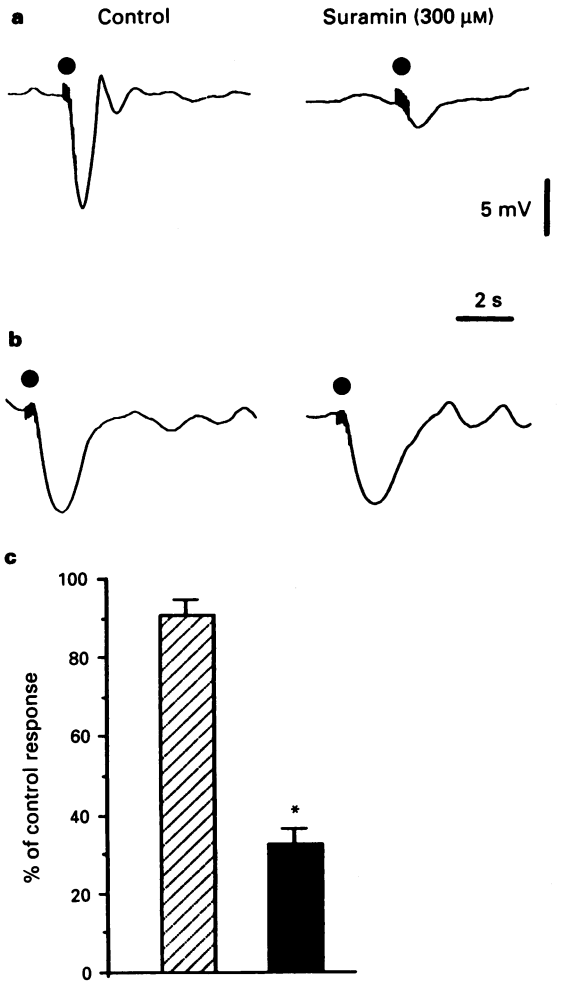


Figure 2 Effect of suramin (300 μM for 40 min) on the apamin-sensitive (a) and apamin-resistant (b) i.j.ps evoked by electrical field stimulation in the circular muscle of the guinea-pig colon; (c) shows the effect of suramin (300 μM) on the amplitude of the apamin-sensitive i.j.p. (solid column) and apamin-resistant i.j.ps (hatched column). Each value is mean ± s.e.mean of three experiments. *Significantly different from control values: $P < 0.05$.

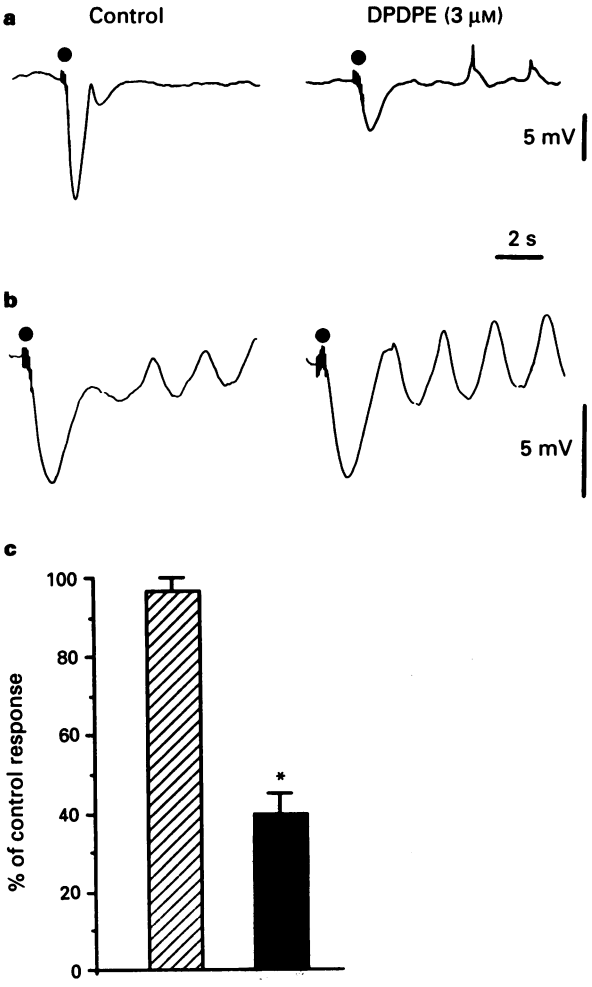


Figure 3 Effect of [D-Pen^2,D-Pen^5]enkephalin (DPDPE, 3 μM for 7 min) on the apamin-sensitive (a) and apamin-resistant (b) i.j.ps evoked by EFS in the circular muscle of the guinea-pig colon; (c) shows the effect of DPDPE (3 μM) on the amplitude of the apamin-sensitive i.j.p. (solid column) and apamin-resistant i.j.ps (hatched column). Each value is mean ± s.e.mean of 4–6 experiments. *Significantly different from control values; $P < 0.05$.

evoked a further inhibition of the apamin-sensitive i.j.p. by 65 ± 11% ($n = 4$), quantitatively similar to the inhibitory effect produced by DPDPE (3 μM) in normal Krebs solution. DPDPE (3 μM) or ω-CgTx (0.3 μM) alone did not change the latency of the apamin-sensitive i.j.p. but, when the two drugs were applied together, the latency of the i.j.p. was about twice (465 ± 54 ms, $n = 4$) that observed in controls (220 ± 20 ms, $n = 4$).

The application of ω-CgTx (0.3 μM for 30 min) did not affect significantly the amplitude of the apamin-resistant i.j.p. (Figure 5).

Nifedipine (1 μM for 30 min) did not significantly affect the amplitude of the apamin-sensitive or apamin-resistant i.j.ps which, in the presence of nifedipine, averaged 93 ± 1.3% and 103 ± 1.2% of the control response, respectively ($n = 3$ each, $P > 0.05$).

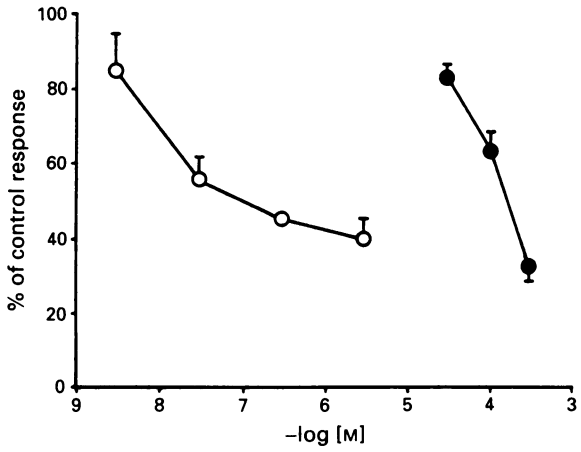


Figure 4 Concentration-dependent inhibition by [D-Pen^2,D-Pen^5] enkephalin (DPDPE, ○) and suramin (●) of the apamin-sensitive i.j.p. evoked by electrical field stimulation in the circular muscle of the guinea-pig colon. Each value is mean ± s.e.mean of 3–6 experiments.

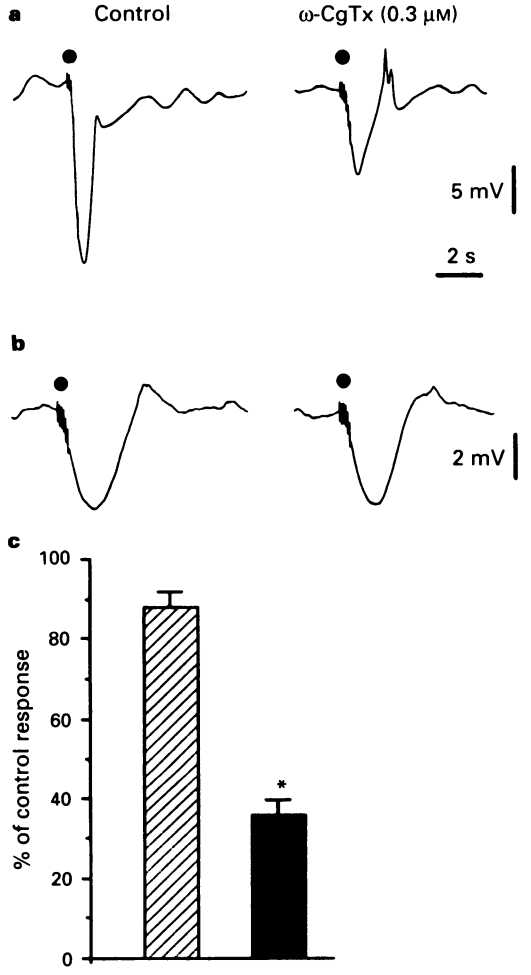


Figure 5 Effect of ω-conotoxin (ω-CgTx, 0.3 μM for 20 min) on the apamin-sensitive (a) and apamin-resistant (b) i.j.ps evoked by electrical field stimulation in the circular muscle of the guinea-pig colon; (c) shows the effect of ω-CgTx (0.3 μM) on the amplitude of the apamin-sensitive i.j.p. (solid column) and apamin-resistant i.j.ps (hatched column). Each value is mean ± s.e.mean of 3–7 experiments. *Significantly different from control values; $P < 0.05$.

Discussion

ATP and NO as mediators of the NANC i.j.p.

In agreement with the findings of a previous study using other antagonists (Zagorodnyuk *et al.*, 1993), we found that the potent tachykinin NK_1 receptor antagonist, SR 140,333 (Emonds-Alt *et al.*, 1993) eliminates the NANC e.j.p. produced by EFS in the circular muscle of the guinea-pig colon. After blockade of tachykinin NK_1 receptors, a pure i.j.p. can be evoked in this preparation: the response is biphasic and two further components can be discerned by the use of apamin which blocks the fast phase of i.j.p., and of L-NOARG which inhibits the second component.

Following desensitization of P_2 purinoceptors by means of stable ATP analogues (Zagorodnyuk & Shuba, 1986; Crist *et al.*, 1992) or by using the purinoceptor antagonists, reactive blue 2 or suramin (Den Hertog *et al.*, 1989; Hoyle *et al.*, 1990b; Crist *et al.*, 1992; Ohno *et al.*, 1993), the proposal has been advanced that ATP is responsible for the fast, apamin-sensitive i.j.p. and part of NANC relaxation in various regions of the guinea-pig intestine (stomach, ileum and taenia coli). This proposal is further supported by the present findings showing that suramin selectively inhibited the fast, apamin-sensitive i.j.p. while leaving unaffected the apamin-resistant response.

It is important to note that, in the present experimental conditions, the apamin-sensitive i.j.p. does not appear to contribute significantly to the evoked relaxation which was almost abolished by L-NOARG instead. Since the aim of this study was to investigate a differential pharmacological modulation of the two components of i.j.p., and L-NOARG alone almost abolished the evoked NANC relaxation, we did not systematically investigate the effect of drugs on this parameter.

The slow i.j.p. evoked by EFS in the presence of apamin was inhibited by the NO-synthase inhibitor, L-NOARG, implying a role of NO, a NO-like, or a NO-generating transmitter(s) in its genesis (cf. Maggi & Giuliani, 1993; Zagorodnyuk *et al.*, 1993). NO-synthase has been shown to be expressed in nerves (Bredt *et al.*, 1990), including neuronal elements of the myenteric plexus of the guinea-pig ileum, where NO synthase-like immunoreactivity co-localizes with vasoactive intestinal polypeptide (VIP) (Costa *et al.*, 1992). Although the application of NO-generating agents, like sodium nitroprusside, produces hyperpolarization and relaxation of the circular muscle of the guinea-pig colon (data not shown), it is as yet uncertain whether the L-NOARG-sensitive i.j.p. and relaxation is produced by NO generated within nerves diffusing to produce its effect in smooth muscle cells. In fact, L-NOARG has been reported to inhibit partially the action of VIP in some intestinal preparations (guinea-pig stomach and ileum, rat stomach) (Li & Rand, 1990; Grider *et al.*, 1992; He & Goyal, 1993). Thus the hypothesis was advanced that VIP released from nerves may induce NO generation in smooth muscle as a contributing and amplifying mechanism to the VIP-induced relaxation (Grider *et al.*, 1992). On the other hand, the action of VIP is unaffected by L-NOARG in certain intestinal preparations (guinea-pig taenia coli, dog

intestine etc.) (Boeckstaens *et al.*, 1990; Sanders & Ward, 1992; Grider *et al.*, 1992) and it is likely that, in the latter, both VIP and NO are released from nerves to produce NANC relaxation via independent mechanisms. It is possible that the circular muscle of the guinea-pig colon belongs to this second group of preparations since preliminary experiments have shown that L-NOARG (30–100 μM) barely affects the relaxation produced by exogenous VIP at this level (Maggi & Zagorodnyuk, unpublished data).

The relatively short latency (about 200 ms) of the slow, L-NOARG-sensitive i.j.p. also points to a non-peptidergic type of transmission since longer latencies have been shown (Zagorodnyuk *et al.*, 1993; Santicioli & Maggi, unpublished data) to be required for peptidergic neuromuscular transmission. We will therefore discuss the effect of ω-CgTx and DPDPE on the apamin-sensitive and apamin-resistant i.j.p. on the assumption that these two junctional events are mediated by ATP and NO, respectively.

Differential modulation of ATP and NO release in guinea-pig colon

The present findings demonstrate that the peptide blocker of N-type calcium channels, ω-CgTx and the selective δ-opioid receptor agonist, DPDPE, both exert a selective inhibitory action on the ATP-mediated, apamin-sensitive i.j.p., without affecting the NO-mediated, apamin-resistant response to EFS.

ω-CgTx has been repeatedly shown to produce a prejunctional blockade of transmitter release at various neuroeffector autonomic junctions in mammals, without any evidence for significant postjunctional effects (Maggi *et al.*, 1988; De Luca *et al.*, 1990; Boeckstaens *et al.*, 1993). The inhibition by ω-CgTx of the ATP-mediated i.j.p. was partial and this may indicate that, in addition to N-type calcium channels, other types of voltage-sensitive calcium channels are involved in ATP release in this preparation. Marino *et al.* (1993) found that both ω-CgTx-sensitive and nifedipine-sensitive calcium channels mediate acetylcholine release in guinea-pig colon; however, nifedipine had no effect on the ATP- or NO-mediated i.j.p., suggesting that L-type calcium channels do not play a role in NANC inhibitory transmission at this level.

Like ω-CgTx, DPDPE produced a selective inhibition of the ATP-mediated i.j.p. For both ω-CgTx and DPDPE, the failure to affect the NO-mediated i.j.p. indicates that their inhibitory effect on the ATP-mediated i.j.p. is not due to nonspecific postjunctional effects on membrane excitability. Furthermore, both agents inhibited the ATP-mediated i.j.p. without affecting the resting membrane potential. The action of DPDPE observed here is in line with the results of previous studies showing a prejunctional modulatory influence of opioid receptor agonists on NANC inhibitory transmission in the human colon (Hoyle *et al.*, 1990a), in which the evoked i.j.p. is mainly ATP-mediated (Zagorodnyuk & Shuba, 1986).

Although opioid receptor agonists are known to reduce N-type calcium currents in neurones (Gross & McDonald, 1987; Akins & McCleskey, 1993) the inhibitory effect of DPDPE on the ATP-mediated i.j.p. (amplitude and latency of i.j.p.) was additive to the inhibitory effect of ω-CgTx, i.e. it occurs independently of the availability of N-type channels to sustain the evoked transmitter release. Since opioid receptor agonists are also known to enhance resting and voltage-gated K currents in neurones (North *et al.*, 1987), we speculate that a similar action may underlie the action of DPDPE on the evoked ATP release in guinea-pig colon. In fact, an effect on K currents could inhibit transmitter secretion independently from the type of voltage-sensitive calcium channels used to sustain transmitter secretion.

In conclusion, the present findings lend support to the purinergic origin of the fast, apamin-sensitive i.j.p. produced by EFS in the circular muscle of the guinea-pig colon and demonstrate that the apamin-sensitive and the apamin-resistant components of the evoked i.j.p. utilize different mechanisms for secretion of the NANC transmitters, ATP and NO, respectively. Whether or not this may indicate that different subsets of enteric neurones mediate the two components of the overall response cannot be decided from the present findings.

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Adaptive changes in 5-HT_{1A} receptor-mediated hippocampal inhibition in the alert rat produced by repeated 8-OH-DPAT treatment

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1 The effect of acute and repeated treatment with 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT), a 5-HT_{1A} receptor ligand, on excitatory amino acid-mediated synaptic transmission was examined in the stratum radiatum CA1 region of the dorsal hippocampus of alert, gently restrained, rats.

2 Acute administration of 8-OH-DPAT transiently reduced the amplitude of the field excitatory postsynaptic potential (e.p.s.p.) in a dose-dependent (25–75 $\mu g\,kg^{-1}$, i.p.) manner. This effect was blocked by the postsynaptic 5-HT_{1A} receptor antagonist, MDL 73005EF (2 and 4 $mg\,kg^{-1}$, i.p.).

3 8-OH-DPAT (25 $\mu g\,kg^{-1}$, i.p.) administered daily for 7 days produced a gradual reduction in the 24 h pre-injection baseline field e.p.s.p. amplitude. The reduction reached its lowest level after 7–8 days and was transiently reversed by acute injection of MDL 73005EF (2 $mg\,kg^{-1}$, i.p.) on day 8. The field e.p.s.p. baseline amplitude recovered fully 5–8 days after cessation of drug treatment.

4 8-OH-DPAT (25 $\mu g\,kg^{-1}$, i.p.) administered daily for 7 days produced a marked reduction in acute response to 8-OH-DPAT (25 and 50 $\mu g\,kg^{-1}$, i.p.) which did not recover until between day 36 and day 80 of the study.

5 It was concluded that repeated treatment with 8-OH-DPAT produced adaptive changes which resulted in a reduction in the dynamic range of 5-HT_{1A} receptor-mediated transmission in the hippocampus.

Keywords: 5-HT (5-hydroxytryptamine, serotonin); 5-HT_{1A} receptors; 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT); hippocampus; excitatory postsynaptic potential (e.p.s.p.); repeated treatment; anxiolytic; antidepressant

Introduction

The clinical anxiolytic and antidepressant effects of partial agonists at 5-HT_{1A} receptors, such as buspirone and gepirone, only become apparent with long-term treatment (Schreiber & De Vry, 1993). In some animal behavioural models there is an enhanced anxiolytic or antidepressant effect following repeated treatment with 5-HT_{1A} receptor agonists (Giral *et al.*, 1988; Schefke *et al.*, 1989) which is consistent with these clinical findings. In contrast, in the case of agonists with relatively high intrinsic activity, tolerance can develop to their other acute behavioural effects such as hyperphagia and the 5-HT syndrome (Kennett *et al.*, 1987; Larsson *et al.*, 1990).

There is considerable controversy in the literature as to the locus of change in 5-HT_{1A} receptor-mediated behavioural effects with repeated agonist dosing. Some authors have reported that there are adaptive changes at presynaptic somatodendritic 5-HT_{1A} autoreceptors in the raphe nuclei, seen as a reduced receptor density and a decrease in 5-HT_{1A} agonist-induced inhibition of 5-HT turnover, whereas others have observed a variable or no effect (Welner *et al.*, 1989; Beer *et al.*, 1990; Bohmaker *et al.*, 1993; Sharp *et al.*, 1993; Söderpalm *et al.*, 1993). Biochemical indices of postsynaptic 5-HT_{1A} receptor binding and function have also produced apparently contradictory results (Larsson *et al.*, 1990; Schechter *et al.*, 1990; Varrault *et al.*, 1991; Newman *et al.*, 1992; van Huizen *et al.*, 1993). These variable findings are probably due in part to differences in the intrinsic activity of the agonist and dose schedule used (Bohmaker *et al.*, 1993; van Huizen *et al.*, 1993).

In electrophysiological studies, chronic treatment with partial agonists such as gepirone, ipsapirone and tandospirone has been shown to decrease 5-HT_{1A} agonist-induced autoreceptor-mediated inhibition of dorsal raphe neurone firing but failed to change postsynaptic 5-HT_{1A} receptor sensitivity in the hippocampus (Blier & de Montigny, 1987; Schechter *et al.*, 1990; Godbout *et al.*, 1991). In apparent agreement with this, previous work in this laboratory (O Connor *et al.*, 1989) has shown that there was a gradual increase in the baseline activation of postsynaptic 5-HT_{1A} receptors in the hippocampus of alert rats during repeated buspirone treatment. This most probably was due to a desensitization of raphe 5-HT_{1A} autoreceptors with a consequent increase in baseline 5-HT output to the postsynaptic receptors. The present work examined the effect of repeated treatment with a selective, high intrinsic activity 5-HT_{1A} receptor agonist, 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT, Hjorth *et al.*, 1982; Leishman *et al.*, 1994) on neurophysiological markers of pre- and postsynaptic 5-HT_{1A} receptor function in the hippocampus of alert animals (O'Connor *et al.*, 1990) using similar methods to those employed in the buspirone study. This is of particular interest since it has been speculated that agents with relatively high intrinsic activity at 5-HT_{1A} receptors may have greater potential therapeutic efficacy and possibly faster onset of action than relatively weak partial agonists such as buspirone (Ansseau *et al.*, 1993).

Methods

Animals and surgery

The procedure adopted was similar to that described previously (O'Connor *et al.*, 1989; 1990). Male Wistar rats which weighed between 180–200 g at the time of surgery were individually housed in a thermoregulated environment (19–23°C) using a 12 h light/dark cycle. Anaesthesia was induced with pentobarbitone sodium (40 $mg\,kg^{-1}$, i.p.) and

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then maintained by means of inhaled halothane (1%) mixed with 100% O_2 at a rate of 1 l min^{-1}.

Three stainless steel screws (1.5 mm diameter) were inserted into the skull via a drill hole without piercing the dura. The ground screw electrode was placed 8 mm posterior to bregma and 4 mm right of the midline while the reference screw electrode was placed 4 mm anterior to bregma and 4 mm right of the midline, over the frontal sinus. A third screw which served as an anchor was placed opposite the ground screw, 8 mm posterior to bregma and 3 mm left of the midline. The electrodes were made of teflon coated tungsten wires (50 μm inner diameter, 75 μm outer diameter). The recording electrode was inserted 3 mm posterior to bregma and 2.8 mm lateral to the midline. The bipolar stimulating electrode was inserted 4 mm posterior to bregma and 3.8 mm lateral to the midline. The dura was pierced through both holes and both the stimulating and recording electrodes were lowered into the stratum radiatum of the CA1 region of the dorsal hippocampus using electrophysiological criteria (Leung, 1979). The entire assembly was sealed and fixed to the skull with dental cement.

Further verification that the electrodes were in the CA1 region of the stratum radiatum was obtained via histological means. When the studies were complete, an overdose of pentobarbitone (1 ml of 60 mg ml^{-1}, i.p.) was administered to the animal and immediately following expiry of the animal the entire brain was removed and immersed in formalin. After a period of at least 48 h had elapsed a block section of the cortex and the hippocampal area in which the electrodes had been placed was prepared. The exact location of the electrodes was determined by light microscopy in thionen-stained sections.

Recordings from alert rats

Before commencing the experiments the animals were allowed between 7–10 days to recover from the surgery. During the recovery period they were rehearsed in the experimental procedure of light restraint in a canvas recording hammock in order to familiarize them with the recording environment. During recording a flexible cable was inserted into the rat's electrode sockets and the animal could easily leave the hammock. The animals were in good health throughout the experiment and gained weight as normal.

The extracellular dendritic population field excitatory post-synaptic potential (field e.p.s.p.) was employed as a measure of excitatory amino acid-mediated synaptic transmission in the dorsal hippocampus. A field e.p.s.p. was generated in the stratum radiatum by stimulating at low frequency (0.025 Hz) with single square wave pulses of 0.1 ms duration. The field e.p.s.p. amplitude was taken as the main indicator of excitatory synaptic transmission, as it was found that there was a direct linear correlation between field e.p.s.p. slope and amplitude. The field e.p.s.p. amplitude was taken as the voltage difference between its point of onset to the peak of the potential. The baseline field e.p.s.p. data were obtained by averaging the response over a 20 min period. The stimulation intensity was adjusted to evoke field e.p.s.ps which were 60–70% (2–3.5 mV) of the maximum amplitude obtainable, a level well below the threshold for eliciting any observable behavioural effect.

Protocols for drug treatment

In acute studies all of the compounds and control vehicles were administered i.p. immediately following measurement of the field e.p.s.p. baseline. The effect was monitored at 5 min intervals (averaging 3 sweeps per sample) until there was clear evidence of onset of recovery or, in the case of controls, a minimum, of 50 min. The peak effect was taken as the maximum reduction observed during this period. The drugs (±)-8-hydroxy-2-(di-*n*-propylamino)tetralin hydrobromide (8-OH-DPAT, Research Biochemicals Inc., Natick) and 8-[2-(2,3-dihydro-1,4-benzodioxin-2-yl-methylamino)ethyl-8-azaspiro[4,5]decane-7,9-dionemethyl sulphonate (MDL 73005EF, Marion Merrell Dow, Strasbourg) were injected in a water vehicle (1 ml kg^{-1}).

Separate acute experiments were carried out in order to determine if the doses of 8-OH-DPAT used to produce an inhibitory effect on the field e.p.s.p. also altered the core temperature. Baseline pre-injection and 30 min post-injection rectal temperatures were measured while the animal was gently manually restrained.

In the chronic study, animals were injected i.p. at the same time each day for 7 days. The 24 h baseline field e.p.s.p. amplitude readings were taken immediately prior to the daily injections on days 1, 2, 3, 5, 6 and 7, and were expressed as a percentage of the field e.p.s.p. amplitude observed on the day prior to the start of the study (day 0). On day 8, MDL 73005EF was administered (24 h after the final chronic injection). The baseline was monitored for recovery on day 12, and the response to the acute administration of 8-OH-DPAT was observed on days 15, 22, 36 and day 80 of the study.

Statistics

Values are expressed as the mean % (or % change from) pre-injection baseline field e.p.s.p. amplitude ± s.e.mean. Statistical significance of the difference between means was estimated by two-tailed paired and unpaired Student's *t* tests in the acute studies. In the chronic studies two-way analysis of variance with repeated measures was carried out followed by *post-hoc t* tests.

Results

Effect of acute administration of 8-OH-DPAT on the field e.p.s.p. amplitude and core temperature

Acute i.p. injection of 8-OH-DPAT produced a marked reduction in the field e.p.s.p. amplitude which was dose-dependent in the range 25–75 μg kg^{-1} ($n = 4$–5; Figure 1a). The effect was transient, reaching a peak at a dose of 50 μg at 20–25 min with full recovery by 60 min post-injection ($n = 4$, $P < 0.001$, compared with water injected controls, Figure 1b,c). When the dose was raised to 100 μg kg^{-1} there appeared to be an attenuation of the effect ($P < 0.05$, compared to that obtained with 75 μg kg^{-1}).

There was no change in the core temperature in animals which received 8-OH-DPAT (-0.05 ± 0.16°C 30 min after 50 μg kg^{-1}, i.p., $n = 4$ or -0.2 ± 0.04°C 30 min after 75 μg kg^{-1}, i.p., $n = 4$) when compared with water-injected controls (-0.23 ± 0.18°C 30 min after 1 ml kg^{-1}, $n = 6$, $P > 0.05$).

Pretreatment for 30 min with MDL 73005EF (2 and 4 mg kg^{-1}) reduced the inhibitory effect of 50 μg 8-OD-DPAT (2 and 4 mg kg^{-1}) on the field e.p.s.p. ($P < 0.01$ compared to 8-OH-DPAT controls, $n = 4$ per group, Figure 1d–f). When injected alone or when given in combination with a separate water injection at the doses of 2 mg kg^{-1} or 4 mg kg^{-1}, MDL 73005EF had no effect on the field e.p.s.p. amplitude ($n = 4$ per group).

Effect of repeated treatment with 8-OH-DPAT (25 μg kg^{-1}) on the 24 h baseline field e.p.s.p. amplitude

An overall significant difference was noted in the amplitude of the field e.p.s.p. between treatment groups ($F(1,13) = 129.6$, $P < 0.001$, Figure 2a,b) and treatment days ($F(1,5) = 39.8$, $P < 0.001$; from day 1 to day 8). The interaction between group and day was also significant indicating a differential effect of 8-OH-DPAT depending on day of repeated treatment ($F(1,5) = 58.2$, $P < 0.001$). A gradual decline in the 24 h baseline amplitude was observed in the 8-OH-DPAT treated rats, which began on day 2 ($P < 0.01$ compared to

the water group on day 2, $n = 6$) and reached a maximum on days 7 and 8 ($P < 0.001$ compared to the water group on days 7 and 8). On day 12, near total recovery was noted, reaching 95 ± 2% of day 0 levels ($P < 0.01$ compared to the water group on day 12). By day 15 there was no difference between 8-OH-DPAT-treated animals and the water-injected controls ($P > 0.05$; Figure 4b).

On day 8 of the chronic study, MDL 73005EF was administered at a dose (2 mg kg^{-1}, i.p., $n = 6$) that was found to antagonize the acute effect of 8-OH-DPAT. Thirty min following its administration, MDL 73005EF reversed the 24 h baseline reduction in the 8-OH-DPAT treated animals ($P < 0.001$, compared to pre-injection levels, Figure 2c). The effect was transient, and full return to the pre-junction baseline values had occurred 50 min post-injection. Injection of this dose of MDL 73005EF in the water control animals had no effect.

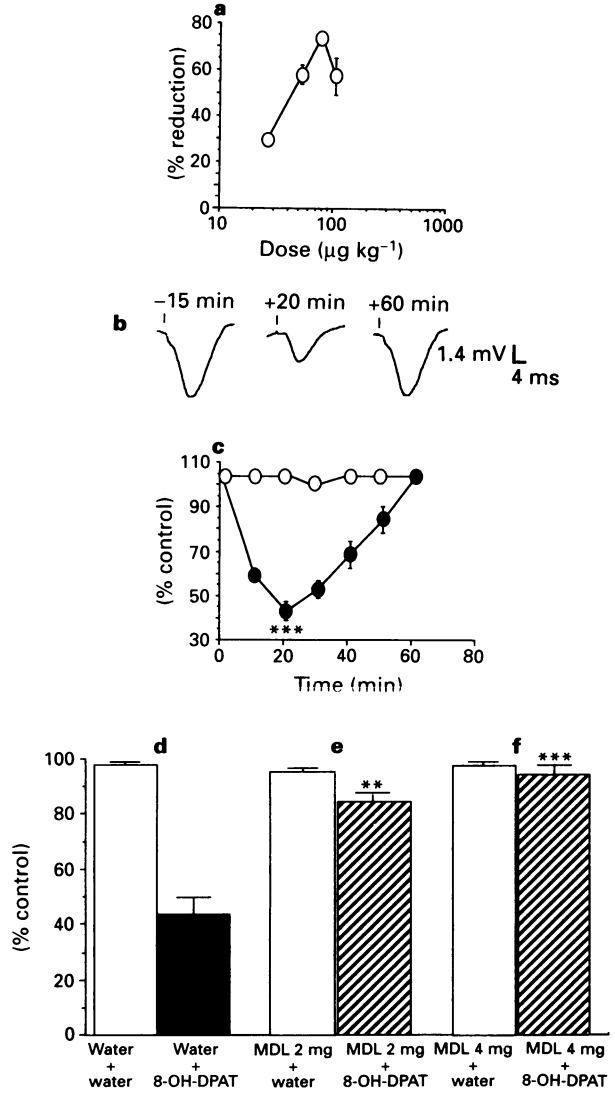


Figure 1 Acute effect of 8-OH-DPAT on hippocampal excitatory synaptic transmission in the alert rat. (a) Dose-response relationship for the inhibitory effect of systemic injection of 8-OH-DPAT (25–100 μg kg^{-1}, i.p., $n = 4$–5 per point) on the amplitude of the electrically evoked field e.p.s.p. recorded in the stratum radiatum of the CA1 region. Values are mean % reduction of the pre-injection field e.p.s.p. amplitude ± s.e.mean. (b) Effect of 8-OH-DPAT (50 μg kg^{-1}) on the field e.p.s.p. (original traces at $t = -15$, + 50 min relative to time of injection). Vertical lines indicate time of stimulus application. (c) Time course for the acute effect of 8-OH-DPAT (50 μg kg^{-1}, $n = 4$, ●) and water (1 ml kg^{-1}, $n = 4$, ○) on the field e.p.s.p. Values are the mean % pre-injection baseline control ± s.e.mean. $^{***}P < 0.001$ versus water control. (d–f) Antagonism by MDL 73005EF (MDL) of the acute effect of 8-OH-DPAT. The acute effect of 8-OH-DPAT (50 μg kg^{-1}, i.p.) on the amplitude of the field e.p.s.p. after pre-treatment with (d) water (solid column, $n = 4$), (e) MDL 73005EF (2 mg kg^{-1}, $n = 4$, hatched column) or (f) MDL 73005EF (4 mg kg^{-1}, $n = 4$, hatched column). Values are the mean % pre-injection baseline control field e.p.s.p. amplitude. $^{**}P < 0.01$ compared with the Water + 8-OH-DPAT group.

Acute response to 8-OH-DPAT in the chronically treated animals

There was a parallel decrease in the acute response to the daily dose of 8-OH-DPAT at the same time as there was a gradual reduction in the 24 h baseline field e.p.s.p. amplitude (Figure 3a). Whereas the field e.p.s.p. reduction from baseline on day 1 was 32 ± 2%, on day 3, 5 and 7 it was 19 ± 1% ($P < 0.01$, $n = 6$), 12 ± 2% ($P < 0.001$, $n = 6$, compared to day 1) and 1 ± 1% ($P < 0.001$, $n = 6$) respectively.

On day 15, 8 days after the final chronic 8-OH-DPAT injection, acute treatment with 50 μg kg^{-1} 8-OH-DPAT pro-

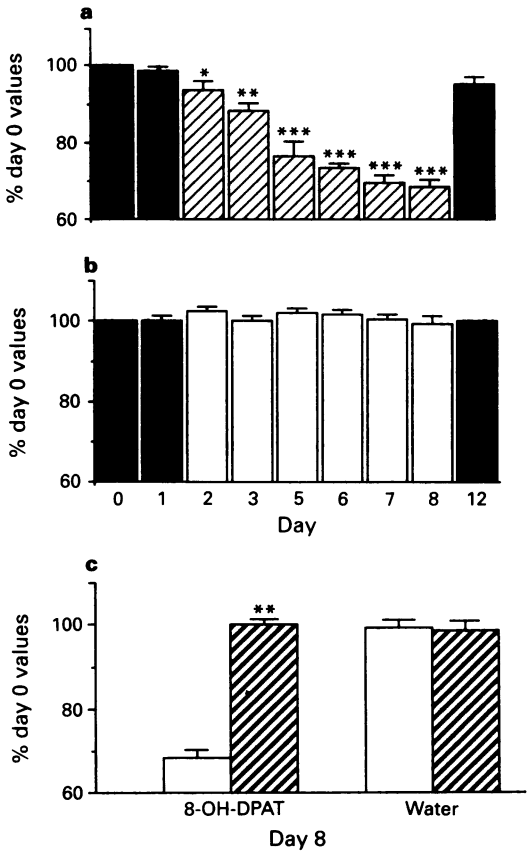


Figure 2 (a,b) The effect of repeated treatment with 8-OH-DPAT on hippocampal excitatory transmission in the alert rat. The amplitude of the field e.p.s.p. was measured 24 h after daily injection on days 1–7 of either (a) 8-OH-DPAT (25 μg kg^{-1}, i.p., $n = 6$; hatched columns) or (b) water ($n = 9$; open columns). Readings were also taken on days 0, 1 and 12 (solid columns) but no injection was given on the previous day. Values are the mean percentage of the day 0 baseline field e.p.s.p. amplitude ± s.e.mean. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to water controls on the same day. (c) MDL 73005EF reversal of the chronic 8-OH-DPAT induced reduction of hippocampal excitatory synaptic transmission in the alert rat. The effect of acute injection of MDL 73005EF (2 mg kg^{-1}, i.p., $n = 6$ per group) on day 8 in animals which had received 8-OH-DPAT (25 μg kg^{-1}, i.p.) or water injections for 7 days (open column: pre-MDL 73005EF, hatched columns: 30 min post-MDL 73005EF). Values are the mean percentage of the day 0 baseline field e.p.s.p. amplitude ± s.e.mean. $^{**}P < 0.01$ versus pre-injection baseline control values.

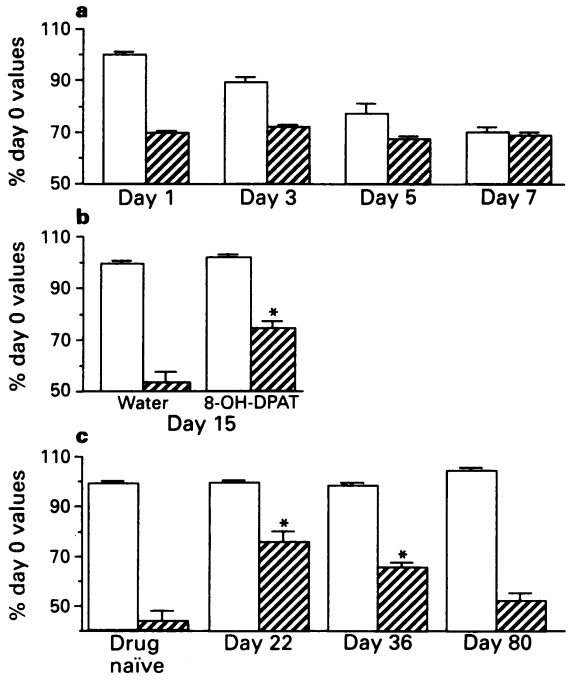


Figure 3 Acute effect of 8-OH-DPAT on hippocampal excitatory synaptic transmission in chronically treated rats. (a) The acute effect of 8-OH-DPAT (25 $\mu g\,kg^{-1}$) on the field e.p.s.p. amplitude on days 1, 3, 5 and 7 of repeated daily treatment with 8-OH-DPAT (25 $\mu g\,kg^{-1}$, i.p., $n = 6$). (b) The acute effect of 8-OH-DPAT (50 $\mu g\,kg^{-1}$) on the field e.p.s.p. amplitude on day 15, 8 days after repeated daily treatment with either 8-OH-DPAT (25 $\mu g\,kg^{-1}$, i.p., $n = 6$) or an equivalent volume of water ($n = 6$). (c) Comparison of the acute effect of 8-OH-DPAT (50 $\mu g\,kg^{-1}$) on the hippocampal field e.p.s.p. amplitude in drug naïve animals and on days 15, 22, 36 and 80 of the study in which rats received repeated daily treatment with 8-OH-DPAT (25 $\mu g\,kg^{-1}$, $n = 4$) from days 1–7. Open columns: pre-injection; hatched columns: post-injection. Values are the mean percentage of the day 0 baseline field e.p.s.p. amplitude ± s.e.mean. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to acute response to 8-OH-DPAT in water-injected or drug naïve controls.

duced a field e.p.s.p. amplitude reduction which was considerably less than that observed in the water-treated controls ($P < 0.001$, $n = 6$, Figure 3b). The time course of the acute response was similar for both groups and to that shown in Figure 1, being transient with a peak effect at approximately 30 min post-injection.

The duration of the loss of the acute response to 8-OH-DPAT was followed in the drug-treated group. On days 22 and 36 the acute response to 50 $\mu g\,kg^{-1}$ 8-OH-DPAT was still greatly reduced ($P < 0.05$, $n = 6$, compared to acute effect of 8-OH-DPAT in drug naïve animals, $n = 4$, Figure 3c). On day 80 the challenge with 50 $\mu g\,kg^{-1}$ 8-OH-DPAT produced a decrease of 50 ± 3% from baseline, a value not significantly different from the acute response to 8-OH-DPAT in drug-naïve animals (Figure 3c).

Discussion

The main findings of the present study are that repeated treatment with 8-OH-DPAT produced a gradual and prolonged reduction in basal excitatory amino acid-mediated synaptic transmission in the hippocampus and that there was an even longer-lasting tolerance to the acute inhibitory effect of 8-OH-DPAT.

Systemically injected 8-OH-DPAT produced a dose-dependent inhibition of the field e.p.s.p. which was probably due to a direct 5-HT_{1A} receptor agonist effect in the hippcampus since it mimicked the effect of intrahippocampal 5-HT_{1A} receptor agonists (O'Connor *et al.*, 1990) and was blocked by MDL 73005EF which is a highly selective 5-HT_{1A} receptor ligand with antagonist properties in this region (Van den Hooff & Galvan, 1991). The observation of a decrease in the inhibitory effect of 8-OH-DPAT at 100 $\mu g\,kg^{-1}$ may be due to its ability to have mixed agonist/antagonist effects at these sites (Colino & Halliwel, 1986; O'Connor *et al.*, 1990). The opening of postsynaptic K^+ channels is believed to underlie the inhibitory effect of 5-HT_{1A} receptor agonists on excitatory amino acid-mediated transmission in the hippocampus (Andrade & Nicoll, 1987; Sprouse & Aghajanian, 1988; O'Connor *et al.*, 1990).

The dose (25 $\mu g\,kg^{-1}$) of 8-OH-DPAT chosen for the chronic study produced a relatively small, transient reduction in the amplitude of the field e.p.s.p. after acute injection. An equivalent reduction in the 24 h baseline value (32%) occurred after repeated treatment for 6–7 days but was much more prolonged in both onset and duration. Indeed full recovery did not occur until 5–8 days after the last dose. It is most unlikely that 8-OH-DPAT accumulated in the body since it is known from *ex vivo* [^{3}H]-8-OH-DPAT binding studies that it is cleared from the hippocampal receptors very rapidly (half life of clearance = 80 min, Sethy & Francis, 1988) and is believed to undergo extensive first-pass metabolism in the liver after i.p. administration (Fuller & Snoddy, 1987; Mason *et al.*, 1992). Assuming that 8-OH-DPAT is not accumulated in the body then we might speculate that the gradual baseline reduction in the field e.p.s.p. amplitude is most likely due to an increase in 5-HT tone to the hippocampal 5-HT_{1A} receptors since the effect was transiently reversed after acute challenge with the postsynaptic 5-HT_{1A} receptor antagonist, MDL 73005EF (Van den Hooff & Galvan, 1991). Larsson *et al.* (1990) reported a 15% increase in the basal turnover of hippocampal 5-HT 48 h after twice daily s.c. injection of rats with 1 mg kg^{-1} 8-OH-DPAT for 7 days although there was only a statistically non-significant elevation in ventral hippocampal extracellular 5-HT levels 18–24 h after either acute (Hjorth, 1991) or repeated (Sharp *et al.*, 1993) 8-OH-DPAT. A decrease in raphe 5-HT_{1A} autoreceptor responsiveness would cause an increase in basal hippocampal 5-HT_{1A} receptor activation. Larsson *et al.* (1990), Hjorth (1991), Sharp *et al.* (1993) and Bohmaker *et al.* (1993) found no change in 5-HT_{1A} receptor-mediated decrease in hippocampal 5-HT synthesis/turnover/levels after prior treatment of rats with 0.4–4 mg kg^{-1} 8-OH-DPAT s.c. although some authors (Kennet *et al.*, 1987; Beer *et al.*, 1990; Kreiss & Lucki, 1992) have obtained evidence of tolerance in other brain regions. One explanation for the discrepancy is that the techniques used were not sensitive enough to detect a small but physiologically important change in 5-HT_{1A} autoreceptor-mediated inhibition of dorsal raphe neurone firing in an area such as the hippocampus which has dual innervation from the dorsal and median raphe nuclei (Schechter *et al.*, 1990; Cox *et al.*, 1993). The report that even 16–24 h after a single s.c. injection of 1 mg kg^{-1} 8-OH-DPAT, there was a reduction in the number of 5-HT_{1A} receptors in the raphe nuclei (Beer *et al.*, 1990) is consistent with the present observation of a small but significant baseline reduction in field e.p.s.p. amplitude on day 2 of the chronic study. A selective reduction in the density of 5-HT_{1A} receptors in the dorsal but not median raphe nucleus was found following chronic treatment with gepirone (Welner *et al.*, 1989), buspirone (Gobbi *et al.*, 1991) and ipsapirone (Fanelli & McMonagle-Strucko, 1992). The 5-hydroxytryptaminergic neurones innervating the stratum radiatum of the CA1 region of the dorsal hippocampus are predominantly of the type with fine axons and minute varicosities and are believed to originate principally from the dorsal raphe nucleus (Mamounas *et al.*, 1991).

As the baseline field e.p.s.p. amplitude gradually diminished during chronic 8-OH-DPAT treatment there was a parallel reduction in the acute response to the drug. Prior occupation of the receptors by an increase in endogenous

5-HT tone would mask the acute effect of 8-OH-DPAT to some extent. However, since the extent of the baseline reduction was always well below the maximum effect of acute 8-OH-DPAT, it is likely that a genuine decrease in postsynaptic 5-HT_{1A} receptor responsiveness developed during the chronic study. Indeed, even on recovery of the baseline 8 days after the last injection of 25 $\mu g\,kg^{-1}$ 8-OH-DPAT the higher dose of 50 $\mu g\,kg^{-1}$ failed to produce a greater inhibition. This indicates that there was a reduction in the maximum effect. Although previous researchers failed to find any change in postsynaptic 5-HT_{1A} receptor density or agonist affinity as measured by [^{3}H]-8-OH-DPAT binding in the rat hippocampus after single or repeated exposure to 8-OH-DPAT (16–48 h after daily injection of 1–2 $mg\,kg^{-1}$, s.c., Beer *et al.*, 1990; Larsson *et al.*, 1990), Newman *et al.* (1992), found an approximately 75% decrease in the maximum 5-HT_{1A} receptor-mediated inhibition of forskolin stimulated adenylate cyclase in the hippocampus 24 h after 8 days (but not 1 day) treatment with 1 $mg\,kg^{-1}$ 8-OH-DPAT s.c. A loss of 5-HT_{1A} receptor-mediated responses downstream of the receptor would account for this and the present finding. The same pertussis toxin sensitive G protein pool may be used to couple 5-HT_{1A} receptors to adenylate cyclase and to K^+ channels (Andrade *et al.*, 1986; Zgombick *et al.*, 1989). Previous electrophysiological studies with chronic gepirone or tandospirone showed no change in 5-HT_{1A} receptor-mediated inhibitory effects on pyramidal cell firing in the hippocampus (Blier & DeMontigny, 1987; Godbout *et al.*, 1991). However, these latter receptors may be of an atypical 'extrasynaptic' type (Blier *et al.*, 1993). Alternatively a lower intrinsic activity of these compounds at 5-HT_{1A} receptors compared to 8-OH-DPAT may be responsible for the difference between the studies. The apparent difference in time for recovery of the acute effect of 8-OH-DPAT and the baseline field e.p.s.p. amplitude after repeated 8-OH-DPAT treatment may be due to the difference in receptor-effector reserve in the dorsal raphe and the hippocampus (Meller *et al.*, 1990; Yocca *et al.*, 1992; Cox *et al.*, 1993).

Overall, repeated 8-OH-DPAT treatment produced a decrease in the dynamic range of 5-HT_{1A} receptor-mediated transmission in the hippocampus with an increase in basal activation and a decrease in acute responsiveness. A reduction in the dynamic range or 'stabilization' of 5-HT_{1A} receptor-mediated transmission may also be responsible for the effects of chronic dosing with the tricyclic antidepressant, imipramine (Rowan & Anwyl, 1985; Rowan *et al.*, 1990; O'Connor *et al.*, 1993) in the CA1 region. Such a change in the dynamics of 5-HT_{1A} receptor-mediated transmissioin in the hippocampus during repeated 8-OH-DPAT treatment will have implications for the interpretation not only of the putative antidepressant (Deakin & Graeff, 1991) and anxiolytic (Stefanski *et al.*, 1993) effects but also anticonvulsant (Wade *et al.*, 1993) and amnesic (Ohno *et al.*, 1993) effects.

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Sodium excretion following central administration of an I_1 imidazoline receptor agonist, moxonidine

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1 Previously we have shown that an intrarenal infusion of moxonidine, an I_1-imidazoline receptor agonist, resulted in a natriuresis which was inhibited by intravenous idazoxan, a selective imidazoline receptor antagonist. Therefore we examined the effects on renal function of intracerebroventricular (i.c.v.) administration of moxonidine with or without i.c.v. idazoxan.

2 Seven days after unilateral nephrectomy, Sprague-Dawley rats had i.c.v. cannulae implanted. Three days later the rats were anaesthetized (pentobarbitone), followed by cannulation of the jugular vein (fluid and drug administration), carotid artery (blood pressure) and the ureter (urine collection).

3 After a 45 min stabilization period, the effect of moxonidine was investigated by the i.c.v. administration of either isotonic saline or moxonidine (0.1, 0.3 or 1 nmol in isotonic saline) administered in 5 μl over 1 min. All doses of moxonidine resulted in an increase in urine flow with a concomitant increase in sodium excretion without affecting blood pressure. The highest dose of moxonidine (1 nmol) also increased free water clearance.

4 In a second series of experiments, the effects of idazoxan on the natriuretic response to i.c.v. moxonidine were determined. Moxonidine (0.3 nmol) again increased sodium and water excretion as compared to the i.c.v. saline control animals. Pretreatment with i.c.v. idazoxan (0.3 nmol), at a dose which alone failed to alter sodium and water excretion, completely attenuated the renal response to moxonidine. These results are consistent with central I_1-imidazoline receptors mediating a moxonidine-induced increase in sodium and water excretion at doses that do not alter blood pressure.

Keywords: Natriuresis; intracerebroventricular; antihypertensive

Introduction

The central nervous system via the sympathetic nervous system has been shown to have an important role in the control and modulation of renal salt and water handling (Kopp & DiBona, 1992). A number of the physiological responses previously attributed to the α_2-adrenoceptor may in fact be mediated through activation of imidazoline receptors (IRs). Several authors have identified IRs in the central nervous system (Ernsberger *et al.*, 1987; Bricca *et al.*, 1989). Two different IR subtypes have been proposed, I_1 and I_2 (Ernsberger, 1992). A clear physiological role involving the interrelationship between central imidazoline receptors and the kidney has yet to be elucidated.

Previous work demonstrated that, with lower doses of clonidine the increase in urine flow rate was primarily due to an increase in free water, while higher doses resulted in an increase in sodium excretion as reflected in osmolar clearance (Blandford & Smyth, 1988). The increase in free water clearance following intrarenal infusions of the α_2-adrenoceptor agonist, clonidine, was secondary to antagonism of vasopressin (Blandford & Smyth, 1990). In our laboratory, using different purported α_2-adrenoceptor agonists, we have demonstrated a dissociation between two physiological functions in the kidney (Smyth *et al.*, 1992). These studies suggested two distinct receptors and the increase in sodium excretion with higher doses of clonidine could be due to activation of the IR. Subsequently, our laboratory demonstrated that the intrarenal administration of moxonidine, a selective I_1-IR agonist, increased sodium and osmolar clearance, an effect that was blocked by intravenous idazoxan, an IR antagonist (Allan *et al.*, 1993). This suggested a physiological role for the IR in the kidney.

Central administration of compounds with high affinity for the imidazoline receptor result in haemodynamic changes (Ernsberger *et al.*, 1988; 1990). In the current study, we examined changes in renal function following the intracerebroventricular (i.c.v.) administration of the selective IR agonist, moxonidine, using doses that were less than are necessary to result in haemodynamic changes. By using clearance techniques, we were able to dissociate water handling from osmolar or solute handling by the kidney. This allowed us to draw conclusions about the effect different i.c.v. doses of moxonidine had on these parameters. Central administration of idazoxan, a selective IR antagonist, was used to inhibit i.c.v. moxonidine directly. Thus, the postulate that the changes in sodium excretion previously reported with central administration of purported α_2-adrenoceptor agonists could be through the activation of the IR, was examined in the current study.

Methods

Experimental preparation

Male Sprague-Dawley rats (200–250 g) underwent unilateral nephrectomy (ether anaesthesia) 1 week prior to placement of an i.c.v. cannula. Animals were housed at 23°C with a 12/12 h light/dark cycle. On the day of the experiment the rats were anaesthetized with pentobarbitone (BDH Chemicals Ltd., Poole, England, 50 mg kg^{-1}). The animals were placed on a heating blanket which was thermostatically controlled to maintain body temperature at 37.5°C. A tracheotomy was performed and the animals were allowed to breath spontaneously. The carotid artery was cannulated for measurement of blood pressure, heart rate and obtaining a blood sample at the end of the experiment. The jugular vein was cannulated for administration of drugs. Blood pressure was monitored with a Statham pressure transducer (model P23Dc) connected to a Grass polygraph model V. A modest

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diuresis was maintained throughout the experiment by administration of isotonic saline (97 μl min^{-1}). The moxonidine and idazoxan, which were dissolved in isotonic saline, were given centrally in a volume of 5 μl over 1 min with a 10 μl Hamilton syringe. The remaining kidney was exposed by a flank incision and the ureter was cannulated with PE-50 for collection of urine. The urine volumes were determined gravimetrically.

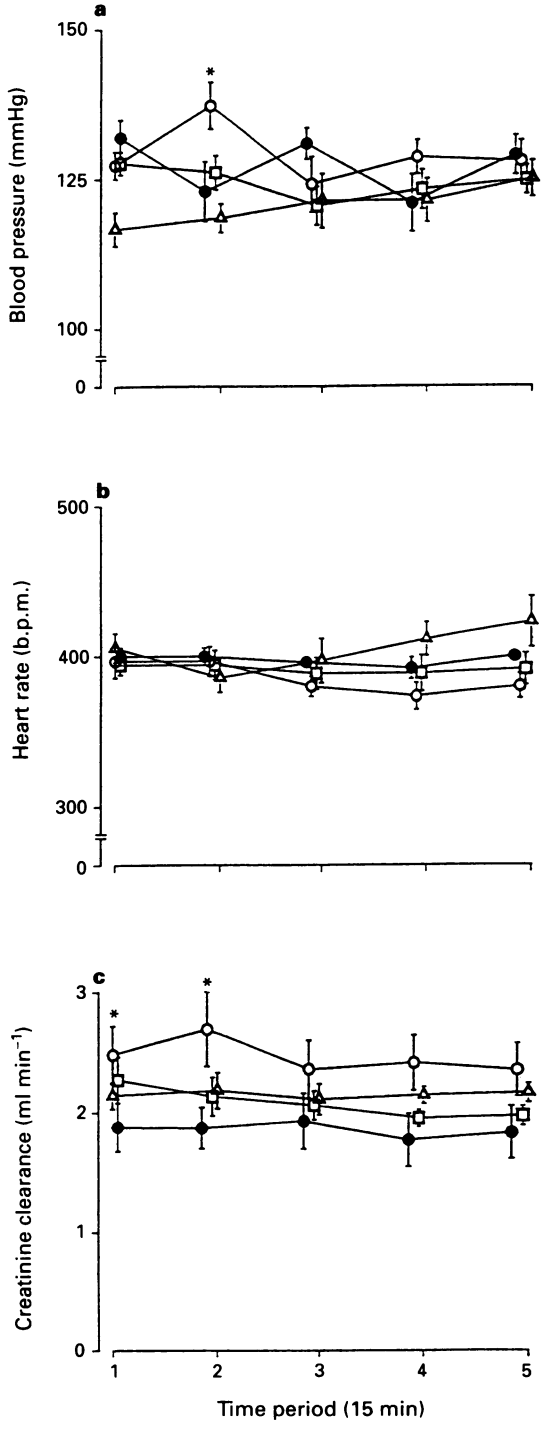


Figure 1 The effects of increasing dose of i.c.v. moxonidine on blood pressure (a), heart rate (b) and creatinine clearance (c) in rats (6 per group). The first time period represents a pretreatment 15 min control urine collection. Time periods 2 to 5 represent 15 min urine collections in the presence of saline vehicle or increasing doses of moxonidine. The different i.c.v. infusions are illustrated as follows: (●) saline control, (○) moxonidine 0.1 nmol; (□) moxonidine 0.3 nmol; (△) moxonidine 1 nmol. The data are presented as the mean ± s.e.mean. *$P<0.05$.

Intracerebroventricular cannula

The method used to implant the cannulae was the same as previously described (Penner *et al.*, 1990). Briefly, the rats were anaesthetized by an intraperitoneal injection of 50 mg kg^{-1} pentobarbitone. The cannula was a modified 23 gauge needle with a solid obturator, threaded through the lumen and extending 0.5 mm beyond the tip of the needle. The head of the rat was placed in a stereotaxic apparatus (model 900, David Kopf, Tujunga, CA, U.S.A.). The coordinates for implantation of the cannula in relation to the skull were 0.3 mm posterior to the bregma, 1.4 mm lateral to the midline and 3.5 mm below the surface of the skull. The cannula was anchored to the skull with three stainless steel jewelry screws in an assemblage of acrylic cement. At the time of the experiment the obturator was removed. A 31 gauge injector tube was inserted which extended 1.0 mm below the end of the guide tube (23 gauge needle) and consequently entered the ventricle. The injector tube was then connected to a 10 μl Hamilton syringe for administration of drug or vehicle. Verification of the cannula location in the cerebral ventricle was performed by injection of dye through the cannula at the end of the experiment, and examination of postmortem brain sections.

Intracerebroventricular moxonidine with or without idazoxan

Three days after the placement of the i.c.v. cannulae the experimental procedure, as outlined above, was performed.

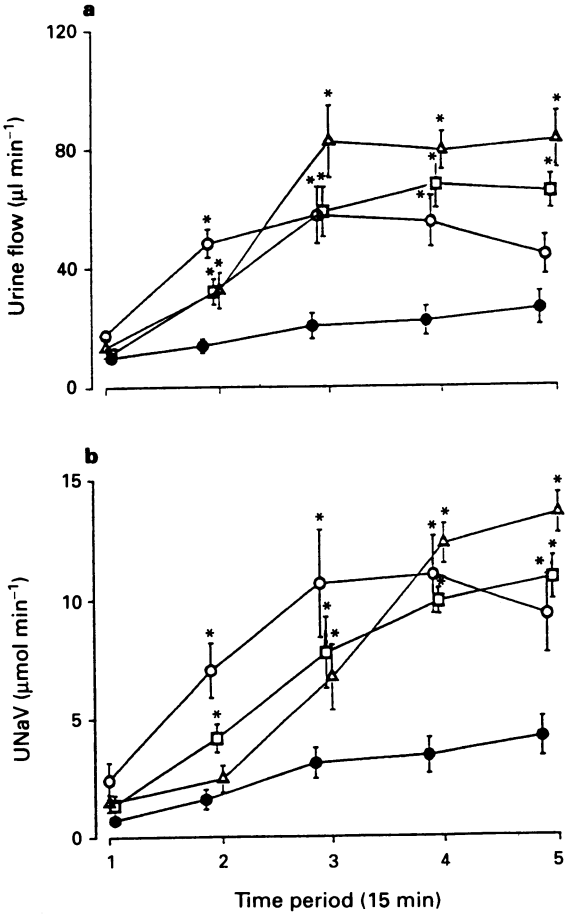


Figure 2 The effects of increasing dose of i.c.v. moxonidine on urine flow (a) and sodium excretion (UNaV) (b) in rats (6 per group). The time periods are as described in Figure 1. The different i.c.v. infusions are illustrated as follows: (●) saline control; (○) moxonidine 0.1 nmol; (□) moxonidine 0.3 nmol; (△) moxonidine 1 nmol. The data are presented as the mean ± s.e.mean. *$P<0.05$.

Following the surgical preparation and a 45 min stabilization period, a 15 min control urine collection was obtained. In the first series of experiments, immediately following this control urine collection, i.c.v. isotonic saline or moxonidine (0.1, 0.3 or 1 nmol) was administered. This was followed by four further 15 min urine collections. In a second series of experiments, the effects of the IR antagonist, idazoxan on the response to moxonidine was assessed. In these experiments, following the control urine collection, idazoxan (0.3 nmol) or isotonic saline was administered i.c.v. followed by moxonidine (0.3 nmol) or isotonic saline. Ten minutes later four post-treatment urine collections of 15 min each were obtained. The four treatment groups for the antagonist study were: saline controls, saline followed by moxonidine, idazoxan followed by saline, and idazoxan followed by moxonidine. Each study was carried out in separate animals. At the completion of the experiment a blood sample was obtained and the plasma frozen for later analysis. Urine and plasma creatinine levels were measured with a Beckman Creatinine 2 Analyzer; sodium was measured with a Beckman Klina Flame Photometer; and osmolality was assessed with a Precision System Micro Osmometer.

Statistical analysis

The data are presented as the mean ± standard error of the mean. Each group contained six animals. Repeated measures of analysis of variance (ANOVA) utilizing SAS System Version 6.07 was used to assess the data. Where significant differences were found with the ANOVA the interactions were analyzed using the Least Squares Means Difference Test. A P value of ≤ 0.05 was used for significance and is denoted by * in the figures.

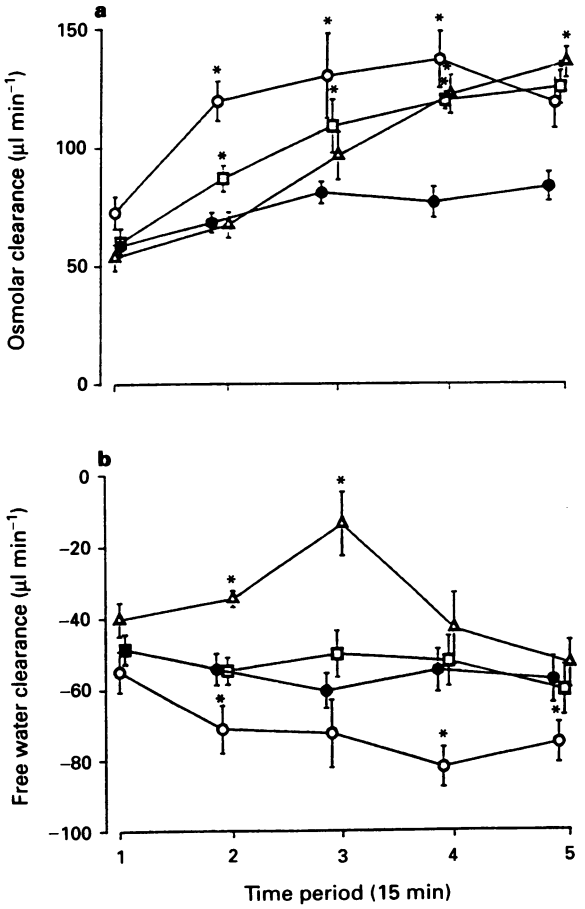


Figure 3 The effects of increasing dose of i.c.v. moxonidine on osmolar clearance and free water clearance in rats (6 per group). The time periods are as described in Figure 1. The different i.c.v. infusions are illustrated as follows: (●) saline control; (○) moxonidine 0.1 nmol; (□) moxonidine 0.3 nmol; (△) moxonidine 1 nmol. The data are presented as the mean ± s.e.mean. *$P<0.05$.

Drugs

Moxonidine was supplied by Berisdorf, AG, Hamburg, FRG and idazoxan was obtained from Research Biochemicals, Inc., Natick, MA.

Results

Dose-response to intracerebroventricular moxonidine

At all doses investigated in the present study, i.c.v. administration of moxonidine had minimal effects on blood pressure and heart rate. The lowest dose of i.c.v. moxonidine investigated (0.1 nmol) produced a transient increase in blood pressure during the first collection immediately following i.c.v. administration. This dose was also associated with a small but significant increase in creatinine clearance (Figure 1). Urine flow rate was increased by all doses of moxonidine. Concomitant with the increase in urine flow was an increase in sodium excretion (Figure 2). Similarly, osmolar clearance was also increased with all three doses of i.c.v. moxonidine (Figure 3). The lowest dose of moxonidine (0.1 nmol) produced the greatest increase in osmolar clearance, which may have been secondary to the increase in creatinine clearance observed with this dose. Free water clearance was decreased by 0.1 nmol of moxonidine, reflecting the greater increase in osmolar clearance for a given urine flow rate. At the highest dose of moxonidine (1 nmol) investigated, free water clearance was increased.

Intracerebroventricular moxonidine and idazoxan

In the second series of experiments, i.c.v. moxonidine alone, i.c.v. idazoxan alone, or i.c.v. moxonidine and idazoxan failed to alter blood pressure, heart rate and creatinine clearance as compared to the animals receiving the saline control vehicle (Figure 4). Idazoxan (0.3 nmol) alone slightly decreased the urine flow rate but did not alter the sodium excretion (Figure 5). Moxonidine (0.3 nmol), as expected from the previous series of animals, increased both the urine flow rate and the sodium excretion. (Figure 5). Administration of idazoxan prior to the moxonidine resulted in a complete attenuation of the diuretic and natriuretic response to moxonidine (Figure 5). Similarly, the increase in osmolar clearance observed following moxonidine was antagonized by the concomitant administration of idazoxan (Figure 6). At the doses chosen in this set of experiments no changes in free water clearance were observed.

Discussion

Initially, clonidine was reported to be a specific α_2-adrenoceptor agonist, and consequently, the blood pressure lowering action of this drug was postulated to be mediated by activation of central α_2-adrenoceptors (Kobinger, 1978). Studies by Bousquet *et al.* (1984) challenged this concept with the examination of the cardiovascular effects of different α_2-adrenoceptor agonists given centrally. In this study by Bousquet *et al.* in 1984, the agonists were not categorized according to previously reported specificity for α_1- and α_2-adrenoceptors, but on structure – imidazoline (i.e. clonidine) versus phenylethylamine (catecholamines). The blood pressure lowering actions of these various agonists were assessed in the nucleus reticularis lateralis. Phenylethylamine-based molecules failed to alter blood pressure, whereas imidazoline-based agonists, whether previously reported to be α_1- (i.e. cirazoline) or α_2- (i.e. clonidine) adrenoceptor-specific, pro-

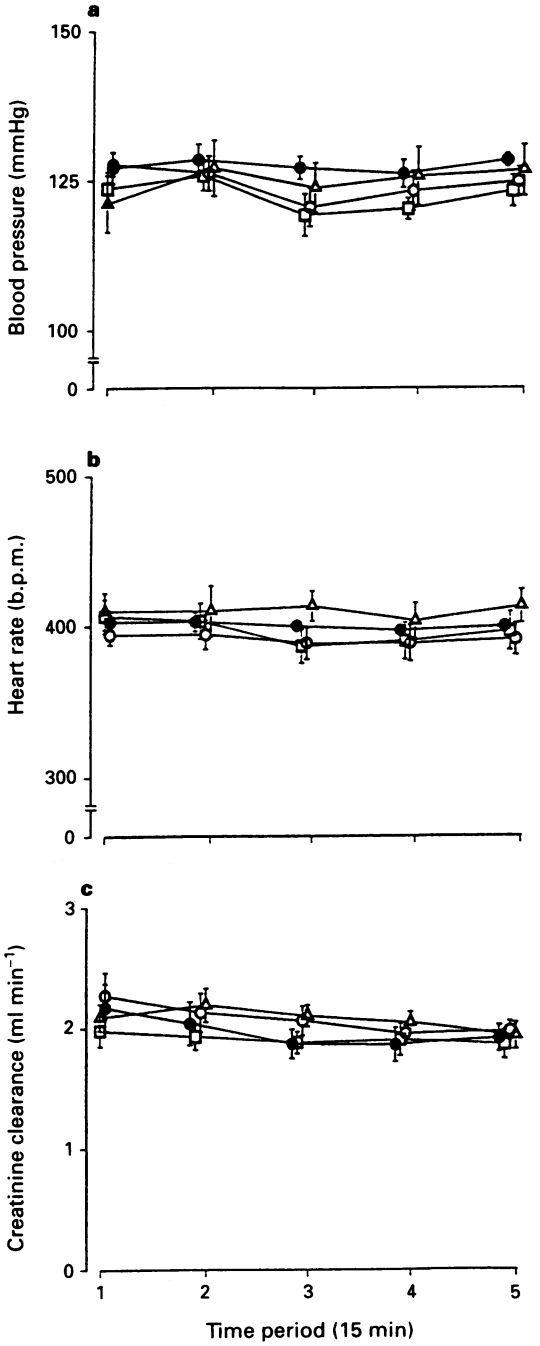


Figure 4 The effects of i.c.v. moxonidine (0.3 nmol) on blood pressure (a), heart rate (b) and creatinine clearance (c) in rats (6 per group) in the presence and absence of idazoxan. The time periods are as described in Figure 1. The different i.c.v. infusions are illustrated as follows: (●) saline control; (○) moxonidine 0.3 nmol and saline; (□) moxonidine 0.3 nmol and idazoxan 0.3 nmol; (△) saline and idazoxan 0.3 nmol. The data are presented as the mean ± s.e.mean. $*P<0.05$.

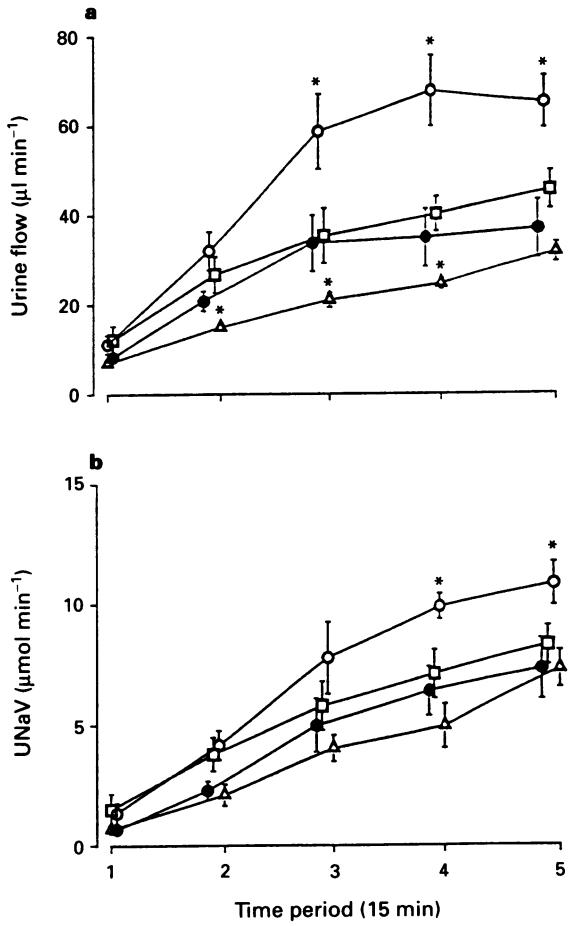


Figure 5 The effects of i.c.v. moxonidine (0.3 nmol) on urine flow rate (a) and sodium excretion (UNaV) (b) in rats (6 per group) in the presence and absence of idazoxan (0.3 nmol). The time periods are as described in Figure 1. The different i.c.v. infusions are illustrated as follows: (●) saline control; (○) moxonidine 0.3 nmol and saline; (□) moxonidine 0.3 nmol and idazoxan 0.3 nmol; (△) saline and idazoxan 0.3 nmol. The data are presented as the mean ± s.e.mean. $*P<0.05$.

duced significant decreases in blood pressure. This indicated that this effect was not mediated by α_2-adrenoceptors *per se* but rather by binding sites which had a higher affinity for imidazoline-based molecules (imidazoline-sites). Paralleling these findings were radioligand binding studies in the central nervous system which demonstrated two populations of receptors, one binding α_2-adrenoceptor compounds, while the second site preferentially bound imidazoline based compounds (Ernsberger *et al.*, 1987; Bricca *et al.*, 1989). Subsequently, the endogenous clonidine-displacing substance was shown to bind to these same receptors with a high affinity and low capacity, thus demonstrating a possible endogenous agonist for this novel receptor (Parini *et al.*, 1989). These studies indicated a potential role of the imidazoline receptor in the regulation of blood pressure.

A number of studies have demonstrated that clonidine and structurally related compounds lower blood pressure through stimulation of imidazoline receptors located centrally (Feldman *et al.*, 1990; Tibirica *et al.*, 1991; Gomez *et al.*, 1991). Clonidine, through what now appears to be imidazoline receptors located in the ventrolateral medulla, has been found to lower blood pressure in a number of different species (Gillis *et al.*, 1985; Sinha *et al.*, 1985; McAuley *et al.*, 1988; Feldman *et al.*, 1990). This blood pressure lowering has been attributed to a suppression of catecholaminergic neurones located centrally which in turn would lower peripheral sympathetic nerve activity (Tibirica *et al.*, 1989; 1992). The central nervous system has also been shown to modulate renal sodium excretion through changes in renal nerve activity irrespective of changes in blood pressure (Koepke *et al.*, 1987). This suggested that stimulation of imidazoline receptors centrally, in addition to having a vasodepressor action, could also function to regulate water and sodium excretion.

In the current study, i.c.v. administration of moxonidine resulted in an increase in urine flow rate and sodium excretion with all doses utilized. This was observed at doses of moxonidine which failed to alter blood pressure, creatinine clearance or heart rate suggesting that the i.c.v. moxonidine

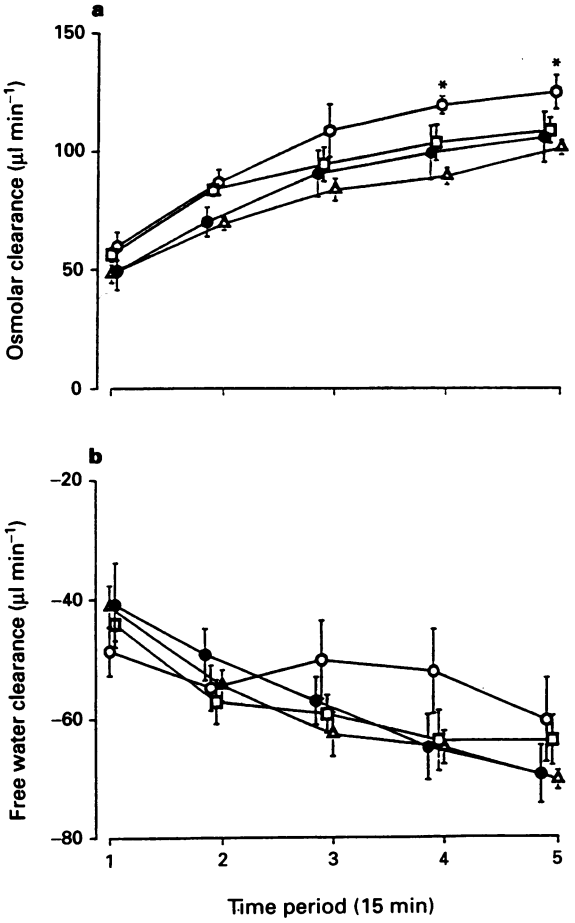


Figure 6 The effects of i.c.v. moxonidine (0.3 nmol) on osmolar clearance (a) and free water clearance (b) in rats (6 per group) in the presence and absence of idazoxan (0.3 nmol). The time periods are as described in Figure 1. The different i.c.v. infusions are illustrated as follows: (●) saline control; (○) moxonidine 0.3 nmol and saline; (□) moxonidine 0.3 nmol and idazoxan 0.3 nmol; (△) saline and idazoxan 0.3 nmol. The data are presented as the mean ± s.e.mean. $*P<0.05$.

was specifically altering renal function, independent of cardiovascular function. The doses of i.c.v. moxonidine were chosen based on preliminary studies in which lower doses were without effect, while higher doses than used in the current study resulted in haemodynamic changes. The doses in the present study of 0.1 to 1 nmol were considerably lower than the total dose of moxonidine used by Allan *et al.* (1993): 1 to 10 nmol kg^{-1} min^{-1} continuously for 1 h. Thus, it is unlikely that the observed changes following i.c.v. moxonidine are due to a direct peripheral effect. As previously observed with the vasodepressor action of imidazoline receptor agonists (Feldman *et al.*, 1990; Tibirica *et al.*, 1991; Gomez *et al.*, 1991), in the current study changes in renal function observed with i.c.v. moxonidine were antagonized with i.c.v. idazoxan. This is consistent with the natriuresis following central administration of moxonidine being secondary to activation of the imidazoline receptors. The highest dose used also increased free water clearance. The mechanism for this observation was unclear, but may be related to this higher dose of moxonidine stimulating α_2-adrenoceptors. This, in turn, may alter the central release of vasopressin or the stimulation of peripheral α_2-adrenoceptors which antagonize the renal actions of vasopressin (Blandford & Smyth, 1990). Further studies will be necessary to elucidate both the mechanism for this finding, as well as, the respective role of the central versus the peripheral activation of the imidazoline receptor in the regulation of sodium excretion.

More recently, radioligand binding studies have identified imidazoline-receptors in the kidney (Bidet *et al.*, 1990). In an initial study, we determined the effect of three different purported α_2-adrenoceptor agonists, clonidine, UK 14,304 and 2,6 dimethyl clonidine, on free water clearance and osmolar clearance. The results for free water clearance demonstrated a rank order of potency of clonidine >> UK 14304 >> 2,6 dimethyl clonidine, which was reversed for osmolar clearance (Smyth *et al.*, 1992). As these were distinct physiological effects, these findings were consistent with two distinct receptors and/or sites. Additional studies, using the selective I_1 imidazoline receptor agonist, moxonidine, infused directly into the renal artery, demonstrated a dose-related natriuresis at doses of 1, 3 and 10 nmol kg^{-1} min^{-1} (Allan *et al.*, 1993). The specific imidazoline receptor antagonist, idazoxan, inhibited the response but rauwolscine (specific α_2-adrenoceptor antagonist) was without effect. This was consistent with the increase in sodium being secondary to activation of renal imidazoline receptors. At present, it is not clear as to whether central and peripheral I_1-imidazoline receptors function exclusively of each other to alter sodium and water excretion. Conceivably, centrally administered moxonidine may have stimulated renal receptors, or peripherally administered moxonidine may have altered function at central sites to alter renal function. Further studies will be required to determine the specific sites of action.

Previous studies have clearly demonstrated a vasodepressor action of centrally administered imidazoline receptor agonists. In the current study, we have demonstrated that moxonidine, a specific I_1-imidazoline receptor agonist, administered i.c.v. produced an increase in urine flow rate and sodium excretion at levels which appeared to have negligible cardiovascular actions. As this appeared at doses lower than those required to alter blood pressure acutely, this centrally mediated natriuresis may represent an additional mechanism by which these agonists function as antihypertensives.

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Investigation of the role of 5-HT_3 and 5-HT_4 receptors in ascending and descending reflexes to the circular muscle of guinea-pig small intestine

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1 The present study was undertaken to ascertain whether 5-hydroxytryptamine (5-HT) acting at either 5-HT_3 or 5-HT_4 receptors plays a significant role in motility reflexes in the guinea-pig small intestine.

2 An isolated segment of small intestine was opened along its mesenteric border and pinned, mucosa uppermost, in a three chambered organ bath so that the oral, middle and anal regions of a single preparation could be separately superfused.

3 Conventional intracellular recording methods were used to monitor the responses of the circular muscle in the oral or the anal end chambers when distension was applied in either of the other two chambers or the mucosal villi were compressed in the middle chamber. Drugs were added to the middle chamber.

4 5-HT_3 receptor antagonists (tropisetron, 0.1–10 μM; granisetron, 1 μM and BRL 46470, 1 μM) depressed the ascending excitatory reflex evoked by these stimuli but had no effect on the descending inhibitory reflex. The depression of the excitatory reflex was observed whether the reflex was evoked from the chamber containing the drug or was simply conducted, via interneurones, through this chamber.

5 The 5-HT_4 receptor antagonist, SDZ 205-557 (1 μM), had no significant effect on either the ascending or descending reflex pathways. However, 5-HT_4 receptors were present as cisapride (0.1 μM) significantly enhanced the ascending excitation without affecting the descending inhibition. This effect of cisapride was converted to a significant depression of the ascending reflex by SDZ 205-557.

6 The results suggest that 5-HT_3, but not 5-HT_4, receptors play an important role in the ascending excitatory reflex and that these receptors may be on interneurones in the reflex pathway.

Keywords: Enteric nervous system; peristalsis; enteric reflexes; 5-hydroxytryptamine (5-HT); 5-HT receptors; small intestine

Introduction

Since the discoveries that 5-hydroxytryptamine (5-HT) occurs in high concentration in the intestine and potently affects intestinal movements (Erspamer & Asero, 1952; Gaddum, 1953), the assumption has been that it is important for the regulation of intestinal movement (Bülbring, 1961; Gershon *et al.*, 1992). However, it has proved extremely difficult to ascertain the physiological role or roles of 5-HT in controlling motility (Tonini *et al.*, 1992; Kirschgessner *et al.*, 1992; Buchheit & Buhl, 1993). The reasons for the difficulty include the fact that two enteric stores of 5-HT exist, endocrine cells and neurones; 5-HT has multiple sites of action; and there are at least four different receptor types for 5-HT or its analogues through which motility can be influenced (Gunning & Humphrey, 1987; Costall & Naylor, 1990; Craig & Clarke, 1990; Tonini *et al.*, 1992). Sites of action began to be identified in the early 1950's. Amongst these were the endings of cholinergic nerve fibres, the cell bodies of excitatory neurones and the muscle (Rocha e Silva *et al.*, 1953; Gaddum & Picarelli, 1957). Further studies indicated that 5-HT stimulates enteric sensory neurones (Bülbring & Crema, 1958) and inhibitory motor neurones (Bülbring & Gershon, 1967). Surprisingly, manipulations to deplete enteric stores of 5-HT (Bülbring & Crema, 1959; Boullin, 1964) or drugs which block 5-HT receptors (Tonini *et al.*, 1992) have failed to identify the physiological roles of 5-HT in motility control. On the other hand, there has been considerable progress in determining the sites and classification of enteric receptors for 5-HT. For example, electrophysiological studies have identified at least two types of excitatory receptors on nerve cell bodies in the guinea-pig gastrointestinal tract, 5-HT_3 and putative 5-HT_{1P} receptors (e.g. Surprenant & Crist, 1988; Mawe *et al.*, 1989; Schemann, 1991). Hyperpolarization of nerve cells is mediated via 5-HT_{1A} receptors (Galligan *et al.*, 1988) and activation of 5-HT_4 receptors on nerve endings enhances transmitter release (Taniyama *et al.*, 1991; Kilbinger & Wolf, 1992).

In view of the number of receptors and multiple sites of action, we have sought to investigate the possible roles of 5-HT using a strategy of preferentially exposing parts of the enteric reflex pathways to drugs acting at receptors for 5-HT. We have done this by using an organ bath in which separate solutions bathe three regions, that from which reflexes are evoked, that in which reflexes are conducted along the intestine and that in which responses in the muscle are recorded. We have used specific receptor agonists and antagonists to evaluate the possible effects of endogenous 5-HT on 5-HT_3 and 5-HT_4 receptors on neurones in ascending and descending reflex pathways.

Methods

Guinea-pigs of either sex weighing between 200 and 400 g were used. The animals were killed by being stunned and bled via the carotid arteries. Segments of small intestine 4–6 cm in length were taken between 10–30 cm oral to the ileo-caecal junction and flushed to remove the content of intestine with physiological saline (composition in mM: Na^+ 151, K^+ 4.7, Ca^{2+} 2.8, Mg^{2+} 0.6, Cl^- 143.7, $H_2PO_4^-$ 1.3, SO_4^{2-} 0.6, HCO_3^- 16.3, glucose 7.7) containing 1 μM nicardipine. In the presence of nicardipine, movement of the muscle is substantially depressed so that cell impalements are more readily retained, but reflex responses can still be

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recorded electrically from the muscle (Smith & Furness, 1988; Yuan et al., 1991). Each segment was opened along its mesenteric border and pinned flat, mucosa uppermost, in a partitioned organ bath.

In order to analyze reflex nerve circuitry and 5-HT receptor locations on the different types of neurones in the pathway, the organ bath was divided into three chambers of different sizes by two plastic partitions (the bottom edge resting against mucosa; 1 mm in thickness). The chambers included one recording chamber (volume 3 ml) and two stimulation chambers (middle; 1 ml and far; 3 ml) each of which had a distending balloon (diameter 5 mm) set into its base. The centres of the distending balloons were separated by 11 mm (Figure 1). Vacuum grease was used to seal the partition area around the preparation and a lower level of superfusion solution in the middle chamber was also used to prevent the drugs in the middle chamber leaking into adjacent chambers. Preliminary experiments were done by applying phenol red (concentration 0.02%) into each of three chambers separately to check the leakage of dye. No leakage was observed. During the experiment, the preparation in the three chambers was superfused individually and continuously by a gravity feed system for the recording and far chambers and by a peristaltic pump for the middle chamber, both flow rates were kept at 0.7 ml min^{-1} except during the rapid application and washout of drugs (2.8 ml min^{-1}) in the middle chamber. The physiological saline was maintained at 37°C and bubbled with carbogen (95% O_2/5% CO_2) on entering each chamber. Drugs were administered into the middle chamber by the peristaltic pump from a reservoir containing the final concentration of drugs in the superfusate.

Reflexes were evoked either by distension (stimulating stretch receptors; 0.1 ml distending volume, duration 9 s) in the middle or far chambers, or by distortion of the mucosa by compression with a compressor (stimulating mucosal mechanoreceptors; distance moving down 1 mm, duration 9 s) in the middle chamber, which was centred over the distending balloon. The distance between the recording electrode in the recording chamber and the centres of both distending balloons and compressor in the middle chamber was 11 mm. The compressor consisted of three parts: a plastic holder on the top, a sponge rubber block in the middle and a sylgard block at the bottom. The sylgard was intended to prevent the lower part of the compressor from absorbing the drugs and to be cleaned easily after each stimulus (Figure 1). The compressor had a compressibility of 0.08 kPa mm^{-1} and the surface of the sylgard block that touched the mucosa was flat and measured 5 mm in the longitudinal axis of the intestine and 11 mm in its circumferential axis. The compressor was mounted in a rigid support that was advanced and withdrawn vertically by hand using a calibrated rackwork bosshead.

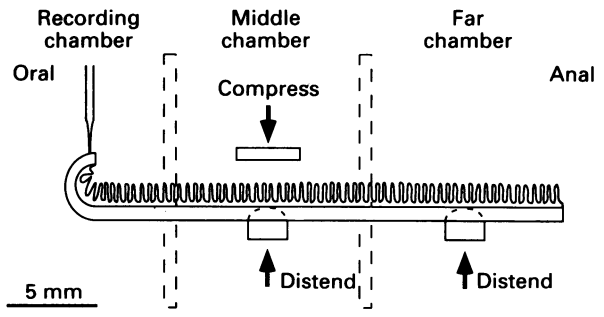


Figure 1 Diagram showing the partitioned organ bath used to evoke the intestinal reflex by distension with a balloon against the serosal surface or mucosal compression with a piece of stiff sponge rubber and to record the reflex responses from the circular muscle. The antagonists or agonists at 5-hydroxytryptamine (5-HT) receptors were added to the middle chamber. The dashed rectangles show the location of the partitions. Note, although the length of the preparation is drawn to scale, the thickness of the preparation is not to scale.

During the experiments, control responses were recorded prior to addition of the drugs to the middle chamber 15 min before a series of test stimuli. Stimuli applied in each of the stimulation chambers tested whether different neuronal components of the reflex circuitry were affected by the drugs. For example, balloon distension in the far chamber was used to test the effect of the drug on the interneurones in the middle chamber which receive inputs from interneurones excited by stretch-sensitive sensory neurones in the far chamber. Balloon distension or mucosal compression in the middle chamber was used to test the effect of drugs on the middle chamber stretch or mucosal distortion-sensitive sensory neurones and some interneurones in the same chamber (Figure 7). Previous studies have shown that responses evoked by mechanical stimuli similar to those used in this present study are completely abolished by tetrodotoxin (Smith & Furness, 1988; Smith et al., 1990) and hence are neurally mediated. The responses of the circular muscle in the recording chamber were recorded by conventional intracellular recording techniques (Yuan et al., 1991). Ascending reflexes were studied by impaling circular muscle cells at the oral end of an opened segment. Descending reflexes were studied in separate preparations whose orientation was reversed so that the anal end was in the recording chamber.

As compression of the mucosal villi can evoke both ascending and descending reflexes, it might have been expected that the partitions dividing the organ bath would also excite such reflexes. However, a previous study (Yuan et al., 1992) has shown that maintaining a constant stimulus causes the reflex responses to decline in amplitude and ultimately to disappear, but it does not suppress reflexes evoked at other sites. Such reflexes can still propagate past the point of a sustained, but ineffective, stimulus.

In several experiments, the mucosa was removed from the wall of the intestine in the middle and far chambers to eliminate the influence of 5-HT released from mucosal enterochromaffin cells. The submucosa and the muscle layers were left intact.

Drugs

The drugs used in this study were: tropisetron (ICS 205-930), granisetron (BRL 43694) and BRL 46470 (endo-N-(8-methyl-8-azabicyclo[3,2,1]oct-3-yl)-2,3-dihydro-3,3-dimethyl-indol-1-carboxamide, hydrochloride), kindly supplied by SmithKline Beecham Pharmaceuticals; SDZ 205-557 (2-methoxy-4-amino-5-chloro-benzoic acid 2-(diethylamino)ethyl ester; gift from Sandoz, Basle, Switzerland). All the drugs above were dissolved in physiological saline. Cisapride (Janssen pharmaceutica, Belgium) was firstly dissolved in DMSO (dimethyl sulphoxide) to make a stock solution (10 mM) and then diluted with saline for the experiment. Vehicle solution was identical except for cisapride. Nicardipine (Sigma) was dissolved in warm distilled water at a concentration of 10 mM, stored in frozen aliquots and dilutions for experiments were freshly prepared every day.

Statistical procedures

Amplitudes of responses were measured at their peaks and statistical comparisons were made using Student's paired t test. All data are expressed as mean ± s.e.mean.

Results

Although consistent responses can be achieved by repeated stimulation when the stimulation intervals are greater than 2 min in conventional organ baths (Yuan et al., 1991), when the partitioned organ bath was used, the amplitudes of res-

ponses to the same amount of stimulation were observed to vary if the time interval between the beginning of the stimulation and the end of the whole experiment was longer than 1 h. Thus, it was necessary to determine the time interval over which consistent responses could be obtained.

Time control experiments

In these experiments, either distension in the far and middle chambers or compression in the middle chamber, were separated by intervals of 2 min (see Yuan *et al.*, 1992). In the middle chamber stimulation by either distension or compression caused consistent compound e.j.ps during the first 30 min of the experiment and the amplitudes of responses were reduced slightly, but not significantly, when the time was extended to 45 min. The amplitudes of e.j.ps evoked by distension in the far chamber were also consistent and reproducible during the first 15 min and then the amplitudes of e.j.ps became depressed with time (Figure 2a). These results indicated that the responses in these time control experiments were consistent and repeatable during the first 30 min. For the comparison of responses with or without drug treatments, the best time interval between control stimuli and stimuli in the presence of drugs was 15 min. Unlike the ascending reflex response, the descending reflex responses evoked by the stimuli either in the middle or in the far chamber were quite consistent and reproducible during the first 30 min of the experiment and, even when the time of experiment was extended to 45 min, the amplitudes of inhibitory junction potentials (i.j.ps) were still not significantly changed (Figure 2b).

Descending inhibitory reflexes

Low concentrations of the 5-HT$_3$ receptor antagonist ICS 205-930 (0.1–1 μM) in the middle chamber had no significant effect on the compound i.j.ps elicited by either distension or compression in both stimulation chambers, only the highest concentration of ICS 205-930 (10 μM) significantly depressed the amplitude of i.j.ps (a fall of 40–50% in four preparations from different animals). This effect was probably not due to a specific action of ICS 205-930 on the descending inhibitory reflexes, because the concentration needed to produce it was 10 times that needed to block either 5-HT$_3$ or 5-HT$_4$ receptors. The amplitudes of the i.j.ps were not significantly affected by the 5-HT$_3$ receptor antagonists, granisetron (1 μM) and BRL 46470 (1 μM), by the 5-HT$_4$ receptor antagonist, SDZ 205557 (0.1 μM), or the mixed 5-HT$_3$ antagonist and 5-HT$_4$ agonist, cisapride (0.1 μM).

Ascending excitatory reflexes

Effect of 5-HT$_3$ receptor antagonists ICS 205-930 in the middle chamber substantially depressed the compound e.j.ps evoked either by distension or by mucosal compression in both stimulation chambers. The effects of ICS 205-930 were dose-dependent. ICS 205-930 0.1 μM reduced the e.j.ps to

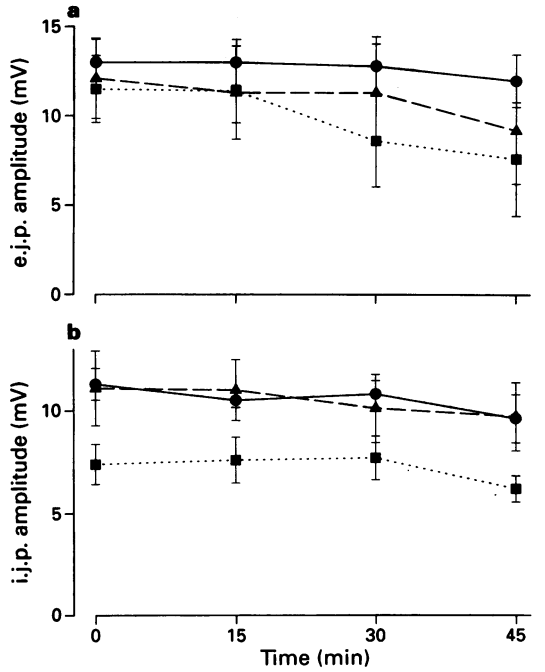


Figure 2 Time control experiments illustrating the consistency of responses evoked by stimuli in different chambers over a 45 min period. (a) Amplitudes of excitatory junction potentials (e.j.ps) recorded from circular muscle oral to the stimuli. (b) Amplitudes of inhibitory junction potentials (i.j.ps) recorded from circular muscle anal to the stimuli: (●) mean responses evoked by distension in the central chamber; (▲) responses evoked by compression of the mucosa in the central chamber; (■) mean responses evoked by distension in the far chamber; s.e.mean are shown. $n = 5$ preparations from different animals for each curve.

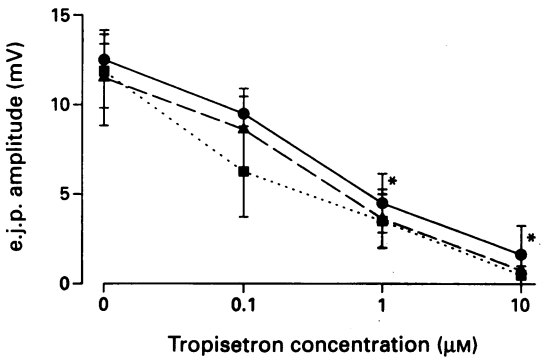


Figure 3 Effect of tropisetron (ICS 205-930) at different concentrations on the ascending excitatory reflex evoked either by distension in the middle or far chamber or by mucosal compression in the middle chamber. Tropisetron at 1 μM and 10 μM markedly reduced the e.j.ps oral to stimuli applied in both stimulation chambers ($*P < 0.05$): (●) distension in central chamber; (▲) mucosal compression in the central chamber; (■) distension in the far chamber. $n = 4$ preparations from different animals.

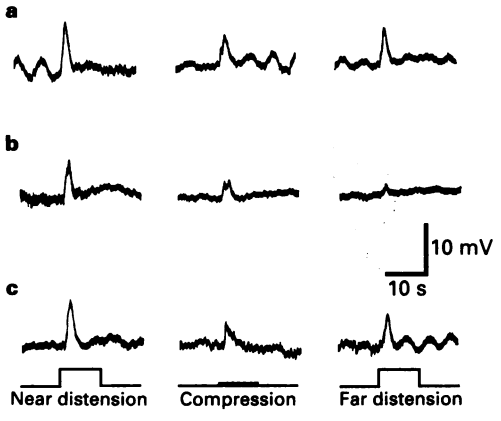


Figure 4 Effects of granisetron on the ascending excitatory reflexes evoked in a single circular smooth muscle cell of the guinea-pig small intestine. (a) Shows compound e.j.ps evoked by near distension (left panel), compression (middle panel) and far distension (right panel), each separated by 2 min. (b) Shows responses evoked by identical stimuli after 1 μM granisetron had been in the central chamber for 15 min. (c) Shows responses evoked by identical stimuli 15 min after beginning to wash out granisetron. The periods during which the stimuli were applied are shown in the bottom panels. Granisetron produced a marked depression of the reflex responses, which reversed after washout.

60–80% of control levels. ICS 205-930, 1 μM further reduced the e.j.ps to 20–30% of control ($P<0.05$) (Figure 3).

Other, more specific, 5-HT_3 receptor antagonists, granisetron (1 μM) (Figure 4) and BRL 46470 (1 μM) also depressed the e.j.ps elicited either by distension or by compression in both stimulation chambers when added to the middle chamber. Each compound reduced the e.j.ps to 50–70% of control levels ($P<0.05$ in the middle chamber; $P<0.01$ in far chamber) (Figure 5a), ($P<0.02$ in the middle chamber; $P<0.01$ in far chamber) (Figure 5b). These observations suggest that endogenously released 5-HT acting on 5-HT_3 receptors plays a role in the ascending excitatory reflex.

To test whether the endogenous 5-HT was released from the enterochromaffin cells of the mucosa or from the neurones in the myenteric plexus, the experiments were repeated after removing the mucosa. Granisetron (1 μM) significantly depressed the e.j.ps evoked by distensions in the middle chambers even when the mucosa was absent ($P<0.05$). After removing the mucosa, the responses to stimulation by compression in the middle chamber were abolished and responses to distension in the far chamber were markedly depressed (Figure 5c).

Effect of a 5-HT_4 receptor antagonist As 1 μM ICS 205-930 might be expected to block 5-HT_4 as well as 5-HT_3 receptors, the effects of a more selective 5-HT_4 receptor antagonist, SDZ 205-557 (0.1 μM) (Buchheit *et al.*, 1991), were examined. However, this compound, when applied in the middle chamber, had no significant effect on the excitatory responses to distension or compression in either stimulation chamber (not illustrated), although it was able to block the effects of the 5-HT_4 receptor agonist, cisapride (see below).

Effect of a 5-HT_4 receptor agonist Cisapride (0.1 μM), a 5-HT_4 receptor agonist, applied in the middle chamber substantially enhanced the responses elicited either by distension or by compression in both stimulation chambers. The amp-

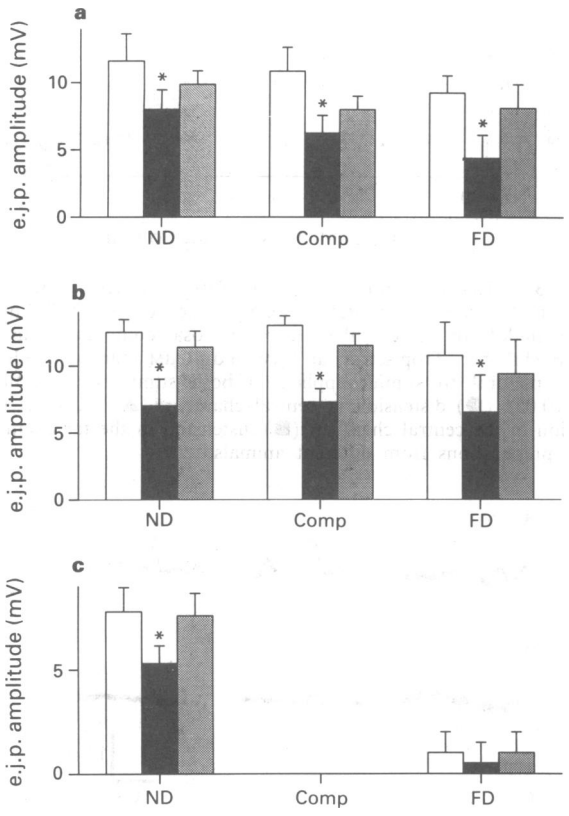


Figure 5 Effect of 5-HT_3 receptor antagonists on ascending excitatory reflexes. (a) Granisetron significantly reduced the amplitudes of e.j.ps evoked by stimuli applied in both stimulation chambers (*$P<0.05$ in middle chamber; $P<0.01$ in far chamber) ($n=4$). (b) BRL 46470 markedly depressed the amplitudes of e.j.ps evoked by stimuli applied in both stimulation chambers (*$P<0.02$ in middle chamber; $P<0.01$ in far chamber) ($n=4$). (c) The depression of e.j.ps amplitudes by granisetron persisted for distensions applied in the near distension chamber after removal of mucosa to prevent the release of 5-HT from enterochromaffin cells (*$P<0.05$) ($n=5$). The reflexes evoked by compression were abolished. Open columns, responses in control solution; solid columns, responses in the presence of the drugs; cross-hatched columns, responses after 15 min washout. ND, distension in central chamber (near distension): Comp, mucosal compression in the central chamber; FD, distension in the far chamber.

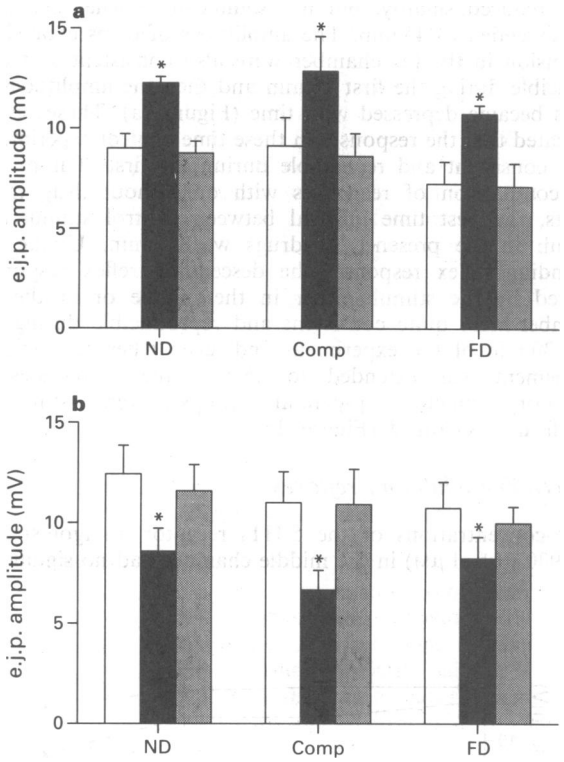


Figure 6 Effect of a 5-HT_4 receptor agonist and an antagonist on ascending excitatory reflexes. (a) Cisapride significantly enhanced the amplitudes of e.j.ps evoked by the stimuli applied in either stimulation chamber (*$P<0.05$ for distension; $P<0.01$ for compression) ($n=4$). (b) In the presence of SDZ 205-557, the effect of cisapride was converted to a significant depression (*$P<0.05$ for distension; $P<0.01$ for compression) ($n=6$). Open columns, responses in control solution; solid columns, responses in the presence of the drugs; cross-hatched columns, responses after 15 min washout. ND, distension in central chamber (near distension); Comp, mucosal compression in the central chamber; FD, distension in the far chamber.

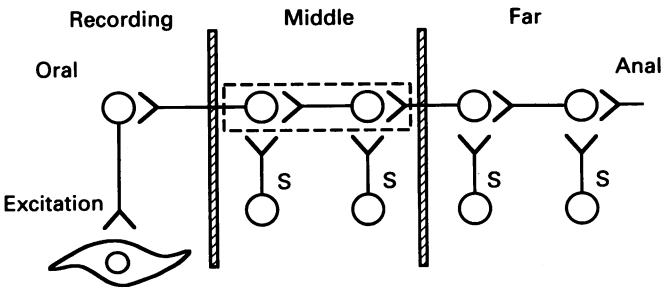


Figure 7 Diagram of the neuronal circuit underlying the ascending excitatory reflex in guinea-pig ileum (Furness & Bornstein, 1993). Sensory neurones (S) provide inputs to ascending interneurones that form chains with outputs to excitatory motor neurones that innervate the circular muscle. The hatched bars show the locations of the partitions that separated the independently perfused compartments. The possible sites of action of the drugs are shown by dashed lines.

litudes of the excitatory responses to the stimuli in both stimulation chambers increased by about 30–50% of control level ($P<0.05$ for distension stimulus; $P<0.01$ for compression stimulus) (Figure 6a).

This effect of cisapride was due to its agonist action on 5-HT$_4$ receptors because it was reversed when these receptors were blocked by SDZ 205-557. Control responses were obtained in the presence of SDZ 205-557 (0.1 μM) to block 5-HT$_4$ receptors in the middle chamber. After cisapride (0.1 μM) was administered in the same chamber for 15 min the responses evoked, either by distensions in middle and far chambers or by compression in middle chamber, were no longer enhanced, but were significantly depressed by cisapride presumably acting via 5-HT$_3$ receptors. Cisapride, in the presence of SDZ 205-557, reduced the excitatory responses to 60–77% of control level ($P<0.05$ for distension stimulus; $P<0.01$ for compression stimulus) (Figure 6b).

Vehicle solution without cisapride was used to repeat the experiments on ascending and descending reflexes. DMSO alone had no effect on i.j.ps or e.j.ps elicited by the stimuli tested. The 5-HT receptor agonist and antagonists used in these experiments did not significantly change the resting membrane potential recorded from the circular muscle or evoke reflex responses.

Discussion

In previous studies, it was difficult to deduce at which points in reflex pathways drugs that affect 5-HT receptors were acting. In contrast, the divided organ bath system used in this work has made it possible for drugs to be applied to separate compartments in such a way that whether a drug acts on sensory neurones, interneurones or motor neurones can be deduced.

The present work revealed consistent effects of antagonists of 5-HT$_3$ receptors on enteric reflexes, despite the fact that previous pharmacological attempts to implicate 5-HT as a transmitter in these reflexes had been inconclusive. Early experiments had tried to uncover a role for 5-HT by depleting its stores in the intestine. 5-HT was depleted by treatment with reserpine (Bülbring & Crema, 1959) or by a tryptophan-free diet (Boullin, 1964). Nevertheless, no effects on intestinal reflexes were found. Although 5-HT receptor antagonists were tested in the years following the first serious suggestion that 5-HT is an enteric transmitter (Gershon *et al.*, 1965; Gershon & Ross, 1966; Bülbring & Gershon, 1967), no convincing pharmacological evidence of a transmitter role of 5-HT in motility reflexes was forthcoming (Furness & Costa, 1982; Furness *et al.*, 1987; Tonini *et al.*, 1991). More recently, immunohistochemical studies have shown that within the small intestine of the guinea-pig, neurones containing 5-HT are descending interneurones which form ultrastructurally identifiable synapses on the cell bodies and dendrites of myenteric neurones (Furness & Costa, 1982; Erde *et al.*, 1985; Young *et al.*, 1993), but no 5-HT terminals were found in the muscle layers. These results indicated that the physiological site of action of neuronal 5-HT was the neurone. Pharmacological evidence suggested that the most likely role of 5-HT would be as an excitatory transmitter, possibly mediating a slow excitatory postsynaptic potential (e.p.s.p) in myenteric neurones (Wood & Mayer, 1978; 1979; Galligan *et al.*, 1988; Mawe *et al.*, 1989). It would thus be anticipated that receptor antagonists for 5-HT would inhibit descending reflexes, because the interneurones that contain 5-HT run anally. We were thus surprised to discover that the inhibitory effects of antagonists for 5-HT$_3$ receptors were selective for orally directed reflex pathways. This suggests that the effects of the antagonists were not a consequence of blocking the actions of 5-HT released from enteric neurones. Indeed, this study provides no support for the idea that 5-HT released from enteric neurones plays a role in motility reflexes.

The lack of correlation of pharmacological observations with the projections of 5-HT neurones suggest that the 5-HT$_3$ receptor antagonists used in our experiments blocked the action of an unknown endogenous ligand released as the synapses in ascending reflex pathways. Three 5-HT$_3$ receptor antagonists all significantly depressed ascending reflex responses but not descending reflexes evoked by either distension or mucosal compression. The extent of the depression was similar whether the drugs were in the middle chamber from which the reflex was initiated or the tissue was exposed to antagonists in the middle chamber and the reflex was evoked from the far chamber (Figure 5a and b). This suggests that the site of the antagonism within the reflex pathway is at neuro-neuronal connections, probably between ascending interneurones (Figure 7). In addition, our results show that the depression of the response to distension by 5-HT$_3$ receptor antagonists remained even after removal of the mucosa, which suggested that 5-HT$_3$ receptors on the nerve terminals in the mucosa did not play a key role in initiating the excitation of the reflex pathways activated by distension. Removal of the mucosa did, however, appear to depress the reflexes evoked by distension in the far stimulation chamber. This observation contrasts with an earlier observation that removal of the mucosa does not affect either initiation of distension reflexes or propagation of reflexes past the region from which the mucosa had been removed (Smith & Furness, 1988; Smith *et al.*, 1990). The major difference between these experiments is in the use of the partitions that divided the organ bath in the present study. It may be that the mucosa normally cushions the partitions, preventing them compressing nerve trunks running in the outer muscle coat, and that when the mucosa is removed the nerve trunks are compressed, thus interrupting the reflex pathways. Although 5-HT$_3$ receptors in the mucosa do not appear to have a role in initiation of the reflexes, our data do not exclude the possibility that putative 5-HT$_{1p}$ receptors on nerve terminals in the mucosa may influence the reflex responses to distension (Kirschgessner *et al.*, 1992). The hypothesized ligand could be an excitatory cotransmitter or a modulator with excitatory presynaptic action.

An earlier study also suggested that a 5-HT-like transmitter might be involved in transmission along the ascending excitatory reflex pathway in the guinea-pig colon (Costa & Furness, 1976). In this region, immunohistochemical studies also show the 5-HT neurones to be in descending pathways (Wardell *et al.*, 1993), suggesting the endogenous transmitter of the ascending pathway in the colon is not 5-HT. If the endogenous substance that acts on 5-HT$_3$ receptors in the ascending pathway is not 5-HT, the failure of depletion of 5-HT stores (Bülbring & Crema, 1959; Boullin, 1964) to affect the peristaltic reflex might be explained.

The 5-HT$_4$ receptor antagonist, SDZ 205-557 (Buchheit *et al.*, 1991) had no action by itself on the peristaltic reflex, clearly implying that endogenous 5-HT acting via 5-HT$_4$ receptors does not play an important role in the peristaltic reflex. However, these receptors can be activated by adding exogenous 5-HT or its agonists (Craig & Clarke, 1991). Cisapride, for example, in the present experiments facilitated the ascending excitatory reflex, consistent with its prokinetic actions (Schuurkes *et al.*, 1988; Tonini *et al.*, 1989). This effect of cisapride can be converted to a significant depression of the ascending excitatory reflexes by SDZ 205-557. The depression is probably via an antagonist action of cisapride at 5-HT$_3$ receptors. Thus, cisapride, but not endogenous 5-HT, had a facilitating action on the peristaltic reflex via 5-HT$_4$ receptors, which probably were located on ascending interneurones.

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Study of the effector mechanism involved in the production of haemorrhagic necrosis of the small intestine in rat passive anaphylaxis

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1 The effector mechanism of intestinal necrosis in rat anaphylaxis was studied following several complementary approaches: (i) the use of monoclonal antibodies (mAb) belonging to different classes (IgG1, IgG2b and IgE anti-DNP), (ii) the assay of mediators, and (iii) the use of pharmacological tools.

2 Lethality and haemorrhagic necrosis of the small intestine were observed in IgE-sensitized rats, whereas IgG mAb produced milder physiological disturbances.

3 Inhibition of leukotriene biosynthesis reduced the drop of systemic blood pressure (BP) and the extent of protein-rich plasma exudation but it did not influence the haemorrhagic component of intestinal necrosis.

4 The antihistamine, pyrilamine, partially diminished the haemorrhagic component of the intestinal necrosis.

5 The involvement of mediators related to platelet-activating factor (PAF) was studied by examining the pharmacological effects of these autacoids and of PAF-receptor antagonists (PCA4248, UR12460 and BB823). PAF induced intestinal lesions similar to those observed in IgE-sensitized rats and PAF-receptor antagonists markedly decreased haemorrhage in IgE-sensitized rats.

6 PAF levels were transiently increased after dinitrophenol (DNP)- bovine serum albumin (BSA) challenge in the small intestine of IgE-sensitized rats.

7 These data stress differences in the outcome of anaphylaxis related to the type of receptors for the Fc portion of immunoglobulins that are involved. IgE is the antibody class that elicits the most severe response due to the activation of mast cells via FcεRI (surface receptors that bind IgE antibodies with high affinity), and the only one able to produce intestinal haemorrhagic necrosis.

8 The mast-cell-derived mediators PAF/acyl-PAF and histamine, most probably associated with tumour necrosis factor α/cachectin (TNF-α), seem to play a central role in the production of the vascular changes required for the extravasation of erythrocytes in the small intestine mucosa.

Keywords: Anaphylaxis; endotoxin; histamine; IgE; ischaemia; leukotrienes; PAF; reversed passive Arthus reaction; shock; tumour necrosis factor

Introduction

The analysis of the cascade of chemical mediators that is triggered following antigen-antibody interaction in anaphylactoid reactions may be hampered by the diversity of potential effector mechanisms, e.g., mediators released by mononuclear phagocytes are important effectors of the vascular disturbances produced by soluble immune complexes and IgG-aggregates (Sánchez Crespo *et al.*, 1985; Doebber *et al.*, 1986; Fernández-Gallardo *et al.*, 1988), and complement-derived anaphylatoxins amplify cardiac anaphylaxis (del Balzo *et al.*, 1989) and activate platelets in the anaphylactoid syndromes elicited by either soluble aggregates of IgG or endotoxin (Fernández-Gallardo *et al.*, 1989; Smedegård *et al.*, 1989). Neither these findings nor the demonstration that mast cells are not required for the cardiopulmonary changes associated with certain anaphylactic reactions (Takeishi *et al.*, 1991) reduce the central role of this cell type in IgE anaphylaxis, that has been emphasized by studies on genetically mast cell-deficient mice. These animals exhibit limited cardiopulmonary changes after challenge with anti-IgE (Martin *et al.*, 1989), as well as significantly decreased neutrophil accumulation, oedema and haemorrhage in the reversed Arthus reaction as compared to congenic controls (Zhang *et al.*, 1991). It has been established that the gut is the main organ of shock in rat anaphylaxis (Sanyal & West, 1958; Fell *et al.*, 1961; Miller *et al.*, 1983). In keeping with this idea, intestinal mast cells are the major source of mast-cell protease II (King *et al.*, 1986) and leukotrienes (Moqbel *et al.*, 1986) in *Nippostrongylus brasiliensis*-primed rats subjected to i.v. challenge of antigen. Moreover, we have reported high amounts of leukotrienes and detectable amounts of platelet activating factor (PAF, 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) in the peritoneal cavity of rats with intestinal necrosis after IgE anaphylaxis (Fernández-Gallardo *et al.*, 1992). The development of hybridoma cell lines (Liu *et al.*, 1980) that produce dinitrophenyl (DNP)-specific murine IgE, IgG1 and IgG2b has made it possible to obtain large amounts of a single class of monoclonal antibodies (mAb) to be used in rats. The use of IgE mAb permits a passive sensitization of circulating basophils and tissue mast cells that spares activation of the complement system; although the activation of mononuclear phagocytes through FcεRII/CD23 may occur (Capron *et al.*, 1977; Rouzer *et al.*, 1982; Wei *et al.*, 1986). Alternatively, the use of either IgG1 or IgG2b allows activation of mononuclear phagocytes through FcγRII (surface receptors that bind IgG1 and IgG2b antibodies), whereas these antibodies show limited ability to activate serosal mast cells (Katz *et al.*, 1992; Pellón *et al.*, 1993). The purpose of this study was to determine the array of chemical mediators involved in the production of intestinal haemorrhagic necrosis in rat passive anaphylaxis. To overcome the difficulties of the analysis, we have designed an experimental approach

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which includes: (i) the use of monoclonal antibodies of the same specificity but belonging to different classes: (ii) the assay of chemical mediators that *a priori* appeared to be involved, and (iii) the use of pharmacological tools.

Methods

In vivo *experimental design*

Male Sprague-Dawley rats of about 200 g body weight were passively sensitized with mAb IgE by i.p. administration in 0.2 ml of a phosphate-buffered isotonic saline solution, pH 7.4, and used for experiments within a period of 24 h (Leng *et al.*, 1988). The solution injected contained, unless otherwise stated, about 3 mg of total protein as assessed by the method of Bradford (1976). After testing several schedules, including the i.p. route, rats sensitized with either IgG1 or IgG2b showed maximal response when the antibody was administered i.v. 1–2 h before i.v. challenge with DNP-BSA. Rats were anaesthetized by i.p. injection of Nembutal at a dose of 50 mg kg^{-1}. A femoral artery was cannulated for mean BP recording. A jugular vein was used for infusion of drugs and collection of blood samples. The experimental protocol was approved by Institutional Review Board. Platelets were counted in blood samples of 44.5 µl anticoagulated with EDTA-K_2 using a Coulter counter.

Evaluation of protein-rich plasma extravasation

Vascular permeability changes were evaluated by the Evans blue (EB) extravasation method, as described by Jancar *et al.* (1991). The EB was injected into the jugular vein together with DNP-BSA, and blood samples were taken at different times to assay the concentration of the dye in plasma. Rats were exsanguinated 15 min later, except when otherwise stated. The peritoneal cavity was washed with 5 ml of saline solution, and the pancreas, duodenum, jejunum-ileum, and thymus, dissected, weighed, and a fragment of each organ put in formamide (4 ml g^{-1} wet tissue at 20°C for 24 h) to extract the EB. The concentration of EB was determined by spectrophotometry at a wavelength of 620 nm. The results were plotted on a standard curve of EB, and the content of each sample was expressed as µg g^{-1} of dry weight tissue or per ml of lavage fluid.

Extraction and quantitation of haemoglobin from intestinal tissue

The concentration of haemoglobin was determined colorimetrically by the cyanomethaemoglobin method (van Kampen & Ziljtra, 1961) using reagents from Sigma, according to the modifications carried out by Tavares de Lima *et al.* (1992). Fragments of the ileal-jejunal portions of the small intestine were excised and minced in 2 ml of potassium cyanide and hexacyanoferrate solution. After 24 h at room temperature in the dark, the tissue was removed, the sample was centrifuged and the optical density of the supernatant was determined spectrophotometrically at 546 nm. The concentration of haemoglobin was calculated by comparison with a standard curve.

PAF assay and characterization

Minced portions of small intestine taken immediately after DNP-BSA challenge were immersed in cold methanol and allowed to stand for 30 min with gentle shaking. After centrifugation at 3,000 *g* for 20 min, the total lipid was extracted and processed as described (Fernández-Gallardo *et al.*, 1992). Briefly, after evaporation to dryness of the lipid extract, this was dissolved in the medium used as a mobile phase for straight-phase h.p.l.c. (high performance liquid chromatography). This was carried out in a Spheri-5 silica column using as a mobile phase A[96% propan-2-ol/hexane, 1/1, supplemented with 0.005% acetic acid] and B[4% water] which was linearly increased to 8% during a 15 min period (Blank & Snyder, 1983). The column was eluted for 60 min at 2 ml min^{-1}, and the fractions collected, evaporated to dryness, and resuspended in 100 µl of a HEPES-saline buffered medium supplemented with 0.25% BSA to carry out a bioassay on rabbit platelets according to the procedure described by Henson (1976). The amount of PAF contained in the samples was corrected for recovery and expressed in ng as assessed from comparison with the activity of the standard. The characterization of the platelet-activating activity as PAF was based on its retention time in h.p.l.c., the lability of the activity to previous treatment with phospholipase A_2, resistance to treatment with phospholipase A_1, and blockade of the platelet-activating ability by the PAF-receptor antagonists used throughout the study.

Assay of haemolytic complement

Total haemolytic activity was measured in rat serum by using sheep erythrocytes and haemolytic serum against sheep erythrocytes raised in rabbits (Hudson & Hay, 1976).

Materials and drugs

Monoclonal anti-DNP IgE, IgG1 and IgG2b were obtained from secreting hybridomas grown as ascites tumours in BALB/c mice and were purified by ammonium sulphate precipitation followed by chromatography. The amount of specific antibody was calculated from classical precipitation reactions using antigen and several dilutions of the antibody solution. In the case of IgE anti-DNP the estimated content of mAb was consistent with the measure of protein in the peak eluting with the M_r of IgE in FPLC gel filtration chromatography using a Superose-12 column (Pharmacia-LKB, Uppsala, Sweden). IgG1 was purified by affinity chromatography in a Protein A-Sepharose CL4B column, and IgG2b was purified by anion exchange FPLC in a MonoS HR5/5 column (Pharmacia LKB, Uppsala, Sweden). 1-Hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF), 1-oleoyl-2-acetyl-*sn*-glycero-3-phosphocholine (acyl-PAF), and pyrilamine were from Sigma, Chemical Company St. Louis, MO, U.S.A. DNP-BSA containing 8 mol of DNP per mol of BSA (bovine serum albumin) was prepared according to the method of Eisen (1964). PCA4248 (2-[phenylthio]ethyl 5-methoxycarbonyl-2, 4, 6-trimethyl-1, 4-dihydropyridine-3-carboxylate) was provided by Alter Laboratories, Madrid, Spain, and solubilized as described (Ortega *et al.*, 1990). UR12460 (1-[(N-diphenylmethyl)aminoacetyl]-4-[(2-methyl-3-pyridyl) cyanomethyl] piperazine) was obtained from URIACH, S.A., Barcelona, Spain, and solubilized in saline solution (Carceller *et al.*, 1993). BB823 (Whittaker *et al.*, 1992), a gift from British Bio-technology, Oxford, U.K., was solubilized by stoichiometric mixture with 1 N HCl to form the hydrochloride salt, and dilution with saline solution. MK886 (3- [1-(4-chlorobenzyl)-3-*t*-butyl-thio-5-isopropylindol-2-yl]-2, 2-dimethylpropanoic acid, [Gillard *et al.*, 1989]) was a gift from Dr C.B. Pickett, Merck Frosst, Pointe Claire-Dorval, Quebec, Canada. MK886 was solubilized in dimethyl sulphoxide and ethanol and diluted in saline solution for i.v. administration.

Statistically analyses

Data are expressed as the mean ± s.e.mean, or mean and range for data points involving three results. For comparison of two groups of samples normally distributed, Student's two tailed *t* test was used to analyse differences for significance. For comparison of two groups of samples not normally distributed, the Mann-Whitney U test was used. Changes of a variable measured along a period of time were analysed by one way analysis of variance (ANOVA). Statistical proce-

dures were performed using a data base and statistical package (Instat, GraphPAD Software Inc., San Diego, CA, U.S.A.), $P<0.05$ was considered significant.

Results

Effect of DNP-BSA challenge on systemic blood pressure (BP)

Animals sensitized with either of the mAb showed a drop of mean BP after i.v. challenge with DNP-BSA. The minimal dose of mAb of either IgG subclass that produced significant hypotension was 3 mg kg^{-1}, and optimal effect was observed with 20 mg kg^{-1}. As regards antigen, optimal hypotension in IgG-sensitized rats required 20 mg kg^{-1}, which taking into account the molar ratio of DNP-substitution and the molecular weight of both antigen and mAb, suggests that optimal expression of the pathophysiological response is observed when the antigen/antibody reaction occurs at antigen excess. This was further confirmed by showing significant hypotensive response when rats were challenged with preformed antigen/antibody complexes in excess of antigen (data not shown). The hypotension observed with IgG1 anti-DNP mAb (Figure 1a) was apparently more prolonged than that produced by the same amount of IgG2b anti-DNP antibody (Figure 1b). In IgE-sensitized rats, challenge with 0.7 mg kg^{-1} DNP-BSA produced a prolonged hypotension which was maintained until the death of the animals (Figure 2). Experiments intended to show pharmacological modulation of the anaphylactic reaction were initially carried out with MK886, an inhibitor of leukotriene biosynthesis, and with PCA4248, a PAF-receptor antagonist. Treatment with either MK886 (5 mg kg^{-1}, i.v., 5 min before antigen challenge) or PCA4248 (2 mg kg^{-1}, i.v., 5 min before antigen challenge), diminished the drop of BP observed in sensitized animals (Figures 1 and 2). The infusion of a bolus injection of PCA4248 after antigen challenge caused a significant increase of mean BP of 28.0 ± 6.1 mmHg ($n = 14$, $P<0.05$). In contrast, MK886 administered after antigen challenge failed to produce this rapid reversal of hypotension, which is consistent with the expected rapidity of effect of a receptor antagonist as compared to an inhibitor of biosynthesis.

Extravasation of EB in peritoneal fluid and selected organs

Accumulation of protein-rich plasma occurred shortly after antigen challenge as judged from both the decay of plasma

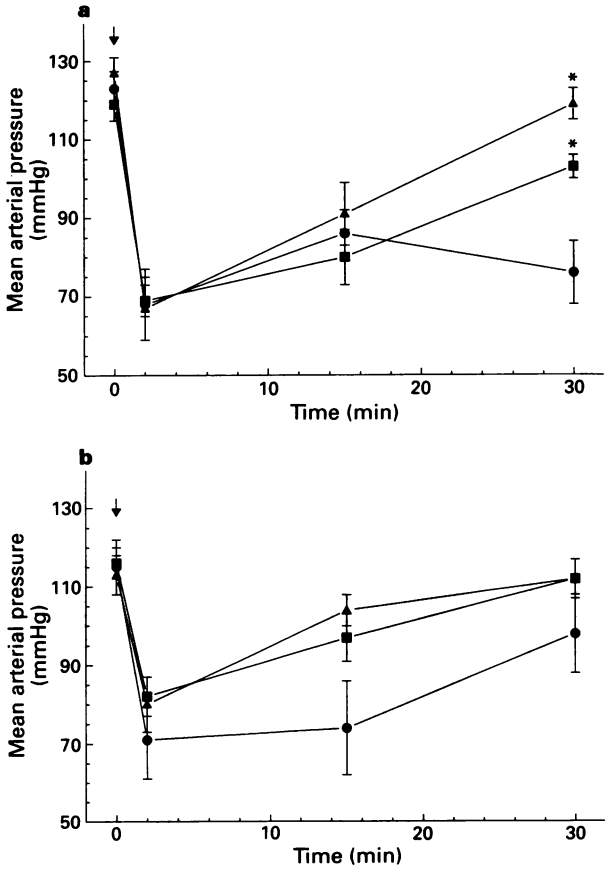


Figure 1 Effect of DNP-BSA on mean arterial pressure in IgG1 (a) and IgG2b (b) sensitized rats, and effect of a previous treatment with MK886 (▲) or PCA 4248 (■). Rats were i.v. sensitized with either IgG class mAb at a dose of 20 mg kg^{-1}, 2 h before challenge with DNP-BSA (20 mg kg^{-1}, arrow). MK886 was i.v. administered (5 mg kg^{-1}), 15 min before antigen challenge. PCA 4248 (2 mg kg^{-1}) or vehicle solutions (●) were i.v. administered 5 min before antigen challenge. Data represent mean ± s.e.mean from 6 to 9 animals in each group. *Indicates $P<0.05$. For abbreviations in this and subsequent legends, see text.

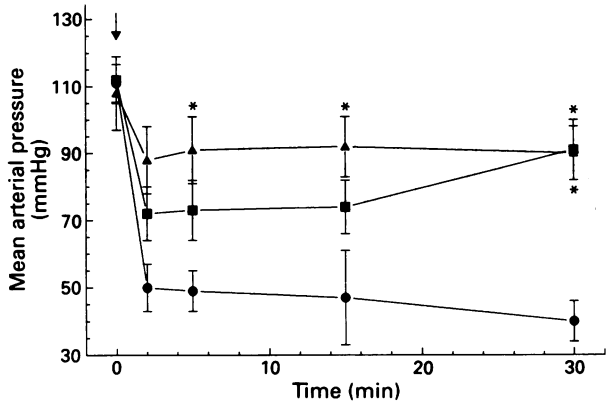


Figure 2 Effect of DNP-BSA on mean arterial pressure in IgE sensitized rats and effect of a previous treatment with MK886 (▲) or PCA 4248 (■). IgE mAb (15 mg kg^{-1}) was i.p. administered 18 h before i.v. challenge with 0.7 mg kg^{-1} DNP-BSA (arrow). See legend to Figure 1 for additional details. Data represent mean ± s.e.mean of 12 to 15 animals in each group.

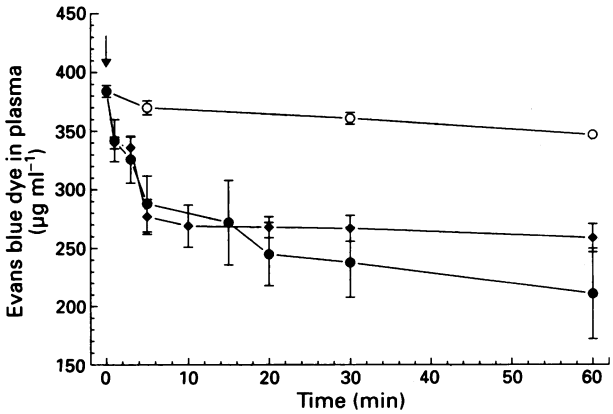


Figure 3 Decay of plasma Evans blue dye (EB) upon DNP-BSA challenge. Rats were sensitized with either IgE mAb (●) or IgG1 mAb (◆). At the times indicated, blood samples of 200 μl were collected and the EB content assessed by spectrophotometric assay; (○) indicate a group of non-sensitized animals challenged with 20 mg kg^{-1} DNP-BSA. The arrow indicates the time of challenge with antigen. Doses of antibodies and antigen are those used for the experiments described in Figures 1 and 2. Data represent mean ± s.e. mean from 6 rats at each point.

EB and its accumulation in tissues. Decay of plasma EB occurred in IgE- and IgG-sensitized rats, but appeared to be longer lasting in the IgE-sensitized rats (Figure 3). The time course of EB accumulation in jejunum-ileum, which is characterized by a rapid increase of dye content up to 10–15 min, and an ensuing drop is shown in Table 1. This can be explained by the accumulation of EB in the peritoneal fluid (Table 2), and in the intestinal lumen because of enhanced mucosal permeability (King & Miller, 1984). Accumulation of EB could be demonstrated in animals sensitized with a dose of IgE mAb as low as 1.5 mg kg^{-1} (not shown). Study of selected organs indicated an increase of the accumulation of EB in mesentery and pancreas up to 60 min, and a reduction after 15 min in thymus (Table 3). To obtain further insight on the chemical mediation of EB accumulation, experiments were carried out with MK886, the antihistamine, pyrilamine, and PAF receptor antagonists (PCA4248, UR-12460 and BB823). EB accumulation was partially inhibited by MK886 at a dose of 5 mg kg^{-1}, i.v., whereas pyrilamine and UR12460 were ineffective (Figure 4 and solid columns in Figure 5).

Table 1 Accumulation of both Evans blue (EB) and haemoglobin in small intestine of rats sensitized with IgE mAb and challenged with DNP-BSA: time-course

Time (min)	*Evans blue dye* (μg g^{-1} dry tissue)	*Haemoglobin* (mg g^{-1} dry tissue)
0	99 (87–116)	33 (31–37)
2	201 (164–269)	89 (70–118)
5	295 (282–317)	93 (80–116)
15	321 (288–361)	80 (72–88)
30	188 (179–196)	78 (67–91)
60	151 (141–162)	85 (74–95)

Rats sensitized with 15 mg kg^{-1} IgE anti-DNP and challenged with 0.7 mg kg^{-1} DNP-BSA were killed at the times indicated and portions of jejunum excised for assay of EB content and haemoglobin accumulation. Data represent mean and range of three animals at each time. EB content and haemoglobin accumulation in rats killed 60 min after challenge with vehicle were similar to those assayed at time 0.

Table 2 Accumulation of Evans blue (EB) in the peritoneum of IgE-sensitized rats: time-course

	Evans blue dye (μg ml^{-1})	
Time (min)	*Antigen challenge*	*Vehicle challenge*
0	0.9 (0–2)	0.9 (0–2)
2	1.1 (0–2)	0.9 (0–2)
5	3 (2–4)	0.9 (1–2)
15	9 (7–12)	–
30	11 (7–14)	1 (1–2)
60	20 (16–22)	1.5 (1–3)

Rats were sensitized with 15 mg kg^{-1} IgE mAb and challenged with either antigen or vehicle. At the times indicated the EB in the peritoneal fluid was assayed. Data represented mean and range of three animals at each time.

Table 3 Accumulation of Evans blue (EB) dye in selected tissues in IgE-sensitized animals: time-course

	Evans blue dye (μg g^{-1} dry tissue)		
Time (min)	*Mesentery*	*Pancreas*	*Thymus*
0	10 (9–12)	52 (40–70)	26 (24–27)
2	22 (11–33)	69 (36–119)	29 (20–41)
5	44 (26–55)	116 (105–129)	160 (145–175)
15	66 (38–90)	228 (179–281)	280 (230–330)
30	78 (58–98)	–	120 (86–157)
60	103 (83–122)	260 (230–295)	121 (102–140)

Data represent mean and range values of groups of three animals sensitized with 15 mg kg^{-1} of IgE mAb killed after challenge with DNP-BSA at the times indicated. Accumulation of EB in the organs of IgE-sensitized rats killed 60 min after challenge with vehicle was 18 (15–23) in the mesentery, 67 (49–81) in the pancreas, and 36 (30–41) in the thymus (mean and range in μg g^{-1} dry tissue).

Small intestine necrosis produced by DNP-BSA

Histological examination of jejunal-ileal portions of small intestine from DNP-BSA challenged rats showed a normal appearance in 8 out of 12 IgG-sensitized rats, and mild necrotic changes in the remainder animals. IgG1 and IgG2b-sensitized rats presented the same histological pattern (not shown). In contrast the lesions were very marked in IgE-sensitized rats. The prominence of haemorrhagic changes prompted us to use Drabkin's cyanomethaemoglobin assay as a quantitative index of the severity of the lesions. The haemoglobin content in jejunum-ileum of IgE-sensitized rats increased very rapidly after antigen challenge, since maximal levels were observed at 2–5 min and remained without significant reduction up to 60 min (Table 1). This pattern of intestinal changes induced us to carry out fixed-time experiments at 15 min after antigen challenge, since at that time both haemorrhage and EB accumulation showed maximal values. To assess the dependency on the dose of antibody, rats were sensitized with IgE mAb in the range 1.5–30 mg kg^{-1}, surprisingly, both EB accumulation and haemorrhagic necrosis were observed even with the lowest dose. Figure 5 shows the effect of several pharmacological treatments on accumulation of both haemoglobin and EB. MK886 at 5 mg kg^{-1}, given i.v. 30 min prior to antigen challenge showed no significant effect on haemoglobin accumulation in the jejunal-ileal portions, suggesting no effect of 5-lipoxygenase products on the pathogenesis of haemorrhagic changes. In contrast, pyrilamine-treated rats showed a significant reduction of haemoglobin accumulation (67.2 ± 4.8 mg g^{-1} dry tissue, $n = 9$). A set of three PAF-

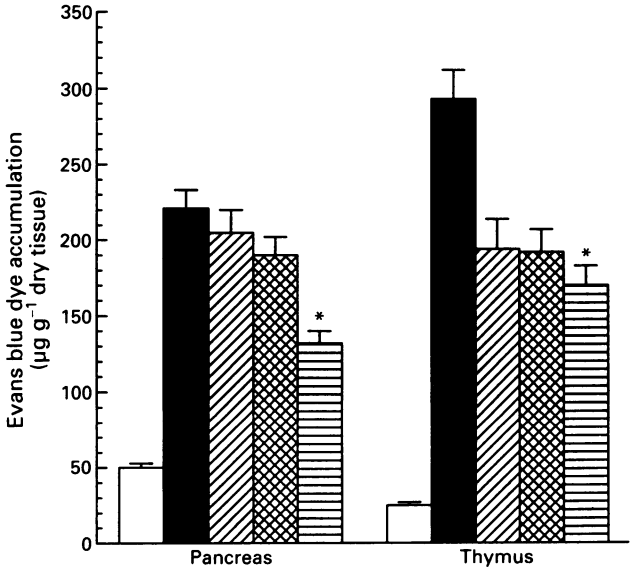


Figure 4 Effect of pharmacological treatments on Evans blue dye (EB) accumulation in pancreas and thymus. Animals were killed 15 min after antigen challenge. Open columns indicate control non-sensitized rats ($n = 10$). Closed columns indicate rats sensitized with 15 mg kg^{-1} IgE mAb ($n = 12$). Cross-hatched columns indicate rats treated with 6 mg kg^{-1} pyrilamine ($n = 9$). Hatched columns indicate rats treated with i.v. UR12460, 5 mg kg^{-1} ($n = 8$). Horizontally lined columns indicate rats treated with 5 mg kg^{-1}, i.v. MK886 ($n = 7$). *Indicates $P < 0.05$ (Student's t unpaired two-tailed test).

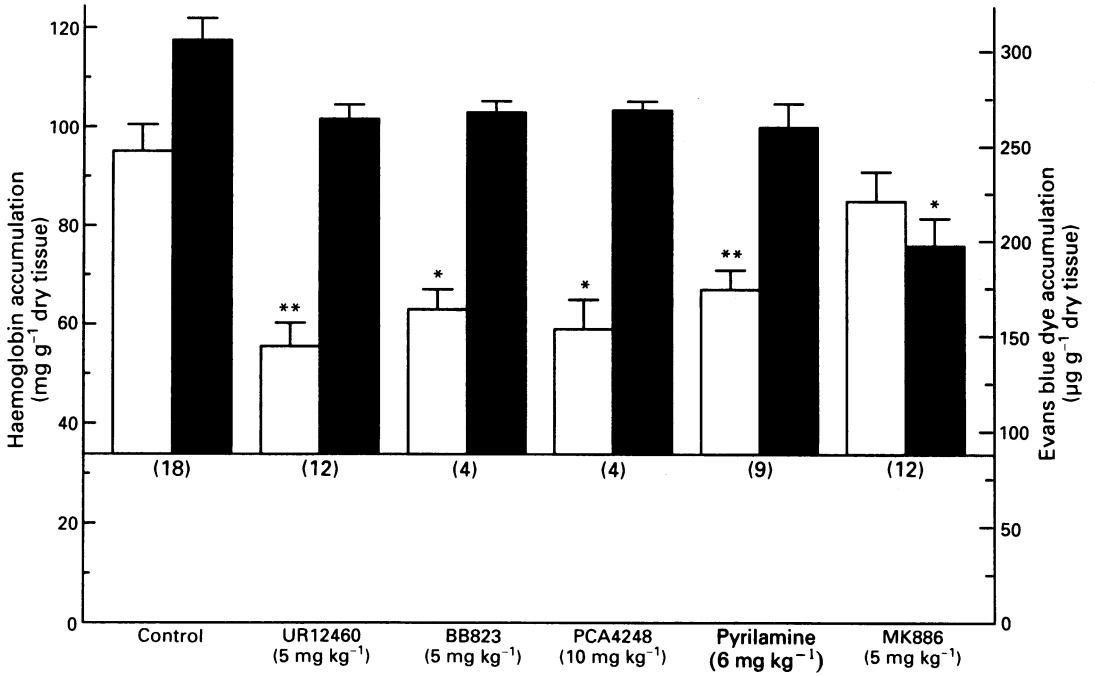


Figure 5 Effect of pharmacological treatments on accumulation of both Evans blue dye (EB) and haemoglobin in the small intestine of IgE-sensitized rats upon antigen challenge. Drugs were administered i.v. prior to antigen challenge at the doses indicated. Figures in parentheses indicate the number of animals in each group. The line at the base of the columns indicates mean control values obtained in 10 IgE-sensitized rats challenged with vehicle. Open columns indicate haemoglobin accumulation. Solid columns indicate EB accumulation. $*P<0.05$; $**P<0.01$.

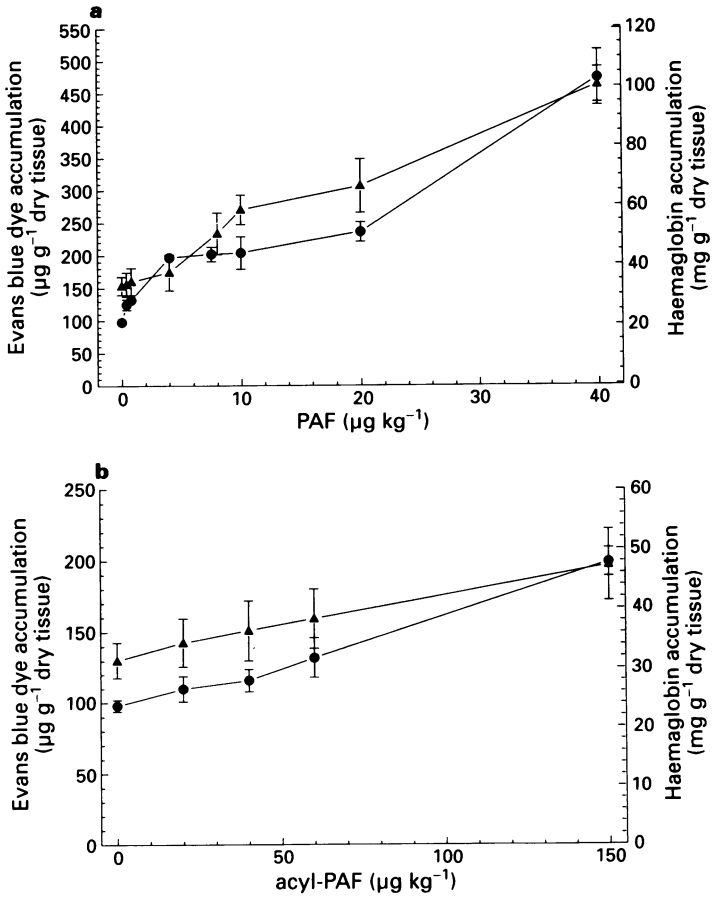


Figure 6 Accumulation of both Evans blue dye (EB) and haemoglobin in small intestine of rats challenged with different doses of PAF (a) and acyl-PAF (b). Animals were killed 15 min after i.v. infusion of either PAF or acyl-PAF. (●) indicate EB content and (▲) indicate haemoglobin accumulation. Data represent mean ± s.e.mean of three animals at each point.

Table 4 Accumulation of Evans blue (EB) dye in selected tissues following i.v. PAF administration: dose-response

PAF (μg kg⁻¹)	*Evans blue dye* (μg g⁻¹ dry tissue) *Mesentery*	*Pancreas*	*Thymus*
Vehicle	10 (9–11)	52 (34–70)	26 (24–28)
0.4	30 (25–33)	155 (114–175)	98 (83–115)
0.8	31 (23–42)	263 (137–337)	110 (94–141)
4	44 (44–45)	256 (216–286)	264 (198–303)
7.5	42 (27–56)	191 (157–221)	203 (160–232)
10	12 (7–17)	136 (121–147)	173 (150–199)
20	14 (8–19)	117 (63–147)	161 (94–217)
40	12 (5–18)	96 (84–108)	106 (59–142)

Data represent mean and range values of groups of three animals killed 15 min after i.v. administration of PAF.

receptor antagonists showed a more significant protection from haemorrhagic changes (55.5 ± 4.8 mg g^{-1} dry tissue, $n = 12$, in the case of UR12460-treated rats). Since the experiments with PAF-receptor antagonists suggested a possible involvement of PAF-like compounds in the production of haemorrhage, the effect of both PAF and acyl-PAF were tested. As shown in Figure 6a, i.v. administration of PAF at doses of 20 μg kg^{-1} caused accumulation of EB and haemoglobin in jejunum-ileum at amounts comparable to those observed in IgE anaphylaxis. A similar result was obtained with 1-acyl-2-acetyl-*sn*-glycero-3-phosphocholine (acyl-PAF), although the study of dose-dependency exhibited a lower pharmacological potency (Figure 6b). As shown in Table 4, maximal accumulation of EB induced by PAF in mesentery, pancreas and thymus was observed with a dose of 4 μg kg^{-1}, which is slightly different from the dose-dependency obtained in small intestine, which showed maximal responses at 40 μg kg^{-1} (Figure 6a).

Assay of PAF and complement consumption

Elevation of PAF levels above those detected in non-challenged rats could be detected only at 30 s and 2 min after

DNP-BSA challenge, whereas they returned to near basal levels 5 min thereafter (Figure 7). Significant complement consumption was observed in rats sensitized with IgE, but not in those sensitized with IgG1 or IgG2b, as judged from the measure of CH_{50} at different times after DNP-BSA challenge. As shown in Figure 8, IgE-sensitized animals showed a drop from 103 ± 13 to 67 ± 12 CH_{50} u ml^{-1}, whereas IgG2b-sensitized animals only showed a drop from 110 ± 4 to 89 ± 17 (Table 5). Platelet count was influenced by antigen challenge in IgG2b sensitized rats ($803,444 \pm 67,550$ cells μl^{-1} before antigen challenge, versus $604,714 \pm 40,696$ cells μl^{-1} 15 min thereafter); however, these changes were nonsignificant (one-way ANOVA).

Discussion

Acute inflammation is characterized by oedema involving extravasation of plasma proteins and cellular infiltration, and only in some circumstances haemorrhage and necrosis occur. This may be the result of complement-dependent neutrophil-mediated injury as in the Arthus reaction (Arthus, 1903), but it may also develop because of an enhanced expression of endothelial-leukocyte adhesion, molecules triggered by cytokines as in the Sanarelli-Shwartzman reaction (Movat et al., 1987). Since these reactions show mechanistic differences with immediate hypersensitivity reactions, it seems of interest to delineate the effector mechanism of intestinal necrosis in anaphylaxis. It is not surprising that haemorrhage and exudation show different pharmacological modulation, because exudation commonly occurs without bleeding, and is impeded by severe vasoconstriction and stasis (Leng et al., 1988; Ramos et al., 1992). In this regard the intravenous injection of PAF into rats produced an accumulation of EB labelled plasma proteins and haemoglobin in the small intestine increasing with increasing doses of PAF up to 40 μg kg^{-1}. In contrast, accumulation of EB in mesentery, pancreas and thymus reached a maximum at doses of 4 μg kg^{-1} and decreased at higher doses. A likely explanation for these findings would be that the rate of extravasation in the small intestine and into the peritoneal cavity at the higher doses of PAF reduced the amount of EB in the blood that was available for accumulation in the other tissues. Alternatively, the haemodynamic changes produced by the highest doses of PAF could influence the distribution of EB.

The results obtained using mAb of different classes indicated that circulatory disturbances can be elicited by activation of either Fcε or Fcγ receptors (Ravetch & Kinet, 1991), but the magnitude of the lesions observed in IgE-sensitized rats points to a central role for FcεRI on mast cells. The rapid appearance of haemorrhage after antigen challenge suggests the involvement of either preformed mediators or secondary mediators rapidly generated upon stimulation of mast cells. Interestingly, the histological study showed strong similarities with the lesions produced by tumour necrosis factor-α/cachectin (TNF-α) (Tracey et al., 1986; Sun & Hsueh, 1988), PAF (González-Crussi et al., 1983) and lipopolysaccharide (LPS) (Hsueh et al., 1986). The potential of acyl-PAF to induce intestinal damage requires more attention: first, this compound is rapidly produced by mast cells upon IgE-dependent activation (Triggiani et al., 1990; 1991; 1992), and also by endothelial cells in response to a variety of autacoids including histamine

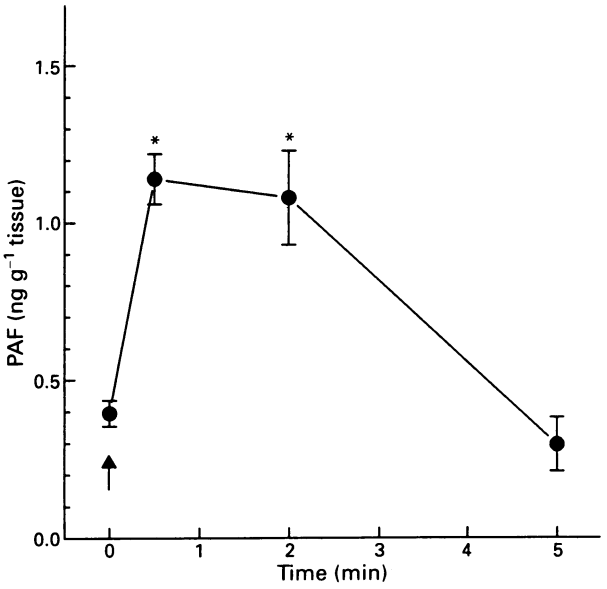


Figure 7 PAF content in small intestine samples from IgE-sensitized rats. Animals were killed before or at different times after DNP-BSA challenge (arrow). A portion of the jejunal-ileal portion of the small intestine was excised, minced and immersed in cold methanol as described under Methods. Data represent mean ± s.e.mean from 4 rats at each time. $*P < 0.05$ (one-way ANOVA).

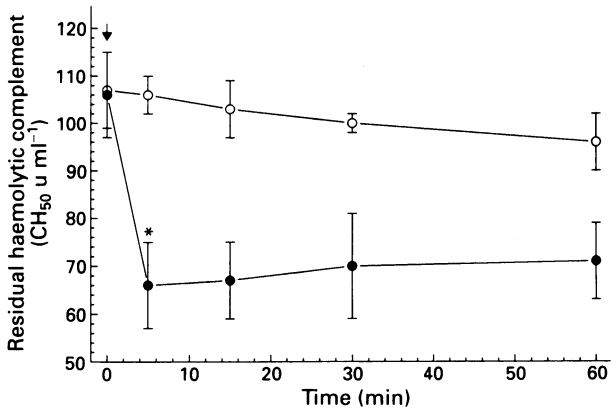


Figure 8 Complement consumption in IgE-sensitized rats after DNP-BSA challenge. Blood samples were collected at the times indicated. (●) represent sensitized-animals challenged with antigen; (○) indicate non-sensitized rats challenged with antigen. The arrow indicates the time of antigen challenge. Data represent mean ± s.e. mean of 8 animals at each time.

Table 5 Effect of antigen challenge on residual complement activity and platelet count in IgG1- and IgG2b-sensitized rats

Time	*IgG1-sensitized*		*IgG2b-sensitized*		*Controls*	
(min)	(CH_{50} u ml^{-1})	(Platelets μl^{-1})	(CH_{50} u ml^{-1})	(Platelets μl^{-1})	(CH_{50} u mol^{-1})	(Platelets μl^{-1})
0	114 ± 4	919,875 ± 58,672	110 ± 4	803,444 ± 67,550	110 ± 5	846,987 ± 47,231
5	93 ± 10	745,625 ± 57,534	89 ± 17	668,625 ± 64,220	107 ± 6	823,654 ± 42,324
15	100 ± 10	717,750 ± 97,786	91 ± 14	605,714 ± 40,696	103 ± 5	815,896 ± 53,987
30	102 ± 9	735,250 ± 97,383	91 ± 16	651,571 ± 65,303	105 ± 7	-
60	105 ± 8	-	88 ± 12	-	107 ± 6	799,564 ± 65,876

Data represent mean ± s.e.mean of 7 to 9 animals in each group. Differences among groups were not significant at any time (ANOVA).

(MacIntyre *et al.*, 1985; Clay *et al.*, 1991); second, the biological actions of acyl-PAF can be blocked by antagonists of the PAF receptor (Smiley *et al.*, 1991); third, acyl-PAF has a reduced activity on the rabbit platelet PAF receptors and this explains why the production of acyl-PAF by either intestinal mucosal mast cells or peritoneal mast cells, which has been mainly assayed with the rabbit platelet bioassay could have been underestimated (Fernández-Gallardo *et al.*, 1992; Hogaboam *et al.*, 1993). Moreover, the probable formation of PAF and/or acyl-PAF by cells in close contact with the vessels and the slow kinetics of accumulation of peritoneal exudate might explain why PAF assay in the peritoneal exudate did not reveal the transient accumulation of PAF in the small intestine shown here. Again, the measure of autacoids in the peritoneum should reflect the secretion products of connective tissue mast cells, whereas the intestinal mucosal mast cells could generate a different array of chemical mediators.

The concurrent involvement of mast cell-derived TNF-α/cachectin in intestinal haemorrhagic necrosis seems likely since mast cells store TNF-α in their granules (Gordon & Galli, 1990), and also because of the ability of TNF-α to induce vessel damage and haemorrhagic necrosis, especially when interactions with other products occur, e.g., bacterial components (Rothstein & Schreiber, 1988) complement factor C5 (Rothstein *et al.*, 1988), or other cytokines (Waage & Espevic, 1988). In addition, an effector role of PAF in the production of intestinal necrosis produced by exogenous TNF-α has been reported (Sun & Hsueh, 1988). However, some differences exist between the lesions produced by TNF-α in rats and those observed in the present experiments. First, the rapid appearance of lesions in IgE-sensitized rats as compared to longer periods in TNF-α challenged rats. Second, the invariable occurrence of infarct in the caecum in TNF-α challenged rats, as compared to a preferential location to the jejunum and ileum in the present experiments. Third, the absence in the present study of occlusion of the vessels by platelet and leukocyte thrombi, which can be partially explained by the lack of PAF receptors in rat platelets (Sánchez Crespo *et al.*, 1982; Humphrey *et al.*, 1984). Nevertheless, as we observed involvement of the caecum in rats challenged with exogenous PAF, it is possible that the different locations of intestinal damage could be explained by the predominant release of endogenous mediators in some intestinal areas, where as exogenous mediators exert their actions throughout the intestine. The involvement of the complement system in rat anaphylactoid syndromes could be suspected in view of the effect of anaphylatoxins on different models of IgE-dependent reactions and the dependence on complement factors of TNF-α and PAF-induced tissue injury (Rothstein *et al.*, 1988; Sun & Hsueh, 1991); however, the results presented would suggest that complement activation is not a sufficient condition for intestinal injury, since the drop of CH_{50} residual complement was less prominent than in the case of infusion of soluble aggregates of IgG (Fernández-Gallardo *et al.*, 1989), and under these conditions there is no evidence of haemorrhagic necrosis. Alternatively, complement consumption could be the consequence of tissue injury rather than its cause, since IgE antibodies have no complement-fixing properties. In summary, our data indicate that different classes of mAb trigger different types of anaphylactoid reactions. IgE is the only antibody class that elicits haemorrhagic necrosis of the small intestine. The release of histamine and PAF/acyl-PAF from mast cells seems to play a central role in the production of necrosis of the small intestine, whereas 5-lipoxygenase-derived products seem involved in leakage of protein-rich plasma. Activation of FcεRII and FcγRII receptors leads to a generation of mediators adequate for the production of vascular disturbances, but not at the amount and/or location required for the production of intestinal haemorrhagic necrosis.

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Modulatory effects of endothelin-1 on purinergic and adrenergic components of sympathetically-mediated contractile activity of rabbit saphenous artery

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1 The present study has been performed to evaluate the modulatory effects of endothelin-1 (ET-1) on the purinergic and adrenergic components of sympathetically-mediated contractile responses of endothelium-free rabbit saphenous artery preparations.

2 ET-1 increased the smooth muscle tone, the pD_2 value being 7.77 ± 0.05.

3 Postjunctionally, ET-1 enhanced the responses to exogenous adenosine 5'-triphosphate (ATP) and did not influence those to exogenous noradrenaline (NA).

4 ET-1 increased the contractile responses to short-lasting and to long-lasting electrical field stimulation at a frequency of 5 or 10 Hz, showing a tendency towards decreasing the prazosin-sensitive component and increasing the mATP-sensitive component of the contractile responses.

5 In prazosin-treated preparations ET-1 increased the residual mATP-sensitive responses and this effect was more pronounced after yohimbine.

6 In mATP-treated preparations ET-1 increased the residual electrically-induced contractions and this increase was abolished after yohimbine.

7 It is suggested that ET-1 modulates co-transmission in the rabbit saphenous artery by potentiating postjunctionally the purinergic component of the contractile responses to both exogenous ATP or electrical stimulation.

Keywords: Endothelin; blood vessels; sympathetic co-transmission; purinergic contractile component; noradrenergic contractile component

Introduction

Endothelin-1 (ET-1), a 21-aminoacid peptide, isolated from porcine aortic endothelial cells (Yanagisawa *et al.*, 1988) has been shown to be one of the most potent vasoconstrictor substances yet discovered. In addition to its direct constrictor effect in vascular (D'Orleans-Juste *et al.*, 1989; Rodman *et al.*, 1989; for reviews see Randall, 1991) and nonvascular (Borges *et al.*, 1989; Maggi *et al.*, 1989; Radomirov & Mutafova-Yambolieva, 1992) smooth muscles, ET-1 appears to have neuromodulatory effects on the autonomic nervous system. In particular, ET-1 suppresses the responses to transmural stimulation in the guinea-pig femoral artery and taenia coli, and in the rat anococcygeus muscle (Wiklund *et al.*, 1988; 1989b) but enhances those in the guinea-pig and rat vas deferens (Maggi *et al.*, 1989; Wiklund *et al.*, 1990; Mutafova-Yambolieva *et al.*, 1992; Mutafova-Yambolieva & Radomirov, 1993) and in the guinea-pig pulmonary artery (Wiklund *et al.*, 1989a). The endothelin-1 action is both prejunctional, since the peptide reduces the outflow of tritiated noradrenaline (NA) from nerve terminals in the guinea-pig femoral artery, pulmonary artery and vas deferens (Wiklund *et al.*, 1988; 1989a,b; 1990) and postjunctional, since ET-1 greatly increases the guinea-pig and rat vas deferens responses to exogenous adenosine 5'-triphosphate (ATP) (Wiklund *et al.*, 1990; Mutafova-Yambolieva & Radomirov, 1993).

There is strong evidence for co-transmission involving a purine (most likely ATP) as well as NA at sympathetic nerve terminals in several blood vessels (Sneddon & Burnstock, 1984; Ishikawa, 1985; Kennedy *et al.*, 1986; Burnstock & Warland, 1987; Bulloch & McGrath, 1988a,b; Bulloch *et al.*, 1991). The question arises as to whether endothelin-1 modulates purinergic and adrenergic components of this transmission process. Investigations into this aspect would add to the knowledge about the ability of endogenous peptide to modulate the processes of sympathetic co-transmission as has been found for neuropeptide Y (Ellis & Burnstock, 1990; Cheung, 1991), angiotensin (Trachte, 1988; Ellis & Burnstock, 1989) and atrial natriuretic peptide (Drewett *et al.*, 1989; Mutafova-Yambolieva *et al.*, 1993).

For this study we have chosen a blood vessel, the rabbit saphenous artery, which exhibits a large purinergic component and where a large α_1-adrenoceptor-mediated component is also evident (Burnstock & Warland, 1987).

Thus, the aim of the present study was to examine the neuromodulatory effects of ET-1 on the purinergic and adrenergic components of the mechanical responses to electrical field stimulation (EFS) or to exogenous ATP and NA in the rabbit isolated saphenous artery.

Methods

General

Male New Zealand white rabbits (2.5–3.0 kg) were killed by a blow to the head and exsanguination. The proximal segment of the saphenous artery was cut into four 4-mm rings. They were cleaned of excessive connective tissue and mounted vertically in 3 ml siliconized organ baths, by inserting two thin threads into the lumen. This procedure has always been shown to damage the endothelium. The preparations were bathed in physiological salt solution (PSS) of the following composition (mM): NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, $MgSO_2.7H_2O$ 1.2, $NaHCO_3$ 24.9, KH_2PO_3 1.2 and glucose

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11.1 (Bulloch *et al.*, 1991), bubbled with 95% O_2 and 5% CO_2 at 37°C. The solution routinely contained ascorbic acid 0.3 mM, to prevent oxidative degradation of NA and indomethacin, 3 mM, to eliminate the influence of cyclo-oxygenase products. Preparations were allowed to equilibrate for 90–120 min under a resting tension of 1.0 g. Contractions of the circular smooth muscle were recorded by a strain gauge (M 1101 B, Microtechna, Prague, Czechia) connected to a recording device (TX 4620, Laboratorni Pristroje, Prague, Czechia).

Control experiments were carried out to examine the effect of the drug vehicles in separate groups of preparations. All the parameters were evaluated comparing the endothelin experiments with the corresponding time control with the relevant vehicle.

In all experiments except for those with cumulative application of ET-1, the peptide was administered only once to each preparation because data of Wiklund *et al.* (1989a) and our preliminary experiments showed that multiple administration of one and the same concentration of ET-1 led to tachyphylaxis.

Experimental design

In all experiments the preparations were initially contracted with NA (1 μM) in order to establish the viability. Once the response had reached a steady state, acetylcholine (1 μM) was added to confirm endothelium denudation. Following washing, the preparations were allowed to equilibrate for 30 min. Then ATP (1 mM) was administered to assess the responsiveness of the preparations to the exogenous purine. Tissues were again washed and allowed to settle for 45 min before being subjected to one of the following treatments.

ET-1-induced contractions Endothelin-1 was applied cumulatively in concentrations of 0.1 to 100 nM at 10–20 min intervals to elicit contractile responses.

Exogenous NA- or ATP-induced contractions Concentration-response curves were constructed to ATP (0.1–4 mM) or NA (0.1–30 μM) in the absence or in the presence of ET-1 at a concentration of 0.1, 10 or 100 nM. At least two equal control responses to ATP or NA were elicited at 30 min intervals with repeated washing in between each concentration-response curve in the absence of ET-1. The peptide was then applied 30 min (0.1 nM) or 10 min (10 or 100 nM) before starting the next concentration-response curve to the relevant agonist. To prevent the possible prejunctional effect of the drugs, the experiments were performed in the presence of 0.3 μM tetrodotoxin (TTX) to block the neuronal input to the smooth muscle cells (Gershon, 1967).

EFS-induced contractions EFS with square-wave pulses delivered from a dual impedance stimulator (Harvard Apparatus Ltd) was applied to the saphenous artery preparations by a pair of platinum ring electrodes placed parallel to, and on either side (top and bottom) of, the vessel. The preparations were electrically stimulated at various frequencies and duration as examples of short-lasting (5 Hz for 1 s and 10 Hz for 1 s) and long-lasting (5 Hz for 10 s and 10 Hz for 20 s) stimuli (50–60 V, 0.3 ms pulse width) to produce contractions. The interval between the pulse trains was 1 min. Stimulations were repeated to ensure consistency of the response. The second component of the biphasic contractions induced by EFS with longer duration was often variable and difficult to assess. In order to standardize the method of calculation, the 'first phase' (I) of the contraction was taken as that response produced in the first 3 s of the onset of stimulation whereas 'the second phase' (II) of the contraction was taken as that maximum response produced after 3 s from the onset of stimulation. The preparations were incubated for 15 min with the α_1-adrenoceptor blocker, prazosin (Cambridge *et al.*, 1977) before any prazosin-resistant responses were elicited and measured. Then the P_{2x}-purinoceptor desensitizing agent α,β-methylene ATP (mATP) (Meldrum & Burnstock, 1983), 5 μM, was added to the organ bath for 15–20 min until the vascular tone was restored to the baseline and subsequently EFS with the above-mentioned parameters was applied again to produce prazosin/mATP-resistant responses. Afterwards, the α_2-adrenoceptor blocker, yohimbine (Starke *et al.*, 1975) (0.1 μM) was administered 15 min before any prazosin/mATP/yohimbine-resistant responses were registered. If any response remained after the treatment, stimulations were repeated following a 10 min incubation with tetrodotoxin (0.3 μM) to determine whether the residual response was neuronal or myogenic. The magnitude of the myogenic component, if any, was then subtracted from the magnitude of all the EFS-induced responses, so that only the changes in the neurogenic component were estimated. Finally, NA (1 μM) and ATP (1 mM) were added to the bath at 3 min intervals to ensure that the α-adrenoceptor- and P_{2x}-purinoceptor-mediated responses were effectively blocked to the end of the experiments.

EFS-induced contractions were recorded in separate groups of experiments during: (i) cumulative ET-1 administration, 10 min after the application of each ET-1 concentration; (ii) single ET-1 administration at a concentration of 0.1 nM for 30 min, 10 nM for 10 min, or 100 nM for 10 min and subsequent administration of prazosin, mATP and yohimbine at the above mentioned time intervals; (iii) single ET-1 administration, followed by mATP-treatment in prazosin-treated preparations; (iv) single ET-1 administration, followed by mATP-treatment in prazosin plus yohimbine-treated preparations; (v) single ET-1 application, followed by prazosin treatment in mATP-treated preparations; (vi) single ET-1 application, followed by prazosin treatment in mATP plus yohimbine-treated preparations.

Drugs

Endothelin-1, adenosine 5'-triphosphate (sodium salt) (ATP), α,β-methylene adenosine 5'-triphosphate (lithium salt) (mATP), guanethidine sulphate, indomethacin, tetrodotoxin (TTX), yohimbine hydrochloride (Sigma Chemical Company, St. Louis, MO, U.S.A.); (−)-noradrenaline bitartrate (Koch-Light Laboratories Ltd., Colnbrook Berks, England); prazosin hydrochloride (Pfizer Ltd., Karlsruhe, Germany). Prazosin was dissolved in propyleneglycol and diluted to a concentration of 100 μM in redistilled water (0.002% final bath concentration of propyleneglycol). Indomethacin was dissolved in phosphate buffer at pH 7.2. All other drugs were dissolved in redistilled water.

Analysis of results

In all experiments concerning contractile responses to exogenous ATP or NA the effects were expressed as percentages of the maximum of the second control concentration-response curve to the relevant agonist. The pD_2 values (negative logarithm of the molar concentration at which half-maximum contraction occurs) for the agonists were calculated for each preparation and averaged within the groups by computer extrapolation (Tallarida & Murray, 1981). The responses to nerve stimulation were expressed either as absolute values (mN) or as percentage of the contractions obtained before the respective treatment. The results were analysed comparing the data of the endothelin experiments with the data of the corresponding time control with the relevant vehicle, using Student's *t* test for unpaired data. Results are expressed as mean ± s.e.mean. Probability of less than 0.05 was considered significant. In all graphs * indicates $P<0.05$; ** $P<0.01$ and *** $P<0.001$. The effect of ET-1 *per se* on the smooth muscle tone was not considered during assessment of the contractions induced either by exogenous ATP or NA or EFS.

Results

Effect of ET-1 on the smooth muscle tone

ET-1, applied cumulatively (0.1–100 nM), produced a concentration-dependent increase of the smooth muscle tone (data not shown), the pD_2 values being 7.77 ± 0.05 nM ($n = 6$). The development of the effect of ET-1 on the tone was slow with concentrations less than 10 nM and required a period of 10–15 min to reach a steady state level. At higher concentrations, the effects of ET-1 developed more rapidly and a steady state level was reached up to the 10th min after drug administration. The maximum contraction achieved by ET-1 100 nM was 26.3 ± 5.0 mN ($n = 6$).

In separate experiments, single application of ET-1 at a concentration of 0.1 nM for 30 min did not change the smooth muscle tone at all, whereas ET-1 at a concentration of 10 nM caused an increase of 3.36 ± 0.83 mN ($n = 4$) and at a concentration of 100 nM caused vasoconstriction of 28.1 ± 0.52 mN ($n = 5$). In all preparations tested acetylcholine, 1 μM, failed to cause vasodilatation in NA-precontracted tissues.

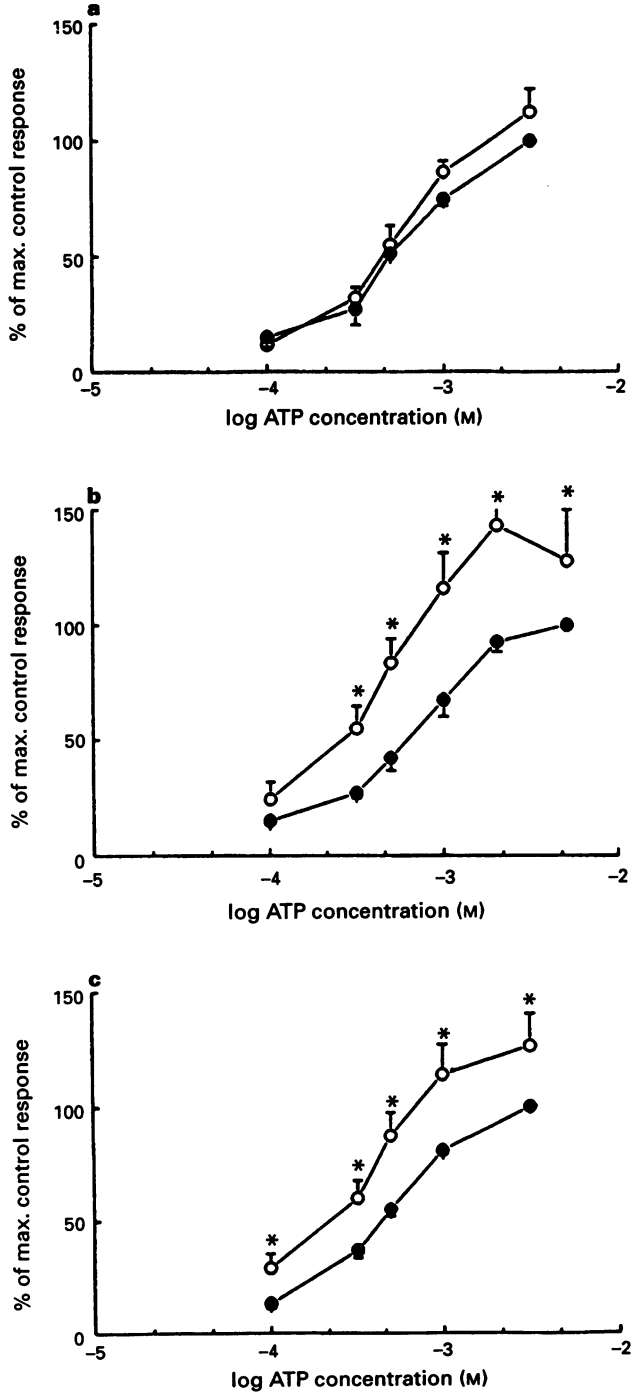


Figure 1 Concentration-response curves to cumulatively applied ATP in the absence of endothelin-1 (ET-1) (●) and in the presence of ET-1 (○) at concentrations of 0.1 nM (a), 10 nM (b), and 100 nM (c). The contractions are expressed as percentages of the maximum control response to ATP. The values are means ± s.e.mean from 4 to 7 experiments.

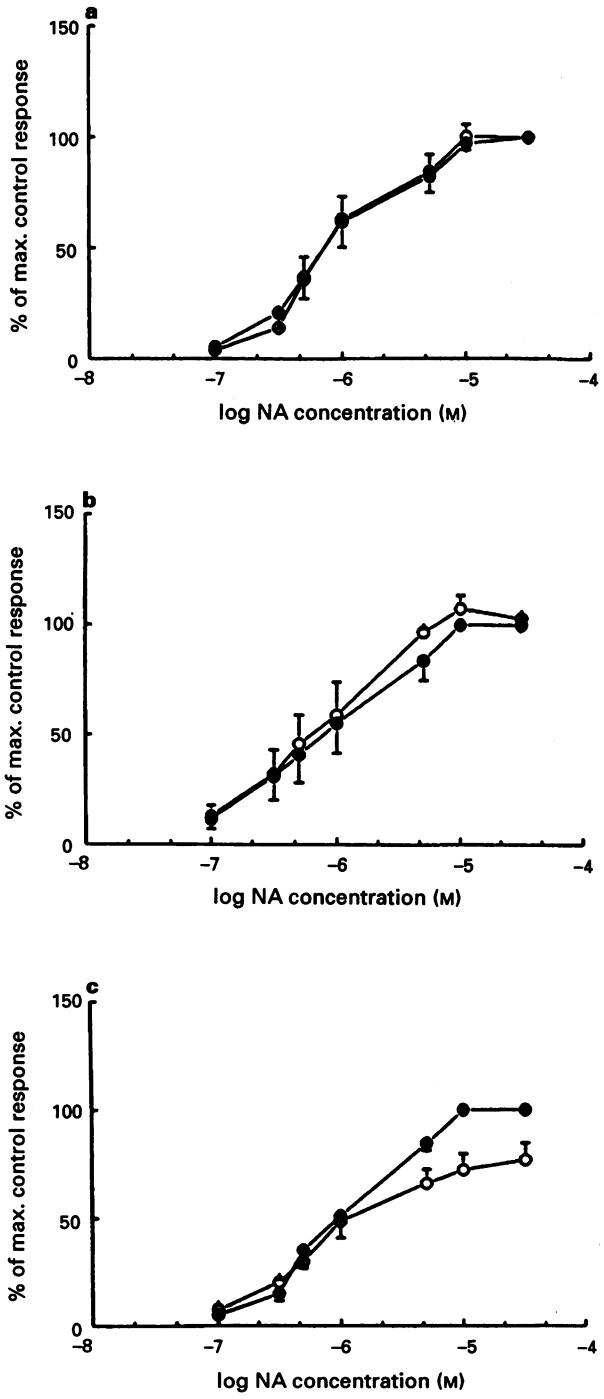


Figure 2 Concentration-response curves to cumulatively applied noradrenaline (NA) in the absence of endothelin-1 (ET-1) (●) and in the presence of ET-1 (○) at concentrations of 0.1 nM (a), 10 nM (b), and 100 nM (c). The concentrations are expressed as percentages of the maximum control response to NA. The values are means ± s.e. mean from 4 experiments.

Effect of ET-1 on ATP-induced contractions

ATP applied cumulatively at concentrations of 0.1 to 30 mM in the presence of 0.3 μM TTX, produced concentration-dependent contractions. The maximum force of the control contractions produced by the highest ATP concentrations was 15.2 ± 1.8 mN ($n = 7$) before 0.1 nM ET-1, 13.7 ± 1.0 mN ($n = 4$) before 10 nM ET-1 and 14.4 ± 2.3 mN ($n = 6$) before 100 nM ET-1. Comparison of the concentration-response curves to exogenous ATP in the presence and in the absence of ET-1 indicated that 0.1 nM ET-1 had no significant effect on ATP-induced contractions, whereas in the presence of ET-1 at a concentration of 10 or 100 nM the concentration-response curves to ATP shifted to the left (Figure 1). In the presence of TTX, ET-1 produced an increase of the smooth muscle tone of 2.94 ± 0.5 ($n = 8$) at a concentration of 10 nM and 22.8 ± 2.1 ($n = 11$) at a concentration of 100 nM.

Effect of ET-1 on NA-induced contractions

NA, applied cumulatively at concentrations of 0.1 to 30 μM in the presence of TTX, produced concentration-dependent contractions. The maximum force of the control contractions produced by the highest NA concentration was 22.2 ± 1.5 mN ($n = 6$) before 0.1 mM ET-1, 17.5 ± 1.0 mN ($n = 4$) before 10 nM ET-1 and 23.87 ± 1.4 mN ($n = 4$) before 100 nM ET-1. Comparison of the curves obtained in response to NA in the presence and in the absence of ET-1 (0.1, 10 or 100 nM) demonstrated that ET-1 had no significant effect on the NA-induced contractions (Figure 2) and produced no significant changes in the pD_2 values of exogenous NA.

EFS-induced contractions to the vessel

Electrical field stimulation applied to the rabbit isolated saphenous artery for 1 s (at 5 and 10 Hz) induced a rapid, monophasic, frequency-dependent contraction (Figure 3). Stimulation at 5 Hz for 10 s or 10 Hz for 20 s elicited a response which varied from preparation to preparation. In all preparations there was an initial rapid transient contraction (first phase) that reached a maximum within 2–3 s. In some preparations this was followed by a sustained contraction that was greater than or almost equivalent to the first phase contraction (Figure 3a,b,c,d) or in others the initial transient contraction was followed by a much smaller contraction. This 'second phase' contraction lasted for the duration of stimulation. All contractions fell back rapidly to base line at the end of the stimulation period and the whole series of stimulations (5 Hz for 1 s, 10 Hz for 1 s, 5 Hz for 10 s, and 10 Hz for 20 s, at 1 min interval between the starts of each stimulation) could be repeated every 15 min without fatigue or desensitization being evident. Figure 4a shows the magnitude of the contractile responses to EFS at all parameters tested. Electrically-induced responses were abolished by TTX (0.3 μM) or guanethidine (10 μM) ($n = 4$) (data not shown), thus demonstrating their neurogenic nature and sympathetic origin.

Neurogenic contractions were compared before (control) and after preincubation of the preparations for 15 min with 1 μM prazosin (Figure 3a, 4b). There was a reduction of all the EFS-induced contractions which was dependent on the parameters of stimulation: at short-lasting stimulation (5 or 10 Hz for 1 s) and in the first phase of the contractions in response to stimulation at 5 Hz for 10 s there was a reduction in the contractions by about 20–40%, whereas at long-lasting stimulation the reduction of the contractions was greater – approximately 70–80%. α,β-Methylene ATP (mATP) almost completely inhibited the residual EFS-induced contractile responses.

Neurogenic contractions were also compared before (control) and after simultaneous administration of prazosin (1 μM) and yohimbine (0.1 μM). The contractile responses to EFS were greater in prazosin plus yohimbine-treated preparations than in preparations treated with prazosin only (Figures 3b, 4c). mATP (5 μM) greatly reduced the residual

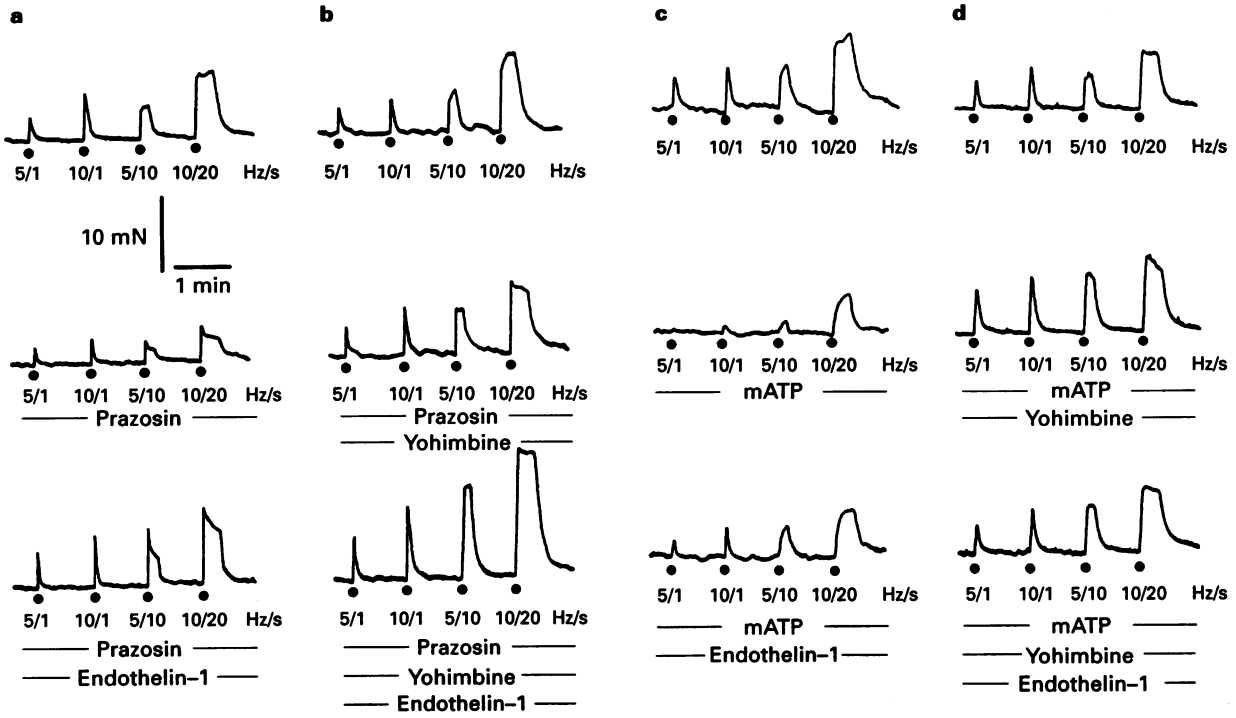


Figure 3 Endothelium-free rabbit saphenous artery. Segments of typical tracings showing the effects of endothelin-1 (ET-1, 10 nM) on the contractions induced by EFS (0.3 ms, 50–60 V, at 5 Hz for 1 s, 10 Hz for 1 s, 5 Hz for 10 s – phase I and II, and 10 Hz for 20 s – phase I and II) in the presence of 1 μM prazosin (a), 1 μM prazosin plus 0.1 μM yohimbine (b), 5 μM α,β-methylene ATP (mATP) (c), and 5 μM mATP plus 0.1 μM yohimbine (d). (●) Onset of the EFS.

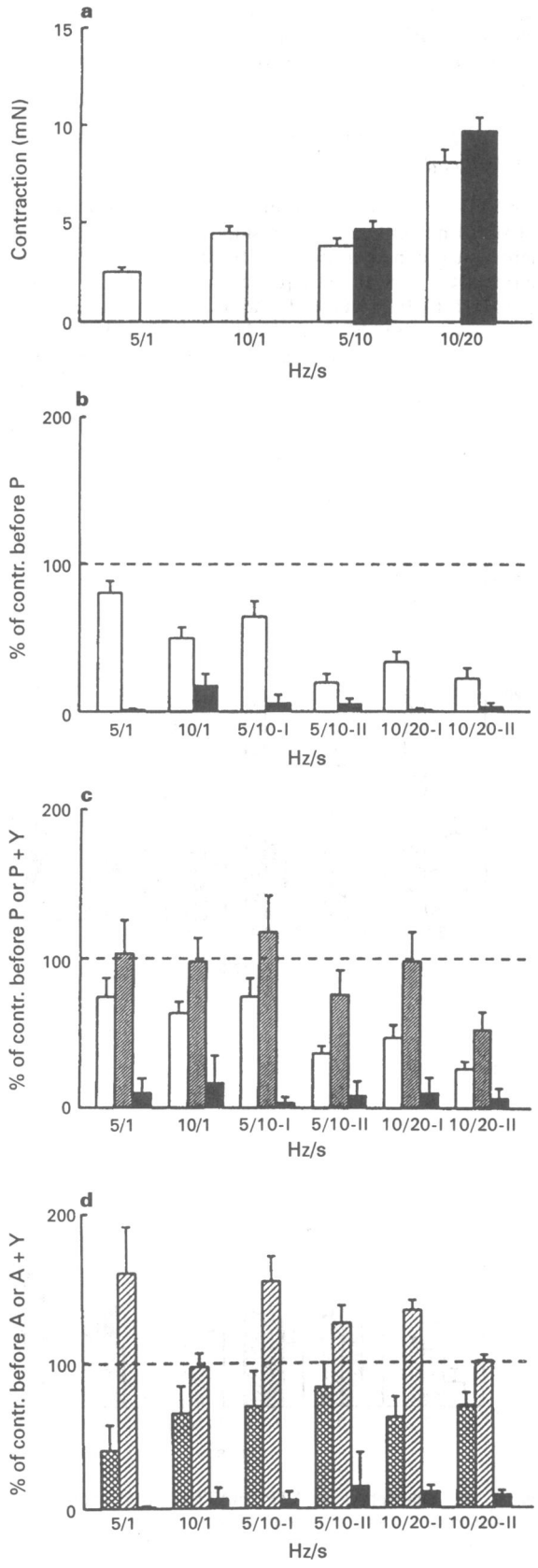


Figure 4 (a) Frequency-dependent contractions to electrical field stimulation in endothelium-free rabbit saphenous arterial rings. Open columns show absolute data from the monophasic contractions to short-lasting pulse trains as well as the first phase of the contractile responses to long-lasting electrical stimulation at frequency of 5 and 10 Hz. Solid columns show the magnitude of the second phase of the contractile responses to long-lasting electrical stimulation. The values are means ± s.e.mean from 98 experiments. (b) The contractile responses to EFS in the presence of 1 μM prazosin, (open columns) followed by 5 μM α,β-methylene ATP (mATP) (solid columns). The responses are presented as percentages of the contractions obtained before the prazosin treatment. The values are means ± s.e.mean from 8 to 12 experiments. (c) EFS-induced contractile responses in the presence of 1 μM prazosin (open columns), 1 μM prazosin plus 0.1 μM yohimbine (hatched columns), and 1 μM prazosin plus 0.1 μM yohimbine plus 5 μM mATP (solid columns), expressed as percentages of the contractions before prazosin (P) alone or prazosin and yohimbine (P + Y) administered together. The values are means ± s.e.mean from 8–13 experiments. (d) EFS-induced contractile responses in the presence of 5 μM mATP, (cross-hatched columns), 5 μM mATP plus 0.1 μM yohimbine (hatched columns), and 5 μM mATP plus 0.1 μM yohimbine plus 1 μM prazosin (solid columns), expressed as percentages of the contractions before mATP (A) alone or mATP and yohimbine (A + Y) applied together. The values are means ± s.e.-means from 9–13 experiments.

contractions in prazosin plus yohimbine-treated preparations.

Exposure to 5 μM mATP caused a transient vasoconstrictor response (approximately 10–15 min in duration) in the rabbit saphenous artery. After restoration of the smooth muscle tone to the baseline, the EFS-evoked contractions were reduced and the reduction was greater at 5 Hz for 1 s, 10 Hz for 1 s and in the first phase of the contraction at 10 Hz for 20 s (Figures 3c, 4d).

Neurogenic contractions were also compared before (control) and after simultaneous administration of mATP (5 μM) and yohimbine (0.1 μM) (Figure 3d). The contractile responses to EFS were much greater in preparations treated with mATP plus yohimbine than in preparations treated with mATP only (Figure 4d). At some parameters of stimulation they were even greater than the initial (before any treatment) contractile responses. Prazosin (1 μM) greatly reduced the residual contractions.

Effect of ET-1 on EFS-induced contractions

ET-1, cumulatively applied at concentrations of 0.1–0.3 nM changed neither the smooth muscle tone nor the EFS-induced contractions. Higher concentrations of ET-1 (above 0.3 nM) caused a concentration-dependent increase of the smooth muscle tone and an increase of the contractile responses to EFS. The neurogenic contractions-enhancing effect of ET-1 was more pronounced at short lasting stimulations (5 Hz and 10 Hz for 1 s), as well as in the first phase of the contractile responses to EFS at 5 Hz for 10 s than at long-lasting stimulations (data not shown).

Based on these results we chose for further examination the following three concentrations of ET-1: 0.1 nM (without visible effect on both the smooth muscle tone and neurogenic contractions); 10 nM (about the EC_{50} value for the effect of ET-1 on the smooth muscle tone); and 100 nM (the maximum contractile effect of ET-1).

The relevant time-control with ET-1 vehicle showed that at all parameters of electrical stimulation the contractions were weaker compared to the contractile responses before the vehicle (Figure 5). In the presence of 0.1 nM ET-1, the contractile responses to EFS were always slightly enhanced and the comparison with the vehicle time control demonstrated these changes to be statistically significant. In the presence of ET-1 at a concentration of 10 or 100 nM the contractile responses to EFS were much more increased.

Effect of ET-1 on the prazosin-resistant component of the EFS-induced contractions

In prazosin-treated preparations, ET-1 at a concentration of 0.1 nM for 30 min did not change significantly the residual EFS-induced response, but at a concentration of 10 nM (10 min incubation) it significantly increased these responses (Figure 3a, 6a). Combined application of prazosin and yohimbine led to a more pronounced increase of the residual

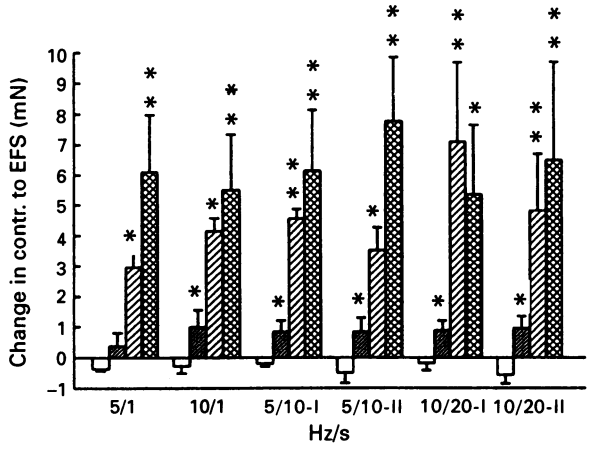


Figure 5 Changes in the contractile responses to EFS (as absolute values) in the presence of endothelin-1 (ET-1) vehicle (open columns), after 30-min incubation with 0.1 nM ET-1 (closely hatched columns), after 10-min incubation with 10 nM ET-1 (widely hatched columns) and after 100 nM ET-1 (cross-hatched columns). The values represent means ± s.e.mean from 10 to 12 experiments.

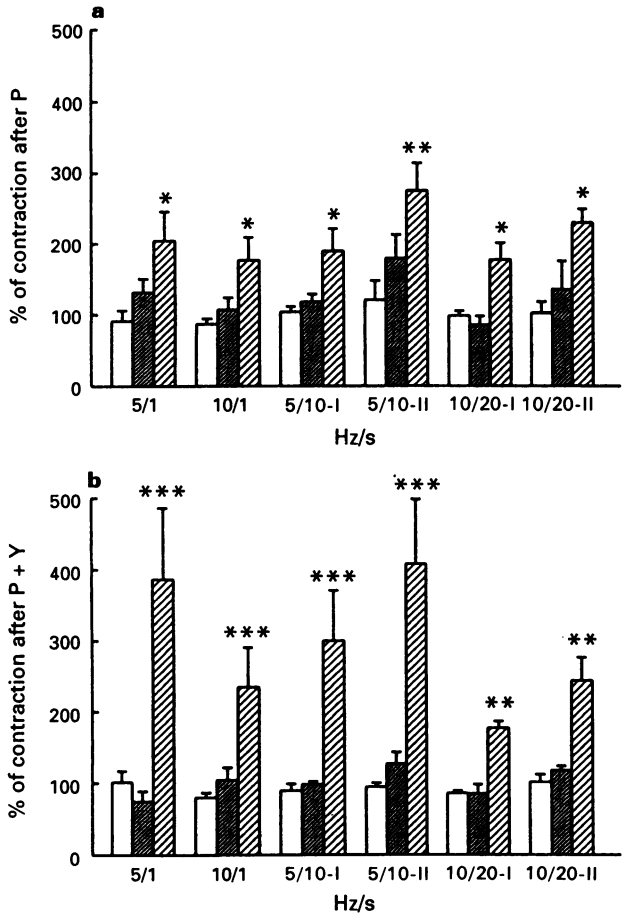


Figure 6 (a) Prazosin-treated endothelium-free rabbit saphenous artery ring preparations. The contractile responses to electrical field stimulation in the presence of endothelin-1 (ET-1) vehicle (open columns), 0.1 nM ET-1 (closely hatched columns), and 10 nM ET-1 (widely hatched columns), expressed as percentage of the responses after prazosin (P)-treatment. The values are means ± s.e.mean from 9 to 12 preparations. (b) Prazosin plus yohimbine-treated endothelium-free rabbit saphenous artery ring preparations. The contractile responses to electrical field stimulation in the presence of ET-1 vehicle (open columns), 0.1 nM ET-1 (closely hatched columns), and 10 nM ET-1 (widely hatched columns), expressed as percentages of the responses after prazosin plus yohimbine (P + Y)-treatment. The values are means ± s.e.mean from 11 to 13 preparations.

response after 10 nM ET-1 (Figure 3b, 6b). The contractile responses to EFS were abolished after 5 μM mATP in both prazosin- and prazosin plus yohimbine-treated preparations.

Effect of ET-1 on the mATP-resistant component of EFS-induced contractions

In mATP-treated preparations, ET-1 at a concentration of 0.1 nM did not influence the residual neurogenic contractions, whereas at a concentration of 10 nM it increased the residual responses to EFS at a frequency of 5 Hz for 1 s, 10 Hz for 1 s, 5 Hz for 10 s (phases I and II) and did not change the contractions after EFS at 10 Hz for 20 s (Figure 3c, 7a). Prazosin, 1 μM, greatly inhibited but did not abolish these responses. In the presence of mATP plus yohimbine, ET-1 exerted no effect on the residual neurogenic contractions at a concentration of 0.1 nM or 10 nM (Figures 3d, 7b), which were abolished after prazosin.

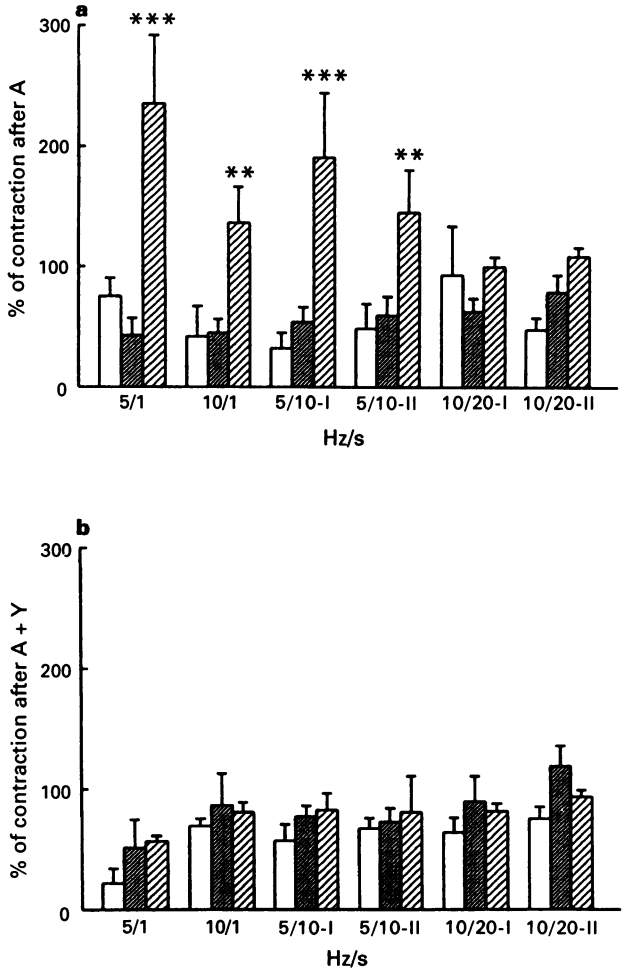


Figure 7 (a) α,β-Methylene ATP (mATP)-treated endothelium-free rabbit saphenous artery ring preparations. The contractile responses to electrical field stimulation in the presence of endothelin-1 (ET-1) vehicle (open columns), 0.1 nM ET-1 (closely hatched columns), and 10 nM ET-1 (widely hatched columns), expressed as percentages of the responses after mATP (A) treatment. The values are means ± s.e.mean from 11 to 12 preparations. (b) mATP plus yohimbine-treated endothelium-free rabbit saphenous artery ring preparations. The contractile responses to electrical field stimulation in the presence of ET-1 vehicle (open columns), 0.1 nM ET-1 (closely hatched columns), and 10 nM ET-1 (widely hatched columns), expressed as percentages of the responses after mATP plus yohimbine (A + Y) treatment. The values are means ± s.e.mean from 9 to 12 preparations.

Discussion

This study provides further evidence that ET-1 is capable of modulating the sympathetic neurotransmission in the periphery.

NA and ATP are now well established as co-transmitters in sympathetic nerves supplying many blood vessels, so that electrical stimulation evokes release of both transmitters from the sympathetic terminals (Sneddon & Burnstock, 1984; Ishikawa, 1985; Kügelgen & Starke, 1985; Kennedy *et al.*, 1986; Vidal *et al.*, 1986; Sedaa *et al.*, 1986). The degree of involvement of either the purinergic or adrenergic components of the response to sympathetic nerve stimulation in blood vessels depends on the parameters of stimulation employed (Kennedy *et al.*, 1986; McGrath *et al.*, 1990; Bulloch *et al.*, 1991). The purinergic component is optimal with short bursts of sympathetic nerve stimulation (1 s or less), while the longer duration of stimulation favours the noradrenaline component of the mechanical responses to EFS (Kügelgen & Starke, 1985; Kennedy *et al.*, 1986; Burnstock, 1988). Under our experimental conditions (endothelium-free preparations, blocked cyclo-oxygenase products), short trains of pulses for a period of 1 s at a frequency of 5 or 10 Hz caused predominantly purinergically-mediated contractions. Long-lasting stimulation for 10 s at a frequency of 5 Hz or for 20 s at 10 Hz elicited biphasic contractile responses. The first phase of the responses to EFS at 5 Hz for 10 s was predominantly purinergic, whereas the second phase of the responses to this stimulation and the two phases of the responses to 10 Hz EFS for 20 s were predominantly adrenergic. Combination of prazosin and mATP virtually abolished the over-all contractile responses to EFS at all parameters, indicating that the responses were mediated by both α_1-adrenoceptors and P_{2x}-purinoceptors that were postjunctional. In some cases, after prazosin plus mATP treatment, weak contractions were observed, but they were both yohimbine- and TTX-resistant showing their myogenic rather than neurogenic nature. Thus, we did not find any postjunctional α_2-adrenoceptor-mediated vasoconstriction under normal (in the absence of pharmacological stimulants) experimental conditions. Burnstock & Warland (1987) also observed that EFS-induced contractions in the rabbit saphenous artery were abolished by the combination of prazosin and mATP. In addition, our findings, in agreement with those of Burnstock & Warland (1987) and McGrath *et al.* (1990), suggest that in this vessel, ATP and NA each contribute to both the monophasic and the biphasic response to EFS.

Yohimbine, administered after prazosin-induced elimination of the α_1-adrenoceptor-mediated component of the contractile responses to EFS, increased the EFS-provoked contractions. This increase is most probably due to ATP (probably increased release), since these enhanced contractions were almost totally inhibited by mATP. More pronounced augmentation of the EFS-induced vascular contractions after yohimbine was observed after preliminary elimination of the purinergic component by mATP. These contractile responses were abolished by prazosin, indicating their adrenergic nature. An increased release of NA due to prejunctional antagonism of yohimbine towards α_2-adrenoceptors might be supposed. In addition, mATP appears to be an antagonist at prejunctional P_3 purinoceptors which tends to increase the release of NA (Shinozuka *et al.*, 1990). Thus, some prejunctional effect of mATP *per se* should not be excluded.

In the present study, ET-1 increased the smooth muscle tone of both non-stimulated and electrically-stimulated preparations, the EC_{50} value being approximately 100 times higher than the EC_{50} value for exogenous NA and approximately 20,000 times higher than the EC_{50} value for exogenous ATP. These findings favour the view that ET-1 is one of the strongest vasoconstrictors yet described and are in accordance with the recent data of Auguet *et al.* (1992) about the vasoconstrictor properties of endothelin in the rabbit saphenous artery. In our study, the peptide showed a potency similar to that reported by Hardebo *et al.* (1989) for the human pial veins, middle meningeal artery and superficial temporal artery with pD_2 values being from 7.61 to 7.80. There is evidence that the vasoconstrictor effect of endothelin-1 is most likely realized via specific endothelin receptors located on the vascular smooth muscle (Randall, 1991; Sumner *et al.*, 1992). Present experimental conditions (endothelium removed, indomethacin treatment) allow interpretation of the effects of endothelin-1, uncomplicated by EDRF or cyclo-oxygenase products.

Besides the direct vasoconstrictor effect of the peptide, we found that ET-1 also increased the contractile responses to EFS at all stimulation parameters. It should be mentioned that both myogenic and neurogenic effects of ET-1 were not directly interrelated. For further and more detailed study we chose three concentrations of ET-1: 'low', 'middle' and 'high'. ET-1 at a concentration of 0.1 nM ('low' ET concentration) did not exert any visible effect on the vascular tone or on the contractile responses to EFS. Elevated levels of circulating ET-1 have been reported in a variety of cardiovascular diseases, including hypertension, renal failure, myocardial ischaemia, etc. (for review see Leppaluoto & Ruskoaho, 1992). Thus, it might be speculated that the 'low' concentration used in the present study might also be pathophysiologically relevant. It is interesting whether a prolonged contact between the vessel and the peptide at such a 'low' concentration could change the mechanical activity of the vessel. Our results showed that even at that concentration, endothelin caused a slight but statistically significant increase of the EFS-induced vasoconstriction after prolonged exposure of the vessel to the peptide. The peptide, at both 'middle' and 'high' concentrations significantly increased the contractile responses to EFS.

The mechanisms of ET-1 modulation of neurotransmission can only be speculated on at present. Although the contractile responses to EFS suggest alterations in neurotransmitter release, it is not clear whether the ET-1 potentiation of the electrically-induced contraction is due to prejunctional influence on the cotransmitter release or to postjunctional interference with the transmitter site(s) of action. Previous studies on vascular and nonvascular tissues (Wiklund *et al.*, 1988; 1989a; Mutafova-Yambolieva *et al.*, 1992; 1993) have shown both prejunctional and postjunctional actions of endothelin-1. For this reason, we examined the ET-1 effects on the contractions evoked by exogenous ATP or NA in the presence of TTX which ensured conditions mainly for studying the postjunctional processes. ET-1 did not affect the contractile response to exogenous NA, suggesting that no postjunctional adrenergic mechanisms underlie the ET-1 potentiating effect on the electrically induced contractile responses. However, ET-1, especially at higher concentrations, potentiated the contractions in response to exogenous ATP, suggesting the involvement of postjunctional purinergic mechanisms in the enhancing effect of ET-1 on the contractile response to EFS. These observations are in agreement with the data of Wiklund *et al.* (1990) and our recent findings (Mutafova-Yambolieva & Radomirov, 1993) showing that ET-1 exerts a potentiating effect on the purinergic component of the sympathetically mediated contractile responses of rodent vas deferens via postjunctional mechanisms.

The reason for the differential effects of ET-1 on the purinergic and the adrenergic components of the neural responses is not clear yet. Some possible mechanisms could be inferred from our understandings of the neurotransmission process in blood vessels. For instance, it is known that ATP acts on P_{2x}-purinoceptors that operate by electromechanical coupling involving voltage-dependent Ca channels, while noradrenaline acts via adrenoceptors that operate by a spike-free mechanism involving receptor-operated Ca channels (Cheung & MacKay, 1986; Burnstock, 1988). Thus, one possible mode of action of ET-1 is to potentiate the purinergic contraction due to the well established enhancing effect of the peptide on the intracellular calcium concentration by acti-

vating the voltage-dependent Ca channels (Yanagisawa *et al.*, 1988). The α-adrenoceptor-mediated contractions are much less dependent on voltage-dependent calcium channels (Cheung, 1984; Fujioka & Cheung, 1987), and ET-1 would not be effective in potentiating these responses. The discrepancy between our findings showing no ET-1 effect on the contractile responses to exogenous NA and those of Wiklund *et al.* (1989a) showing a significant ET-1 induced increase of the NA effect in the guinea-pig pulmonary artery, indicates again the tissue specificity (Masaki, 1991) of the ET-1 effects.

As expected, in the presence of prazosin the residual purinergic component of the EFS-induced vasoconstriction was significantly increased by 10 nM ET-1 and this increase was more pronounced after simultaneous administration of yohimbine and prazosin. It might be assumed that the increased concentration of ATP in the synaptic cleft results in augmented mechanical responses because of the potentiating effect of ET-1 on the purinergic component. A presynaptic release of ATP induced by ET-1 is also possible. Generally, the relative participation of both the purinergic and the adrenergic components of the neurogenic response was not substantially changed after ET-1. The peptide tended to decrease the prazosin-sensitive component and to increase the mATP-sensitive component but this effect was significant only after the highest ET-1 concentration at some stimulation parameters.

In the present study, although ET-1 did not change the contractile responses to exogenous noradrenaline, it increased the electrically-induced contractile responses after elimination of the purinergic component by mATP. This increase was eliminated after yohimbine treatment, thus suggesting participation of α_2-adrenoceptors. The residual response after ET-1 plus mATP plus yohimbine was mediated by α_1-adrenoceptors. Therefore, it could be speculated that the incubation of the vessel with ET-1 might reveal a postjunctional α_2-adrenoceptor-mediated vasoconstrictor component. Combination of increased sympathetic tone and increased circulating ET-1 levels may promote increased expression of prolonged α_2-adrenoceptor-mediated regional vasoconstriction in disease states, which implicate high levels of circulating endothelin, or high local levels of the peptide. MacLean & McGrath (1990) also reported pressor responses to α_2-adrenoceptor activation, uncovered by ET-1-induced preconstriction in the isolated vascular bed of the rat tail.

In conclusion, it is suggested that endothelin, apart from being a potent vasoconstrictor, can act as a modulator of the sympathetic neuroeffector transmission in the rabbit isolated saphenous artery. ET-1 exerts an enhancing effect on the purinergic component of the mechanical response to exogenous ATP or electrical stimulation. The long-lasting exposure to low ET-1 concentrations, which causes no visible changes in the smooth muscle tone may influence the sympathetically-mediated contractility of the vessel.

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Blockade of the inward rectifier potassium current by the Ca^{2+}-ATPase inhibitor 2′,5′-di(tert-butyl)-1,4-benzohydroquinone (BHQ)

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1 We have investigated the effect of 2′,5′-di (tert-butyl)-1,4-benzohydroquinone (BHQ) and thapsigargin, inhibitors of the intracellular Ca^{2+}-ATPase, on ionic currents in rat basophilic leukaemia (RBL-2H3) cells under whole cell voltage clamp.

2 The whole cell current was inwardly rectifying and reversed at -35 ± 6 mV ($n = 16$). The conductance of the inward current increased as the concentration of extracellular K^+ was raised from 2.7 to 5.4, 10.8 and 21.6 mM. $BaCl_2$ (100 μM) reduced the current to a small linear component and shifted the reversal potential to -4 ± 3 mV ($n = 6$). A concentration of 50 μM $BaCl_2$ produced $45 \pm 10\%$ ($n = 4$) blockade of the inward current.

3 BHQ and thapsigargin were examined for their effects on the inwardly rectifying current. A maximal blockade of inward current was obtained within 6 min after perfusion with 10 μM BHQ. The small current remaining after blockade with BHQ had a linear voltage-dependence and reversed direction at -6 ± 9 mV ($n = 6$). Thapsigargin (up to 3 μM) was without effect on the inward rectifier.

4 In contrast to the blockade of the inward rectifier produced by $BaCl_2$ which was predominantly on the steady state current, particularly at the very hyperpolarized holding potentials (− 120 mV), blockade by BHQ was equally strong on the instantaneous as well as the steady state current.

5 Blockade of the inward rectifier by BHQ may cause depolarization of the cell which will affect Ca^{2+} influx during investigations with BHQ. Thapsigargin does not block the inward rectifier and will not inhibit Ca^{2+} influx in this way.

Keywords: Inward rectifier; 2′,5′-di(tert-butyl)-1,4-benzhydroquinone (BHQ); RBL-2H3; calcium-influx

Introduction

In non-excitable cells the influx of extracellular Ca^{2+} is closely related to depletion of intracellular Ca^{2+} stores (Mason *et al.*, 1991; Dolor *et al.*, 1992; Schilling *et al.*, 1992; Vaca & Kunze, 1993). This relationship between Ca^{2+} influx and the intracellular Ca^{2+} stores has been frequently investigated by use of drugs such as 2′,5′-di(tert-butyl)-1,4-benzohydroquinone (BHQ) and thapsigargin. These drugs inhibit the endoplasmic and/or sarcoplasmic reticulum Ca^{2+}-ATPase, thus preventing reuptake of leaked Ca^{2+} and consequently depleting intracellular stores without an apparent increase of phosphoinositide hydrolysis (Goeger *et al.*, 1988; Jackson *et al.*, 1988; Goeger & Riley, 1989; Seider *et al.*, 1989; Takemura *et al.*, 1989; Foder *et al.*, 1989; Kass *et al.*, 1989; Oldershaw & Taylor, 1990; Koshiyama & Tashjian, 1991).

The 'capacitative' coupling model stipulates that the plasmalemmal Ca^{2+} permeability is controlled by the degree of filling of intracellular Ca^{2+} storage compartments (Putney, 1986; Putney, 1990). According to this model, BHQ should evoke influx of extracellular Ca^{2+} and many studies have found this (Mason *et al.*, 1991; Dolor *et al.*, 1992; Schilling *et al.*, 1992), but BHQ has also been shown to deplete effectively internal Ca^{2+} stores without increasing plasmalemmal Ca^{2+} permeability (Kass *et al.*, 1989). This cannot be attributed to tissue specificity, since thapsigargin increased Ca^{2+} influx in the same cells (Thastrup, 1990; Thastrup *et al.*, 1990). Such a finding would be consistent with the capacitative model if concurrent with inhibition of the intracellular Ca^{2+}-ATPase, BHQ prevented Ca^{2+} influx after depleting intracellular stores of Ca^{2+}.

To investigate whether BHQ has any effect on plasmalemmal ionic channels we examined the effect of BHQ on whole cell currents in rat basophilic leukaemia (RBL-2H3) cells, focusing on the inwardly rectifying potassium current which is the main component present under resting conditions (Lindau & Fernandez, 1986; McCloskey & Cahalan, 1990; Piguet & North, 1992).

Methods

Culture and preparation of rat basophilic leukaemia cells (RBL-2H3)

The rat basophilic leukaemia (RBL) 2H3 subline (Barsumian *et al.*, 1981; Siraganian, 1988) was maintained in RPMI 1640 solution (Gibco Cat No. 430-3400EA) supplemented with 10% foetal bovine serum (FBS), 20 mM $NaHCO_3$ and 100 u ml^{-1} penicillin-streptomycin. The cells were incubated under 5% CO_2 at 37°C in microtest II plates and were subcultured daily by adding 1 ml of the cell suspension to a sterile tissue culture dish containing 1 ml of new medium. It has been shown by use of negative staining with Trypan Blue that 30% of the suspended cells are alive. All experiments were conducted on adherent cells 24–48 h after passage.

Whole cell patch clamp recording

Glass pipettes were prepared from capillary tubes (No. 7052) and had a resistance of 3 MΩ when filled with electrolyte. The pipettes were used to make high-resistance 1.7 ± 0.3 GΩ ($n = 23$) seals with the cell membrane for whole cell voltage-clamp studies at room temperature (~25°C). Recordings were obtained using an Axopatch-1C amplifier (Axon Instruments) and an indifferent electrode which was an Ag-AgCl plug connected to the bath via a 150 mM KCl agar

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bridge. Voltage protocols as well as data acquisition were carried out using a digital interface (TL-1 DMA; Axon Instruments) coupled to an IBM compatible computer. Programmed pulse protocols were applied and the resulting cell currents were digitized at 2 kHz, and stored for later analysis using the pClamp software (Axon Instruments). Currents were recorded without compensation for capacitance or series resistance.

Reagents and solutions

The standard extracellular physiological salt solution (EPSS) which was used for all recordings contained in mM: NaCl 137, KCl 5.4, $MgCl_2$ 1, $CaCl_2$ 2, D-glucose 10 and HEPES 10 with pH adjusted to 7.3 using 1 M NaOH. The pipette solution contained in mM: KCl 145, HEPES 10, ethylene-glycol-*bis*(β-amino-ethyl-ether)*N'*,*N*,*N'*-tetraacetic acid (EGTA) 2.2 and $CaCl_2$ 0.2, with pH adjusted to 7.3 using 1 N KOH.

For test solutions, concentrations of $BaCl_2$ were prepared daily by adding the salt to EPSS, and stock solutions of 2',5'-di(tert-butyl)-1,4-benzohydroquinone (BHQ) or thapsigargin were prepared daily from powder respectively to a 10 mM and 300 μM concentration. Aliquots of this stock solution were diluted in EPSS to produce the required concentrations. When the inwardly rectifying current was tested under different concentrations of extracellular potassium, the

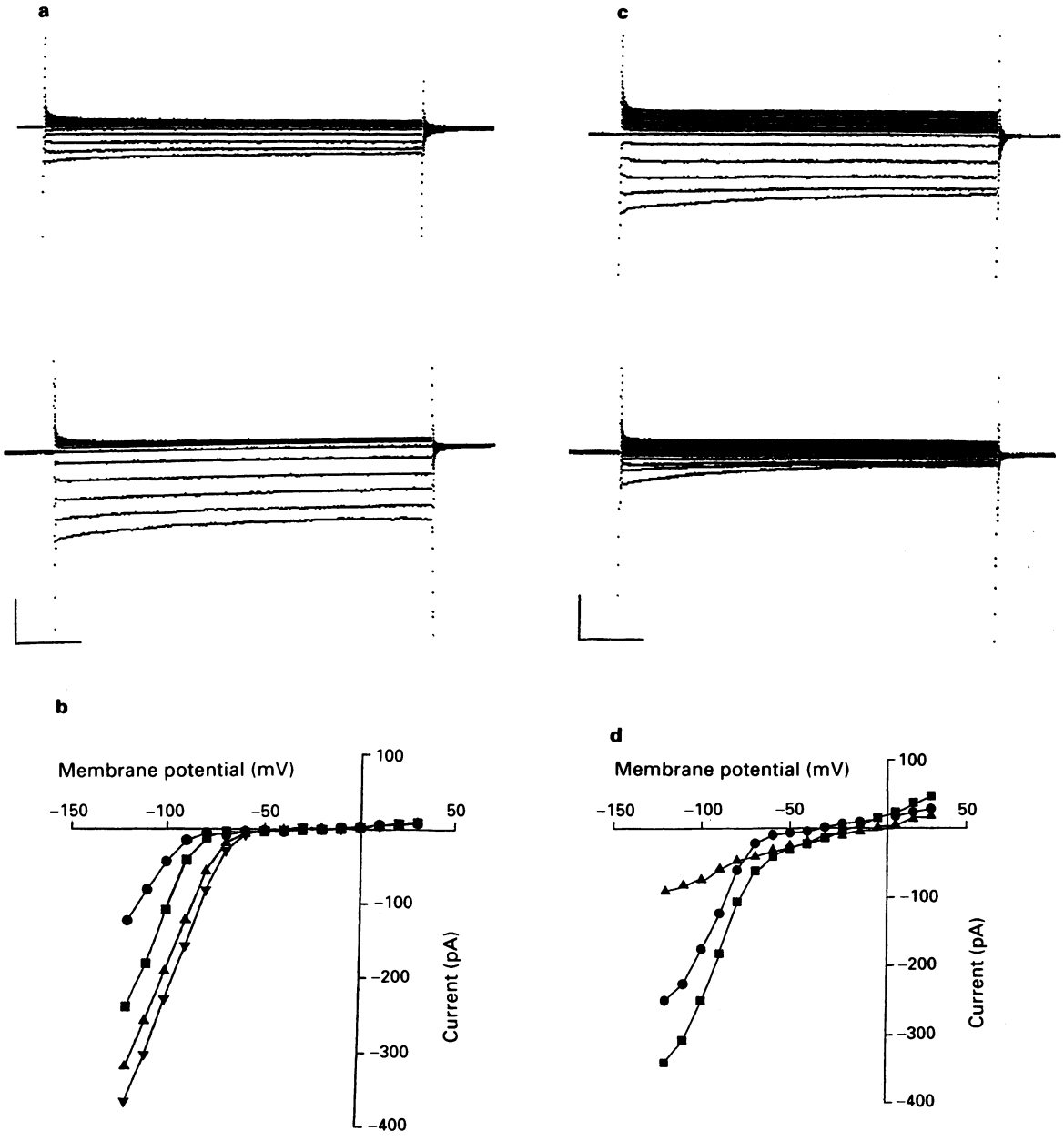


Figure 1 Characteristics of the inward rectifier current: (a) The current recorded in response to 300 ms steps of 10 mV increments from − 120 to + 30 mV, starting from a holding potential of − 70 mV, recorded from a single cell in 2.7 (top) and 21.6 (bottom) mM K^+. (b) Steady-state current-voltage relationship measured 250 ms after the start of the pulse, and recorded in extracellular solutions of 2.7 (●), 5.4 (■), 10.8 (▲) and 21.6 (▼) mM K^+ concentration demonstrating higher conductance of the inward rectifier with increasing concentration of extracellular K^+. Shown are representative curves, taken from a single cell. Similar data were obtained in four other experiments. (c) Representative traces from a single cell of the current recorded in response to 300 ms steps of 10 mV increments from − 120 to + 30 mV before (top) and 1 min after (bottom) perfusion with 100 μM $BaCl_2$ showing the greater blockade of the steady state current than the instantaneous current. (d) The steady state current-voltage relationship, recorded from a single cell, measured 250 ms after the start of the step, before (■) and after (●) subtraction of residual current of unknown nature remaining after blockade with 100 μM $BaCl_2$ (▲). Similar data were obtained in four other experiments. The scale bars indicate 200 pA and 60 ms.

concentration of NaCl was changed to maintain constant osmolarity. The $BaCl_2$, KCl, thapsigargin or BHQ solutions were applied by gravity driven perfusion with a flow rate of 10 ml min^{-1}. A vacuum suction was stationed at the opposite end of the dish to the inflow. A fluid volume of about 1 ml was set for the culture dish by adjusting the height of the vacuum catheter in the dish. The culture dish was flushed with 5 ml of test solution. Time $t = 0$ was considered to be the start of superfusion with test solution. Any effect on the currents of changing perfusate was tested by flushing 5 ml of extracellular solution, running the step protocol, and repeating this procedure four times. Concentration-response curves depicted in Figure 4 were constructed using cumulative concentrations of BHQ.

Statistics

All values are expressed as means ± s.e. The group means were compared using an ANOVA and a Student's paired *t* test. Multiple comparisons with one mean were conducted with an ANOVA followed by the Dunnett test. A difference between means was considered to be significant when $P < 0.05$.

Results

Profile of the inwardly rectifying current of RBL-2H3 cells

From a holding potential of − 70 mV, a step protocol from − 120 to + 30 mV, produced a predominantly inwardly rectifying current (Figure 1). The inward current had two components, an instantaneous component that relaxed to a steady component which was − 312 ± 35 pA ($n = 17$) at − 120 mV (Figure 1a). The relaxation of the instantaneous current in RBL-2H3 cells has been shown to be due to blockade by Na^+ ions (Lindau & Fernandez, 1986). The slope conductance of the inward current, calculated from the steady state current 50 ms before the end of the step, increased with higher extracellular concentrations of K^+ (Figure 1b). This is consistent with the behaviour of inwardly rectifying currents in RBL-2H3 cells reported earlier (Lindau & Fernandez, 1986; Piquet & North, 1992).

$BaCl_2$ was used to block the inwardly rectifying K^+ current leaving a linear current over this voltage range. The steady state inward current was reduced to 30 ± 6% ($n = 6$) of its original value by 100 μM $BaCl_2$ (Figure 1c), and was not reduced further by 1 mM $BaCl_2$ (34 ± 11%, $n = 5$). A concentration of 50 μM $BaCl_2$ produced about 50% blockade (45 ± 10%, $n = 4$). Maximum blockade of the inward current could be achieved within 3 min with 100 μM $BaCl_2$, and blockade of the steady state current was greater than blockade of the instantaneous current, particularly at the most negative potentials (Figure 1c). The whole cell current reversal potential of − 35 ± 6 ($n = 16$) was shifted to − 4 ± 3 mV ($n = 6$) after treatment with $BaCl_2$ (Table 1). After blockade of the inward rectifier with $BaCl_2$ only a small inward current with a linear current-voltage relationship remained (Figure 1d). This current of unknown nature has been reported by others (Lindau & Fernandez, 1986; Piguet & North, 1992) and was not studied further by us.

Attenuation of the inward rectifier current by BHQ

Continuous repetition of the voltage protocol to elicit the inwardly rectifying current at 30 s intervals, over a 7 min period, did not affect the magnitude of the inward current (Figure 2). When the cells were perfused with 300 nM thapsigargin ($n = 5$), a concentration that inhibits the Ca-ATPase, the inward current was not changed (Figure 2). Increasing the concentration to 3 μM thapsigargin also had no effect ($n = 5$, result not shown). On the other hand, BHQ clearly inhibited the inward current. In the presence of 10 μM BHQ the inward current was attenuated with a maximum inhibition produced about 6–7 min after application of BHQ (Figure 2). Hence, all measures of blockade by BHQ were taken 7 min after perfusion with BHQ. Blockade of the inward current by 10 μM BHQ shifted the reversal potential of the current to − 6 ± 9 mV ($n = 6$) (Table 1). The BHQ-evoked blockade of the inward rectifier was concentration-dependent with only 10 ± 5% ($n = 6$) and 15 ± 14% ($n = 3$) of the total inward current remaining after blockade with respectively 10 and 100 μM BHQ (Figure 3c,d). The block by BHQ was present over the entire duration of the step protocol (Figure 3a,b). This is in contrast to the type of blockade produced by Na^+ or Ba^{2+} where the effects are more marked later in the hyperpolarizing step than at the beginning of the step.

Discussion

We found that whole cell currents of RBL-2H3 cells, in physiological extracellular solution, include an inwardly rectifying K^+ current and a small current with an approximately linear current-voltage relationship that reverses near zero mV, consistent with the findings of others (Lindau & Fer-

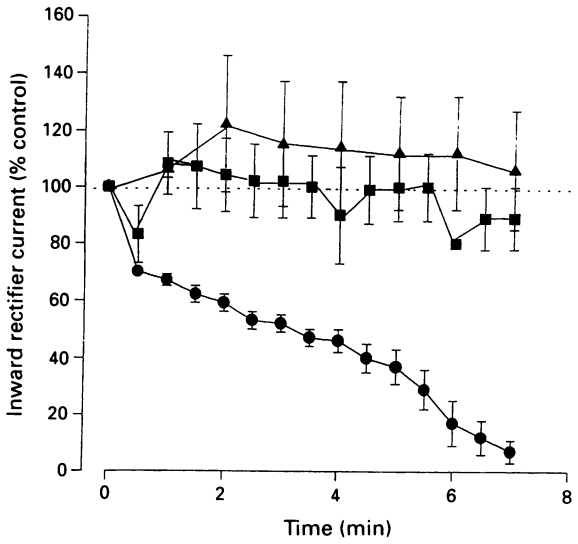


Figure 2 The time course of inward rectifier blockade by 10 μM BHQ; reproducibility of the inward rectifier current was investigated by repeating the step protocol every 30 s for at least 7 min (■, $n = 4$). The inward rectifier current did not change in the presence of 300 nM thapsigargin (▲, $n = 5$) although 10 μM BHQ attenuated the inward rectifier current with maximum blockade achieved 6 min after the start of perfusion (●, $n = 8$). The residual current, after $BaCl_2$ blockade, of unknown nature has been subtracted to isolate the effect of these drugs directly on the inward rectifier. The blockade is significant ($P < 0.05$) starting 2 min after perfusion as determined by an ANOVA and Dunnett's test *a posteriori*.

Table 1 Shift in resting potential after treatment with drug

Group	n	*Resting potential* (mV)
Control	16	− 35 ± 6
$BaCl_2$ 100 μM	6	− 4 ± 3*
BHQ 10 μM	6	− 6 ± 9*

Values are means ± s.e.mean of measurements obtained from *n* cells in each group. Statistical differences in the means between the control and the treated groups were determined by an ANOVA followed by the Dunnett test: *$P < 0.05$.

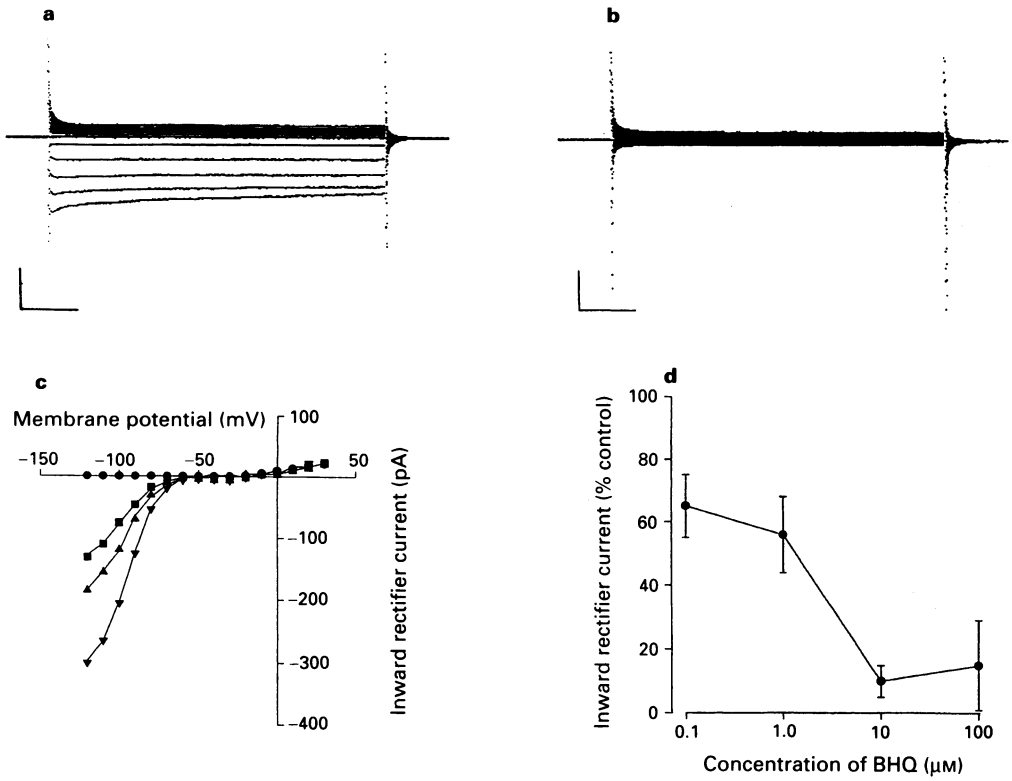


Figure 3 Inhibition of the inward rectifier by BHQ: representative traces from a single cell recorded before (a) and 7 min after 10 μM BHQ (b). (c) Representative steady-state current voltage relationship of $n = 8$ experiments recorded from control (▼) and in the presence of 0.1 (▲), 1.0 (■) and 10 (●) μM BHQ. (d) The concentration-dependent blockade of the inward rectifier current measured 7 min after the start of perfusion with each concentration of BHQ. The residual current of unknown nature has been subtracted to isolate the effect of BHQ on the inward rectifier. The scale bars indicate 150 pA and 50 ms.

nandez, 1986; Piguet & North, 1992). The K^+ conducting properties of the inward rectifier were verified by the dependence of the inward conductance on the concentration of extracellular K^+ and by block of the current with $BaCl_2$.

BHQ completely blocked the inwardly rectifying K^+ current and shifted the reversal potential toward a more depolarized value as did $BaCl_2$; the effects of BHQ and $BaCl_2$ were different in that $BaCl_2$ blocked predominantly the steady state current evoked by large hyperpolarizing voltage steps. This is consistent with the finding of others (Lindau & Fernandez, 1986; Piguet & North, 1992). The blockade by $BaCl_2$ is favoured by hyperpolarization (Fukushima, 1982), and is thought to require entry of the blocking ion into the channel without completely traversing the plasma membrane (Hille & Schwarz, 1978). In contrast, the blockade by BHQ was present over the entire duration of the voltage step. Although the time course of the BHQ block is slow (minutes), it is not possible with the present data to distinguish between an intracellular and extracellular site of action of BHQ which is a membrane permeable compound.

The results of the experiments presented here can explain the seemingly conflicting reports on the effects of BHQ on changes in intracellular Ca^{2+}, as well as some of the differences between thapsigargin and BHQ. The concentrations of BHQ we investigated are those regularly used to block intracellular Ca^{2+}-ATPase. The usual profile of $[Ca^{2+}]_i$ in response to BHQ is a rise followed by a sustained increase (Mason *et al.*, 1991; Schilling *et al.*, 1992). It is thought that this profile has two components, the initial Ca^{2+} rise due to block of reuptake of Ca^{2+} leaked from intracellular stores and the second phase that is a sustained influx of extracellular Ca^{2+} in response to the continued store depletion. In rat thymic lymphocytes, a time and dose-dependent relationship between the maximum increase of $[Ca^{2+}]_i$ and BHQ was shown, where 5 μM BHQ produced a sustained increase of $[Ca^{2+}]_i$ while a higher concentration of 50 μM BHQ produced only a transient increase in $[Ca^{2+}]_i$ (Mason *et al.*, 1991). BHQ (25 μM) has also been shown to deplete effectively internal Ca^{2+} stores without increasing plasmalemmal Ca^{2+} permeability (Kass *et al.*, 1989) in rat hepatocytes. In another study, it was found that the sustained increase of $[Ca^{2+}]_i$ produced by 10 μM BHQ changed to only a transient increase of $[Ca^{2+}]_i$ when the cell was depolarized with KCl (Schilling *et al.*, 1992). Furthermore, depolarization with high external K^+ of stimulated RBL-2H3 cells has been shown to reduce uptake of $^{45}Ca^{2+}$ (Hide & Beaven, 1991). Together with the data presented here these studies suggest that BHQ at high concentrations may depolarize the cell and thus inhibit calcium influx. Hence, a transient change in intracelluar Ca^{2+} rather than a sustained influx can occur in response to BHQ if the inwardly rectifying potassium current is blocked concurrently with the Ca^{2+}-ATPase.

The ability of BHQ in concentrations above 1 μM to block the inward rectifier and possibly reduce Ca^{2+} influx due to the depolarization it would cause, must be considered when BHQ is used to deplete intracellular Ca^{2+} stores to evoke influx of extracellular Ca^{2+}. Thapsigargin, at concentrations which evoke maximal increases of $[Ca^{2+}]_i$ (Mason *et al.*, 1991; Takemura *et al.*, 1991), is free of such an effect on the inward rectifier.

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Evidence for a capsaicin-sensitive, tachykinin-mediated, component in the NANC contraction of the rat urinary bladder to nerve stimulation

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1 In the presence of atropine (1 μM) and guanethidine (3 μM), electrical field stimulation (EFS) of the rat isolated urinary bladder for 30 s induced a frequency-dependent (1–30 Hz) nonadrenergic noncholinergic (NANC) triphasic contraction characterized by a peak response (within 10 s from onset of stimulation), a late response (determined as the tension developed at the end of the stimulation period) and a prolonged post-stimulus 'off' response. The latter peaked at 2–6 min from the end of the stimulation period. At 10 Hz, the amplitude of the three responses averaged 89 ± 6, 76 ± 6 and 18 ± 3% of the response to 40 mM KCl, respectively. Tetrodotoxin (1 μM) abolished all contractile responses to EFS.

2 In capsaicin-pretreated bladder strips (10 μM for 15 min) the amplitude of the peak response to EFS (1–30 Hz for 30 s) was unchanged, the 'late' response to EFS was significantly reduced as compared to controls, and the post-stimulus response was absent, being replaced by a transient relaxation.

3 When varying train duration from 1 to 120 s at a frequency of 10 Hz, the differences between control and capsaicin-treated strips became evident for periods of stimulation > 10 s.

4 The tachykinin NK_1 receptor antagonist, SR 140,333 (0.1–1 μM) had no effect on the peak response to EFS (10 Hz for 30 s) while it decreased significantly the late response at both concentrations tested (16 ± 3 and 33 ± 3% inhibition). At 1 μM, SR 140,333 also significantly reduced (29 ± 9% inhibition) the peak of the post-stimulus contraction. The tachykinin NK_2 receptor antagonist, MEN 10,627 (0.1–1 μM) had no significant effect on the peak response to EFS (10 Hz for 30 s), and decreased the late response at 1 μM only (32 ± 4% inhibition). MEN 10,627 inhibited the post-stimulus response at both concentrations tested and almost abolished it at 1 μM.

5 The combined administration of SR 140,333 and MEN 10,627 (1 μM each) produced a small reduction (22 ± 3% inhibition) of the peak response to EFS, a marked reduction (48 ± 3% inhibition) of the late response and the abolition of the post-stimulus response which was replaced by a post-stimulus relaxation as observed in capsaicin-pretreated strips.

6 SR 140,333 (0.1 and 1.0 μM) produced a large rightward shift in the concentration-response curve to the NK_1 receptor agonist, [Sar^9]substance P sulphone (apparent pK_B 8.97 ± 0.14), without affecting the response to the NK_2 receptor-selective agonist, [βAla^8]neurokinin A (4–10). MEN 10,627 (0.1 and 1 μM) produced a large rightward shift of the concentration-response curve to [βAla^8]neurokinin A (4–10) (apparent pK_B 8.95 ± 0.16) without affecting the response to [Sar^9]substance P sulphone. SR 140,333 and MEN 10,627 (1.0 μM each) did not affect the contraction produced by exogenous ATP (1 mM).

7 These findings provide evidence that the NANC contraction of the rat isolated urinary bladder to transmural nerve stimulation has two components, which are sharply differentiated by blockade of the efferent function of sensory nerves following *in vitro* capsaicin administration. The first component, probably mediated by endogenous ATP, is fully activated during short periods of nerve activity (< 10 s) and does not involve capsaicin-sensitive nerve afferents. The second component, which is capsaicin-sensitive and tachykinin-mediated, is evident as a late 'on' response during nerve stimulation and as a post-stimulus 'off' response for periods of stimulation > 10 s. Activation of both NK_1 and NK_2 receptors contributes to the capsaicin-sensitive responses.

Keywords: Rat urinary bladder; capsaicin-sensitive primary afferents; tachykinin receptors; atropine-resistant neurotransmission; nonadrenergic, noncholinergic neurotransmission

Introduction

After the early observation that part of the excitatory response of the mammalian urinary bladder to nerve stimulation is atropine-resistant (Langley & Anderson, 1895; Ursillo & Clark, 1956), the neurotransmitter(s) responsible for this phenomenon has remained unknown for a considerable time. In the 1970s the concept of nonadrenergic noncholinergic (NANC) neurotransmission developed in full and adenosine triphosphate (ATP) was proposed as the main transmitter responsible for the atropine-resistant excitation of the mammalian bladder (Burnstock *et al.*, 1972; 1978; Brown *et al.*, 1979). This idea gained wide acceptance in subsequent years (Hoyle & Burnstock, 1985; Fujii, 1988; Brading & Mostwin, 1989; Brading & Williams, 1990; Luheshi & Zar, 1990; Maggi, 1991). Some investigators have considered the possibility that peptides, especially substance P or related tachykinins, may be involved in the atropine-resistant NANC neurotransmission to the urinary bladder. However, early studies on this topic have negated this possibility, mainly because of lack of mimicry by, e.g. substance P, of the fast atropine-resistant contraction of the bladder smooth muscle to nerve stimulation (e.g. Husted *et al.*, 1981; MacKenzie & Burnstock, 1984; Longhurst *et al.*, 1984; Callahan & Creed, 1986). Since the mid '80s, the role of substance P and

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tachykinins in regulating urinary bladder function has been re-evaluated: Sharkey *et al.* (1983) showed that substance P is present in a population of sensory nerves innervating the rat urinary bladder which are sensitive to the action of capsaicin. The functional changes in bladder function produced by the acute application of capsaicin, which stimulates sensory nerves, and by pretreatment with large doses of capsaicin, which inactivates sensory nerves (capsaicin densensitization), were consistent with the idea that substance P and related tachykinins may act as afferent transmitters in the micturition reflex pathway (Sharkey *et al.*, 1983; Maggi *et al.*, 1984; 1986). At the present time, anatomical, neurochemical, pharmacological and physiological evidence implicates an important role for these afferent neurones in regulating micturition and mediating bladder pain (Maggi, 1993 for review). A peculiar feature of the capsaicin-sensitive afferents is their ability to exert, in addition to their sensory function, an 'efferent' function produced through the release of neuropeptides, such as tachykinins, from their peripheral endings (Szolcsanyi, 1984; Maggi & Meli, 1988). The application of capsaicin, which acutely releases tachykinins from sensory nerves, produces a contraction of the rat isolated urinary bladder, sensitive to blockade by tachykinin receptor antagonists (Maggi *et al.*, 1985; 1991a; Santicioli *et al.*, 1986), suggesting that these peptides may participate in the local regulation of bladder tone and motility. Notwithstanding this, no evidence for a capsaicin-sensitive contribution in the contractile response of the rat isolated urinary bladder to electrical nerve stimulation has yet been provided. Previous studies on this topic have failed to detect any difference in the amplitude of the atropine-resistant contraction of the rat isolated bladder to nerve stimulation between control and capsaicin-pretreated animals (e.g. Sharkey *et al.*, 1983; Santicioli *et al.*, 1986). Therefore, any conclusion about the role of tachykinins as 'efferent' neurotransmitters released from sensory nerves in the urinary bladder mainly relies on experiments in which the acute application of capsaicin has been shown to produce tachykinin release (Maggi *et al.*, 1990) or a contraction of the isolated bladder (Santicioli *et al.*, 1986; Maggi *et al.*, 1991a).

In the present study, we provide the first evidence indicating the existence of a capsaicin-sensitive component in the atropine-resistant NANC excitatory motor innervation of the rat urinary bladder. The use of prolonged periods of nerve stimulation is shown to be a key factor in disclosing the existence of a sensory nerve-mediated component to the NANC contraction of the rat isolated bladder. In addition, by using newly developed potent and selective NK_1 and NK_2 receptor antagonists, we have provided evidence that tachykinins are the main mediators of the capsaicin-sensitive component of the atropine-resistant response.

Methods

Male albino rats of Wistar strain (300–350 g b.wt.) were killed by a blow on the back of the head and exsanguination. The whole urinary bladder was excised through a cut just above the proximal urethra and placed in oxygenated and warmed (95% O_2 and 5% CO_2, pH 7.4 at 37°C) modified physiological Krebs solution which was used in all experiments. The Krebs solution had the following composition (mmol l^{-1}): NaCl 119, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.5, KCl 4.7, $CaCl_2$ 2.5 and glucose 11. A longitudinal cut was made on the anterior wall of the urinary bladder from the base to the dome and the bladder was pinned flat in a Petri dish for further dissection. A 1 cm long longitudinal strip of the detrusor muscle was excised just adjacent to the midline of the posterior wall. For experiments involving a comparison between normal and capsaicin-pretreated preparations, two parallel strips were excised from the same bladder. The strips were transferred to 5 ml organ baths and prepared for isotonic recording (load 3 mN) of mechanical activity which was displayed on Basile 7050 pen recorder, as described previously (Meini *et al.*, 1994).

All experiments commenced after a 45 min equilibration period. At this time the response to KCl (40 mM, added to the bath) was recorded. In some strips, after washout of KCl, capsaicin (10 μM) was added to the bath and left in contact for 15 min to produce inactivation of the efferent function of sensory nerves (*in vitro* capsaicin-desensitization). Capsaicin itself produced a long lasting contraction averaging 50–70% of the response to 40 mM KCl. A second application of capsaicin, 30 min later, had no effect ($n = 4$) indicating complete desensitization. After 15 min contact time, capsaicin was washed out and the strips allowed to equilibrate for further period of 30 min or until a stable baseline was obtained.

Experimental protocol

Effect of varying frequency of stimulation In a first series of experiments two strips from the same bladder were studied in parallel, one of which was pre-exposed to capsaicin (10 μM for 15 min, *in vitro* capsaicin desensitization) and the other served as control. Electrical field stimulation (pulse width 0.5 ms, 100–120 V) was performed for 30 s at frequencies between 1 and 30 Hz. The cycles of stimulation were performed with increasing frequencies of stimulation at 30 min intervals.

Three phases of the response to EFS were quantitated: (a) the 'peak' response to EFS which occurred within 10 s from the onset of stimulation; (b) the 'late' response to EFS, measured as the tension recorded at the end of the period of stimulation (30 s); (c) the 'post-stimulus' response (see results) (Figure 1). The amplitude of the peak, late and post-stimulus response to EFS was calculated as % of the response to KCl recorded in each strip. The amplitude of the post-stimulus response was measured at 3.5 min after the end of the stimulation period. The area under the curve (AUC) of

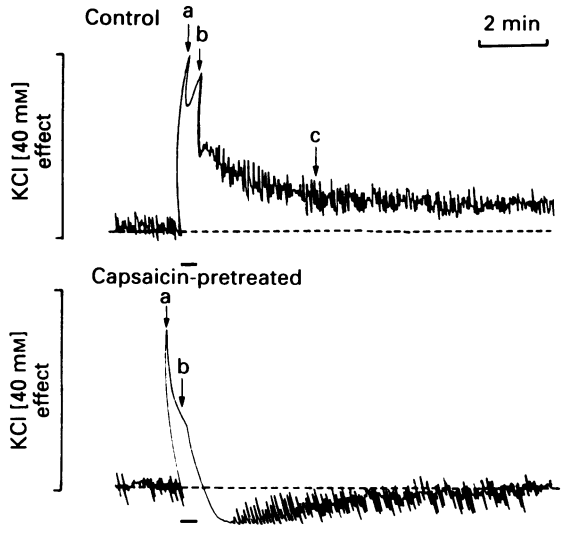


Figure 1 Typical tracings showing the NANC contractile response of the rat isolated bladder to prolonged EFS (10 Hz for 30 s) in control and capsaicin-pretreated (10 μM for 15 min) strips. In the control strip three distinct phases of contraction were observed: a peak response to EFS (a), a late response to EFS, measured as the tension developed at the end of train of stimuli (b) and a post-stimulus prolonged response (c). In the capsaicin-pretreated bladder strip, the peak response to EFS was similar to that in controls, the late response to EFS was lower than in controls and the post-stimulus contraction was replaced by a relaxation. Dotted lines indicate resting baselines. Horizontal bars indicate the period of stimulation.

the post-stimulus response, measured from 0.5 to 10.5 min after the end of the stimulation period, was also quantitated as % of the AUC of the overall contractile response recorded during the 30 s of EFS.

Effect of varying train duration In a second series of experiments, performed in parallel on control and capsaicin-pretreated strips, the response to EFS at 10 Hz (pulse width 0.5 ms, 100–120 V) was determined by increasing train duration from 1 to 120 s. Stimulation cycles were performed every 30 min and the various parameters of the response were quantitated as described above.

Effect of SR 140,333 and MEN 10,627 on the response to EFS In a third series of experiments we determined the effect of the tachykinin receptor antagonists, SR 140,333 (0.1 and 1.0 μM) for NK_1 receptors (Emonds-Alt *et al.*, 1993) and MEN 10,627 (0.1 and 1 μM) for NK_2 receptors (Patacchini *et al.*, 1993), alone or in combination, on the response to a train of stimuli delivered for 30 s at a frequency of 10 Hz. The stimulation cycles were performed at 30 min intervals from each other and the contact time for antagonists was 15 min. In control experiments ($n = 4$), three consecutive stimulation cycles were performed at 30 min intervals in control or capsaicin-pretreated strips; in these experiments no significant changes in the amplitude of the various phase of the NANC response to EFS were detected. The effect of the combined administration of the two antagonists was also investigated on the response to EFS obtained in capsaicin-pretreated bladder strips.

Effect of SR 140,333 and MEN 10,627 on the response to NK_1 and NK_2 receptor agonists and to ATP In a fourth series of experiments, cumulative concentration-response curves to the NK_1 receptor selective agonist, [Sar^9]substance P sulphone and the NK_2 receptor selective agonist, [βAla^8]neurokinin A (4–10), were constructed as described previously (Meini *et al.*, 1994). The effects of SR 140,333 and MEN 10,627 on the response to [Sar^9]substance P sulphone and [βAla^8]neurokinin A (4–10) were investigated after a 15 min contact time.

In separate experiments, two reproducible responses to ATP (1 mM) were established at 30 min intervals and the effect of SR 140,333 and MEN 10,627 (1.0 μM each) on the response to ATP was determined after 15 min contact time.

Statistical analysis

All data in the text and figures are mean ± s.e.mean. Statistical analysis was performed by means of Student's *t* test or analysis of variance when appropriate. Linear regression was performed by the least squares method: EC_{50} and 95% confidence limits were calculated accordingly. Throughout the text, % inhibition values of the effects produced by SR 140,333 and MEN 10,627 have been calculated toward the control responses obtained before addition of the antagonists. A P level <0.05 was considered statistically significant.

Drugs

Drugs used were: capsaicin, ATP and guanethidine (Sigma), tetrodotoxin (Sankyo), atropine HCl (Serva), [Sar^9]substance P sulphone (Peninsula). SR 140,333 or (S)1-{2-[3-(3,4-dichlorophenyl) -1-3 (3-isopropoxyphenylacetyl) piperidine-n-3-yl] ethyl}4 phenyl-1-azoniabicylo[2,2,2]octane chloride, was a kind gift of Dr X. Emonds-Alt, Sanofi Research, Montpellier, France. [βAla^8]neurokinin A (4–10) and MEN 10,627 or cyclo(Met-Asp-Trp-Phe-Dap-Leu)cyclo(2β–5β) were synthesized at Menarini Pharmaceuticals.

Results

A capsaicin-sensitive component in the atropine-resistant NANC response to EFS

In a first series of experiments ($n = 12$ for each group) EFS (pulse width 0.5 ms, 100–120 V) was performed for 30 s at frequencies between 1 and 30 Hz. In both control and capsaicin-pretreated strips EFS produced a rapidly developing phasic contraction which peaked within 10 s from the beginning of stimulation (Figure 1). The amplitude of this response was not significantly affected by *in vitro* capsaicin pretreatment (Figure 2a).

After the initial peak, and during continuous stimulation for 30 s the strips continued to contract but a qualitative difference was evident between control and capsaicin-pretreated strips (Figure 1): in control strips, the developed tension was sustained throughout the 30 s stimulation period and, in 8 out of 12 strips, a second distinct component of contraction became evident after the first 15 s of stimulation. On the other hand, in capsaicin-pretreated strips, the developed tension declined evenly toward baseline during the 30 s period of stimulation ('fading') (Figure 1). As a consequence of this different behaviour, the tension measured at the end of the 30 s stimulation period ('late' response to EFS) was significantly greater, at 10 and 30 Hz, in control than in capsaicin-pretreated group (Figure 2b).

The fading of the 'late' response to EFS observed in capsaicin-pretreated strips was frequency-dependent: when expressing the 'late' response as % of the peak response to EFS it averaged 84 ± 2, 68 ± 2, 41 ± 3 and 53 ± 4% at 1, 3, 10 and 30 Hz, respectively ($n = 12$). This phenomenon was not evident in control strips in which the 'late' response averaged 83 ± 2, 83 ± 4, 84 ± 3 and 82 ± 4% of the peak response at 1, 3, 10 and 30 Hz, respectively ($n = 12$). A second major difference was observed between control and capsaicin-pretreated strips in the post-stimulus period (Figure 1). In both groups, tension rapidly recovered toward baseline

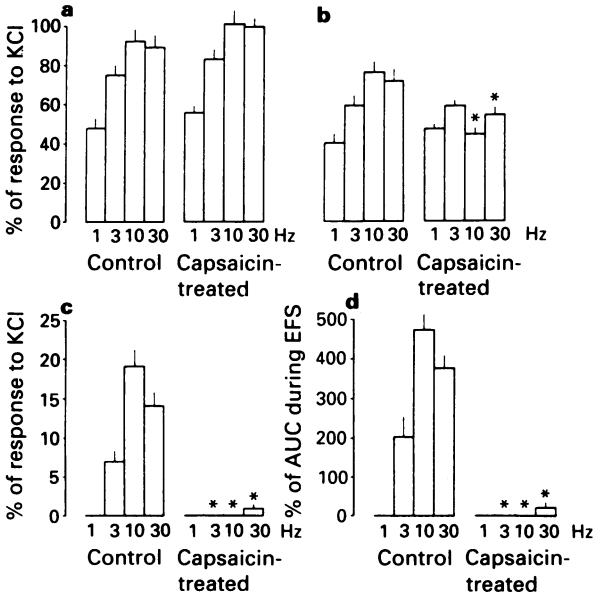


Figure 2 Frequency-dependent NANC contractile responses to prolonged (30 s) EFS of the rat isolated bladder in control and capsaicin-pretreated strips. The amplitude of the peak (a) and late (b) responses to EFS and of the post-stimulus response (c) were calculated as % of the response to 40 mM KCl. The area under the curve (AUC) of the post-stimulus response was also calculated as % of the AUC of the response obtained during EFS at each frequency (d). Each value is the mean ± s.e.mean of 12 experiments. Significantly different from control strips; $*P<0.05$.

upon cessation of the stimulus: in capsaicin-pretreated strips, the recovery toward baseline was complete within 30 s from the end of the stimulation period and, in all cases, a transient (2–4 min) after-relaxation below the pre-stimulus baseline was observed (Figure 1). In control strips, however, tension recovery toward baseline was much slower and, at frequencies of 3–30 Hz, a long lasting tonic contraction was observed in all strips (Figure 1 and 2c,d). Recovery to baseline of the post-stimulus response was complete within 15 min. The amplitude of the post-stimulus response to EFS (measured at 3.5 min from the end of stimulation) was relatively small in absolute values, not exceeding, on average, 20% of the response to KCl (Figure 2c). Quantitatively, its duration appears to be more relevant than its amplitude: the AUC of the post-stimulus response, calculated from 0.5 to 10.5 min after the 30 s stimulation period, ranged between 200 and 500% of the AUC of the atropine-resistant contraction recorded during EFS (Figure 2d).

All contractile responses produced by EFS (10 Hz for 30 s) in control or capsaicin-pretreated strips were abolished by 1 μM tetrodotoxin ($n = 6$), indicating their neural origin. The only exception was a small (<20% of the response to KCl) and quick 'off' response to EFS which was observed in some strips after tetrodotoxin. This response was clearly distinct from the post-stimulus response described above: it ensued immediately after the end of the stimulation period, peaked in 1–2 s and returned to baseline within 10–15 s.

From these experiments, a frequency of stimulation of 10 Hz was selected for further analysis since it produced maximal effect on the three components of the response observed in control strips.

Effect of varying train duration on the atropine-resistant NANC response to EFS

In these experiments ($n = 6$ for each group), trains of pulses were delivered at frequency of 10 Hz for 1–120 s in control and capsaicin-pretreated bladder strips. The peak response to EFS did not show much variation with changes in train duration and its amplitude was not significantly different between control and capsaicin-pretreated strips (Figure 3a). For periods of stimulation <10 s at a frequency of 10 Hz, no clear evaluation of the late response to EFS was possible.

The amplitude of the late response to EFS was slightly, but not significantly, lower in capsaicin-pretreated than in control strips for train duration of 10 s (Figure 3b). For periods of stimulation of 30–120 s, the amplitude of the late response was significantly lower in the capsaicin-treated group (Figure 3b). The fading of the 'late' response to EFS observed in capsaicin-pretreated strips was dependent on train duration: when expressing the late response to EFS as % of the peak response observed for each value of train duration, it averaged 69 ± 3, 51 ± 4, 38 ± 4 and 21 ± 2% at train duration of 10, 30, 60 and 120 s respectively ($n = 6$). This fading was not observed in control strips, in which the 'late' responses averaged 80 ± 4, 84 ± 8, 84 ± 9 and 72 ± 9% of the peak response at train duration of 10, 30, 60 and 120 s, respectively ($n = 6$). For train duration of 1–5 s, no distinct post-stimulus contraction was observed; at 10–120 s of train duration a post-stimulus contraction was observed in all control strips tested while an after relaxation was observed in capsaicin-pretreated strips (Figure 3c).

Effect of SR 140,333 and MEN 10,627 on the atropine-resistant NANC response to EFS

From the experiments described in the previous sections it appears that a capsaicin-sensitive component is present in the atropine-resistant contraction of the rat isolated bladder to EFS, which becomes especially evident for prolonged periods (>10 s) of stimulation. The following experiments were performed to assess the possible contribution of tachykinins by investigating the effect of the potent and selective NK_1 receptor antagonist, SR 140,333 and NK_2 receptor antagonist, MEN 10,627, alone and in combination. Three consecutive cycles of stimulation (10 Hz for 30 s) were performed at 30 min intervals and the antagonists were added 15 min before of the next cycle. SR 140,333 (0.1–1.0 μM) and MEN 10,627 (0.1–1.0 μM) did not affect resting tension of the rat bladder. At 1.0 μM, MEN 10,627 produced a small (<20% of the response to KCl) tonic contraction in six out of 12 cases tested. When both antagonists were administered, a tonic contraction was also observed in five out of eight cases tested, which was qualitatively and quantitatively similar to that observed with MEN 10,627 alone.

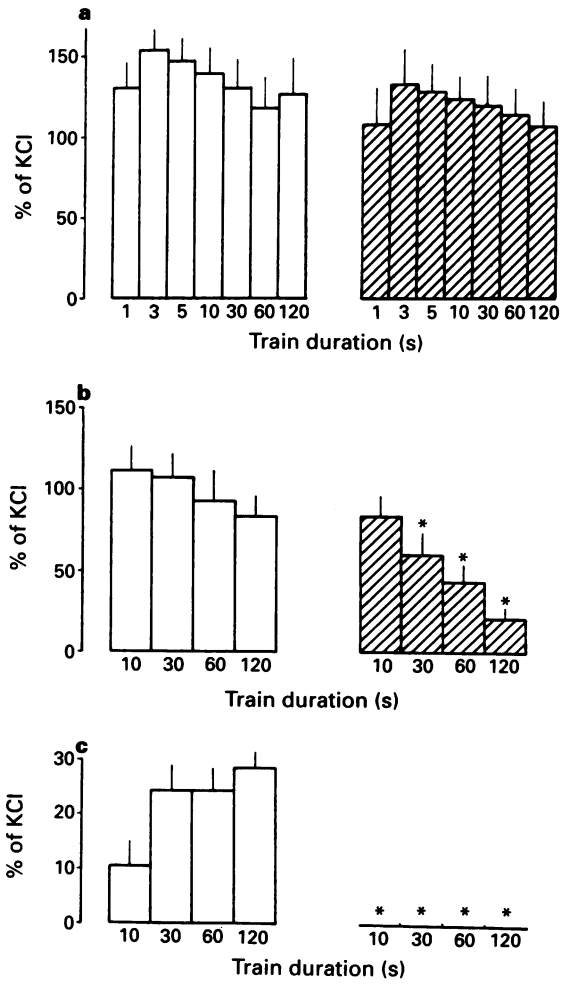


Figure 3 Relationship between train duration and the amplitude of the peak (a), late (b) and post-stimulus (c) NANC contractile responses in control and capsaicin-pretreated strips of the rat isolated bladder to EFS, expressed as % of the response to 40 mM KCl. Open columns = control strips; hatched columns = capsaicin-pretreated (10 μM for 15 min) strips. Each value is the mean ± s.e.mean of six experiments. Significantly different from control strips; $*P < 0.05$.

As shown in Figures 4 and 5, SR 140,333 had no effect on the peak response to EFS, while it decreased significantly the late response at both 0.1 and 1 μM (16 ± 3 and 33 ± 3% inhibition, respectively, $n = 12$). At 1 μM, SR 140,333 also significantly reduced (27 ± 9% inhibition, $n = 12$) the peak of the post-stimulus response but the effect on the AUC was not significant. MEN 10,627 had no significant effect on the peak response to EFS, and significantly decreased (32 ± 4% inhibition, $n = 12$) the late response at 1 μM only. MEN 10,627 inhibited the post-stimulus response (both as peak and as AUC) at both concentrations tested and, at 1 μM, almost abolished it.

The combined administration of SR 140,333 and MEN

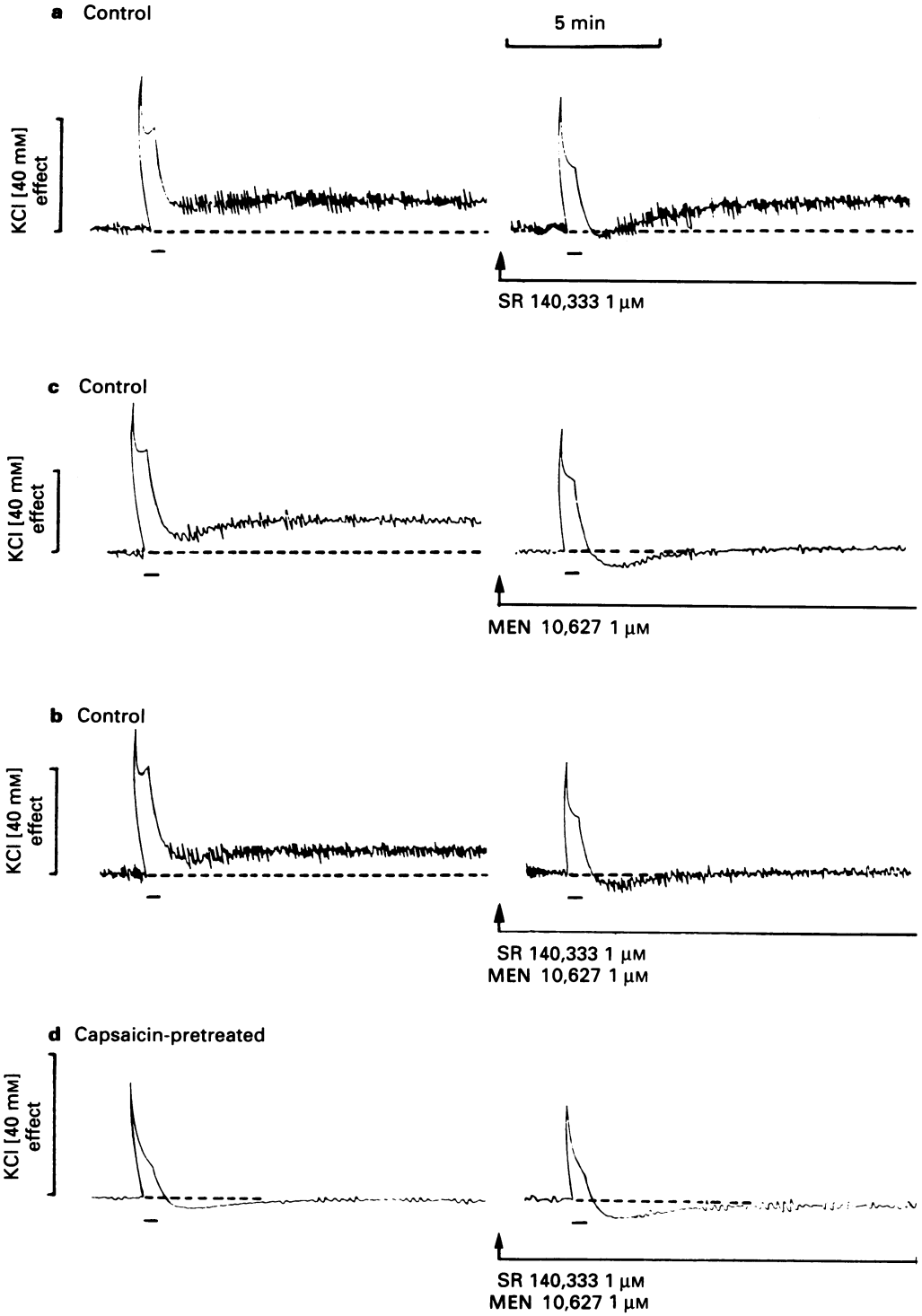


Figure 4 Effect of the tachykinin NK_1 receptor antagonist SR 140,333 (1 μM, a), of the tachykinin NK_2 receptor antagonist MEN 10,627 (1 μM, b) or of the combined administration of the two antagonists (1 μM each, c) on the NANC contractile responses in control (a, b and c) and capsaicin-pretreated (d) strips of the rat isolated bladder to prolonged EFS (10 Hz for 30 s). In (d) is shown the effect of the combined administration of SR 140,333 and MEN 10,627 on the response to EFS in a capsaicin-pretreated bladder strips. Note that SR 140,333 produced a marked decrease of the late response to EFS and had a minor effect on the post-stimulus response, while MEN 10,627 had a minor effect on the late response to EFS and abolished the post-stimulus response.

10,627 produced a small (22 ± 3% inhibition, $n = 12$) but statistically significant reduction of the peak response to EFS, a marked reduction of the late response (48 ± 3% inhibition, $n = 12$) and abolition of the post-stimulus response (Figures 4 and 5) which was replaced by a post-stimulus relaxation, similar to that observed in capsaicin-pretreated strips.

The effect of SR 140,333 plus MEN 10,627 (both at 1 μM) was also investigated on the peak and late responses to EFS produced in capsaicin-pretreated bladder strips (Figure 5). As shown in Figure 5, the combined administration of the two antagonists slightly but significantly reduced the peak response to EFS. The extent of this effect (18 ± 2% inhibition, $n = 9$) was comparable to that observed after administration

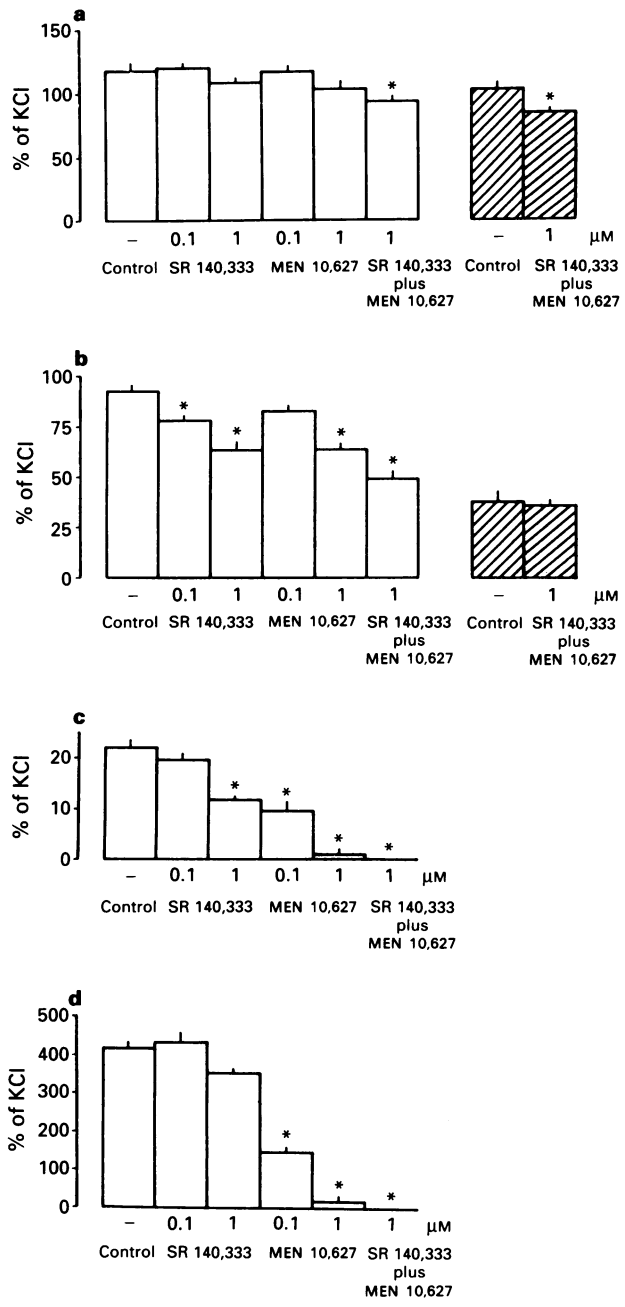


Figure 5 Effect of the tachykinin receptor antagonists SR 140,333 (0.1 and 1 μM) and MEN 10,627 (0.1 and 1 μM), alone or in combination, on the three components of the NANC response of the rat isolated bladder to EFS: (a) peak response; (b) late response; (c) post-stimulus response and (d) post-stimulus response (AUC). Open columns = control strips; hatched columns = capsaicin-pretreated strips. Each value is the mean ± s.e.mean of 9–24 experiments. Significantly different from control strips; $*P<0.05$.

of both antagonists on the peak response to EFS in bladder strips not exposed to capsaicin (22 ± 3% inhibition, $n = 12$). By contrast, the combined administration of SR 140,333 and MEN 10,627 did not significantly affect the late response to EFS in capsaicin-pretreated bladder strips (Figure 5).

Effect of SR 140,333 and MEN 10,627 on the response of the rat isolated urinary bladder to selective tachykinin NK_1 and NK_2 receptor agonists

Both [Sar^9]substance P sulphone and [βAla^8]neurokinin A (4–10) produced a concentration-dependent contraction of the rat isolated bladder: their EC_{50} values and 95% c.l. (in brackets) were 42 nM (30–54 nM) and 46 nM (33–58 nM), respectively. SR 140,333 (0.1 and 1.0 μM) produced a large rightward shift of the concentration-response curve to [Sar^9]substance P sulphone without affecting the response to the NK_2 receptor agonist, [βAla^8]neurokinin A (4–10) (Figure 6a and b). From the dose-ratios (DR) of EC_{50s} of [Sar^9]SP sulphone in the absence and presence SR 140,333 (105 ± 23 and 2257 ± 743 fold increase in EC_{50} of the agonist at 0.1 and 1.0 μM, $n = 8$ and 4, respectively) an apparent pK_B value of 8.97 ± 0.14 ($n = 12$) was calculated, assuming competitive antagonism.

MEN 10,627 (0.1 and 1 μM) produced a large rightward shift of the concentration-response curve to the NK_2 receptor agonist without affecting the response to [Sar^9]substance P sulphone (Figure 6c and d). From the DR of EC_{50s} of [βAla^8]neurokinin A (4–10) in the absence and presence of 0.1 μM MEN 10,627 (117 ± 51 fold increase in EC_{50} of the agonist, $n = 4$) an apparent pK_B value of 8.95 ± 0.16 ($n = 4$) was calculated, assuming competitive antagonism. At 1.0 μM, MEN 10,627 produced a further shift of the concentration-response curve to [βAla^8]neurokinin A (4–10), which at the highest concentration of agonist tested (30 μM), did not exceed 60% of maximal response obtained during the control curve (Figure 6).

ATP (1 mM) produced a quick phasic contraction of the bladder strips which, on two consecutive administrations (30 min apart from each other) averaged 41 ± 4 and 40 ± 4% of the response to KCl ($n = 4$, NS). In the presence of SR 140,333 plus MEN 10,627 (1.0 μM each, 15 min before) the response to ATP averaged 41 ± 2% of KCl (NS as compared to controls).

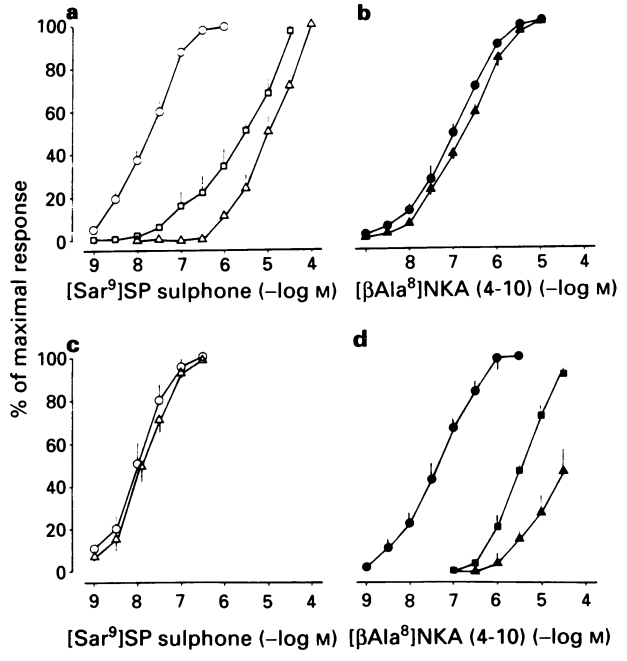


Figure 6 Effects of SR 140,333 (NK_1 receptor selective antagonists) and MEN 10,627 (NK_2 receptor selective antagonist) on the response to the NK_1 receptor selective agonist [Sar^9]substance P sulphone and to the NK_2 receptor selective agonist [βAla^8]neurokinin A(4–10) in the rat isolated bladder. (a) Effect of SR 140,333 on the concentration-response curve to the [Sar^9]substance P sulphone: (○) control; (□) SR 140,333 0.1 μM; (△) SR 140,333 1 μM. (b) Effect of SR 140,333 on the concentration-response curve to [βAla^8]neurokinin A(4–10): (●) control; (▲) SR 140,333 1 μM. (c) Effect of MEN 10,627 on the concentration-response curve to [Sar^9]substance P sulphone: (○) control; (△) MEN 10,627 1 μM. (d) Effect of MEN 10,627 on the concentration-response curve to [βAla^8]neurokinin A(4–10): (●) control; (■) MEN 10,627 0.1 μM; (▲) MEN 10,627 1 μM. Each value is the mean ± s.e.mean of 4–12 experiments.

Discussion

The results of this study demonstrate the existence of a capsaicin-sensitive component in the atropine-resistant NANC contraction of the rat isolated urinary bladder to nerve stimulation and, by the use of receptor antagonists, provide evidence that the capsaicin-sensitive fraction of the atropine-resistant NANC response is mediated through the release of endogenous tachykinins from peripheral endings of primary afferent neurons.

A crucial factor leading to the identification of a capsaicin-sensitive, tachykinin-mediated component in the response to EFS is the duration of the train of pulses applied to excite intramural nerves: the requirement of a relatively prolonged (>10 s) period of stimulation may be linked to the slow character of the contractile action of tachykinins, as opposed to the fast action of ATP, the transmitter responsible for the peak response to EFS in the presence of atropine. As determined in a previous study (Maggi *et al.*, 1991b) the response to low concentrations of tachykinins, substance P and neurokinin A, develops with a slower time course as compared to ATP and it is also long lasting. By using *in vitro* capsaicin desensitization to block the efferent function of sensory nerves, we conclude that the demonstration of a tachykinin-mediated component in the response to nerve stimulation of the rat isolated bladder requires periods of stimulation >10 s (Figure 3) at frequencies >3 Hz (Figure 2). By comparing the effect *in vitro* capsaicin desensitization and that of blockade of tachykinin receptors on the NANC contraction, we conclude that: (a) the peak response to EFS is entirely capsaicin-resistant; (b) the late response to EFS has a mixed contribution of capsaicin-resistant and capsaicin-sensitive nerves and (c) the post-stimulus response is totally mediated by capsaicin-sensitive afferents.

The identity of the transmitter responsible for the fast, capsaicin-resistant, NANC contraction has not been investigated in this study but, according to previous studies on the rat isolated bladder (Brown *et al.*, 1979; Brading & Williams, 1990; Luheshi & Zar, 1990; Maggi, 1991), it is most likely to be ATP. The capsaicin-resistant response to EFS showed a marked 'fading' during a prolonged train of stimuli: this fading was proportional to both frequency of stimulation (train duration set at 30 s) and to duration of the train (at a frequency of 10 Hz). This may reflect the fast and transient nature of the postjunctional response to ATP, perhaps involving some degree of desensitization of purinoceptors during prolonged stimulation by endogenous ATP and/or a prejunctional inhibitory feedback on transmitter release by e.g. products of ATP breakdown (Parija *et al.*, 1991; Acevedo *et al.*, 1992). It is important to remember that, in addition to their direct contractile effect, exogenously administered tachykinins potentiate the contractile response of the rat isolated bladder to EFS (Maggi *et al.*, 1988; Maggi, 1991). It is possible that the capsaicin-sensitive, tachykinin-mediated contribution to the 'late' response to EFS involves a facilitation of ATP release and/or a facilitation of ATP action at postjunctional level rather than a direct contractile effect of tachykinins on smooth muscle. Another possibility may be that ATP induces or facilitates the release of tachykinins from sensory nerves: in future studies, it will be necessary to use drugs or procedures which totally eliminate the purinergic contraction to investigate the putative relationships between ATP and tachykinin released during EFS.

The long lasting post-stimulus response to EFS is a pure capsaicin-sensitive and tachykinin-mediated event; the peak amplitude of this response is not quantitatively large, a character which had been anticipated on the basis of quantitative differences in the magnitude of the cholinergic and purinergic contractile responses of the rat bladder vs. the magnitude of the maximal response to capsaicin, considered as the most effective stimulus to induce tachykinin release from sensory nerves (cf. Maggi, 1993 for further discussion on this point). Since a post-stimulus after-relaxation to EFS was evident after *in vitro* capsaicin administration or following blockade of tachykinin receptors, we cannot discount the possibility that the amplitude of the post-stimulus contractile response was underestimated because of a concomitant relaxation through a capsaicin-resistant mechanism. The nature of the capsaicin-resistant post-stimulus relaxation remains speculative at present: even in this case, products of ATP breakdown may be involved, since both AMP and adenosine are known to relax the rat bladder smooth muscle (Brown *et al.*, 1979). Irrespective of this point, the prolonged duration is the most impressive character of the capsaicin-sensitive post-stimulus contraction, which, when calculated as area under the curve, exceeds the overall NANC contractile response produced during nerve stimulation.

Previous binding, autoradiographic and functional experiments had shown that tachykinin NK_1 and NK_2 receptors are both present in the rat urinary bladder and mediate contraction of the smooth muscle to tachykinins (Burcher & Buck, 1986; Maggi *et al.*, 1986). This notion has been further supported by the demonstration that high levels of mRNAs encoding for NK_1 and NK_2 receptors are expressed in the rat bladder (Tsuchida *et al.*, 1990). Furthermore, both receptors appear to be involved in the contractile response to capsaicin, with a preferential contribution of NK_1 and NK_2 receptors in the 'early' and 'late' components of the response to selective sensory nerve stimulation, respectively (Maggi *et al.*, 1991b). This is in keeping with the present results, whereby the NK_1 receptor selective antagonist, SR 140,333, preferentially inhibited the 'late' response to EFS, while the selective NK_2 receptor antagonist, MEN 10,627, preferentially inhibited the post-stimulus response.

SR 140,333 (Emonds-Alt *et al.*, 1993) and MEN 10,627 (Patacchini *et al.*, 1993) have been characterized as highly potent, selective and competitive antagonists at tachykinin NK_1 and NK_2 receptors, respectively. This profile is further supported by the present data, whereby SR 140,333 produced a remarkable rightward shift of the curve to the NK_1 receptor selective agonist [Sar^9]substance P sulphone without affecting that to the NK_2 receptor selective agonist, [βAla^8] neurokinin A (4–10), while MEN 10,627 antagonized the response to [βAla^8]neurokinin A (4–10) but not that to [Sar^9]substance P sulphone. Since the primary aim of these experiments was to unravel a putative involvement of endogenous tachykinins in the NANC response to EFS, we did not perform a complete pharmacological analysis of the antagonist activity of SR 140,333 and MEN 10,627; notwithstanding this, the concentrations of antagonists and the incubation time selected for this study appear appropriate to produce a full blockade of NK_1 and NK_2 receptors in the rat urinary bladder smooth muscle. The selectivity of SR 140,333 and MEN 10,627 at postjunctional level was further verified by demonstrating their lack of inhibitory action toward the response to exogenous ATP.

In the presence of both antagonists, the NANC response to EFS in control strips became almost superimposable to that observed in capsaicin-pretreated bladder strips, suggesting an exclusive mediator role of tachykinins in the capsaicin-sensitive response observed in control strips. The small reduction of the peak response to EFS observed in the presence of both SR 140,333 and MEN 10,627 most likely represents a non specific, possibly prejunctional, effect since it was equally observed in capsaicin-pretreated bladder strips and the two drugs do not affect the postjunctional response to ATP. In addition to tachykinins, capsaicin-sensitive afferents innervating the rat urinary bladder also contain and release calcitonin gene-related peptide (CGRP) (Maggi, 1993 for review). However, CGRP has little if any effect on the motility of the rat isolated bladder smooth muscle (Maggi *et al.*, 1987), where it may serve other functions, such as regulation of blood flow.

A basic question raised by the present findings is: what is the physiological significance of the capsaicin-sensitive, tachykinin-mediated, excitatory motor innervation of the rat blad-

der? Since the early *in vivo* studies on this topic it was evident that capsaicin-pretreatment, at doses which impair the afferent arm of the micturition reflex and deplete the bladder content of tachykinins, produce little changes in the ability of the bladder to contract and empty during micturition (Maggi *et al.*, 1984; 1986). This concept has received substantial confirmation by more recent studies showing that the administration of antagonists to block tachykinin receptors at bladder level has little, if any, effect on the amplitude of micturition contraction in normal rats (Maggi *et al.*, 1991a; Lecci *et al.*, 1993b), while the blockade of tachykinin receptors at spinal cord level exerts a modulatory influence on the afferent arm of the micturition reflex (Lecci *et al.*, 1993a). It is theoretically possible that, at the high pressure values developed during the micturition contraction, some tachykinins are released by mechanical excitation of capsaicin-sensitive bladder afferents, but their contribution to the detrusor contraction producing normal bladder voiding seems minor, at best. The present results are in full agreement with these concepts since the contribution of tachykinins to the peak neurogenic contraction produced during the period of electrical stimulation is quantitatively small as compared to the overall atropine-resistant response. On the other hand, a peripheral release of tachykinins from sensory nerves certainly occurs during cystitis or anyway during bladder irritation (Maggi, 1993 for review) and provides a neurogenic contribution to the inflammatory processes of the lower urinary tract (Eglezos *et al.*, 1991; Giuliani *et al.*, 1993. Ahluwalia *et al.*, 1994). The prolonged increase in bladder tone produced by tachykinins, as demonstrated here as a post-stimulus response to EFS, may contribute to generate symptoms of urgency and frequency during cystitis, since even small changes in bladder tone can enhance the afferent input to the spinal cord.

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Effects of cyclopiazonic acid on rhythmic contractions in uterine smooth muscle bundles of the rat

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1 We studied the effects of cyclopiazonic acid (CPA) on rhythmic contractions and on Ca^{2+} uptake by the intracellular stores in longitudinal muscle strips of the rat uterus at 30°C.

2 Oxytocin (1 μM) in Ca^{2+}-free solution induced a transient rise in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and contraction after Ca^{2+} loading of the stores in high-K^+- and Ca^{2+}-containing solution. CPA inhibited oxytocin-induced Ca^{2+} release and contraction, the half and full inhibitory concentrations of CPA being 0.3 and 10 μM, respectively. In contrast, addition of CPA after Ca^{2+} loading exerted no significant inhibitory effects.

3 Oxytocin (10 nM) applied in Ca^{2+}-containing solution induced rhythmic increases in both force and $[Ca^{2+}]_i$. CPA (10 μM) had no effect on oxytocin-induced rhythmic contractions.

4 At a high concentration (300 μM), CPA inhibited the rhythmic contractions induced by 10 nM oxytocin; the frequency and the peak height were decreased, and in many bundles contractions were completely abolished. These inhibitory effects were reversed after CPA washout.

5 CPA (300 μM) inhibited the rate of rise of $[Ca^{2+}]_i$ due to depolarization induced by high-K^+-containing solution.

6 These results suggest that low concentrations of CPA inhibit the loading of Ca^{2+} into intracellular stores in intact tissue strips, and that the Ca^{2+} stores are not directly involved in the uterine rhythmic contractions. It is also suggested that a high concentration of CPA inhibits the mechanism that is responsible for the generation of rhythmic contractions as well as voltage-dependent Ca^{2+} channels.

Keywords: Cyclopiazonic acid; Ca^{2+}; uterine smooth muscle; oxytocin

Introduction

Uterine smooth muscles undergo spontaneous rhythmic contractions without neuronal or hormonal stimulation *in vitro*. Changes in the membrane potentials were recorded from the myometrial cells during these contractions, and the rhythmic contractions were found to be accompanied by groups of action potentials (Kleinhaus & Kao, 1969). The inward currents mediating the upstroke of the action potentials are due mainly to Ca^{2+} influx through voltage-dependent Ca^{2+} channels (Mironneau, 1973; Kao & McCullough, 1975). L-type Ca^{2+} channels were also shown to be responsible for the inward currents which were blocked by a dihydropyridine Ca^{2+} channel antagonist (Jmari *et al.*, 1986; Honore *et al.*, 1989). Agonists like oxytocin and prostaglandins can increase the force and frequency of rhythmic contractions (Mironneau, 1976), which are also suppressed by L-type Ca^{2+} channel blockers or by removal of extracellular Ca^{2+} (Edwards *et al.*, 1986; Kawarabayashi *et al.*, 1986; Tasaka *et al.*, 1991). Therefore, Ca^{2+} influx through L-type Ca^{2+} channels is important in Ca^{2+} regulation in both spontaneous and agonist-stimulated rhythmic contractions of uterine smooth muscle cells.

Recently, the primary structure of the human oxytocin receptor was determined by molecular cloning of its complementary DNA and the receptor molecule was predicted to have seven transmembrane stretches, typical of G-protein-coupled receptors (Kimura *et al.*, 1992). Indeed, oxytocin has been shown to activate phospholipase C in the myometrium through a G-protein-mediated process. This results in the rapid formation of inositol 1,4,5-trisphosphate (IP_3) (Marc *et al.*, 1986; 1988), which releases Ca^{2+} from the sarcoplasmic reticulum (SR) vesicles of the uterine smooth muscle (Carsten & Miller, 1985). Coleman and colleagues (1988) also showed that oxytocin causes contractions in the myometrium in the absence of extracellular Ca^{2+} due to Ca^{2+}-release from the intracellular stores. These results suggest that oxytocin can increase $[Ca^{2+}]_i$ both by enhancing Ca^{2+} influx through L-type Ca^{2+} channels and by causing IP_3-induced Ca^{2+} release from the intracellular Ca^{2+} stores. However, the relative roles of extracellular and intracellular sources of Ca^{2+} to the contractile actions of oxytocin are unclear.

CPA is one of the mycotoxins and was first reported as a potent inhibitor of the Ca^{2+} uptake and ATPase activity of rat skeletal muscle SR vesicles by Goeger *et al.* (1988). Subsequently, CPA was shown to be a specific inhibitor of the Ca^{2+}-ATPase of skinned skeletal muscle SR (Kurebayashi & Ogawa, 1991) and skinned smooth muscle bundles (Uyama *et al.*, 1992). These workers showed that smooth muscle contractile responses to caffeine or IP_3 were reduced or abolished when the preceding Ca^{2+} uptake was performed in the presence of 0.1–10 μM CPA, whereas the addition of CPA after Ca^{2+} uptake had no significant effects (Uyama *et al.*, 1992). Therefore, CPA may be used as a pharmacological agent to inhibit the function of Ca^{2+} stores of smooth muscle cells. To determine the role of the intracellular Ca^{2+} stores in the oxytocin-induced rhythmic contractions, we have now studied the effect of CPA on contractions of uterine smooth muscle cells.

Methods

Non-pregnant female Wistar rats, weighing 200–300 g, were stunned and exsanguinated. A vaginal smear test was performed, and only rats in oestrus were used. Uteri were excised, and longitudinal muscle bundles (1.5 mm in length, 150–200 μm in width, and 150 μm in thickness) were carefully dissected in physiological salt solution (PSS) with the composition given below. The dissection was carried out at room temperature, and the experiments were performed at 30°C.

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Measurement of tension and fluorescence intensity

To load the smooth muscle cells with a fluorescent Ca^{2+} indicator, Fura-2, muscle bundles were incubated in 20 μM Fura-2AM in PSS with 0.005% Pluronic F-127 for 3–5 h at 30°C. Then each end of the muscle bundle was tied with silk filament and the bundle was attached to a pair of stainless steel hooks, with one end connected to an isometric tension transducer (AE801, Akers, Norway) and the other to a micromanipulator for the adjustment of muscle length. The muscle strips were placed in an experimental trough (capacity 300 μl) mounted on a fluorometer (CAF-100, JASCO, Japan), and were illuminated alternately by 340 and 380 nm lights at 50 Hz. Fluorescence signals from the muscle strips were collected by a photomultiplier tube through a 500 nm interference filter. When $[Ca^{2+}]_i$ rose, the changes in the fluorescence intensity (photomultiplier current) at 340 and 380 nm excitations showed mirror-image features. Therefore, the ratio of the two fluorescence intensities (F340/F380) was used as an indicator of $[Ca^{2+}]_i$. To change solutions, the solution in the trough was first aspirated and a new solution was then pumped in. The procedure was controlled by a personal computer (PC9801, NEC, Japan) and was accomplished within 5 s. The two fluorescence intensity signals as well as the isometric tension signal were collected using an analog-to-digital converter board in the computer and stored on floppy disks for later analysis and plotting using another computer (Quadra 800, Apple, U.S.A.). The ratio of the fluorescence intensities and the tension record were also displayed on a pen recorder (SR6221, Graphtec, Japan).

When the rate of rise of $[Ca^{2+}]_i$ induced by a high concentration of K^+ solution was to be compared, the muscle bundle was attached to a stainless steel wire (100 μm in diameter) and was placed in a capillary cuvette with an internal diameter of 400 μm. The solution was changed within 1 s by a rapid flow of solutions through the cuvette. F340/F380 of the muscle bundles was measured with a fluorometer (CAF-110, JASCO, Japan).

The solutions used were as follows (in mM). Physiological salt solution (PSS): NaCl 150, KCl 4, $CaCl_2$ 2, $MgCl_2$ 1, HEPES (N-2-hydroxyethylpiperazine-N'-ethanesulphonic acid) 5, glucose 5.6. High-K^+ solution: KCl, 154, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 5, glucose 5.6, sucrose 44. High-K^+ Ca^{2+}-free solution: KCl 154, $MgCl_2$ 3, HEPES 5, EGTA 5, glucose 5.6, sucrose 44. The pH of all solutions was adjusted to 7.4 with NaOH.

Materials

Chemicals used were: Fura-2AM from Dojin (Japan), Pluronic F-127 from BASF (U.S.A.), CPA, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), carbachol and nicardipine from Sigma (U.S.A.) and oxytocin from Peptide Institute, Inc. (Japan).

Control study and statistical analysis

CPA was prepared in 10 mM and 100 mM stock solutions in dimethyl sulphoxide (DMSO), which at the highest concentration used (0.3%) had no effect on oxytocin-induced Ca^{2+} release, rhythmic contractions and high-K^+ induced contractions in the time-match control experiments; also had no effects on fluorescence signals of Fura-2 in cuvette calibration.

All values are expressed by means ± s.e.mean, and n is the number of observations. We obtained only one preparation from one animal.

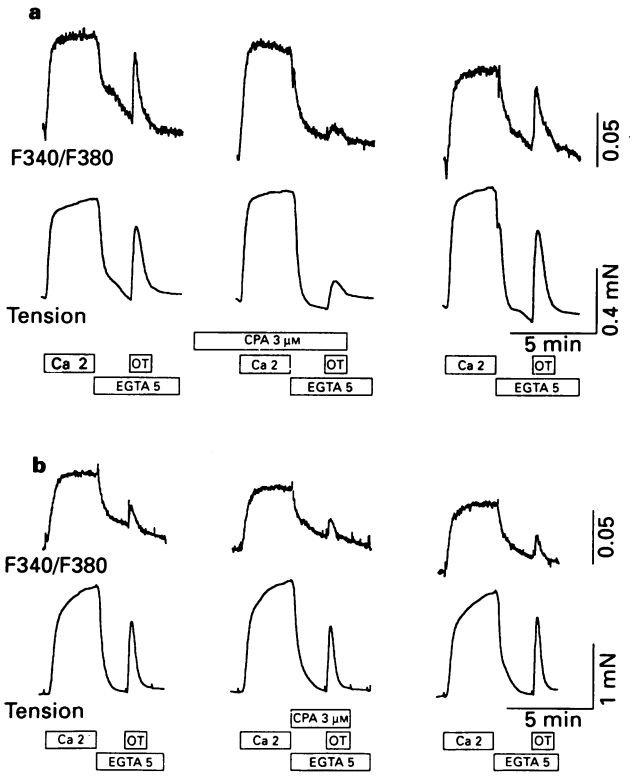


Figure 1 Effects of CPA on Ca^{2+} release from intracellular Ca^{2+} stores. Ca^{2+} was loaded into the stores during high-K^+ (154 mM K^+, 2 mM Ca^{2+})-induced contraction for 3 min, then oxytocin (OT, 1 μM) was applied in the absence of extracellular Ca^{2+} (EGTA 5 mM). Changes in $[Ca^{2+}]_i$ (upper traces) and isometric tension (lower traces) were simultaneously measured. (a) Three consecutive runs obtained from the same bundle ($n = 6$). CPA (3 μM) was applied 3 min before Ca^{2+} loading until the end of oxytocin application in the second run as shown by the box below the traces. In the presence of CPA, the response to oxytocin was markedly reduced (middle figure). The effect of CPA was reversible and the response induced by oxytocin recovered in the subsequent control run. (b) Similar to (a), but 3 μM CPA was added after Ca^{2+} loading in the second run. There was no significant effect of CPA with this protocol ($n = 3$).

Results

Effects of CPA on Ca^{2+} release from intracellular Ca^{2+} stores

CPA inhibits Ca^{2+} uptake by the sarcoplasmic reticulum in the skinned fibres of both skeletal muscle and smooth muscle (Kurebayashi & Ogawa, 1991; Uyama *et al.*, 1992). We studied whether CPA could also inhibit Ca^{2+} uptake by the stores in intact myometrial muscle strips. As shown in Figure 1, Ca^{2+} was loaded into the stores during high-K^+ (154 mM K^+, 2 mM Ca^{2+})-induced contraction for 3 min, then extracellular Ca^{2+} was removed with 5 mM EGTA-containing solution in the continued presence of a high concentration of K^+. After 2 min in the Ca^{2+}-free solution, oxytocin (1 nM–1 μM) was applied and it induced transient $[Ca^{2+}]_i$ increase and contraction (Figure 1a, left). This response was due to Ca^{2+} release from the stores since extracellular Ca^{2+} was absent. We were able to obtain the same response repeatedly from one strip when oxytocin was applied every 11–12 min after the same Ca^{2+} loading procedure. The peak size of the transient contraction was dependent on the concentration of oxytocin; 14.6 ± 10.4% (1 nM), 57.1 ± 8.7% (10 nM) and 81.5 ± 7.0% (100 nM) of that for 1 μM oxytocin, and the rise of $[Ca^{2+}]_i$ was 8.3 ± 10.2% (1 nM), 48.4 ± 22.1% (10 nM), 68.8 ± 13.7% (100 nM) of that for 1 μM (average ± s.e.mean, $n = 3$; all the concentrations of oxytocin were tested in each muscle). In subsequent experiments, we used 1 μM oxytocin as the standard stimulus to examine the effect of CPA on the function of the Ca^{2+} stores.

If 3 μM CPA was applied 3 min before the Ca^{2+} loading and remained until the end of oxytocin application, the response to oxytocin was markedly reduced (Figure 1a, mid-

dle). However, if CPA was added after Ca^{2+} loading, no significant suppression was observed (Figure 1b, middle). The effect of CPA was reversible and the response induced by oxytocin recovered in the following control run (Figure 1a and b, right). These results show that CPA inhibits Ca^{2+} uptake, thereby reducing subsequent Ca^{2+} release from the Ca^{2+} stores.

The open symbols in Figure 2 show concentration-dependent effects of CPA on the Ca^{2+} release. Peak responses obtained in a test run with CPA application during the Ca^{2+} loading were normalized by the average of the control runs obtained before and after the test run. In the presence of 10 μM CPA during the Ca^{2+} loading, the rise of $[Ca^{2+}]_i$ due to oxytocin application was completely inhibited (open triangle). The relative value of the corresponding contractile response was also reduced to 0.09 ± 0.02 (open circle). The remaining contraction was slow in onset and was very probably due to the increase in Ca^{2+} sensitivity of the contractile proteins induced by agonists (see Karaki, 1989).

Rhythmic contractions of uterine smooth muscle bundles

Figure 3 shows the response of a uterine smooth muscle bundle to 10 nM oxytocin in the presence of 2 mM Ca^{2+}. A rapid rise in tension which lasted for approximately 2 min occurred, followed by a spontaneous partial relaxation and subsequent rapid contraction. This partial relaxation and contraction cycle went on a few times until constant rhythmic contractions from the baseline tension developed (Figure 3, lower trace). The frequency of the rhythmic contractions was $0.75 \pm 0.03\ min^{-1}$ (mean ± s.e.mean, $n = 21$). These changes in the tension synchronized with the rise of $[Ca^{2+}]_i$ (Figure 3, upper trace). After drug washout, both tension and $[Ca^{2+}]_i$ signals returned to basal levels. Similar rhythmic contractions were sometimes seen spontaneously and were observed when activated by 1 μM $PGF_{2\alpha}$ ($0.75 \pm 0.09\ min^{-1}$, $n = 3$) or 10 μM carbachol ($0.70 \pm 0.16\ min^{-1}$, $n = 3$).

Effect of CPA on rhythmic contractions

We then examined the effects of 10 μM CPA, which is enough to inhibit the function of Ca^{2+} stores, on the rhythmic contractions induced by 10 nM oxytocin. Figure 4a illustrates

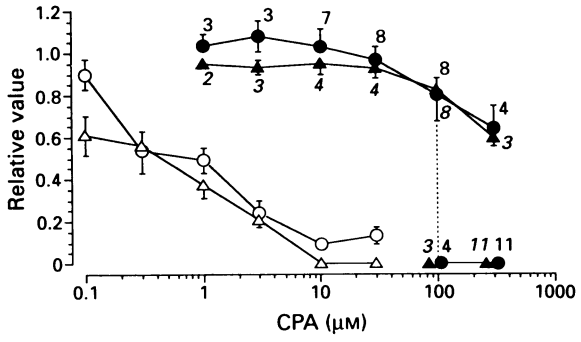


Figure 2 Concentration-response relationship of the effect of CPA on Ca^{2+} release induced by 1 μM oxytocin and rhythmic contractions by 10 nM oxytocin. Shown here are peak sizes of oxytocin-induced contraction (○) and $[Ca^{2+}]_i$ rise (△) following Ca^{2+} loading in the presence of CPA at the concentrations shown on the abscissa scale (see Figure 1). Results were expressed relative to the average of the corresponding values of control runs obtained before and after the test run (mean ± s.e.mean, $n = 7-8$ for ≤ 10 μM, $n = 3$ for 30 μM). Peak sizes of the rhythmic contractions (●) or $[Ca^{2+}]_i$ change (▲) from 10 min to 15 min after the application of CPA were normalized by the corresponding peak values prior to the drug application (see Figure 4, mean ± s.e.mean). At 100 and 300 μM CPA, data from the muscle bundles with complete loss of rhythmic contraction were pooled separately to obtain averages. Numbers beside symbols indicate the number of observations. In some bundles $[Ca^{2+}]_i$ was not measured.

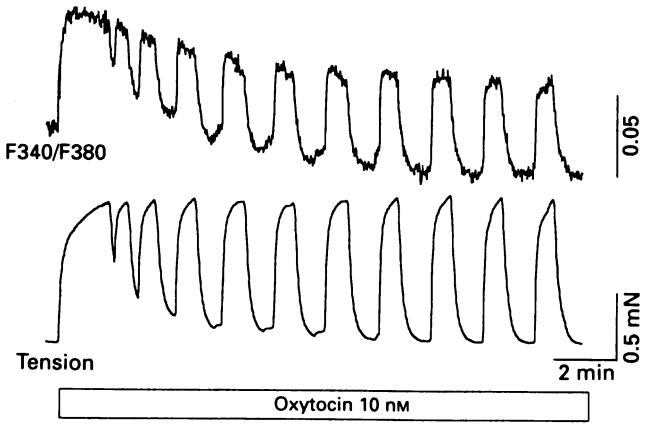


Figure 3 Rhythmic contractions of a uterine smooth muscle bundle induced by 10 nM oxytocin in the presence of 2 mM Ca^{2+}. The resting tension was 0.5 mN, and both tension and $[Ca^{2+}]_i$ remained quiescent until application of 10 nM oxytocin, upon which rhythmic contractions started. The change in the tension (lower trace) synchronized with the rise of $[Ca^{2+}]_i$ expressed by F340/F380 (upper trace).

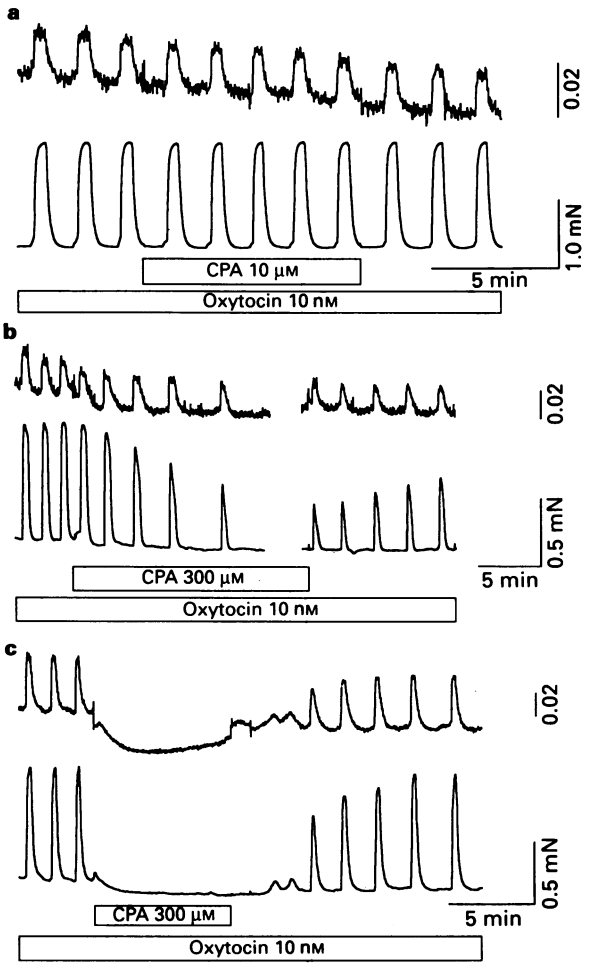


Figure 4 Effect of CPA on the rhythmic contractions induced by 10 nM oxytocin. In each panel, upper and lower traces show F340/F380 and tension, respectively. The gradual decrease in F340/F380 was probably due to dye bleaching. (a) CPA (10 μM) was added during the rhythmic contractions induced by 10 nM oxytocin ($n = 7$). There were two types of inhibition by 300 μM CPA. (b) In some preparations the frequency and the peak of rhythmic contractions were gradually decreased ($n = 4$). The break in the traces was due to a temporary halt of data acquisition necessary for the transfer of accumulated data to the floppy disk. Both traces showed no change in the chart record of the pen recorder. (c) In other muscle bundles, the rhythmic contractions were inhibited immediately after the drug application ($n = 11$). The suppression was reversed after the washing out of CPA.

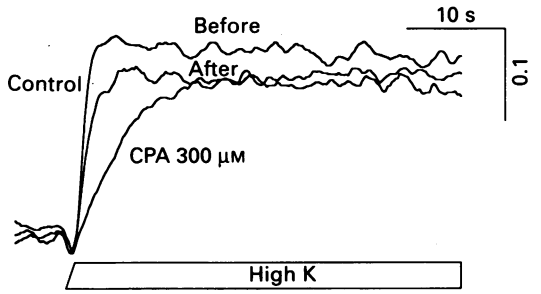


Figure 5 Effect of 300 μM CPA on the depolarization-induced $[Ca^{2+}]_i$ rise. The change in $[Ca^{2+}]_i$ (F340/F380) induced by high-K^+ solution was compared in the presence and absence of 300 μM CPA. CPA was added 3 min before the application of high-K^+ solution. CPA decreased the rate of initial rise of $[Ca^{2+}]_i$. As the CPA induced a slight rise in the resting level of $[Ca^{2+}]_i$ in PSS under these conditions, curves were shifted vertically to facilitate comparison of the rate of rise of $[Ca^{2+}]_i$. The dips in the traces before the onset of $[Ca^{2+}]_i$ rise were because of movement due to flushing of solution. These results are representative of 5 muscles.

that 10 μM CPA had no effect either on the amplitude or frequency of the rhythmic contractions. However, when 300 μM CPA was added during the rhythmic contractions induced by oxytocin, we observed clear inhibitory effects, which were of two types. Sometimes, both the amplitude and frequency of $[Ca^{2+}]_i$ and tension transients gradually decreased (Figure 4b, $n = 4$). Alternatively, both responses were suppressed immediately after the CPA application (Figure 4c, $n = 11$). All inhibitory effects of CPA were reversed after washing out of the drug.

The solid symbols in Figure 2 show the concentration-dependent inhibitory effect of CPA on the rhythmic contractions induced by 10 nM oxytocin. The peak size of the rhythmic rise in tension (circle) or $[Ca^{2+}]_i$ (triangle) 10 min to 15 min after the application of CPA was normalized by the respective peak values prior to the drug application. CPA had no effect on the rhythmic contractions at or below 30 μM where significant inhibition of Ca^{2+} stores was observed. At 100 μM CPA, we observed complete inhibition in 4 samples as in Figure 4c, and in 8 muscles the peak values of tension and $[Ca^{2+}]_i$ change as well as their frequency were decreaed (Figure 4b). When there was no effect of CPA on the peak tension, we did not observe a significant effect on the frequency of rhythmic contractions. These results suggest that the Ca^{2+} stores are not directly involved in the rhythmic tension changes induced by oxytocin. At higher concentrations above 100 μM, CPA inhibited the rhythmic contractions.

Effect of CPA on high-K^+-induced contractions

We then examined whether the high concentration of CPA has any effect on the depolarization-induced $[Ca^{2+}]_i$ rise. To change solutions as quickly as possible, we placed the muscle strip in a glass capillary cuvette (see Methods). Therefore, in this type of experiment, we were not able to measure isometric tension simultaneously, but we could observe rapid change in $[Ca^{2+}]_i$ more closely. We examined the high-K induced $[Ca^{2+}]_i$ change first during a control run and then when CPA was added 3 min before the second high-K-induced depolarization. We then performed a second control run. As shown in Figure 5, the extent of the $[Ca^{2+}]_i$ change due to high-K^+-induced depolarization was almost identical with or without application of 300 μM CPA. The relative value of the response with CPA normalized by the average of the control runs obtained before and after the test run was 0.97 ± 0.06 (mean ± s.e.mean, $n = 3$). The most conspicuous effect of CPA was on the rate of rise of $[Ca^{2+}]_i$, which was reduced in the presence of 300 μM CPA. The relative value of the half time of $[Ca^{2+}]_i$ rise to the peak was 3.91 ± 0.80 ($n = 3$). This inhibition was reversed after wash out of CPA. These data suggest that high concentrations of CPA have a partial inhibitory effect on voltage-dependent Ca^{2+} channels.

Discussion

In this paper, we examined the role of Ca^{2+} stores in oxytocin-induced rhythmic contractions using CPA as a pharmacological agent to inhibit the function of Ca^{2+} stores. In the presence of 10 μM CPA during the Ca^{2+} loading, the rise of $[Ca^{2+}]_i$ due to subsequent oxytocin application was completely inhibited, but not when CPA was added after Ca^{2+} loading. Therefore, CPA ($\leqslant 10$ μM) has rather specific effects on Ca^{2+} uptake and almost no direct effect on the Ca^{2+} release mechanism *per se* or on the Ca^{2+}-induced contractile reaction. However, CPA at 10 μM did not change the oxytocin-induced rhythmic contractions (Figure 4a). These results clearly suggest that there is little contribution of Ca^{2+} stores in the rhythmic contractions of uterine smooth muscle cells induced by oxytocin.

Thapsigargin, a tumour-promoting sesquiterpene lactose, was found to discharge intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase in rat hepatocytes (Thastrup *et al.*, 1990). We also found that 10 μM thapsigargin inhibited Ca^{2+} uptake by the Ca^{2+} stores of uterine smooth muscle cells and that it did not affect the rhythmic contraction in accordance with the results obtained with CPA (data not shown). Because it was difficult to remove the effect of thapsigargin by washout, we did not carry out a systematic study using this agent.

At higher concentrations ($\geqslant 100$ μM), CPA decreased the frequency of the rhythm and reversibly inhibited the rhythmic contractions (Figures 2 and 4b). This inhibitory effect of CPA on uterine rhythmic contraction is not related to its inhibitory effect on the Ca^{2+} uptake by the stores, because the required concentration was 10 times greater than that for complete inhibition of Ca^{2+} uptake (present study). We studied the effect of CPA on the high-K^+-induced increase in $[Ca^{2+}]_i$ in order to examine any possible action on voltage-dependent Ca^{2+} channels. The rate of rise of $[Ca^{2+}]_i$ induced by high-K^+ solution was inhibited by 300 μM CPA (Figure 5). Therefore, high concentrations of CPA seem to have inhibitory effects on the voltage-dependent Ca^{2+} channels. Further studies are required to clarify whether the inhibition of the rhythmic contractions by CPA is entirely due to the inhibition of voltage-dependent Ca^{2+} channels or whether other mechanisms are involved.

Oxytocin activates phospholipase C which cleaves phosphatidyl inositol 4,5-bisphosphate into IP_3 and diacylglycerol (Marc *et al.*, 1986; 1988). Our results indicate that one of the two branches of the phosphatidyl inositol pathway, i.e. IP_3-induced Ca^{2+} release, is not directly related to the oxytocin-induced rhythmic contraction. Therefore, future studies should examine the role of either the diacylglycerol-protein kinase C pathway (Nishizuka, 1984) or of other receptor-coupled mechanisms.

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Role of cyclic AMP-induced Cl conductance in aqueous humour formation by the dog ciliary epithelium

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1 The effects of isoprenaline, a forskolin derivative NKH-477, and dibutyryl cyclic AMP (db cyclic AMP) on the membrane potential, conductance and cell volume of the dog non-pigmented ciliary epithelium (NPE) were investigated by intracellular potential recording, nystatin-perforated patch clamp technique and videomicroscopic cytometry.

2 The resting membrane potential of NPE was about −70 mV in physiological saline and was depolarized by isoprenaline in a dose-dependent manner with an ED_{50} of about 3 nM. This depolarization was competitively antagonized by the β-adrenoceptor antagonist, timolol (pA_2 = ca. 9) and almost completely blocked by the Cl transport blocker, DIDS.

3 In single dissociated NPE cells, 10 μM isoprenaline induced an inward current and caused a concomitant decrease in cell volume. The reversal potential measurement indicated that this inward current was carried mainly by Cl ion. DIDS (10 μM) abolished both the current and cell volume decrease.

4 NKH-477 (10 μM) or db cyclic AMP (1 mM) also induced an inward current together with a cell volume decrease, the properties of which were similar to those caused by isoprenaline.

5 These results suggest that β-adrenoceptor stimulation in NPE leads to an increased rate of aqueous humour production by increasing Cl^- efflux via an elevation of cyclic AMP and this effect is efficiently blocked by timolol.

Keywords: β-Adrenoceptor; cyclic AMP; Cl current; aqueous humour; dog ciliary epithelium

Introduction

The intraocular pressure (IOP) is critically controlled by the balance between aqueous humour production in the ciliary epithelium and its outflow from the anterior chamber, and disorders disrupting this balance are believed to form the main aetiological basis for glaucoma. Although the physiology of these processes is not fully understood due to technical difficulties arising from the complex morphology of this epithelium, it is generally agreed that the non-pigmented layer of the ciliary epithelium (NPE) plays a central role in the formation of aqueous humour (Okisaka *et al.*, 1974; Caprioli, 1987; Bartels, 1989).

It has been suggested that production of aqueous humour involves primarily the active secretion of Na ion to the aqueous side, and this process is attributed largely to the Na^+,K^+-ATPase which is densely distributed on the basolateral membrane of NPE cells (Caprioli, 1987; Usukura *et al.*, 1988; Bartels, 1989; Sears, 1991). According to this hypothesis, the standing Na^+ gradient created by Na^+,K^+-ATPase osmotically drives water into the intercellular invaginated spaces of NPE cells and subsequently into the posterior chamber (Diamond & Bossert, 1967; Cole, 1961; Caprioli, 1987; Bartels, 1989). There is, however, evidence arguing that other ionic transport systems also participate in the production of aqueous humour. In several species including the dog, the transepithelial potential is found to be negative on the aqueous side and depends on chloride and bicarbonate concentrations (Holland & Gipson, 1970; Kishida *et al.*, 1981; Iizuka *et al.*, 1984). Furthermore, a net flux from the stromal to the epithelial side has been proved for Cl^- but not for Na^+ (Kishida *et al.*, 1983). These findings are not consistent with the dominance of Na^+ secretion, but suggest a possible role of Cl^- transport in aqueous humour production.

β-Adrenoceptor antagonists have recently been introduced in the treatment of glaucoma. Many clinical investigations have found that these drugs, especially timolol, are useful in lowering the IOP without affecting the outflow facility (Katz *et al.*, 1976; Zimmerman *et al.*, 1977). The mechanism underlying this effect is supposed to be related in part to the modulatory role of the sympathetic nervous system on the rate of humour production. There is however confounding controversy over the effects of β-adrenoceptor agonists such as adrenaline and isoprenaline, depending on the species and experimental conditions. For example, it has been reported that β-adrenoceptor stimulation or application of agents which increase adenosine 3′:5′-cyclic monophosphate (cyclic AMP) leads to the reduction in both the IOP and aqueous flow in the rabbit (Caprioli *et al.*, 1989; Sears, 1991), whereas the opposite evidence has been presented in the monkey and human being (Miichi & Nagataki, 1983; Schenker *et al.*, 1981). Further, a stimulatory role of adrenergic activity on humour production and IOP and its blockade by timolol have been demonstrated under conditions where the circadian rhythm of neural activities is preserved (Topper & Brubaker, 1985; Yoshitomi *et al.*, 1991). Some explanations have been attempted to resolve these discrepancies (Sears, 1991), but we feel that more simplified experimental conditions are preferable to gain more straightforward information about the modulatory role of β-adrenoceptor agents on the aqueous humour production. To this end, in the present experiments, we decided to measure both the net ionic and water movement across the NPE cell membrane of dogs using single isolated NPE cells, and to examine their causal relationship. As a result of the work, we have found that a net Cl^- efflux induced by an increase in the intracellular cyclic AMP is likely to produce a concomitant outflow of water from NPE cells.

Methods

Cell isolation

All procedures used here accord with the ARVO resolution. Mongrel dogs of either sex weighing 10∼15 kg were exsan-

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guinated under anaesthesia with intravenous administration of sodium pentobarbitone (30 mg kg^{-1}). Both eye balls were quickly enucleated and transferred to physiological salt solution (PSS). For isolating the ciliary epithelium, we adopted the method described by Sears *et al.* (1991) with a minor modification. In brief, the eye was cleaned of attached connective tissue under a binocular microscope so that the two posterior ciliary arteries became clearly visible. After soaking in 100% methanol for several minutes to reduce contaminating microorganisms, 5 ml phosphate buffered saline (PBS) containing dispase (Gohdoh Shusei, Tokyo, Japan; 1000 p.u. ml^{-1}) was injected into the ciliary artery at a rate of about 0.5–1 ml min^{-1} through a 27-gauge subcutaneous needle. Thereafter, the eye was opened posteriorly and the ciliary segment was excised. A ring of ciliary body corresponding to the pars plicata was excised and then incubated in the Ca,Mg-free PSS containing dispase (1000 p.u. ml^{-1}) for 30–45 min at 35°C equilibrated with 5% CO_2. At completion of chemical digestion, a thin double layer of ciliary epithelium was almost detached from the stroma; thus we could easily strip it off from the stroma with fine forceps. The resultant epithelial sheet was then fixed to the rubber of a dish with the non-pigmented epithelial side up. A stream of PSS was ejected through a 23-gauge needle along the epithelial surface, resulting in release of a sufficient number of cells. In some experiments, a digested sheet of epithelium was minced and triturated with a blunt tipped Pasteur pipette. Dispersed cells were stored in Dulbecco's modified Eagle medium complemented with 33 mM bicarbonate and 10% foetal bovine albumin. Cells dissociated from the non-pigmented epithelial layer were readily identified by the absence of black pigment inside. Staining with 0.3% trypan blue revealed that nearly 90% of yielded cells were viable.

Patch clamp experiments

The experimental set-up used for generation of voltage commands and acquisition of data was the same as described by Chen *et al.* (1993). The liquid junction potential arising at the interface between the external PSS and the Cs-rich, Cl-reduced internal solution was about 5 mV. This potential was corrected *a posteriori* by shifting the current-voltage relationship by 5 mV toward the hyperpolarizing direction. Details of the nystatin perforated and conventional whole cell recordings have been described by Chen *et al.* (1993).

Videomicroscopic cytometry

Changes in cell size were monitored under an inverted phase-contrast microscope (Diaphot TMD, Nikon, Japan) equipped with a CCD camera (CS3130, Teli, Japan) and a TV monitor (12M310, Teli, Japan). Images were stored on a video tape connected through a casette tape recorder (AG-7350, National, Japan) and replayed later for off-line analysis: two cell diameters along the horizontal (D1) and vertical (D2) axes on the TV monitor screen were measured at an interval of 10 s using a width analyser (C3161, Hamamatsu, Japan). During about the first 10 s after the start of solution change, video images were distorted by stirred surface of bath solution. The measurement was therefore started at a point of 10 s from the beginning of solution change. Measured values were calibrated using an objective micrometer (Nikon, Japan). The change in relative cell volume was then calculated according to the following equation:

$$V/V_0 = (D1*D2)^{3/2}/(D1_0*D2_0)^{3/2}$$

where V_0, $D1_0$ and $D2_0$ represent the initial volume, vertical and horizontal diameters of the cell respectively, and V represents the cell volume at subsequent measurements. During application of drugs tested, the cell size also changed in the direction perpendicular to the floor of the experimental chamber, which obscured the maximum diameter of the cell. We tried to minimize this error by refocussing manually throughout the recording. All cytometric measurements shown in the figures were carried out under electrically unclamped conditions, since the patch electrode blurred the image of NPE cells.

Intracellular recording

After opening the eye posteriorly, a thin small ridge of the pars plicata was dissected from the ciliary body and tightly pinned on the rubber bottom of the experimental chamber with the aqueous side up. In some experiments, an isolated sheet of double-layered ciliary epithelium was used. To equilibrate the tissue, the chamber was continously perfused with PSS (see below) which was aerated with 5% CO_2 and 95% O_2 and prewarmed at 32–34°C for 1 h. Microelectrodes of 40–100 MΩ resistance were fabricated with a puller (PG-1, Narishige, Japan) and filled with 3 M KCl, and a Ag/AgCl electrode was used as the ground. The microelectrode was connected to the head stage of a high-impedance preamplifier (MEZ-7101, Nihon Kohden, Japan) and voltage signals were monitored on a high gain oscilloscope (VC-9, Nihon Kohden, Japan) and a brush pen recorder (RJG-4024, Nihon Kohden, Japan). Impalement of the ciliary epithelium cells was achieved by use of a three-dimensional manipulator (Narishige, Japan) with the aid of a binocular microscope. Recordings were only started if the potential after impalement had stabilized.

Solutions

The composition of the solutions used in the present experiments was as follows (mM): PSS: Na^+ 142.2, K^+ 4.7, Mg^{2+} 1.2, Ca^{2+} 1.8, Cl^- 118.4, HCO_3^- 33.2, $H_2PO_4^-$ 1.2, glucose 6.9; PBS: Na^+ 153.1, K^+ 4.2, Cl^- 139.6, $H_2PO_4^-$ 1.5, HPO_4^{2-} 8.1; KCl internal solution for nystatin-perforated recording: K^+ 140, Mg^{2+} 2, Cl^- 44, EGTA 1 and HEPES 10 (titrated to pH 7.2–7.25 by Tris base); Cs-rich, Cl-reduced internal solution: Cs^+ 140, Mg^{2+} 2, Cl^- 24, aspartate$^-$ 110, Na_2ATP 2, EGTA 10/4~5Ca^{2+} and HEPES 10 (titrated to pH 7.2–7.25 with Tris base). PSS was aerated with 5% CO_2 and 95% O_2, and prewarmed to 32–34°C, unless otherwise stated. The pH of PSS equilibrated with 5% CO_2 was about 7.5. For preparing Ca,Mg-free PSS, Ca^{2+} and Mg^{2+} were simply omitted from PSS.

Chemicals

Isoprenaline hydrochloride, acetylcholine chloride, phenylephrine hydrochloride, noradrenaline hydrochloride, timolol maleate and dibutyryl cyclic AMP (Na salt), were purchased from Wako (Osaka, Japan). Tetraethylammonium chloride (TEA), 4-aminopyridine (4-AP), $BaCl_2$, CsCl and 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS) were purchased from Sigma (St. Louis, U.S.A.). 3-(Dimethylaminopropionyl)-forskolin (NKH-477) was kindly given by Dr Takeo Itoh, Department of Pharmacology, Kyushu University. All drugs used were freshly prepared from the powder for each experimental day.

Statistics

Results were expressed as mean ± standard deviation. Statistical difference was evaluated by Student's *t* test.

Results

Isoprenaline-induced depolarization of the non-pigmented epithelial membrane

The mean resting membrane potential of the non-pigmented layer of dog ciliary epithelium (Vm) was -71.0 ± 1.6 mV ($n = 20$) when superfused with physiological saline the com-

position of which was similar to the aqueous humour (e.g., Caprioli, 1987). Vm depended on the extracellular potassium concentration ($[K]_o$) (Figure 1). A ten fold increase in $[K]_o$ resulted in about 50 mV depolarization of the epithelium. Vm was also found to be sensitive to a number of drugs which are known to affect K and Cl transports (Table 1). Amongst the drugs listed in Table 1, quinine, Ba^{2+}, caesium and ouabain significantly depolarized the membrane, whereas very small or no effects were observed for tetraethylammonium, procaine, 4-AP and pinacidil even at high concentrations. DIDS caused no significant change in Vm at 10 μM (Table 1), but produced a small hyperpolarization at a concentration of 100 μM. This pharmacological profile suggests

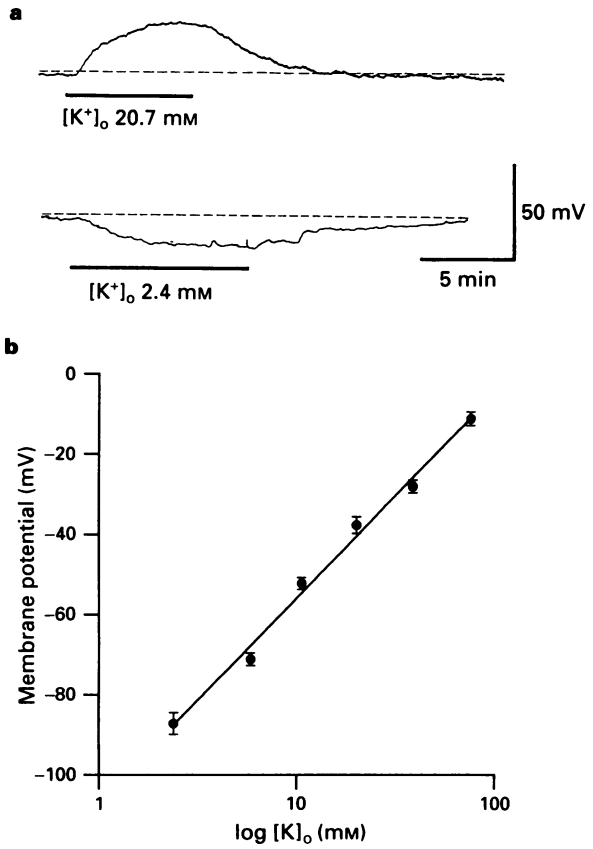


Figure 1 Effect of the external potassium concentration on the membrane potential (mV) of ciliary epithelium. The bath was continuously perfused with the gassed and prewarmed PSS. (a) Effect on Vm when the concentration of $[K]_o$ was changed from 4.7 mM. Dashed lines indicate − 70 mV. (b) The relationship between $[K]_o$ and Vm. Each datum point represents the mean with s.d. from 10–20 cells. The solid line is the best fit of data points according to the equation, $V_m = V_o{*}\log([K]_o/[K]_i)$, where V_o and $[K]_i$ = 50.8 mV and 127 mM, respectively ($r = 0.996$).

Table 1 Effects of various agents on the membrane potential of ciliary epithelium

Agent	*Membrane potential* (mV)
Control	− 71.0 ± 1.6 (20)
TEA 10 mM	− 71.4 ± 1.0 (9)
Procaine 10 mM	− 70.1 ± 1.0 (9)
4-AP 10 mM	− 70.7 ± 1.1 (7)
Quinine 0.1 mM	− 61.7 ± 1.4 (6)*
Ba^{2+} 0.1 mM	− 60.6 ± 0.9 (5)*
# Cs^+ 10 mM	− 41.5 ± 1.0 (4)*
DIDS 10 μM	− 70.1 ± 1.3 (10)
Ouabain 10 μM	− 60.8 ± 0.9 (10)*
Pinacidil 10 μM	− 71.6 ± 1.4 (7)

The values indicate mean ± s.d., and the number of experiments in parentheses.
Data obtained from isolated epithelial sheets. Statistically significant difference (*$P < 0.01$) from the value in the absence of drugs (control).

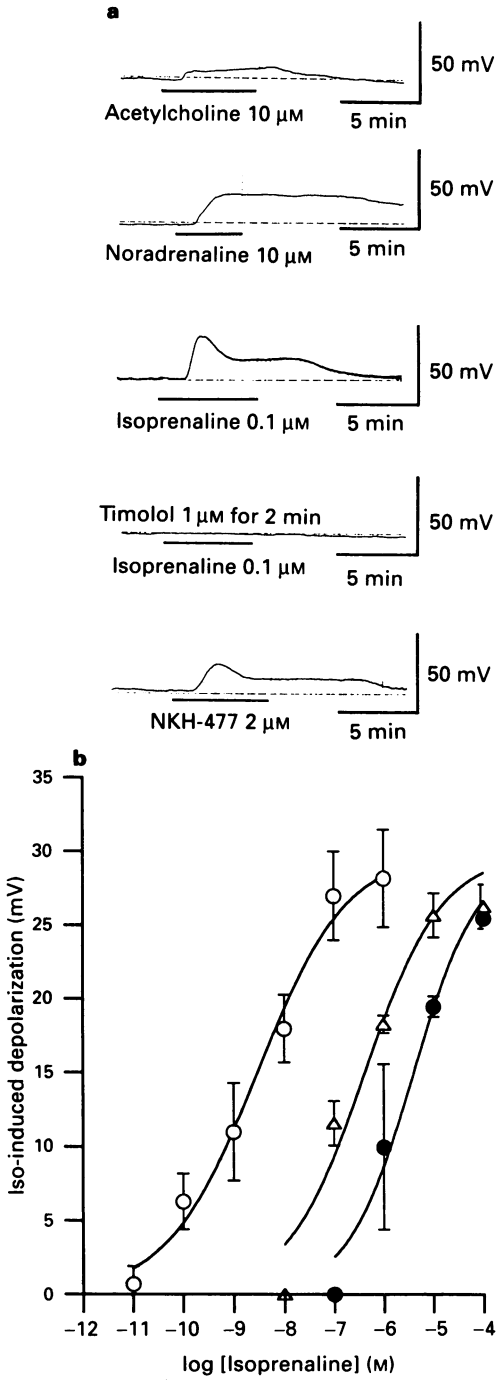


Figure 2 Effects of agents affecting intraocular pressure on the membrane potential of ciliary epithelium and the antagonism by timolol of isoprenaline. (a) Actual traces of membrane depolarization produced by 10 μM acetylcholine, 10 μM noradrenaline, 2 μM NKH-477 and 0.1 μM isoprenaline and antagonism of isoprenaline by 1 μM timolol. The drugs were present at the times indicated by the bars. Dashed lines indicate − 70 mV. (b) The peak of the isoprenaline-induced depolarization (measured from the resting membrane potential) plotted against the concentration of isoprenaline, in the absence (○) and presence of 0.1 (△) and 1 μM timolol (●). The curves are drawn according to the equation $V_o/(1 + (K_d/[Iso])^n)$, where V_o, K_d and n denote the maximum depolarization, the concentration of isoprenaline causing 50% of V_o and the Hill coefficient. The best fit values are 30 mV and 0.5 for V_o and n, and 3 nM (no timolol), 0.3 μM (0.1 μM timolol) and 4 μM (1 μM timolol) for the K_d values.

that K and Cl transporters and a Na^+/K^+ pump are functionally important in the control of Vm of ciliary epithelia.

It is well known that the intraocular pressure (IOP) is controlled by various neurotansmitters, autacoids and hormones (Caprioli, 1987). We therefore tested several representative drugs which are known to cause changes in IOP. As demonstrated in Figure 2a, acetylcholine, noradrenaline and isoprenaline all depolarized the ciliary epithelium, but the potency of isoprenaline was more than 100 times greater than the others. The depolarization induced by isoprenaline was blocked by β-adrenoceptor antagonists such as propranolol and timolol (Figure 2a). Figure 2b shows the relationship between the concentration of isoprenaline and the membrane potential of NPE. The threshold of depolarization was around 0.01 nM and half-maximum and maximum depolarization occurred at about 3 nM and 100 nM, respectively. Pretreatment with timolol shifted this dose-response curve to the right in a dose-dependent manner. The mode of this antagonism seems to be competitive with a pA_2 value of about 9. The effect of isoprenaline did not appear to result from stimulating nerve terminals on the stromal side of the epithelium, because quantitatively similar results were obtained with isolated sheets of ciliary epithelium (Table 2).

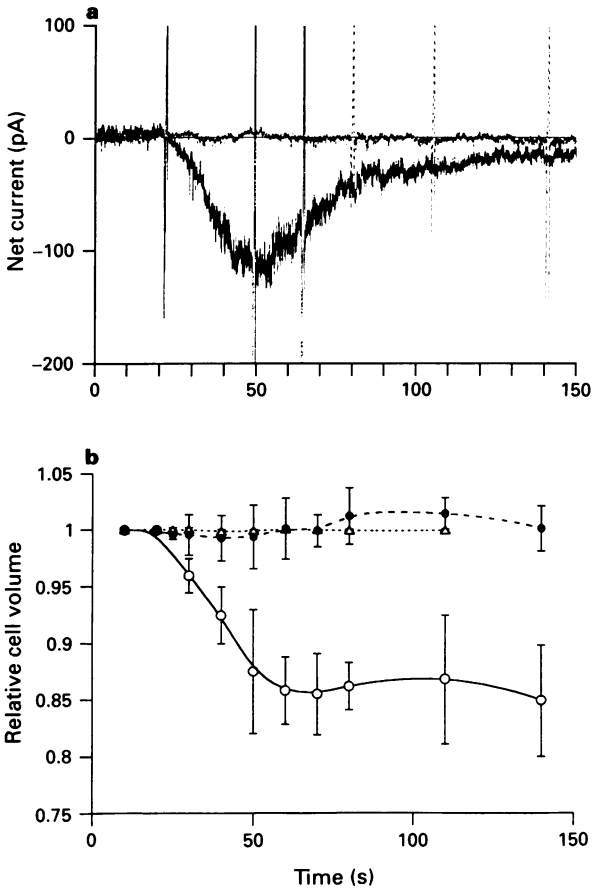


Figure 3 Time course of the isoprenaline-induced inward current and cell volume decrease. (a) Typical records of isoprenaline-induced inward currents in the absence (solid curve) and presence (dotted curve) of 10 μM DIDS, with nystatin-perforated recording (KCl internal solution in the pipette). 20 μM isoprenaline was introduced into the bath at time zero. The holding potential was −70 mV. Vertical deflections represent the currents responding to ramp voltages. (b) The time-dependent profile of cell volume change in the absence (○) and presence of 10 μM DIDS (●) or of 1 μM timolol (△). Isoprenaline (20 μM) was applied at time zero. Symbols represent the mean with s.d. from 5–11 cells. For the definition of relative cell volume, see the Methods and the legends to Table 3.

It has been reported that in the ciliary epithelium β_2-adrenoceptor stimulation elevates the intracellular cyclic AMP level by activating adenylate cyclase (Waitzman & Wood, 1971; Caprioli *et al.*, 1987). We therefore tested a newly synthesized derivative of forskolin, NKH-477, which is water-soluble and very potent at increasing intracellular cyclic AMP by directly activating the adenylate cyclase (Shafiq *et al.*, 1992). As shown in Figure 2a, superfusion with 2 μM NKH-477 produced a depolarization comparable in size to that induced by isoprenaline.

The pharmacological nature of the isoprenaline- or NKH-477-induced depolarization was investigated in the presence of various drugs listed in Table 2. Ouabain and Ba^{2+} reduced only partially but DIDS almost fully abolished the isoprenaline-induced depolarization. Furthermore, the extent of the depolarization was strongly attenuated by lowering the temperature of the superfusate to below 30°C.

These results suggest that isoprenaline applied at the aqueous side of non-pigmented epithelium is very efficacious in particular in producing a DIDS-sensitive ionic movement, presumably anions like Cl^-. It is however equivocal whether this is due solely to changes in the NPE cells or to combined actions of the two epithelial layers. To gain more insight, we explored the correlation between ionic and water movements by directly measuring the transmembrane current and cell volume change induced by isoprenaline, in single isolated NPE cells.

Isoprenaline induced inward current associated with a cell volume decrease

Single NPE cells were cuboidal or spherical in shape with an approximate diameter of 9～18 μm (13.4 ± 3.3, $n = 20$) and had a capacitance of 6.2 ± 0.8 pF (4～8, $n = 21$) when

Table 2 Effect of various drugs on the isoprenaline- and NKH-477-induced depolarization

	Membrane potential (mV)	
Drugs	*Intact ciliary body*	*Epithelial sheet*
Control	− 71.0 ± 1.6 (13)	− 67.9 ± 2.7 (16)
Isoprenaline 10 μM	− 52.7 ± 2.3 (9)*	− 57.0 ± 1.0 (5)*
+ TEA 10 mM	− 53.0 ± 1.0 (2)*	− 58.0 ± 6.0 (2)*
+ Cs^+ 10 mM	–	− 31.0 ± 1.7 (3)*
+ Ba^{2+} 1 mM	− 55.5 ± 1.5 (2)*	–
+ Ouabain 10 μM	− 48.7 ± 1.3 (10)*	− 53.3 ± 1.2 (3)*
+ DIDS 10 μM	− 65.9 ± 1.3 (10)*	− 68.0 ± 1.0 (3)
28～30°C	− 70.8 ± 1.3 (4)	–
NKH-477 10 μM	− 55.8 ± 1.1 (5)*	− 53.0 ± 1.7 (3)*
+ Ouabain 10 μM	− 57.4 ± 0.9 (5)*	–
+ DIDS 10 μM	–	− 68.0 ± 3.5 (3)

The values indicate mean ± s.d., and the number of experiments in parentheses. Statistically significant difference (*$P < 0.01$) from the values of controls for the intact ciliary body and isolated epithelial sheet, respectively.

placed in PSS containing the HCO_3^-/CO_2 buffer. In the absence of HCO_3^- and at room temperature, however, they showed a progressive decrease in size and deterioration. To minimize this, it was necessary to perfuse the cells continuously with the bicarbonate-containing PSS gassed with 5% CO_2 and 95% O_2 and prewarmed at 32~34°C throughout the experiment.

Using the nystatin perforated whole-cell recording, administration of 20 μM isoprenaline into the bath evoked an inward current associated with an increased noise level at a holding potential of −70 mV (Figure 3a). The current reached a peak within a few tens of seconds and persisted after washout of isoprenaline. The peak amplitude of this inward current varied greatly from cell to cell (−5~−123 pA; −29.3 ± 14.4, $n = 35$), and this may have been due to the different extent of enzymic digestion of NPE cells. The isoprenaline-induced current was almost completely blocked by pretreatment with 1 μM timolol or 10 μM DIDS (Figure 3a) and was dependent on the temperature. When the temperature of the superfusate was lowered to 25–30°C, isoprenaline failed to evoke measurable inward currents (Table 3).

Administration of isoprenaline brought about a reduction in size of NPE cells and the time course appeared to parallel the development of the inward current (Figure 3). This cell volume decrease was sensitive to the same procedures used above. Namely, pretreatment with 1 μM timolol or 10 μM DIDS or lowering the temperature resulted in great attenuation of cell volume decrease (Figure 3b and Table 3).

Isoprenaline-induced current is a Cl current

Figure 4 shows the current-voltage relationships ($I-V$-curves) with the nystatin perforated recording. In most NPE cells examined, the $I-V$ curve was nearly ohmic or slightly inward rectifying at potentials between −150 and −50 mV, but outward-rectifying at more positive potentials. The leak conductance of NPE cells calculated from the linear portion of $I-V$ curve near the resting membrane potential (−70 mV) was 0.53 ± 0.24 nS ($n = 10$), a value similar to that reported elsewhere (Gooch *et al.*, 1992). TEA (10 mM) or quinine (0.1 mM) strongly suppressed the outward current at potentials positive to −50~−40 mV (Figure 4a), whereas Cs^+ (10 mM) (Figure 4b) or Ba^{2+} (1 mM) caused a substantial decrease in the membrane conductance near the resting membrane potential. Isoprenaline (10 μM) shifted this $I-V$ curve in the inward direction (Figure 5a). Figure 5b shows that for the current sensitive to 10 μM isoprenaline, there is no apparent reversal potential. Although at more negative potentials the difference current became more inward, it is difficult to

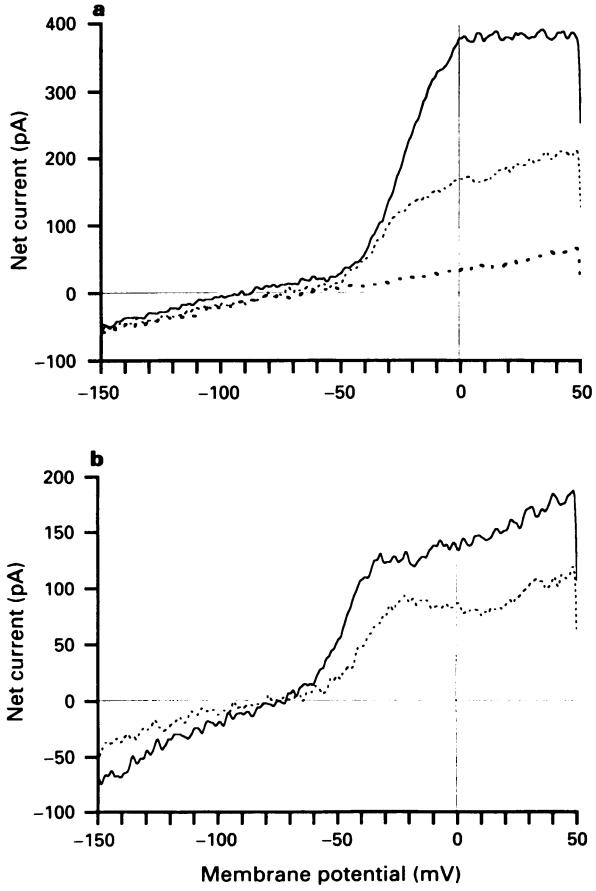


Figure 4 Current-voltage relationships (I-V curves) of non-pigmented epithelial (NPE) cells. Nystatin-perforated recordings with KCl internal solution. From a holding potential of −70 mV, ramp voltages (−150~+50 mV, 0.25 V/s) were applied for 800 ms. This minimized the change in NPE cell size due probably to perturbation of intracellular ionic content, which usually occurred on repeated application of voltage pulses. (a) I-V curves in the absence (solid curve) and presence of 10 mM TEA (thin dotted curve) or 0.1 mM quinine (thick dotted curve). (b) I-V curve in the absence (solid curve) and presence of 10 mM Cs^+ (dotted curve).

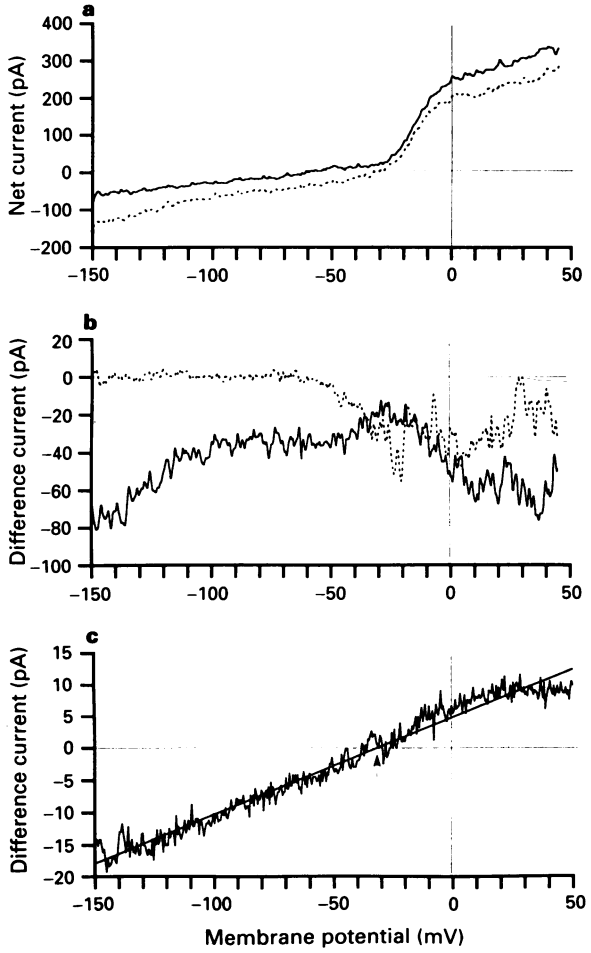


Figure 5 I-V curves of the isoprenaline-induced current. Nystatin-perforated recordings with KCl internal solution. The ramp protocol used was the same as in Figure 4. (a) Net currents in the absence (solid curve) and presence (dotted curve) of 10 μM isoprenaline are plotted against the membrane potential. (b) The difference in net currents recorded in the presence and absence of 10 μM isoprenaline (hereafter referred to as the isoprenaline-sensitive current) is plotted against membrane potential without (solid curve) and with (dotted curve) pretreatment of 10 μM DIDS. (c) The I-V curve for the isoprenaline (10 μM)-sensitive current in the presence of 10 mM TEA. An arrow indicates −31 mV.

define the reversal potential. Pretreatment with 10 μM DIDS selectively blocked the portion below − 50 mV of the *I–V* curve (dotted curve in Figure 5b). We considered these results to be a consequence of a complex effect of isoprenaline on the membrane conductance of the NPE cell, i.e. induction of inward current and suppression of outward current. Therefore, we repeated the same experiment in the presence of 10 mM TEA or 10 mM CsCl to separate the two effects, because these drugs have been found to suppress the K outward current at positive potentials (TEA) and the inward rectifying K current at very negative potentials (CsCl), respectively (Gooch *et al.*, 1992). As shown in Figure 5c, in the presence of 10 mM TEA, the isoprenaline-sensitive current reversed near − 30 mV, mean − 23.3 ± 6.1 mV ($n = 5$). Similar values of reversal potential were obtained with 10 mM Cs^+ (− 20.4 ± 3.6 mV, $n = 5$). However, in this case, suppression of the remaining outward current by isoprenaline at very positive potentials was sometimes observed. Such data were excluded from the evaluation. It should be noted that the reversal potentials of about − 20 mV accords with the Cl^- equilibrium potential of intact ciliary epithelium that can be estimated from intracellular Cl^- concentration measurements (ca. 60 mM, Wiederholt & Zadunaisky, 1986).

In the next series of experiments, the cells were dialysed with the Cs-rich, Cl-reduced internal solution, in order to suppress K current and shift the theoretical equilibrium potential for Cl^- towards more negative potentials. Under these conditions, isoprenaline still evoked inward currents at a holding potential of − 70 mV (Figure 6a). The *I–V* curve of the isoprenaline-sensitive current is slightly outward-rectifying, and reversed polarity at − 42.3 ± 7.1 mV ($n = 5$) (solid line in Figure 6b). This reversal potential is in good agreement with the theoretical equilibrium potential of Cl^- under these experimental conditions (− 42.4 mV, at 34°C). Pretreatment with 10 μM DIDS almost completely abolished the ability of isoprenaline to induce the inward current (dotted line in Figure 6b). The chord conductance of isoprenaline-induced current at − 70 mV with the Cs pipette was 0.79 ± 0.29 nS ($n = 5$), which is somewhat smaller than that obtained with the nystatin-perforated recording (0.94 ± 0.47 nS; Table 3). This value, however, is reasonable to account for the maximum depolarizing effect of isoprenaline (ca. 30 mV, see Discussion). These results strongly indicate that the main conductance which contributes to the inward current induced by isoprenaline can be ascribed to Cl^-, thus suggesting that near the resting membrane potential isoprenaline produces efflux of Cl^- from NPE cells.

Pretreatment with 10 μM ouabain significantly reduced the conductance induced by isoprenaline recorded with the nystatin perforated patch electrode ($P < 0.01$; the values marked

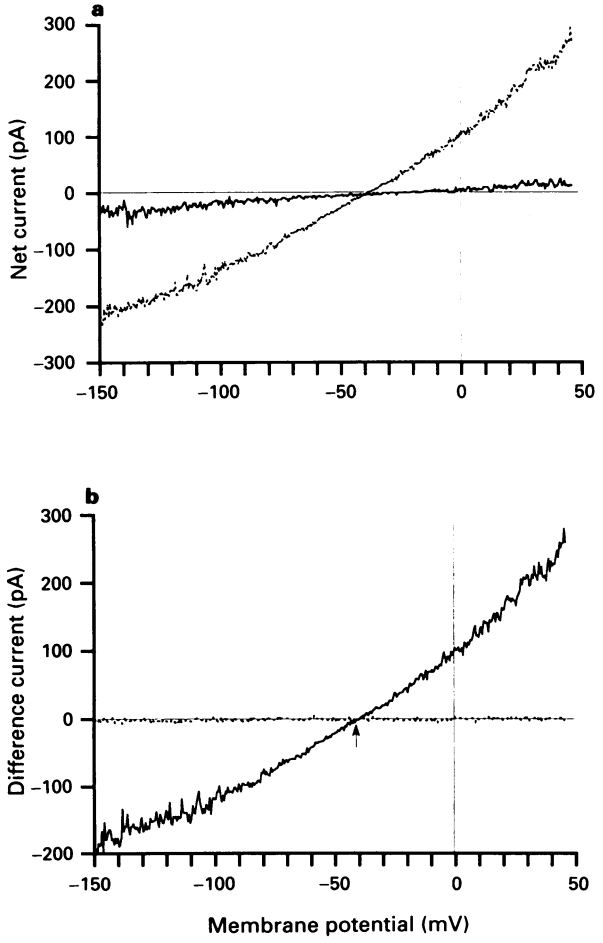


Figure 6 *I-V* curves of isoprenaline-induced currents recorded from Cs^+-loaded NPE cells. Experimental conditions were the same as in Figure 4 except that cells were dialysed with Cs-rich, Cl-reduced internal solution (see Methods) via a patch electrode. (a) Net currents recorded in the absence (solid curve) and presence (dotted curve) of 20 μM isoprenaline. (b) The *I-V* curve of the isoprenaline-sensitive current from (a) (solid curve) and that in the presence of 10 μM DIDS (dotted curve). An arrow indicates the equilibrium potential for Cl^- under these experimental conditions (− 42 mV at 34°C).

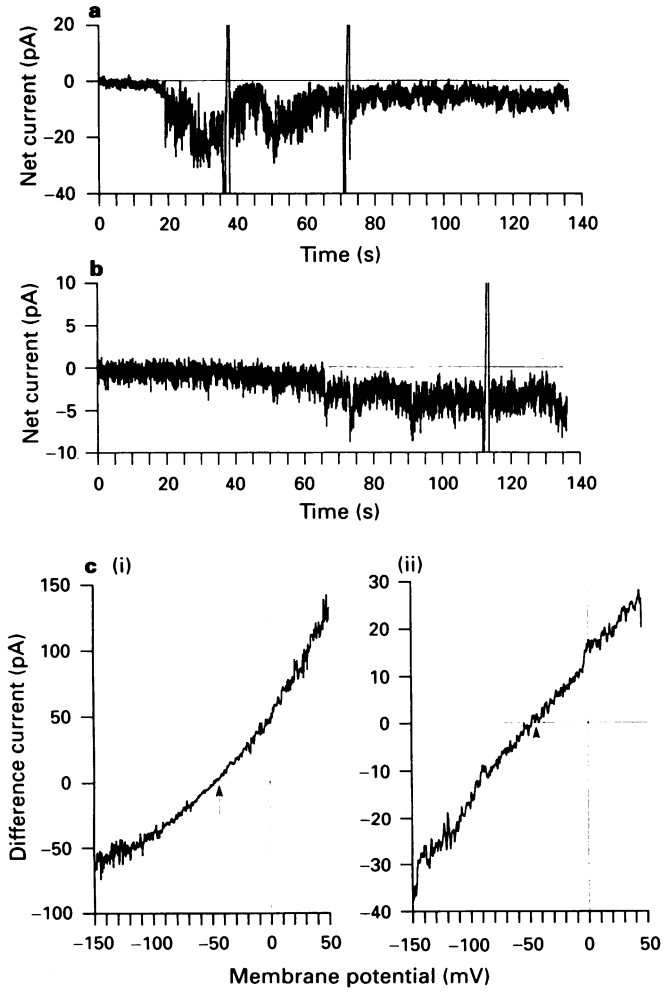


Figure 7 Membrane currents induced by NKH-477 and db cyclic AMP, and their *I-V* curves. The cell was dialysed with the Cs-rich, Cl-reduced internal solution. (a) and (b) At a holding potential of − 70 mV, 10 μM NKH-477 (a) or 1 mM db cyclic AMP (b) were applied at time zero. Small inward currents developed slowly. Vertical deflections represent ramp currents. (c) *I-V* curves for 10 μM NKH-477- (i) and 1 mM db cyclic AMP- (ii) sensitive currents. Arrows indicate the equilibrium potential for Cl^-.

with #, Table 3). However, this reduction became very small ($P > 0.05$) when the cell was dialysed with the Cs-rich, Cl-reduced internal solution. This may suggest a permissive role of the Na^+/K^+ pump in the control of isoprenaline-induced inward current.

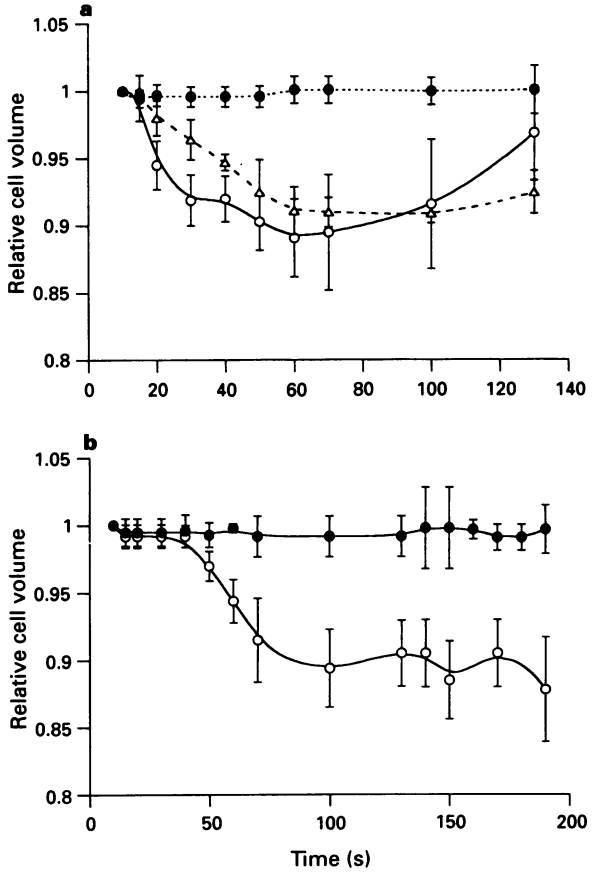


Figure 8 Cell volume decrease induced by NKH-477 and db cyclic AMP. NKH-477 or db cyclic AMP were applied at time zero. (a) Time course of cell volume decrease induced by 10 μM NKH-477, in the absence (○) and presence of 10 μM DIDS (●) or 1 μM timolol (△). (b) Time course of cell volume change induced by 1 mM db cyclic AMP, in the absence (○) and presence of 10 μM DIDS (●). Symbols represent the mean ± s.d. Data were obtained from 3–8 cells.

Induction by a forskolin derivative, NKH-477 and dibutyryl cyclic AMP of cell volume decrease and a current with similar properties

Similar results to the above were obtained when NKH-477 or a membrane permeable analogue of cyclic AMP, db cyclic AMP was administered. As shown in Figure 7, 10 μM NKH-477 or 1 mM db cyclic AMP induced an inward current which reversed near the Cl^- equilibrium potential (Figure 7c) and was blocked by 10 μM DIDS (Table 3). The reversal potentials of NKH- and db cyclic AMP induced currents with the Cs-rich, Cl-reduced internal solutions were -40.7 ± 6.7 mV ($n = 6$) and -46.2 ± 6.3 mV ($n = 4$), respectively. The cell volume decrease induced by 10 μM NKH or 1 mM db cyclic AMP was also greatly reduced by pretreatment with 10 μM DIDS, but not with 1 μM timolol (Figure 8).

Isoprenaline-induced cell volume decrease shows a similar pharmacological profile

Table 3 summarises the effects of various blockers and low temperature on the isoprenaline-induced inward current and cell volume decrease. Most noteworthy here is that DIDS and low temperatures are very efficacious in suppressing both the isoprenaline-induced conductance and cell volume decrease.

Discussion

The present study clearly shows that isoprenaline and other agents that increase intracellular cyclic AMP cause the cell volume to decrease along with activation of a Cl^- inward current in the dog NPE. These two cellular changes seem to be causally related because they occur with a similar time course and show similar sensitivities to the drugs tested (e.g. DIDS). The data obtained from the intact and isolated ciliary epithelia with intracellular potential recording also suggest that this could be extrapolated to the multicellular condition.

Historically, most of work regarding the aqueous humour production has emphasized the importance of a steady state standing Na^+ gradient existing between the interdigitated space of NPE cells and the posterior chamber. This gradient is thought to be maintained by the Na^+/K^+-ATPase localized on the basolateral membrane of NPE and to cause

Table 3 Summary of effects of isoprenaline, NKH-477 and db cyclic AMP on the membrane conductance and cell volume

	Relative cell volume (%)	*Conductance induced* (nS)
Isoprenaline 20 μM	85 ± 4 (11)	0.79 ± 0.29 (5) (#0.94 ± 0.47 (15))
+ TEA 10 mM	89 ± 1 (4)	0.42 ± 0.07 (5)**
+ Cs^+ 10 mM	92 ± 1 (3)**	0.28 ± 0.05 (5)*
+ Ouabain 10 μM	91 ± 2 (4)**	0.58 ± 0.15 (6) (#0.25 ± 0.1 (5))*
+ DIDS 10 μM	101 ± 1 (5)*	NC (4) (#NC(9))
25–30°C	97 ± 2 (6)*	NC (5)
NKH 10 μM	89 ± 3 (8)	0.41 ± 0.28 (5)
+ Ouabain 10 μM	95 ± 2 (5)*	–
+ DIDS 10 μM	95 ± 2 (5)*	NC (4)
db cyclic AMP 1 mM	88 ± 4 (3)	0.27 ± 0.13 (6)
+ DIDS 10 μM	99 ± 2 (4)*	NC (3)

The values indicate mean ± s.d., and the number of experiments (in parentheses). The relative cell volume is the percentage of the initial cell volume (just before drug application) 1 ~ 2 min after drug application.
#Data obtained with nystatin-perforated recording. Otherwise the conventional whole-cell recording with Cs-rich, Cl-reduced internal solution was used. The conductance induced by isoprenaline, NKH-477 or db cyclic AMP was measured as the chord conductance at − 70 mV for Cs-rich, Cl-reduced internal solution, and as the conductance estimated from the slope between − 150 ~ − 100 mV for the nystatin-perforated recording. NC: no measurable conductance change. Statistically significant difference (*$P < 0.01$ or **$P < 0.02$) from the values obtained in the presence of isoprenaline, NKH-477 or db cyclc AMP.

the osmotic outflow of water into the posterior chamber (Caprioli, 1987; Bartels, 1989). In line with this hypothesis, it has been postulated that the main target of β-adrenoceptor stimulation in humour production is the Na^+/K^+-ATPase *per se*, although the contribution of active and passive Cl^- transport to the aqueous humour production has not been entirely excluded (Sears, 1991). If Na^+/K^+ ATPase is the main and sole target of β-stimulation in NPE, the membrane depolarization induced by isoprenaline should reflect a reduced activity of the ATPase (Delamere *et al.*, 1990) and thus result in retention of water inside the NPE cell, i.e. swelling of the cell, since the net current carried by the ATPase is outward-going over a wide range of membrane potentials (DeWeer *et al.*, 1988; Guyton, 1991). However, the observed effect of isoprenaline is the opposite of this, a decrease in cell volume. The simplest explanation for this observation is that anions flow out from NPE cells along with the water in response to isoprenaline, as widely found in other types of epithelia (Stetson *et al.*, 1985; McCann & Welsh, 1990; Dawson, 1991; Reuss *et al.*, 1991). We have confirmed this by identifying a cyclic AMP-dependent and DIDS-sensitive Cl^- current in parallel with the cell volume decrease. Furthermore, the magnitude of this current seems almost to account fully for the extent of isoprenaline-induced depolarization observed in the multicellular preparation. By using a quantitative treatment proposed by Ginsborg (1967), the magnitude of agonist-induced depolarization can be estimated from the following equation:

$$\Delta E = (E_{agonist} - E_{rest})^*(\Delta G/(G + \Delta G)$$

where ΔE, E_{rest}, $E_{agonist}$, ΔG and G denote the magnitude of agonist-induced depolarization, the resting membrane potential, the reversal potential of the agonist-induced current, the conductance induced by the agonist and the resting (leak) conductance, respectively. If we use the values of -70 mV, 0.8 nS and 0.5 nS for E_{rest}, ΔG and G, respectively (taken from the present study), and assume $E_{agonist}$ as the equilibrium potential of Cl^- in the intact unstimulated NPE (ca. -20 mV, calculated from $[Cl^-]_i$ = ca. 60 mM; Wiederholt & Zadunaisky, 1986), then a value of about 30 mV for ΔE is obtained. This value is comparable to the maximum depolarization attained by isoprenaline (Figure 2b).

The cyclic AMP-dependent Cl^- current has been found in a broad range of epithelia and is thought to be involved in secretory process (McCann & Welsh, 1990; Dawson, 1991; Hughes & Segawa, 1993). In general, Cl^- channels in epithelia are thought to be contained in the apical and/or basolateral membranes of epithelia and serve as electrodiffusional pathways: Cl ions exit passively via this route along the electrochemical gradient and are accompanied by an outflow of water. This pathway is often found to be tightly coupled to other Cl^- transport systems in a variety of complex ways. For example, in the tracheal epithelium (McCann & Welsh, 1990), a coordinated interplay of Na^+/K^+ pump, Na^+/Cl^- cotransporter and Cl^- channels has been envisaged: the Na^+,Cl^- or Na^+, K^+, Cl^- cotransporter accumulates Cl^- inside the cell by utilizing the Na^+ gradient which is maintained by a Na^+/K^+ pump. This increases the electrochemical force for Cl^- diffusion and drives Cl^- out of the cell through the Cl^- channels. The elevation of cyclic AMP facilitates these mechanisms, especially by inducing a cyclic AMP-dependent Cl^- conductance on the apical membrane of the epithelium. The ciliary epithelium is double-layered, so net movements of solutes and water must occur across the two layers of epithelium to produce the aqueous flow into the posterior chamber. However, very little is yet known about how the two layers are coordinated for this secretory process. The normal operation of the Na^+/K^+ pump is a prerequisite for the aqueous humour production (Bartels, 1989), but it is still unclear whether the pump controls the whole process or is involved merely as a primary initiator of aqueous secretion. We postulate that Cl^- channels, especially cyclic AMP-dependent Cl^- channels, may be localized predominantly on the aqueous side of NPE and serve as a final step of aqueous secretion from the NPE. Further investigation is required to explore the interaction of cyclic AMP-dependent Cl^- channels with other transport systems such as Cl^-/HCO_3^- exchange (Wolosin *et al.*, 1989; Saito, 1992), Na^+, K^+, Cl^- cotransport (Mito *et al.*, 1993) and Na^+/K^+ pump (Usukawa *et al.*, 1988; Martin-Vasallo *et al.*, 1989), in aqueous humour production. A close link of cyclic AMP-dependent Cl^- conductance to the Na^+/K^+ pump has been reported in rabbit corneal epithelium (Klyce & Wong, 1977).

Recently, Gooch *et al.* (1992) showed that in bovine NPE cells, isoprenaline did not change the membrane conductance despite its ability to increase the intracellular cyclic AMP level. However, this may not be simply a species difference. Firstly, they tested the effect of isoprenaline on the membrane conductance at 30°C or lower temperatures, whereas they measured the cyclic AMP level at 37°C. In our experiments, we have observed a clear dependence on temperature of the inward current as well as cell volume change induced by isoprenaline. At 30°C or less, the effects of isoprenaline on the intracelllar potential recording, patch clamp recording and cell volume measurement were almost undetectable. Secondly, in our preliminary experiments, we noticed that the use of unphysiological buffers such as HEPES and low temperatures caused progressive shrinkage of NPE cells. Normal cells could be restored by using a bicarbonate-CO_2 buffering system with oxygen supply and a temperature of 37°C. NPE cells are thought to contain many mitochondria and vigorously produce ATP (Caprioli, 1987). Provided that the induction of Cl^- current is mediated by phosphorylation of the channel proteins by cyclic AMP-dependent protein kinase as in other epithelia (McCann & Welsh, 1990), it is tenable to conceive the essential requirement of ATP to maintain normal function of NPE cells. In this sense, it is necessary to optimize the conditions for NPE cells when their functions are studied.

The rate of aqueous humour production stimulated by 10 μM isoprenaline in individual NPE cells can be estimated from the initial phase of cell volume decrease (Figure 3b). The maximum rate is approximately 0.19 pl min^{-1} per cell, assuming the initial diameter and the percentage rate of cell volume decrease to be on average 13.4 μm and 15% min^{-1}, respectively. This gives an estimated increased rate of aqueous flow by isoprenaline of 0.8 μl min^{-1}, by further assuming that the cross-sectional area of a single NPE cell is 1.41×10^{-6} cm^2 and the total surface area of the ciliary body is about 6 cm^2 (Farahbakhsh & Fain, 1987; Guyton, 1991). This implies that considerable elevation in IOP could result from β-adrenoceptor stimulation, considering that the basal rate of aqueous humour production is about 2.5 μl min^{-1} (Guyton, 1991). However, *in vivo*, the consequences of elevated cyclic AMP in the eye are not confined to modulation of the rate of aqueous humour production, but may include the decreased resistance to outflow (Bartels *et al.*, 1982) or the altered activity of the sympathetic nervous system (Potter *et al.*, 1985). Recent experiments with *in vivo* fluorophotometry suggest that the β-antagonist, timolol, produces a significant reduction in the rate of aqueous flow in the dark, in which the cyclic AMP level in the aqueous humour is increased due to the increased activity of sympathetic nerves (Yoshitomi & Gregory, 1991; Yoshitomi *et al.*, 1991). Since the threshold of the isoprenaline effect is as low as 0.01 nM (the concentration-depolarization curve in Figure 2b), a small alteration in the basal activity of the sympathetic nerves that occurs during the circadian rhythm might have considerable implications in the regulation of aqueous flow. This would partly provide a basic mechanism for the observation that at low sympathetic tone the aqueous

flow is stimulated while at high tone it is rather reduced, due presumably to antagonistic inhibition of adenylate cyclase by α_2-adrenoceptor activation (Sears, 1991).

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Potentiation by forskolin of both SNP- and ANP-stimulated cyclic GMP accumulation in porcine isolated palmar lateral vein

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1 The aim of this study was to examine the effect of modulation of adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels (by using forskolin, a direct activator of adenylyl cyclase, or rolipram, a cyclic AMP selective phosphodiesterase inhibitor) on basal and stimulated guanosine 3':5'-cyclic monophosphate (cyclic GMP) levels in the porcine isolated palmer lateral vein by use of a [^{3}H]-guanine prelabelling technique.

2 Sodium nitroprusside (SNP; 10^{-5}–10^{-3} M) and atrial natriuretic peptide (ANP; 10^{-8}–10^{-6} M), produced concentration-dependent increases in [^{3}H]-cyclic GMP levels via stimulation of soluble and particulate guanylyl cyclase respectively. The SNP-stimulated [^{3}H]-cyclic GMP response peaked after 5 min in the presence and absence of the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX).

3 In the absence of IBMX, forskolin (3×10^{-5} M) significantly increased [^{3}H]-cyclic GMP levels to $118.5 \pm 8.7\%$ of basal values ($P<0.05$, $n=8$), and significantly increased both the SNP- and ANP-stimulated [^{3}H]-cyclic GMP accumulation at all concentrations of SNP and ANP used. For example, effects at the maximal SNP (10^{-3} M) and ANP (10^{-6} M) concentrations were: SNP: $154.7 \pm 15.4\%$ of basal; SNP + forskolin: $191.3 \pm 14.8\%$ of basal ($P<0.05$, $n=4$); ANP: $161.4 \pm 17.4\%$ of basal; ANP- + forskolin: $220.0 \pm 20.0\%$ of basal ($P<0.05$, $n=4$).

4 The cyclic AMP-selective phosphodiesterase inhibitor, rolipram (10^{-5} M), had no effect on basal or SNP-stimulated [^{3}H]-cyclic GMP levels; however, the combination of forskolin and rolipram produced an increase in the basal ($158.7 \pm 27.1\%$ of basal) and SNP-stimulated [^{3}H]-cyclic GMP accumulation (SNP (10^{-3} M): $165.3 \pm 8.7\%$ of basal; SNP + forskolin + rolipram: $510.7 \pm 64.8\%$ of basal; $P<0.05$, $n=5$), greater than either forskolin or rolipram alone. The phosphodiesterase inhibitor, IBMX (10^{-3} M) significantly raised [^{3}H]-cyclic GMP levels, and forskolin (3×10^{-5} M) in the presence of IBMX had no significant effect on either basal or SNP-stimulated [^{3}H]-cyclic GMP levels (e.g. in the presence of IBMX: SNP (10^{-3} M): $660 \pm 90\%$ of basal; SNP + forskolin: $790 \pm 86\%$ of basal, $n=3$).

5 The data indicate the presence of both soluble and particulate guanylyl cyclase in the porcine isolated palmar lateral vein. The ability of forskolin to potentiate SNP- and ANP-stimulated [^{3}H]-cyclic GMP accumulation may suggest a cyclic AMP-cyclic GMP interaction at the level of the phosphodiesterases. Further, the ability of cyclic AMP to influence cyclic GMP levels may indicate that the two nucleotides, as well as having independent mechanisms to induce smooth muscle relaxation, could produce vasodilatation via a common mechanism.

Keywords: Cyclic GMP; SNP; ANP; forskolin; rolipram; IBMX; porcine vascular smooth muscle

Introduction

Nitric oxide, a potent vasodilator, is known to stimulate soluble guanylyl cyclase which catalyses the formation of guanosine 3':5'-cyclic monophosphate (cyclic GMP) from GTP. The effects of cyclic GMP are mediated through several mechanisms, amongst which are activation of cyclic GMP-dependent protein kinases, cyclic GMP-gated ion channels, and cyclic GMP-regulated phosphodiesterases (Lincoln & Cornwell, 1993). The cyclic GMP-dependent protein kinases are found at the highest densities in platelets, cerebellum, and vascular smooth muscle, where they may play a role in vasodilatation (Lincoln & Cornwell, 1993). Using a [^{3}H]-guanine prelabelling technique to study cyclic GMP levels in guinea-pig cerebellar slices, Hernandez *et al.* (1994) have recently reported a potentiation of stimulated cyclic GMP accumulation by adenosine 3':5'-cyclic monophosphate (cyclic AMP). Although it was proposed originally that cyclic AMP and cyclic GMP had antagonistic roles, i.e. the Ying-Yang hypothesis (Goldberg *et al.*, 1975), many agents which elevated cyclic AMP formation also induced cyclic GMP accumulation in mouse (Ferrendelli *et al.*, 1972; 1973; 1974; 1975) and guinea-pig cerebellar slices (Ohga & Daly, 1977; Saito, 1977). Further, in a study of β-adrenoceptors, known to be positively linked to adenylyl cyclase, the non-selective β-adrenoceptor agonist, isoprenaline, produced vasorelaxation in rat thoracic aorta, and elevated cyclic GMP levels to a greater extent (12 fold) than those of cyclic AMP (4 fold) (Gray & Marshall, 1992). Cyclic AMP has been reported to activate directly cyclic GMP-dependent protein kinase (Lincoln *et al.*, 1990; Jiang *et al.*, 1992), although this may not be the mechanism through which cyclic GMP levels become elevated. Hernandez and co-workers (1994) showed that forskolin, an activator of adenylyl cyclase, potentiated both basal and SNP-stimulated cyclic GMP accumulation in guinea-pig cerebellar slices, and suggested that because forskolin has no direct effect on either isoform of guanylyl cyclase the cyclic AMP-cyclic GMP interaction might occur at the level of the phosphodiesterases, the enzymes responsible for cyclic AMP and cyclic GMP catabolism. The aim of this study was to adapt the prelabelling technique used by Hernandez *et al.* (1994) for use with vascular smooth muscle and then to study any modulation by forskolin of cyclic GMP stimulation via soluble and particulate guanylyl cyclase activation, in the porcine isolated palmar lateral vein; a vascular tissue in which cyclic AMP studies have already been reported (Wright *et al.*, 1993).

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Some of these results were presented in a preliminary form at the Manchester Meeting of the British Pharmacological Society (Wright *et al.*, 1994a).

Methods

Tissue preparation

Male porcine fore-trotters were obtained from a local abattoir, shortly after the animals had been killed, and transported to our laboratory in ice-cold Krebs solution. The palmar lateral vein was dissected free from the trotters (the location of this vessel has been reported previously, Wright *et al.*, 1993). Tissues were stored overnight in Krebs solution containing 2% Ficoll (to promote tissue integrity). Vessels were cut into 5 mm lengths and then incubated for 60 min at 37°C in a shaking waterbath. After this period of time, segments of vessel were incubated with [^{3}H]-guanine (specific activity = 8.75 Ci mmol^{-1}; 20 μCi 10 ml^{-1}) for 60 min at 37°C. Tissues were washed by resuspension three times and transferred to incubation tubes (1 per tube) with an assay volume of 300 μl; each experiment was carried out in quadruplicate. Twenty minutes was allowed for equilibrium to be reached (either in the presence or absence of either 3-isobutyl-1-methylxanthine (IBMX; 10^{-3} M) or rolipram (10^{-5} M)), and the agonists sodium nitroprusside (SNP; 10^{-5}–10^{-3} M) and atrial natriuretic peptide (ANP; 10^{-8}–10^{-6} M) added for a period of 5 min. In studies using the adenylyl cyclase activator, forskolin (3×10^{-5} M), this was added 10 min prior to the addition of either SNP or ANP. Incubation tubes were resealed under O_2:CO_2 (95%: 5%) after each addition; 5 min after the addition of either SNP or ANP the incubation was stopped by the addition of 200 μl 1 M HCl, followed by 750 μl of distilled water; they were then allowed to stand for 30 min. [^{3}H]-cyclic GMP was separated from [^{3}H]-guanine and other [^{3}H]-products using sequential Dowex/alumina chromatography (based on Salomon *et al.*, 1974), adjusting for variation in recovery by the addition of [^{14}C]-cyclic GMP (ca, 30 Bq per tube). Some of the sample (0.9 ml) was taken from each incubation tube and 100 μl [^{14}C]-cyclic GMP added; this was then applied to Dowex columns and the eluate discarded. The Dowex columns were placed over alumina columns and 4 ml distilled water added to the Dowex columns, the eluants dripped onto the alumina columns and were then discarded. One ml distilled water was added to each column and eluate discarded. The Dowex columns were removed and 5 ml imidazole (0.1 M) was added to the alumina columns and the eluants collected into vials. Measurement of total ^{3}H taken up into each individual tissue allowed for correction for variation in tissue size and [^{3}H]-guanine uptake. [^{3}H]-cyclic GMP, [^{14}C]-cyclic GMP, and total ^{3}H taken up into each individual tissue were determined using liquid scintillation counting.

Preliminary experiments were carried out to study the effect of different incubation times on the response of cyclic GMP accumulation to one concentration of SNP (10^{-3} M), either in the presence or absence of the phosphodiesterase inhibitor, IBMX (10^{-3} M).

Data analysis

In studies using either forskolin or rolipram, a control concentration response curve to the agonist and the effects of forskolin or rolipram were carried out within the same experiment, hence all data of this type were statistically analysed using a paired Student's *t* test.

Drugs

The composition of the Krebs solution was (mM): NaCl 118.4, KCl 4.7, $CaCl_2$ 1.3, $MgSO_4.7H_2O$ 1.2, $NaHCO_3$ 24.9, KH_2PO_4 1.2 and glucose 11.1.

[^{3}H]-guanine was supplied by Rotem Industries, Israel, and [^{14}C]-cyclic GMP supplied by Amersham International, Amersham, Bucks. Sodium nitroprusside dihydrate and atrial natriuretic peptide (rat) were obtained from Sigma and were prepared fresh each day in Krebs solution; forskolin was obtained from Sigma and dissolved in absolute ethanol (to a concentration of 10^{-2} M) and diluted in Krebs solution on the day of the experiment. Rolipram was a gift from Schering, and was also prepared fresh each day in Krebs solution.

Results

Figure 1 shows a time course of SNP- (10^{-3} M) stimulated [^{3}H]-cyclic GMP accumulation in the presence and absence of the phosphodiesterase inhibitor, IBMX (10^{-3} M) in porcine isolated palmar lateral vein. IBMX (10^{-3} M) raised levels of [^{3}H]-cyclic GMP to 193 ± 13% ($n = 3$) of basal, thus all results are expressed as % of basal values either with or without IBMX to allow comparisons to be made. In the presence of IBMX, SNP stimulated [^{3}H]-cyclic GMP accumulation to approximately 384 ± 26% of basal values ($n = 3$, with each experiment being carried out in quadruplicate) and the response peaked at 5 min after which time there was a decline in the amount of [^{3}H]-cyclic GMP accumulation (after 40 min levels were 291 ± 25% of basal) (Figure 1). In the absence of IBMX, SNP stimulated [^{3}H]-cyclic GMP accumulation to only 233 ± 32% of basal ($n = 3$). The response also peaked after 5 min and then declined with time (after 40 min levels were 143 ± 10% of basal) (Figure 1). Based on the results obtained from the time course study, concentration-response curves to SNP and ANP were carried out in the presence of IBMX, although, in studies investigating the interaction of forskolin with cyclic GMP, IBMX was excluded because inhibition of cyclic GMP metabolism would further complicate the interpretation of the results. However, IBMX was included in one study to determine the effects of forskolin after inhibition of cyclic GMP metabolism.

Both SNP (10^{-5}–10^{-3} M) and ANP (10^{-8}–10^{-6} M) produced concentration-dependent increases in [^{3}H]-cyclic GMP in the presence of 10^{-3} M IBMX (Figure 2a and b respectively), with the response at the highest concentrations of SNP and ANP being 692 ± 82% and 325 ± 12% of basal values respectively ($n = 3$ for both).

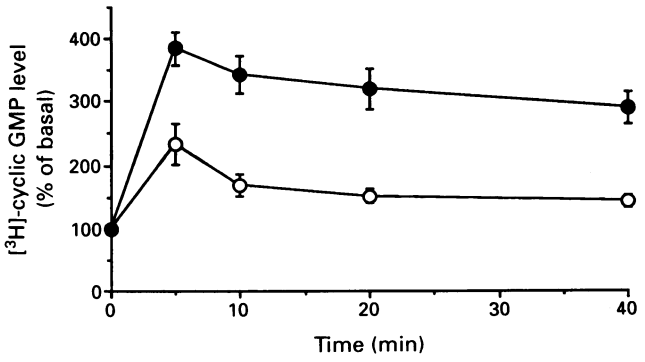


Figure 1 Effect of sodium nitroprusside (SNP, 10^{-3} M) on [^{3}H]-cyclic GMP accumulation either in the absence (○) or presence (●) of the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX, 10^{-3} M) at different times of incubation in porcine isolated palmar lateral vein. All results are expressed as a percentage of basal level for that particular experiment; each experiment was carried out in quadruplicate, time courses to SNP in the absence or presence IBMX were carried out within the same experiment. Data are expressed as the mean ± s.e.mean of [^{3}H]-cyclic GMP levels at that particular time, and are the result of 3 separate experiments. Basal [^{3}H]-cyclic GMP levels = 1387 ± 251 d.p.m.; basal + IBMX = 2677 ± 484 d.p.m.

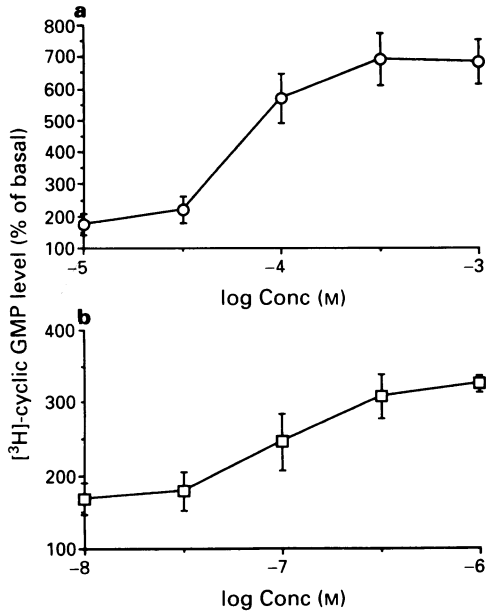


Figure 2 Effect of increasing concentrations of (a) sodium nitroprusside (SNP) and (b) atrial natriuretic peptide (ANP) on [^{3}H]-cyclic GMP accumulation in porcine isolated palmar lateral vein. All experiments were carried out in the presence of IBMX and incubation with either SNP and ANP was for a 5 min period. Results are expressed as a percentage of basal [^{3}H]-cyclic GMP levels for that particular experiment; each experiment was carried out in quadruplicate. Data are expressed as the mean ± s.e. mean, and are the result of 3 separate experiments. Basal [^{3}H]-cyclic GMP levels for SNP = 3496 ± 847 d.p.m.; basal [^{3}H]-cyclic GMP levels for ANP = 2721 ± 323 d.p.m.

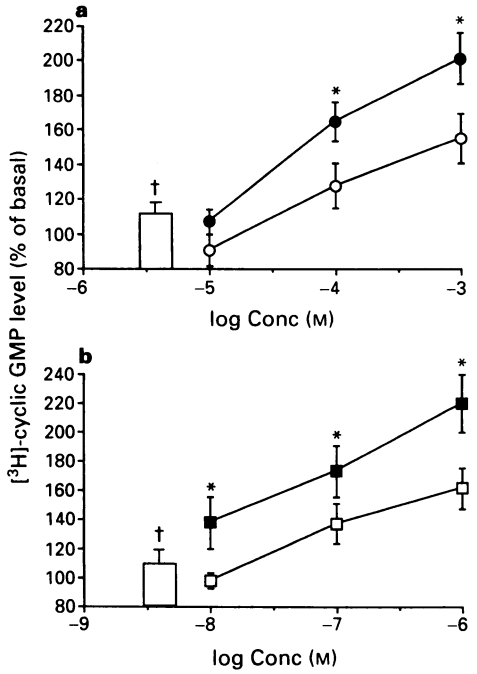


Figure 3 Effect of forskolin (3×10^{-5} M) (solid symbols) on basal (open column) and either (a) sodium nitroprusside (SNP)- or (b) atrial natriuretic peptide (ANP)- (open symbols) stimulated [^{3}H]-cyclic GMP accumulation in porcine isolated palmar lateral vein. All experiments were carried out in the absence of 3-isobutyl-1-methylxanthine (IBMX) and forskolin was added 10 min prior to either SNP or ANP which was incubated for a 5 min period. All results are expressed as a percentage of basal level for that particular experiment; each experiment was carried out in quadruplicate, the effects of the absence or presence of forskolin on the SNP or ANP stimulated [^{3}H]-cyclic GMP accumulation were carried out within the same experiment. Data are expressed as the mean ± s.e.mean, and are the result of 4 separate experiments. For SNP study: basal [^{3}H]-cyclic GMP levels = 2662 ± 496 d.p.m.; forskolin = 3601 ± 822 d.p.m.; ANP study: basal [^{3}H]-cyclic GMP levels = 2086 ± 486 d.p.m.; forskolin = 2480 ± 678 d.p.m. *$P<0.05$ compared to SNP or ANP alone, Student's 2 tailed paired t test. †$P<0.05$ compared to basal Student's 2 tailed paired t test.

Forskolin (3×10^{-5} M) significantly increased [^{3}H]-cyclic GMP levels to 118.5 × 8.7% of basal values ($P<0.05$, 2 tailed paired Student's t test, $n = 8$). In the absence of IBMX, forskolin significantly increased both the SNP and ANP-stimulated cyclic GMP accumulation at all concentrations of SNP and ANP used (except 10^{-5} M SNP) (Figure 3a and b respectively). For example, effects at the maximal SNP (10^{-3} M) and ANP (10^{-6} M) concentrations were: SNP: 154.7 ± 15.4% of basal; SNP + forskolin: 191.3 ± 14.8% of basal ($P<0.05$, $n = 4$); ANP: 161.4 ± 17.4% of basal; ANP + forskolin: 220.0 ± 20.0% of basal ($P<0.05$, $n = 4$).

The cyclic AMP-selective phosphodiesterase inhibitor, rolipram (10^{-5} M), had no effect on basal or SNP-stimulated [^{3}H]-cyclic GMP levels (Figure 4a), however the combination of forskolin (3×10^{-5} M) and rolipram (10^{-5} M) produced significant increases in the basal (154.9 ± 27.1% of basal) and SNP-stimulated [^{3}H]-cyclic GMP accumulation at all concentrations of SNP (Figure 4b). The effects at the maximal concentration of SNP (10^{-3} M) were: SNP: 165.3 ± 8.7% of basal; SNP + forskolin + rolipram: 510.7 ± 64.8% of basal; $P<0.05$, $n = 5$. The phosphodiesterase inhibitor, IBMX (10^{-3} M) significantly raised [^{3}H]-cyclic GMP levels (as reported earlier) and in the presence of IBMX, forskolin (3×10^{-5} M) had no significant effect on either basal or SNP-stimulated [^{3}H]-cyclic GMP levels (e.g. in the presence of IBMX: SNP (10^{-3} M): 660 ± 90% of basal; SNP + forskolin: 790 ± 86% of basal, $n = 3$) (Figure 4c).

Discussion

This study demonstrates that it is possible to measure (using a [^{3}H]-guanine prelabelling technique) and pharmacologically manipulate cyclic GMP in porcine isolated palmar lateral vein. SNP stimulated [^{3}H]-cyclic GMP accumulation and the response reached a peak after 5 min, either in the presence or absence of the phosphodiesterase inhibitor, IBMX. In the presence of IBMX the basal [^{3}H]-cyclic GMP level was raised, as were the SNP-stimulated [^{3}H]-cyclic GMP values at all time points compared to values without IBMX. It is interesting to note that in the absence of IBMX there was a fall in SNP stimulated [^{3}H]-cyclic GMP levels after 5 min which appeared almost to level off after 20 min, suggesting that there is some ongoing metabolism of [^{3}H]-cyclic GMP and that the phosphodiesterases are saturated after this time. However, it was unexpected to observe a similar profile in the presence of IBMX. This suggests that either the concentration of IBMX used did not inhibit all of the phosphodiesterases, that [^{3}H]-cyclic GMP activates a group of phosphodiesterases which are not inhibited by IBMX, or, perhaps because IBMX is a competitive antagonist, that the elevated cyclic GMP was able to compete with IBMX for binding to the phosphodiesterase and subsequently be metabolised, or that [^{3}H]-cyclic GMP was being metabolised by another pathway. In a study on a neuronal cell line (NG-108), Reiser (1992) found that even in the presence of IBMX, bradykinin- and 5-hydroxytryptamine-stimulated [^{3}H]-cyclic GMP levels declined after a period of several minutes.

Concentration-dependent stimulation of [^{3}H]-cyclic GMP levels by SNP indicates the presence of a responsive soluble guanylyl cyclase in the porcine isolated palmar lateral vein, and this is consistent with other vascular smooth muscle and neuronal preparations such as rabbit aortic segments (Schini

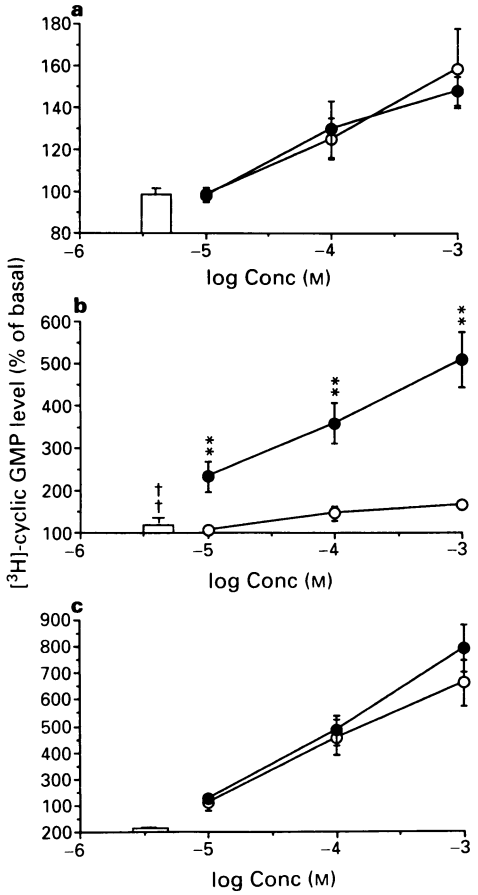


Figure 4 Effect of (a) rolipram (10^{-5} M), (b) rolipram (10^{-5} M) + forskolin (3×10^{-5} M) or (c) 3-isobutyl-1-methylxanthine (IBMX, 10^{-3} M) + forskolin (3×10^{-5} M) (solid symbols; sodium nitroprusside (SNP) alone – open symbols) on basal (open column) and SNP-stimulated [^{3}H]-cyclic GMP accumulation in porcine isolated palmar lateral vein. Forskolin was added 10 min prior to SNP which was incubated for a 5 min period. All results are expressed as a percentage of basal level for that particular experiment; each experiment was carried out in quadruplicate, the effects of the absence or presence of either rolipram, rolipram + forskolin, or forskolin (in the presence of IBMX) on basal and SNP-stimulated [^{3}H]-cyclic GMP accumulation were carried out within the same experiment. Data are expressed as the mean ± s.e.mean, and are the result of 3 to 5 separate experiments. For rolipram study: basal [^{3}H]-cyclic GMP levels = 2486 ± 468 d.p.m.; rolipram = 2507 ± 305 d.p.m.; rolipram + forskolin study: basal [^{3}H]-cyclic GMP levels = 1508 ± 94 d.p.m.; rolipram + forskolin = 2336 ± 170 d.p.m.; basal [^{3}H]-cyclic GMP levels = 1794 ± 129 d.p.m.; IBMX = 2678 ± 332 d.p.m.; IBMX + forskolin = 2959 ± 410 d.p.m. $^{**}P<0.01$ compared to SNP alone, Student's 2 tailed paired t test. $\dagger\dagger P<0.05$ compared to basal, Student's 2 tailed paired t test.

et al., 1989), guinea-pig thoracic aorta and coronary artery dispersed smooth muscle cells (Zhang *et al.*, 1993), and guinea-pig cerebellar slices (Hernandez *et al.*, 1994). ANP also produced concentration-dependent elevations of [^{3}H]-cyclic GMP, agreeing with results of other studies of vascular smooth muscle (Murad *et al.*, 1987; Zhang *et al.*, 1993), and indicating the presence of a responsive particulate guanylyl cyclase which is independent of the soluble isoform.

Forskolin, an activator of adenylyl cyclase, enhanced basal and SNP-stimulated levels of [^{3}H]-cyclic GMP in the porcine isolated palmar lateral vein, agreeing with a similar study carried out in guinea-pig cerebellar slices (Hernandez *et al.*, 1994). Forskolin also enhanced ANP-stimulated [^{3}H]-cyclic GMP accumulation, indicating that the mechanism(s) by which forskolin potentiates basal and stimulated cyclic GMP levels does not depend upon whether it is soluble or particulate guanylyl cyclase that is activated. Reiser (1992) and Gray & Marshall (1992) both suggested that the potentiating effect of cyclic AMP elevation was via activation of the enzyme, nitric oxide synthase (NOS), thus increasing levels of nitric oxide leading to soluble guanylyl cyclase activation and cyclic GMP elevation. Particulate guanylyl cyclase is not affected by NOS activity and hence should not theoretically be affected by forskolin if the forskolin effect depends upon a cyclic AMP activation of NOS. Also, a cyclic GMP response to a maximally effective concentration of SNP would not be expected to be influenced by an enhancement of NOS activity since the soluble guanylyl cyclase would already be saturated by SNP-derived NO. Hernandez *et al.* (1994) have suggested that the cyclic AMP-cyclic GMP interaction occurs at the level of catabolism, i.e. that the two cyclic nucleotides compete for a common phosphodiesterase, and this theory is supported by the present data as forskolin potentiated soluble and particulate guanylyl cyclase independently. However, the cyclic AMP-selective phosphodiesterase inhibitor, rolipram, at a concentration known to increase cyclic AMP levels in porcine isolated palmar lateral vein by 1.7 fold (Wright *et al.*, 1994b) did not have any effect on basal and SNP-stimulated [^{3}H]-cyclic GMP accumulation. It is probable that this small increase in cyclic AMP was not sufficient to saturate all of the phosphodiesterases, thus allowing continuing metabolism of [^{3}H]-cyclic GMP. This seems a realistic explanation as the combination of rolipram and forskolin had a much greater effect than either agent alone. Further support comes from the use of the non-selective phosphodiesterase inhibitor, IBMX; when all catabolism is inhibited by IBMX, forskolin no longer potentiates basal or SNP-stimulated [^{3}H]-cyclic GMP accumulation, indicating that this is the site of interaction of the two nucleotides. Because the phenomenon of cyclic AMP potentiation of cyclic GMP appears to exist in neuronal cell lines (Reiser, 1992), rat aorta (Gray & Marshall, 1992), guinea-pig cerebellar slices (Hernandez *et al.*, 1994) and porcine isolated palmar lateral vein (present study), it might be that this interaction is a normal physiological regulatory mechanism in a number of different tissues.

It is well established that α_2-adrenoceptors are negatively coupled to adenylyl cyclase, resulting in an inhibition of cyclic AMP formation in non-vascular preparations (Duman & Enna, 1986; Bylund, 1988), and it has been postulated that this could be one of the mechanisms through which vascular α_2-adrenoceptors produce contractile responses. Recently, Wilson & Warren (1993) reported that the importance of adenylyl cyclase-mediated vascular relaxation increased with decreasing vessel size. Further, endothelium-dependent relaxations in porcine pulmonary arteries increased as the vessel diameter decreased (probably as a result of increases in endothelium:smooth muscle ratio with decrease in diameter) (Elbishti & Pollock, 1994). Thus, it is possible that cyclic AMP, as well as directly activating cyclic GMP-dependent protein kinase (Lincoln *et al.*, 1990; Jiang *et al.*, 1992), may also potentiate the cyclic GMP-mediated cellular events, and thus the two cyclic nucleotides, as well as having independent mechanisms to induce smooth muscle relaxation, may produce vasodilatation via a common mechanism.

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Comparative studies on the affinities of ATP derivatives for P_{2X}-purinoceptors in rat urinary bladder

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1 Radioligand binding assays have been used to determine the affinities of a series of ATP derivatives with modifications of the polyphosphate chain, adenine and ribose moieties of the ATP molecule for [^{3}H]-α,β-methylene ATP ([^{3}H]-α,β-MeATP) binding sites in rat urinary bladder.

2 The replacement of the bridging oxygen in the triphosphate chain of ATP ($pIC_{50} = 5.58$) with a methylene or imido group markedly increased the affinity (691 fold in IC_{50} values for β,γ-imidoATP, 15 fold for β,γ-methylene ATP), and the replacement of an ionized oxygen on the γ-phosphate with a sulphur (ATPγS) also led to increased affinity (5623 fold in IC_{50} values).

3 Modifications at N^6, N^1, and C-8 positions on the purine base usually reduced the affinity of ATP (a decrease of 2.8 fold in IC_{50} values for N^6-methylATP and 8.9 fold for 8-bromo ATP), while the attachment of an alkylthio group to the C-2 position greatly increased the affinity for P_{2X}-purinoceptors (from 3.5 to 98 fold increase in IC_{50} values).

4 Replacement of the 3′-hydroxyl group on the ribose with substituted amino or acylamino groups produced more potent P_{2X}-purinoceptor agonists (an increase of 447 fold in IC_{50} values for 3′-deoxy-3′-benzylamino ATP and 28 fold for 3′-deoxy-3′-(4-hydroxyphenylpropionyl)amino ATP.

5 Diadenosine polyphosphates (Ap[n]A) were also shown to displace the [^{3}H]-α,β-MeATP binding. The rank order of potency was Ap6A > Ap5A > Ap4A ≫ Ap3A ≫ Ap2A.

6 Suramin, PPADS, and reactive blue 2 could competitively displace the binding of [^{3}H]-α,β-MeATP to P_{2X}-purinoceptors, with pIC_{50} values of 6.26, 5.35, and 6.22, respectively.

Keywords: P_{2X}-purinoceptors; urinary bladder; [^{3}H]-α,β-methylene ATP; ATP derivatives; suramin; reactive blue 2; PPADS; radioligand binding assay

Introduction

Recent years have witnessed a significant expansion in the field of the physiological functions of extracellular purines. ATP, ADP, adenosine and some other purine derivatives were found to play the roles of neurotransmitter, co-transmitter, and neuromodulator in various types of tissues including both the central and peripheral nervous system (Burnstock, 1993). The receptors which mediate the effects of extracellular purines have been classified into two major types, i.e., P_1- and P_2-purinoceptors, mainly on the basis that the principal endogenous ligands were adenosine and ATP, respectively (Burnstock, 1978). P_2-purinoceptors have been further divided into P_{2X}-, P_{2Y}-, (Burnstock & Kennedy, 1985), P_{2T}-, P_{2Z}-purinoceptors (Gordon, 1986). Recently, some further subtypes of P_2-purinoceptors, P_{2U}-, P_{2N}-, have also been proposed (for review see Abbracchio *et al.*, 1993). The classification of P_2-purinoceptors was mainly based on the rank order of potencies of a series of purine compounds in evoking certain biological responses such as the contraction or relaxation of smooth muscles. Therefore, researchers in this field are concerned to develop more selective and more potent agonists for each subtype of P_2-purinoceptors, which would provide further evidence for the classification. Such work has been summarized by Cusack (1993).

P_{2X}-purinoceptors are mainly located on smooth muscle, where they mediate contraction. Recently, P_{2X}-purinoceptors have also been identified at neuro-neuronal synapses (Evans *et al.*, 1992; Edwards *et al.*, 1992; Silinsky *et al.*, 1992). In early as well as more recent investigations, rodent urinary bladder and vas deferens have been the two main organs used for pharmacological studies on P_{2X}-purinoceptors. Many studies have been carried out to explore the structure-activity relationships of purine compounds to elicit the P_{2X}-purinoceptor-mediated contraction (Lukacsko & Krell, 1982; Fedan *et al.*, 1982; 1986; Welford *et al.*, 1987; Howson *et al.*, 1988; Satchell, 1988). A lot of important information has been obtained from these studies. However, some factors, such as metabolic transformation and uptake of exogeneously applied ligands and the existence of multiple subtypes of receptors, may significantly influence the results from experiments on living tissues. Radioligand binding assay, on the other hand, deals directly with the interaction of a ligand and its receptors, offering an important tool to determine the structure-affinity relationship between ligands and receptors.

α,β-Methylene ATP is one of the most potent and relatively selective agonists for P_{2X}-purinoceptors. A few years ago, the tritiated form [^{3}H]-α,β-methylene ATP ([^{3}H]-α,β-MeATP) was found to bind preferentially to P_{2X}-purinoceptors. The distribution of the binding sites is also in accordance with the distribution of P_{2X}-purinoceptors proven by pharmacological experiments (Bo & Burnstock, 1990; 1993b). Displacement experiments using several purine compounds showed that their rank order of potency in displacing the binding was in good agreement with that obtained from pharmacological experiments (Bo & Burnstock, 1990). Now several other laboratories have also begun to employ this radioligand to study P_{2X}-purinoceptors (Michel & Humphrey, 1993).

Recently, many new ATP derivaties with modifications of the triphosphate chain, purine base and the D-ribose have been synthesized in Dr. Jacobson's laboratories in NIH (Fischer *et al.*, 1993; Burnstock *et al.*, 1994). Functional studies like *in vitro* recording of smooth muscle responses and P_{2Y}-purinoceptor-promoted phospholipase C activity have shown that some of the compounds are more potent and more

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selective or specific for either P_{2X}- or P_{2Y}-purinoceptors (Fischer et al., 1993; Burnstock et al., 1994). In this study we used [^{3}H]-α,β-MeATP as a tool to examine the affinities of these new purine compounds and some obtained commercially for the P_{2X}-purinoceptors in rat urinary bladder. Several putative P_2-purinoceptor antagonists and a UTP derivative have also been tested. The results help to build up a profile of the structure-affinity relationship of the P_2-purinoceptor ligands with the receptors, which may provide some hints for the design of new P_{2X}-purinoceptor-selective compounds.

Methods

Male Wistar rats (200–250 g) were killed by asphyxiation with CO_2. Urinary bladders were removed immediately and placed in modified Krebs solution of the following composition (mM): NaCl 133, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 0.6, $NaHCO_3$ 16.3, NaH_2PO_4 1.4, glucose 7.7, (pH 7.4). They were trimmed free from surrounding adipose and connective tissues, minced and homogenized in 10 volume of ice-cold 50 mM Tris/HCl buffer containing 1 mM EGTA, 1 mM benzamidine hydrochloride, 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 0.01% (w/v) bacitracin, and 0.002% (w/v) soybean trypsin inhibitors, pH 7.4 (buffer A), with a Polytron PT-3000 (Kinematica, Switzerland) twice in 15 s bursts at 30,000 r.p.m. The homogenate was centrifuged at 170 *g* for 5 min at 4°C. The supernatant was passed through double layers of nylon mesh and centrifuged at 105,000 *g* for 50 min at 4°C. The pellets were washed and suspended in buffer A and the protein content was determined by the method of Peterson (1977).

For saturation assays, serial concentrations of [^{3}H]-α,β-MeATP (from 0.55 to 160 nM) were incubated with bladder membrane preparations (about 30 μg protein per assay tube) in buffer A at 4°C for 120 min. At the end of the incubation the assay tubes were put on ice and the reaction mixture was rapidly filtered through a Whatman GF/B filter (presoaked in 20 mM disodium pyrophosphate solution) under vacuum. The filters were washed with 2 aliquots of 5 ml ice-cold 50 mM Tris/HCl buffer, pH 7.4, dried under an infrared lamp, and then the radioactivity trapped in the filters was measured in a Beckman SC6100I scintillation counter with an efficiency of about 56 to 61%. Non-specific binding was determined by including 100 μM β,γ-methylene ATP (β,γ-MeATP) in the incubation mixture. The assay was carried out in triplicate and each experiment was repeated five times.

Displacement experiments were carried out by incubating the membrane preparations (about 30 μg proteins per assay tube) with 10 nM [^{3}H]-α,β-MeATP and serial concentrations of an unlabelled ligand in buffer A at 4°C for 120 min (final volume 0.5 ml). The other procedures were the same as those for saturation assays.

Chemicals

[^{3}H]-α,β-MeATP was custom-synthesized by Amersham International plc. (Amersham, U.K.) with a specific activity of 19.2 Ci mmol^{-1} and chemical purity of 98–99%. The names, abbreviations, and sources of the non-labelled compounds used in this study are listed in Table 1. The chemical structures of some of the compounds are shown in Figure 1. Amongst the 50 compounds tested, all of them are putative agonists for P_2-purinoceptors except suramin, PPADS, and RB-2, which are regarded as P_2-purinoceptor antagonists.

Statistics

The data were expressed as mean ± standard error (s.e.mean). The data were analysed with the computer programme EBDA-LIGAND (Biosoft, Cambridge, UK) using non-linear curve fitting (Munson & Rodbard, 1980). The one or two binding site model was chosen based on the results of an *F* test on the weighted sum of squares. Student's *t* test was employed to compare the means of the pIC_{50} values. A *P* value less than 0.05 was considered as significant.

Results

The specific binding of [^{3}H]-α,β-MeATP to rat bladder membrane preparations was saturable and reversible. Computer-assisted non-linear least squares curve fitting of the binding data revealed two affinity sites, with K_d of 6.8 ± 1.9 nM and B_{max} of 9.4 ± 2.2 pmol mg^{-1} protein ($n = 5$) for the high-affinity sites, and K_d of 62.3 ± 18.8 nM and B_{max} of 47.5 ± 16.3 pmol mg^{-1} protein ($n = 5$) for the low-affinity sites. The specific binding in the displacement experiments was 6.9 ± 1.3 pmol mg^{-1} protein, and the non-specific binding (determined by 100 μM β,γ-MeATP) was about 10–15% of the total binding. The data from the displacement experiments have also been analysed with the computer programme LIGAND for one or two binding site fitting. The fittings for two binding sites were not significantly better than the fitting for one binding site. The main reason for the discrepancy with the two binding site fitting of the saturation assay may be that these compounds have no selectivity for the two affinity states of [^{3}H]-α,β-MeATP binding sites in rat bladder; therefore, near monophasic displacement curves were produced.

As shown in Table 2 and Figure 2 the potencies of the compounds tested in displacing the [^{3}H]-α,β-MeATP binding to P_{2X}-purinoceptors in rat bladder vary significantly. In the first group of compounds with modifications on the triphosphate chain, the rank order of potency was the same as reported before (Burnstock & Kennedy, 1985), i.e., ATP > ADP ≫ AMP > AD, suggesting that the binding sites are P_2-purinoceptors. Replacement of an ionized oxygen in the triphosphate chain of ATP or the diphosphate group in ADP by a sulphur (ATPγS, ATPαS, and ADPβS) led to an increase of the affinities for P_{2X}-purinoceptors. ATPγS displayed the highest potency in displacing the binding of [^{3}H]-α,β-MeATP to P_{2X}-purinoceptors amongst the compounds tested in this study, which was about one thousand times higher than those of ADPβS and ATPαS ($P < 0.001$, Table 2, Figure 2a). However, the attachment of a big moiety to the phosphate at the γ-position like γ-(1[2-nitrophenyl]ethyl)ester (ATP-γ-E), did not significantly affect the affinity of ATP for the receptor. Some other moieties like ramidate (ApR, and AppR), morpholidate (ApM), and ribose (ADP-ribose), when attached to the end phosphate of AMP or ADP, abolished or significantly reduced (117 fold for ADP-ribose, $P < 0.001$ compared with ADP) their potencies in displacing the [^{3}H]-α,β-MeATP binding to P_{2X}-purinoceptors. Attachment of a sulphate to the phosphate of AMP (AMP-SO_4) markedly increased its affinity for the receptors (407 fold in IC_{50} values, $P < 0.001$).

In the group of compounds with the phosphate-linking oxygen being replaced by methylene or imido, β,γ-imidoATP showed the highest affinity, followed by α,β-MeATP, β,γ-MeATP, and L-β,γ-MeATP (Table 2, Figure 2b). α,β-MeADP was almost without effect on the binding. Cyclization of the C-3′ and C-5′ of β,γ-MeATP to form cAMPPCP abolished the affinity of β,γ-MeATP for P_{2X}-purinoceptors.

Sodium tetraphosphate (PPPP) was almost as potent as ATP in displacing the binding, but sodium metatriphosphate (mPPP), a cyclic triphosphate structure, was about 113 fold less potent than the open chain triphosphate structure (sodium tripolyphosphate).

For the dinucleotides the pattern was quite evident: the longer the polyphosphate chain between the two adenosine groups, the more potent the compound in displacing the binding (Table 2, Figure 2c). The difference between the IC_{50} values of Ap6A and Ap2A is more than seven million times.

Table 1 The full names, abbreviations, and sources of the purine compounds and P_2-purinoceptor antagonists used in this study

Abbreviation	*Name*	*Source*
ATP	Adenosine 5'-triphosphate	Sigma
ADP	Adenosine 5'-diphosphate	Sigma
AMP	Adenosine 5'-monophosphate	Sigma
AD	Adenosine	Sigma
α,β-MeATP	Adenosine 5'-(α,β-methylene)triphosphate	Sigma
α,β-MeADP	Adenosine 5'-(α,β-methylene)diphosphate	Sigma
β,γ-MeATP	Adenosine 5'-(β,γ-methylene)triphosphate	Sigma
L-β,γ-MeATP	L-Adenosine 5'-(β,γ-methylene)triphosphate	Fisons
cAMPPCP	3',5'-cyclic adenosine 5'-(β,γ-methylene)triphosphate	NIH
β,γ-ImidoATP	Adenosine 5'-(β,γ-imido)triphosphate	Sigma
PPPP	Sodium tetraphosphate	Sigma
mPPP	Sodium metatriphosphate	Sigma
ATPγS	Adenosine 5'-O-(3-thiotriphosphate)	Sigma
ATPαS	Adenosine 5'-O-(1-thiotriphosphate)	Sigma
ATP-γ-E	Adenosine 5'-triphosphate γ-(1[2-nitrophenyl]ethyl)ester	Sigma
ADPβS	Adenosine 5'-O-(2-thiodiphosphate)	Sigma
ApR	Adenosine 5'-monophosphoramidate	Sigma
AppR	Adenosine 5'-diphosphoramidate	Sigma
ApM	Adenosine 5'-monophosphomorpholidate	Sigma
AMP-SO_4	Adenosine 5'-monophosphosulphate	Sigma
ADP-ribose	Adenosine 5'-diphosphoribose	Sigma
Ap6A	P^1,P^6-diadenosine hexaphosphate	Sigma
Ap5A	P^1,P^5-diadenosine pentaphosphate	Sigma
Ap4A	P^1,P^4-diadenosine tetraphosphate	Sigma
Ap3A	P^1,P^3-diadenosine triphosphate	Sigma
Ap2A	P^1,P^2-diadenosine pyrophosphate	Sigma
N-O-ATP	Adenosine-N^1-oxide 5'-triphosphate	Sigma
AAHeATP	8-(6-aminohexyl)aminoadenosine 5'-triphosphate	NIH
8-BrATP	8-Bromoadenosine 5'-triphosphate	NIH
1,N^6-E-ATP	1,N^6-Ethenoadenosine 5'-triphosphate	Sigma
N^6-MeATP	N^6-Methyladenosine 5'-triphosphate	NIH
5-F-UTP	5-Fluorouridine 5'-triphosphate	NIH
2-MeSATP	2-Methylthioadenosine 5'-triphosphate	RBI
2-HeSATP	2-Hexylthioadenosine 5'-triphosphate	NIH
2-HexSADP	2-(5-Hexenyl)thioadenosine 5'-diphosphate	NIH
2-HexS-N^6-MeATP	2-(5-Hexenyl)thio-N^6-methyladenosine triphosphate	NIH
2-CyaHeSATP	2-(6-Cyanohexyl)thioadenosine 5'-triphosphate	NIH
2-CyaHeSAMP	2-(6-Cyanohexyl)thioadenosine 5'-monophosphate	NIH
2-cHeSATP	2-Cyclohexylthioadenosine 5'-triphosphate	NIH
2-NPESAMP	2-(2-*p*-Nitrophenylethyl)thioadenosine 5'-monophosphate	NIH
2'd-ATP	2'-deoxyadenosine 5'-triphosphate	Sigma
3'd-ATP	3'-deoxyadenosine 5'-triphosphate	Sigma
2',3'dd-ATP	2',3'-dideoxyadenosine 5'-triphosphate	Sigma
3'BH-NH-ATP	3'-deoxy-3'-(4-hydroxy-phenylpropionyl)aminoadenosine 5'-triphosphate	NIH
3'dBAATP	3'-deoxy-3'-benzylaminoadenosine 5'-triphosphate	NIH
2',5'-ADP	Adenosine 2',5'-diphosphate	Sigma
2'3'-iPIAMP	2',3'-isopropylidene adenosine 5'-monophosphate	NIH
PPADS	Pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid	Lambrecht
RB-2	Reactive blue 2	Sigma
Suramin		Bayer

Sigma: Sigma Chemicals UK Ltd., Poole, UK; RBI: Research Biochemicals Incorporated, Natick, USA; Bayer: Bayer AG UK Ltd., Strawberry Hill, Newbury, UK; NIH: Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Disease, Bethesda, U.S.A.; Lambrecht: Prof. Lambrecht, University of Frankfurt, Germany.

Modifications to the purine base of ATP molecule usually lead to a reduction of ATP affinity for P_{2X}-purinoceptors except at the C-2 position (Table 2, Figure 2d). N-O-ATP, 1,N^6-E-ATP, N^6-MeATP, and 8-BrATP all showed reduced affinities relative to ATP (in IC_{50} values: 2.8 fold for N-O-ATP and N^6-MeATP, 4.5 fold for 1,N^6-E-ATP, and 8.9 fold for 8-BrATP), and their displacement curves were usually rather steep (Hill coefficients n_H from 1.67 to 2.15) (Figure 2) except 8-BrATP, which has a n_H of 0.7. AAHeATP has a (6-aminohexyl)amino group attached to the C-8 position, however, its potency was increased by 4.7 fold compared to ATP ($P<0.01$). The attachment of a thioether group to the C-2 position increased the affinity of ATP for P_{2X}-purinoceptors. The 2-hexylthio derivative 2-HeSATP possessed higher affinity than the 2-methylthio derivative 2-MeSATP (Table 2). The addition of a cyano group at the end of the hexyl chain of 2-HeSATP did not change its affinity. However, cyclization of the hexyl chain did reduce its potency by 6 times ($P<0.01$). The only UTP derivative tested in this study, 5-F-UTP, showed a 10 fold lower affinity for P_{2X}-purinoceptors than that of ATP.

Modifications to the ribose of ATP molecule also produced some potent ligands (Table 2, Figure 2e). Attachment of a large benzyl or phenylpropionyl moiety to the C-3' position via an amino group (3'BH-NH-ATP and 3'dBAATP) significantly increased the affinity of ATP for P_{2X}-purinoceptors (28 and 447 fold, respectively, $P<0.01$). 3'd-ATP was about 4 fold more potent than 2'd-ATP in displacing [^{3}H]-α,β-MeATP binding ($P<0.05$), while the potency of 2',3'dd-ATP fell in-between those of 2'd-ATP and 3'd-ATP and close to that of ATP. The addition of a phosphate group at the C-2' position of AMP (2',5'-ADP) significantly increased the affinity of AMP (9.3 fold in IC_{50} values).

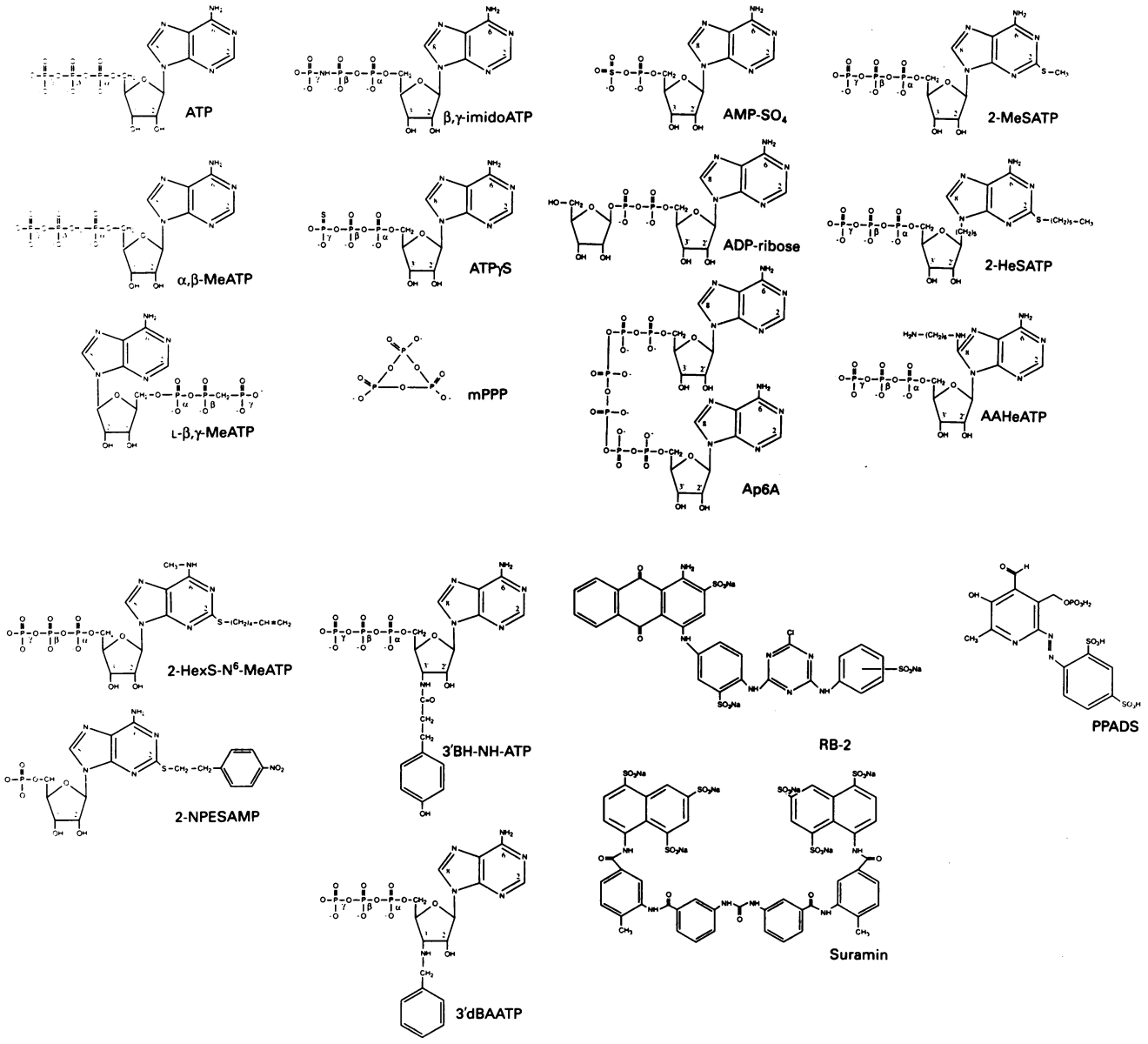


Figure 1 Chemical structures of the ATP derivatives and the P_2-purinoceptor antagonists.

Among the three putative P_2-purinoceptor antagonists, suramin and RB-2 were nearly equipotent in displacing the binding of [^{3}H]-α,β-MeATP. PPADS was about 7–8 times less potent in displacing the binding than suramin or RB-2 (Table 2). The displacement curve of RB-2 was rather steep (n_H = 1.46), while suramin and PPADS showed flatter sigmoid curves (n_H = 0.61 and 0.85, respectively) (Figure 2f).

Discussion

Studies on structure-activity relationship have shown that the triphosphate group in ATP molecule is responsible for the efficacy in activating P_{2X}-purinoceptors while the purine base and the D-ribose are associated with affinity (Lukacsko & Krell, 1982; Fedan *et al.*, 1986; Welford *et al.*, 1987; Howson *et al.*, 1988). Results from binding studies indicate that the triphosphate chain in the ATP molecule is also the key structure responsible for the affinity for P_{2X}-purinoceptors (Bo & Burnstock, 1993a). In this study, 46 newly synthesized or commercially available purine compounds with modifications at different positions of ATP, and one UTP analogue were tested, and remarkable differences in the affinities of the compounds for P_{2X}-purinoceptors have been observed.

First the compounds which are commonly used to distinguish P_1-, P_2-, P_{2X}-, and P_{2Y}-purinoceptors were examined again, since the experimental conditions in this study were different from those reported in a previous paper (Bo & Burnstock, 1990). Divalent cations can facilitate the degradation of ATP and its derivatives and influence the interaction of purine compounds with P_{2X}-purinoceptors (Bo & Burnstock, 1990); therefore, the incubation in this study was carried out in a media free of exogenous divalent cations and in the presence of 1 mM EGTA at 4°C. Such changes produced a significant increase in the potencies of the purine nucleotides for displacing the [^{3}H]-α,β-MeATP binding to P_{2X}-purinoceptors. For α,β-MeATP, β,γ-MeATP, ATP and ADP, 10 to 12 fold increases in IC_{50} values were noted. The IC_{50} value of suramin was increased by 4 times, while the IC_{50} value of 2-MeSATP was increased by 127 times. The order of potency of these compounds is: α,β-MeATP > β,γ-MeATP > 2-MeSATP > ATP > ADP >> AMP >> AD, which indicates that the [^{3}H]-α,β-MeATP binding sites under these experimental conditions still conform to the criteria for P_{2X}-purinoceptors according to Burnstock & Kennedy (1985).

Table 2 The pIC_{50} values and Hill coefficients of purinoceptor ligands in displacing the binding of [^{3}H]-α,β-methylene ATP to rat urinary bladder membranes

Ligand	pIC_{50}	n_H
ATP and triphosphate modification		
ATP	5.58 ± 0.17	0.85 ± 0.11
ADP	5.32 ± 0.24	0.90 ± 0.13
AMP	4.09 ± 0.45	1.41 ± 0.21
AD	> 3.00	
α,β-MeATP	8.03 ± 0.11	0.97 ± 0.05
α,β-MeADP	> 3.00	
β,γ-MeATP	6.75 ± 0.04	1.07 ± 0.05
L-β,γ-MeATP	5.68 ± 0.15	0.67 ± 0.11
cAMPPCP	> 2.00	
β,γ-ImidoATP	8.42 ± 0.03	0.94 ± 0.20
PPPP	5.56 ± 0.09	0.87 ± 0.08
mPPP	2.98 ± 0.09	1.49 ± 0.65
ATPγS	9.33 ± 0.23	0.90 ± 0.04
ATPαS	6.39 ± 0.36	1.05 ± 0.04
ATP-γ-E	5.41 ± 0.05	1.19 ± 0.12
ADPβS	6.41 ± 0.12	0.90 ± 0.11
ApR	> 2.00	
AppR	> 2.00	
ApM	> 2.00	
AMP-SO_4	6.70 ± 0.13	1.62 ± 0.07
ADP-ribose	3.25 ± 0.01	1.12 ± 0.02
Ap6A	8.88 ± 0.07	0.71 ± 0.06
Ap5A	8.05 ± 0.14	0.96 ± 0.03
Ap4A	7.57 ± 0.27	0.76 ± 0.06
Ap3A	5.16 ± 0.06	0.95 ± 0.10
Ap2A	> 2.00	
Base modification		
N-O-ATP	5.14 ± 0.07	1.76 ± 0.21
AAHeATP	6.25 ± 0.15	1.04 ± 0.09
8-BrATP	4.63 ± 0.21	0.70 ± 0.23
1,N^6-E-ATP	4.93 ± 0.03	1.67 ± 0.38
N^6-MeATP	5.14 ± 0.11	2.15 ± 0.13
5-F-UTP	4.60 ± 0.01	1.61 ± 0.09
2-MeSATP	6.33 ± 0.17	0.71 ± 0.12
2-HeSATP	7.57 ± 0.24	0.83 ± 0.04
2-HexSADP	6.01 ± 0.17	0.72 ± 0.16
2-HexS-N^6-MeATP	6.13 ± 0.15	0.72 ± 0.22
2-CyaHeSATP	7.24 ± 0.13	0.96 ± 0.10
2-CyaHeSAMP	4.34 ± 0.18	1.37 ± 0.16
2-cHeSATP	6.79 ± 0.23	1.06 ± 0.12
2-NPESAMP	5.42 ± 0.14	0.81 ± 0.11
Ribose modification		
2'd-ATP	5.28 ± 0.08	1.68 ± 0.22
3'd-ATP	5.92 ± 0.11	1.11 ± 0.13
2',3'dd-ATP	5.70 ± 0.14	1.14 ± 0.20
3'BH-NH-ATP	7.03 ± 0.23	0.76 ± 0.10
3'dBAATP	8.23 ± 0.19	1.12 ± 0.05
2'5'-ADP	5.06 ± 0.05	0.59 ± 0.05
2'3'-iPIAMP	5.33 ± 0.25	0.60 ± 0.09
Antagonists		
PPADS	5.35 ± 0.17	0.85 ± 0.08
RB-2	6.22 ± 0.04	1.46 ± 0.20
Suramin	6.26 ± 0.07	0.61 ± 0.09

It should also be mentioned that the Hill coefficients for the displacement curves of ATP and ADP have been reduced greatly (from 1.53 to 0.85 for ATP, and from 1.45 to 0.90 for ADP). The reason may be that the degradation of ATP and ADP had been inhibited under the experimental conditions used in this study so their effects at lower concentrations were revealed.

As has been reported by many researchers, the replacement of the bridging oxygen between the phosphates in ATP molecule with methylene or imido leads to an increase in the potency in activating P_{2X}-purinoceptors (Brown *et al.*, 1979; Lukacsko & Krell, 1982; Fedan *et al.*, 1982; 1986; Welford *et al.*, 1987; Howson *et al.*, 1988). α,β-MeATP, β,γ-MeATP, and β,γ-imidoATP all showed higher affinity for P_{2X}-purinoceptors than ATP. It was thought that α,β-MeADP would be more potent than ADP in displacing the binding of [^{3}H]-α,β-MeATP to P_{2X}-purinoceptors. α,β-MeADP, however, displayed almost no affinity for P_{2X}-purinoceptors. Results from pharmacological experiments also showed that α,β-MeADP produced lesser responses than ADP, and the characteristics of the responses are different from either those of ADP or those of α,β-MeATP (Fedan *et al.*, 1982), suggesting a different mechanism was involved. α,β-MeADP has been reported to be an inhibitor of ectonucleotidases (Bruns, 1980).

The L-enantiomer of β,γ-MeATP has been reported to be more selective for P_{2X}-purinoceptors and to be more resistant to dephosphorylation (Cusack *et al.*, 1987; Welford *et al.*, 1987). However, the potency of L-β,γ-MeATP in activating P_{2X}-purinoceptors was much less than β,γ-MeATP, and about equipotent with ATP (Welford *et al.*, 1987). This is in agreement with the binding results, which indicate that the IC_{50} value of L-β,γ-MeATP is 11 times higher than that of β,γ-MeATP ($P < 0.01$) and nearly equal to that of ATP ($P > 0.05$). Another modification of β,γ-MeATP, cyclization of C-2' and C-5' of the ribose (cAMPPCP), totally abolished the affinity of β,γ-MeATP for P_{2X}-purinoceptors. This phenomenon may be due to a significant change of conformation caused by the cyclization, thus, cAMPPCP may be unable to fit into the binding pocket. Pharmacological experiments proved that cAMPPCP activates neither P_{2X}- nor P_{2Y}-purinoceptors (Burnstock *et al.*, 1994).

The replacement of an ionized oxygen on the phosphate chain of ATP or ADP by a sulphur atom produces ATPγS, ATPαS, and ADPβS. These compounds showed enhanced affinities for P_{2X}-purinoceptors relative to ATP. It seems that the position of the sulphur on the phosphate chain is a determinant for the affinity. Thus, the γ-sulphur derivative, ATPγS, was a thousand times more potent than the α-sulphur derivative, ATPαS. Pharmacological experiments also showed that ATPγS was far more potent than ATP in eliciting contraction of guinea-pig bladder (Fedan *et al.*, 1982) and cat bladder (Theobald, 1992). However, this compound is weaker when compared to β,γ-MeATP and β,γ-imidoATP (Fedan *et al.*, 1982), which may be due to its non-selectivity for P_{2X}- and P_{2Y}-purinoceptors; the latter may mask the P_{2X}-purinoceptor-mediated responses in *in vitro* recording experiments. The attachment of a large group like a [1-(2-nitrophenyl)ethyl]ester at the γ-position did not affect the affinity of ATP for P_{2X}-purinoceptors. However, attachment of a large group like ribose, ramidate, or morpholidate to the terminal phosphate group of AMP or ADP significantly reduced the affinities (ADP ribose), or totally abolished their affinities (ApR, AppR, ApM) for P_{2X}-purinoceptors suggesting the property of the substituting group or atom is also a determinant for the affinity. These phenomena have also been observed in pharmacological experiments (Fedan *et al.*, 1982; 1986; Hoyle & Edwards, 1992). AMP-SO_4, a compound with a sulphate attached to the terminal phosphate of AMP, displayed a much higher affinity for P_{2X}-purinoceptors than ATP. However, in pharmacological experiments AMP-SO_4 was less potent than ATP in eliciting the contraction of guinea-pig vas deferens (Fedan *et al.*, 1982). It is unclear whether this discrepancy is due to a difference in the P_{2X}-purinoceptors in guinea-pig vas deferens versus rat urinary bladder, or because this compound is a partial agonist. The Hill coefficient of AMP-SO_4 displacement curve was significantly higher than unity which supports the assumption that another mechanism may be involved.

In a previous experiment we observed that sodium triphosphate displaces the binding of [^{3}H]-α,β-MeATP to rat bladder with a potency nearly equal to that of ATP (Bo & Burnstock, 1993a). In the present study a similar effect was observed in the displacement experiment with sodium tetraphosphate. Sodium metatriphosphate, a cyclic structure of triphosphate, was much less potent than triphosphate in

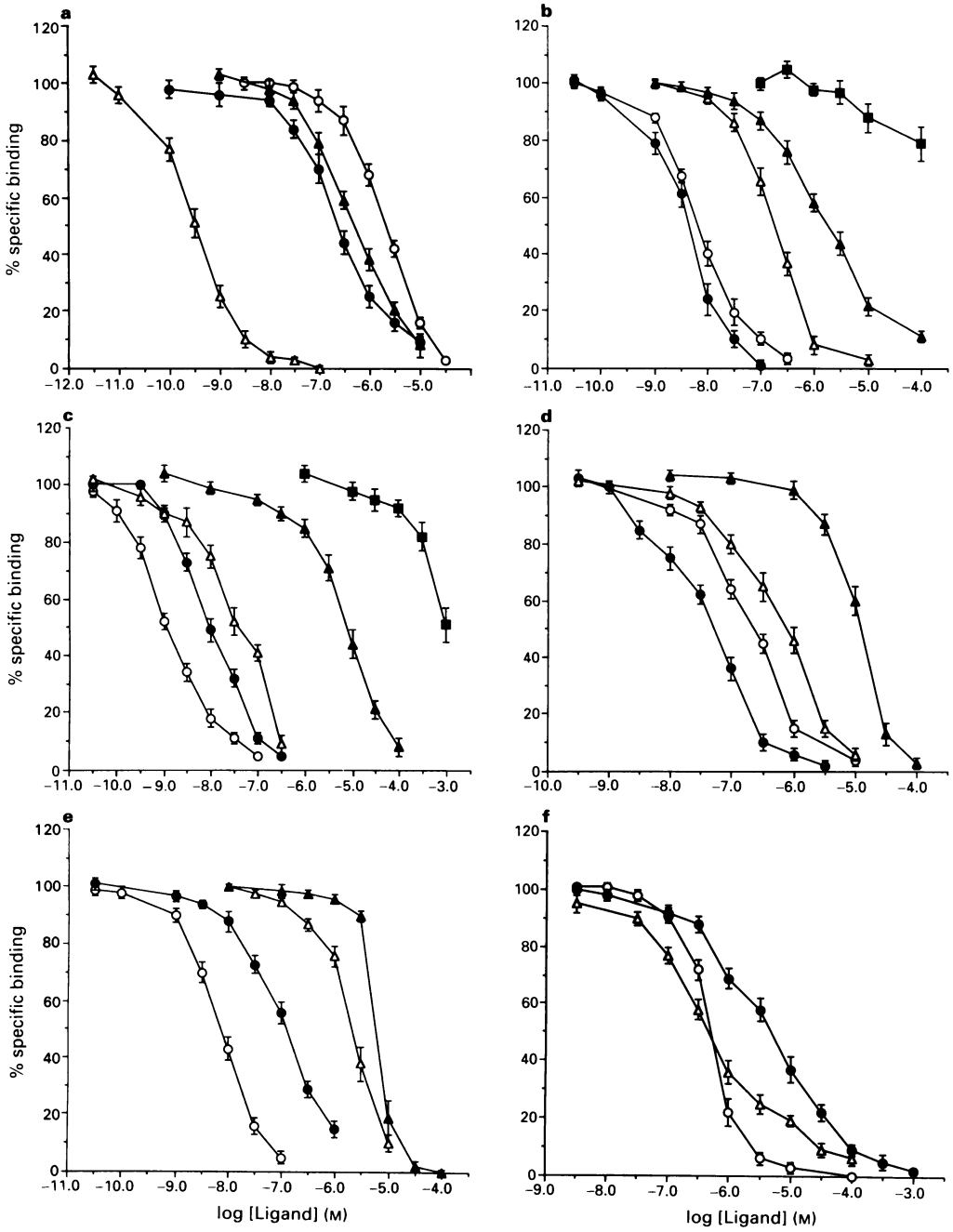


Figure 2 Displacement of [^{3}H]-α,β-methylene ATP binding to rat urinary bladder membranes by ATP derivatives and the P_2-purinoceptor antagonists. Rat urinary bladder membrane preparations were incubated with about 10 nM [^{3}H]-α,β-methylene ATP in the presence of the unlabelled ligand at 4°C for 2 h. (a) (○) ATP, (●) ADPβS, (▲) ATPαS, (△) ATPγS; (b) (○) α,β-MeATP. (●) β,γ-imidoATP, (△) β,γ-MeATP, (▲) L-β,γ-MeATP, (■) α,β-MeADP; (c) (○) Ap6A, (●) Ap5A, (△) Ap4A, (▲) Ap3A, (■) Ap2A; (d) (○) 2-MeSATP, (●) 2-CyaHeSATP, (△) 2-HexS-N^6-MeATP, (▲) 1,N^6-E-ATP; (e) (○) 3'dBAATP, (●) 3'BH-NH-ATP, (△) 3'd-ATP, (▲) 2'd-ATP; (f) (○) RB-2, (●) PPADS, (△) suramin. The points are the means with s.e.mean of five triplicated experiments.

displacing [^{3}H]-α,β-MeATP binding, suggesting that the triphosphate group in ATP molecule has to be a chain structure.

α,ω-Adenine dinucleotide polyphosphates have been found to exist in many mammalian tissues like hepatocytes (Rapaport & Zamecnik, 1976), adrenal medullary chromaffin granules (Rodriguez-del-Castillo *et al.*, 1988), and platelets (Lüthje & Ogilvie, 1983), which can be released upon stimulation. Their physiological function is unclear at present. Possible roles as neurotransmitters, cotransmitters, or trophic factors have been suggested (Hoyle, 1990). In pharmacological experiments these compounds have been reported to activate both P_{2X}- and P_{2Y}-purinoceptors (Stone, 1981; Busse *et al.*, 1988; Busshardt *et al.*, 1989; Stone & Paton, 1989; Hoyle *et al.*, 1989; Pintor *et al.*, 1991). The binding results from the present study demonstrated a clear order of affinities associated with the length of the polyphosphate chain linking the two adenosine moieties: Ap6A > Ap5A > Ap4A >> Ap3A >> Ap2A. This is in agreement with the results from pharmacological experiments which showed that Ap6A and Ap5A are as potent as α,β-MeATP in eliciting constrictions of guinea-pig vas deferens (MacKenzie *et al.*, 1988) and human urinary bladder (Hoyle *et al.*, 1989), and more potent than β,γ-MeATP in guinea-pig

urinary bladder (Stone, 1981). Therefore, it is possible that diadenine nucleotides may have a role as endogenous ligands for P_{2X}-purinoceptors as well as for their own specific receptors.

ATP derivatives with various modifications on the purine base have also been tested pharmacologically. These compounds, like N^6-phenyl ATP, N^6-MeATP, 8-BrATP, 8-azidoATP, N-O-ATP, 1,N^6-E-ATP, and formacyn-ATP, usually have diminished efficacy at P_{2X}-purinoceptors (Fedan *et al.*, 1982; 1986; Welford *et al.*, 1987; Howson *et al.*, 1988). In this study, N-O-ATP, 8-BrATP, and N^6-MeATP showed lower affinities for P_{2X}-purinoceptors than ATP, and their displacement curves were usually steep (with Hill coefficients significantly higher than unity except 8-BrATP), which suggests that their interaction with P_{2X}-purinoceptors may be different from those typical P_{2X}-purinoceptor agonists like α,β-MeATP and β,γ-MeATP. AAHeATP is an exception in this group of compounds. It did not show any agonist activity at the P_{2X}-purinoceptors in guinea-pig bladder and vas deferens, and rabbit saphenous artery (Burnstock *et al.*, 1994), yet its potency was 5 times higher than that of ATP. It is possible that this compound possesses some antagonistic property at P_{2X}-purinoceptors.

So far the only position on the purine base where modification can produce potent P_2-purinoceptor agonists is the C-2. 2-MeSATP has been known for a long time to be a potent P_{2Y}-purinoceptor agonist (Gough *et al.*, 1973; Satchell & Maguire, 1975; Burnstock *et al.*, 1983) although it can also activate P_{2X}-purinoceptors at a higher concentration (Howson *et al.*, 1988; Theobald, 1992). Further modifications on the C-2 position produced 2-HeSATP, 2-CyaHeSATP, 2-phenylethylthio ATP, and 2-(2-*p*-nitrophenylethyl)thio ATP, which did not show significantly increased efficacy on P_{2Y}-purinoceptors, while the potency on P_{2X}-purinoceptors have been greatly enhanced (Fischer *et al.*, 1993). Binding results from the present experiments showed that both 2-HeSATP and 2-CyaHeSATP had significantly higher potencies than 2-MeSATP ($P<0.01$). Cyclization of the hexyl group (2-cHeSATP) reduced the affinity of 2-HeSATP slightly. Addition of a methyl group at the N^6-position of 2-HexSATP (2-HexS-N^6-MeATP) reduced its affinity for P_{2X}-purinoceptors by 28 times. Pharmacological experiments showed that this compound had a higher selectivity for P_{2Y}-purinoceptors than 2-HexSATP (Fischer *et al.*, 1993). Taken together the observation that N^6-MeATP was selective for P_{2Y}-purinoceptors in guinea-pig taenia coli and inactive at P_{2X}-purinoceptors, suggested that the combination of the N^6 and C-2 modification may produce more selective and more potent agonists for P_{2Y}-purinoceptors (Fischer *et al.*, 1993).

5-F-UTP, like UTP ($pIC_{50}=4.67$, Bo & Burnstock, 1993a), was not very potent in displacing the [^{3}H]-α,β-MeATP binding to P_{2X}-purinoceptors in rat bladder. However, in pharmacological experiments, it was very potent in inducing contraction of guinea-pig bladder detrusor (Burnstock *et al.*, 1994). Such response to 5-F-UTP may be mediated by the putatively termed P_{2U}-purinoceptors or 'pyrimidinoceptor' (Seifert & Schultz, 1989).

Modification on the ribose of ATP molecule also produced some compounds which displayed different potencies at P_{2X}-purinoceptors. Early pharmacological studies showed that the removal of the 2'-hydroxyl group of ATP led to a reduced potency for P_{2X}-purinoceptors, while removal of the 3'-hydroxyl group led to a slight increase in potency (Fedan *et al.*, 1982; 1986; Howson *et al.*, 1988). Such observations have been confirmed by the present experiments, showing 3'd-ATP had a potency about 4 fold higher than that of 2'd-ATP. Attachment of a (4-hydroxyphenylpropionyl)amino group at the C-3' position of 3'd-ATP (3'BH-NH-ATP) significantly enhanced the potency of 3'd-ATP in displacing the [^{3}H]-α,β-MeATP binding to P_{2X}-purinoceptors. Another 3'd-ATP derivative which has a benzylamino group attached to the C'-3 position (3'dBAATP) was shown to be very potent in activating P_{2X}-purinoceptors in guinea-pig vas deferens and urinary bladder, while it was devoid of efficacy for P_{2Y}-purinoceptors (Burnstock *et al.*, 1994). Displacement experiments showed this compound had a very high affinity for P_{2X}-purinoceptors. Therefore, the replacement of the 3'-hydroxyl group with other specific groups does open a new area to search for more selective and more potent P_{2X}-purinoceptor agonists.

The development of specific antagonists for P_2-purinoceptors has not been so fruitful. For many years RB-2 has been used as an antagonist for P_{2Y}-purinoceptors (Kerr & Krantis, 1979) and suramin for P_{2X}-purinoceptors (Dunn & Blakeley, 1988). It was later found that their selectivity for either P_{2Y}- or P_{2X}-purinoceptor is not high (Choo, 1980; Fedan & Lamport, 1990; Hoyle *et al.*, 1990). PPADS has recently been reported as an antagonist for P_{2X}-purinoceptors (Lambrecht *et al.*, 1992). In this study, all three compounds could competitively displace the [^{3}H]-α,β-MeATP binding to rat bladder membranes (Figure 2f), however, the Hill coefficient of the RB-2 displacement curve was much higher than those of suramin and PPADS. The reason for such differences is unclear at present. It should be mentioned that in another similar experiment carried out on rabbit urinary bladder membranes PPADS was shown to interact with two affinity states of [^{3}H]-α,β-MeATP binding sites with K_i values of 22 nM and 48 μM, respectively (Ziganshin *et al.*, 1993), while in rat bladder membranes the one binding site model was preferred, which indicates that the properties of [^{3}H]-α,β-MeATP binding sites in rabbit and rat urinary bladder are somewhat different.

In this study, the order of potency of most of the ligands tested is in agreement with that obtained from pharmacological experiments. However, one question that may be raised is why the K_i values of many potent P_{2X}-purinoceptor agonists are in the nanomolar range, while their EC_{50} values in eliciting contractile responses in smooth muscle are in the micromolar range? Ideally, the pharmacology of the binding should correlate quantitatively with the pharmacology of receptor-mediated effects. In practice, such quantitative correlation is difficult to achieve, especially when agonists are used as radioligands (Ehlert *et al.*, 1984; Burt, 1985), because of some unknown sequence of events between agonist receptor occupation and measured pharmacological responses. A pharmacological response depends on transmitter-receptor-effector coupling, while the binding assay only deals with transmitter-receptor interaction. The influence of environmental factors also have important implications in the interpretation of agonist binding data. Pharmacological experiments on the P_{2X}-purinoceptor-mediated responses were carried out on intact tissues or cells in the presence of divalent cations at 37°C, while the binding assays were performed on separated membranes in Ca^{2+}-free media in the presence of EGTA at 4°C. For many P_{2X}-purinoceptor-active agonists, the situation is more complicated because accurate EC_{50} values are difficult to obtain due to their ability to desensitize the receptor. A proportion of P_{2X}-purinoceptors would be turned into an inactive state after exposure to these agonists, while the pharmacological responses can only be displayed by the receptors in their active state. The rapid degradation of some of the agonists by the ectonucleotidases abundantly present on the cell membranes may partially account for their low efficacy in activating P_{2X}-purinoceptors.

In conclusion, modifications on the triphosphate chain, adenine and ribose of ATP molecules can significantly change the affinity of ATP for P_{2X}-purinoceptors in rat urinary bladder. The replacement of the bridging oxygen in the triphosphate chain with a methylene or imido group markedly increased the affinity and selectivity, and the replacement of an ionized oxygen on the γ-phosphate with a sulphur also leads to increase of affinity. Modifications at N^6, N^1, and C-8 positions on the purine base usually reduces the affinity of ATP, while the attachment of an alkylthio group to the C-2 position greatly increases the affinity for P_{2X}-purinoceptors. Replacement of 3'-hydroxyl group on the

ribose with some specific groups produces more potent P_{2X}-purinoceptor agonists. Suramin, PPADS, and RB-2 can competitively displace the binding of [^{3}H]-α,β-MeATP for P_{2X}-purinoceptors, but their affinities are moderate.

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Muscarinic acetylcholine response in pyramidal neurones of rat cerebral cortex

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1 The effects of acetylcholine (ACh) on pyramidal neurones acutely dissociated from the rat cerebral cortex were studied in the whole-cell mode, by use of the nystatin-perforated patch recording configuration.

2 ACh induced a net inward current (I_{ACh}) accompanied by a membrane conductance decrease at a holding potential (V_H) of -40 mV. I_{ACh} increased in a concentration-dependent manner with a half-maximum concentration (EC_{50}) of 8.7×10^{-7} M.

3 I_{ACh} mainly resulted from the suppression of the voltage- and time-dependent K^+ current (M-current).

4 Muscarine and muscarinic agonists such as McN-A-343, oxotremorine and oxotremorine-M mimicked the ACh response. The potency was in the order of oxotremorine-M > McN-A-343 ≥ muscarine > oxotremorine.

5 Pirenzepine shifted the concentration-response curve for ACh to the right and the corresponding Schild plot yielded a pA_2 value of 7.81. Other muscarinic antagonists also reversibly blocked I_{ACh} in a concentration-dependent manner. The inhibitory potency was in the order of atropine > 4-DAMP > pirenzepine > AF-DX-116.

6 I_{ACh} could be induced normally even after pre-incubation of dissociated neurones in external solution with 200 ng ml^{-1} pertussis toxin (PTX) for 8 h, whereas the inhibitory effect of ACh on high-voltage-activated Ca^{2+} channels was completely abolished by the PTX treatment.

Keywords: Rat cortical pyramidal neurone; muscarinic acetylcholine receptor; M-current

Introduction

The cholinergic innervation to the cerebral cortex has intrigued investigators for many years because of its possible roles in higher brain functions such as arousal, memory and learning (Squire & Davis, 1981). Interest in the cortical cholinergic system has been stimulated further by the fact that there are deficits of this system in patients with Alzheimer's disease (Coyle *et al.*, 1983). One major step toward understanding this system was the study of the muscarinic acetylcholine receptors in the central nervous system (McKinney & Richelson, 1984). The activation of muscarinic receptors has been shown to evoke a wide variety of responses, suggesting that multiple subtypes might exist. Molecular biological studies have confirmed the existence of at least five distinct subtypes (m1–m5) of muscarinic receptor genes (Bonner, 1989). The m1–m4 genes correspond most closely to the pharmacologically defined M_1–M_4 subtypes, respectively (Hulme *et al.*, 1990; Lazareno *et al.*, 1990).

Many biochemical and electrophysiological studies have addressed the ACh response in the mammalian cortex. In the rat cortex, the activation of muscarinic receptors stimulates phosphoinositide hydrolysis (Downes, 1982) and evokes a synaptic inhibition and a slow excitation (Krnjevic *et al.*, 1971; Krnjevic, 1974). In the guinea-pig cortex, the muscarinic receptor with high affinity for pirenzepine depolarizes the pyramidal neurones, while that with low affinity for pirenzepine is involved in the activation of inhibitory interneurones (McCormick & Prince, 1985). The ACh-induced depolarization of pyramidal neurones is mainly due to a decrease in a voltage-dependent K^+ current known as the M-current (I_M; Brown & Adams, 1980). However, the muscarinic receptor subtypes and the pharmacological properties involved in these responses have not been fully elucidated in this brain area.

In the present study, therefore, we investigated quantitatively the ACh-induced response and its pharmacological properties in mammalian cortical neurones under voltage-clamp conditions. For this purpose we used pyramidal neurones acutely dissociated from the rat cerebral cortex. The nystatin perforated patch recording configuration was used to maintain, intact, the intracellular second messenger systems.

Methods

Preparation

Single pyramidal neurones of the cerebral cortex were acutely dissociated as reported previously (Ito *et al.*, 1991). Briefly, 1- to 2-week-old Wistar rats were decapitated under deep ether anaesthesia. The area between 2 and 4 mm from the anterior tip of the frontal lobe was cut into coronal slices (350 μm thick) with a microslicer (D.S.K., Model DTK-1000), and pre-incubated in incubation solution saturated with 95% O_2:5% CO_2 for 40 min at room temperature (22–25°C). Thereafter, the slices were treated with enzymes. The enzyme treatments were made first in an oxygenated standard external solution with 0.015% pronase for 15 min at 31°C and successively in 0.015% thermolysin under the same conditions. The slice preparations were then rinsed with Ca^{2+}-free EGTA external solution and incubated for another 15 min in standard solution. The dorsomedial portion of the cortex was micro-punched out and dissociated mechanically with fine fire-polished glass Pasteur pipettes in a small plastic culture dish (Falcon) filled with the standard solution. Dissociated neurones adhered to the bottom of the dish within 30 min, allowing electrophysiological studies to be conducted. The neurones having the original morphological features of pyramidal neurones such as the pyramidal somatic shape and the prominent apical dendritic process were used.

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Electrical measurement

Electrical recordings were performed by use of the nystatin perforated patch recording with some modifications (Wakamori *et al.*, 1993). Patch pipettes were fabricated from glass capillaries (Narishige, 1.5 mm outer diameter) on a two-state puller (Narishige, PB-7). The resistance between the patch-pipette filled with the internal soluton and the reference electrode was 4 to 8 MΩ. After formation of the stable perforated patch, the series resistance ranged from 16 to 20 MΩ. Ionic currents and voltages were measured with a patch-clamp amplifier (List Electronic, EPC-7). Signals were filtered with a low-pass filter (NF Electronic Instruments, FV-665) at a cut off frequency of 1 kHz and were monitored simultaneously on a storage oscilloscope (Iwatsu, MS-5100A) and a pen-recorder (San-ei, RECTI HORIZ 8K). Records were stored on a video cassette recording system (Mitsubishi, HV-F93) via a pulse coded modulation processor (SONY, PCM 501) for subsequent analysis using the pCLAMP system (Axon Instruments). All the experiments were performed at room temperature (22–25°C).

Statistical analysis

The data are presented as the mean ± standard error of the mean (s.e.mean) in the text, and the s.e.mean is indicated by a vertical bar in figures.

The continuous theoretical curves for concentration-response relationships were constructed according to a modified Michaelis-Menten equation (1) using a least-squares fitting routine (Kijima & Kijima, 1980):

$$I = I_{max} \frac{C^n}{C^n + EC_{50}{}^n} \qquad (1)$$

where I is the drug-induced current amplitude and C is the corresponding drug concentration. EC_{50} and n denotes the half-maximum concentration and Hill slope, respectively. The equation for the concentration-inhibition curve is the mirror image of the Michaelis-Menten equation:

$$I = 1 - \frac{C^n}{C^n + IC_{50}{}^n} \qquad (2)$$

where I is the current amplitude normalized by that of the control and IC_{50} denotes the half-inhibition concentration of antagonists.

Solutions and their application

The ionic composition of the standard external solution was (in mM): NaCl 150, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid (HEPES) 10 and glucose 10. The pH was adjusted to 7.4 with tris (hydroxymethyl) aminomethane base (Tris-base). The ionic composition of the incubation medium was (in mM): NaCl 125, KCl 5, KH_2PO_4 1.2, $MgSO_4$ 1.3, $CaCl_2$ 2.4, $NaHCO_3$ 26 and glucose 10, aerated with 95% O_2:5% CO_2 gas to a final pH of 7.4. The ionic composition of the external solution with 10 mM Ba^{2+} was (in mM): NaCl 138, CsCl 5, $MgCl_2$ 1, $BaCl_2$ 10, HEPES 10, glucose 10 and tetrodotoxin 0.001. The pH was adjusted to 7.4 with Tris-base. The ionic composition of the pipette solution was (in mM): KCl 150 and HEPES 10 for recording the ACh-induced current, and CsCl 150 and HEPES 10 for recording the high-voltage activated Ba^{2+} current (HVA I_{Ba}). The pH was adjusted to 7.2 with Tris-base. A fresh stock solution of 10 mg ml^{-1} nystatin in methanol was prepared every day and kept at − 20°C. This was then dissolved into the pipette solution to produce a final nystatin concentration of 150 μg ml^{-1}. The final pipette solution was made fresh every 2 or 3 h.

Drugs were applied by a rapid application system termed the 'Y-tube' method, as described elsewhere (Murase *et al.*, 1990). With the use of this technique, the solution surrounding a dissociated neurone could be completely exchanged within 10 to 20 ms.

Drugs

The drugs used in the present experiments were acetylcholine chloride (ACh), muscarine chloride, nicotine, nystatin and thermolysin (Sigma), tetrodotoxin (TTX) (Sankyo), atropine sulphate (Merck), pertussis toxin (PTX) (Seikagaku Kougyo), pronase (Calbiochem), oxotremorine-M, 4-diphenylacetoxy-N-methylpiperidine (4-DAMP) methiodide and 4-(m-chlorophenylcarbamoyloxy)2-butynyltrimethylammonium chloride (McN-A-343) (RBI). 11-2[[2-[(dietylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepine-6-one (AF-DX-116) was kindly given by Prof. T. Kamiya (Fukuoka University, Japan). All drugs were dissolved in external solution just before use.

Results

ACh-induced currents

In the perforated patch recording configuration, 10^{-5} M ACh evoked a net inward current (I_{ACh}) associated with a 30 to 60% decrease in membrane conductance in 75 out of 110 cortical pyramidal neurones at a holding potential (V_H) of − 40 mV (Figure 1a, upper panel). The inward currents were long-lasting and typically persisted for 4 to 5 min after the application of ACh for 30 to 40 s. The lower panel in Figure 1a illustrates the effect of ACh on currents evoked by 300 ms hyperpolarizing step pulses from − 40 to − 50 mV. The step pulse represented a leak current plus the M-current (I_M; Brown & Adams, 1980; Constanti & Brown, 1981) before the application of ACh. During the steady-state of I_{ACh}, the step pulse represented the deactivation of I_M. The I_M was reactivated by the removal of ACh. A small transient outward current was observed in 35 out of 110 neurones (31.8%), which was followed by a slow inward current (Figure 1b).

During the perforated patch whole-cell recording, the application of ACh at 10 min intervals could elicit I_{ACh} with stable current amplitudes over 1 h (Figure 1c(i)), whereas the conventional whole-cell recording (Akaike *et al.*, 1978; Hamill *et al.*, 1981) caused a considerable 'run-down' of I_{ACh} within 15 min after rupture of the membrane patch (Figure 1c(ii)). It is known that I_M in rat sympathetic ganglion cells slowly declines over a period of 15 min in a conventional whole-cell patch recording (Brown *et al.*, 1989). ATP added to the pipette solution can reduce the run-down (Pfaffinger, 1988). However, this may alter the responsiveness for ACh since the muscarinic ACh response is mediated by multiple steps of the intracellular signal transduction system. In the present study, therefore, the nystatin perforated patch recording configuration was used in subsequent experiments to prevent wash-out of the ACh response.

Current-voltage (I-V) relationship for ACh response

The concentrations of K^+ in the external and internal (pipette) solutions were 5 and 150 mM, respectively. The voltage-dependent Ca^{2+} and Na^+ channels were suppressed by adding 10^{-5} M La^{3+} and 10^{-6} M tetrodotoxin, respectively. Figure 2a illustrates the inward currents evoked by 10^{-5} M ACh at various V_Hs. As shown in Figure 2a and summarized in Figure 2b, the I_{ACh} had distinct voltage-dependency. The I_{ACh} increased as the membrane depolarized and was not observed at potentials more negative than − 80 mV without showing any reversal of current direction, being consistent with that of I_M. Figure 2c shows the current-voltage (*I-V*) relationships to asymmetrical hyperpolarizing ramp voltage between − 20 and − 100 mV from a V_H of − 40 mV, before (control) and during the application of ACh. In this case, the *I-V* property was also identical to that of I_M.

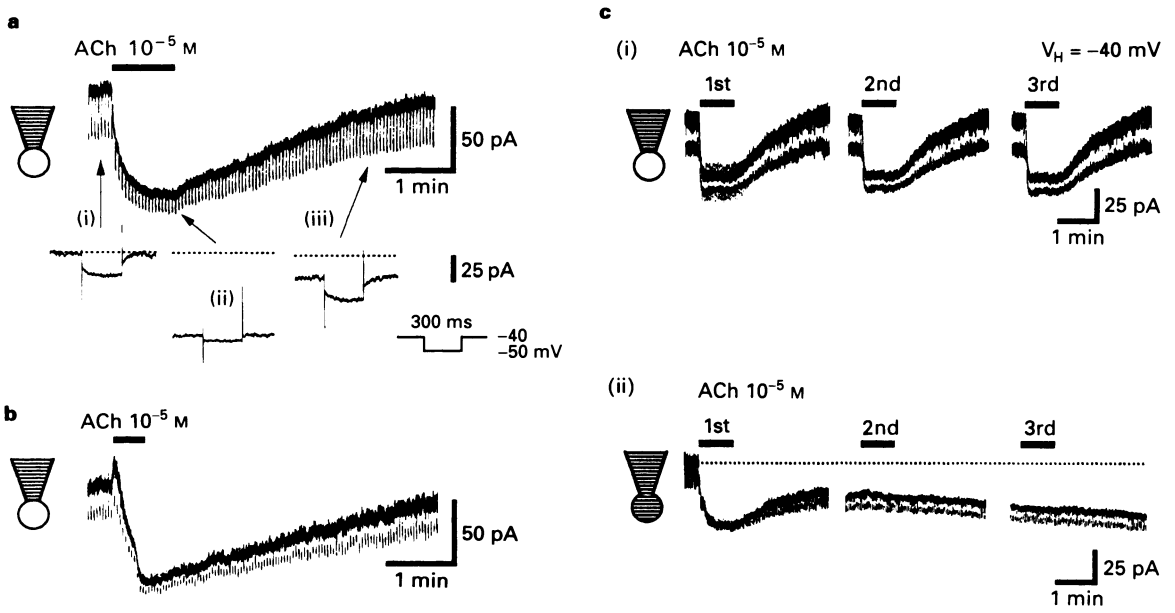


Figure 1 Effects of ACh (10^{-5} M) on the pyramidal neurones freshly dissociated from the rat cerebral cortex. (a) ACh-induced inward current (I_{ACh}) recorded by the nystatin perforated patch technique. The neurone was held at a holding potential (V_H) of − 40 mV and a brief hyperpolarizing pulse of 10 mV with 300 ms duration was applied every 2 s. ACh was applied during the period indicated by the horizontal bar above each current recording. Inward current relaxations produced by a hyperpolarizing voltage command from − 40 to − 50 mV, before, during and after the application of 10^{-5} M ACh are expanded for detail and shown in the lower panel of (a). (b) ACh elicited a small transient outward current followed by a long-lasting inward current in 31.8% of neurones tested. (c) (i) In nystatin perforated patch mode, I_{ACh} was constantly recorded by successive applications of ACh. (c) (ii) I_{ACh} recorded in the conventional whole-cell mode at a V_H of − 40 mV. The I_{ACh} showed run-down with time. Recordings (i) and (ii) were obtained from different neurones.

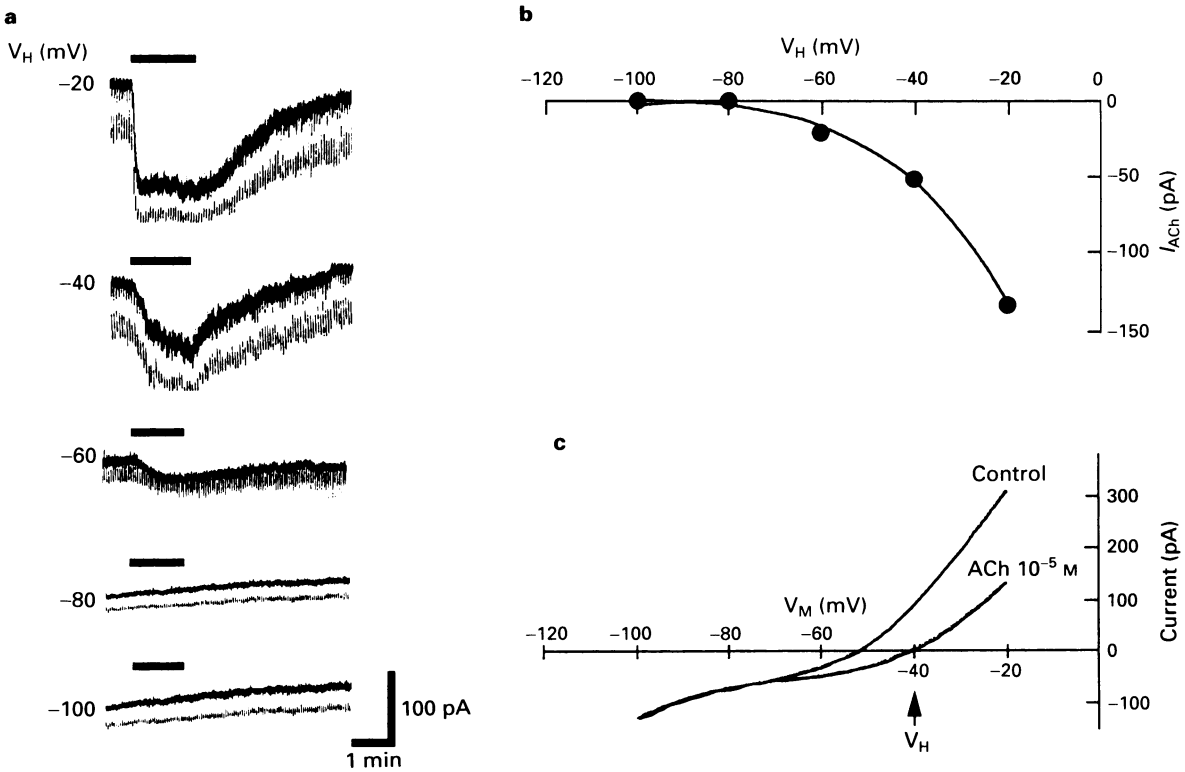


Figure 2 ACh response and its current-voltage (*I-V*) relationship. (a) ACh-induced currents at various V_Hs. Horizontal bars indicate the period of application of 10^{-5} M ACh. (b) *I-V* relationship of the I_{ACh} at various V_Hs. The data were obtained from (a). Similar results were obtained from five cells. (c) *I-V* relationships obtained by ramp-commands between − 20 and − 100 mV in 2 s in the presence or absence of 10^{-5} M ACh. V_M; membrane potential. Similar results were obtained from six cells.

Concentration-response relationships for ACh and its agonists

Figure 3a(i) and (ii) show typical inward currents induced by ACh and muscarine of various concentrations at a V_H of -40 mV, respectively. Nicotine evoked no response at concentrations up to 10^{-3} M. Figure 3b summarizes the concentration-response relationships for ACh and its agonists. The ACh and muscarine responses increased in a concentration-dependent manner. Muscarine was approximately 10 times less potent than ACh. The half maximum concentration (EC_{50}) of ACh in the concentration-response curve was 8.7×10^{-7} M. The Hill coefficient was 0.9. The EC_{50} value and Hill coefficient of the muscarine response were 8.6×10^{-6} M and 0.8, respectively. These results suggest that the ACh response is mediated by the muscarinic ACh receptors. Therefore, the effects of muscarinic agonists such as McN-A-343, oxotremorine and oxotremorine-M were tested in the cortical pyramidal neurones. Figure 3a(iii) shows the inward currents evoked by McN-A-343 in a concentration-dependent manner. Figure 3b also summarizes the concentration-response relationships for McN-A-343 and two other muscarinic agonists. The EC_{50} of McN-A-343 was 4.5×10^{-6} M. Oxotremorine, a cardiac M_2 agonist (Brann *et al.*, 1987), was about 30 times less potent than muscarine. The order of potency was ACh > oxotremorine-M > McN-A-343 $\geqslant$ muscarine > oxotremorine.

Effects of muscarinic antagonists

The effects of muscarinic antagonists (atropine, pirenzepine, 4-DAMP and AF-DX-116) on the I_{ACh} were examined. Pirenzepine shifted the concentration-response curve for ACh to the right. Figure 4a(i) shows the concentration-response relationships for ACh obtained under control condition (●), and in the presence of 10^{-7} (▲), 3×10^{-7} (◆) and 10^{-6} M (■) pirenzepine ($n = 4-5$ in each case), with EC_{50} values of 8.49×10^{-7}, 7.17×10^{-6}, 1.64×10^{-5} and 7.64×10^{-5} M, respectively. Figure 4a(ii) illustrates the corresponding Schild plot (Arunlakshana & Schild, 1959). The estimated pA_2 value was 7.81. Figure 4b summarizes the concentration-inhibition curves of various antagonists on I_{ACh} elicited by 10^{-5} M ACh. Atropine, pirenzepine, 4-DAMP and AF-DX-116 reversibly blocked I_{ACh} in a concentration-dependent manner. The half-inhibition concentrations (IC_{50}) were 2.49×10^{-9} M for atropine, 1.18×10^{-8} M for 4-DAMP, 2.07×10^{-7} M for pirenzepine and 3.35×10^{-6} M for AF-DX-116.

Effect of pertussis toxin (PTX)

It is well established that muscarinic receptors are linked to G-proteins (Bonner, 1989). To investigate what kind of G-proteins may mediate the I_{ACh}, the dissociated cortical neurones were incubated in standard external solution with or without PTX 200 ng ml^{-1} for 8 h, because PTX is able to ADP-ribosylate the α-subunit of G_i and G_o proteins, resulting in the inhibition of their activation by the receptors (Kurose *et al.*, 1983; Murayama & Ui, 1983). The PTX-treated and non-treated neurones were voltage-clamped at a V_H of -40 mV and tested with 10^{-5} M ACh. The amplitude and response pattern of I_{ACh} in the PTX-treated neurones did not differ from those of the control without PTX treatment (Figure 5a, $n = 8$). To ensure that PTX was effective, the effect of ACh on the high-voltage activated Ba^{2+} inward

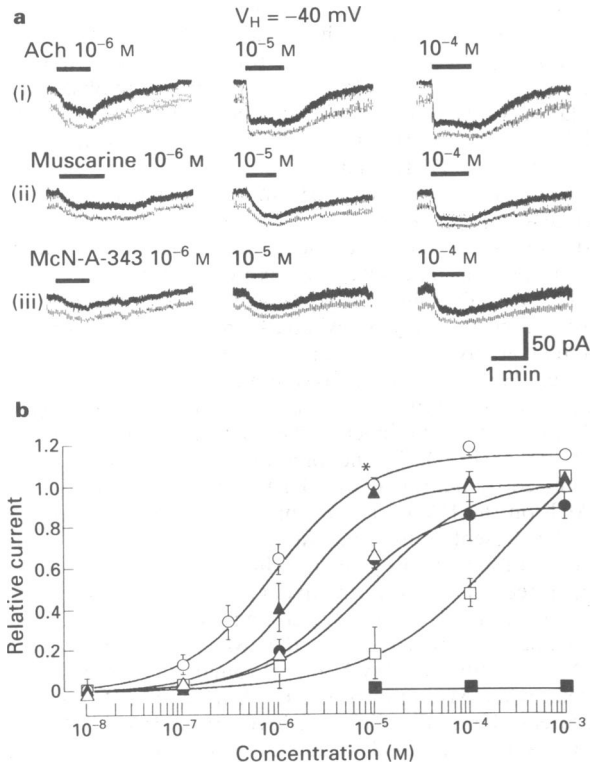


Figure 3 Concentration-response relationships for ACh agonists. (a) The inward currents induced by ACh, muscarine and McN-A-343 of various concentrations at a V_H of -40 mV. The neurones were exposed to each agonist for the period indicated by a horizontal bar. (b) Concentration-response relationships for the peak inward current induced by ACh (○), muscarine (△), McN-A-343 (●), oxotremorine (□) and oxotremorine-M (▲). Nicotine (■) elicited no response. All currents were normalized to the peak current amplitude induced by 10^{-5} M (*). Each point represents the mean (± s.e.mean) of six to eight neurones.

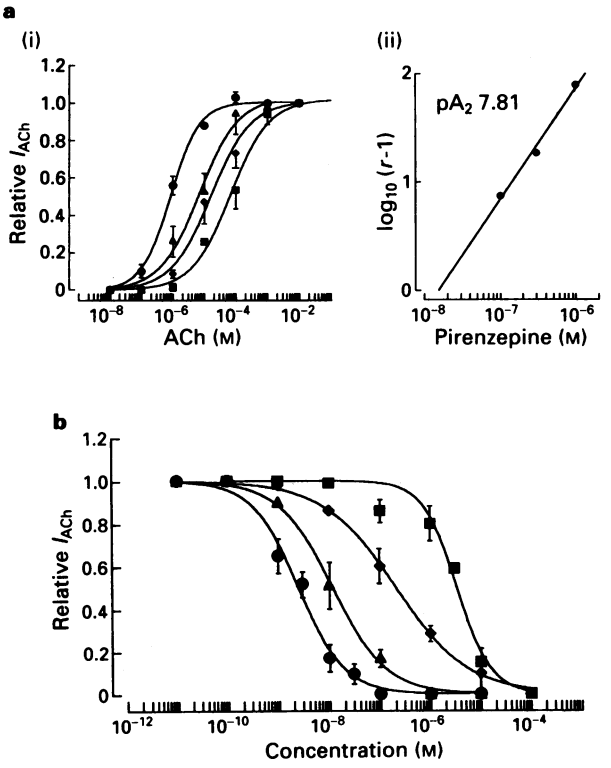


Figure 4 Effects of muscarinic antagonists on I_{ACh}. (a) (i) Shift of the concentration-response curves for ACh (●) by 10^{-7} (▲), 3×10^{-7} (◆) and 10^{-6} M (■) pirenzepine. (ii) Corresponding Schild plot where r is the agonist concentration ratio. The line was fitted by linear regression and yielded a pA_2 for pirenzepine of 7.81 with a unconstrained slope of 1.07 ($R^2 = 0.99$). (b) Concentration-dependent inhibition curves of I_{ACh} in the presence of atropine (●), 4-DAMP (▲), pirenzepine (◆) and AF-DX-116 (■). Neurones were pre-treated for 2 min with each antagonist. Each symbol represents the mean (± s.e.mean) of five to seven neurones.

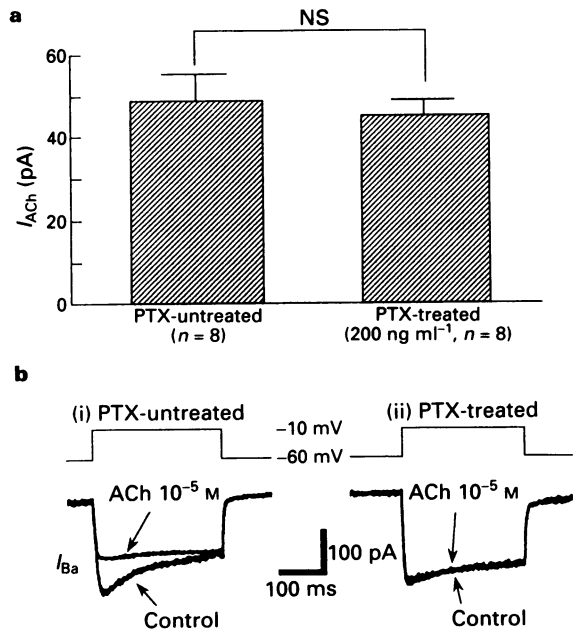


Figure 5 Effects of pertussis toxin (PTX) on I_{ACh} and HVA I_{Ba}. (a) Dissociated neurones were immersed in external solution with or without PTX for 8 h. NS: no significant difference. *n*: the number of neurones tested. (b) HVA I_{Ba} was evoked by a 300 ms step pulse to − 10 mV from a V_H of − 60 mV. In these experiments, neurones were pretreated with ACh for at least 2 min. (b) (i) ACh 10^{-5} M suppressed HVA I_{Ba} in neurones without PTX treatment. (ii) ACh had no effect on HVA I_{Ba} in neurones treated with 200 ng ml^{-1} PTX for 8 h ($n = 12$).

current (HVA, I_{Ba}) passing through the Ca^{2+} channel was simultaneously examined in K^+-free Cs^+ external solution containing 10 mM-Ba^{2+} in the presence or absence of PTX (Figure 5b). The HVA I_{Ba} was induced by a 300 ms depolarizing command step to − 10 mV from a V_H of − 60 mV, at which potential the low-voltage activated Ca^{2+} channel was inactivated (Akaike *et al.*, 1989). Here, 10^{-5} M ACh suppressed the activation phase of I_{Ba} in control neurones 8 h after the dissociation ($n = 8$), whereas ACh had no effect on I_{Ba} in neurones treated with PTX for 8 h ($n = 12$).

Discussion

In the present study, ACh induced a long-lasting net inward current (I_{ACh}) in rat cortical pyramidal neurones. This inward current resulted from the decrease in a voltage- and time-dependent K^+ conductance known as the M-current (I_M; Brown & Adams, 1980). This reduction of I_M is widely observed in various neurones such as neurones in the sympathetic ganglia (Brown & Adams, 1980; Constanti & Brown, 1981), the hippocampus (Benardo & Prince, 1982; Halliwell & Adams, 1982), the cerebral cortex (McCormick & Prince, 1985; Constanti & Sim, 1987) and the amygdala (Womble & Moises, 1992). The inhibition of I_M by ACh in these neurones may greatly enhance the neuronal excitability, thereby regulating functions of neuronal systems.

Molecular cloning studies have identified five genes for muscarinic ACh receptors denoted as m 1–m 5 (Bonner *et al.*, 1988) and the receptors expressed for m 1–m 4 may correspond pharmacologically to M_1–M_4 (Hulme *et al.*, 1990). Although muscarinic receptors cannot be differentiated exclusively with the agonists and antagonists, the receptors do show different sensitivity to these drugs. In the present study, pirenzepine effectively inhibited I_{ACh}, and the pA_2 value for pirenzepine was 7.81. This high affinity of pirenzepine is closer to those of M_1 and M_4 than that of M_2, M_3 and the receptor expressed for m 5. Atropine, 4-DAMP and AF-DX-116 also suppressed I_{ACh}. The sensitivity of I_{ACh} to these antagonists was in the order of atropine > 4-DAMP > pirenzepine > AF-DX-116, whereas AF-DX-116 has higher affinity for M_2 than pirenzepine (Micheletti *et al.*, 1987; Buckley *et al.*, 1989). Oxotremorine, the cardiac M_2 agonist, could induce inward current only at high concentrations. Thus, M_2 was not involved in the present response. McN-A-343 is functionally potent in stimulating the M_1 receptor and has less activity at M_2 and M_3, though this compound displays little selectivity for muscarinic receptors in the binding assay (Birdsall *et al.*, 1983; Freedman *et al.*, 1988; Lambrecht *et al.*, 1993). McN-A-343 mimicked the ACh response in the present preparation, while this agonist could not activate M_3 in CA1 pyramidal neurones (Wakamori *et al.*, 1993). Therefore, the candidate subtypes responsible for the I_{ACh} may be M_1 or M_4. In transfected NG108-15 cells, expressed receptors for m 1 and m 3 are capable of inhibiting I_M, while m 2 and m 4 are not (Fukuda *et al.*, 1988; Robbins *et al.*, 1991). In rat sympathetic ganglion cells M_1 also could couple to I_M but M_4 could not (Marrion *et al.*, 1989; Bernheim *et al.*, 1992). In addition, M_4 is known to link to PTX-sensitive G-protein, while the present response was PTX-insensitive. Taking these facts into account, the M_1 subtype may be predominantly responsible for the reduction of I_M in rat cortical pyramidal neurones. Immunocytochemistry (Levey *et al.*, 1991; Wall *et al.*, 1991) and *in situ* hybridization studies (Buckley *et al.*, 1988) showed the presence of multiple subtypes of muscarinic ACh receptors in the rat neocortex. The m 1 subtype, most abundant in this brain area, may regulate I_M. The m 2 protein was also recognized by immunocytochemistry, whereas mRNA for the m 2 gene is low. This is consistent with the suggestion that this subtype possibly functions as an autoreceptor at the ends of cholinergic fibres projected from the basal forebrain (Levey *et al.*, 1991). The proteins and mRNA for m 3 and m 4 were also detected. Although the M_3 receptor can be involved in the regulation of I_M (Fukuda *et al.*, 1988; Robbins *et al.*, 1991) and M_4 can be coupled to the high-voltage-activated (HVA) Ca^{2+} channel (Bernheim *et al.*, 1992), the functional significances of these subtypes in the cortex remain to be elucidated.

Multiple G-proteins are known to mediate the muscarinic ACh responses. The receptors expressed for m 1, m 3 and m 5 link to a PTX-insensitive G-protein and regulate inositol phospholipid hydrolysis, whereas those for m 2 and m 4 inhibited adenylyl cyclase through PTX-sensitive G-protein (Bonner, 1989). In electrophysiological studies, the inhibition of I_M in various types of neurones is PTX-insensitive (Dutar & Nicoll, 1988; Pfaffinger, 1988; Brown *et al.*, 1989; Bernheim *et al.*, 1992). In the present study, the I_{ACh} was not suppressed by treatment with PTX, while the inhibitory effect of ACh on the HVA Ca^{2+} channel was abolished, suggesting that the present muscarinic ACh receptor also regulated I_M via PTX-insensitive G-protein, while the HVA Ca^{2+} channel is operated by a PTX-sensitive G-protein. Recently, Bernheim *et al.* (1992) demonstrated that M_1 and M_4 coexist in rat sympathetic neurones and that M_1 suppresses I_M and the HVA Ca^{2+} current (HVA I_{Ca}) through PTX-insensitive G-protein, while M_4 inhibited the HVA I_{Ca} with a PTX-sensitive G-protein. Thus, the PTX-sensitive muscarinic inhibition of the HVA Ca^{2+} channel in cortical pyramidal neurones may be also mediated by another co-existing receptor subtype such as M_2 or M_4 rather than by M_1. In the present study, a PTX-insensitive reduction of the HVA Ca^{2+} channel was not observed. This may reflect differences between species or the brain region.

Of the neurones tested, 31.8% showed a small transient outward current, although its physiological implication is unknown. In hippocampal CA1 neurones, many inositol hydrolysis-mediated responses were accompanied by a transient outward K^+ current (Uneyama *et al.*, 1993; Wakamori *et al.*,

1993; Shirasaki *et al.*, 1994). Here, ACh and 5-hydroxytryptamine facilitated inositol triphosphate formation and thereby mobilized Ca^{2+} from intracellular stores. Then, Ca^{2+}/calmodulin-dependent protein kinase activated a K^+ conductance, resulting in an outward current. In the cortical region, ACh facilitated inositol hydrolysis via a muscarinic receptor with high affinity for pirenzepine, possibly M_1 (Downes, 1982). Hence, the present outward current may be activated through the inositol hydrolysis pathway.

Alzheimer's disease is considered to be associated with a marked depletion of cortical acetylcholinesterase and the loss of cortical cholinergic innervation (Mesulam & Geula, 1988). Furthermore, M_1-selective antagonists impair memory in animals (Messer *et al.*, 1990). Therefore, the understanding of the actions of ACh on cortical pyramidal neurones may facilitate the development of pharmacological therapies for Alzheimer's disease and other neurological disorders in this brain area.

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Heterogeneity of α_1-adrenoceptor subtypes involved in adrenergic contractions of dog blood vessels

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1 We determined the α_1-adrenoceptor subtypes involved in adrenergic contractions of eight different blood vessels isolated from the dog.

2 Noradrenaline produced concentration-dependent contractions in all the blood vessels tested, which were competitively inhibited by prazosin, WB4101, HV723 and 5-methylurapidil. However, there was considerable difference between the vessels with regard to the pK_B values for all the antagonists. The α_1-adrenoceptors of dog vertebral and carotid arteries had high affinity for prazosin ($pK_B > 9.0$) but low affinity for WB4101 (< 8.5), 5-methylurapidil (< 7.5) and HV723 (≤ 8.5). By contrast, HV723 had higher affinity (> 9.0) than prazosin (< 8.3), WB4101 (< 8.7) and 5-methylurapidil (< 8.2) in the portal vein, mesenteric artery and vein, and renal artery. In the femoral artery and vein, however, the four antagonists showed pK_B values in the range 8.0–8.7.

3 Chloroethylclonidine (10 μM) produced a remarkable reduction of the contractile responses to noradrenaline in the vertebral and carotid arteries as compared with those in the other vessels. Nifedipine inhibited the responses to noradrenaline in all the tissues tested, and had marked effects in the portal vein.

4 Sympathetic adrenergic contractions induced by transmural electrical stimulation were also inhibited by prazosin and HV723 at different potencies among tissues. The relative potencies of both the antagonists paralleled the relationship in inhibiting the responses to exogenous noradrenaline in each vessel.

5 According to recent α_1-adrenoceptor subclassification, the present results suggest that the contractions of blood vessels induced by endogenous and exogenous noradrenaline are mediated through different α_1-adrenoceptor subtypes heterogeneously distributed in each vessel; presumably, the α_{1B} subtype in the carotid and vertebral arteries, the α_{1N} subtype in the visceral region and the α_{1L} subtype in the femoral region. Regionally different expression of α_1-adrenoceptor subtypes may be in part associated with the regional heterogeneity of sympathetic responses in the blood vessels.

Keywords: α-Adrenoceptors; noradrenaline-induced contraction; dog blood vessels; α_1-adrenoceptor subclassification

Introduction

Heterogeneity of postjunctional α_1-adrenoceptors has been demonstrated in mammalian blood vessels, where different sensitivities to agonists and antagonists have been observed (McGrath, 1982; Drew, 1985; Bevan *et al.*, 1986; Takayanagi *et al.*, 1988; Docherty, 1989). The heterogeneity may be associated with different α_1-adrenoceptor subtypes (Flavahan & Vanhoutte, 1986; Minneman, 1988; Muramatsu *et al.*, 1990). Binding and/or molecular biological studies have revealed the existence of at least 4 α_1-adrenoceptor subtypes (α_{1A}, α_{1B}, α_{1C} and α_{1D}) with high affinity for prazosin (1993 Receptor Nomenclature supplement, *Trends in Pharmacological Sciences*). The α_{1A} subtype shows high affinity for WB4101 (2-(2,6-dimethoxy-phenoxyethyl)aminomethyl-1,4-benzodioxane) and 5-methylurapidil, while the α_{1B} subtype is not sensitive to the drugs. The α_{1C} and α_{1D} subtypes are also highly sensitive to WB4101 but the α_{1D} subtype may be distinguished by its lesser sensitivity to 5-methylurapidil than the α_{1C} and α_{1A} subtypes (Morrow & Creese, 1986; Han *et al.*, 1987; Lomasney *et al.*, 1991; Perez *et al.*, 1991; Schwinn & Lomasney, 1992). On the other hand, another subclassification has been proposed from functional and binding studies, whereby the α_1-adrenoceptors can be separated into three subtypes (α_{1H}, α_{1L} and α_{1N}) (Muramatsu *et al.*, 1990; Oshita *et al.*, 1991; 1993; Ohmura *et al.*, 1992). Prazosin has a higher affinity for the α_{1H} subtype than for the α_{1L} and α_{1N} subtypes. The third α_{1N}-adrenoceptor subtype is distinguished by its higher affinity for HV723 (α-ethyl-3,4,5-trimethoxy-α-(3-((2-(2-methoxyphenoxyl)ethyl)-amino)-propyl)benzeneacetonitrile fumarate). The α_{1A}, α_{1B}, α_{1C} and α_{1D} subtypes mentioned above may be included in the α_{1H} group of the latter subclassification because of their high affinity for prazosin.

The objectives of this study were to identify the α_1-adrenoceptor subtypes involved in adrenergic contractions in various blood vessels of dogs according to the α_1-adrenoceptor subclassifications and to characterize the regional heterogeneity of sympathetic adrenergic responses with special reference to α_1-adrenoceptor subtypes.

Methods

Mongrel dogs of either sex (7–15 kg) were killed under pentobarbitone anaesthesia. Thereafter, the blood vessels listed in Table 1 were isolated and helically cut under a dissecting microscope (Muramatsu *et al.*, 1990), except for longitudinal preparations of the portal vein. To avoid the involvement of endothelium-derived relaxing factor in the mechanical response (Furchgott, 1981), the endothelial cells in the blood vessels were removed by rubbing them with filter paper. The functional loss of endothelial cells was confirmed by the loss of the relaxing response to acetylcholine (1 μM) or substance P (0.1 μM) in noradrenaline-precontracted arteries or veins (Muramatsu *et al.*, 1990). Each strip was mounted vertically in an organ bath containing 20 ml of modified 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 equilibrated with a gas mixture consist-

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Table 1 EC_{50} values and relative contractions caused by noradrenaline before and after 10 μM or 50 μM chloroethylclonidine (CEC)-treatment

	EC_{50}[a]			Relative contraction[b]		
Blood vessel	Before CEC	Without CEC[c]	After 10 μM CEC	After 50 μM CEC	Without CEC	After 10 μM CEC	After 50 μM CEC
Vertebral artery	6.95 ± 0.06	6.95 ± 0.08	4.68 ± 0.48[d]	4.19 ± 0.08[d]	1.10 ± 0.07	0.58 ± 0.06[d]	0.55 ± 0.03[d]
Carotid artery	6.53 ± 0.12	6.40 ± 0.18	5.06 ± 0.37[d]	3.93 ± 0.19[d]	1.08 ± 0.03	0.72 ± 0.08[d]	0.46 ± 0.08[d]
Portal vein	7.35 ± 0.15	7.28 ± 0.45	7.64 ± 0.28	7.11 ± 0.22	1.00 ± 0.06	0.95 ± 0.03	0.90 ± 0.03
Mesenteric artery	6.45 ± 0.10	6.47 ± 0.25	6.25 ± 0.15	5.66 ± 0.30	1.03 ± 0.08	0.91 ± 0.07	0.79 ± 0.05[d]
Mesenteric vein	6.70 ± 0.13	6.51 ± 0.21	6.34 ± 0.08	6.14 ± 0.07	0.99 ± 0.07	0.93 ± 0.08	0.77 ± 0.03[d]
Renal artery	6.20 ± 0.08	6.19 ± 0.07	5.89 ± 0.15	5.87 ± 0.28	1.03 ± 0.10	0.93 ± 0.02	0.92 ± 0.06
Femoral artery	6.26 ± 0.06	6.26 ± 0.22	5.91 ± 0.09	5.40 ± 0.43	0.98 ± 0.04	0.94 ± 0.02	0.75 ± 0.08[d]
Femoral vein	6.50 ± 0.12	6.33 ± 0.34	6.32 ± 0.08	5.96 ± 0.28	1.02 ± 0.10	0.92 ± 0.07	0.82 ± 0.05[d]

[a]The concentrations of noradrenaline which produced a contraction to the level of a half maximal response before CEC treatment are represented as negative logarithm concentrations.
[b]The relative value of the maximal contraction induced by 100 μM noradrenaline after treatment with vehicle or CEC against the maximal contraction before the treatment in the same blood vessel.
[c]The vessels were treated by the same procedure as for CEC-treatment but the vehicle was used instead of CEC.
[d]Significantly different from the value without CEC ($P<0.05$).
Means ± s.e. of 4–16 experiments.

ing 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.

Noradrenaline was used as the agonist in all tissues. Desmethylimipramine (0.1 μM), deoxycorticosterone (5 μM) and propranolol (1 μM) were added to the bath solution to block neuronal and extraneuronal uptake of noradrenaline and to block β-adrenoceptors, respectively. Cumulative concentration-response curves for noradrenaline were obtained 5 or 6 times from the same strip and the third concentration-response curve was used as a control. In preliminary experiments, the reproducibility of the concentration-response curves obtained from the third to the sixth trial in the absence of the α-antagonist was confirmed. Three increasing concentrations of α-antagonist were added for 30 min before the fourth to sixth concentration-response curves. In each preparation, the pA_2 value was estimated from the ratio of median effective concentrations of noradrenaline in the presence or absence of blocking agents. When the straight line yielded a slope with unity, the pA_2 value estimated was represented as pK_B (Arunlakshana & Schild, 1959). In chloroethylclonidine (CEC) experiments, 10 or 50 μM CEC was added for 20 min after recording the third concentration-response curve for noradrenaline; then concentration-response curves were repeated after a 30 min washout period. Similar treatment with vehicle of CEC had no effect on the concentration-response curve for noradrenaline (i.e. less than 2 fold shift in the curve).

Transmural electrical 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 them being about 2 mm. The stimulus parameters were 0.3 ms in duration and the supramaximum voltage (10 V) for 10 s. The stimulus frequency was 20 Hz in all preparations except the portal vein, in which 5 Hz was used. 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). When studying the dog mesenteric artery and portal vein, α,β-methylene ATP (10 μM) was present throughout in order to block the sympathetic purinergic component (Machaly *et al.*, 1988; Muramatsu *et al.*, 1989). Atropine (1 μM) blocked muscarinic responses in the portal vein (Ichikawa *et al.*, 1979).

Experimental values are given as means ± standard error of mean (s.e.mean). 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 hydrochloride, atropine sulphate (Nacalai Tesque, Kyoto, Japan), prazosin (Taito-Pfizer, Tokyo, Japan), chloroethylclonidine dihydrochloride (CEC), WB4101 (2-(2,6-dimethoxyphenoxyethyl)aminomethyl-1,4-benzodioxane), 5-methylurapidil (Funakoshi, Tokyo, Japan), HV723 (α-ethyl-3,4,5-trimethoxy-α-(3-((2-(2-methoxyphenoxyl)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 prazosin, WB4101, HV723 and 5-methylurapidil on the contractile responses to noradrenaline

Noradrenaline produced concentration-dependent contractions in the arteries and veins isolated from dogs. The pD_2 values for noradrenaline were within the range from 6.0 to 7.5 (Table 1). The concentration-response curves were attenuated by prazosin, WB4101, HV723 and 5-methylurapidil, resulting in a parallel shift of the curves to the right. The slopes of the Schild plot for all antagonists were not significantly different from unity, indicating that the antagonists competitively inhibited the contractile responses induced by noradrenaline in all tissues (Table 2). Figure 1 shows the representative Schild plots for prazosin, WB4101 and HV723 in the vertebral artery, femoral artery and portal vein. Prazosin inhibited the responses to noradrenaline at lower concentrations than WB4101 or HV723 in the vertebral and carotid arteries, thus higher pK_B values than those for WB4101 and HV723 were calculated. In the femoral artery and vein, the antagonist affinities did not differ among these four antagonists. On the other hand, the pK_B values for HV723 were higher than those for prazosin, WB4101 or 5-methylurapidil in the portal vein, mesenteric artery and vein, and renal artery. The pK_B values and slopes of the Schild plot for the α_1-adrenoceptor antagonists in these blood vessels are summarized in Table 2.

Effects of chloroethylclonidine (CEC) on the contractile responses to noradrenaline

Since CEC is thought to inactivate the α_{1B}-adrenoceptor subtype (Han *et al.*, 1987), we examined its effects on the contractile responses to noradrenaline in various vascular tissues. As shown in Figure 2, pretreatment with CEC (10 and 50 μM) inhibited the concentration-response curves for noradrenaline in the dog vertebral artery. Similar results were obtained with the carotid artery. However, the attenuation by CEC was much less in the other blood vessels tested (Table 1). In the portal vein, CEC itself produced an irreversible contraction which lasted after CEC removal.

Effects of nifedipine on the contractile responses to noradrenaline

Nifedipine (1 μM) slightly but significantly attenuated the contractile responses to noradrenaline in all the tissues tested (Figure 3, Table 3). The maximum inhibition (47% reduction at 100 μM noradrenaline) was seen in the portal vein in which the spontaneous rhythmic contraction disappeared after exposure to nifedipine. The pD_2 values for noradrenaline were slightly (less than 4 times in affinity) reduced by nifedipine in the portal vein, mesenteric, renal and femoral arteries.

Effects of prazosin and HV723 on the contractile responses to transmural electrical stimulation

Transmural electrical stimulation produced transient contractions in the presence of propranolol, DG-5128, α,β-methylene ATP and/or atropine (see Methods for experimental details). These responses were abolished by guanethidine (3 μM) or tetrodotoxin (0.5 μM) ($n = 3$, for each drug and each vessel), suggesting sympathetic neurogenic responses. The contractions were also inhibited by prazosin and HV723 in concentration-dependent manner. However, the inhibitory potencies of both drugs varied among the tissues. Prazosin was more potent in inhibiting the neurogenic response than HV723 in the vertebral and carotid arteries, whereas the converse relationship was observed in the portal vein, mesenteric artery and vein, and renal artery (Table 4). In the femoral artery the inhibition by both prazosin and HV723 was equipotent, but the EC_{50} values were relatively low. These high potencies of both drugs in the femoral artery may be related to low concentrations of noradrenaline which diffuse from the sympathetic nerve terminals to the postjunc-

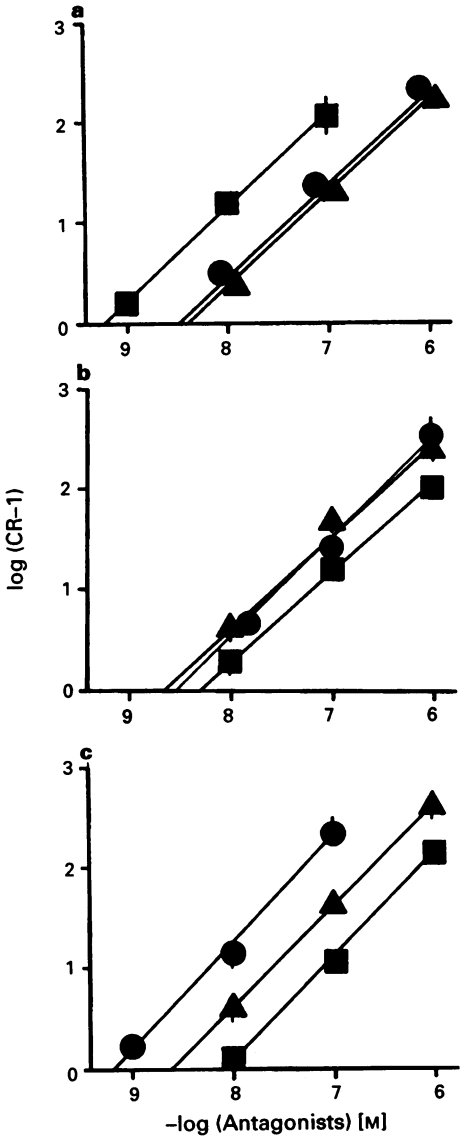


Figure 1 Representative Schild plots for competitive inhibition of noradrenaline-induced contraction by HV723 (●), WB4101 (▲) and prazosin (■) in the dog vertebral artery (a), femoral artery (b) and portal vein (c).

Table 2 α_1-Adrenoceptor affinities for prazosin, WB4101, HV723 and 5-methylurapidil in dog blood vessels

	pK_B/*Slope* (95% CL)			
Blood vessel	*Prazosin*	*WB4101*	*5-Methylurapidil*	*HV723*
Vertebral artery	9.24 ± 0.13	8.41 ± 0.11	7.29 ± 0.10	8.49 ± 0.15
	0.93 (0.75–1.12)	0.93 (0.79–1.07)	0.97 (0.81–1.13)	0.92 (0.75–1.10)
Carotid artery	9.55 ± 0.14	8.15 ± 0.15	7.21 ± 0.11	8.52 ± 0.10
	0.98 (0.73–1.13)	0.92 (0.81–1.03)	0.87 (0.73–1.00)	0.90 (0.77–1.04)
Portal vein	8.05 ± 0.07	8.63 ± 0.10	8.16 ± 0.06	9.11 ± 0.09
	1.03 (0.91–1.15)	0.97 (0.86–1.11)	1.08 (0.98–1.18)	1.09 (0.96–1.23)
Mesenteric artery	8.12 ± 0.10	8.76 ± 0.10	8.05 ± 0.10	9.15 ± 0.05
	0.94 (0.84–1.04)	0.95 (0.88–1.02)	1.14 (0.96–1.33)	0.96 (0.87–1.02)
Mesenteric vein	7.95 ± 0.10	8.51 ± 0.07	7.73 ± 0.16	9.04 ± 0.07
	0.95 (0.79–1.11)	0.97 (0.88–1.07)	1.04 (0.82–1.26)	0.96 (0.84–1.08)
Renal artery	8.05 ± 0.09	8.67 ± 0.10	8.21 ± 0.13	9.14 ± 0.12
	1.07 (0.91–1.23)	0.98 (0.86–1.11)	0.96 (0.78–1.15)	1.04 (0.85–1.23)
Femoral artery	8.29 ± 0.11	8.70 ± 0.16	8.43 ± 0.16	8.62 ± 0.13
	0.90 (0.75–1.04)	0.88 (0.73–1.03)	0.85 (0.66–1.05)	0.95 (0.70–1.20)
Femoral vein	8.33 ± 0.15	8.43 ± 0.11	8.00 ± 0.14	8.21 ± 0.18
	0.86 (0.68–1.04)	1.00 (0.84–1.16)	1.01 (0.74–1.27)	1.09 (0.75–1.42)

Data shown are means ± s.e. of 4–7 experiments.
All data were obtained from Schild plot analysis.

tional α_1-adrenoceptors and produce the contraction (Bevan & Su, 1973). The sympathetic contraction in the femoral artery developed slowly (time to peak: 115 ± 10 s in the femoral artery vs 18 ± 2 s in the carotid artery, $n = 5$). The femoral vein was not examined because the sympathetic adrenergic contraction was small and unstable.

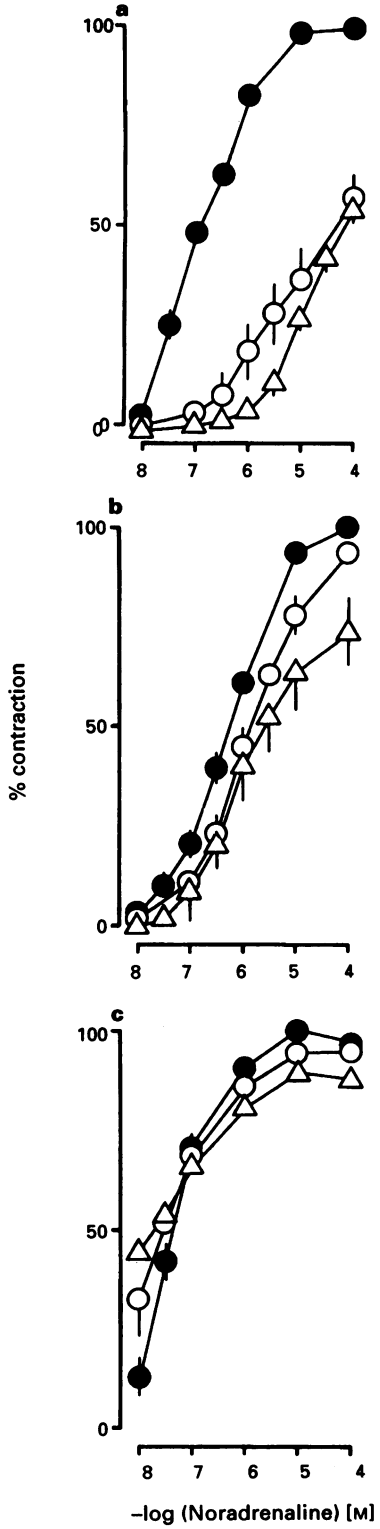


Figure 2 Effect of pretreatment with chloroethylclonidine (CEC) on noradrenaline-induced contractions of the dog vertebral artery (a), femoral artery (b) and portal vein (c). CEC (10 or 50 μM) was added for 20 min, then removed from the bath. The test response was recorded 30 min after washing out the CEC. The maximum contraction induced by noradrenaline before CEC treatment was taken as 100%. (●) Control; (○) 10 μM CEC-pretreated; (△) 50 μM CEC-pretreated. Each value is the mean with the s.e.mean of 6 experiments shown by vertical lines.

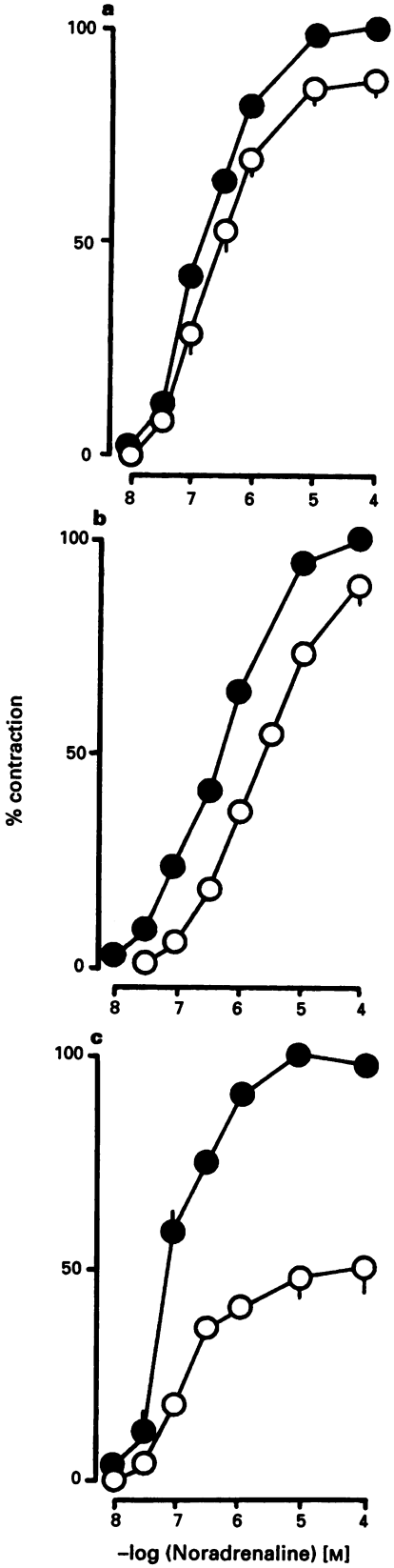


Figure 3 Effects of nifedipine (1 μM) on the concentration-response curves for noradrenaline in the dog vertebral artery (a), femoral artery (b) and portal vein (c). (●) Control; (○) in the presence of nifedipine. Means ± s.e.mean of 6 experiments.

Table 3 pD_2 values and relative contraction to noradrenaline before and after nifedipine-treatment

Blood vessel	pD_2 Before nifedipine	pD_2 Without nifedipine[b]	pD_2 After nifedipine	Relative contraction[a] Without nifedipine	Relative contraction[a] After nifedipine
Vertebral artery	6.82 ± 0.05	6.75 ± 0.12	6.70 ± 0.09	1.12 ± 0.07	0.87 ± 0.04[c]
Carotid artery	6.95 ± 0.03	6.86 ± 0.04	6.82 ± 0.02	1.12 ± 0.08	0.83 ± 0.06[c]
Portal vein	7.10 ± 0.03	7.03 ± 0.06	6.73 ± 0.05[c]	1.05 ± 0.05	0.53 ± 0.06[c]
Mesenteric artery	6.61 ± 0.06	6.53 ± 0.10	6.06 ± 0.09[c]	0.98 ± 0.03	0.78 ± 0.03[c]
Mesenteric vein	6.33 ± 0.08	6.25 ± 0.10	6.07 ± 0.07	0.98 ± 0.06	0.81 ± 0.02[c]
Renal artery	6.23 ± 0.06	6.22 ± 0.05	5.66 ± 0.06[c]	1.10 ± 0.10	0.70 ± 0.03[c]
Femoral artery	6.33 ± 0.04	6.29 ± 0.07	5.77 ± 0.06[c]	1.07 ± 0.04	0.87 ± 0.04[c]
Femoral vein	6.72 ± 0.08	6.67 ± 0.10	6.51 ± 0.08	1.03 ± 0.04	0.90 ± 0.04[c]

[a]Relative value of maximal contraction against the maximal contraction induced by 100 μM noradrenaline before nifedipine (1 μM) or vehicle.
[b]The vessels were treated with vehicle instead of nifedipine.
[c]Significantly different from the value without nifedipine ($P<0.05$).
Means ± s.e. of 4–12 experiments.

Table 4 EC_{50} values for prazosin and HV723 in inhibiting the sympathetic adrenergic contraction induced by transmural electrical stimulation

Blood vessel	EC_{50} (nM) Prazosin	EC_{50} (nM) HV723
Vertebral artery	1.31 ± .015	5.25 ± 0.56[a]
Carotid artery	2.14 ± 0.47	10.4 ± 3.88[a]
Portal vein	50.0 ± 8.29	8.09 ± 2.67[a]
Mesenteric artery	9.81 ± 4.38	0.97 ± 0.33[a]
Mesenteric vein	6.24 ± 1.28	1.15 ± 0.33[a]
Renal artery	26.1 ± 18.0	1.82 ± 0.23[a]
Femoral artery	1.06 ± 0.79	2.09 ± 0.15

[a]Significantly different from the value for prazosin ($P<0.05$).
Means ± s.e. of 4–6 experiments.

Discussion

Noradrenaline produced concentration-dependent contractions in 8 different blood vessels isolated from the dog, and the responses were inhibited by prazosin, WB4101, HV723 and 5-methylurapidil. The slopes of Schild plots were close to unity, indicating that the contractile response to noradrenaline in each blood vessel is mediated through a single population of α_1-adrenoceptors. However, the pK_B values for the antagonists (especially prazosin, 5-methylurapidil and HV723) varied among the tissues. This suggests regional heterogeneity of the α_1-adrenoceptors involved in noradrenaline-induced contractions.

Recently, α_1-adrenoceptors were found not to be homogeneous. Binding and molecular biological studies have demonstrated that the α_1-adrenoceptors having high affinity for prazosin ($pK_B>9$) are subdivided into at least 4 subtypes (α_{1A}, α_{1B}, α_{1C} and α_{1D}), where the α_{1A}, α_{1C} and α_{1D} subtypes are more sensitive to WB4101 ($pK_B>9$) as compared with the α_{1B} subtype. 5-Methylurapidil shows higher affinity toward the α_{1A} ($pK_B>9$) and α_{1C} (pK_B close to 8.5) subtypes than α_{1B} and α_{1D} subtypes ($pK_D<8.0$) (Morrow & Creese, 1986; Han *et al.*, 1987; Cotecchia *et al.*, 1988; Voigt *et al.*, 1990; Schwinn *et al.*, 1990; Lomasney *et al.*, 1991; Perez *et al.*, 1991). On the other hand, another subclassification (α_{1H}, α_{1L}, α_{1N} subclassification) was proposed from functional and binding studies, where the α_1-adrenoceptors were first separated into prazosin-high and low affinity sites (Drew, 1985; Flavahan & Vanhoutte, 1986). The prazosin-high sites (α_{1H}) may include the α_{1A}, α_{1B}, α_{1C} and α_{1D} subtypes because of their high affinity for prazosin, while the low sites were further subdivided into α_{1L} and α_{1N} subtypes by HV723 (α_{1N}-high affinity drug) (Muramatsu *et al.*, 1990; Muramatsu, 1992; Ohmura *et al.*, 1992). WB4101 and 5-methylurapidil cannot discriminate between α_{1L} and α_{1N} subtypes. CEC produces a remarkable inactivation of the α_{1B} and α_{1C} subtypes (more than 70% inactivation at 10 μM CEC), but the other subtypes were less sensitive to CEC alkylation (Lomasney *et al.*, 1991; Perez *et al.*, 1991; Ohmura *et al.*, 1992).

The present study shows that the α_1-adrenoceptors of dog vertebral and carotid arteries have high affinity for prazosin but low affinity for WB4101, 5-methylurapidil and HV723, and that CEC more potently inhibits the contractile responses to noradrenaline in both the arteries as compared with the other vessels tested. These results are consistent with the characteristics of α_{1B} subtype mentioned above, indicating that the contractile response to noradrenaline is mediated through the α_{1B} subtype in the vertebral and carotid arteries (Muramatsu *et al.*, 1991).

By contrast, the results obtained in the other six blood vessels were complex. Unlike carotid and vertebral arteries, the α_1-adrenoceptors of the visceral and femoral vessels were less sensitive to CEC and showed lower affinity for prazosin (pK_B: approximately 8.0). The affinities for WB4101 were almost the same among the vessels tested and were lower than the affinities (pK_B close to 9.5) of α_{1A}, α_{1C} and α_{1D} subtypes. 5-Methylurapidil also showed higher affinities in the visceral and femoral vessels than the carotid and vertebral arteries (presumably α_{1B} subtype). These results suggest that the α_1-adrenoceptors in the visceral and femoral vessels cannot be fully determined by the criteria presently proposed for α_{1A}, α_{1B}, α_{1C} and α_{1D} subtypes. Rather, the results seem to fit the α_{1H}, α_{1L} and α_{1N} subclassification, suggesting the involvement of α_{1N} subtype in the portal vein, mesenteric artery and vein, and renal artery, and of α_{1L} subtype in the femoral artery and vein.

In the original α_{1A}, α_{1B} subclassification, nifedipine was proposed to produce a selective inhibition of α_{1A}-mediated responses because the α_{1A} subtype was considered to be coupled to calcium channels (Han *et al.*, 1987; Minneman, 1988). In this study, the α_{1A} subtype with high affinity for prazosin, WB4101 and 5-methylurapidil and insensitive to CEC was not detected. However, nifedipine slightly but significantly inhibited the responses to noradrenaline in all tissues tested. Previously, we suggested that contractile responses mediated through α_1-adrenoceptor subtypes cannot be classified strictly by a difference in signal transduction mechanisms (Muramatsu *et al.*, 1991; Ohmura *et al.*, 1992). Therefore, it is more likely that α_1-adrenoceptors involved in noradrenaline-induced contraction in the dog blood vessels cannot be distinguished by nifedipine, as suggested in other species (Muramatsu *et al.*, 1990; 1991; Oriowo & Ruffolo, 1992; Oriowo *et al.*, 1992; Oshita *et al.*, 1993; Sayet *et al.*, 1993).

Sympathetic adrenergic contractions induced by trans-

mural electrical stimulation were also inhibited by prazosin and HV723 with variable potency among tissues. The variation in the inhibitory potencies between both the antagonists was similar to that in inhibiting the response to noradrenaline. Thus, it is likely that endogenous noradrenaline released from sympathetic nerve terminals acts upon the same α_1-adrenoceptor subtypes as does exogenous noradrenaline, producing adrenergic neurogenic contractions (Muramatsu, 1991a).

Regional heterogeneity of sympathetic transmission in blood vessels has been demonstrated (Lundberg *et al.*, 1984; Burnstock, 1988; Muramatsu, 1991b). In the dog, sympathetic contractions of the carotid and femoral arteries are adrenergic in nature, while both adrenergic and purinergic components are involved in the sympathetic contractions in the portal vein, mesenteric artery and vein, and saphenous vein (Muramatsu *et al.*, 1984; 1989; Machaly *et al.*, 1988). This evidence, together with the results of this study, suggests that the regional heterogeneity of sympathetic responses may be caused by differences not only among the transmitters released but also in the α_1-adrenoceptor subtypes distribution.

In conclusion, there is evidence of regional differences in the distribution of α_1-adrenoceptor subtypes, which suggests regionally variable expression of α_1-adrenoceptor subtypes.

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(−)-Discretamine, a selective α_{1D}-adrenoceptor antagonist, isolated from *Fissistigma glaucescens*

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1 The selectivity of (−)-discretamine for α_1-adrenoceptor subtypes was investigated by use of functional and binding studies in rat vas deferens, spleen and aorta, and in cultured DDT_1MF-2 and A10 cells.

2 In prostatic portions of rat vas deferens, the competitive antagonists (−)-discretamine, 5-methylurapidil (5-MU) and prazosin inhibited contractions to noradrenaline (NA) with pA_2 values of 6.21, 8.71 and 9.27, respectively. The irreversible antagonist, chloroethylclonidine (CEC, 100 μM) failed to affect contractions to NA while nifedipine (1 μM) blocked them almost completely.

3 In rat spleen, the competitive antagonists (−)-discretamine, 5-MU and prazosin inhibited contractions to phenylephrine with pA_2 values of 6.44, 7.19 and 9.45, respectively. CEC (100 μM) significantly reduced the maximum contraction to phenylephrine while nifedipine (1 μM) did not affect it.

4 In rat aorta, the competitive antagonists (−)-discretamine, 5-MU and prazosin inhibited contractions to NA with pA_2 values of 7.60, 8.00 and 9.40, respectively. CEC also antagonized the contractions to NA in a competitive manner with a pA_2 value of 6.10.

5 The specific binding of [^{3}H]-prazosin to DDT_1MF-2 and A10 cells was concentration-dependent and saturated at 3–5 nM with K_D values of 0.24 ± 0.02 and 0.20 ± 0.02 nM, respectively. (−)-Discretamine, 5-MU, CEC and prazosin inhibited specific [^{3}H]-prazosin binding to DDT_1MF-2 and A10 cells in a concentration-dependent manner with IC_{50} values of 390.8 ± 20.6, 43.6 ± 3.9, 200.0 ± 30.0 and 0.8 ± 0.1 nM, respectively in DDT_1MF-2 cells, and 25.0 ± 3.2, 8.6 ± 1.4, 1000.0 ± 30.8 and 0.52 ± 0.03 nM, respectively in A10 cells.

6 Pretreatment of A10 cells with CEC (10 μM) for 30 min and then washed out thoroughly, reduced specific [^{3}H]-prazosin binding by 30%. The CEC-insensitive [^{3}H]-prazosin binding was inhibited by (−)-discretamine with an IC_{50} value of 7.0 ± 0.3 nM.

7 5-MU (100 nM), CEC (1 μM) and prazosin (10 nM) markedly inhibited NA (3 μM)-induced [^{3}H]-inositol monophosphate formation in DDT_1MF-2 and A10 cells, while (−)-discretamine (100 nM) inhibited NA-induced [^{3}H]-inositol monophosphate formation only in A10 cells.

8 In conclusion, (−)-discretamine is a selective α_{1D}-adrenoceptor antagonist in vascular smooth muscle. Its selectivity among various α_1-adrenoceptor subtypes is α_{1A}:α_{1B}:α_{1D} = 0.04:0.07:1.0.

Keywords: α_{1D}-Adrenoceptor antagonist; rat thoracic aorta; (−)-discretamine; *Fissistigma glaucescens*

Introduction

Two major subtypes (α_{1A} and α_{1B}) of α_1-adrenoceptors have been identified on the basis of binding experiments and the ability of the alkylating agent chloroethylclonidine (CEC) to inactivate the α_{1B}- but not the α_{1A}-subtypes (Morrow & Creese, 1986; Han *et al.*, 1987; Hanft & Gross, 1989). Based on this classification, it has been suggested that contractions of rat vas deferens to exogenous noradrenaline (NA) or adrenaline are mediated predominantly by α_{1A}-adrenoceptors (Han *et al.*, 1987; Hanft & Gross, 1989), whereas contractions to NA in rat spleen are mediated predominantly by α_{1B}-adrenoceptors (Han *et al.*, 1987). Thus, contractions of the rat vas deferens to NA have been used as a model for α_{1A}-adrenoceptors and contractions of the rat spleen to NA or phenylephrine have been used as a model for α_{1B}-adrenoceptors. However, molecular cloning techniques have confirmed the existence of at least three (Cotecchia *et al.*, 1988; Schwinn *et al.*, 1990; Lomasney *et al.*, 1991) or four (Perez *et al.*, 1991) genes coding for α_1-adrenoceptor subtypes.

The subtype of α_1-adrenoceptor present in the rat aorta has been variously classified as α_{1B} (Han *et al.*, 1990), both α_{1A} and α_{1B} (Tian *et al.*, 1990; Piascik *et al.*, 1991) or atypical (Muramatsu *et al.*, 1991; Aboud & Docherty, 1992; Oriowo & Ruffolo, 1992). In the last three studies, CEC failed to reduce the maximum response to NA, but caused a parallel shift in the concentration-response curve. Recently, Aboud *et al.* (1993) re-examined the α_1-adrenoceptor subtypes present in rat aorta and confirmed that contractions of rat aorta to NA appear to be mediated by CEC sensitive non-α_{1A}-, non-α_{1B}-adrenoceptors. Furthermore, from the rat hippocampus, Perez *et al.* (1991) cloned a cDNA encoded a novel α_{1D}-adrenoceptor subtype, which also exists in rat aorta.

(−)-Discretamine is a selective α_1-adrenoceptor antagonist, isolated from the plant *Fissistigma glaucescens* (Ko *et al.*, 1993). Its selectivity for α_1-adrenoceptor subtypes was elucidated in this study by use of functional and binding experiments. The α_1-adrenoceptor subtype(s) mediated contractions of rat aorta to NA was also investigated.

Methods

Rat aortic contraction

Wistar rats of either sex, 250–300 g, were humanely killed. The thoracic aorta was isolated and excess fat and connective

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tissue were removed. The vessels were cut into rings of about 5 mm in length and the endothelium was removed by rubbing with a cotton ball; the absence of acetylcholine-induced relaxation was taken as an indicator that vessels were denuded successfully. Aortic rings were then mounted in organ baths containing 5 ml Krebs solution of the following composition (mM): NaCl 118.4, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 11.7, $CaCl_2$ 1.9 and $NaHCO_3$ 25.0. The tissue bath solution was maintained at 37°C and gassed with 95% O_2:5% CO_2. Two stainless steel hooks were inserted into the aortic lumen, one was fixed while the other was connected to a transducer. Aortae were equilibrated in the medium for 90 min with three changes of Krebs solution and maintained under an optimal tension of 1 g before specific experimental protocols were initiated. Aortae were then contracted with NA administered cumulatively (for 30 min at 3 min intervals) and contractions were recorded isometrically via a force-displacement transducer connected to a Grass polygraph. Once a maximum response to NA had been obtained, aortae were washed and bathing solution was changed every 15 min for at least 60 min. Various concentrations of competitive antagonists were added and incubated for either 15 min ((−)-discretamine, 5-methyl-urapidil or prazosin) or 30 min (chloroethylclonidine) before the generation of a cumulative concentration-response curve with NA for 30 min at 3 min intervals in a single preparation. Results are expressed as percentages of the maximal control response of NA before the addition of antagonists.

Rat spleen contraction

Spleens were bisected transversely into two portions, and experiments were carried out as for the rat aorta except that phenylephrine was used as the contractile agent.

Rat vas deferens contraction

In these studies NA was used as the agonist. After the equilibration period, prostatic portions of rat vas deferens were contracted twice to noradrenaline (10 μM) and washed for 60 min. Noncumulative concentration-response curves for NA-stimulated contractions were determined in the absence or presence of the indicated antagonists.

Cell culture

DDT_1MF-2 (hamster vas deferens smooth muscle) and A10 (rat thoracic aortic smooth muscle) cells provided by American Type Cell Collection were cultured at 37°C in a humidified air/CO_2 (95:5) atmosphere in 100 mm dishes. The growth medium was Dulbeccos modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum, 100 units of penicillin and 100 μg of streptomycin sulphate ml^{-1}.

Binding of [^{3}H]-prazosin

For binding assay, cells were finally grown in 24 well plates. At confluence, monolayer cells were washed with Krebs-Henseleit solution (KHS) (composition mM: NaCl 117.5, KCl 5.4, NaH_2PO_4 1.2, $NaHCO_3$ 25.0, $CaCl_2$ 2.5, $MgSO_4$ 1.2, glucose 5.5, HEPES 25.0) pH 7.4, and then 250 μl of various concentrations of [^{3}H]-prazosin (0.01–5 nM) was added for 30 min at 37°C. The incubation was terminated by washing the monolayer cells rapidly with 2 ml of ice-cold KHS three times. Subsequently, 0.5 ml of 1 N NaOH was added to solubilize the cell monolayer. The solubilized cells were counted for bound radioactivity by liquid scintillation counter (Beckman 5000 TC). The equilibrium saturation binding of [^{3}H]-prazosin and competition with antagonists were conducted in triplicate. Nonspecific binding was defined as binding in the presence of 10 μM phentolamine. Protein content of cells was measured by the method of Lowry *et al.* (1951) with bovine serum albumin used as a standard.

[^{3}H]-inositol monophosphate accumulation

Cells for inositol phospholipid hydrolysis were finally grown in 12 well plates. At confluence, monolayer cells were loaded with [^{3}H]-*myo*-inositol (5 μCi ml^{-1}) for 24 h in inositol-free DMEM. Prelabelled cells were then washed twice with KHS solution and incubated for 15 min in the presence of 10 mM LiCl. NA was added and incubation continued for 30 min. Incubation was terminated by stop buffer and addition of 0.1 N HCl (1:1 v/v). Cells were left a minimum of 30 min at − 20°C before isolation of total [^{3}H]-inositol phosphates by anion exchange chromatography (Alexander *et al.*, 1989). Briefly, 800 μl of the supernatant was neutralized by addition of 300 μl of 0.1 N NaOH, 1 ml of 50 mM Tris-HCl and added to an AG1-X8 column. The resin was washed with a total

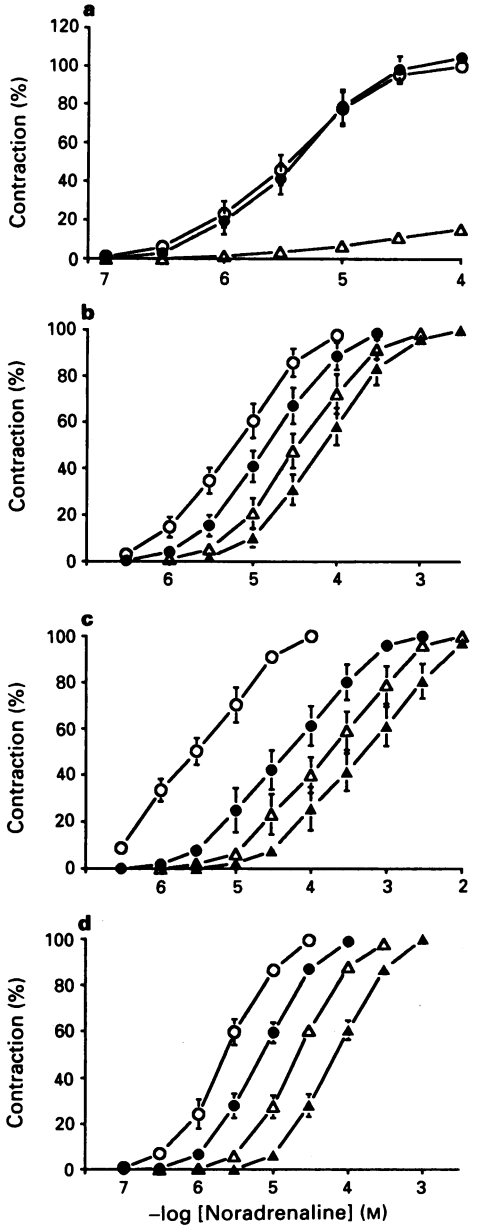


Figure 1 Concentration-response curves to noradrenaline in rat vas deferens. Dimethylsulphoxide (0.1%, control) (○) or various concentrations of inhibitors were preincubated with vas deferens for 15 min except for chloroethylclonidine which was preincubated for 30 min. (a) Nifedipine (1 μM, △) or chloroethylclonidine (100 μM, ●); (b) (−)-discretamine, 1 μM (●), 3 μM (△) and 10 μM (▲); (c) 5-methyl-urapidil, 0.03 μM (●), 0.1 μM (△) and 0.3 μM (▲); (d) prazosin, 1 nM (●), 3 nM (△) and 10 nM (▲). Each point represents the mean with s.e.mean ($n = 6$).

Table 1 Potencies of α_1-adrenoceptor antagonists against contractions to noradrenaline in rat vas deferens and aorta, and to phenylephrine in rat spleen

	(−)-Discretamine	*5-MU*	*CEC*	*Prazosin*
Vas deferens	6.21	8.71	No effect	9.27
(α_{1A})	(5.52–6.90)	(7.95–9.47)		(8.90–9.64)
Spleen	6.44	7.19	Effective	9.45
(α_{1B})	(6.19–6.69)	(6.66–7.72)		(9.14–9.76)
Aorta	7.60	8.00	6.10	9.40
(α_{1D})	(7.42–7.78)	(7.69–8.31)	(5.81–6.39)	(9.02–9.78)

Potencies are expressed as pA_2 values (and 95% confidence limits) from the Schild plots of Figures 1, 2 and 3. The effectiveness of chloroethylclonidine (CEC, 100 μM) on agonist responses is also shown. Abbreviations: 5-MU, 5-methyl-urapidil.

25 ml of H_2O. The [^{3}H]-inositol monophosphate was then eluted with 3 ml of 0.2 M ammonium formate/0.1 M formic acid. Radioactivity was determined by scintillation counting after addition of scintillation cocktail.

Data analysis

In each experiment, agonist dose-response curves in the presence of antagonists were related to the control dose-response curve, of which the maximum response was taken as 100%. In most experiments, three or four concentrations of antagonists were tested and the slopes of the resulting Schild plots were used to assess competitive antagonism. The pA_2 values were calculated for each concentration of antagonist according to: $pA_2 = -\log([\text{antagonist}]/[\text{dose ratio} - 1])$ (Mackay, 1978).

Saturation and displacement binding data were analyzed by the weighted least-squares iterative curve fitting programme LIGAND (Munson & Rodbard, 1980). The data were first fitted to a one- and then a two-site model, and if the residual sums of squares were statistically less for a two-site fit of the data than for a one-site, as determined by *F*-test comparison, then the two-site model was accepted.

The experimental results are expressed as the mean ± s.e. mean and accompanied by the number of observations. Statistical significance was assessed by Student's *t* test and *P* values less than 0.05 were considered significant.

Drugs

(−)-Discretamine was isolated from the plant *Fissistigma glaucescens* as previously described (Lu *et al.*, 1985). The following drugs were used: noradrenaline HCl, prazosin HCl, phentolamine HCl, acetylcholine HCl and nifedipine were obtained from Sigma Chemical Co.; phenylephrine HCl was from Denmarks Apotekerforening; chloroethylclonidine HCl and 5-methyl-urapidil were from Research Biochemicals International; AG1-X8 (100–200 mesh) resin was from Bio-Rad Lab.; *myo*-[2-^{3}H]inositol (10–20 Ci mmol^{-1}) was purchased from Amersham. If drugs were dissolved in dimethylsulphoxide (DMSO), the final concentration of DMSO in the bathing solution did not exceed 0.1% and had no effect on the muscle contraction.

Results

Rat vas deferens

Contractions of the rat vas deferens to NA are mediated predominantly by α_{1A}-adrenoceptors, but in the presence of nifedipine a second CEC-sensitive component can be identified, particularly in the epididymal portion (Aboud *et al.*, 1993). Thus, the prostatic portion of rat vas deferens was used in this study. In prostatic portions of rat vas deferens, noradrenaline (NA) produced transient contractions with a pD_2 of 5.40 ± 0.21 (mean and s.e.mean, − log M) and a

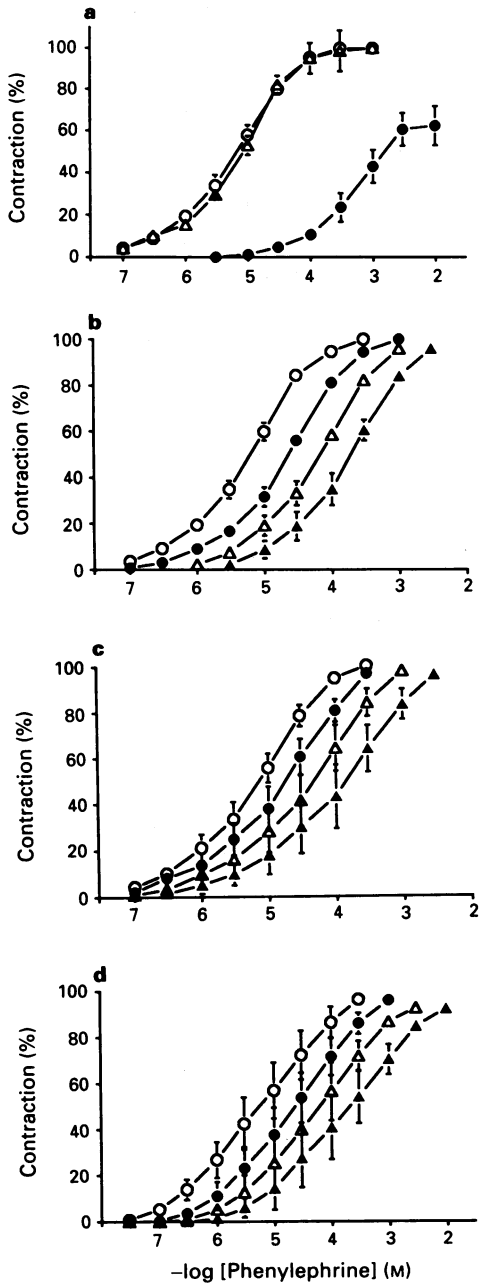


Figure 2 Cumulative concentration-response curves to phenylephrine in rat spleen. Dimethylsulphoxide (0.1%, control) (○) or various concentrations of inhibitors were preincubated with spleen for 15 min except for chloroethylclonidine which was preincubated for 30 min. (a) Nifedipine (1 μM, △) or chloroethylclonidine (100 μM, ●); (b) (−)-discretamine, 1 μM (●), 3 μM (△) and 10 μM (▲); (c) 5-methyl-urapidil, 0.1 μM (●), 0.3 μM (△) and 1 μM (▲); (d) prazosin, 1 nM (●), 3 nM (△) and 10 nM (▲). Each point represents the mean with s.e.mean ($n = 6$).

maximum contraction of 1.19 ± 0.11 g ($n = 30$). Nifedipine (1 μM) almost completely blocked this contraction while chloroethylclonidine (CEC, 100 μM) did not affect it (Figure 1a). (−)-Discretamine (1−10 μM), 5-methyl-urapidil (5-MU, 0.03−0.3 μM) and prazosin (1−10 nM) produced parallel, rightward shifts of the concentration-response curve of NA, consistent with competitive blockade (Figure 1b,c and d). The pA_2 values of (−)-discretamine, 5-MU and prazosin were 6.21, 8.71 and 9.27, respectively (Table 1). In all cases the slopes of Schild plots were not significantly different from 1.0.

Rat spleen

In rat spleen, it proved impossible to obtain competitive interactions between NA and the selective α_1-adrenoceptor antagonists such as prazosin. This may be due to the involvement of α_2- as well as α_1-adrenoceptors in this response (Kenakin & Novak, 1988). Thus, phenylephrine was used in this experiment. Phenylephrine caused isometric contractions with pD_2 value of 5.24 ± 0.35 and maximum contractions of 0.21 ± 0.01 g ($n = 30$) in rat spleen. Nifedipine (1 μM) did not affect this contraction while the irreversible α_{1B}-adrenoceptor antagonist, CEC (100 μM) reduced the maximum responses by 40% and shifted the concentration-response curve 138 fold to the right ($pD_2 = 3.10 \pm 0.20$, Figure 2a). (−)-Discretamine (1−10 μM), 5-MU (0.1−1 μM) and prazosin (1−10 nM) produced shifts of the curve of phenylephrine without reducing the maximum response (Figure 2b,c and d). The pA_2 values of (−)-discretamine, 5-MU and prazosin were calculated to be 6.44, 7.19 and 9.45, respectively (Table 1). The slopes of Schild plots did not differ significantly from 1.0.

Rat aorta

NA produced isometric contractions of rat aorta with a pD_2 of 7.79 ± 0.17 and a maximum contraction of 2.07 ± 0.12 g ($n = 24$). (−)-Discretamine (0.1−3 μM), 5-MU (0.1−1 μM) and prazosin (10−300 nM) produced concentration-dependent rightward shifts in the concentration-response curve of NA without reducing the maximum response (Figure 3a,b and d). The pA_2 values of (−)-discretamine, 5-MU and prazosin were calculated to be 7.60, 8.00 and 9.40, respectively (Table 1).

The irreversible antagonist CEC (3−100 μM) failed to reduce significantly the maximum response to NA but produced parallel rightward shifts in the curve of NA (Figure 3c) with a pA_2 value of 6.10 (Table 1). Again, all the slopes of Schild plots did not differ significantly from 1.0.

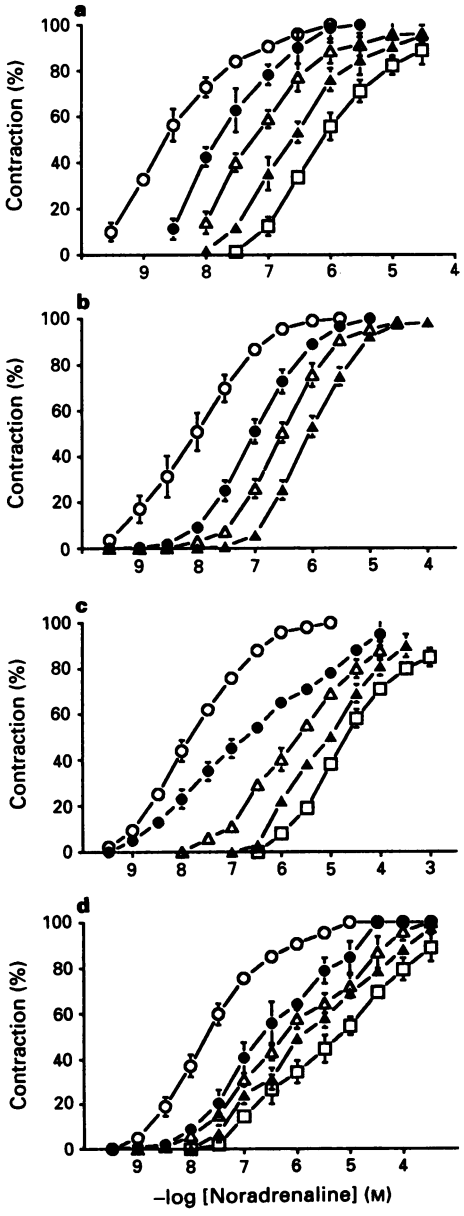


Figure 3 Cumulative concentration-response curves to noradrenaline in rat aorta. Dimethylsulphoxide (0.1%, control) (○) or various concentrations of inhibitors were preincubated with aorta for 15 min except for chloroethylclonidine which was preincubated for 30 min. (a) (−)-Discretamine, 0.1 μM (●), 0.3 μM (△), 1 μM (▲) and 3 μM (□); (b) 5-methyl-urapidil, 0.1 μM (●), 0.3 μM (△) and 1 μM (▲); (c) chloroethylclonidine, 3 μM (●), 10 μM (△), 30 μM (▲) and 100 μM (□); (d) prazosin, 10 nM (●), 30 nM (△), 100 nM (▲) and 300 nM (□). Each point represents the mean with s.e.mean ($n = 6$).

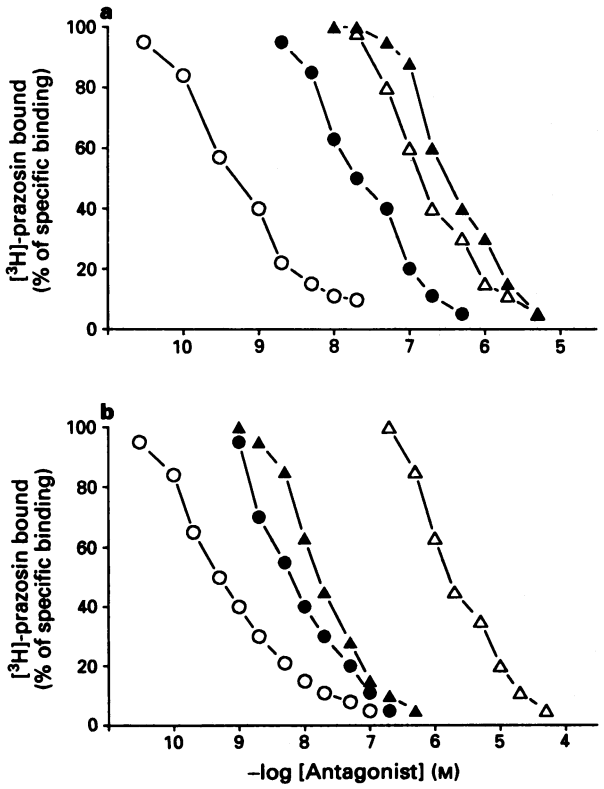


Figure 4 Displacement of [³H]-prazosin binding from DDT₁MF-2 (a) and A10 (b) cells by prazosin (○), 5-methyl-urapidil (●), (−)-discretamine (▲) and chloroethylclonidine (△). The concentration of [³H]-prazosin used was 0.2 nM. The figure represents a single experiment for each drug, where each point is the mean of triplicate determinations.

Table 2 Relative potencies (IC_{50}) of α_1-adrenoceptor antagonists in competing for [^{3}H]-prazosin binding to hamster vas deferens smooth muscle (DDT_1MF-2) and rat aortic smooth muscle (A10) cells

	DDT_1MF-2 (α_{1B})		A_{10} (α_{1D})	
	IC_{50} (nM)	*Hill coefficient*	IC_{50} (nM)	*Hill coefficient*
(−)-Discretamine	390.8 ± 20.6	0.98 (0.96–1.00)	25.0 ± 3.2	0.88 (0.79–0.98)
5-MU	43.6 ± 3.9	0.84 (0.80–0.88)	8.6 ± 1.4	0.98 (0.92–1.04)
CEC	200.0 ± 30.0	0.89 (0.84–0.94)	1000.0 ± 30.8	0.94 (0.89–0.98)
Prazosin	0.8 ± 0.1	1.16 (1.12–1.20)	0.52 ± 0.03	0.92 (0.83–1.00)

IC_{50} values are expressed as means ± s.e.mean of six observations conducted in triplicate. Hill coefficients are expressed as means (and range). CEC, chloroethylclonidine; 5-MU, 5-methyl-urapidil.

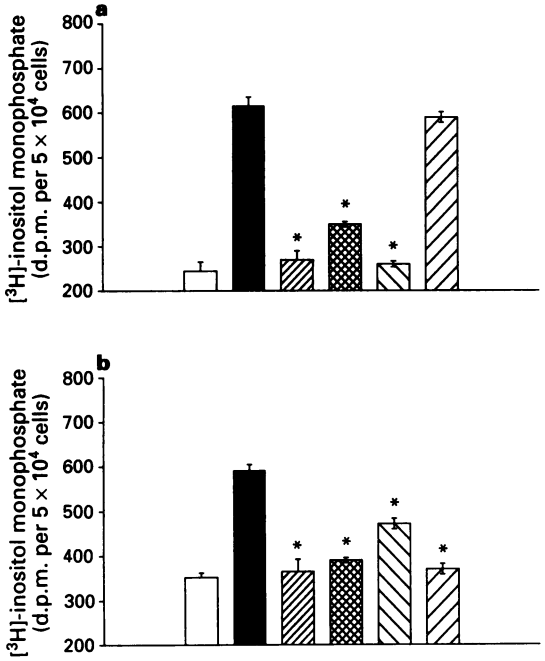


Figure 5 Effects of (−)-discretamine, 5-methyl-urapidil, chloroethylclonidine and prazosin on noradrenaline-stimulated inositol monophosphate accumulation in DDT_1MF-2 (a) and A10 (b) cells. Cells were pretreated with dimethylsulphoxide (0.1%, resting and noradrenaline control), prazosin (10 nM), 5-methyl-urapidil (100 nM) or (−)-discretamine (100 nM) for 15 min, or with chloroethylclonidine (1 μM) for 30 min. Noradrenaline (3 μM) was then added for another 30 min. Each column represents the mean with s.e.mean ($n = 6$). Resting (□); noradrenaline control (■); + prazosin (▨), + 5-methyl-urapidil (▩), + chloroethylclonidine (▧), + (−)-discretamine (▨).

Radioligand binding

[^{3}H]-prazosin at concentrations ranging from 0.01 to 5 nM was used to label α_1-adrenoceptors of hamster vas deferens smooth muscle (DDT_1MF-2) and rat aortic smooth muscle (A10) cells. The specific binding of [^{3}H]-prazosin was approximately 90% of the total binding at 0.2 nM [^{3}H]-prazosin and showed a saturable tendency at the concentrations of 3–5 nM. Scatchard plots of the binding data in the DDT_1MF-2 cells were linear, resulting in a better fit to a one-site model in computerized analysis with a K_D value of 0.24 ± 0.02 nM and a B_{max} of 80 ± 2 fmol mg^{-1} protein (Hill coefficient = 0.99). On the other hand, Scatchard plots of the binding data from A10 cells were curvilinear, suggesting more than a single class of binding sites. LIGAND analysis fitted the data to a two-site model. The high affinity site showed a B_{max} of 20.1 ± 5.3 fmol mg^{-1} protein with a K_D value 0.20 ± 0.02 nM, while the low affinity site showed a B_{max} of 72.2 ± 9.2 fmol mg^{-1} protein and K_D value of 2.51 ± 0.24 nM. Thus, [^{3}H]-prazosin 0.2 nM was used in the further studies. (−)-Discretamine, 5-MU, CEC and prazosin displaced specific [^{3}H]-prazosin binding to DDT_1MF-2 and A10 cells in a concentration-dependent manner (Figure 4) with IC_{50} values 390.8 ± 20.6, 43.6 ± 3.9, 200.0 ± 3.00 and 0.8 ± 0.1 nM, respectively in DDT_1MF-2 cells, and 25.0 ± 3.2, 8.6 ± 1.4, 1000.0 ± 30.8 and 0.52 ± 0.03 nM, respectively in A10 cells (Table 2). Pretreatment of cells with CEC (10 μM) for 30 min and thorough washing, reduced specific [^{3}H]-prazosin binding by 30% in A10 cells and 95% in DDT_1MF-2 cells. The CEC-insensitive [^{3}H]-prazosin binding in A10 cells was inhibited by (−)-discretamine with an IC_{50} value 7.0 ± 0.3 nM (Hill coefficient = 0.99; range 0.88–1.10).

[^{3}H]-inositol monophosphate formation

NA (3 μM) markedly stimulated [^{3}H]-inositol monophosphate (IP) accumulation in DDT_1MF-2 and A10 cells (Figure 5). 5-MU and prazosin at concentrations of 100 nM and 10 nM that almost completely inhibited [^{3}H]-prazosin binding to DDT_1MF-2 and A10 cells also markedly blocked NA-induced IP formation in these two cultured cells. CEC at a concentration of 1 μM almost completely blocked [^{3}H]-prazosin binding to and NA-induced IP formation of DDT_1MF-2 cells. In contrast, (−)-discretamine (100 nM) markedly inhibited [^{3}H]-prazosin binding to and NA-induced IP formation of A10 cells, without affecting [^{3}H]-prazosin binding to and NA-induced IP formation of DDT_1MF-2 cells (Figures 4 and 5).

Discussion

Molecular cloning techniques have revealed the existence of four genes coding for α_1-adrenoceptors, α_{1A} (rat: Lomasney *et al.*, 1991), α_{1B} (hamster: Cotecchia *et al.*, 1988; rat: Voigt *et al.*, 1990), α_{1C} (bovine: Schwinn *et al.*, 1990) and α_{1D} (rat: Perez *et al.*, 1991). Among them, the α_{1C}-adrenoceptor is not present in any rat tissue (Schwinn *et al.*, 1991). The subtype of α_1-adrenoceptor present in the rat aorta has been variously classified as α_{1B} (Han *et al.*, 1990), both α_{1A} and α_{1B} (Tian *et al.*, 1990; Piascik *et al.*, 1991) or atypical (Muramatsu *et al.*, 1991; Aboud & Docherty, 1992; Oriowo & Ruffolo, 1992). In the last three studies, CEC failed to reduce the maximum response to NA, but caused a parallel shift in the concentration-response curve. Recently, Aboud *et al.* (1993) suggest this receptor is a non-α_{1A}-, non-α_{1B}-adrenoceptor, and may represent an α_{1D}-adrenoceptor which is expressed in rat aorta (Perez *et al.*, 1991). The α_1-adrenoceptor subtypes possess different susceptibilities to 5-methyl-urapidil (5-MU), WB-4101 and CEC. The order of affinity of 5-MU and WB4101 for α_1-adrenoceptor subtypes were $\alpha_{1A} > \alpha_{1D}$, $\alpha_{1C} > \alpha_{1B}$ (Perez *et al.*, 1991). The alkylating agent CEC selectively inactivates the α_{1B} subtype in a non-competitive manner (Han *et al.*, 1987; Minneman, 1988). Unlike α_{1A}-adrenoceptors, α_{1D}-adrenoceptors could be extensively blocked by CEC (Perez *et al.*, 1991; Aboud *et al.*, 1993). In the present studies, contractions of rat aorta to NA possessed intermediate susceptibility to

5-MU blockade (Table 1). Furthermore, contractions of rat aorta to NA were inhibited by CEC in a competitive manner with a pA_2 value of 6.10 (Table 1). Thus, our data clearly support the idea that the non-α_{1A}-, non-α_{1B}-adrenoceptor (the putative α_{1D}-adrenoceptor) exists in rat aorta.

We have reported that (−)-discretamine is a selective α_1-adrenoceptor antagonist, which was isolated from *Fissistigma glaucescens* (Ko *et al.*, 1993). In this paper, we have further characterized its selectivity for various α_1-adrenoceptor subtypes. Contractions of the rat vas deferens to NA are usually used as a model for α_{1A}-adrenoceptors and contractions of the rat spleen are used as a model for α_{1B}-adrenoceptors. (−)-Discretamine antagonized NA-induced contractions of rat vas deferens and phenylephrine-induced contractions of rat spleen in a competitive manner with pA_2 values of 6.21 and 6.44, respectively (Table 1). In contrast, (−)-discretamine potently antagonized NA-induced contractions of rat aorta with a pA_2 value of 7.60. This implies that (−)-discretamine is a selective α_{1D}-adrenoceptor antagonist. This conclusion is further supported by the ligand binding studies. The α_1-adrenoceptor subtype of DDT_1MF-2 cell is α_{1B} subtype (Cotecchia *et al.*, 1988; Han *et al.*, 1992). (−)-Discretamine inhibited specific [^{3}H]-prazosin binding to DDT_1MF-2 and A10 cells with IC_{50} values of 390.8 ± 20.6 and 25.0 ± 3.2 nM, respectively, which are close to its pA_2 values, antagonized phenylephrine-induced rat spleen contractions and NA-induced rat aorta contractions. Furthermore, pretreatment of A10 cells with CEC (10 μM) for 30 min followed by thorough washing out of CEC, irreversibly inactivated α_{1B}-adrenoceptors. The CEC-insensitive [^{3}H]-prazosin binding in A10 cells was inhibited by (−)-discretamine with an IC_{50} value of 7.0 ± 0.3 nM. These data indicate that (−)-discretamine is indeed a selective α_{1D}-adrenoceptor antagonist.

The α_{1A}-adrenoceptor subtype has been suggested to gate Ca^{2+} influx through dihydropyridine-sensitive channels in smooth muscle while the α_{1B}-adrenoceptor subtype has been associated with formation of inositol 1,4,5-trisphosphate and mobilization of intracellular Ca^{2+} (Han *et al.*, 1987; 1990; Hanft & Gross, 1989; Tsujimoto *et al.*, 1989; Suzuki *et al.*, 1990). NA induced [^{3}H]-inositol monophosphate (IP) formation in both DDT_1MF-2 and A10 cells (Figure 5). (−)-Discretamine at a concentration of 100 nM, which blocked specific [^{3}H]-prazosin binding to A10 cells by 90%, almost completely suppressed NA-induced IP formation. On the other hand, (−)-discretamine (100 nM) only slightly but not significantly inhibited NA-induced IP formation in DDT_1-MF-2 cells. These data not only support the idea that (−)-discretamine is a selective α_{1D}-adrenoceptor antagonist but also imply that α_{1D}-adrenoceptor may cause phosphoinositides hydrolysis in rat aorta.

In conclusion, (−)-discretamine is a selective α_{1D}-adrenoceptor antagonist in vascular smooth muscle. Its selectivity among various α_1-adrenoceptor subtypes is α_{1A}:α_{1B}:α_{1D} = 0.04:0.07:1.0 based on the pA_2 values of functional studies (Table 1) and assumes the pA_2 value of aorta is 1.0. (−)-Discretamine may be a useful research tool for characterizing α_1-adrenoceptor subtypes

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Cutaneous vasodilatation induced by nitric oxide-evoked stimulation of afferent nerves in the rat

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1 The site of action at which nitric oxide (NO) may contribute to neurogenic vasodilatation in the hindpaw skin of urethane-anaesthetized rats was examined by the use of N^G-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO synthase.

2 Skin blood flow was measured by laser Doppler flowmetry, and neurogenic vasodilatation was evoked either by topical application of mustard oil (5%) or antidromic electrical stimulation of the saphenous nerve (antidromic vasodilatation).

3 L-NAME (60 μmol kg^{-1}, i.v.) attenuated the hyperaemia evoked by mustard oil in an enantiomer-specific manner but failed to reduce antidromic vasodilatation and the vasodilatation due to i.v. injected calcitonin gene-related peptide (CGRP) and substance P (0.1–1 nmol kg^{-1} each), two proposed mediators of neurogenic vasodilatation.

4 Pretreatment of rats with capsaicin (125 mg kg^{-1}, s.c. 2 weeks beforehand), to defunctionalize afferent neurones, reduced the hyperaemic response to mustard oil and prevented L-NAME from further decreasing the vasodilatation evoked by mustard oil.

5 Intraplantar infusion of sodium nitroprusside (SNP, 0.15 nmol in 1 min), a donor of NO, induced hyperaemia which was significantly diminished by the CGRP antagonist $CGRP_{8-37}$ (50 nmol kg^{-1}, i.v.) and by capsaicin pretreatment. The ability of $CGRP_{8-37}$ to inhibit the vasodilator response to SNP was lost in capsaicin-pretreated rats.

6 Taken together, these data indicate that NO does not play a vasorelaxant messenger role in neurogenic vasodilatation but can contribute to activation of, and/or transmitter release from, afferent nerve fibres in response to irritant chemicals.

Keywords: Nitric oxide; N^G-nitro-L-arginine methyl ester (L-NAME); capsaicin; mustard oil; sodium nitroprusside; calcitonin gene-related peptide (CGRP); substance P; afferent nerve stimulation; skin blood flow; antidromic vasodilatation

Introduction

Electrical or chemical stimulation of afferent nerves in the skin causes an inflammatory reaction that begins with a marked rise of cutaneous blood flow. The chemical irritant mustard oil has long been used in the study of neurogenic inflammation because its effect to increase skin blood flow and vascular permeability involves, at least in part, afferent nerves (Bruce, 1910; Jancsó *et al.*, 1977; Lembeck & Holzer, 1979; Lynn & Shakhanbeh, 1988; Louis *et al.*, 1989). Calcitonin gene-related peptide (CGRP) and substance P have been established as primary mediators of neurogenic inflammation in the skin (Holzer, 1992), and experiments involving immunoneutralization of CGRP and substance P have indicated that both peptides contribute to the vasodilator effect of mustard oil in the rat hindpaw skin (Louis *et al.*, 1989).

A previous study from this laboratory (Lippe *et al.*, 1993) has shown that the hyperaemic response to mustard oil is significantly attenuated by N^G-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide (NO) synthase (Gardiner *et al.*, 1990; Rees *et al.*, 1990). This finding as well as other observations (Hughes *et al.*, 1990; Khalil & Helme, 1992; Ralevic *et al.*, 1992) support the contention that neurogenic vasodilatation in the skin depends in part on the formation of NO as a vasorelaxant messenger. The precise role of NO remains enigmatic. In view of the reports that NO does not participate in the hyperaemic effect of CGRP (Ralevic *et al.*, 1992; Hughes & Brain, 1994) which is the chief mediator of antidromic and capsaicin-evoked vasodilatation (Delay-Goyet *et al.*, 1992; Escott & Brain, 1993; Hughes & Brain, 1994) the present study was undertaken to examine systematically a possible participation of NO in the hyperaemic responses to antidromic nerve stimulation and to application of mustard oil, CGRP and substance P. The possible site of action of NO has also been evaluated. The latter study involved sodium nitroprusside (SNP), a donor of NO, which has been reported to dilate cerebral arterioles in part via release of CGRP (Wei *et al.*, 1992).

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Methods

Animal preparation

The experiments in this study were approved by the Austrian Ministry of Science and Research. Female Sprague-Dawley rats (Versuchstierzuchtanstalt Himberg, Austria) weighing 180–220 g were anaesthetized with 1.5 g kg^{-1} urethane injected s.c. and fitted with a tracheal cannula. A catheter in the left jugular vein was used for the continuous infusion of saline (1.5 ml h^{-1}), to avoid dehydration and for the i.v. injection of drugs. Mean arterial blood pressure (MAP) was recorded from a catheter inserted in the right carotid artery and connected to a pressure transducer. Rectal temperature was maintained at 37–38°C by automatic control of a heating pad.

Capsaicin pretreatment

To destroy the function of fine afferent neurones, rats were pretreated s.c. with a total dose of 125 mg kg^{-1} capsaicin 2 weeks before the experiments (Holzer *et al.*, 1992). This dose of capsaicin was given in 4 injections over 2 days (first day: 25 mg kg^{-1} in the morning and 25 mg kg^{-1} in the late after-

noon, second day: 25 mg kg^{-1} in the morning and 50 mg kg^{-1} in the late afternoon). The control rats received equal volumes (2 + 2 + 2 + 4 ml kg^{-1}) of vehicle (10% ethanol, 10% Tween 80 and 80% saline; vol/vol/vol). All injections of capsaicin and its vehicle were performed under deep ether anaesthesia. To counteract the respiratory impairment associated with the administration of capsaicin, the rats received an intraperitoneal injection of atropine (0.2 mg kg^{-1}), terbutaline (0.2 mg kg^{-1}) and aminophylline (20 mg kg^{-1}) 15 min before the first and third capsaicin and vehicle injections (Esplugues *et al.*, 1989).

Antidromic stimulation of the saphenous nerve

Antidromic stimulation of the cut saphenous nerve was performed as described previously (Lembeck & Holzer, 1979; Delay-Goyet *et al.*, 1992; Escott & Brain, 1993). Briefly, the right saphenous nerve was carefully dissected in the leg and cut, and the peripheral stump placed on bipolar platinum electrodes and immersed in paraffin oil. Electrical stimulation was performed with pulses of 15 V strength and 1 ms duration delivered at 2 Hz for a period fo 30 s (Delay-Goyet *et al.*, 1992; Escott & Brain, 1993).

Topical and intraplantar administration of substances

Mustard oil (5% in paraffin oil, wt/wt) was applied with a cotton bud, to the dorsal side of the hindpaw close to the laser optic probe. For intraplantar administration of drugs a catheter fitted with an injection needle (outside diameter 0.3 mm) was inserted from the distal aspect of the hindpaw into the middle of the plantar subcutaneous space. Substances were administered by infusion of a volume of 15 µl over a period of 1 min with an infusion pump. Thus it was possible to record blood flow continuously, without injection artifacts, during and after the intraplantar administration of drugs.

Laser Doppler flowmetry

Blood flow in the skin of the hind paw was recorded with a laser Doppler flowmeter (model MBF3D, Moor Instruments, Devon, U.K.). To this end, a stainless steel laser optic probe (model P1, Moor Instruments) was taped perpendicularly to the skin of the hindpaw, the topographical position depending on the type of experiment. In order to measure blood flow changes in response to mustard oil or electrical stimulation of the saphenous nerve, the laser probe was positioned on the medio-lateral aspect of the plantar side of the hindpaw. When the effect of intraplantar drugs was studied, the probe was placed on the centre of the plantar side of the hindpaw in close vicinity to the tip of the infusion needle. After completion of the experimental preparations an equilibration period of at least 20 min was allowed before the experiments commenced. Blood flow was monitored as flux, the time constant being 0.5 s, and changes in blood flow were expressed as percentage changes in flux (Escott & Brain, 1993) relative to the average values recorded during the 2 min-period immediately before the changes were induced.

Statistics

All data are expressed as means ± s.e.mean. Statistical evaluation of the results was performed with the Mann-Whitney U test, when two independent groups of data were compared with each other, or with the Kruskal-Wallis H test followed by the Mann-Whitney U test, when more than two independent groups of data were compared with each other. The Wilcoxon test for pair differences was used when data obtained before and after drug treatment were compared with each other. Probability values $P<0.05$ were regarded as significant.

Substances and solutions

The saline solution was 0.9% NaCl (wt/wt). Aminophylline (20 mg ml^{-1}; Sigma, Deisenhofen, Germany), atropine (0.2 mg ml^{-1}; Merck, Darmstadt, Germany) and terbutaline (0.2 mg ml^{-1}; Astra, Södertälje, Sweden) were dissolved in saline at the concentrations indicated. Capsaicin (Serva, Heidelberg, Germany) was dissolved in absolute ethanol, followed by addition of Tween 80 and saline, the final concentration being 12.5 mg ml^{-1} capsaicin in 10% ethanol, 10% Tween 80 and 80% saline (v/v/v). The vehicle solution, without capsaicin, was prepared in the same manner. Mustard oil (allyl isothiocyanate; Fluka, Buchs, Switzerland) was diluted (5%, v/v) with paraffin oil. Urethane (Fluka, Buchs, Switzerland) was dissolved in water (25%, wt/wt).

D-NAME (N^G-nitro-D-arginine methyl ester) and L-NAME (N^G-nitro-L-arginine methyl ester) were obtained from Bachem (Bubendorf, Switzerland) and dissolved in saline to give solutions of 60 mM which were injected i.v. at a volume of 1 ml kg^{-1}. Sodium nitroprusside (SNP, 1 mM; Merck, Darmstadt, Germany) was also dissolved in saline. Rat α-CGRP (calcitonin gene-related peptide), human $CGRP_{8-37}$ and substance P (Bachem, Bubendorf, Switzerland) were dissolved in 0.02 M acetic acid and diluted with saline. The tachykinin NK_1 receptor antagonist RP-67,580 ([3aR,7aR]-7,7-diphenyl-2-[1-imino-2-(2-methoxyphenyl)ethyl] perhydroisoindol-4-one, Rhône-Poulenc Rorer, Vitry-sur-Seine, France) was dissolved in one part of 0.1 M HCl, to which 9 parts of distilled water were added to give a stock solution of 10 mM RP-67,580. For intravenous administration of RP-67,580, the stock solution was diluted five fold with Tyrode buffer solution (composition, mM NaCl 136.9, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.0, $NaHCO_3$ 11.9, NaH_2PO_4 0.4). The vehicle solution was prepared in the same manner.

Results

Effect of intraplantar versus intravenous L-NAME on cutaneous blood flow

Initial experiments were carried out to compare the effects of intraplantar and i.v. administration of L-NAME on cutaneous blood flow and mean arterial blood pressure (MAP). Intraplantar L-NAME (95 nmol) led, after a brief initial rise, to a 45% reduction of plantar blood flow (Table 1), an effect that became maximal after 19 ± 3 min ($n = 6$). This pronounced attenuation of blood flow was associated with a modest increase in MAP. I.v. injection of L-NAME, at a dose (60 µmol kg^{-1}) that had previously been found to be maximally effective in causing hypertension (Holzer *et al.*, 1993), caused a marked rise of MAP which was accompanied by a small increase, rather than a decrease, in cutaneous blood flow. Pretreatment of rats with capsaicin or its vehicle did not alter the ability of i.v. L-NAME to elevate MAP and blood flow (Table 1). The enantiomer D-NAME (60 µmol kg^{-1}, i.v.) did not change blood flow but led to a delayed and gradual rise of MAP (Table 1) whereas the hypertensive response to L-NAME was immediate in onset and sustained. Saline (1 ml kg^{-1}, i.v.) was without effect on cutaneous blood flow and MAP.

Effect of L-NAME and capsaicin pretreatment on mustard oil-induced hyperaemia

Topical administration of mustard oil (5%) caused a rapid rise of cutaneous blood flow that reached a peak within a few minutes and then stayed roughly constant during the observation period of 15 min. This hyperaemic response was accompanied by a transient increase of MAP (Figure 1). The experiments designed to examine the effects of saline, D-NAME and L-NAME on the mustard oil-induced hyperaemia were initiated by applying mustard oil to the

Table 1 Effect of saline, N^G-nitro-D-arginine methyl ester (D-NAME) and N^G-nitro-L-arginine methyl ester (L-NAME) on cutaneous blood flow (CBF) and mean arterial blood pressure (MAP)

Pretreatment	*Acute treatment*	*CBF* (% of control) n	*after*	*MAP* (mmHg) n	*before*	*after*
None	Saline intraplantar	9	105.2 ± 8.5	9	107 ± 2.6	109 ± 2.9
	L-NAME intraplantar	6	54.4 ± 9.8**	6	106 ± 6.6	118 ± 5.1*
	Saline i.v.	23	96.8 ± 3.0	21	96 ± 3.2	96 ± 3.3
	D-NAME i.v.	20	106.2 ± 4.5	17	92 ± 2.2	105 ± 2.3**
	L-NAME i.v.	25	135.7 ± 14.0*	22	92 ± 2.6	141 ± 2.5**
Vehicle for capsaicin	L-NAME i.v.	8	129.5 ± 12.7	8	103 ± 6.3	142 ± 4.2**
Capsaicin	L-NAME i.v.	8	133.9 ± 12.9*	8	103 ± 3.5	139 ± 5.5**

The changes of CBF and MAP induced by intraplantar infusion of saline (15 µl given during a period of 1 min) and L-NAME (95 nmol) reflect the maximal changes that were observed in the period 0–30 min post-infusion. The changes induced by i.v. injection of saline (1 ml kg^{-1}), D-NAME and L-NAME (60 µmol kg^{-1} each) refer to the changes measured 30 min post-injection. Rats were pretreated with capsaicin (125 mg kg^{-1}, s.c.) or its vehicle (10% ethanol, 10% Tween 80 and 80% saline; v/v/v) 2 weeks before the experiments. The values shown are means ± s.e.mean of *n* animals as indicated. $*P<0.05$; $**P<0.01$ versus before acute treatment (Wilcoxon test for pair differences).

right hindpaw and recording blood flow and MAP for a period of 15 min. Then saline (1 ml kg^{-1}), D-NAME or L-NAME (60 µmol kg^{-1} each) was injected i.v. followed 15 min later by application of mustard oil to the left hindpaw and recording blood flow in this paw. The rise of blood flow and MAP evoked by application of mustard oil did not differ significantly between the right and left hindpaw when saline or D-NAME was injected i.v. in between the two applications of mustard oil. In contrast, L-NAME diminished the cutaneous vasodilator reaction to mustard oil by 54% (Figure 2). The average hypotensive reaction to mustard oil was also lessened by L-NAME but this change, which is most probably related to the sustained hypertension caused by L-NAME (Table 1), was not statistically significant.

Responses of rats pretreated with the vehicle for capsaicin did not differ from those of untreated rats with respect to basal cutaneous blood flow (data not shown), basal MAP (Table 1), the vasodilatation and transient hypertension due to mustard oil and the ability of L-NAME to attenuate the mustard oil-evoked hyperaemia (Figure 2). Capsaicin pretreatment caused a 47% reduction of the effect of mustard oil to increase cutaneous blood flow when compared with the respective effect in vehicle-pretreated rats (Figure 2). I.v. injection of L-NAME to capsaicin-pretreated animals failed to inhibit further the hyperaemic reaction to mustard oil (Figure 2). The transient rise of MAP evoked by mustard oil was totally absent in capsaicin-pretreated rats (Figure 2) whereas basal MAP (Table 1) and basal cutaneous blood flow (not shown) were not altered by capsaicin pretreatment.

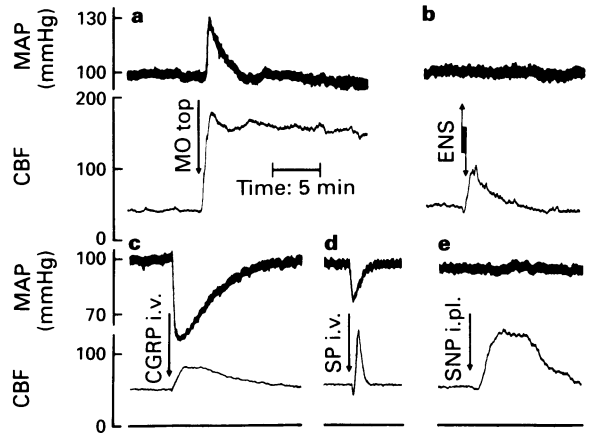


Figure 1 Recordings of the effects of (a) mustard oil (MO, 5% in paraffin oil) applied topically (top) to the skin, (b) antidromic electrical stimulation of the saphenous nerve (ENS, pulses of 15 V and 1 ms delivered at 2 Hz for 30 s), (c) i.v. injection of calcitonin gene-related peptide (CGRP, 0.3 nmol kg^{-1}) and (d) substance P (SP, 0.3 nmol kg^{-1}) and (e) intraplantar (i.pl.) administration of sodium nitroprusside (SNP, 0.15 nmol in 15 µl infused over a period of 1 min) on cutaneous blood flow (CBF) in the rat hindpaw and mean arterial blood pressure (MAP). CBF was measured by laser Doppler flowmetry and expressed in arbitrary flux units. Each tracing is from a separate rat.

Effect of L-NAME on antidromic vasodilatation

Antidromic vasodilatation was elicited by electrical stimulation of the saphenous nerve for a period of 30 s at 25 min-intervals. This procedure increased cutaneous blood flow (Figure 1) in a manner similar to that seen in other studies (Delay-Goyet *et al.*, 1992; Escott & Brain, 1993). Fifteen minutes after the recording of two episodes of antidromic vasodilatation, saline (1 ml kg^{-1}) or L-NAME (60 µmol kg^{-1}) was injected i.v. and the cycle of electrical stimulation of the saphenous nerve continued after a wait of 10 min. As is shown in Table 2, antidromic vasodilatation did not change significantly after the i.v. administration of saline or L-NAME, and the hyperaemic response to electrical nerve stimulation stayed constant for a period of 60 min post-treatment.

Effect of L-NAME on the hyperaemic responses to CGRP and substance P

The effect of L-NAME on the dermal hyperaemia evoked by CGRP and substance P was tested with a previously reported protocol (Holzer *et al.*, 1993). After priming the animals with three i.v. injections of rat α-CGRP or subtance P (300 pmol kg^{-1}) at 15-min intervals, to ensure reproducible

Table 2 Effect of N^G-nitro-L-arginine methyl ester (L-NAME) on antidromic vasodilatation evoked by electrical stimulation of the saphenous nerve (pulses of 15 V and 1 ms delivered at 2 Hz for 30 s)

Treatment	*Antidromic vasodilatation* (% of control) after 10 min	35 min	60 min
Saline	91 ± 6.9	90 ± 6.5	101 ± 10.8
L-NAME	117 ± 15.1	111 ± 15.9	122 ± 22.0

Antidromic vasodilatation was evoked at 25-min intervals before and after the i.v. injection of saline (1 ml kg^{-1}) or L-NAME (60 µmol kg^{-1}). The vasodilator response measured 15 min before the injection of saline or L-NAME was taken as 100%, and the responses measured post-treatment were expressed as a percentage of that control response. The values shown are means ± s.e.mean of 5 rats in each group.

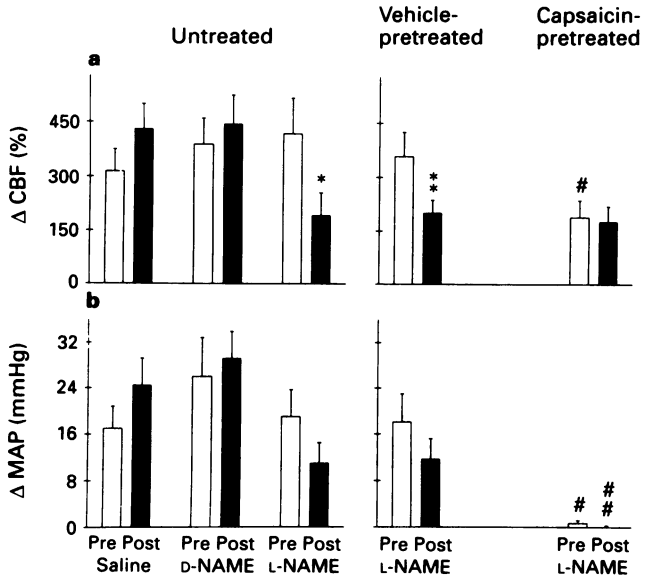


Figure 2 Effect of i.v. injection of saline (1 ml kg^{-1}), N^G-nitro-D-arginine methyl ester (D-NAME) and N^G-nitro-L-arginine methyl ester (L-NAME; 60 μmol kg^{-1} each) on the increment of cutaneous blood flow (ΔCBF) (a) and mean arterial blood pressure (ΔMAP) (b) evoked by topical application of mustard oil (5% in paraffin oil) to the rat hindpaw. The responses 'pre'-treatment were evoked by mustard oil application to the right hindpaw and are shown by the open columns. The responses 'post'-treatment were evoked by mustard oil application to the left hindpaw 15 min after the injection of saline, D-NAME or L-NAME and are shown by the solid columns. The experiments were carried out with untreated rats and rats that had been pretreated with vehicle (10% ethanol, 10% Tween 80 and 80% saline; v/v/v) or capsaicin (125 mg kg^{-1}, s.c.) 2 weeks before the experiments. The values shown are means with s.e.mean; $n = 6-8$. $^*P<0.05$; $^{**}P<0.01$ versus 'pre'-treatment (Wilcoxon test for pair differences); $^{\#}P<0.05$; $^{\#\#}P<0.01$ versus respective values in vehicle-pretreated rats (evaluated by the Mann-Whitney U test after the existence of a statistically significant difference between all groups had been revealed by the Kruskal-Wallis H test).

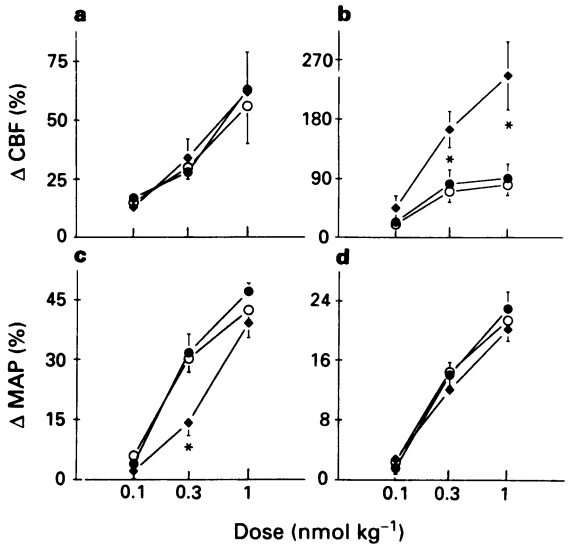


Figure 3 Dose-dependent changes in cutaneous blood flow (Δ CBF) and mean arterial blood pressure (Δ MAP) evoked by i.v. injection of calcitonin gene-related peptide (CGRP) (a, c) and substance P (b, d) to rats treated with either saline (○), N^G-nitro-D-arginine methyl ester (●, D-NAME) or N^G-nitro-L-arginine methyl ester (◆, L-NAME). Saline (1 mg kg^{-1}), D-NAME and L-NAME (60 μmol kg^{-1} each) were injected i.v. 15 min before the recording of the dose-response curves for CGRP and substance P was begun. The values shown are means with s.e.mean; $n = 5-8$. $^*P<0.05$ versus respective values recorded in rats treated with saline (evaluated by the Mann-Whitney U test after the existence of a statistically significant difference between rats treated with saline, D-NAME and L-NAME had been revealed by the Kruskal-Wallis H test).

vasodilator effects on repeated injection of the peptides (Holzer *et al.*, 1993), saline (1 ml kg^{-1}), D-NAME or L-NAME (60 μmol kg^{-1} each) was given by i.v. injection. After a wait of 15 min, dose-response curves for the hyperaemic effects of CGRP or substance P were recorded by i.v. injection of increasing doses of CGRP and substance P (0.1–1 nmol kg^{-1} in volumes of 1 ml kg^{-1}) at 15-min intervals. CGRP and substance P caused dose-dependent increases of cutaneous blood flow which were accompanied by dose-dependent decreases of MAP (Figures 1 and 3). The two peptides were roughly equipotent but their effects differed with respect to magnitude and duration (Figures 1 and 3). I.v. injection of the vehicle (saline, 1 ml kg^{-1}) was devoid of any effect on blood flow and MAP ($n = 12$, data not shown).

L-NAME failed to alter the potency of CGRP in augmenting blood flow in the skin when compared with the activity of CGRP in rats treated with saline. However, the ability of CGRP to lower MAP was attenuated by L-NAME to a minor but significant extent as deduced from a parallel rightward shift of the pertinent dose-response curve (Figure 3). In contrast, D-NAME was without influence on the CGRP-evoked hyperaemia and hypotension (Figure 3). The effects of substance P in causing cutaneous hyperaemia remained unchanged by D-NAME but were significantly enhanced by L-NAME (Figure 3). The hypotension which accompanied the hyperaemic effect of substance P was not modified by L- or D-NAME.

Effects of $CGRP_{8-37}$, RP-67,580 and capsaicin pretreatment on the cutaneous hyperaemia induced by sodium nitroprusside

The haemodynamic effect of sodium nitroprusside (SNP) was explored by repeated intraplantar infusion (15 μl in 1 min) of the substance at 15 min-intervals. Initially two or three infusions of the drug were performed to establish reproducible blood flow responses. SNP caused a transient rise of cutaneous blood flow in the absence of any change in MAP (Figure 1). Preliminary experiments had established that the vasodilator effect of SNP was dose-dependent, 0.015 nmol SNP enhancing cutaneous blood flow by 15 ± 5% ($n = 7$, $P<0.05$) compared with a rise of +115 ± 15% ($n = 7$, $P<0.01$) evoked by 0.15 nmol SNP. The latter dose was chosen as test dose in the further experiments. Intraplantar administration of the vehicle (saline, 15 μl) enhanced blood flow to an insignificant extent (+12 ± 6%, $n = 9$, $P>0.05$) and had no effect on MAP.

The effect of $CGRP_{8-37}$ (50 nmol kg^{-1}; Delay-Goyet *et al.*, 1992) on the vasodilator response to SNP was tested by injecting this CGRP antagonist i.v. 2 min before administration of SNP. $CGRP_{8-37}$ led to a small but significant elevation of skin blood flow (+20 ± 4%, $n = 24$, $P<0.01$) and MAP (+5 ± 1 mmHg, $n = 24$, $P<0.01$) whereas blood flow and MAP did not change significantly after i.v. injection of the vehicle (saline, 1 ml kg^{-1}; $n = 7$). The SNP-induced hyperaemia was attenuated by 41% after administration of $CGRP_{8-37}$ but was left unaltered by the vehicle (Figure 4). The ability of intraplantar SNP to augment cutaneous blood flow and the effect of $CGRP_{8-37}$ to inhibit partially the SNP-induced hyperaemia did not differ between untreated rats and rats pretreated with the vehicle for capsaicin (Figure 4). Pretreatment of animals with capsaicin, however, decreased the vasodilator reaction to SNP by 53% when

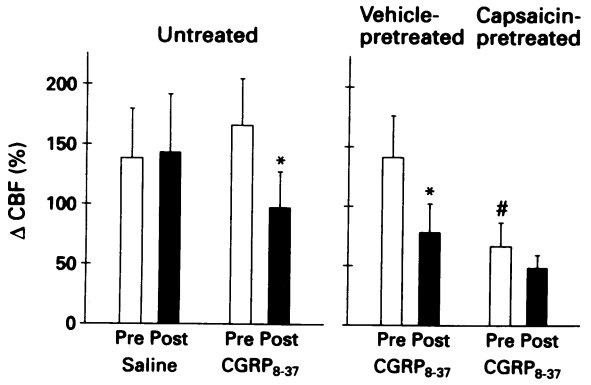


Figure 4 Effect of i.v. injection of saline (1 ml kg^{-1}) and $CGRP_{8-37}$ (50 nmol kg^{-1}) on the increment of cutaneous blood flow (ΔCBF) evoked by intraplantar administration of sodium nitroprusside (SNP, 0.15 nmol infused over a period of 1 min). $CGRP_{8-37}$ was injected 2 min before SNP. The experiments were carried out with untreated rats and rats that had been pretreated with vehicle (10% ethanol, 10% Tween 80 and 80% saline; v/v/v) or capsaicin (125 mg kg^{-1}, s.c.) 2 weeks before the experiments. The values shown are means with s.e.mean; $n = 6-7$. $^{*}P<0.05$ versus 'pre'-treatment (Wilcoxon test for pair differences); $^{\#}P<0.05$ versus respective value in vehicle-pretreated rats (evaluated by the Mann-Whitney U test after the existence of a statistically significant difference between all groups had been revealed by Kruskal-Wallis H test).

compared with that found in vehicle-pretreated rats (Figure 4). $CGRP_{8-37}$ was no longer able to inhibit significantly the SNP-evoked hyperaemia in capsaicin-pretreated rats (Figure 4).

The substance P antagonist, RP-67,580 (2 μmol kg^{-1}, i.v.; Moussaoui *et al.*, 1993) caused a small elevation of MAP (+6 ± 2 mmHg, $n = 18$, $P<0.01$) and cutaneous blood flow (+10 ± 3%, $n = 18$, $P<0.01$) as measured 5 min post-treatment. However, RP-67,580 failed to alter significantly the SNP-evoked hyperaemia in the skin, the increase in blood flow before RP-67,580 being +110 ± 25% and that measured after RP-67,580 being +95 ± 30% ($n = 6$).

The specificity and effectiveness of $CGRP_{8-37}$ and RP-67,580 were evaluated by the i.v. injection of test doses of CGRP and substance P (0.3 nmol kg^{-1}) at 15 min-intervals. After establishing a reproducible hyperaemic response to these agonist doses, $CGRP_{8-37}$ was injected 2 min, and RP-67,580 5 min, before another application of the respective agonists. $CGRP_{8-37}$ reduced the CGRP-evoked increment of cutaneous blood flow from +80 ± 13% to +22 ± 5% ($n = 6$, $P<0.01$) but failed to affect that to substance P (+143 ± 19% before $CGRP_{8-37}$, +151 ± 19% after $CGRP_{8-37}$, $n = 5$). Conversely, RP-67,580 decreased the substance P-induced rise of cutaneous blood flow from +141 ± 28% to +13 ± 8% ($n = 6$, $P<0.01$) but did not alter that evoked by CGRP (+55 ± 7% before RP-67,580, +60 ± 7% after RP-67,580, $n = 5$). The effects of CGRP and substance P on MAP were also specifically inhibited by $CGRP_{8-37}$ and RP-67,580, respectively.

Discussion

The present findings with the NO synthase inhibitor L-NAME confirm that the cutaneous hyperaemia evoked by topical mustard oil depends in part on the formation of endogenous NO (Lippe *et al.*, 1993). The inhibitory effect of L-NAME was enantiomer-specific as D-NAME which is said to be inactive on NO synthase (Gardiner *et al.*, 1990; Rees *et al.*, 1990) did not interfere with mustard oil-induced vasodilatation. D-NAME, however, was not totally inactive since it gave rise to a slowly and gradually developing hypertension, an unexplained effect that has been noted previously (Holzer *et al.*, 1993). L-NAME was given systemically since this route of administration did not diminish, but rather enhanced, cutaneous blood flow whereas local application of the drug reduced blood flow as has been observed in other studies (Hughes *et al.*, 1990; Lawrence & Brain, 1992). The use of s.c. L-NAME was regarded as impractible, therefore, given that local L-NAME could inhibit vasodilator responses solely by virtue of its vasoconstrictor effect.

The vascular responses to topical mustard oil are thought to arise in part from stimulation of afferent nerve fibres and release of vasodilator transmitter substances from their peripheral endings (Bruce, 1910; Jancsó *et al.*, 1977; Lembeck & Holzer, 1979; Louis *et al.*, 1989). Pretreatment of rats with a neurotoxic dose of capsaicin (Holzer, 1991) confirmed that a component of the vasodilator response to mustard oil was mediated by fine afferent nerve fibres. This observation is consistent with other findings indicating that the ability of mustard oil to augment vascular permeability in the skin is reduced but not abolished by pretreatment of adult animals with capsaicin (Jancsó *et al.*, 1977; Lynn & Shakhanbeh, 1988). In contrast, the transient rise of MAP evoked by mustard oil was abolished by capsaicin pretreatment, which suggests that the pressor response to mustard oil is another reflection of the involvement of capsaicin-sensitive afferent nerve fibres in cardiovascular reflexes activated by noxious stimuli (Holzer, 1992).

The combination of capsaicin pretreatment and i.v. administration of L-NAME established that it is the neurogenic component of the mustard oil-evoked hyperaemia that depends on the formation of NO. This conclusion is in keeping with the hypothesis that, while CGRP and substance P are the primary neurogenic mediators of mustard oil-induced vasodilatation (Louis *et al.*, 1989), NO is a secondary vasorelaxant messenger of neurogenic vasodilatation in the skin (Lippe *et al.*, 1993). However, this hypothesis was not supported by the inability of L-NAME to inhibit the increase of cutaneous blood flow elicited by antidromic stimulation of the saphenous nerve. NO has likewise been ruled out as a contributor to antidromic vasodilatation in the pulp and lip of the rat, which is not reduced but augmented by L-NAME (Kerezoudis *et al.*, 1993). The chief, if not exclusive, mediator of antidromic vasodilatation evoked by saphenous nerve stimulation (Delay-Goyet *et al.*, 1992; Escott & Brain, 1993) or local application of capsaicin (Hughes & Brain, 1991; 1994) is CGRP. It was not unexpected, therefore, to find that the cutaneous hyperaemia due to CGRP was also independent of NO as has been noted in other studies (Ralevic *et al.*, 1992; Hughes & Brain, 1994). However, L-NAME caused a slight depression of the hypotensive effect of CGRP, an effect that reflects the participation of NO in the vasodilator action of CGRP in certain non-cutaneous vascular beds (Gardiner *et al.*, 1991; Holzer *et al.*, 1993). In contrast, the ability of substance P to lower MAP remained unaltered by L-NAME (this study) and N^{G}-nitro-L-arginine (Santicioli *et al.*, 1993) but was attenuated by N^{G}-monomethyl-L-arginine (Whittle *et al.*, 1989). These discrepant data as well as the ability of L-NAME to enhance the vasodilator activity of substance P in the hind-paw skin in a way similar to that seen in the pulp and oral mucosa (Kerezoudis *et al.*, 1993) are not understood, and it was beyond the scope of the present study to explore the interaction between L-NAME and substance P in more detail. Here it was important to know that the hyperaemic action of substance P, another supposed neurogenic mediator of the mustard oil-evoked hyperaemia (Louis *et al.*, 1989), did not depend on the formation of NO.

The data discussed thus far indicate that NO participates in the neurogenic component of the vasodilator response to the chemical irritant mustard oil, but has no role in the vasodilatation elicited by electrical nerve stimulation and in the vasodilatation evoked by the neuropeptides CGRP and substance P. Provided that antidromic vasodilatation and the

neurogenic component of the mustard oil-induced hyperaemia arise from the same neurovascular transmission process it would appear, therefore, that NO is not a vasorelaxant messenger of neurogenic vasodilatation but is involved in the mustard oil-evoked excitation of, or peptide release from, afferent nerve fibres. An identical conclusion has been reached with regard to the hyperaemic action of another chemical irritant, capsaicin, in the rabbit skin (Hughes & Brain, 1994). The emerging hypothesis, that NO plays an important regulatory role in peptide release from afferent nerve fibres in response to chemical irritants, raises the question as to the cellular location of the NO synthase that is stimulated by mustard oil and capsaicin. As discussed by Hughes & Brain (1994) it seems most probable that NO is formed either in cells adjacent to afferent nerve fibres or in the nerve fibres themselves. This question cannot be settled on the basis of the available data, but it is worth considering that NO can be formed in mast cells (Salvemini *et al.*, 1991), which could be a non-neural source of NO generated in response to chemical irritants, and in afferent neurones themselves (Morris *et al.*, 1992; Verge *et al.*, 1992; Vizzard *et al.*, 1994). Important in the latter context is the finding that the bradykinin- and capsaicin-induced production of guanosine 3':5'-cyclic monophosphate in cultured primary afferent neurones is blocked by an inhibitor of NO synthase (Bauer *et al.*, 1993), which demonstrates that NO can be formed in stimulated afferent neurones and may play an important role in the chemical activation of sensory neurones and, further on, release of peptide transmitters from them.

The proposed role of NO in the chemical activation of neurogenic vasodilator responses was substantiated by the observation that the hyperaemia caused by the NO donor, SNP, was in part inhibited by the specific CGRP antagonist, $CGRP_{8-37}$ and by capsaicin-induced defunctionalization of afferent nerve fibres. This and the finding that $CGRP_{8-37}$ was no longer able to inhibit the vasodilator effect of SNP in capsaicin-pretreated animals indicate that SNP can dilate blood vessels in the rat skin by releasing CGRP from afferent nerve fibres, a conclusion that also holds true for the SNP-evoked dilatation of cerebral arterioles in the cat (Wei *et al.*, 1992). It is important to consider that the mechanism of SNP-evoked hyperaemia may be subject to species and tissue differences, since the vasodilator effect of SNP in the rabbit skin was not antagonized by $CGRP_{8-37}$ (Hughes & Brain, 1994). Unlike CGRP, substance P does not seem to play a role in the hyperaemic response to SNP in the rat skin, because the tachykinin NK_1 receptor antagonist, RP-67,580 (Moussaoui *et al.*, 1993), failed to antagonize SNP. RP-67,580 was chosen because the microvascular effects of substance P appear to be mediated by NK_1 receptors and RP-67,580 is a potent and selective antagonist at the rat variant of the NK_1 receptor (Maggi *et al.*, 1993; Moussaoui *et al.*, 1993). The significance of the small hyperaemic and hypertensive effects of RP-67,580 and $CGRP_{8-37}$, which were observed here, and their possible physiological implications await further clarification.

In conclusion, the present study has shown that NO or a related product of the L-arginine:NO pathway participates in the neurogenic component of the cutaneous vasodilator response to the irritant mustard oil. NO, however, does not play a vasorelaxant messenger role but contributes to the irritant-elicited excitation of, or release of vasodilator peptides from, afferent nerve fibres. The source of NO generated in response to chemical irritants remains to be identified.

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ET_B but not ET_A receptor-mediated contractions to endothelin-1 attenuated by respiratory tract viral infection in mouse airways

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1 The current study investigated the effects of respiratory tract viral infection on the density of ET_A and ET_B receptors in murine tracheal smooth muscle and on the contractile response to endothelin-1 mediated by these receptors.

2 Quantitative autoradiographic studies using [^{125}I]-endothelin-1 revealed that tracheal smooth muscle from control mice contained ET_A and ET_B receptors in the ratio of 42%:58% ($\pm$ 4%, $n = 10$ mice), respectively. In contrast, tracheal smooth muscle obtained from mice 2 days post-inoculation with Influenza A/PR-8/34 virus contained 23 $\pm$ 2% fewer receptors for [^{125}I]-endothelin-1 ($n = 10$, $P < 0.01$). This reflected a selective reduction in ET_B receptor density and a change in the ratio of ET_A and ET_B receptors to 77%:23% ($\pm$ 5%, $n = 10$ mice), respectively.

3 The ET_B receptor-selective agonist, sarafotoxin S6c, was a potent spasmogen of murine isolated tracheal smooth muscle and the EC_{50} for contraction was similar in preparations from control (3.6 nM [95% confidence limits, 2.7–4.8 nM], $n = 16$ preparations from 8 mice) and virus-inoculated mice (3.0 nM [2.4–3.7 nM], $n = 16$ preparations from 8 mice). However, the maximum contractions induced by sarafotoxin S6c (100 nM) in the preparations from virus-inoculated mice (37 $\pm$ 5% C_{max}, where 100% C_{max} was the response to 10 μM carbachol) were significantly smaller than those from control mice (85 $\pm$ 4% C_{max}, $P < 0.01$).

4 Contractions induced by endothelin-1 in tracheal smooth muscle preparations obtained from virus-inoculated mice (EC_{50} for contraction, 6.5 nM [95% confidence limits, 2.7–16 nM]; maximum contraction, 112 $\pm$ 5% C_{max}; $n = 4$) were similar to those induced by endothelin-1 in control preparations (EC_{50} 9.3 nM (4.2–21); maximum contraction, 110 $\pm$ 3% C_{max}; $n = 4$). Endothelin-1-induced contractions in control preparations were only marginally inhibited by the ET_A receptor-selective antagonist BQ-123 (in the presence of 3 μM BQ-123; EC_{50} for contraction, 5.9 nM [4.1–8.5]; maximum contraction, 82 $\pm$ 4% C_{max}; $n = 4$). In contrast, 3 μM BQ-123 caused a 50 fold rightward shift (17–160, $n = 4$) of the concentration-effect curve to endothlin-1 in preparations obtained from virus-inoculated mice (measured at the 30% C_{max} level of contraction).

5 Tracheal smooth muscle preparations exposed to 100 nM sarafotoxin S6c for 30 min (followed by a 30 min washout period) did not contract to subsequently administered sarafotoxin S6c (1–100 nM; $n = 8$), but contracted normally in response to endothelin-1 (EC_{50} 6.5 nM (2.3–18); maximum contraction, 109 $\pm$ 2% C_{max}; $n = 4$). Endothelin-1-induced contractions in these ET_B receptor desensitized preparations were markedly inhibited by 3 μM BQ-123, irrespective of whether the preparations were obtained from control (63 fold shift (10–400) at the 30% C_{max} level of contraction, $n = 4$) or virus-inoculated mice (46 fold shift (18–120), $n = 4$).

6 In summary, tracheal smooth muscle obtained from mice infected with a respiratory tract virus, Influenza A/PR-8/34 had a reduced density of ET_B receptors and an attenuated ET_B receptor-mediated contractile response to sarafotoxin S6c and endothelin-1. Virus-inoculation was also associated with a modest increase in tracheal smooth muscle ET_A receptor density, although no significant change in ET_A receptor-mediated contractile activity was seen. Thus, virus infection in murine airways produced profound alterations in endothelin receptor density, some of which were associated with changes in receptor-mediated contractile activity.

Keywords: Respiratory tract virus; Influenza A virus; endothelin-1; ET_A receptors; ET_B receptors; airway smooth muscle; mice; sarafotoxin S6c; BQ-123

Introduction

Respiratory tract viral infection is frequently associated with airway hyperreactivity to spasmogens in both asthmatic and otherwise healthy individuals (Empey *et al.*, 1976; Beasley *et al.*, 1988; Lemanske *et al.*, 1989; Laitinen *et al.*, 1991). The mechanisms by which respiratory tract viruses induce airway hyperreactivity are multifactorial and not yet fully resolved. However, virus-induced airway hyperreactivity appears to involve a complex combination of the direct actions of respiratory tract viruses on cells residing within the airway wall, such as epithelial cells and the secondary actions of inflammatory cells which are recruited and activated during viral infection (for review see Bjornsdottir & Busse, 1992). For example, viral upper respiratory tract infections cause transient and reversible damage to airway epithelium which may contribute to hyperreactivity by (1) eliminating epithelium-derived relaxant factors, (2) inactivating degradative enzymes such as neutral endopeptidase, (3) sensitizing afferent vagus nerve fibres or (4) removing a diffusion barrier to inhaled bronchoconstrictor agents (Buckner *et al.*, 1985; Holroyde, 1986; Saban *et al.*, 1987; Jacoby *et al.*, 1988; Bjornsdottir & Busse, 1992). Recently studies indicate that respiratory tract viruses may also alter the balance of the respiratory autonomic nervous system by inhibiting the func-

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tion of neuronal M_2 muscarinic autoreceptors and thereby increasing the release of acetylcholine from parasympathetic nerves (Fryer & Jacoby, 1991) and by inhibiting β-adrenoceptor function in airway smooth muscle and inflammatory cells (Lee, 1980; Henry *et al.*, 1991).

Endothelin-1 is a 21 amino acid peptide synthesized within and released from several cell types, including airway epithelial cells (MacCumber *et al.*, 1989; Black *et al.*, 1989; Rozengurt *et al.*, 1990). The *de novo* synthesis of endothelin-1 within epithelial cells is regulated by various cytokines known to be involved in epithelial cell injury and repair (Endo *et al.*, 1992). Once released from epithelial cells, endothelin-1 may exert several actions within the airway wall including airway smooth muscle proliferation (Noveral *et al.*, 1992) and contraction (Uchida *et al.*, 1988; Henry *et al.*, 1990b; Hay *et al.*, 1993b) and sensitization of parasympathetic nerves (McKay *et al.*, 1993). There is accumulating evidence that these effects contribute to the airway obstruction observed in asthma (Springall *et al.*, 1991; Hay *et al.*, 1993a). Importantly, it is now established that asthma, which is an inflammatory airway disease, is associated with elevated expression and release of endothelin-1-like material from the bronchial epithelium (Springall *et al.*, 1991; Vittori *et al.*, 1992). It is conceivable that virus-induced changes in epithelial function may lead to the enhanced release of endothelin-1 and that the neuromodulatory and spasmogenic actions of endothelin-1 may contribute to virus-induced airways hyperreactivity.

We have recently demonstrated that both endothelin$_A$ (ET_A) and ET_B receptors can mediate endothelin-1-induced contractions in airway smooth muscle (Henry, 1993). Thus, in the current study we examined the influence of respiratory tract viral infection on the distribution and density of ET_A and ET_B receptors and on endothelin-1-induced contractions mediated via these pathways in a murine model (Henry *et al.*, 1991).

Methods

Respiratory tract virus stock

Influenza A/PR-8/34 virus was grown in the allantoic fluid of 10-day-old embryonated chicken eggs at 37°C for 3 days as described previously (Williams & MacKenzie, 1977). The crude allantoic fluid was harvested and contained 4×10^7 egg infectious doses (EID_{50}) of virus per ml as determined by the method of allantois-on-shell titration for infectivity (Fazekas de St. Groth & White, 1958). The virus stock was stored in 0.5 ml aliquots at − 70°C.

Tissue preparation

Four week old male CBA/CaH mice, specified pathogen-free, were obtained from the Animal Resources Centre (Perth, Australia), housed in a controlled environment (Microbiology Animal House, University of Western Australia) and received food and water *ad libitum*. Mice were anaesthetized (50 mg kg^{-1} pentobarbitone sodium, i.p.) and intranasally inoculated with 2000 EID_{50} doses of Influenza A/PR-8/34 virus in sterile saline or 25 µl sterile saline alone (control mice). Two days post-inoculation, mice were lightly anaesthetized with ether, cervically dislocated and exsanguinated. The trachea was removed and placed in Krebs bicarbonate solution (KBS) of the following composition (in mM): NaCl 117, KCl 5.36, $NaHCO_3$ 25, KH_2PO_4 1.03, $MgSO_4 \cdot 7H_2O$ 0.57, $CaCl_2$ 2.5 and glucose 11.1. The 4–5 mm length of trachea was carefully cleaned of adherent fat and connective tissue.

Autoradiographic studies

Slide-mounted transverse sections of mouse trachea were prepared as described previously (Henry *et al.*, 1990a). Serial frozen transverse sections (10 µm) were cut at − 20°C and thaw-mounted onto gelatin/chrom alum-coated glass slides. Slide-mounted tracheal sections were washed twice for 10 min in a Tris-HCl buffer (50 mM Tris-HCl, 100 mM NaCl, 0.25% bovine serum albumin, 10 µM phenylmethylsulphonyl fluoride) and incubated for 2 h with 0.2 nM [^{125}I]-endothelin-1 alone (total binding) or in the presence of an ET_A receptor-selective ligand (1 µM BQ-123) or ET_B receptor-selective ligand (100 nM sarafotoxin S6c or 300 nM BQ3020). Non-specific [^{125}I]-endothelin-1 binding was determined in the combined presence of ET_A and ET_B receptor-selective ligands. Slides were washed twice for 20 min in Tris-HCl buffer, rinsed in water and dried with a stream of cold, dry air. Emulsion (NTB-2, Kodak)-coated coverslips were attached to the non-tissue end of the slide with cyanoacrylate adhesive and exposed for 90 h at 4°C in X-ray cassettes. The emulsion was developed (Kodak Dektol, 1:1, 3 min), rinsed for 15 s in 2% acetic acid containing hardener (Ilford) and fixed (Ilford Hypam, 1:4, 2.75 min). Tissue sections were stained with haematoxylin, dehydrated in ethanol, cleared in xylene and mounted in Depex (BDH) for light microscopy.

Autoradiographic grain densities were determined by a grain detection and counting system (Henry *et al.*, 1990a,b). In this study, a total of 21 slides were assessed (6 × total binding, 3 × BQ-123, 3 × sarafotoxin S6c, 3 × BQ3020, 3 × BQ-123 plus sarafotoxin S6c and 3 × BQ-123 plus BQ3020). In each slide, 20 tracheal sections were examined; one section from each of ten virus-inoculated and ten control mice. Four measurements of grain density were made from each tracheal section; 3 over the tracheal smooth muscle band and one over a non-tissue area in the airway lumen (background binding). Thus, a total of 1680 fields were analysed (21 slides × 20 sections × 4 fields). Autoradiographic grain densities were expressed as grains per 1000 µm^2 (grains 1000 µm^{-2}). Data are presented as the mean grain density ± s.e.mean of 10 animals.

Functional studies

In each experiment, a 4–5 mm length of trachea was obtained from a virus-inoculated mouse and another from a paired control mouse. Tracheae were cut in half to generate a total of four tracheal segments, approximately 2 mm in length. Each segment was suspended under 0.5 g tension in a 3 ml organ bath containing KBS at 37°C, bubbled with 5% CO_2 in O_2. Changes in isometric tension were recorded on a Grass Polygraph via FTO3 force-displacement transducers. Following a 45 min equilibration period, tracheal smooth muscle viability was tested by the cumulative addition of 0.2 and 10 µM carbachol, a cholinoceptor agonist. Upon reaching a contraction plateau with 10 µM carbachol, preparations were repeatedly washed and rested for a further 30 min. All subsequent contractions were expressed as a percentage of the contraction to 10 µM carbachol (100% C_{max}).

The primary aim of these experiments was to assess the contractile responses mediated by the ET_A and ET_B receptor-effector systems in virus-inoculated and control mice. Thus for each set of 4 tracheal smooth muscle preparations one of three protocols was followed;

Protocol 1 To assess ET_B receptor-mediated contractions in control and virus-inoculated preparations, contractile responses to the ET_B receptor-selective agonist sarafotoxin S6c were measured. A cumulative concentration-effect curve to sarafotoxin S6c (1–100 nM, 0.5 log-concentration increments) was completed in each preparation. This protocol was also used to assess the sensitivity of the preparation to sarafotoxin S6c after ET_B receptor desensitization. Thus, at the completion of the sarafotoxin S6c concentration-effect curve, preparations were not washed and remained in contact with sarafotoxin S6c for a further 30 min. Following this desensitization period, preparations were washed repeatedly for

30 min with KBS and a second concentration-effect curve to sarafotoxin S6c completed.

Protocol 2 To assess ET_A receptor-mediated contractions, contractile responses to endothelin-1 were determined in the presence and absence of the ET_A receptor-selective antagonist, BQ-123, in ET_B receptor-desensitized preparations from control and virus-inoculated mice. Thus, the initial cumulative concentration-effect curve to sarafotoxin S6c and the 30 min desensitization period were completed in each preparation as described in Protocol 1. After a 15 min washout period, control and virus-inoculated preparations were incubated with 3 μM BQ-123 for a further 15 min. Paired preparations were incubated with BQ-123 vehicle (9 μl of 100 mM Na_2CO_3 in 3 ml KBS). All four preparations were then exposed to the cumulative addition of endothelin-1 (1–300 nM, 0.5 log-concentration increments).

Protocol 3 To determine the extent to which endothelin-1-induced contractions in control and virus-inoculated preparations were mediated by ET_A receptors, contractile responses to endothelin-1 were completed in the presence or absence of BQ-123. In these experiments, control and virus-inoculated preparations were incubated for 15 min with 3 μM BQ-123. Paired preparations were incubated with vehicle (as for Protocol 2). Cumulative concentration-effect curves to endothelin-1 were then completed as described above. Unless otherwise stated, each set of concentration-effect data was fitted to a logistic (sigmoidal) function and computer-assisted least-squares regression analysis used to estimate agonist potency (EC_{50}) and maximum effect (as a percentage of C_{max}).

Statistical analyses

Unless otherwise stated, grouped data are presented as the arithmetic mean ± s.e.mean (n animals). Differences between treatment means were assessed by analysis of variance (SigmaStat) and considered statistically significant when $P < 0.05$. Agonist potency (EC_{50}) is presented as the geometric mean with 95% confidence limits (n animals).

Drugs

Drugs used were; endothelin-1, sarafotoxin S6c, BQ3020 ([Ala^{11,15}]Ac-endothelin-1(6-21)), [^{125}I]-endothelin-1 (Auspep, Melbourne, Australia), carbamylcholine chloride (carbachol), bovine serum albumin (Sigma Chemical Company, St. Louis, U.S.A.), phenylmethylsulphonyl fluoride (Calbiochem, La Jolla, U.S.A.), BQ-123 (cyclo[D-Trp-D-Asp-L-Pro-D-Val-L-Leu]; (generous gift from Dr D.W.P. Hay, SmithKline Beecham Pharmaceuticals, King of Prussia, U.S.A.).

Results

Quantitative autoradiography

Light microscopic autoradiographic studies of mouse isolated tracheal sections incubated with 0.2 nM [^{125}I]-endothelin-1 identified high densities of autoradiographic grains over the tracheal smooth muscle band (Figure 1b). Quantitative analyses indicate that the grain density associated with total [^{125}I]-endothelin-1 binding was lower in the tracheal smooth muscle band of virus-inoculated mice than in control mice (Figure 2a). Grain densities associated with non-specific binding of [^{125}I]-endothelin-1 were low (less than 10% of total) and were similar in virus-inoculated and control sections (Figure 2a). Thus, the mean grain density associated with specific [^{125}I]-endothelin-1 binding to tracheal smooth muscle of virus-inoculated mice was only 77 ± 2% of that estimated in paired control sections (Figure 2).

The density of autoradiographic grains associated with specific [^{125}I]-endothelin-1 binding was reduced in the presence of the ET_A receptor-selective ligand, BQ-123 and also

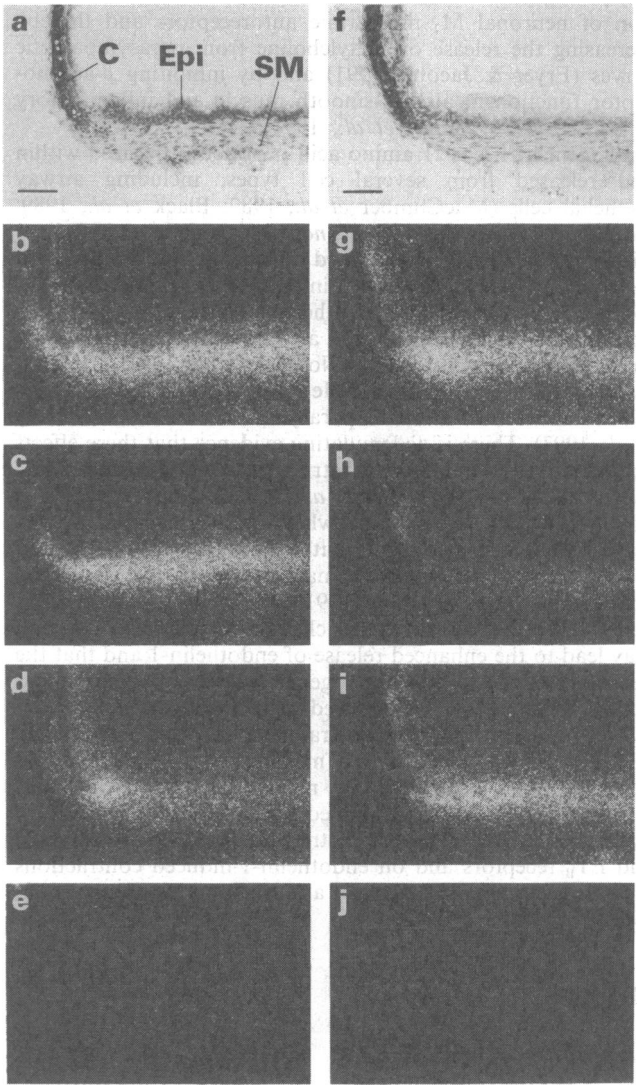


Figure 1 Light microscopic autoradiographs showing [^{125}I]-endothelin-1 binding to transverse tracheal sections obtained from control (a–e) and virus-inoculated (f–j) mice. (a & f) Lightfield autoradiographs of tracheal sections depicting major tissue structures. SM, tracheal smooth muscle; C, cartilage; Epi, epithelium. Bar = 100 μm. (b & g) Darkfield view of tracheal sections (a & f) incubated with 0.2 nM [^{125}I]-endothelin-1 showing autoradiographic grains associated with total binding. The majority of the autoradiographic grains are located over SM. (c & h) [^{125}I]-endothelin-1 binding in the presence of the ET_A receptor-selective ligand, BQ-123 (1 μM). As shown, [^{125}I]-endothelin-1 binding to virus-inoculated sections was particularly sensitive to inhibition by BQ-123; indicative of a predominance of ET_A receptors in the SM of virus-inoculated mice. (d & i) [^{125}I]-endothelin-1 binding in the presence of the ET_B receptor-selective ligand BQ3020 (300 nM). (c & j) Nonspecific binding of [^{125}I]-endothelin-1 determined in the combined presence of 1 μM BQ-123 and 300 nM BQ3020.

in the presence of either of the ET_B receptor-selective ligands, sarafotoxin S6c or BQ3020; indicative of the co-existence of both ET_A and ET_B receptors in murine tracheal smooth muscles (Figures 1 and 2b). Competition binding studies using these receptor-selective ligands indicated that on average, the proportion of ET_A:ET_B receptors in tracheal smooth muscle from control mice was 42%:58% (± 4%, $n = 3$ competing ligands, Table 1), respectively. In contrast, specific [^{125}I]-endothelin-1 binding to tracheal smooth muscle from virus-inoculated mice was noticeably more sensitive to inhibition by BQ-123 (compare Figures 1c and h), indicative of a larger proportion of ET_A receptors. Indeed, subsequent quan-

titative autoradiographic analyses (Figure 2b, Table 1) demonstrated that the proportion of ET_A:ET_B receptors in tracheal smooth muscle from virus-inoculated mice was 77%:23%, respectively. Thus, for any given area of tracheal smooth muscle, virus-inoculated mice contained 40% more ET_A receptors and 69% fewer ET_B receptors than control mice.

Functional studies

Consistent with our previous observations (Henry *et al.*, 1991), contractions induced by 10 μM carbachol were of similar magnitude in preparations obtained from control mice (1.5 ± 0.1 g, $n = 24$) and in mice 2-days after inoculation with virus (1.6 ± 0.1 g, $n = 24$).

The ET_B receptor-selective agonist, sarafotoxin S6c, induced concentration-dependent contractions in preparations from control and virus-inoculated mice (Figure 3), and the EC_{50}s for contraction were similar (3.6 nM in control preparations (95% confidence limits, 2.7–4.8 nM, $n = 16$ preparations from 8 mice; combined results from protocols 1 and 2) versus 3.0 nM in virus-inoculated preparations (2.4–3.7 nM, $n = 16$ preparations from 8 mice). However, as shown in Figure 3, the maximum contraction induced by sarafotoxin S6c was significantly smaller in tracheal smooth muscle preparations obtained from virus-inoculated mice (37 ± 5% C_{max}, $n = 16$ preparations from 8 mice) than in those preparations obtained from control mice (85 ± 4% C_{max}, $n = 16$ preparations from 8 mice, $P < 0.01$).

Endothelin-1 was equipotent in contracting preparations from control and virus-inoculated mice (9.3 nM (95% confidence limits, 4.2–21 nM) versus 6.5 nM (2.7–16 nM), respectively; $n = 4$) and produced similar maximum contractions in these preparations (Protocol 3, Figure 4a and b). However, the ET_A receptor-selective antagonist, BQ-123, was markedly more potent against endothelin-1-induced contractions in virus-inoculated preparations than in control preparations (Protocol 3, Figure 4). In control preparations, BQ-123 (3 μM) caused a small reduction in the maximum contraction induced by endothelin-1, but produced no significant change in contractile potency (EC_{50} for contraction in the presence of BQ-123, 5.9 nM [4.1–8.5], $n = 4$; Figure 4a). In contrast, endothelin-1 concentration-effect curves were displaced 50 fold (95% confidence limits, 17–160 fold, $n = 4$) to the right by BQ-123 in preparations from virus-inoculated mice (Figure 4b). Together, the data presented in Figures 3 and 4 indicate hypofunction of the ET_B receptor-effector system in virus-inoculated preparations.

To isolate and characterize the function of the ET_A receptor-effector system, endothelin-1-induced contractions were examined in preparations in which the ET_B receptor-effector system was desensitized (Protocol 2). No preparation previously exposed to sarafotoxin S6c contracted in response to subsequent sarafotoxin S6c administration, demonstrating successful desensitization of the ET_B receptor-effector system (Protocol 1, $n = 8$). Despite its marked effects on tracheal

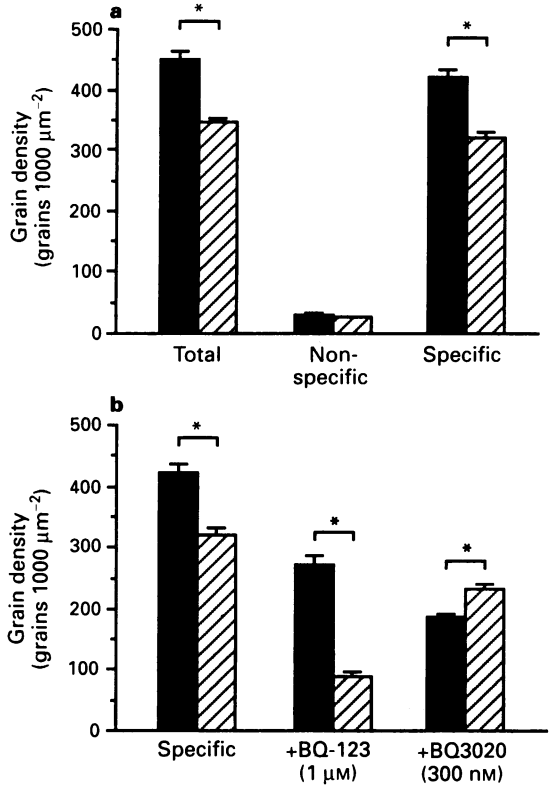


Figure 2 (a) Autoradiographic grain densities associated with total, specific and nonspecific binding of 0.2 nM [^{125}I]-endothelin-1 to the tracheal smooth muscle band of control (solid columns) and virus-inoculated (hatched columns) mice. Nonspecific binding was determined in the combined presence of 1 μM BQ-123 and 300 nM BQ3020. Specific grain density was estimated by subtracting nonspecific grain density from the corresponding total grain density for 10 mice. The tracheal smooth muscle band from virus-inoculated mice contained significantly fewer (*$P < 0.05$) specific binding sites for [^{125}I]-endothelin-1. (b) Specific [^{125}I]-endothelin-1 binding determined in the presence of the ET_A receptor-selective ligand BQ-123 (1 μM) or the ET_B-receptor-selective ligand, BQ3020 (300 nM) in control (solid columns) or virus-inoculated mice (hatched columns). Vertical bars represent the s.e.mean of $n = 10$ mice. *$P < 0.05$.

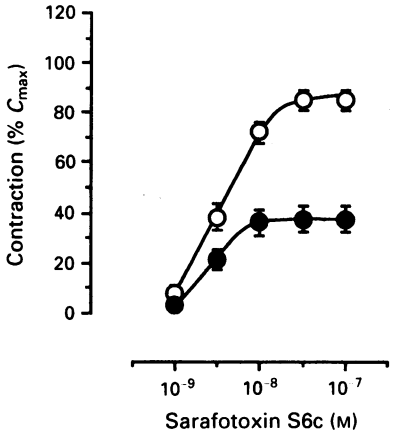


Figure 3 Cumulative concentration-effect curves to the ET_B receptor-selective agonist, sarafotoxin S6c, in mouse isolated tracheal smooth muscle preparations from control (○) and virus-inoculated (●) mice. Values are mean with s.e.mean of 16 preparations from 8 mice.

Table 1 Estimated ratios of ET_A:ET_B receptors in tracheal smooth muscle from control and virus-inoculated mice, determined from autoradiographic studies using [^{125}I]-endothelin-1 and selective ligands for ET_A (BQ-123) and ET_B (BQ3020, sarafotoxin S6c) receptors

	Receptor-selective ligand			
	BQ-123	*BQ3020*	*Sarafotoxin S6c*	*Average*
Control	36:64	44:56	47:53	42:58 (± 4)
Virus-inoculated	73:27	72:28	85:15	77:23 (± 5)

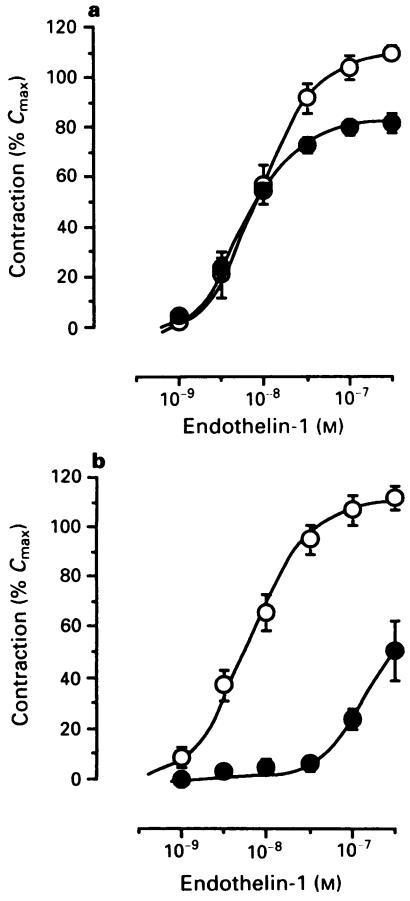


Figure 4 Cumulative concentration-effect curves to endothelin-1 in mouse isolated tracheal smooth muscle preparations from (a) control and (b) virus-inoculated mice. Concentration-effect curves to endothelin-1 were completed in the absence (○) or presence (●) of the ET_A receptor-selective antagonist, BQ-123 (3 μM, see Protocol 3). Values are mean with s.e.mean of 4 preparations from different mice.

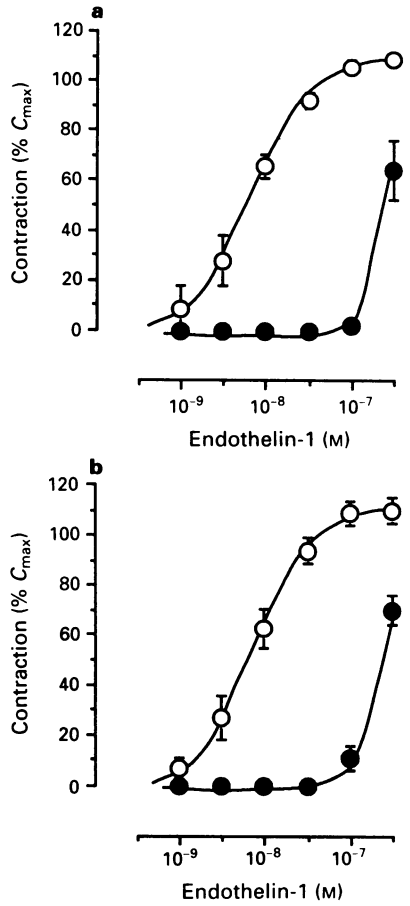


Figure 5 Cumulative concentration-effect curves to endothelin-1 in sarafotoxin S6c desensitized preparations from (a) control and (b) virus-inoculated mice. Concentration-effect curves to endothelin-1 were completed in the absence (○) or presence (●) of 3 μM BQ-123 (see Protocol 2). Values are mean ± s.e.mean of 4 preparations from different mice.

smooth muscle responsiveness to sarafotoxin S6c, this ET_B receptor desensitization protocol had no significant effect on responsiveness to endothelin-1 (compare Figures 4 and 5). BQ-123 (3 μM) markedly inhibited endothelin-1-induced contractions in ET_B receptor desensitized preparations from control mice (63 fold rightward shift at the 30% C_{max} level of contraction, [10–400 fold]; $n = 4$) and virus-inoculated mice (45 fold [18–120 fold]; $n = 4$) (Figure 5). Concentration-effect curves to endothelin-1 in ET_B receptor-desensitized preparations (Figure 5b) were not significantly different from those obtained in virus-inoculated preparations (without ET_B receptor desensitization, Figure 4b).

Discussion

The current study has clearly demonstrated that ET_A and ET_B receptors coexist in murine tracheal smooth muscle and that both receptor subtypes mediate endothelin-1-induced contractions. Moreover, profound alterations in both the density and function of these receptor subtypes were observed during respiratory tract viral infection. Specifically, Influenza A/PR-8/34 virus infection produced a marked reduction in the density of tracheal smooth muscle ET_B receptors and in the contractile function of the ET_B receptor-effector system. In stark contrast, ET_A receptor density was increased in tracheal smooth muscle from virus-inoculated mice, but this was not associated with any significant change in the contractile function of the ET_A receptor-effector system.

Quantitative autoradiographic studies using [^{125}I]-endothelin-1 have previously revealed the existence of specific binding sites for [^{125}I]-endothelin-1 in murine tracheal smooth muscle (Henry *et al.*, 1990a). In the current study, competition binding experiments between [^{125}I]-endothelin-1 and several recently developed ET_A-receptor-selective (BQ-123) and ET_B receptor-selective (sarafotoxin S6c, BQ3020) ligands have demonstrated that murine tracheal smooth muscle contains both ET_A and ET_B receptors in the ratio of 40%:60%, respectively. In this respect, tracheal smooth muscle of mice is similar to that of rat, which also contained ET_A and ET_B receptors in about equal proportions (Henry, 1993). Functional studies, which measured isometric tension development, revealed that both ET_A and ET_B receptors were linked to murine tracheal smooth muscle contraction. For example, sarafotoxin S6c was a potent spasmogen of this preparation, demonstrating a link between ET_B receptors and tracheal smooth muscle contraction. Selective ET_A receptor agonists are not currently available for study. However, the potential role of ET_A receptors in contraction was assessed using the ET_A receptor-selective antagonist, BQ-123 in control and ET_B receptor-desensitized preparations. In control preparations, BQ-123 produced only minor attenuation of endothelin-1-induced contractions, presumably because ET_B receptor-mediated contraction remained uncompromised. However, in preparations in which the ET_B receptor-effector system was first desensitized by prolonged incubation with sarafotoxin S6c, BQ-123 produced considerable inhibition of endothelin-1-induced contractions. This is compelling evidence that ET_A,

as well as ET_B receptors are linked to contraction in murine tracheal smooth muscle.

In the current study, respiratory tract viral infection was associated with several striking changes in the densities of ET_A and ET_B receptors in murine airways. Overall, tracheal smooth muscle from virus-inoculated mice consistently contained 25% fewer endothelin receptors than tracheal smooth muscle from paired control mice. This reduction in total endothelin receptor density was primarily due to a 70% reduction in the density of the normally slightly larger ET_B receptor population. The marked reduction in ET_B receptor density was counteracted, to some extent, by a 40% increase in the density of the usually smaller population of ET_A receptors. Thus, whereas tracheal smooth muscle from control mice contained more ET_B than ET_A receptors, tracheal smooth muscle from virus-inoculated mice contained primarily ET_A receptors (ratio of ET_A:ET_B, about 75%:25%).

The dramatic reduction in ET_B receptor density in tracheal smooth muscle from virus-inoculated mice was consistent with functional data which demonstrated a reduced contractile response to ET_B receptor stimulation in these preparations. In functional studies, contractile responses to the ET_B receptor-selective agonist sarafotoxin S6c were diminished in tracheal smooth muscle preparations from virus-inoculated mice. Furthermore, the ET_A receptor antagonist BQ-123 markedly inhibited contractions to endothelin-1 in preparations from virus-inoculated mice, but not in control mice. This latter finding supports the idea that endothelin-1 contracts tracheal smooth muscle from virus-inoculated mice primarily by activating the now dominant ET_A receptor-effector system. This is in stark contrast to endothelin-1-induced contractions in control mice which were less sensitive to inhibition by BQ-123 since they were mediated via approximately equiactive ET_A and ET_B receptor-effector systems.

In the current study, respiratory tract viral infection was associated with a marked reduction in the density of ET_B receptors and this may be the basis for the reduced contractile responsiveness of these preparations to ET_B receptor stimulation. It remains to be established whether this apparent loss of tracheal smooth muscle ET_B receptors is the result of a direct effect of Influenza A/PR-8/34 virus on ET_B receptors or of the inflammatory processes within the airway wall which accompany respiratory tract viral infection. Recent studies have suggested that respiratory tract viruses may alter the binding of acetylcholine to M_2 cholinoceptors (Fryer *et al.*, 1990) and of substance P to its receptors (Jarreau *et al.*, 1992) either directly via viral neuraminidase activity or secondarily by releasing inflammatory cell products such as major basic protein (Fryer & Jacoby, 1993). The influence of these processes on endothelin receptors remains to be determined.

In view of the findings that endothelin-1-induced contractions were mediated via activation of the ET_A and ET_B receptor-effector systems and that respiratory tract viral infection was associated with ET_B receptor-effector system hypofunction, it was somewhat surprising that endothelin-1-induced contractions were not significantly attenuated by virus infections (current study; Henry *et al.*, 1991). Findings from quantitative autoradiographic studies, which demonstrated a modest increase in ET_A receptor density in tracheal smooth muscle from virus-infected mice raised the possibility that virus-infection may be associated with ET_A receptor-effector system hyperfunction, which counteracts the observed ET_B receptor-effector system hypofunction. However, functional data were not consistent with this concept. For example, ET_A receptor-mediated contractions induced by endothelin-1 in ET_B receptor-desensitized preparations obtained from virus-inoculated were identical to those from control mice. Furthermore, these ET_A receptor-mediated contractions in control and virus-infected mice were equisensitive to inhibition by BQ-123. A more likely explanation for the findings that endothelin-1-induced contractions were not significantly attenuated by virus infection (in the face of virus-induced ET_B receptor-effector system hypofunction) is that significant inhibition of endothelin-1-induced contractions occurs only during concomitant inhibition of both ET_A and ET_B receptor-effector systems. This is in accord with the findings in control preparations that endothelin-1-induced contractions were only significantly inhibited during simultaneous blockade of the ET_A (BQ-123) and ET_B (desensitization) receptor-effector systems.

Another possible explanation for the observed reduction in ET_B receptor density is that viral infection stimulates the release of endothelin-1 from airway epithelial cells which causes selective down-regulation of ET_B receptors in neighbouring airway smooth muscle cells. It is not known whether respiratory tract viral infection causes the enhanced release of endothelin-1 from airway epithelial cells, although there is some circumstantial evidence that this process may occur. Firstly, respiratory tract viral infection in mice is associated with the generation and release of numerous mediators and cytokines (Hennet *et al.*, 1992) some of which stimulate the enhanced synthesis and release of endothelin-1 from airway epithelial cells (Endo *et al.*, 1992). Secondly, enhanced synthesis and release of endothelin-1 from the airway wall has been demonstrated in inflammatory diseases of the airways, such as asthma and in animal models of airway inflammation (Springall *et al.*, 1991; Vittori *et al.*, 1992; Andersson *et al.*, 1992). Like asthma, respiratory tract viral infection is associated with epithelial cell damage and airway wall inflammation which may trigger enhanced release of endothelin-1. According to this hypothesis, enhanced release of endothelin-1 causes the selective down-regulation of airway smooth muscle ET_B receptors. Consistent with this, isolated tension studies have demonstrated that the ET_B receptor-effector system is very sensitive to desensitization by the ET_B receptor-selective agonist, sarafotoxin S6c. However, it is presently not known whether endothelin-1 also causes ET_B receptor-effector system desensitization or indeed if desensitization is associated with receptor down-regulation in this system. Moreover, it remains to be established that ET_B receptors are intrinsically more sensitive to down-regulation than ET_A receptors. Clearly, further studies are warranted to determine the underlying mechanisms of virus-induced ET_B receptor-effector system hypofunction.

The question as to whether the effects of viral infection on the synthesis, release and actions of endothelin-1 within the airway wall contribute significantly to virus-induced airway hyperreactivity, remains unanswered. However, endothelin-1 is a potent spasmogen of airway smooth muscle and elevated bronchomotor tone induced by the enhanced release of endothelin-1 during viral infection may well augment the actions of other endogenous bronchoconstrictor agents. Furthermore, the recently identified neuromodulatory role of endothelin-3 in the airways (McKay *et al.*, 1993) may also contribute to virus-induced hyperreactivity. The ability of endothelin-3 to potentiate the effects of parasympathetic nerve stimulation may explain, at least in part, the enhanced bronchoconstriction induced by exercise or by inhalation of histamine or cold air observed during and following respiratory viral infections (Empey *et al.*, 1976; Aquilina *et al.*, 1980).

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Characterization of the angiotensin II AT_1 receptor subtype involved in DNA synthesis in cultured vascular smooth muscle cells

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1 This study was undertaken in cultured vascular smooth muscle cells to characterize the angiotensin II (AII) AT_1 receptor subtype involved in DNA synthesis because (i) the AII receptor involved in vascular proliferation has previously been characterized *in vitro* in rat aortic cells and identified as an AT_1 subtype and (ii) molecular cloning and biochemical studies have provided evidence for the existence of different AT_1 receptor subtypes.

2 In cultured rat aortic vascular smooth muscle (VSMC), exposure to AII (0.1 to 100 nM) resulted in a concentration-dependent increase in [^{3}H]-thymidine incorporation with an EC_{50} of 1.41 ± 0.51 nM. Maximal stimulation was observed in the presence of 100 nM AII and corresponded to 271 ± 40% of basal [^{3}H]-thymidine incorporation.

3 To characterize the AII AT_1 receptor subtype involved in this effect, cells were exposed to AII (3 nM) in the absence or presence of increasing concentrations of various AII receptor antagonists. The stimulatory effect of AII (3 nM) on [^{3}H]-thymidine incorporation in VSMC was antagonized by the non-selective AT_1/AT_2 receptor antagonist, [Sar^1,Ile^8]-AII (IC_{50} = 5.6 nM), by the AT_{1A}/AT_{1B} receptor antagonist, losartan (IC_{50} = 10.5 nM) and the AT_1 receptor antagonist, L-158809 (IC_{50} = 0.20 nM). The selective AT_2 receptor ligand, CGP 42112A, antagonized AII-induced [^{3}H]-thymidine incorporation with an IC_{50} of 6.3 ± 1.3 μM while the AT_2/AT_{1B} receptor antagonist, PD 123319, was found to be almost inactive ($IC_{50} > 10$ μM).

4 Under the same experimental conditions, angiotensin III (AIII) was found to be at least 50 times less potent than AII with an apparent EC_{50} of 81.6 ± 7.7 nM. At the highest concentration tested (10 μM), the effect of AIII corresponded to 327 ± 61% of basal [^{3}H]-thymidine incorporation.

5 These results confirm that AII can stimulate DNA synthesis in VSMC through an AT_1 receptor. Furthermore, the pharmacological characterization of this AT_1 receptor is compatible with the AT_{1A} receptor subtype recently described on cultured mesangial cells since (i) the AT_{1A}/AT_{1B} receptor antagonist losartan is active at nanomolar concentrations, (ii) micromolar concentrations of the AT_2/AT_{1B} receptor antagonist PD 123319 are ineffective at antagonizing the AII-induced [^{3}H]-thymidine incorporation and (iii) AII is at least 50 times more potent than AIII in stimulating DNA synthesis.

Keywords: Vascular smooth muscle cells; angiotensin II; DNA synthesis; [^{3}H]-thymidine; AT_1 receptor subtypes

Introduction

The role of angiotensin II (AII) in some forms of hypertension and the observation that angiotensin converting enzyme (ACE) inhibitors and AII receptor antagonists are more effective than β-blockers in reducing vascular hypertrophy in hypertensive rats (Wang & Prewitt, 1990; Osterrieder *et al.*, 1991) have led to the hypothesis that AII, in addition to its vasoconstrictor effects, may have mitogenic properties at the level of the vascular wall. In rats, AII infused at low doses induces vascular hypertrophy partly by a non pressor mechanism (Griffin *et al.*, 1991). The hypothesis of a trophic effect of AII has been strengthened by the observations that, *in vitro*, AII induces the expression of proto-oncogenes such as c-*fos* or c-*myc* (Kawahara *et al.*, 1988; Taubman *et al.*, 1989) involved in cell replication, as well as platelet-derived growth factor (PDGF)-A chain mRNA (Naftilan *et al.*, 1989). The effects of AII have been studied *in vitro* on vascular smooth muscle cells (VSMC) (Berk *et al.*, 1989; Owens, 1989) where both hypertrophic and hyperplasic responses have been observed, depending on the experimental conditions used (Dzau *et al.*, 1991, for discussion).

Recently, the existence of AII receptor subtypes has been described based on differences in affinities for the non-peptide AII receptor antagonists, losartan, PD 123319 or PD 123177, or the peptide CGP 42112A (Bumpus *et al.*, 1991; Bottari *et al.*, 1993 for review). According to the current nomenclature (Bumpus *et al.*, 1991), the designation AT_1 has been proposed for the receptor subtype sensitive to losartan, and AT_2 for the subtype sensitive to PD 123177 (Blankley *et al.*, 1991) and CGP 42112A (Whitebread *et al.*, 1989). Functional studies have revealed that the AT_1 receptor subtype is responsible for the well described effects of AII on vasoconstriction, aldosterone and adrenaline release as well as water intake (Criscione *et al.*, 1990; Wong *et al.*, 1990; Herblin *et al.*, 1991). In cultured VSMC, there is now good evidence that the trophic effects of AII are mediated through the stimulation of an AT_1 receptor subtype (Chiu *et al.*, 1991; Bunkenburg *et al.*, 1992). On the other hand, the pathophysiological role of the AT_2 receptor subtype is not yet well understood (Criscione *et al.*, 1990; Dudley *et al.*, 1990; Herblin *et al.*, 1991; Timmermans *et al.*, 1991; Bottari *et al.*, 1993).

More recently, development of the expression cloning technique has made it possible to identify at least three different cDNAs coding for type 1 AII receptors, a rat renal and vascular smooth muscle cell receptor now termed AT_{1A}, a rat adrenal receptor AT_{1B} (Inagami *et al.*, 1992) and an AT_{1C} receptor subtype abundant in cultured VSMC and mesangial cells (Hahn *et al.*, 1993). Thus far, one class of AT_1 receptor gene has been identified for bovines and man (Sasaki *et al.*, 1991; Furuta *et al.*, 1992; Takayanagi *et al.*, 1992) with a high degree of homology to the AT_{1A} sequence (Mauzy *et al.*, 1992). The question of the existence of AT_1 receptor subtypes

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in human tissues is still under debate (Yoshida et al., 1992; Mauzy et al., 1992), while a cDNA coding for an AT_1 different from the AT_{1A} subtype has been recently isolated from a human placental cDNA library (Kuroda et al., 1993). Therefore, it seems likely that subtypes of AT_1 receptors can also be expressed in man.

Although the different AT_1 receptor subtypes identified so far have been expressed in COS-7 cells, their pharmacological profiles have been difficult to distinguish, essentially because of the limited number of agonists and antagonists used for comparison in these experiments (Murphy et al., 1992). On the other hand, in a recent study aimed at characterizing the AII receptor subtypes present in cultured rat renal mesangial cells (Ernsberger et al., 1992; Zhou et al., 1993), two different AT_1 receptor subtypes also termed AT_{1A} and AT_{1B} have been described on the basis of differences in their pharmacological profile. Of particular interest was the fact that the AT_2 receptor ligand, PD 123319, exhibited a nanomolar affinity for a site labelled with [^{125}I]-AII, sensitive to losartan but showing no affinity for the peptide-selective AT_2 receptor ligand, CGP 42112A (Ernsberger et al., 1992). Moreover, this receptor subtype appeared to be coupled to adenylate cyclase through a G_i protein, further supporting the hypothesis of an AT_1 receptor subtype, different from the AT_{1A} and tentatively classified as an AT_{1B} receptor (Zhou et al., 1993). It should be emphasized that this pharmacologically based AT_{1A}/AT_{1B} distinction is in no way similar to the $AT_{1A}/AT_{1B}/AT_{1C}$ classification derived from the molecular cloning results.

Therefore, the objectives of the present work were to establish experimental conditions under which AII is able to induce DNA synthesis, and to characterize the AT_1 receptor subtype involved in this effect in the light of the recent AT_{1A}/AT_{1B} pharmacological characterization proposed in cultured renal mesangial cells (Ernsberger et al., 1992).

Methods

Cell culture

Rat thoracic aortic smooth muscle cells were isolated and cultured by a modification of the method of Chamley-Campbell et al. (1979). Briefly, 11 week old male Wistar Kyoto rats (Charles River, France) were killed by cervical dislocation. Thoracic aortae were excised and placed in Hanks balanced salt solution (HBSS, Gibco, France). Aortic strips were cleaned of fatty tissue, opened longitudinally and pre-digested in collagenase (87.5 u ml^{-1}, Intermed, France). Endothelial cells were removed by scraping the inner surface of the vessels with forceps. The adventitia was carefully removed with forceps and medial cells were dissociated by collagenase (87.5 u ml^{-1}) and elastase (10 μM, Biosys, France) during gentle stirring. The resulting pellet was resuspended in Minimum Essential Medium (Gibco) containing 10% foetal calf serum (Gibco), penicillin (100 u ml^{-1}, Gibco) and streptomycin (100 μg ml^{-1}, Gibco). Cells were seeded in 48 multiwell plates (Costar) at 1×10^4 cells/well for proliferation studies, and in 25 cm^2 plastic culture flasks (Costar) for subsequent subcultures. Cells were harvested at one week intervals with trypsin-EDTA (0.05% trypsin, 0.02% EDTA, Gibco) and were used between passages 4 and 10.

Quiescence was induced by replacing culture medium in multiwell plates with Dulbecco's Modified Eagle's Medium mix HAM's F12 (Gibco), supplemented with insulin (1 μM, Sigma), transferrin (5 μg ml^{-1}, Gibco) and ascorbic acid (100 μM, Sigma). This medium has been reported to maintain cells in a nondividing state which resembles that of cells in the normal arterial wall (Libby & O'Brien, 1983). Smooth muscle cells were identified by indirect immunofluorescent staining with a monoclonal anti-α-smooth muscle actin antibody (Sigma).

Proliferation studies

DNA synthesis in VSMC was assessed in cells maintained in quiescent medium for 72 h.

After a 20 h pre-incubation of cells with various agonists, [^{3}H]-thymidine (0.5 μCi/well, 5.0 Ci mmol^{-1}, Amersham, U.K.) was added to the culture medium for a 4 h incubation period in the continuing presence of agonists. When studied, antagonists, or vehicles were incorporated 1 h before agonists.

At the end of the incubation, cells were washed twice with 0.5 ml cold phosphate-buffered-saline (PBS, Gibco). [^{3}H]-DNA was precipitated with ice cold trichloracetic acid (10%), solubilized in sodium hydroxide (0.5 M) at 37°C and quantified by liquid scintillation counting.

Drugs

The following drugs were used: angiotensin II (human, Sigma), angiotensin III (Sigma), [Sar^1,Ile^8]-angiotensin II ([Sar^1,Ile^8]-AII) (Sigma); losartan, DuP 753 (2-n-butyl-4-chloro-5-hydroxymethyl-1-[(2'-(1H-tetrazol-5-yl) biphenyl-4yl) methyl] imidazole, potassium salt) was kindly provided by Dupont de Nemours (U.S.A.); PD 123319 (1-[[4-(dimethylamine)-3-methylphenyl]methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazo[4,5-c]pyridine-6- carboxylic acid) kindly provided by Parke-Davis laboratories; L-158809 ((5,7-dimethyl-2-ethyl-3[[2'-(1H-tetrazol-5-yl)[1,1'-biphenyl]-4-yl] methyl]-3H-imidazo [4,5-b] pyridine, monohydrate, Merck) was synthesized by the Department of Chemistry, Synthélabo Recherche (Bagneux, France). CGP 42112A (nicotinic acid-Tyr-(N$^\alpha$-benzyl-oxycarbonyl-Arg)Lys-His-Pro-Ile-OH, Ciba-Geigy), recombinant human PDGF-AA and PDGF-BB were obtained from Bachem Laboratories (Switzerland).

To prevent adherence of AII related peptides to plastic wares and degradation by peptidases in culture medium, stock solutions and subsequent dilutions of AII, AIII, [Sar^1, Ile^8]-AII and CGP 42112A were prepared in distilled water containing 1% bovine serum albumin (BSA, Sigma) and 5 mg ml^{-1} bacitracin (Sigma). Final concentrations of BSA and bacitracin in multiwell plates were 0.1% and 0.5 mg ml^{-1}, respectively.

Other drug solutions were prepared in dimethylsulphoxide (DMSO) at 1 mM and subsequent dilutions were made in distilled water. DMSO concentrations in multiwell plates never exceeded 0.1%, which is the highest concentration tolerated by cells without damage.

Statistics

In the figures, results are expressed as mean ± s.e.mean. Student's t test for unpaired data was used to compare the effects of AII and PDGF-AA or -BB with respective control values, and probabilities below the 0.05 level were considered significant. The concentration-response curves and the interactions between antagonists and agonists were analysed by the Allfit programme according to the extra sum of squares principle (De Lean et al., 1978). The EC_{50} values obtained by fitting the logistic equations are expressed as parameter estimates ± approximate standard errors.

Results

Time course of AII-induced [^{3}H]-thymidine incorporation

The kinetics of [^{3}H]-thymidine incorporation in response to AII (3 nM) were examined over a 36 h period at 4–6 h intervals. Addition of AII to cultured cells induced a time-dependent increase in [^{3}H]-thymidine incorporation (Figure 1). The rate of incorporation was maximal between the 20th

and 24th hour of incubation as shown by the steeper slope of the response curve during this period of time.

Effects of AII and AIII on VSMC [^{3}H]-thymidine incorporation

Exposure of rat thoracic aortic cells to AII (0.1 nM–0.1 μM) 20 h before the [^{3}H]-thymidine pulse increased [^{3}H]-thymidine incorporation in a concentration-dependent manner, with an EC_{50} value of 1.41 ± 0.51 nM ($n = 6$) (Figure 2). The effect of AII was maximal at a concentration of 100 nM and averaged 271 ± 40% of basal [^{3}H]-thymidine incorporation. Under the same experimental conditions, AIII (1 nM–10 μM) increased [^{3}H]-thymidine incorporation in a concentration-dependent manner. However, the maximum effect of AIII was not reached even at the highest concentration tested (10 μM). At 10 μM, the effect of AIII corresponded to 327 ± 61% of basal [^{3}H]-thymidine incorporation. The apparent EC_{50} value obtained by fitting this concentration-response curve was 81.6 ± 7.7 nM ($n = 6$).

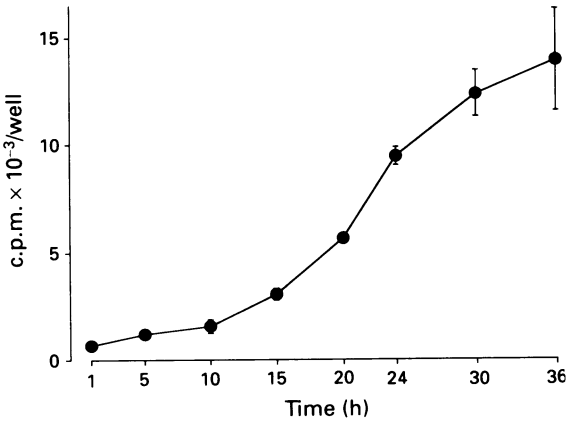


Figure 1 Time course of angiotensin II (AII)-induced [^{3}H]-thymidine incorporation. AII (3 nM) and [^{3}H]-thymidine were added to vascular smooth muscle cells at the time 0. The medium was removed at the times indicated and labelled DNA was extracted in trichloracetic acid-insoluble, NaOH-soluble material. Each point is the mean of eight determinations performed on two different cultures.

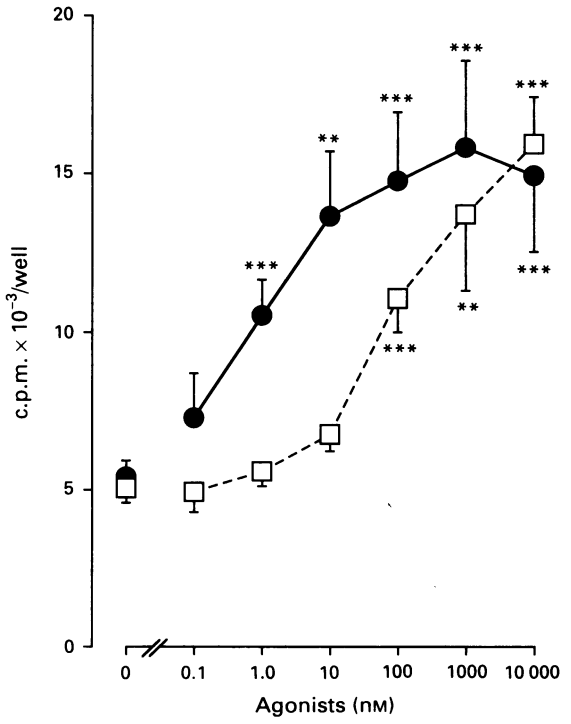


Figure 2 Effects of angiotensin II (AII) and AIII on vascular smooth muscle cells (VSMC) [^{3}H]-thymidine incorporation. VSMC were incubated for 20 h with AII (0.1 nM–1 μM, ●) or AIII (0.1nM–100 μM, □) before [^{3}H]-thymidine was added for 4 h. Results are expressed in counts per minute (c.p.m. × 10^{-3}) per well. Data are mean ± s.e.mean of 6 concentration-response curves carried out on cells cultured from passages 4 to 10. $^{**}P < 0.01$; $^{***}P < 0.001$ when compared with control value.

Effects of PDGF-AA and PDGF-BB on the [^{3}H]-thymidine incorporation in cultured VSMC

Exposure of cultured VSMC to PDGF-AA and PDGF-BB (10–100 ng ml^{-1}) significantly increased [^{3}H]-thymidine incorporation in a concentration-dependent manner, but maximum effects were not reached, even at the highest concentrations tested (100 ng ml^{-1}). The apparent EC_{50} values obtained by fitting these concentration-response curves were 16.6 ± 0.8 (PDGF-AA, $n = 4$–6) and 25.3 ± 4.7 ng ml^{-1} (PDGF-BB, $n = 5$) (Figure 3).

Effects of AII receptor antagonists on the AII-induced [^{3}H]-thymidine incorporation

The effects of antagonists were tested against the [^{3}H]-thymidine incorporation produced by AII (3 nM). The addition of the octapeptide [Sar^1,Ile^8]-AII (0.3–100 nM), a potent but non selective AII receptor antagonist, antagonized the AII-induced [^{3}H]-thymidine incorporation in a concentration-dependent manner with an $IC_{50} = 5.6 \pm 1.2$ nM (Figure 4).

Exposure to the selective AT_1 receptor antagonist, losartan (Chiu *et al.*, 1990) (0.1 nM–1 μM) inhibited in a concentration-dependent manner the [^{3}H]-thymidine incorporation induced by AII with an IC_{50} value of 10.5 ± 1.8 nM (Figure 4). Under the same experimental conditions, exposure to L-158809, a potent and selective AT_1 receptor antagonist (Mantlo *et al.*, 1991) (0.01–3 nM) also antagonized the AII-induced [^{3}H]-thymidine incorporation with an IC_{50} value of 0.20 ± 0.02 nM.

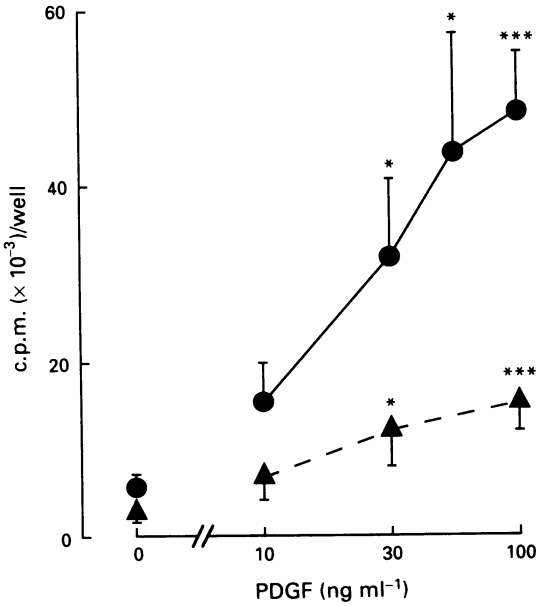


Figure 3 Effect of platelet-derived growth factor (PDGF)-AA and PDGF-BB on vascular smooth muscle cells [^{3}H]-thymidine incorporation. Cells were exposed to either PDGF-AA (●) or PDGF-BB (▲) for 24 h. Results are expressed in c.p.m. (× 10^{-3})/well. Each point represents the mean ± s.e.mean of 4 to 6 (PDGF-AA) or 5 (PDGF-BB) experiments. $^{*}P < 0.05$; $^{***}P < 0.001$ when compared with control value.

In order to establish the type of competition involved in this interaction, a complete concentration-response curve to AII was constructed in the absence and in the presence of 0.01 μM losartan. Under these experimental conditions, losartan significantly shifted to the right the entire concentration-response curve to AII ($EC_{50} = 2.4 \pm 0.6$ nM in control experiments; $EC_{50} = 6.3 \pm 1.0$ nM in the presence of losartan, 0.01 μM) (Allfit comparison $P < 0.05$ when comparing both curves). A pK_B value of 8.2 for losartan was calculated from the concentration-ratios obtained in these experiments. The maximum effect of AII was not depressed, suggesting competitive antagonism (Figure 5).

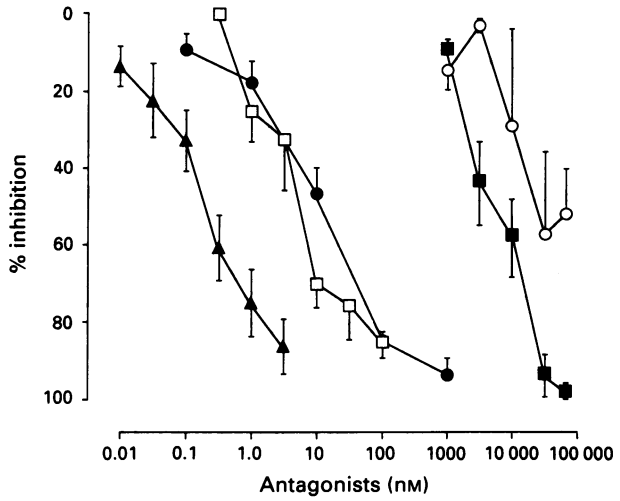


Figure 4 Effects of antagonists on angiotensin II (AII)-induced [^{3}H]-thymidine incorporation in vascular smooth muscle cells (VSMC). [Sar^1,Ile^8] AII (0.3–100 nM, □), losartan (0.1nM–1 μM, ●), L-158809 (0.1–3 nM, ▲), CGP 42112A (1–50 μM, ■) and PD 123319 (1–50 μM, ○) were added to quiescent VSMC 1 h before AII (3 nM). Results are expressed as percentage inhibition of AII-induced [^{3}H]-thymidine incorporation and are mean ± s.e.mean of 6 to 8 determinations (except for CGP 42112A, $n = 3$).

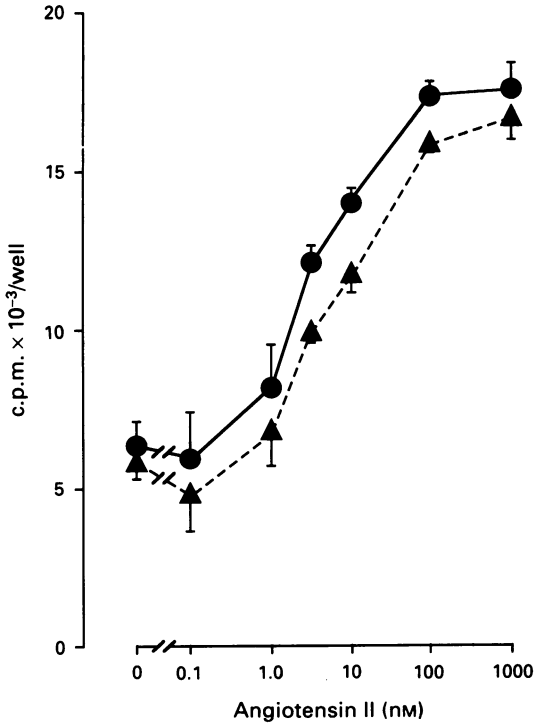


Figure 5 Antagonism by losartan of the angiotensin II (AII)-induced [^{3}H]-thymidine incorporation in vascular smooth muscle cells (VSMC). The effects of AII (0.1nM–1 μM) were determined in cultured VSMC in the absence (●) or in the presence of losartan (0.01 μM) (▲). Results are expressed in c.p.m. ($\times 10^{-3}$)/well and are the mean ± s.e.mean of 3 experimental values. The two concentration-response curves were compared according to the extra sum of squares principle (De Lean *et al.*, 1978) and were found significantly different at the $P < 0.05$ level. A pK_B value of 8.2 for losartan was calculated from the concentration-ratios.

In contrast to the potency of AT_1 selective antagonists at antagonizing the AII-induced response, the selective AT_2 receptor ligand, CGP 42112A (Criscione *et al.*, 1990) inhibited the AII-induced [^{3}H]-thymidine incorporation only at micromolar concentrations ($IC_{50} = 6.3 \pm 1.3$ μM). Under the same experimental conditions, the AT_2 receptor antagonist, PD 123319 (Dudley *et al.*, 1990) was found to be almost inactive ($IC_{50} > 10$ μM) (Figure 4).

Effects of adrenoceptor antagonists on the AII-induced [^{3}H]-thymidine incorporation

In order to test the selectivity of the AII-induced effect on [^{3}H]-thymidine incorporation, the effects of the α-adrenoceptor antagonist, phenoxybenzamine (0.1 nM–1 μM) and the β-adrenoceptor antagonist, propranolol (0.1 nM–1 μM) were evaluated. Neither of these antagonists modified the AII-induced [^{3}H]-thymidine incorporation under these experimental conditions (data not shown).

Discussion

In cultured VSMC, AII at nanomolar concentrations induces a significant and concentration-dependent increase in [^{3}H]-thymidine incorporation with a maximum effect of AII (100 nM) corresponding to 271% of basal [^{3}H]-thymidine incorporation. Results concerning the ability of AII to induce DNA synthesis are conflicting (Campbell-Boswell & Robertson, 1981; Geisterfer *et al.*, 1988). Significant increases of [^{3}H]-thymidine incorporation have been reported (177% in the presence of 500 nM AII; Paquet *et al.*, 1990), as well as a modest increase of 20–30% (Geisterfer *et al.*, 1988) or no effect at all even at the high concentration of 100 nM (Bobik *et al.*, 1990). These discrepancies can be partially explained by differences in the experimental protocols used, among which the duration of incubation with AII as well as the labelling period with [^{3}H]-thymidine appear to be determinant factors to observe an increase in DNA synthesis. Under our experimental conditions, an incubation period of VSMC of less than 15 h duration in the presence of AII, did not induce any significant increase in DNA synthesis, while the most efficient period of [^{3}H]-thymidine incorporation was observed between the 20th and 24th hour of incubation with AII. Therefore it is likely that the reported lack of effect of AII in inducing an increase in DNA synthesis (Bobik *et al.*, 1990) could be explained by a too short duration of incubation, since AII was applied for only 16 h with 1 h [^{3}H]-thymidine labelling. Moreover, the observation that PDGF but not AII was able to increase DNA synthesis under these experimental conditions (Bobik *et al.*, 1990), is consistent with the ability of PDGF to shorten the lag time before the start of DNA synthesis, indicating an acceleration of the induction of competence as previously reported (Kimura *et al.*, 1992). On the other hand, in studies reporting a significant although weak effect of AII on DNA synthesis (Geisterfer *et al.*, 1988), it should be emphasized that the experimental procedure did not mention the presence of any antipeptidase, such as bacitracin, in the culture medium, therefore raising the question of a possible degradation of AII during prolonged exposure to VSMC.

It is important to note that an increase in DNA synthesis does not always reflect hyperplasia but can also suggest polyploidy, without true mitosis. There is now increasing evidence to indicate that AII acts preferentially as an hypertrophic agent in VSMC derived from normotensive rats but

can induce hyperplasia in some situations such as culture of VSMC derived from SHR rats (Millar *et al.*, 1990; Bunkenburg *et al.*, 1992) or in the presence of low concentrations of serum. Therefore, AII could play a role as a 'partial' growth factor leading to enhanced cellular protein and DNA synthesis but lacking the properties to induce a real mitosis.

The mechanism by which AII induces DNA synthesis in cultured VSMC remains to be defined. Under our experimental conditions, PDGF-AA and PDGF-BB both induce an increase in [^{3}H]-thymidine incorporation, the maximum effect of PDGF-BB being higher than that of PDGF-AA. Since AII induces the expression of proto-oncogenes such as c-*fos*, c-*jun* or c-*myc* involved in cell replication as well as PDGF-A chain mRNA in cultured vascular smooth muscle cells (Kawahara *et al.*, 1988; Taubman *et al.*, 1989; Naftilan *et al.*, 1989), it is possible that the mitogenic effect of AII could be mediated through an increase in synthesis and/or release of PDGF-AA. Such an autocrine effect of AII has in fact been described (Itoh *et al.*, 1990). In contrast, it has been reported that the increase in [^{3}H]-thymidine incorporation induced by interleukin-1α but not by AII in cultured VSMC can be antagonized by an antibody specific for PDGF-AA (Ikeda *et al.*, 1990), although it was not clear whether the cells used in these studies were responsive by themselves to PDGF-AA. In fact, an AII-induced increase in DNA synthesis could be limited by the ability of cells to respond to PDGF-AA. On the other hand, the secretion of transforming growth factor β1 (TGF-β1) and/or basic fibroblast growth factor (bFGF) may be critical for determining the magnitude of the trophic responses to AII (Dzau *et al.*, 1991; Itoh *et al.*, 1993). Whether the AII-induced trophic effect is the result of a balance between proliferative (bFGF) and antiproliferative (TGF-β1) autocrine production needs further investigation.

Recently, the development of non peptide AII receptor antagonists has enabled the characterization of two AII receptor subtypes, AT_1 and AT_2. In cultured VSMC, most of the trophic effects of AII have been attributed to the AT_1 subtype (Chiu *et al.*, 1991; Bunkenburg *et al.*, 1992). Under our experimental conditions, AII-induced DNA synthesis is antagonized by nanomolar concentrations of the selective AT_1 receptor antagonists, losartan and L-158809 and micromolar concentrations of CGP 42112A while PD 123319 was almost inactive. Losartan (0.01 μM) significantly shifts to the right the concentration-response curve of angiotensin II without decreasing the maximal effect of AII, supporting the view that it acts as a competitive antagonist of the AII-induced [^{3}H]-thymidine incorporation. The pK_B value of 8.2 for losartan agrees well with the pA_2 value of 8.4 reported previously in rabbit isolated aorta contracted by AII (Chiu *et al.*, 1990). Taken together, these results are compatible with the involvement of an AT_1 receptor located on VSMC, as previously described, mediating the AII-induced [^{3}H]-thymidine incorporation indicative of DNA synthesis. These results do not rule out the possibility that VSMC in culture could express AT_2 receptors but suggest that this receptor subtype does not significantly contribute to the increase in DNA synthesis in response to AII in this experimental model.

Recently, molecular biological approaches have provided evidence for the existence of AT_1 receptor subtypes (Inagami *et al.*, 1992). In rat, VSMC and adrenal cells express different AT_1 mRNA coding for different receptors named AT_{1A} and AT_{1B} respectively. The tissue distribution of these AT_{1A} and AT_{1B} receptor subtypes is heterogeneous, suggesting possible differences in the functions mediated through these receptors, although little is known about their pharmacology. It should be pointed out that the currently available AII receptor antagonists do not discriminate between these AT_1 subtypes. Recently, by means of binding and functional techniques in cultured rat renal mesangial cells, it has been possible to characterize two AII AT_1 receptors, both coupled to a G-protein and exhibiting differences in their affinity for known AT_1 or AT_2 receptor antagonists (Ernsberger *et al.*, 1992). The proposed 'AT_{1A}' receptor subtype shows a nanomolar affinity for losartan, a micromolar affinity for PD 133319 and is more sensitive to AII than to AIII. The proposed 'AT_{1B}' receptor subtype exhibits a micromolar affinity for losartan, a high nanomolar affinity for PD 123319 and AIII, but is nevertheless distinct from an AT_2 receptor since (i) it is coupled to a G protein (Bottari *et al.*, 1991), (ii) it shows a micromolar affinity for CGP 42212A as opposed to the nanomolar affinity of this ligand for the AT_2 receptor (Whitebread *et al.*, 1989) and (iii) losartan is active at micromolar concentrations compared to its millimolar affinity for the AT_2 receptor (Timmermans *et al.*, 1991).

In light of the pharmacologically described AT_1 receptor subtypes present in cultured rat mesangial cells (Ernsberger *et al.*, 1992), the profile of the AT_1 receptor involved in DNA synthesis modulation in VSMC (present results) is clearly not compatible with an AT_{1B} subtype since DNA synthesis in cultured VSMC (i) is antagonized by nanomolar concentrations of losartan, but (ii) is not antagonized even by micromolar concentrations of PD 123319. Moreover the observation that DNA synthesis in VSMC is more sensitive to AII than to AIII is compatible with the involvement of an AT_{1A} subtype. However, it should be emphasized that the classification proposed by Ernsberger *et al.* (1992) has not been confirmed and is not in agreement with the current nomenclature (Bumpus *et al.*, 1991; Inagami *et al.*, 1992). Evolution in this domain is very rapid, and re-evaluation of the classification would certainly be of interest.

In this context, it is interesting to note that in addition to the 'cloned receptor' proposed classification (AT_{1A}/AT_{1B}, Inagami *et al.*, 1992), a novel AT_{1C} receptor subtype has been identified in rats (Hahn *et al.*, 1993). The AT_{1C} receptor seems to be abundant in cultured VSMC and mesangial cells and weakly expressed in brain and kidney. The nucleotide sequence of the AT_{1C} receptor presents a higher degree of homology (90%) with the AT_{1A} receptor sequence than with the AT_{1B} receptor sequence previously described (Inagami *et al.*, 1992). These findings raise the interesting possibility that the pharmacologically described 'AT_{1A}' receptor in cultured mesangial cells (Ernsberger *et al.*, 1992) could be identical to the AT_{1C} receptor subtype obtained by molecular biology techniques in the same cell type (Hahn *et al.*, 1993). Furthermore, considering the presence of an AT_{1C} receptor subtype in cultured rat VSMC and mesangial cells (Hahn *et al.*, 1993) but not in rat aortae and the high degree of homology (90%) between the AT_{1C} and AT_{1A} nucleotide sequence, it is tempting to speculate whether the AT_{1A} receptor subtype identified by molecular cloning techniques in rat vascular tissues could be modified by tissue-culture conditions into the AT_{1C} receptor subtype which has been identified to a high level in cultured cells. Interestingly, a recombinant rat AT_{1A} receptor has been expressed in Chinese hamster ovary cells and confers to these cells the ability to synthesize DNA in response to AII (Teutsch *et al.*, 1992). Furthermore in these transfected cells, AII induces a real proliferation (increase in cell number) in the absence of any other growth factor indicating in these conditions that AII can act as a growth factor and not simply as a cofactor.

Although similarities exist between the AT_{1A} receptor (Ernsberger *et al.*, 1992) and the AT_{1C} receptor (Hahn *et al.*, 1993), clearly more studies are necessary to correlate the $AT_{1A}/AT_{1B}/AT_{1C}$ classification based on molecular cloning and the pharmacologically described AT_{1A}/AT_{1B} receptors present in cultured mesangial cells.

In conclusion, our results provide evidence that AII induces DNA synthesis through an AT_1 receptor which shares the same pharmacological properties as the AT_{1A} subtype pharmacologically described in cultured mesangial cells and could possibly be identical to the AT_{1C} receptor subtype sequence identified in cultured VSMC or mesangial cells. These results support the hypothesis of a role of AII as a hypertrophic agent in cardiovascular pathological situations. The ability of AII receptor antagonists to prevent the 'growth factor-like' effect of AII both *in vitro* and *in vivo*

suggests that these molecules could be useful not only in the treatment of hypertension but also in the prevention of the myointimal proliferation observed in some pathological situations.

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Role of R-type calcium channels in the response of the perfused arterial and venous mesenteric vasculature of the rat to platelet-activating factor

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1 The vasoactive properties of platelet-activating factor (PAF) were studied in the arterial and venous vasculature of the rat double-perfused mesenteric bed. Although PAF (0.01–0.3 pmol) induced a dose-dependent vasodilatation of the arterial mesenteric vasculature, it triggered only vasoconstrictions on the venous side, with an intact endothelium as bradykinin induced a significant venodilatation.

2 N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM), a nitric oxide synthase inhibitor, markedly reduced the vasodilatation induced by PAF in the arterial mesenteric vasculature and potentiated the contractile responses of the venous side to the same agent.

3 The PAF antagonist, WEB-2170, markedly reduced the response to PAF on both sides of the mesenteric vasculature. However, the IC_{50} of WEB-2170 against PAF was reached at a much higher concentration (1×10^{-8} M) on the arterial side than on the venous side (5.3×10^{-11} M). Furthermore, a second antagonist of PAF receptors, SRI-63441, although being less potent on the venous vasculature than WEB-2170, was equipotent in antagonizing the venoconstriction and the arterial dilatation induced by PAF (IC_{50} of SRI-63441, arterial side: 2.9×10^{-9} M; venous side: 3.1×10^{-9} M).

4 The dual L- and R-calcium channel blocker, isradipine (PN 200-110), but not the L-type calcium channel blocker, nifedipine, markedly reduced the PAF-induced vasoactive properties on both sides of the mesenteric vasculature.

5 Our results illustrate the differential vasoactive properties of PAF in the mesenteric vasculature of the rat. These vasoactive responses occur following activation of specific receptors for PAF or, alternatively, through activation of R-type calcium channels.

Keywords: Platelet-activating factor; WEB-2170; nitric oxide; N^G-nitro-L-arginine methyl ester (L-NAME); receptors, mesenteric artery and vein; R-type calcium channels

Introduction

Intravascular administration of platelet-activating factor (PAF) triggers a marked hypotensive response in the rat (Braquet *et al.*, 1987). However, besides its potent hypotensive properties, PAF is also a potent enhancer of plasma extravasation which does not appear to be platelet-mediated in the same animal model (Sanchez-Crespo *et al.*, 1982; Pirotzky *et al.*, 1984; Filep *et al.*, 1991). This latter property of PAF may be greatly influenced by regional differences in the responses of various vasculatures to the phospholipid (Benveniste *et al.*, 1983; Pirotzky *et al.*, 1985; Martins *et al.*, 1987; Chu *et al.*, 1988; Lagente *et al.*, 1988). Indeed, opposite effects of PAF in the pre- and post-capillary circuits in various organs may favour or alternatively counteract plasma leakage induced by PAF.

In the present study, we were interested to assess the effect of PAF in the endothelium-intact, perfused arterial and venous mesenteric vasculature of the rat. An endothelium-dependent relaxation induced by PAF in the perfused mesenteric arterial vasculature of the rat has been previously shown (Chiba *et al.*, 1990). Furthermore, other studies have reported that the rat portal vein contracted to PAF in a concentration-dependent fashion (Hellegouarch *et al.*, 1988). However, the contribution of the venous mesenteric endothelium in the response to PAF had yet to be investigated.

In addition, we have recently reported that PAF induces a concentration-dependent and sustained increase of intracellular calcium in human and canine cultured endothelial cells (Bkaily *et al.*, 1993) via the specific activation of the recently reported R-type calcium channel (Bkaily *et al.*, 1992). Indeed, we have shown that PAF increases calcium via an isradipine-sensitive calcium channel whose activation was unaffected by nifedipine, an L-type calcium channel blocker. As suggested by Bkaily *et al.* (1992, 1993), R-type calcium channels may have an important role in sustained contraction of vascular smooth muscle and in excitation-secretion coupling mechanisms in vascular endothelial cells and subsequent release of vasoactive factors, such as endothelium-derived relaxing factor (EDRF). Hence, in the present study, the dual L- and R-type calcium channel blocker, isradipine (Bkaily *et al.*, 1992), as well as the L-type calcium channel blocker, nifedipine, were compared for their blocking properties on the response induced by PAF on both sides of the mesenteric vasculature of the rat.

Finally, the inhibitory effects of the PAF receptor antagonists, WEB-2170 (Casals-Stenzel, 1987) and SRI-63441 (Handley *et al.*, 1987), were assessed against the PAF-induced vasoactive responses on both sides of the mesenteric vasculature. Our results suggest that PAF induces an endothelium-dependent arterial dilatation and an endothelium-independent venoconstriction of the mesenteric vasculature. Moreover, R-type calcium channel activation importantly contributes to the vasoactive properties of PAF in the mesenteric vasculature of the rat.

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Methods

The simultaneously-perfused superior mesenteric arterial and venous vascular bed of the rat

The simultaneously-perfused arterial and venous vessels of the superior mesenteric vascular bed of the rat were prepared as described previously (Warner, 1990). Male albino Wistar rats (250–350 g) were killed by stunning and exsanguinated. The abdomen was opened and the ileocolic and colic branches of the superior mesenteric artery were tied. The portal and superior mesenteric veins were separated from the connecting tissue in the region of the portal vein. A plastic cannula (Portex, size 3FG, external diameter 1.02 mm, internal diameter 0.61 mm) was inserted retrogradely into the portal vein and passed into the superior mesenteric vein 1–1.3 cm distal to the portal-mesenteric junction. The superior mesenteric artery was cannulated, as described previously (McGregor, 1965), with an identical cannula. The superior mesenteric vascular bed was perfused via the artery for 5 min at 2 ml min^{-1} with Krebs solution (at room temperature) containing heparin (100 u ml^{-1}). During this period, the effluent from the bed passed out from the venous cannula. At the end of this period, the intestine was separated from the mesentery by cutting close to the intestinal border and the preparation supported on a Petri dish while the arteries and veins were perfused independently at a constant flow of 2 ml min^{-1} with warm (37°C), oxygenated (95% O_2; 5% CO_2) Krebs solution containing indomethacin (5 μM). When the arterial side was precontracted with methoxamine (100 μM), the flow was increased to 4 ml min^{-1} to reduce the marked spontaneous activity of the preparation (Warner, 1990). Changes in perfusion pressures were measured with a Statham pressure transducer (Model P-23A) and recorded on a Grass physiograph (Model 7-D).

Vasodilatation responses of the arterial and venous mesenteric vasculature

Following an equilibration period of 45 min, the vasoactive effect of PAF, acetylcholine (ACh) or bradykinin (BK) was evaluated. The perfusion pressure on both sides of the mesenteric circulation was increased by infusing either a sympathomimetic, methoxamine (100 μM), on the arterial side or a thromboxomimetic, U46619 (0.5 μM), on the venous side, as previously described (Warner, 1990; Claing *et al.*, 1992; D'Orléans-Juste *et al.*, 1993). When a plateau was reached, the agents were administered by bolus injections (1 to 5 μl). Vasodilator responses were expressed as % reduction of induced tone on both sides of the mesenteric vasculature, as previously reported (Warner, 1990). In some experiments, the effects of inhibitors and antagonists were evaluated. They were all infused 15 min before the administration of PAF, bradykinin, or acetylcholine, except for N^G-nitro-L-arginine methyl ester (L-NAME) which was infused 30 min before. The apparent affinities of WEB-2170 and SRI-63441 were obtained by monitoring the vasoactive responses of the mesenteric vasculature to PAF (arterial: 0.1 pmol; venous: 5 pmol) in the presence of increasing concentrations of the antagonists and their IC_{50}, calculated from a linear regression analysis.

Vasoconstrictor responses on the venous mesenteric vasculature

The vasoconstrictor properties of PAF and angiotensin II were evaluated on the venous mesenteric vasculature perfused at basal pressure. Bolus doses of PAF (0.1–5 pmol) were administered in the absence and presence of the various inhibitors or antagonists. To avoid tachyphylaxis, the consecutive doses were administered at 1 h time intervals or when the perfusion pressure was returned to basal value. Vasoconstrictor responses were expressed as the increase of the perfusion pressure.

All studies using nifedipine, isradipine, SRI-63441 and WEB-2170 were carried out in the dark. The effects of isradipine were studied in mesenteric vasculatures pretreated with nifedipine in order to discount any influence at L-type calcium channels.

Materials

The Krebs solution had the following composition (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4.7H_2O$ 1.2, $CaCl_2.6H_2O$ 2.5, $NaHCO_3$ 25 and glucose 5.5 Indomethacin, methoxamine, heparin, sodium nitroprusside, N^G-nitro-L-arginine methyl ester (L-NAME), nifedipine and acetylcholine were purchased from Sigma Chemical Co. (St-Louis, U.S.A.). Angiotensin II and bradykinin were synthesized in the laboratory of Dr D. Regoli (Université de Sherbrooke, Sherbrooke, Québec, Canada). U46619 (11α, 9α-epoxymethane PGH_2) was purchased from Cayman Chemical Company (Ann Arbor, U.S.A.). C_{16}-PAF was obtained from Bachem (U.S.A.) and WEB-2170 (3-[4-(2-chlorophenyl)-9-methyl-6H-thieno[3,2-F] [1,2,4]triazolo-4[,3-a] [1,4]-diazepin-2-Y1)-1-(4-morpholimyl)-1-propanone was a generous gift from Dr H. Heuer (Boehringer-Ingelheim, Germany). Isradipine (PN 200–110) was obtained from Sandoz Canada Inc. and SRI-63441 (cis(±)-1-[2-[hydroxy[[tetrahydro-5-[(octa-decyl-aminocarbonyl)oxy]-methyl]furan-2-yl]-methoxy-phosphinyl-oxy]ethyl]-quinolinium hydroxide was a generous gift from Dr D. Handley (Sandoz, East Hanover, NJ).

All drugs were dissolved in phosphate buffered saline (PBS; PH: 7.4), except for nifedipine which was dissolved in 10% dimethylsulphoxide (DMSO), isradipine in 60% ethanol and indomethacin in Trizma base (pH 7.4; 0.2 M, Sigma).

Statistics

Results are shown as mean values ± s.e.mean for n experiments. All data are subjected to comparison and analysis by a one-way analysis of variance (ANOVA), followed by a Mann-Whitney U-test to assess the statistical significance of the results, except for the experiment with L-NAME. In this case, Student's t test was used. A P value less than 0.05 was considered statistically significant.

Results

Effect of PAF on arterial and venous mesenteric vasculature of the rat perfused at basal pressure or precontracted

The basal perfusion pressures of arterial and venous sides of the mesenteric vasculature were 5.1 ± 0.3 mmHg (n = 40) and 1.7 ± 0.2 mmHg (n = 35), respectively, when perfused at 2 ml min^{-1}. An increase in perfusion flow to 4 ml min^{-1} also increased the perfusion pressure of the arterial vasculature to 12.1 ± 0.9 mmHg (n = 25). The infusion of methoxamine (100 μM, arterial side) and U46619 (0.5 μM, venous side) increased the perfusion pressures by 39.1 ± 3.6 mmHg (n = 27) and 7.1 ± 1.2 mmHg (n = 8), respectively.

Typical traces of Figure 1 illustrate the vasoactive properties of PAF on the arterial and venous vasculatures, precontracted respectively with methoxamine (100 μM) and U46619 (0.5 μM) or on the venous vasculature perfused at basal pressure. Acetylcholine (100 pmol) induced a transient vasodilatation on the arterial side and bradykinin (1000 pmol) induced a vasodilatation of the venous vasculature. PAF (0.1 pmol) induced only a vasodilatation of the arterial mesenteric vasculature. In venous preparations perfused at basal pressure, angiotensin II (100 pmol) and PAF (5 pmol) induced an increase of the perfusion pressure (Figure 1c).

Figure 2 illustrates the dose-dependent vasodilatation by

PAF (0.01–0.3 pmol) on the arterial side precontracted with the methoxamine (Figure 2a), the effect of PAF (0.1 and 1 pmol) on the venous side in the precontracted vasculature (U46619, 0.5 μM) (Figure 2b) and the effect of PAF (1 and 5 pmol) on the venous vasculature perfused at basal pressure (Figure 2c).

Effect of L-NAME on the vasodilator and venoconstrictive properties of PAF

Acetylcholine and PAF induced a vasodilatation in the arterial mesenteric vasculature precontracted with methoxamine. In Figure 3a, a 30 min preinfusion of L-NAME (100 μM) reduced the vasodilatation induced by acetylcholine (100 pmol) by 82% ($n = 7$, $P < 0.01$) and PAF (0.1 pmol) by 99% ($n = 8$, $P < 0.01$) when compared to the responses to these vasoactive agents in the absence of L-NAME, without affecting the vasodilatation induced by sodium nitroprusside (1000 pmol) ($n = 8$). Furthermore, as illustrated in Figure 3b, L-NAME significantly potentiated the constrictive properties of PAF (5 pmol) ($n = 6$, $P < 0.05$) and angiotensin II (100 pmol) ($n = 6$, $P < 0.05$) when compared to the effect of these two constrictor agents in the absence of L-NAME in the venous mesenteric vasculature perfused at basal pressure. Infusion of L-NAME increased the perfusion pressure of the precontracted arterial side and non-precontracted venous vasculature by 42.0 ± 5.8 ($n = 8$) and 3.6 ± 1.2 mmHg ($n = 6$), respectively.

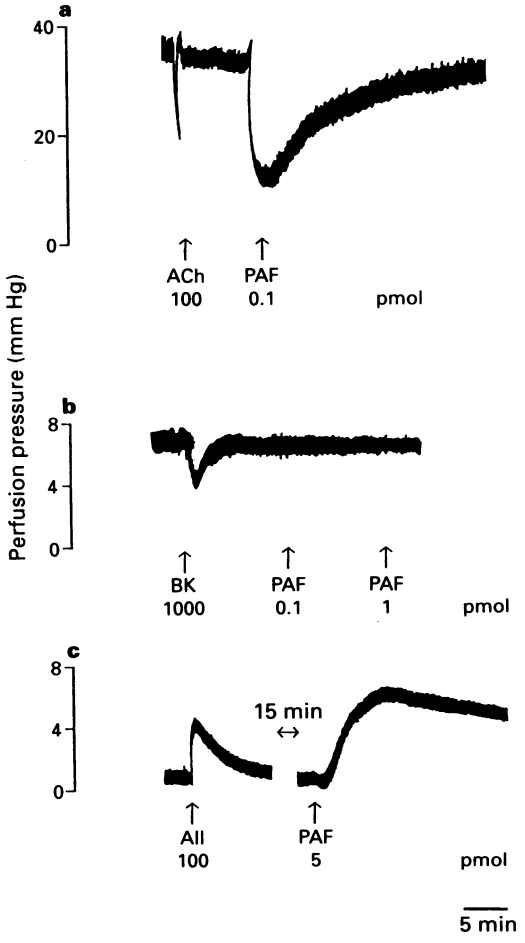


Figure 1 Typical trace illustrating the effect of acetylcholine (ACh) (100 pmol), bradykinin (BK) (1000 pmol) and PAF (0.1–1 pmol) on the arterial (a) and venous (b) vasculature precontracted respectively with methoxamine (100 μM) and U46619 (0.5 μM). This trace illustrates also the effect of angiotensin II (AII 100 pmol) and PAF (5 pmol) on the venous vasculature perfused at basal pressure (c). This trace is representative of five other experiments.

Effect of WEB-2170 and SRI-63441 on the vasoactive properties of PAF in the arterial and venous mesenteric vasculatures

Figures 4 and 5 illustrate, respectively, the effects of WEB-2170 and SRI-63441, on the vasodilator and venoconstrictor responses to PAF of the mesenteric vasculature. The PAF antagonists significantly reduced the vasodilator properties of PAF in the arterial mesenteric vasculature. WEB-2170 was inactive at 10^{-10} M (not shown) and a concentration ten times higher was necessary to reduce significantly the vasodilator properties of PAF (0.1 pmol); the IC_{50} of the antagonist was estimated at 1×10^{-8} M. The IC_{50} value of the second antagonist, SRI-63441, against PAF (0.1 pmol)-induced vasodilatation was 2.9×10^{-9} M. In contrast, a concentration of 1×10^{-7} M of WEB-2170, which was sufficient to block the vasodilator properties of PAF by 88%, did not affect the endothelium-dependent vasodilatation induced by acetylcholine (100 pmol) (Figure 4a).

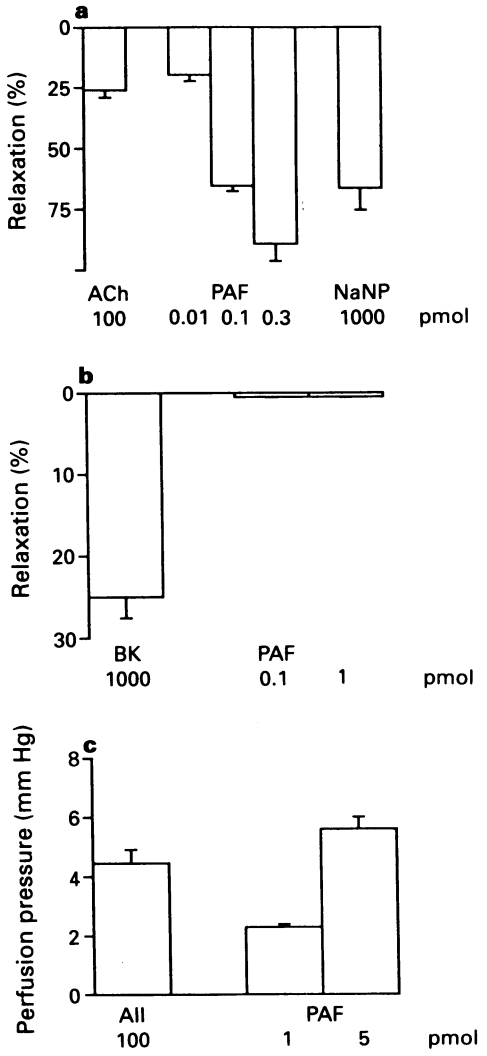


Figure 2 (a) Dose-dependent vasodilatation induced by PAF (0.01–0.3 pmol) on the arterial side precontracted with methoxamine (100 μM), (b) constriction of the venous side induced by PAF (0.1 and 1 pmol) in precontracted (U46619, 0.5 μM) mesenteric vasculature or (c) constriction by PAF (1 and 5 pmol) of the venous vasculature perfused at basal pressure. The vasodilator effect of acetylcholine (ACh) (100 pmol) and sodium nitroprusside (NaNP) (1000 pmol) is also determined on the arterial side (a) and that of bradykinin (BK) (1000 pmol) on the venous side (b). The vasoconstrictor effect of angiotensin II (AII) (100 pmol) is determined on the venous side (c). Each column represents the mean ± s.e.mean of four to 10 determinations.

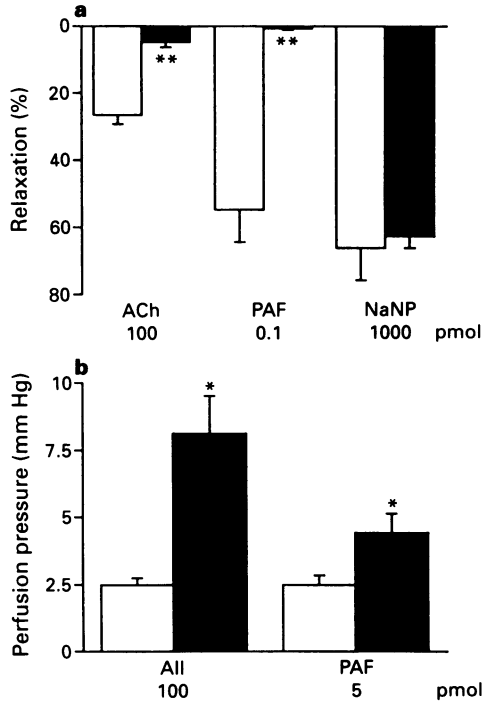


Figure 3 Effect of N^G-nitro-L-arginine methyl ester (L-NAME) (100 μM, 30 min) (solid columns) on the response of the arterial (a) mesenteric vasculature (precontracted with methoxamine) to acetylcholine (ACh) (100 pmol), PAF (0.1 pmol) and sodium nitroprusside (NaNP) (1000 pmol) and on the venous side perfused at basal pressure (b) to angiotensin II (AII) (100 pmol) and PAF (5 pmol). Each column represents the mean ± s.e.mean of 6 to 8 determinations. $*P<0.05$, $**P<0.01$, when compared to control values (open columns).

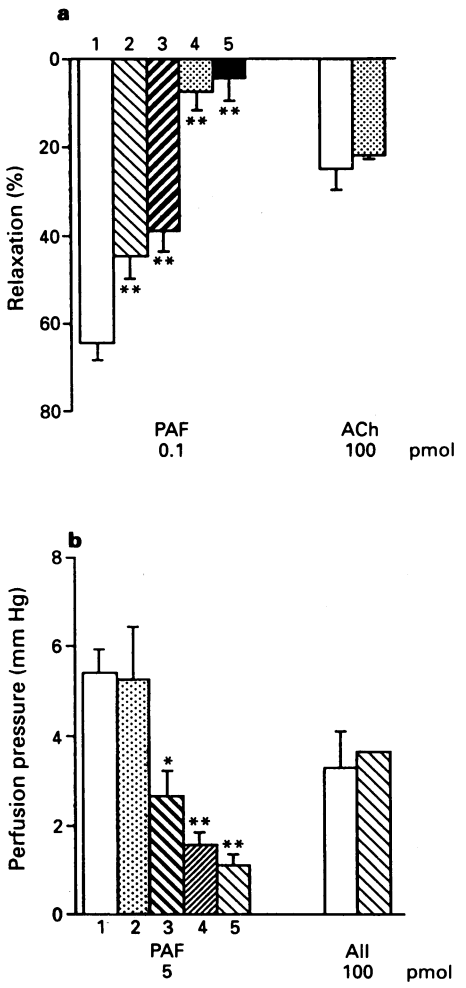


Figure 4 Effect of PAF (0.1 pmol) and acetylcholine (ACh) (100 pmol) in the absence of ((1), open columns) and presence of different concentrations of WEB-2170 ((2): 0.001 μM; (3): 0.01 μM; (4): 0.1 μM; (5): 1 μM) on the precontracted arterial (a) mesenteric vasculature. The vasoconstrictor effect of PAF (5 pmol) and angiotensin II (AII) (100 pmol) was also assessed on the venous (b) vasculature perfused at basal pressure in absence (open columns) and in presence of various concentrations of WEB-2170 ((2): 0.001 nM; (3): 0.01 nM; (4): 0.1 nM; (5): 1 nM). Each column represents the mean ± s.e.mean of 5 to 6 determinations. $*P<0.05$; $**P<0.01$ when compared to control values (□).

WEB-2170 and SRI-63441 also markedly reduced the venoconstrictive properties of PAF (5 pmol) in the mesenteric vasculature at threshold concentrations of 1×10^{-11} M and 1×10^{-9} M, respectively (Figure 4b and 5b). In terms of antagonism of PAF-induced venoconstriction, the IC_{50} value of WEB-2170 was lower (5.3×10^{-11} M) than that of SRI-63441 (3.1×10^{-9} M).

Effect of isradipine on the vasoactive properties of PAF

The effects of a L-type calcium channel blocker, nifedipine, and a dual L- and R-type calcium channel blocker, isradipine, were tested for their inhibitory properties on the response of PAF and other vasoactive agents in the mesenteric vasculature. Figures 6 and 7 illustrate that the L-type calcium channel blocker, nifedipine (0.1 μM), did not affect the vasodilator properties of PAF on the arterial side nor the venoconstrictor properties of the same agent. In contrast, in the presence of nifedipine (0.1 μM), treatment with isradipine ((+) or (−)enantiomer) significantly reduced the vasoactive effects of PAF on the arterial and venous mesenteric vasculature, without affecting the vasodilator properties of acetylcholine on the arterial side and the venoconstrictor properties of angiotensin II (Figures 6 and 7). Finally, the co-infusion of isradipine enantiomers and WEB-2170 did not further reduce the vasoactive effect of PAF when compared to mesenteric vasculatures treated only with isradipine (Figures 6 and 7).

Discussion

The present study illustrates the heterogeneity of the vasoactive properties of PAF in the mesenteric vasculature of the rat. Our results support the report by Chiba *et al.* (1990) who have suggested that PAF induced an endothelium-dependent relaxation in the rat mesenteric arterial vasculature. In contrast, PAF was found to be a potent vasoconstrictor of the venous mesenteric vasculature in which the functional endothelium was monitored through the vasodilator properties of bradykinin (Warner, 1990; Claing *et al.*, 1992). This heterogeneity of the response of the mesenteric vasculature to the potent vasoactive factor, PAF, is of interest considering that this compound is a potent enhancer of vascular permeability changes in conscious rats (Sirois *et al.*, 1988). Indeed, assuming that PAF induces a marked endothelium-dependent vasodilator effect in the precapillary vasculature as well as a marked endothelium-independent constriction of the postcapillary circulation, one can suggest that hydrostatic pressure changes play an important role in the plasma leakage induced by PAF in the mesenteric vasculature. The contribution of venoconstriction in the plasma leakage induced by PAF has been previously discussed by Heffner *et al.* (1989) and Sirois *et al.* (1990) who suggested that the complex phenomenon involved in capillary extravasation was com-

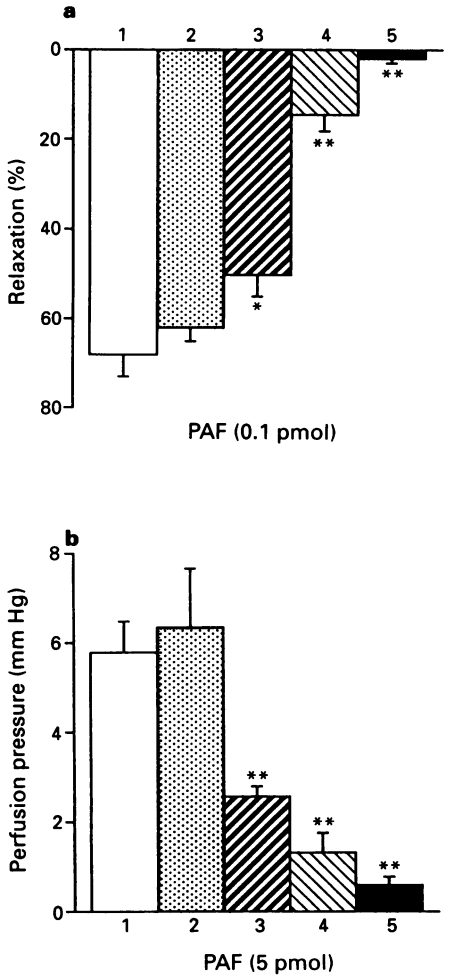


Figure 5 Effect of PAF (0.1–5 pmol) in the absence ((1), open column) and presence of different concentrations of SRI-63441 ((2): 0.0001 μM; (3): 0.001 μM; (4): 0.01 μM; (5): 0.1 μM) on the precontracted arterial (a) and venous (perfused at basal pressure) (b) mesenteric vasculature. Each column represents the mean ± s.e.mean of 5 to 6 determinations. $*P<0.05$; $**P<0.01$ when compared to control values (open columns).

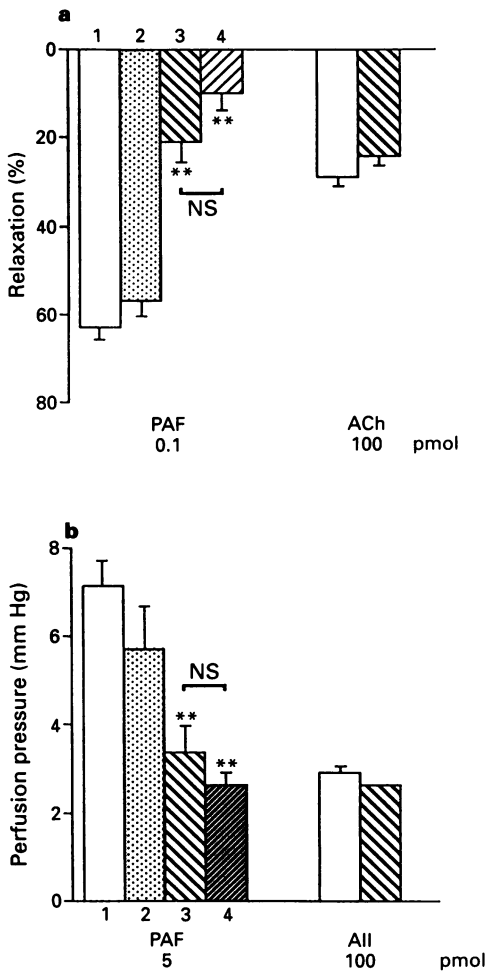


Figure 6 Effect of PAF (0.1 and 5 pmol on the arterial and venous sides, respectively) in the absence ((1) open column) or presence of nifedipine (0.1 μM) (2) or nifedipine (0.1 μM) and (+)-isradipine (1 μM) (3) or nifedipine (0.1 μM), (+)-isradipine (1 μM) and WEB-2170 (arterial: (a) 0.1 μM: (4): venous; (b): 0.1 nM: (4)). The same mixture of antagonists was tested against acetylcholine (ACh) (100 pmol) on the arterial and angiotensin II (AII) (100 pmol) on the venous mesenteric vasculature. Each column represents the mean ± s.e.mean of 5 to 8 determinations. NS: not significant. $**P<0.01$ when compared to control values (open columns).

posed, among other mechanisms, of both post-capillary disjunction of endothelial cells and constriction.

EDRF, as previously suggested (Chiba *et al.*, 1990), greatly contributes to the vasodilator properties of PAF in the arterial mesenteric vasculature. Indeed, these authors have shown that methylene blue, an inhibitor of guanylate cyclase, markedly blunted the response of the arterial mesenteric vasculature to PAF and acetylcholine. We have confirmed these data by the use of a specific nitric oxide synthase inhibitor, L-NAME (Moore *et al.*, 1990).

On the venous side, although PAF did not induce an endothelium-dependent vasodilatation, L-NAME still markedly potentiated the increase of perfusion pressure triggered by PAF, suggesting that basally-released EDRF may contribute as a physiological antagonist of the vasoconstrictor properties of PAF as well as those of other agonists, such as angiotensin II, neurokinins and endothelin-1 in the rat mesenteric vasculature (D'Orléans-Juste *et al.*, 1991, 1993; Claing *et al.*, 1992).

The PAF antagonist, WEB-2170, markedly blocked the response of both sides of the mesenteric vasculature to PAF, suggesting that this mediator may very well activate specific receptors to induce its vasodilator effects as well as its vasoconstrictive properties. The IC_{50} of WEB-2170 as an antagonist of the arterial vasodilator properties of PAF was lower (2.5 log units) when compared to the IC_{50} of the same antagonist to block PAF-induced venoconstriction. The discrepancy in concentrations found with this antagonist was not seen with another PAF receptor antagonist (SRI-63441; Handley *et al.*, 1987). Indeed, the latter antagonist markedly reduced the arterial vasodilator properties as well as the venoconstrictor properties of PAF at similar concentrations (IC_{50} values of 2.9×10^{-9} M and 3.0×10^{-9} M, respectively). The fact that WEB-2170 was more sensitive as a blocker of the venoconstriction than of the arterial vasodilatation may explain the heterogeneity of the antagonist properties of that compound in PAF-induced plasma extravasation (Sirois *et al.*, 1988; Henriques *et al.*, 1990), as pre- and post-capillary vasculatures may be affected differently by the antagonist. The discrepancy may be further explained by the presence of high affinity receptors for PAF in the venous smooth muscle as opposed to lower affinity receptors in the arterial endothelium. It is of interest that WEB-2170 has been used to discriminate between high and low affinity receptors for PAF in human HL-60 myeloid leukaemia cells (Rola-Pleszczynski & Stankova, 1992). By the use of another PAF antagonist BN52021, those authors were unable to distinguish between the two receptor populations. We suggest that SRI-63441, as

opposed to WEB-2170, is unable to discriminate between the two receptor populations in the mesenteric vasculature. In addition, Henriques *et al.* (1990), by the use of two heterazepine PAF antagonists, have shown that distinct receptor populations were involved in paw oedema and pleurisy in the mice.

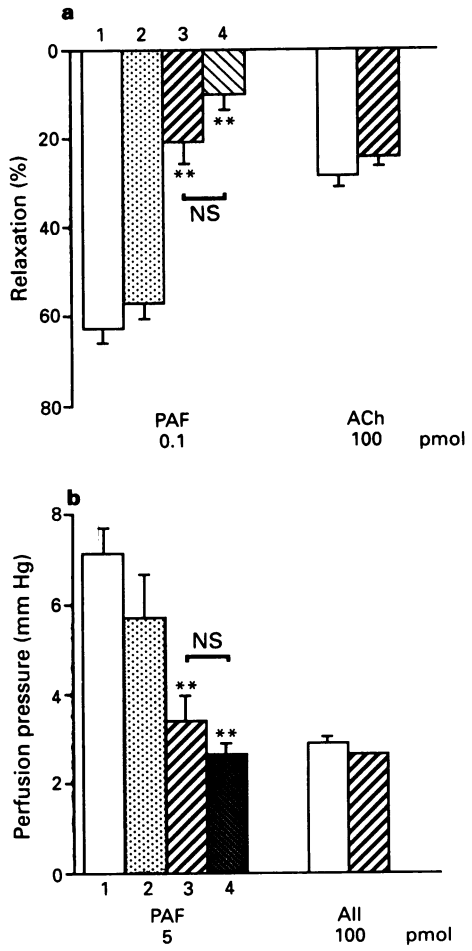


Figure 7 Effect of PAF (0.1 and 5 pmol on the arterial and venous sides, respectively) in the absence ((1) open columns) or presence of nifedipine (0.1 μM) (2) or nifedipine (0.1 μM) and (−)-isradipine (1 μM) (3) or nifedipine (0.1 μM), (−)-isradipine (1 μM) and WEB-2170 (arterial: (a) 0.1 μM: (4): venous; (b): 0.1 nM: (4)). The same mixture of antagonists was tested against acetylcholine (ACh) (100 pmol) on the arterial and angiotensin II (AII) (100 pmol) on the venous mesenteric vasculature. Each column represents the mean ± s.e.mean of 5 to 8 determinations. NS: not significant. $^{**}P<0.01$ when compared to control values (open columns).

On the other hand, it is suggested that WEB-2170 interferes with entities other than PAF receptors to counteract the effect of that phospholipid, in accordance with a previous study in which we have shown that PAF induced a significant increase of intracellular calcium in human and canine endothelial cells via the opening of calcium channels (Bkaily *et al.*, 1993). This effect was unaltered by nifedipine, yet reversed by isradipine, suggesting that PAF induced a sustained increase of intracellular calcium via the activation of a recently described calcium channel, the resting potential calcium channel (R-type calcium channel) (Bkaily *et al.*, 1992, 1993). In one of those studies, we had also reported that WEB-2170 also reversed not only the sustained increase of Ca^{2+} induced by PAF, but also by high $[K]_o$ depolarization in human and canine endothelial cells, suggesting that the PAF antagonist may directly interfere with the opening of R-type Ca^{2+} channels (Bkaily *et al.*, 1993).

Since we have shown in the present study that isradipine, but not nifedipine, markedly reduced both the vasodilator and venoconstrictor properties of PAF, we suggest that this particular agonist may well increase the probability of opening the R-type calcium channels prior to inducing its vasoactive effects in the mesenteric vasculature. The activation of L-type calcium channels was excluded as nifedipine did not alter the vasoactive properties of PAF. Furthermore, Warner *et al.* (1989) have previously shown that Bay K 8644, a dihydropyridine activator of L-type calcium channels, did not trigger the release of endothelium-derived relaxing factor (EDRF) from the arterial mesenteric vasculature of the rat.

In conclusion, our results illustrate the opposite effect of PAF on the arterial and venous mesenteric vasculature of the rat. The use of such a model as the double-perfused mesenteric bed allows a better understanding of the pre- and post-capillary vasoactive properties of PAF and their contribution in capillary leakage. Furthermore, the use of an R-type calcium channel blocker, such as isradipine, should give us further insight into the contribution of ionic channels in pro-inflammatory conditions.

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Mechanism of bile salt vasoactivity: dependence on calcium channels in vascular smooth muscle

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1 The vasoactive mechanisms of bile salts have been investigated in rat isolated portal venous and superior mesenteric arterial rings and perfused mesentery.

2 The isolated perfused mesentery was precontracted with a selective α_1-adrenoceptor agonist, cirazoline. Incremental doses of tauroursodeoxycholate (TUDC), taurochenodeoxycholate (TCDC) and taurodeoxycholate (TDC) caused a dose-dependent vasorelaxation. The order of potency of the vasodilator effect was TDC > TCDC > TUDC.

3 The effect of TDC (1.9×10^{-8}–1.9×10^{-6} mol) was examined before and after propranolol (3 μM), tetraethylammonium (5 mM), ouabain (10^{-5} M), N^G-nitro-L-arginine methyl ester (10^{-4} M) and capsaicin (50 mg kg^{-1}) to block, respectively, β-adrenoceptors, K^+-channels, Na^+, K^+-ATPase, nitric oxide synthase, and primary sensory nerves. The vasodilator effect of TDC was not affected by any of these blocking agents or by denuding vascular endothelium with distilled water.

4 Infusion of TDC (1.9×10^{-8}–1.9×10^{-6} mol) with K^+-free or high K^+ (60 mM) physiological salt solution (PSS) did not affect the vasodilator effect of TDC.

5 Contractions induced by KCl (0.01–1.0 M), arginine vasopressin (AVP, 10^{-10}–10^{-7} M) or cirazoline ($10^{-7} \times 10^{-5}$ M) were all inhibited by TDC (300 μM).

6 TDC (10^{-6} to 10^{-3} M) also inhibited the basal tension and the development of spontaneous contractions in the isolated portal vein.

7 TDC (300 μM), however, did not affect noradrenaline-induced phasic contractions elicited in Ca^{2+}-free PSS by Ca^{2+} release from intracellular stores.

8 We conclude that TDC inhibits Ca^{2+} entry through both voltage-operated and receptor-operated calcium channels, whereas intracellular Ca^{2+} release is not affected.

Keywords: Bile salts; vascular smooth muscle; calcium channels; taurodeoxycholate; haemodynamics; cirrhosis; splanchnic circulation

Introduction

Bile salts are amphiphilic end products of cholesterol metabolism which have multiple physiological functions. Under normal conditions, bile salts are restricted to the enterohepatic circulation, maintaining a serum level below 5 μmol l^{-1} (Ahlberg *et al.*, 1977). In addition to their well-known effects in the intestinal tract, bile salts have effects on the cardiovascular system.

Both intraluminal placement and intra-arterial infusion of bile salts elicit an increase in ileal blood flow, suggesting a possible role in mediating postprandial intestinal hyperaemia (Kveitys *et al.*, 1981). Previously, we showed that bile salts are directly vasoactive, dilating vessels *in vitro* and inducing systemic hypotension *in vivo* (Pak & Lee, 1993). In conditions where serum bile salt levels are elevated up to 100 fold, such as in liver cirrhosis, the circulation becomes hyperkinetic: cardiac output is increased, and systemic vascular resistance and arterial pressure are decreased (Kowalski & Abelman, 1953). Bile salts have been suggested as possible mediators of this hyperdynamic circulation in cirrhosis (Bomzon *et al.*, 1984; Pak & Lee, 1993). Thus, they appear to be vasoactive in various physiological and pathological conditions. However, to date little is known regarding the mechanism(s) whereby bile salts mediate vasoactivity.

Various theories have been proposed. Based on direct or circumstantial evidence, a role for membrane α- or β-adrenoceptors (Joubert, 1978; Jacob *et al.*, 1993), K^+ channels (Binah *et al.*, 1987), Na^+, K^+-ATPase (Harries & Sladen, 1971; Seda *et al.*, 1984), endothelium-dependent relaxing factors (Lee *et al.*, 1992), and sensory afferent fibres (Rozsa & Jacobson, 1989) in the mechanism of bile salt vasoactivity has been suggested.

Despite the foregoing suggestions, the exact mechanism of bile salt vasoactivity remains unclear. Hence, in the present study we tried to clarify the vasoactive mechanisms by systematically investigating the effects of pharmacological blockade of the membrane pumps, ion channels, adrenoceptors and sensory afferent nerves on the vasodilator action of bile salts. The possible involvement of intact vascular endothelium was also tested.

Methods

Male Sprague-Dawley rats (Charles River, St. Constant, QC, Canada), weighing 300–350 g were studied. The protocol was approved by the University of Calgary Animal Care Committee. All rats were allowed free access to food and water until 14–16 h before the experiment when food was withdrawn.

The effect of bile salts was tested in an isolated mesenteric bed which contains resistance arteries, as described by McGregor (1965). Rats were killed by decapitation and a midline laparotomy was performed. The aorta was ligated both proximally and distally to the superior mesenteric artery. After a small cannula (PE-50, Clay Adams, Parsippany, NJ, U.S.A.) was inserted into the superior mesenteric artery at its confluence with the aorta, the vessels were

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flushed with warmed physiological salt solution (PSS, 10 ml) to wash out blood. The composition of PSS was as follows (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 12.5, KH_2PO_4 1.2, D-glucose 11.1. After the gut was resected close to the mesenteric border, the whole mesenteric bed was isolated, and placed in a thermostatically controlled (37°C) organ bath and perfused with oxygenated, warm PSS (37°C, pH 7.4, aerated with 95% O_2 and 5% CO_2) at a rate of 5 ml min^{-1} by a peristaltic pump (LKB Bromma, model 2132, Sweden). Changes in perfusion pressure were measured with a Statham pressure transducer (P23 DB transducer, Gould Inc., Oxnard, CA, U.S.A.) and recorded on a polygraph recorder (model 7E, Grass instruments, Quincy, MA, U.S.A.). The effluent of the perfused vessels was continuously drained from the organ bath in order to prevent any contact of vessels with that fluid. The preparation was equilibrated for 1 h before starting any experiment. The mesenteric bed was precontracted by continuous infusion of a selective α_1-adrenoceptor agonist, cirazoline (3×10^{-6} M). Tauroursodeoxycholate (TUDC), taurochenodeoxycholate (TCDC) or taurodeoxycholate (TDC) was administered as a single dose (1.9×10^{-8} to 10^{-6} mol) in the precontracted mesenteric bed and noncumulative dose-response curves were obtained.

In order to plot vascular response curves to bile salt concentrations, a different series of experiments was performed. Each bile salt was added to the PSS reservoir supplying the peristaltic pump. In this way, responses to bile salts at concentrations from 10^{-6} to 10^{-3} M were obtained. Cumulative dose-response curves for the three types of bile salt were constructed. Only one type of bile salt was examined per mesenteric bed preparation. Changes in perfusion pressure were calculated as a percentage of the maximal pressure induced by cirazoline (3×10^{-6} M).

To measure the contractile responses in isolated vascular rings, rats were anaesthetized by inhalation of halothane, and the superior mesenteric artery, carotid artery and portal vein were isolated and cleaned *in situ*. These vessels were removed and the rats killed by exsanguination. Vascular rings of 4 mm length were excised and mounted in an organ bath containing PSS. This length was shown in preliminary studies to provide optimum contractile responses. The preparation was continuously aerated with 95% O_2 and 5% CO_2. The temperature of the bath was kept at 37°C and the pH adjusted to 7.4. The vessels were allowed to equilibrate for at least 60 min before the start of any experiment; meanwhile PSS was replaced every 20 min. An initial 0.5 g tension for portal vein and 1.5 g tension for arteries were applied by changing the position of the force displacement transducer (Model FT 03, Grass Instruments, Quincy, MA, U.S.A.). The changes in tension were recorded on a polygraph recorder (model 2400S, Gould Inc., Cleveland, OH, U.S.A).

Involvement of ion channels or membrane pumps

The vascular tension and the spontaneous contractile activity were compared before and after TDC application on isolated portal venous rings. Cumulative dose-response curves to TDC (10^{-6} to 10^{-3} M) were constructed.

Contractions induced by cirazoline, vasopressin, and KCl were obtained in the perfused mesenteric bed before and after TDC treatment. The mesenteric bed was isolated as described above. Control contractile responses for each agonist (cirazoline 10^{-7} to 3×10^{-5} M; vasopressin 10^{-10} to 10^{-7} M; KCl 0.01 to 1.0 M) were obtained at the beginning of the experiment. The tissue was then incubated with TDC (300 μM) for a 30 min period before adding the contractile agonists. For this study, only one agonist was used for each experiment. The control contractile response to each agonist in the absence of TDC was compared with that in the presence of TDC.

To study the effects of TDC on the intracellular store(s) of Ca^{2+} mobilized by noradrenaline, a previously described experimental protocol was used (Karaki *et al.*, 1979). Rings of superior mesenteric and carotid artery were incubated in Ca^{2+}-free PSS (containing EGTA 1.0 mM) for 10 min. Then the tissues were stimulated by successive additions of 10^{-6} M noradrenaline for 5 min ('Ca^{2+}-washing') in the absence of Ca^{2+} until no further contraction was observed. This indicated that complete depletion of intracellular Ca^{2+} stores sensitive to noradrenaline had been achieved. After this, the tissues were exposed to PSS containing 2.7 mM Ca^{2+} for 20 min to load the intracellular Ca^{2+} stores ('Ca^{2+}-refilling') in the absence of noradrenaline. With the internal Ca^{2+} stores refilled with Ca^{2+}, the extracellular Ca^{2+} was removed again by washing the tissues with Ca^{2+}-free PSS for 5 min. The tissues were then stimulated by 10^{-6} M noradrenaline. This contraction in a phasic mode reflects Ca^{2+} mobilization from intracellular Ca^{2+} stores. For control groups, the above protocol was performed without addition of TDC. The same experimental protocol was repeated in the presence of TDC (300 μM) applied at the end of the 'Ca^{2+}-refilling' period. Results are expressed as the percentage of the control group contractions in the absence of TDC.

The contribution of K^+ channels to the vasodilator actions of the bile salts was assessed by use of high K^+-PSS (60 mM) which interrupts the K^+ efflux. High K^+ PSS was prepared by substituting an equimolar amount of K^+ for Na^+. It is possible that high K^+ induced-depolarization may cause the release of noradrenaline from adrenergic nerve terminals. This undesired event was blocked by adding prazosin (10 nM) to the high K^+-PSS reservoir. Since high K^+-PSS contracts vessels by depolarization, the isolated mesenteric bed perfused with this solution does not require any other contractile agonist. When the contractile response was maximal, TDC was added as a single dose (1.9×10^{-8} to 1.9×10^{-6} mol). The results were expressed as a percentage of the maximal contraction induced by high K^+-PSS. The dose-response curve to TDC in this high K^+-PSS was compared to that obtained in normal PSS.

The role of the K^+ channels in the action of bile salts was also tested using the non-selective potassium-channel blocker, tetraethylammonium (TEA, 5 mM). TEA was perfused for 1 h and the mesenteric arterial bed isolated from control rats was contracted with cirazoline (3×10^{-6} M). The effect of TEA was determined on the vasodilator effect of TDC (1.9×10^{-8} to 1.9×10^{-6} mol) in this preparation.

PSS without potassium inhibits Na^+,K^+-ATPase activity (Lawson & Cavero, 1989). In order to evaluate the possible contribution of Na^+,K^+-ATPase in mediating vasodilatation caused by bile salts, the vasodilator effects of TDC were tested during perfusion with either normal or K^+-free PSS. K^+-free PSS was prepared by an equimolar replacement of KCl with NaCl and KH_2PO_4 with NaH_2PO_4. The perfused mesentery was precontracted by cirazoline (3×10^{-6} M) and then the response to TDC (1.9×10^{-8} to 1.9×10^{-6} mol) was recorded during perfusion with normal K^+ PSS or K^+-free PSS.

In other experiments, the mesenteric bed isolated from control rats was perfused with normal PSS containing the Na^+,K^+-ATPase blocker, ouabain (10^{-5} M), for 30 min. The selectivity of ouabain for Na^+,K^+-ATPase was reported against K^+-induced vasorelaxation (Webb & Bohr, 1978). Then dose-response curves to TDC (1.9×10^{-8} to 1.9×10^{-6} mol) with or without ouabain pretreatment were constructed and compared.

Importance of the endothelium for the effect of bile salts

Dose-response curves to TDC in precontracted arteries were constructed with or without endothelium. The endothelium was denuded by instillation of distilled water for 10 min (Criscione *et al.*, 1984). This denuded mesenteric bed was equilibrated with normal PSS for 1 h and was contracted with cirazoline (3×10^{-6} M). Dose-response curves to TDC (1.9×10^{-8} to 1.9×10^{-6} mol) were established in these

denuded vessels and compared with those obtained from the mesentery with intact endothelium.

In some arteries with intact endothelium, the involvement of nitric oxide (NO) in bile salt-induced vasodilatation was tested by using the NO synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME). The mesenteric arterial perfusion pressure was increased by infusing 3×10^{-6} M cirazoline, and the response to TDC (1.9×10^{-8} to 1.9×10^{-6} mol) with or without L-NAME (10^{-4} M, incubated for 30 min) was plotted.

In both types of experiments, the adequacy of removal of endothelium and the blockade of NO were tested by comparing the vasodilator effect of acetylcholine (ACh, 10^{-6} M) before and after either endothelial denudation or L-NAME treatment.

Neurally-mediated mechanisms

The effects of bile salts were tested in rats without primary sensory nerves. To denervate the primary afferent nerves, rats were treated with capsaicin when they were neonates. Capsaicin treatment in the neonate causes permanent degeneration of capsaicin-sensitive neurones throughout the body and desensitization to capsaicin (Jancso *et al.*, 1977).

Under halothane anaesthesia, rat pups (Charles River Canada, St. Laurent, QC, Canada) were injected with capsaicin 50 mg kg^{-1} (dissolved in a vehicle of 80% physiological saline, 10% Tween 80 and 10% ethanol), s.c., on the second day of life and allowed to grow (Jancso *et al.*, 1977; Lee & Sharkey, 1993). Three months later, when their body weights were between 200–250 g, a mesenteric perfusion study was performed as described earlier. TDC was administered as a single dose (1.9×10^{-8} to 1.9×10^{-6} mol) in a cirazoline (3×10^{-6} M)-induced precontracted mesenteric bed and noncumulative dose-response curves were obtained. The efficacy of sensory afferent denervation was confirmed the day before the experiment by the absence of a corneal reflex in response to capsaicin (0.1% capsaicin, dissolved in distilled water) instilled into the eye. In addition, after finishing the experiment, the effect of capsaicin (10^{-7} M) was tested in the precontracted mesenteric arterial bed to confirm the inhibition of capsaicin-induced vasorelaxation (Manzini & Perretti, 1988).

The involvement of β-adrenoceptors was tested by using propranolol. The effect of TDC (10^{-6} to 10^{-3} M) was tested before and after propranolol (3 μM, incubated for 10 min) in isolated portal venous and superior mesenteric arterial rings.

Drugs

The following drugs were purchased from Sigma Chemicals, St. Louis, MO, U.S.A.: noradrenaline bitartrate, acetylcholine bromide, arginine vasopressin, tetraethylammonium, capsaicin, propranolol, prazosin, N^G-nitro-L-arginine methyl ester, ethylene glycol-*bis* (β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid, phentolamine hydrochloride and ouabain. Cirazoline hydrochloride was obtained from Synthelabo Laboratories, Paris, France. Sodium salts of tauroursodeoxycholic acid, taurochenodeoxycholic acid and taurodeoxycholic acid were purchased from Calbiochem Corporation (La Jolla, CA, U.S.A.). Noradrenaline stock solution (10^{-2} M) was prepared in 0.1 M hydrochloric acid and diluted with distilled water on the day of the experiment.

Statistical analysis

The alterations in perfusion pressure were calculated as a percentage change. The changes in tension in isolated vascular rings were depicted as differences in absolute values. The responses to cirazoline, vasopressin, and KCl were also expressed as pressure changes in absolute values. All results are expressed as mean ± s.e.mean. The data were analysed by the Student's *t* test and one-way analysis of variance with a Tukey correction for multiple comparisons. Differences were considered significant at $P<0.05$.

Results

Mesenteric perfusion study

The basal perfusion pressure in mesenteric arteries of control rats perfused with PSS was 15 ± 1 mmHg ($n = 30$). Infusion of cirazoline (3×10^{-6} M) resulted in a sustained perfusion pressure (59 ± 5 mmHg) and bile salts infused as a single dose (1.9×10^{-8} to 1.9×10^{-6} mol) induced a dose-dependent vasorelaxation (Figure 1). TUDC had no effect; TCDC induced moderate relaxation whereas with TDC relaxation was greatest, virtually eliminating the cirazoline-induced pressure at 5.8×10^{-7} mol. The vasodilator effect was rapid and almost completely reversible.

Responses to infusions of bile salts at 10^{-6} to 10^{-3} M showed a similar concentration-dependent relaxation and the

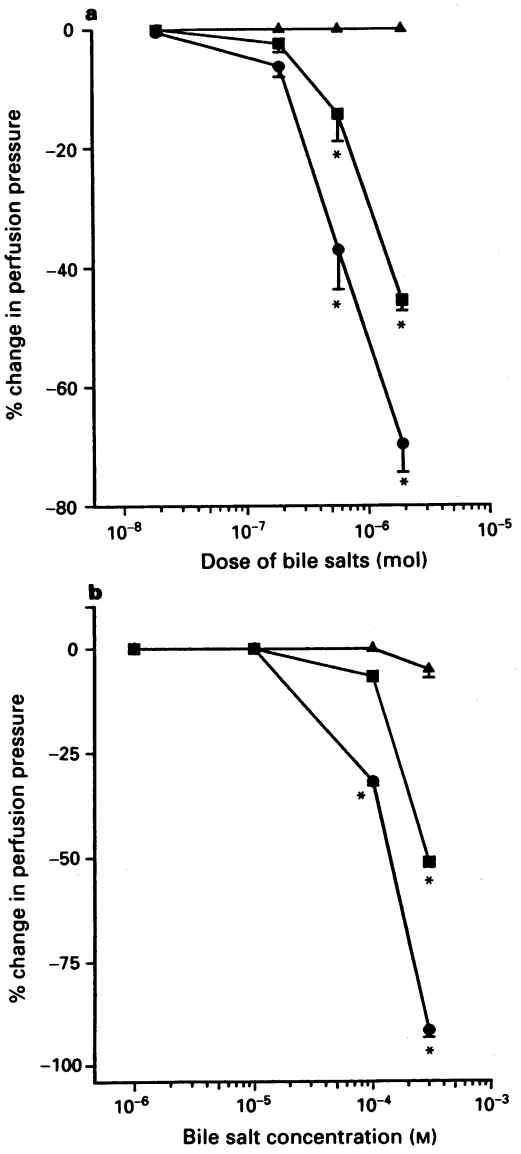


Figure 1 Percentage relaxation effects of taurodeoxycholate (●), taurochenodeoxycholate (■), and tauroursodeoxycholate (▲) on isolated mesenteric arterial bed of the rat precontracted with cirazoline (3×10^{-6} M): (a) shows the responses of bile salts in mol dose (1.9×10^{-8}–1.9×10^{-6} mol) ($n = 8$) and (b) shows the responses to molar concentrations of bile salts (10^{-6}–5×10^{-4} M) ($n = 6$). *$P<0.05$ vs. contractile tone induced by cirazoline.

potency of three different bile salts were of the same order (Figure 1).

Involvement of Ca^{2+} channels

Effects of TDC on resting tension and myogenic portal vein activity In portal venous rings (0.5 g basal tension), the amplitude of spontaneous contractions was 287 ± 10 mg ($n = 7$) and the frequency was 3–4 min^{-1}. TDC decreased the basal tension of portal venous rings in a concentration-dependent manner between 10^{-5} and 10^{-3} M (Figure 2). In addition, the amplitude and rate of the spontaneous contractions were considerably depressed in response to TDC (Figure 2) resulting in complete abolition at 5×10^{-4} M. After a washout period of 45–60 min, the frequency and amplitude of spontaneous contractions had almost completely recovered.

TDC effect on contractions induced by cirazoline, vasopressin and KCl The contractile agonists cirazoline (10^{-7} to 3×10^{-5} M), vasopressin (10^{-10} to 10^{-7} M) and KCl (0.01 to 1.0 M) induced contractions in a concentration-dependent manner in the isolated perfused mesentery. This contractile activity was attenuated by incubating the mesenteric arteries with TDC (300 μM) (Figure 3).

Effects of TDC on the membrane stores of Ca^{2+} that are mobilized by noradrenaline Stimulation with 10^{-6} M nora-

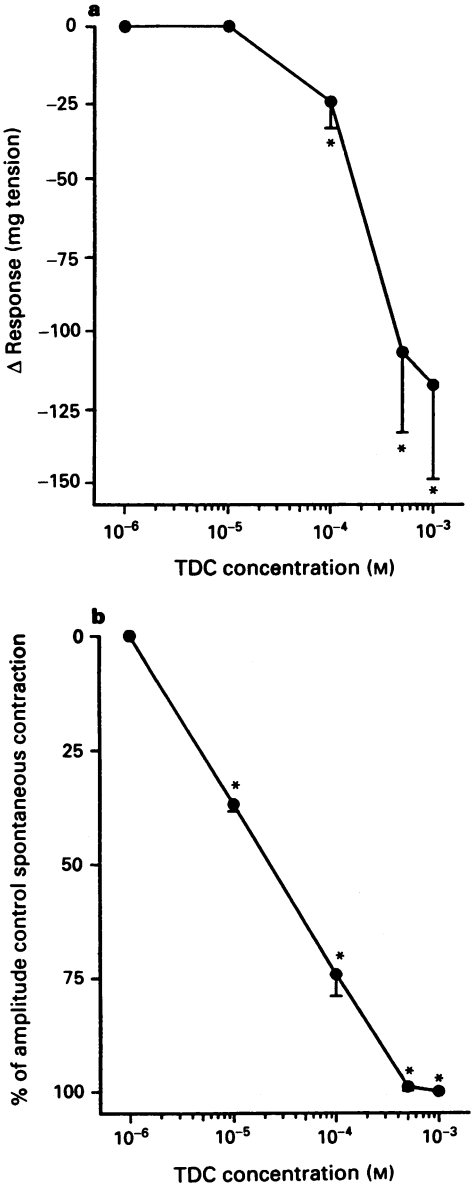


Figure 2 The effect of taurodeoxycholate (TDC; 10^{-6}–10^{-3} M) on isolated rings of portal vein from control rats. Taurodeoxycholate induced a concentration-dependent relaxation of the basal tension (a, $*P < 0.05$ vs. initial tension) and inhibition of spontaneous contractile amplitude (b, $*P < 0.05$ vs. initial amplitude of spontaneous contraction). $n = 7$ for each group.

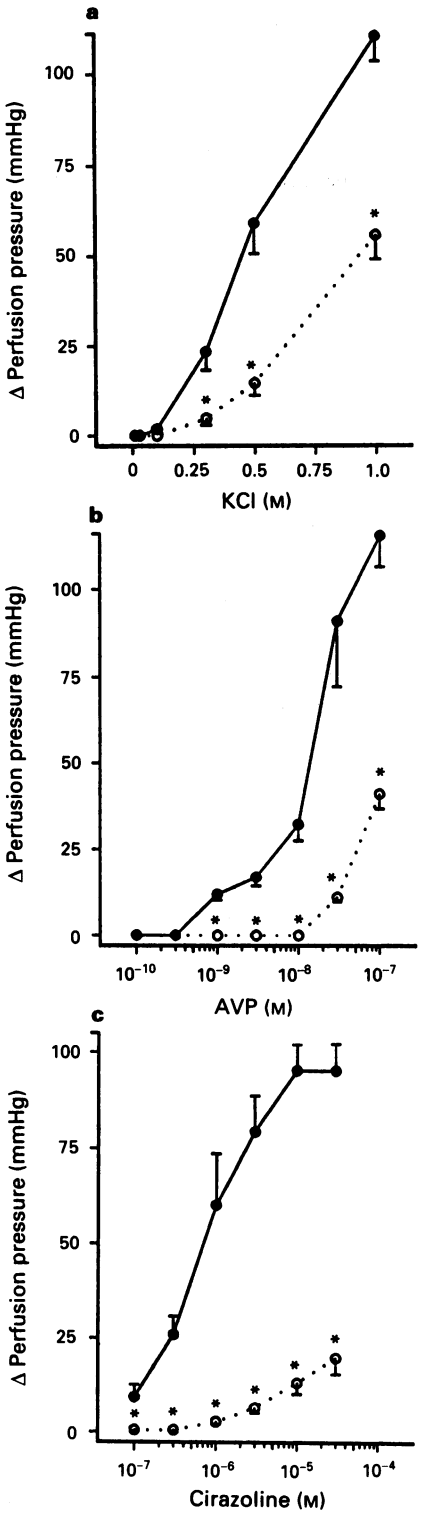


Figure 3 Dose-response curves to KCl (0.01–1.0 M; a), arginine vasopressin (AVP, 10^{-10}–10^{-7} M; b) and cirazoline (10^{-7}–3×10^{-5} M; c) in perfused mesenteric arteries. In all panels, (●, $n = 6$) represent control responses to the agonist, and (○, $n = 6$) represent the responses after preincubation with taurodeoxycholate (300 μM). $*P < 0.05$ vs. control group (●).

drenaline in the absence of Ca^{2+} induced phasic contractions of the vessels. The amplitude was 609 ± 116 mg for the superior mesenteric artery ($n = 8$) and 160 ± 28 mg for the carotid artery ($n = 7$). This reflects Ca^{2+} mobilization from an intracellular store (Karaki *et al.*, 1979). When TDC (3×10^{-4} M) was applied at the end of the 'Ca^{2+}-refilling' period, TDC did not change the amplitude of the noradrenaline-induced phasic contraction (12 ± 8% reduction in the superior mesenteric artery and 6 ± 17% increase in the carotid artery; both changes were not significantly different from their respective control values) (Figure 4).

Involvement of K^+ channels

Infusion of 60 mM K^+ PSS with 10 nM of prazosin produced a stable tone of 101 ± 3 mmHg. In this preparation, there was also a dose-dependent fall in perfusion pressure in response to TDC infusion (1.9×10^{-8} to 1.9×10^{-6} mol) (Figure 5). This effect of TDC was not attenuated by pretreatment with TEA, 5 mM (Figure 5).

Involvement of Na^+,K^+-ATPase

Perfusion with K^+-free PSS did not change the basal tone of the mesentery. In K^+-free PSS, however, the cirazoline-induced contraction was enhanced compared to normal PSS (80 ± 5 mmHg for K^+-free PSS and 60 ± 2 mmHg for normal PSS, $n = 6$ for each group). TDC (1.9×10^{-8} to 1.9×10^{-6} mol)-induced vasodilatation was not changed significantly in K^+-free PSS (Figure 5). The decrease in perfusion pressure induced by TDC (1.9×10^{-8} to 1.9×10^{-6} mol) was not attenuated by ouabain (10^{-5} M) infusion (Figure 5).

Importance of the endothelium for the effect of bile salts

Removal of the endothelium by treatment with distilled water did not change the basal perfusion pressure in the mesenteric bed. However, infusion of cirazoline (3×10^{-6} M) through denuded vessels generated significantly higher tone (95 ± 14 mmHg) than with intact endothelium (57 ± 2 mmHg). Endothelium denudation caused complete abolition of ACh (10^{-6} M)-induced reduction in perfusion pressure. TDC dilated the mesenteric bed without endothelium in a similar pattern to the mesenteric arteries with intact endothelium (Figure 5).

L-NAME (10^{-4} M)-treatment enhanced the cirazoline-induced tone. The tone was increased from 58 ± 7 mmHg to 89 ± 1 mmHg (each, $n = 5$). The vasorelaxant effect of TDC (1.9×10^{-8} to 1.9×10^{-6} mol) was not affected by L-NAME treatment (Figure 5). The adequacy of the NO synthase inhibition was also tested with ACh (10^{-6} M). The vasodilatation induced by ACh (43 ± 3 mmHg reduction in control) was significantly reduced in L-NAME-treated vessels compared with that in control mesentery (25 ± 1% of control; $P < 0.05$).

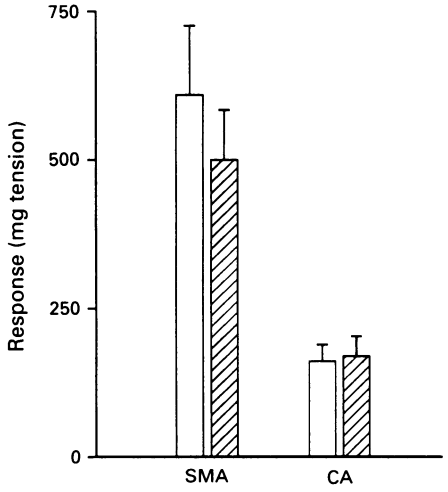


Figure 4 Effects of taurodeoxycholate (300 μM) on membrane stores of Ca^{2+} mobilized by noradrenaline (NA; 10^{-6} M). Isolated rings of superior mesenteric artery (SMA) and carotid artery (CA) were incubated in Ca^{2+}-free medium, and NA-sensitive intracellular Ca^{2+} stores were depleted by stimulation with NA in the absence of Ca^{2+}. After the intracellular calcium stores were refilled by exposure to Ca^{2+} (2.7 mM × 20 min), contraction was induced by NA in the absence of Ca^{2+}. This phasic contraction induced by NA (open columns) was compared with contraction (hatched columns) from the tissues which were incubated with taurodeoxycholate applied at the end of Ca^{2+}-refilling period. There were no significant differences between the taurodeoxycholate-pretreated (hatched columns) and nonpretreated (open columns) groups. $n = 8$ for the superior mesenteric artery and $n = 7$ for the carotid artery.

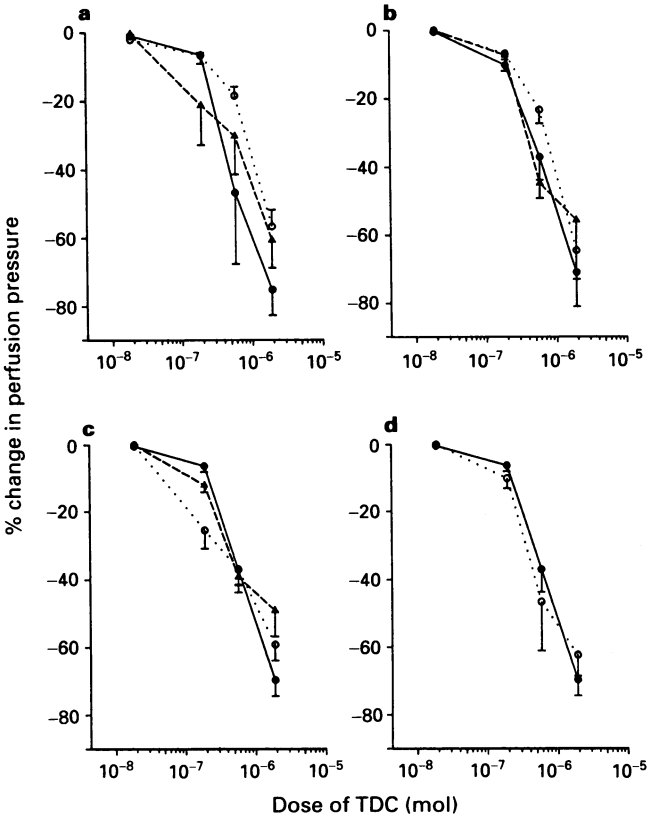


Figure 5 Effects of variations in physiological salt solution K^+ concentration, ouabain, tetraethylammonium, endothelial denudation, nitric oxide synthase inhibition, and capsaicin on the vasodilatation induced by taurodeoxycholate (TDC; 1.9×10^{-8}–1.9×10^{-6} mol) in precontracted perfused mesenteric arteries. In (a) the vasodilator effects of taurodeoxycholate (1.9×10^{-8}–1.9×10^{-6} mol) on the isolated mesenteric bed perfused with normal K^+ (●) as a control, K^+-free (○), and high K^+ (▲) physiological salt solution are shown. In (b) the vasodilator effect of taurodeoxycholate (1.9×10^{-8}–1.9×10^{-6} mol) was compared before (●) and after ouabain, 10^{-5} M (▲) and tetraethylammonium, 5 mM (○) infusions. There were no significantly different points compared to the corresponding values in the control group (●). $n = 6$ in all groups (a and b). In (c), neither denudation of endothelium (○) nor N^G-nitro-L-arginine methyl ester, 10^{-4} M (▲) had any effect on the vasodilatation induced by taurodeoxycholate (1.9×10^{-8}–1.9×10^{-6} mol). $n = 5$ for each point in all groups. $^*P < 0.05$ vs. control with intact endothelium (●). In (d), the effects of taurodeoxycholate (1.9×10^{-8}–1.9×10^{-6} mol) on the mesenteric beds isolated from control rats (●) ($n = 4$) and capsaicin-treated rats (○, 50 mg kg^{-1}) ($n = 6$) are shown. There were no significantly different points compared to the corresponding values in the control group (●).

Dependence of bile salt effect on the sensory afferent nerves

The basal perfusion pressure of the capsaicin-treated mesenteric bed was 13 ± 1 mmHg ($n = 6$). This pressure remained stable throughout the experiment. In capsaicin-treated mesenteric vessels, the vasodilator effect of TDC (1.9×10^{-8} to 1.9×10^{-6} mol) remained unchanged (Figure 5). The efficacy of capsaicin denervation was complete because the vasodilator effect of acute capsaicin (10^{-7} M) administration was completely blocked in the capsaicin-treated mesenteric bed (data not shown).

Involvement of β-adrenoceptors

The portal vein pretreated with propranolol (3 μM) continued to undergo spontaneous contractions but the basal tone increased slightly. TDC (10^{-6} to 10^{-3} M) added after the propranolol incubation reduced the basal portal venous tone significantly (107 ± 26 mg for control and 73 ± 8 mg for propranol pretreated group) and abolished the spontaneous contractions at the highest concentration (500 μM).

In isolated superior mesenteric arterial rings ($n = 6$), vasorelaxation induced by TDC (10^{-6} to 10^{-3} M) was completely unaffected by pretreatment with propranolol (3 μM). The dose-response curves of TDC vasorelaxation with and without propranolol pretreatment were virtually identical: at the 500 μM concentration of TDC, there was 42 ± 5% relaxation without propranolol, whilst with propranolol pretreatment, the % relaxation was 41 ± 3%.

Discussion

The cardiovascular effects of bile salts have been well described (Joubert, 1978; Bomzon *et al.*, 1984; Binah *et al.*, 1987; Pak & Lee, 1993), but few studies have examined their mechanism of action. The vasodilator effect of bile salts was confirmed in the present study using the isolated perfused mesentery. Since the contribution of small arteries (< 200 μm diameter) to the peripheral vascular resistance is much more important than that of large arteries, the effects of bile salts in a perfused mesentery which contains small arteries, provide more convincing evidence that bile salts are indeed vasodilator. In addition, the vasoactivity of different subtypes of bile salts agrees with previous results; TDC > TCDC > TUDC (Pak & Lee, 1993). Since the hydrophobic: hydrophilic ratio also decreases in the order TDC > TCDC > TUDC (Coleman, 1987), vasoactive potency seems to relate to their degree of hydrophobicity. To delineate further the mechanism of bile salt-induced vasodilatation, the most potent vasoactive bile salt, TDC, was used. Even though the vasodilator potencies of TDC and TCDC were different, their vasoactive properties were similar. Therefore we believe that the mechanism underlying this TDC effect can be extrapolated to the other bile salts.

Noncumulative dose-response curves were constructed by injecting a single dose of each bile salt into the perfused mesenteric bed. Thus this dose was expressed as mol dose. To allow comparison with other studies that report their data in molar concentration, we also examined cumulative concentration-dependent curves with molar concentration. When the two curves were compared, the vasorelaxant effects of 1.9×10^{-6} mol of bile salts (TDC and TCDC) were similar to those of concentrations between 10^{-4} and 3×10^{-4} M. In other words, the effect of the highest dose of bile salts may correspond to a serum concentration less than 3×10^{-4} M which is still within the concentration frequently seen in liver disease.

The vasoactivity of bile salts did not involve β-adrenoceptors, sensory afferent nerves, endothelium or nitric oxide synthase, or Na^+,K^+-ATPase.

Recently, inhibition of ATP-sensitive K^+ channels with glibenclamide has been studied in portal hypertensive rats (Moreau *et al.*, 1992). This treatment significantly diminished the increased portal pressure and hyperkinetic circulation. This implies that under basal conditions of portal hypertension, ATP-sensitive K^+ channels are activated and lead to K^+ efflux and consequently vasodilatation. Since portal hypertensive rats have high bile salt levels, we wondered if bile salts may dilate the vessels via K^+ channel activation. However, the present data argue against this hypothesis, as nonspecific K^+ channel blockade by TEA did not affect TDC-induced vasodilatation.

Most vasoconstriction requires an increase in intracellular Ca^{2+} concentration. Bile salts may interfere with the elevation of Ca^{2+} levels, thus inducing vasodilatation. Evidence favouring this idea was seen in the isolated portal venous ring which has spontaneous contractions under normal conditions. TDC reduced the basal tone and inhibited the spontaneous myogenic activity of this vein. Since these spontaneous contractions are triggered by bursts of propagated action potentials which activate the voltage-operated calcium channels (VOC) (Johansson & Ljung, 1967), it appears that TDC inhibits Ca^{2+} influx via VOC, hindering generation of spontaneous contractile activity.

Also in agreement with this hypothesis is the finding that TDC inhibited the contraction caused by high K^+ depolarization. High K^+-induced vasocontraction is caused by an increased Ca^{2+} entry via voltage-operated L-type Ca^{2+} channels (Bolton, 1979), and is noncompetitively and specifically blocked by calcium antagonists (Weiss, 1981). Thus vasodilatation induced by TDC is due to the inhibition of Ca^{2+} entry through VOC.

The possible involvement of other types of Ca^{2+} channels was tested by use of cirazoline and vasopressin. TDC decreased the contraction induced by both agonists. Contraction caused by cirazoline is thought to result from an increased release of intracellular Ca^{2+} from sarcoplasmic reticulum, plus an increased Ca^{2+} entry through receptor-operated Ca^{2+} channels (ROC) (Van Meel *et al.*, 1981). Since the relative contribution of Ca^{2+} mobilization from two different sources on cirazoline-induced contraction is unknown in mesenteric vessels, this experiment does not provide a clear answer as to which process is more effectively inhibited by TDC. The vasoconstrictor effect of vasopressin is mediated by the vasopressin V_1 receptor subtype (Fox *et al.*, 1987). Activation of this receptor is followed by inositol trisphosphate production, Ca^{2+} release from intracellular stores and augmentation of Ca^{2+} entry through ROC. Inhibition of vasopressin-induced contractions by TDC provides additional evidence of possible involvement of ROC. It seems, therefore, that TDC interferes with the Ca^{2+} entry in a nonspecific way, by inhibiting both ROC and VOC.

The tonic component of noradrenaline-induced contractions has been attributed to an increase in Ca^{2+} influx via ROC (Bolton, 1979). The phasic component has been attributed to the release of Ca^{2+} from intracellular stores, since this transient contraction was persistent in Ca^{2+}-free medium (Karaki *et al.*, 1979). This phasic contraction induced by noradrenaline was not affected by TDC. This again supports the notion that TDC dilates vessels not by affecting the Ca^{2+} release from intracellular stores but by inhibiting Ca^{2+} entry through Ca^{2+} channels.

Bile salts can enter certain cells in the liver and intestine. They are taken up either by a sodium-dependent transport system or by simple passive diffusion in those cells. Can bile salts also enter vascular smooth muscle cells? This may be possible for at least unconjugated, hydrophobic di- and monohydroxy bile salts, because they can enter the cell by passive, nonsaturable diffusion (Van Dyke *et al.*, 1982). However, the target of the bile salt action on the vascular smooth muscle cells is probably the plasma membrane. In this regard, bile salts have been reported to exert effects on the plasma membranes of the hepatocyte and erythrocyte by changing membrane fluidity or phospholipid composition.

Both decreased and increased membrane fluidity have been observed in cholestasis (reviewed in Sellinger & Boyer, 1990). In chronic biliary cirrhotic rats, however, cardiac membrane fluidity is reduced (Ma *et al.*, 1994). Therefore, TDC may affect the Ca^{2+} channels by changing the plasma membrane fluidity and further investigation of the interaction between bile salts and Ca^{2+} channels in the plasma membrane is therefore necessary.

The relationship between Ca^{2+} and bile salts has been studied actively, but mostly regarding gallstone formation and cytotoxic effects of bile salts. Those studies show that bile salts elevate the intracellular Ca^{2+} level by promoting Ca^{2+} uptake or increasing Ca^{2+} release from the intracellular stores (Anwer *et al.*, 1988). This increase in cytosolic Ca^{2+} accounts for the cytotoxicity of bile salts, especially monohydroxy bile salts. However, the data have been drawn from either erythrocytes or hepatocytes, and the conditions in vascular smooth muscle may be different.

It has also been reported that bile salts can bind Ca^{2+}. The calcium binding capacity is correlated to the concentrations of bile salts – the higher the concentration of bile salt, the stronger binding to calcium (Gleeson *et al.*, 1990). Furthermore, the calcium binding at a bile salt level less than 10 mM was essentially negligible (Gleeson *et al.*, 1990). Hence, our results are extremely unlikely to be related to the calcium-binding property of bile salts.

In conclusion, the vasodilator effects of bile salts have been confirmed in the isolated perfused rat mesentery which contains resistance vessels. The potency of the vasoactive effect of three bile salts coincides with the order of hydrophobicity. Vasodilatation induced by bile salts is mediated by their Ca^{2+} antagonistic property. Our results suggest that TDC inhibits Ca^{2+} entry through both VOC and ROC, whereas intracellular Ca^{2+} release is not affected.

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Effects of bipyridylium compounds on calcium release from triadic vesicles isolated from rabbit skeletal muscle

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1 The effects of 1,1'-diheptyl-4,4'-bipyridinium dibromide (DHBP), a viologen for electrochromic memory display agent, on calcium release and ryanodine binding were studied with triad-rich sarcoplasmic reticulum (SR) vesicles isolated from rabbit skeletal muscle.

2 DHBP inhibited the calcium release induced by 2 mM caffeine and 2 $\mu g\ ml^{-1}$ polylysine with an IC_{50} value of 5 $\mu g\ ml^{-1}$ and 4 $\mu g\ ml^{-1}$ respectively.

3 DHBP inhibited [^{3}H]-ryanodine binding in a dose-dependent manner with an IC_{50} of 2.5 $\mu g\ ml^{-1}$ and 90–100% inhibition at 20–30 $\mu g\ ml^{-1}$.

4 Calcium uptake by SR was inhibited in the presence of caffeine and this inhibition was antagonized by concomitant addition of DHBP.

5 The effect of DHBP on muscle twitches was studied on the mouse diaphragm. Muscle twitches elicited by direct electrical muscle stimulation and contractions induced by either 10 mM caffeine or 1 μM ryanodine were blocked by pretreatment with DHBP.

6 Data from this study provided evidence that DHBP blocked the calcium release from SR by direct interaction with the calcium release channel, also known as the ryanodine receptor. A possible use of this agent as a specific inhibitor for calcium release and as a muscle relaxant was suggested.

Keywords: Skeletal muscle; SR; calcium release; bipyridylium

Introduction

Muscle contraction is initiated by membrane depolarization which, by an unknown mechanism, triggers calcium release from the sarcoplasmic reticulum (SR) into the myoplasma and causes the muscle to contract. How transient changes in the T-tubule membrane potential lead to Ca^{2+} release from SR is one of the most important unresolved questions in muscle physiology (for a recent review, see McPherson & Campbell, 1993). SR is the major internal calcium storage site of the muscle cell, the uptake and release of the calcium ion from and into the myoplasma are controlled by two molecules located on SR membrane, namely, Ca^{2+}-ATPase and the calcium release channel (also known as the ryanodine receptor) respectively. Several chemicals have been shown to affect the calcium release process by direct interaction with the calcium release channel (Palade *et al.*, 1989) and defects of the calcium release channel may be associated with some clinical diseases, such as malignant hyperthermia (Mickelson *et al.*, 1988).

Pyridine compounds, such as dihydropyridines and 4-aminopyridine, have been shown to affect the physiological muscle contraction by acting on L-type calcium channels located on the transverse tubule (Romey *et al.*, 1988) and potassium channels on the transverse tubule or Ca^{2+}-ATPase on the SR (Ishida & Honda, 1993), respectively. Nagura *et al.* (1988) have shown that 4,4'-dithiodipyridine binds to certain SH groups of the Ca^{2+}-induced Ca^{2+} release channels in the SR membrane and thus induces Ca^{2+} release. Recently, we have shown that 2,2'2''-tripyridine, a byproduct found in paraquat manufacturing, produced a curare-like action in the mouse phrenic nerve-diaphragm preparation (Lin-Shiau *et al.*, 1992) and blocked the nicotinic acetylcholine receptor channels in embryonic *Xenopus* muscle cells (Hsu *et al.*, 1993).

In this study, we have investigated the effect of several bipyridylium compounds (also known as viologens) widely used as oxidation-reduction indicators in biological systems or as nonselective contact herbicides, on calcium release and muscle contraction. We have found that 1,1'-diheptyl-4,4'-bipyridinium dibromide (DHBP) but not other bipyridylium compounds tested, can effectively block the calcium release from SR vesicles isolated from mammalian skeletal muscle and inhibit the ryanodine binding to its receptor on the SR membrane by a direct interaction with the calcium release channel. Part of this work has been published in an abstract form (Kang *et al.*, 1993).

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Methods

Preparation of heavy SR fraction

The triad enriched heavy fraction of SR (HSR) was prepared from rabbit leg and back muscles by differential centrifugation as described by Ikemoto *et al.* (1984) with modifications. Briefly, the muscles were homogenized in three times their volume of ice cold MOPS 20 mM, EDTA 0.1 mM, EGTA 0.1 mM and PMSF 0.2 mM, pH 7.0 buffer and the homogenates were centrifuged at 10,000 *g* for 5 min in a JA-14 rotor (Beckman). The supernatant fraction was filtered through eight layers of cheese cloth and then centrifuged at 17,000 *g* for 50 min. The sedimented fraction was homogenized in a solution containing sucrose 0.3 M, KCl 150 mM, PMSF 0.2 mM and MOPS 20 mM (pH 6.8), and centrifuged at 17,000 *g* for 40 min in a JA-20 rotor (Beckman). The sedimentable fraction was homogenized in the above solution at a final protein concentration of 20–30 mg ml^{-1}. The preparation was quickly frozen in liquid nitrogen and stored at −70°C.

Mouse phrenic diaphragm preparation

Mice (ICR strain) of either sex, weighing 20–25 g, were used. The diaphragm preparation was isolated according to the method of Bülbring (1946). A modified Krebs solution of the following composition (mM) was used: NaCl 130.6, KCl 4.8, $MgSO_4$ 1.2, $NaHCO_3$ 12.5 and glucose 11.1, pH 7.4, with $CaCl_2$ 2.5 or with EGTA 2.5. The diaphragm prepara-

tion was placed in a 10 ml organ bath that was constantly gassed with 95% O_2 + 5% CO_2 at 37.0 ± 0.5°C. Contractions of the diaphragm were elicited either by direct stimulation of the muscle with a pulse of 0.5 ms at 0.2 Hz and or by the addition of 10 mM caffeine, 1 μM ryanodine (drug-induced) or 50 mM KCl (depolarization-induced) with no electrical stimulation. The muscle was loaded with a resting tension of 0.5 g, and the changes of tension were recorded via an isometric transducer (Grass FT.03) on a Grass Model 7 polygraph (Grass Instrument Co., Quincy, MA, U.S.A.).

Mitochondria preparation and calcium uptake measurement

Mitochondria were prepared from rat liver according to the method of Cain & Skilleter (1985). The calcium uptake was measured with a dual wavelength spectrophotometer using antipyrylazo III as the calcium probe in a medium containing KCl 10 mM, sucrose 0.25 M, Tris-HCl 10 mM, pH 7.1 and 1 mg ml^{-1} of freshly prepared mitochondria. The reaction was initiated by using succinate (5 mM) as substrate of oxidative phosphorylation reaction and $CaCl_2$ (30 μM) was added after 5 min incubation to measure the calcium uptake by mitochondria. The calcium ionophore, A23187 (2 μM; Sigma), was added at the end of reaction to calculate the total calcium release.

Endoplasmic reticulum preparation

Rat liver endoplasmic reticulum (ER) microsomes were prepared by the method of Alvares & Mannering (1970). Briefly, rat liver was homogenized with four times its volume of 1.15% KCl and centrifuged at 9,000 *g*, 4°C for 20 min and the supernatant was then further centrifuged at 100,000 *g*, 4°C for 1 h. The final pellet (the microsomes) after washing once with 1.15% KCl was resuspended in 0.1 M potassium phosphate buffer, pH 7.4 and stored at − 70°C.

Synaptic plasma membrane (brain plasma membrane)

Synaptic plasma membranes were prepared by the modified method of Hakim *et al.* (1982). About 1.5 g of brain from 8 rats was washed and homogenized in 10 times its volume of sucrose 0.32 M, Tris-HCl 10 mM, pH 7.4 (homogenization buffer) with a motor driven pestle. The homogenate was then centrifuged at 3,000 *g*, 4°C for 20 min and then the supernatant was centrifuged at 40,000 *g*, 4°C for 30 min to obtain a crude microsome pellet. After resuspending in Tris-HCl 10 mM, pH 7.4 (resuspension buffer), the microsome was layered on a discontinuous sucrose gradient consisting of equal volumes of 10%, 28.5% and 34% sucrose and centrifuged with the SW41 rotor (Beckman) at 100,000 *g*, 4°C for 1 h. The purified synaptic plasma membrane which appeared at the interface of 28.5% and 34% gradient layers was removed and centrifuged at 100,000 *g*, 4°C for 1 h after dilution with the resuspension buffer and the pellet resuspended with the homogenization buffer and stored at − 70°C.

Ca^{2+} release assay of SR vesicles

The time course of Ca^{2+} release from SR vesicles was investigated with a calcium sensitive probe, antipyrylazo III, in a dual wavelength spectrophotometer (Model DW-2000, Aminco) with no addition of precipitating agent according to the method modified from Palade (1987a). SR vesicles (0.5 mg ml^{-1}) were actively loaded by adding ATP 0.5 mM into a reaction mixture containing KCl 150 mM, $MgCl_2$ 0.5 mM, antipyrylazo III 100 μM, MOPS 20 mM (pH 6.8); more Ca^{2+} was then added until the SR vesicles were saturated. A release inducer, such as polylysine (2 μg ml^{-1}, mol. wt. = 3,800 Da; Sigma) or caffeine 2 mM (Sigma), was added to induce Ca^{2+} release from SR. For inhibition studies, DHBP (Aldrich) at concentration indicated was added prior to the addition of the calcium release inducer.

[^{3}H]-ryanodine binding measurement

Ryanodine binding was measured according to the method described by Pessah *et al.* (1986). In order to mimic the calcium release conditions, triadic membranes (0.5 mg ml^{-1}) were incubated at 37°C for 2 h in a medium containing KCl 250 mM, NaCl 15 mM, [^{3}H]-ryanodine 10 nM (NEM, 87 Ci $mmol^{-1}$), Tris 20 mM, pH 7.1 with the test compounds at the concentration indicated in each experiment. Non-specific binding was measured in the presence of 1 μM cold ryanodine (Calbiochem). At the end of the incubation, 100 μl of each reaction mixture was withdrawn and added to 5 ml of ice cold buffer to quench the reaction and rapidly filtered through the Whatman GF/B glass filter and rinsed once with 5 ml ice cold buffer. A value of 2 pmol mg^{-1} SR protein for [^{3}H]-ryanodine binding to SR was obtained for our SR vesicles. The data shown are the average of triplicate determinants of at least two different preparations.

Cation-dependent ATPase activity

ATPase activity was determined with a coupled-enzyme spectrophotometric ADP-release assay (Warren *et al.*, 1974) by measuring the oxidation of NADH at 340 nm in a medium containing MOPS 20 mM, pH 6.8, NADH 0.3 mg ml^{-1}, $MgCl_2$ 5 mM, EGTA 0.2 mM, PEP 0.45 mM, pyruvate kinase 5 units ml^{-1} and lactate dehydrogenase 10 units ml^{-1} (the assay mixture). Mg^{2+}-ATPase activity was measured by incubating 5–10 μg of protein in a 1 ml assay mixture at 37°C for 5 min, ATP (1 mM) was then added to start the reaction. Ca^{2+}-ATPase was measured with the addition of $CaCl_2$ (0.2 mM) and calcium ionophore, A23187 (2 μM). Na^+,K^+-ATPase was measured in the presence of NaCl 100 mM and KCl 20 mM with ouabain 1 μM used as the inhibitory control. To unmask the latent Na^+,K^+-ATPase activity, the reaction was carried out in the presence of saponin 0.2 mg ml^{-1} (or 0.003% SDS).

Lipid peroxidation measurements

Thiobarbituric acid (TBA) test described by Begin & Ells (1992) was used for lipid peroxidation determination. Microsomes (ER, 10 μg; SR, 200 μg) were incubated with NADPH 6.25 mM in 250 μl 0.1 M K-phosphate buffer, pH 7.4 at 37°C with or without the test compounds. After the reaction was stopped by addition of 1 ml of 15% TCA at the time indicated and centrifuged at 15,000 *g* for 15 min, 1 ml of supernatant was withdrawn and reacted with 1 ml of 0.67% 2-thiobarbituric acid-0.25 N HCl at 85°C for 10 min. The reactant was cooled and degassed before absorbance measurement at 532 nm.

Protein determination

Protein was determined by the method of Lowry *et al.* (1953) with bovine serum albumin used as standard.

Results

Effects of DHBP on calcium release from SR vesicles

Figure 1 shows a typical experiment using a metallochromic calcium indicator dye, antipyrylazo III, to monitor the calcium uptake and release by isolated membrane vesicles, e.g. triad rich heavy sarcoplasmic reticulum (HSR) and mitochondria in this study. As shown in Figure 1a, calcium in the reaction medium was rapidly and actively translocated into the SR upon addition of Mg^{2+} ATP by the Ca^{2+}-ATPase located on the SR membrane, as indicated by the decrease of

optical absorbance difference at 710 nm and 790 nm; aliquots of 10 nmol $CaCl_2$ were added sequentially to the reaction medium until a near saturated level of calcium was loaded (usually 80–100 nmol Ca^{2+} mg^{-1} protein) and further addition of $CaCl_2$ would have initiated a Ca^{2+}-induce Ca^{2+} release. Addition of 2 $\mu g\ ml^{-1}$ polylysine (trace 1, Figure 1a), a calcium release inducer (Cifuentes *et al.*, 1989), after loading induced a rapid release of 80% of the loaded calcium and total release was obtained upon addition of the calcium ionophore, A23187 (2 μM). This polylysine-induced calcium release from HSR was blocked by prior addition of 2 μM ruthenium red (trace 6, Figure 1a).

DHBP blocked the calcium release induced by 2 $\mu g\ ml^{-1}$ polylysine in a dose-dependent manner (traces 2–5, Figure 1a) with an IC_{50} of 5 $\mu g\ ml^{-1}$. This inhibition was not due to the interaction of DHBP with antipyrylazo III or calcium ion chelation by DHBP since the optical signal of total released calcium was not affected when the calcium ionophore was added. Unlike ruthenium red, which can also block calcium uptake by mitochondria (trace 1, Figure 1b), DHBP (50 $\mu g\ ml^{-1}$) did not affect the calcium uptake by mitochondria through the uniporter (trace 3, Figure 1b).

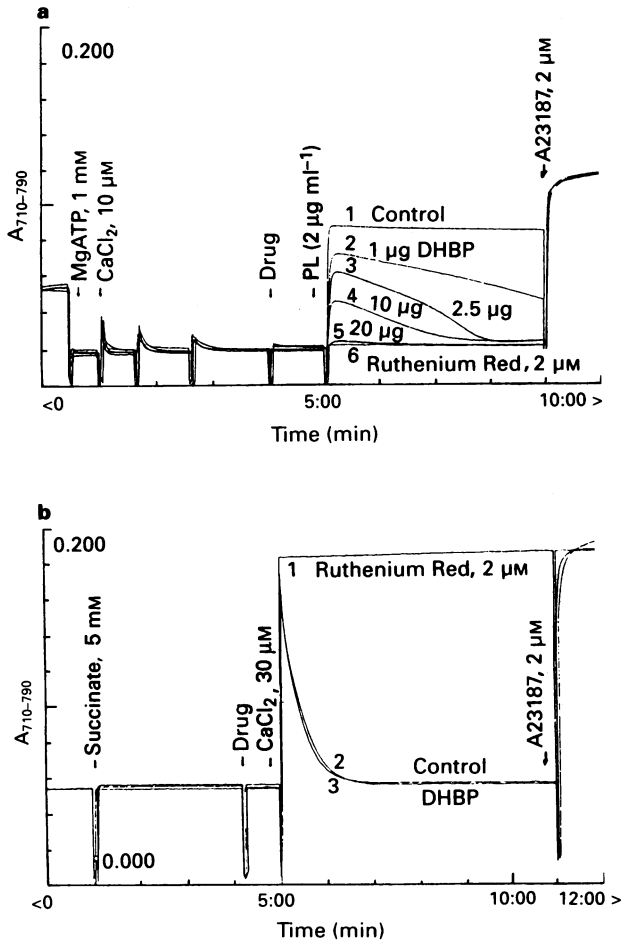


Figure 1 Effects of DHBP on calcium release of SR and uptake of mitochondria. In (a) SR vesicles (0.5 mg ml^{-1}) were loaded with calcium as described in the text and the calcium concentration was monitored by the absorbance difference at 710 nm and 790 nm according to the procedure outlined in Methods. Trace 1, calcium release induced by polylysine (PL) without any addition of drug; traces 2–5, calcium release induced by polylysine with prior addition of DHBP at the concentration indicated; trace 6, calcium release with prior addition of 2 μM ruthenium red. In (b) mitochondria (1 mg ml^{-1}) were incubated for 5 min with succinate (5 mM) to initiate the respiratory chain reaction. Ruthenium red (trace 1) and DHBP (50 $\mu g\ ml^{-1}$) (trace 3) was then added prior to addition of $CaCl_2$ (30 μM). Trace 2 was the control with no drug addition.

DHBP can also block calcium release induced by 2 mM caffeine (Figure 2) and 5 μM silver ion (data not shown) in a dose-dependent manner with IC_{50} of 4 $\mu g\ ml^{-1}$ and 6 $\mu g\ ml^{-1}$, respectively.

Effect of DHBP on ryanodine binding

Since both polylysine (Cifuentes *et al.*, 1989) and caffeine (Palade, 1987a) induced calcium release from SR through a direct interaction with the ryanodine receptor and this induced calcium release was blocked by DHBP, suggesting that DHBP might exert its inhibitory effect by direct interaction of ryanodine receptor resulting in a blockade of the release channel. We then tested the DHBP effect on ryanodine binding of HSR. DHBP inhibited the ryanodine binding in a dose-dependent manner as shown in Figure 2. An IC_{50} of 2.5 $\mu g\ ml^{-1}$ DHBP was observed with 90–100% inhibition of ryanodine binding at 20–30 $\mu g\ ml^{-1}$.

Effects of other dipyridyl analogues on calcium release and ryanodine binding

The effects of other dipyridyl analogues, including diethyl bipyridinium (DEBP), dibenzyl bipyridinium (DBBP), dimethyl bipyridinium (paraquat), 4,4'-dipyridine and 2,2'-dipyridine, on calcium release and ryanodine binding were tested. No significant effects on either calcium release or ryanodine binding were observed with all compounds tested at concentrations up to 100 $\mu g\ ml^{-1}$, suggesting that DHBP effects were specific.

Effects of DHBP on calcium uptake and ATPase activity

The effects of DHBP on active calcium uptake and Ca^{2+}-ATPase of SR were also tested. The amounts of calcium sequestered by SR vesicles without and with 10 $\mu g\ ml^{-1}$ DHBP measured by the use of $^{45}Ca^{2+}$ as described in Methods were 70 ± 5 nmol mg^{-1} and 72 ± 5 nmol ng^{-1} respectively. The uptake capacity was greatly reduced to 25 ± 2 nmol mg^{-1} in the presence of 2 mM caffeine with no added DHBP due to the activation of the calcium release channel on the SR by caffeine. DHBP at 10 $\mu g\ ml^{-1}$ can effectively antagonize the effect of caffeine and restore the

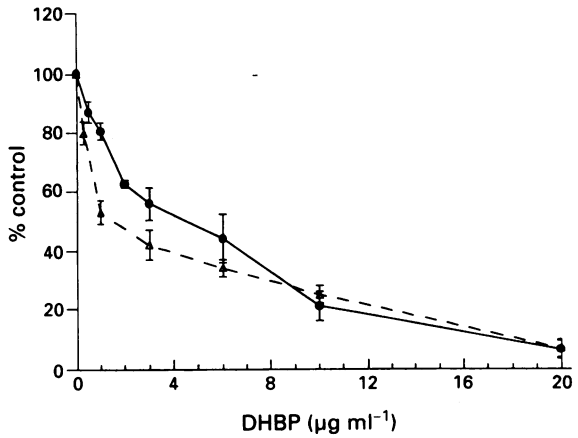


Figure 2 Dose-response of DHBP on SR calcium release and [^{3}H]-ryanodine binding: (●) total calcium release of SR induced by 2 mM caffeine with addition of DHBP at concentration indicated. Data are presented as percentage of control with at least three duplicates of three different preparations. IC_{50} value of 4 $\mu g\ ml^{-1}$ was determined by fitting y = b + alog(x). (Δ) DHBP effect on the specific binding of 10 nM [^{3}H]-ryanodine (2 pmol mg^{-1}) according to the procedure outlined in Methods. An IC_{50} of 2.5 $\mu g\ ml^{-1}$ DHBP was observed with 90–100% inhibition of ryanodine binding at 20–30 $\mu g\ ml^{-1}$.

calcium uptake capacity of the SR to 72 ± 2 nmol mg^{-1} when added at the same time as caffeine.

When ATPase activity of the SR was measured by a coupling assay, DHBP at concentrations up to 50 $\mu g\ ml^{-1}$ neither inhibited nor stimulated Ca^{2+}-ATPase activity (data not shown), but at higher concentrations (> 100 $\mu g\ ml^{-1}$), a slight activation (~10%) was observed, suggesting that the effect of DHBP on calcium uptake at low concentration was not due to the stimulation of Ca^{2+}-ATPase.

No effects of DHBP on ATPase activity from other sources, such as Na^+,K^+-ATPase of synaptic plasma membrane and Mg^{2+}-ATPase of mitochondria, was observed at 100 $\mu g\ ml^{-1}$ DHBP (data not shown).

Lipid peroxidation of SR of skeletal muscle and of liver microsomes

Recently, Dinis *et al.* (1993) have reported that lipid peroxidation of the SR membrane can affect the Ca^{2+} pump activity through alteration of physical properties in the lipid phase or lipid-protein interface. By using thiobarbituric acid-reactive substances (TBARs) production as an indicator (Begin & Ells, 1992), the effect of DHBP on the level of lipid peroxidation of SR membrane and liver microsome was examined. Unlike paraquat and CCl_4, both of which enhance the level of lipid peroxidation of liver microsomes, DHBP at concentrations up to 100 $\mu g\ ml^{-1}$ did not enhance or produce lipid peroxidation of liver microsomes. All the compounds tested did not produce lipid peroxidation of the SR membrane suggesting that the effect of DHBP seen on the calcium channel was not due to alteration of lipids in the SR membrane.

Effect of DHBP on muscle contraction of mouse diaphragm

The effects of DHBP on muscle contraction were tested on the mouse diaphragm preparation as described in Methods. As shown in Figure 3a, biphasic contraction (a phasic contraction followed by a slowly developing tonic contraction) was induced upon addition of 10 mM caffeine in the presence of 2.5 mM calcium. The tonic contraction was greatly reduced (>90%) leaving a small and slow relaxing tonic contraction upon removal of the external calcium (Figure 3b) while the phasic contraction was only partially reduced (~25%) suggesting that the tonic contraction was induced by the influx of extracellular calcium and the phasic contraction was induced mainly by the calcium released internally from the SR. The remaining small tonic contraction might be the consequence of incomplete removal of the external calcium such as in the lumen of the T-tubule since this tonic contraction can be prevented by using a higher concentration of EGTA but this also accelerated the inactivation of the muscle. Pretreatment with DHBP 0.1 mg ml^{-1} inhibited the phasic contraction induced by caffeine with little effect on the tonic contraction (Figure 3c). Muscle contraction induced by ryanodine 1 μM in the presence of 2.5 mM calcium (Figure 4a, upper trace) was also blocked by pretreatment with DHBP 0.1 mg ml^{-1} (Figure 4a, lower trace).

Dose-dependence of DHBP inhibition of caffeine- and potassium-induced muscle contraction are summarized in Figure 5, IC_{50} values being 54 $\mu g\ ml^{-1}$ and 1 mg ml^{-1}, respectively. DHBP blocked the potassium-induced contraction at a much higher concentration, with only 20% inhibition at 0.25 mg ml^{-1}, suggesting a higher sensitivity of DHBP to calcium-induced (caffeine) or drug-induced (ryanodine) calcium release than to depolarization-induced (high potassium) calcium release; this was supported by the fact that a high concentration of DHBP (2 mg ml^{-1}) was needed to block completely the twitches induced by electrical stimulation (Figure 4b). Addition of a high dose of ryanodine after the twitch had been completely blocked (Figure 4b) was unable to induce contraction suggesting a complete blockade of the calcium release channel. This twitch inhibition at a high concentration of DHBP was not due to the inhibitory effect on contractile proteins since permeabilization of the sar-

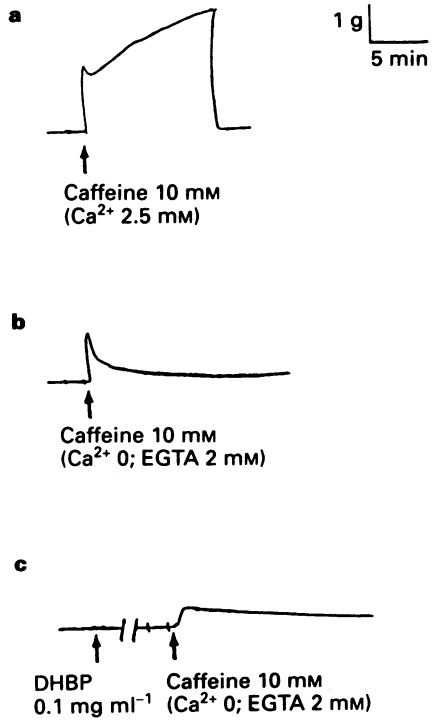


Figure 3 Effects of DHBP on muscle contraction of mouse diaphragm induced by caffeine. Mouse diaphragm was prepared as described in Methods and equilibrated in calcium 2.5 mM (a) or calcium-free (b) medium for 30 min before addition of caffeine. In (c) DHBP (0.1 mg ml^{-1}) was added prior to addition of caffeine.

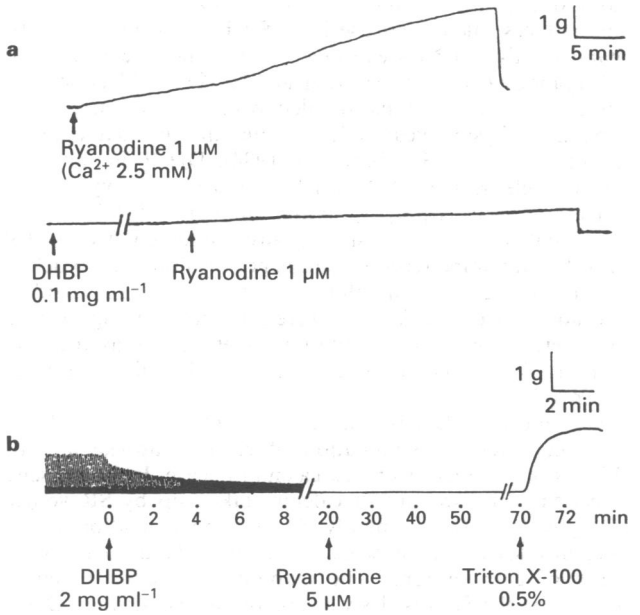


Figure 4 Effects of DHBP on muscle contraction of mouse diaphragm induced by ryanodine or direct electrical stimulation. Mouse diaphragm was prepared as described in Methods and equilibrated in Krebs solution containing 2.5 nM calcium for at least 30 min. In (a) ryanodine (1 μM) was added without (upper trace) or with (lower trace) preincubation with DHBP (0.1 mg ml^{-1}). In (b) DHBP, at a high concentration of 2 mg ml^{-1} was added after the twitch became stable and ryanodine (5 μM) and 0.5% Triton X-100 were added after the twitch had been completely blocked.

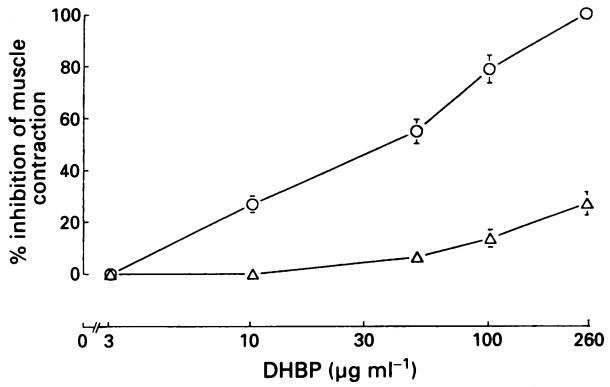


Figure 5 Dose-response to DHBP of muscle contraction induced by caffeine or high potassium. DHBP at concentrations indicated was added and incubated with muscle in calcium-free medium for 15 min before challenge with 10 mM caffeine (○) or 50 mM KCl (△). Data are presented as percentage inhibition relative to control. Data are presented as means ± s.e.mean ($n \geq 6$).

colemma by 0.5% Triton X-100 still caused the muscle to contract in response to the influx of external calcium.

Discussion

In this study, we have presented evidence showing that DHBP can inhibit the calcium release from skeletal muscle induced by caffeine, polylysine, ryanodine and low concentrations of silver ion. These calcium release inducers have been shown to activate specifically the calcium release channel, also known as the ryanodine receptor, of the SR. For example, polylysine at low concentrations has been shown to induce calcium release from SR by a direct interaction with the ryanodine receptor (Cifuentes *et al.*, 1989; Kang *et al.*, 1992), while at high concentrations, polylysine actually blocked the calcium release (Palade, 1987b). Caffeine, not only induces calcium release from SR but also modulates the channel activity (Rousseau *et al.*, 1988) and alters the K_d of [^{3}H]-ryanodine binding (Pessah *et al.*, 1987). Silver ions, at low concentrations, induced calcium release by acting at the apparent physiological release site in the sarcoplasmic reticulum (Salama & Abramson, 1984). DHBP inhibited the calcium release from SR vesicles induced by polylysine or caffeine as well as [^{3}H]-ryanodine binding to SR in a dose-dependent manner, suggesting a direct interaction of DHBP with the ryanodine receptor and hence resulting in a blockage of the channel and an inhibitory effect on ligand binding. The addition of blocker concurrently with the triggering of Ca^{2+} release producing inhibition as effectively as the addition of blocker before triggering, would indicate a direct action of DHBP.

The effect of DHBP on calcium uptake correlated well with the extent of stimulation of calcium uptake and the abilities of the agent to block calcium release. In the presence of caffeine, the amount of calcium taken up by SR vesicles was greatly reduced, mainly due to the activation of the calcium release channel which resulted in the decrease of net influx of calcium ions. The amount of calcium taken up actively by the SR vesicles was greatly increased when DHBP was added at the same time as caffeine and stimulation of transport by DBHP was due to closure of the channel activated by caffeine. This conclusion is consistent with the finding that DHBP does not alter Ca^{2+}-ATPase activity.

Recently, Dinis *et al.* (1993) have reported that lipid peroxidation of the SR membrane can affect the Ca^{2+} pump activity through alteration of physical properties in the lipid phase of lipid-protein interface. Nonselective contact herbicides, such as paraquat and related bipyridylium compounds, produce toxicity through oxygen-derived radicals formed by the reaction of oxygen with the bipyridylium radical cations (DeGray *et al.*, 1991) and cause lipid peroxidation (Steffen & Netter, 1979; Ruiz-Gutierrez *et al.*, 1991) or release of iron from ferritin (Thomas & Aust, 1986). By using TBARs as the indicator of lipid peroxidation, we have found that DHBP did not induce lipid peroxidation of SR membrane or liver microsomes, suggesting that the effect of DHBP on SR calcium transport is not due to the alteration of physical properties of the SR membrane. It is interesting to note that although CCl_4 induced an increase of lipid peroxidation in liver microsomes it had no effect on SR, suggesting that SR might not contain metabolic enzymes, such as cytochrome P450, and this could explain why none of the bipyridylium compounds, including paraquat, affected the Ca^{2+}-ATPase activity of SR.

Caffeine contraction is biphasic in calcium-containing bathing solution and several sites of action have been suggested (Endo, 1977; Røed, 1991). We showed that, in a calcium-free buffer, caffeine induced a small phasic contraction of mouse diaphragm through direct action of caffeine at the terminal cisternae of the SR (Kumbarachi & Nastuk, 1982) resulting in calcium release from the SR. Pretreatment with DHBP can effectively block this caffeine-induced contraction suggesting that DHBP can enter the muscle cell and interact with the calcium release channel located on the terminal cisternae of SR resulting in a blockade of calcium release. The IC_{50} value of DHBP for blockade of caffeine-induced contraction of the diaphragm is higher than that for blockade of calcium release from SR vesicles. It is possible that this apparent discrepancy is due to the differences in the experimental conditions between the *in situ* (e.g. the use of EGTA) and *in vivo* studies or it could reflect the different sensitivity of preparations to DHBP or protein and lipid concentration. Although easy diffusion of drug into the muscle strip of the diaphragm makes it the preparation of choice in most pharmacological studies, a large amount of drug was always needed compared to the dose tested with isolated membrane vesicles, possibly due to the presence of a large amount of proteins and multimembrane barriers which made it difficult to estimate the free drug concentration, especially when a hydrophobic compound was used. Blockade of the ryanodine-induced muscle contraction by DHBP further suggested a direct effect of DHBP on ryanodine receptor.

An even higher concentration of DHBP was needed to block the potassium-induced contraction (also in calcium-free solution), suggesting a different sensitivity of DHBP to different calcium release mechanisms. Endo (1977) has suggested that the two classes of Ca^{2+} release, namely, Ca^{2+}-induced Ca^{2+} release including drug-induced Ca^{2+} release and depolarization-induced Ca^{2+} release are governed by different mechanisms, as indicated by the fact that they differ in several criteria, such as Ca^{2+}-loading requirements and sensitivities to Mg^{2+}, ATP, sugars and procaine. In contrast to this observation, Antoniu *et al.* (1985) found that procaine inhibited both Ca^{2+}-induced and depolarization-induced Ca^{2+} release in isolated SR vesicles. We have not tested the DHBP effect on depolarization-induced Ca^{2+} release in isolated SR vesicles; it is therefore difficult to compare the sensitivity of DHBP on these two types of calcium release inducing mechanism. Data in this study, however, have shown a different sensitivity to DHBP of calcium-induced (caffeine) or drug-induced (ryanodine) and depolarization-induced (high potassium) muscle contraction. This is further supported by the fact that a high concentration (2 mg ml^{-1}) of DHBP was needed to block completely the muscle twitch induced by direct electrical stimulation.

Effects of other bipyridylium compounds, including positively charged bipyridylium analogues (e.g. DEBP, DBBP, paraquat) and neutral bipyridines (e.g. 2,2'-bipyridine, 4,4'-bipyridine), on calcium release and ryanodine binding were compared and found to be ineffective both on release blockade and ligand binding inhibition. This result suggested,

beside the specificity of DHBP, that a positive charge on the nitrogen atom of pyridine and a long alkyl chain are both needed to produce the inhibitory effects on the calcium release channel thus resembling the action of procaine on caffeine-contracture proposed by Bianchi (1968, cf. Figure 10). Several unrelated cations, such as neomycin and spermine (Palade, 1987b), ruthenium red (Miyamoto & Racker, 1982) and Mg^{2+} (Nagasaki & Kasia, 1981) have been shown to inhibit calcium release from SR. We cannot conclude; at this point, whether these cations and DHBP interact at the Ca^{2+}-regulatory domain of the ryanodine receptor which binds Ca^{2+} with μmolar affinity for gating the Ca^{2+} channel and is inhibited by millimolar Mg^{2+} by direct competition for the activator site as Mg^{2+} (Pessah *et al.*, 1987) or other ligand binding domains.

Although ruthenium red is an effective calcium release blocker of skeletal muscle (Miyamoto & Racker, 1982) by reacting with the calcium release channel (Antoniu *et al.*, 1985), it was reported to inhibit functions of many different systems: e.g., the spontaneous miniature endplate potential at synaptic junctions (Rahamimoff & Alnaes, 1973), mitochondrial Ca^{2+} transport (Reed & Bygrave, 1974), and SR Ca^{2+} binding (Vale & Carvalho, 1973). In contrast to ruthenium red, which blocks both the calcium release from SR (trace 6, Figure 1a) and calcium uptake by mitochondria (trace 1, Figure 1b), DHBP did not inhibit the calcium uptake by mitochondria (trace 3, Figure 1b). DHBP at concentrations tested also had no effect on Ca^{2+}-ATPase of SR, Na^+,K^+-ATPase of synapsomal membrane and Mg^{2+}-ATPase of mitochondria. These results further suggested a specific effect of DHBP on the ryanodine receptor of skeletal muscle and that DHBP may be a more useful pharmacological agent than ruthenium red for intact tissue studies, besides its specificity, since unlike ruthenium red, it is membrane permeable.

Data in this study provided evidence that DHBP blocked the calcium release from SR and the ligand binding through a direct interaction with the ryanodine receptor. Use of this agent as a specific antagonist for the calcium release channel of skeletal muscle for biochemical study and possibly as a muscle relaxant clinically is suggested.

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Antagonism by lipophilic quaternary ions of the K^+ channel opener, levcromakalim, in vascular smooth muscle

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1 The aim of this study was to characterize the interaction between the K^+ channel opener levcromakalim (LKM) and several quaternary ions, in vascular smooth muscle, *in vitro*. Segments of isolated, thoracic aorta of the rat were suspended in organ baths filled with Krebs solution at 37°C. Cumulative concentration-response curves to LKM were obtained in the absence and in the presence of increasing concentrations of quaternary ions using a number of agents to pre-constrict the vessel. The ions tested were tetraphenylphosphonium (chloride TPP-Cl and bromide TPP-Br salts), tetrapentylammonium (TPeA), tetraethylammonium (TEA), tetraphenylarsonium (TPAs) and tetraphenylboron (TPB).

2 For the compounds which antagonized the vasorelaxation responses of LKM, 'apparent pK_B' values were estimated on the basis of a single concentration of antagonist. These were then used to obtain the following order of potency: TPP-Br (7.22 ± 0.25) = TPAs (7.12 ± 0.04) = TPP-Cl (7.11 ± 0.15) > TPeA (6.23 ± 0.20). TEA and TPB were both found to be inactive at the maximum concentrations used.

3 The interaction between the cationic TPP and anionic TPB was also investigated. The shift in the LKM concentration-response curve constructed in the presence of both of these compounds was compared to that when each agent was present separately. We found that TPB, at concentrations greater than 1 μM, reversed the blockade of the LKM-mediated relaxation induced by TPP (3 μM).

4 Similar experiments were undertaken combining TPB with either alinidine or glibenclamide (both functional antagonists of K^+ channel openers). It was found that TPB (10 μM) partially reversed the antagonism induced by alinidine (30 and 100 μM) but had no effect on the action of glibenclamide (3 μM).

5 These studies show that lipophilic cations such as TPP and TPAs are potent antagonists of levcromakalim-mediated vasorelaxation responses in the rat thoracic aorta. The mechanism by which these compounds cause their antagonism is not known. However, given the lipophilicity of these compounds, it is possible they may act at a number of sites including the K_{ATP} channel itself or possibly via some other intracellular mechanism.

Keywords: Levcromakalim; smooth muscle relaxation; quaternary ions; K^+ channels; K^+ channel antagonists; rat thoracic aorta; tetraphenylphosphonium; tetraphenylboron

Introduction

Levcromakalim is representative of a new class of smooth muscle relaxants (Edwards & Weston, 1993), which act to open a type of K^+ channel present in the cell membrane resulting in membrane hyperpolarization. These K^+ channels have been likened to an ATP-sensitive K^+ (K_{ATP}) channel similar to the ATP-sensitive K^+ channel first identified in the heart (Noma, 1983). This idea has come about through the observation that potent sulphonylurea-based antagonists of the ATP sensitive K^+ channel in the pancreatic β-cell, such as glibenclamide (see Ashford, 1990), are also able to antagonize the vasorelaxant response to the K^+ channel openers in vascular smooth muscle (see McPherson, 1993; Edwards & Weston, 1993). Several years ago we reported that phentolamine (an imidazoline), and alinidine (an imidazolidine) and other structurally related compounds, are also able to antagonize the relaxation induced by the K^+ channel openers in vascular smooth muscle (McPherson & Angus, 1989; 1990). Subsequent studies have shown that a number of chemically diverse compounds are able to antagonize the functional effects of K^+ channel openers in vascular smooth muscle and other tissues (see McPherson, 1993; Edwards & Weston, 1993).

Quaternary ions, in particular tetraethylammonium (TEA), have also played an important role in the characterization and classification of various K^+ channels (Cook & Quast, 1990), although this particular quaternary ion has a low potency (> 1 mM) on the K_{ATP} channels described above. Another area where quaternary ions have been extensively used is in the measurement of membrane potential. It has long been known that some quaternary ions, such as tetraphenylphosphonium (TPP), can partition into cells due to their lipophilic nature. This property has allowed researchers to use [^{3}H]-tetraphenylphosphonium ([^{3}H]-TPP) as a tool for assessing membrane potential in cells because [^{3}H]-TPP partitions between the inside and outside of the cell in proportion to the charge difference (Grinius *et al.*, 1970; Haydon & Hladky, 1972). We recently applied this technique to measure the effect of levcromakalim on membrane potential in cultured smooth muscle cells. It was noted however, in control experiments, that TPP antagonized the functional effects of this compound. Consequently we have performed more detailed studies using a number of selected quaternary ions and found that two lipophilic quaternary ions, TPP and tetraphenylarsonium (TPAs) are potent antagonists of levcromakalim-mediated vasorelaxation responses in the rat thoracic aorta. In addition, we have shown that the blockade of levcromakalim-mediated responses caused by TPP can be reversed by the negatively charged quaternary ion, tetraphenylboron (TPB). The studies show that lipophilic quaternary ions such as TPP and TPAs are some of the most potent antagonists of levcromakalim-mediated actions.

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Methods

Tissue source

Male Wistar-Kyoto rats (WKY), aged between 10–14 weeks, were killed by 20% O_2 and 80% CO_2 narcosis and exsanguination. The thoracic aorta was rapidly removed and placed in ice-cold Krebs solution (composition in mM: NaCl 119, KCl 4.7, $MgSO_4.7H_2O$ 1.17, KH_2PO_4 1.18, $CaCl_2$ 2.5, $NaHCO_3$ 25, and glucose 5.5). The aorta was cleared of connective tissue and cut into 3 mm long segments. Each segment was then suspended on two stainless steel wire hooks, 400 μm in diameter, in 25 ml jacketed glass organ baths filled with Krebs solution aerated with 5% CO_2 in O_2 and maintained at 37°C. The lower hook was fixed to a support leg attached to a micrometer, while the upper wire hook was suspended from a Grass FT03C force transducer through which changes in isometric force were recorded. Force recordings were displayed on a 2-channel flat-bed chart recorder (Model 320 W + W Scientific Instruments, Switzerland). Vessels were left to equilibrate under zero force for 30 min after which an initial force of 5 g was then applied. After another 30 min the force was re-adjusted to 5 g and the tissues were left for a further 30 min. A potassium depolarizing solution (KPSS: composition in mM: KCl 123, $MgSO_4.7H_2O$ 1.17, KH_2PO_4 1.18, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 5.5) was then added to obtain a maximal contractile response of the vessel. This response was used as the standard vasoconstrictor response. After a plateau to KPSS was reached, vessels were washed twice and left until the response returned to the initial baseline, before commencing the experiment.

Each aortic ring was submaximally constricted with a vasoconstrictor (in most studies this was KCl, 25 mM). Once a plateau response to the constrictor was reached, a cumulative concentration-response curve to levcromakalim (LKM) (0.01–30 μM, 0.5 log increments) was then constructed. Concentrations of the relaxant were added when the response to the previous concentration had reached a plateau. In some cases LKM did not cause maximal tissue relaxation, especially if an antagonist was present. Consequently, sodium nitroprusside (SNP; 100 μM) was added at the end of each curve to obtain full tissue relaxation. In studies investigating antagonist action the test compound was added 20 min prior to constricting the tissue with KCl (25 mM; unless specified otherwise). Only one concentration-response curve was constructed on any one aortic ring segment.

Analysis of results

The contraction to the submaximal constrictor (e.g. KCl) was taken as 100% response and relaxation produced by different concentrations of the vasodilator (i.e. LKM or SNP) was expressed as a percentage of this response. The percentages obtained were then represented graphically and the pD_2 ($-\log EC_{50}$) value calculated as the concentration of dilator required to cause 50% relaxation of the maximal relaxant response. In the cases where the maximal response had not been obtained (i.e. in the presence of high concentrations of the antagonists) the response to the highest concentration of the dilator was taken as the maximal response. To determine whether the antagonists directly affected the tissue's ability to contract, the response to the constrictor was expressed as a percentage of the maximal KPSS contraction.

It was observed that at high concentrations, most of the active quaternary ions shifted the LKM concentration-response curve in a non-parallel fashion. Also, the slope of the curve was reduced and the maximum response to LKM appeared to be depressed. This suggested that the type of antagonism displayed by the quaternary ions was non-competitive, preventing us from the use of Schild analysis (Jenkinson, 1991) as a means of determining the potency of the active ions. Instead, an 'apparent pK_B' was estimated, based on a single concentration of antagonist.

The following equation was used:

$$\text{'Apparent } pK_B\text{'} = -\log([\text{antagonist Conc., M}]/[\text{concentration ratio} - 1]) \quad (1)$$

The single concentration of the antagonist used to calculate the 'apparent pK_B' was selected on the basis that it produced a 'parallel' shift in the LKM concentration-response curve without overtly affecting the maximal LKM response.

One-way repeated measures analysis of variance (ANOVA) was used for comparison of several dependent groups and one-way ANOVA was used to test several values which were independent. Either Student-Newman-Keuls test (for all pairwise multiple comparisons) or Dunnett's method (for comparisons for all groups vs control) were used as post-ANOVA tests. All statistical calculations were performed using SigmaStat (Jandal Scientific, U.S.A.). Results in the text are given as mean ± s.e.mean.

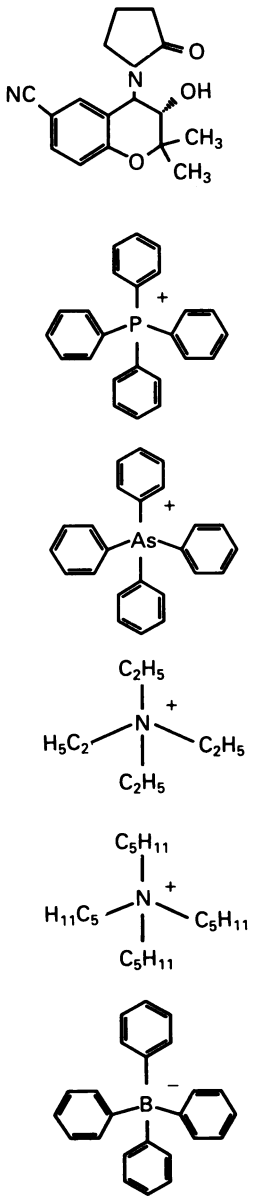


Figure 1 Chemical structures of the quaternary ions and the K^+ channel opener, levcromakalim. They are (from top to bottom) levcromakalim, tetraphenylphosphonium, tetraphenylarsonium, tetraethylammonium, tetrapentylammonium and tetraphenylboron.

Drugs

Drugs used and their sources were: levcromakalim (Beecham, UK); sodium nitroprusside (David Bull, Australia); methoxamine hydrochloride (Sigma, U.S.A.); 11α, 9α-epoxymethano-prostaglandin H_2 (U46619, The Upjohn Co., Kalamazoo, U.S.A.); glibenclamide (Hoechst, Australia); alinidine hydrobromide (Boehringer-Ingelheim, Germany); tetraethylammonium chloride hydrate, tetrapentylammonium iodide, tetraphenylphosphonium chloride, tetraphenylphosphonium bromide (Aldrich, USA); tetraphenylarsonium chloride, tetraphenylboron sodium (Sigma, U.S.A.).

Results

Antagonism of levcromakalim responses by quaternary ions

Six quaternary ions were tested for the ability to antagonize LKM-mediated vasorelaxation. The structures of these compounds are shown in Figure 1. The potency of each active ion was estimated by calculating the 'apparent pK_B' value. The results from these calculations are summarized in Table 1. Four of the compounds: tetraphenylphosphonium bromide (TPP-Br), tetraphenylphosphonium chloride (TPP-Cl), tetraphenylarsonium (TPAs) and tetrapentylammonium (TPeA), were able to block LKM-induced vasorelaxation (Figure 2). The two tetraphenylphosphonium salts, TPP-Br and TPP-Cl (Figure 2a), at concentrations as low as 1 μM, produced a significant shift in the LKM concentration-response curve. Statistically significant shifts in the LKM curve were also associated with 1 and 3 μM TPAs (Figure 2b and Table 1).

Tetrapentylammonium (TPeA) produced a significant shift in the LKM concentration-response curve at a concentration of 10 μM (Figure 2c); however, this response was markedly less than that produced by the two TPP salts and TPAs (see Table 1). It should be noted that TPeA, at 3 μM and greater, acted directly on the smooth muscle to constrict it. This was reflected by a change in tone generated to KCl (25 mM), which increased from approximately 30–40% to 60% of the KPSS contraction. In contrast, none of the other quaternary ions tested directly affected the basal tone or that induced by KCl (25 mM) which remained constant at between 30–40%.

The negatively charged tetraphenylboron (TPB), at concentrations ranging from 0.1 to 10 μM, was found to be inactive as was tetraethylammonium (TEA) at concentrations up to 100 μM (Table 1).

Characterization of tetraphenylphosphonium chloride (TPP-Cl)-induced antagonism

Since TPP-Cl was found to be quite a potent antagonist of the vascular actions of LKM, we performed a number of additional studies with this compound to characterize the type of antagonism displayed by the quaternary ions.

Time to equilibrium and reversibility studies In separate studies, the optimal incubation time for the quaternary ions was determined. TPP-Cl (3 μM) was selected as the represen-

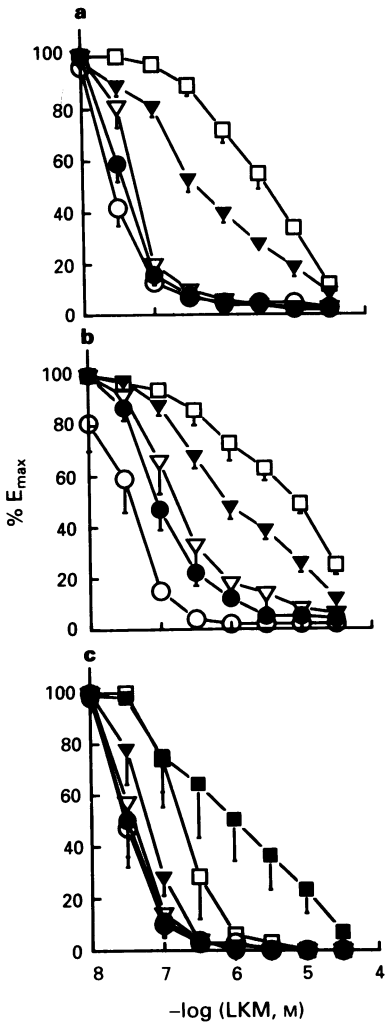


Figure 2 Mean levcromakalim (LKM) concentration-response curves obtained in the rat isolated thoracic aorta in the absence (○) and in the presence of increasing concentrations of the quaternary ions. (a) Tetraphenylphosphonium chloride; (b) tetraphenylarsonium; (c) tetrapentylammonium. The concentrations used were 0.1 (●), 0.3 (▽), 1 (▼), 3 (□) and 10 (■) μM. Error bars are ± s.e.mean, and if not visible are contained within the symbol ($n = 4$).

Table 1 Summary of the pD_2 and 'apparent' pK_B values obtained for the quaternary ions tested for levcromakalim (LKM) antagonistic activity, in rat isolated thoracic aorta constricted with KCl (25 mM) or methoxamine (•) (3 μM)

Compound	*Control* pD_2[1]	*Antagonist* (μM)[2]	*+ Antagonist* pD_2[3]	*'Apparent'* pK_B[4]
TPP-Br	7.34 ± 0.14	1	6.08 ± 0.30	7.22 ± 0.25
TPP-Cl	7.55 ± 0.07	1	6.40 ± 0.13	7.11 ± 0.15
•TPP-Cl	7.10 ± 0.08	3	5.57 ± 0.22	7.03 ± 0.12
TPAs	7.34 ± 0.07	1	6.19 ± 0.09	7.12 ± 0.04
TEA	7.50 ± 0.12	100	7.43 ± 0.03	NA
TPeA	7.53 ± 0.12	3	6.72 ± 0.15	6.23 ± 0.20*
TPB	7.63 ± 0.02	10	7.54 ± 0.04	NA

[1]pD_2 value to LKM obtained in the absence of antagonist.
[2]Concentration of the quaternary ion used to calculate 'apparent pK_B'.
[3]pD_2 values obtained in presence of the antagonist, at the concentration given in the previous column.
[4]'Apparent' pK_B values estimated for each of the active compounds, calculations based on the single concentration of antagonist given.
*Mean significantly different from other means ($P < 0.05$, one way ANOVA and Student-Newman-Keuls test). $n = 4$.
NA: not active.

tative quaternary ion in this series of experiments and was left in the organ bath for a period of 10, 20, 30, 45 and 60 min prior to constricting the vessel with KCl (25 mM). The concentration-effect curves to LKM, obtained in the presence of TPP-Cl (3 μM), were superimposable at all times of pre-incubation studied, indicating that equilibrium antagonism was achieved rapidly (< 10 min). A 20 min pre-incubation was chosen in subsequent experiments including those described above.

We were also interested in testing the reversibility of the TPP-Cl antagonism. TPP-Cl (3 μM) was incubated for 20 min and then the vessels were repeatedly washed (at intervals of 5 min) for a period of 30, 60 and 120 min, before the tissues were preconstricted and LKM concentration-response curves constructed. It was found that even after extensive washing for a period up to 120 min, TPP-Cl (3 μM) antagonism was still evident (Figure 3).

Vasoconstrictor and vasodilator dependency Methoxamine (3 μM) was also used to constrict the aorta to determine whether the antagonism produced by TPP-Cl was dependent on the constrictor agent used. TPP-Cl, at concentrations as low as 1–3 μM, produced a significant shift in the LKM concentration-response curve in these vessels (Figure 4) in a manner similar to that observed in KCl constricted tissues (Table 1). As in the previous study, the pretreatment of the tissue with TPP did not alter the ability of methoxamine to constrict the tissue which was approximately 60–70% of the maximum response to KPSS in all cases.

We also wanted to determine whether TPP-Cl inhibited only LKM responses or whether it affected the actions of other vasodilators as well. The same protocol as described above was followed, except that sodium nitroprusside (SNP) was used to obtain stepwise tissue relaxation responses. TPP-Cl at concentrations ranging from 0.1 μM to 10 μM, had no significant effect on the action of SNP on KCl (25 mM) or methoxamine (3 μM) preconstricted tissues ($n = 4$).

Effect of the negatively charged tetraphenylboron (TPB) on selective levcromakalim antagonists

Effect of TPB on TPP-induced levcromakalim antagonism While performing experiments with TPP-Cl, we noticed that the negatively charged quaternary ion in our group, TPB, was able to reverse the antagonism produced by TPP-Cl. A typical experiment showing the interaction between the two ions is shown in Figure 5. In KCl (25 mM)-constricted tissues, TPB at concentrations greater than 1 μM, reversed the antagonism caused by TPP-Cl (3 μM). At concentrations of 10 μM and 100 μM, TPB produced a full reversal of TPP-Cl action (Figure 6). In another series of experiments, we constricted the vessels with methoxamine (3 μM) and again found that TPB (10 μM) was able to reverse fully the blockade of LKM action produced by TPP-Cl (3 μM).

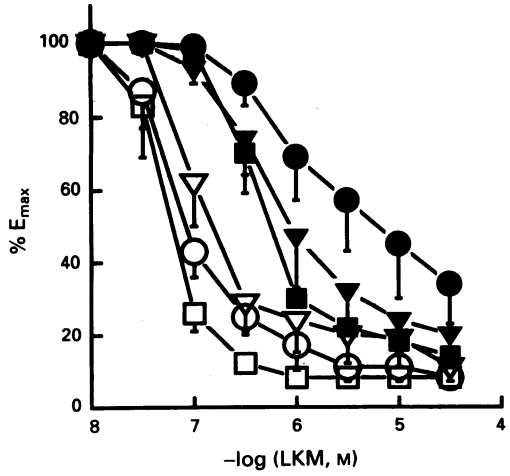


Figure 3 Reversibility of tetraphenylphosphonium chloride (TPP-Cl) antagonism: mean levcromakalim (LKM) concentration-response curves in the absence (open symbols) and in the presence (closed symbols) of TPP-Cl (3 μM) incubated for 20 min followed by washing at 5 min intervals for 30 (●), 60 (▼) and 120 (■) min ($n = 4$). Control curves constructed in the absence of TPP where treated identically to those obtained in the presence of TPP in terms of washing. Error bars are ± s.e.mean and if not visible are contained within the symbol.

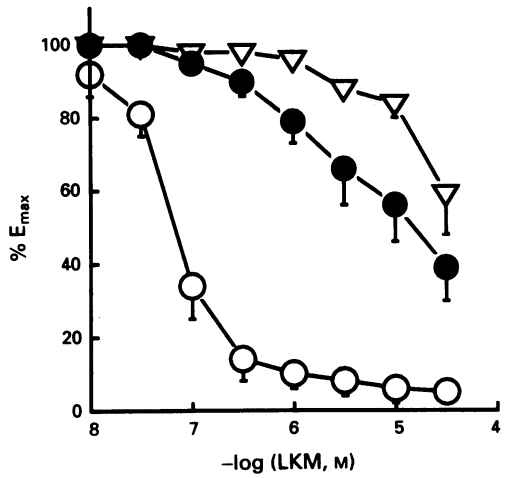


Figure 4 Mean levcromakalim (LKM) concentration-response curves obtained in the absence (○) and in the presence of increasing concentrations of tetraphenylphosphonium chloride (TPP-Cl; 3 (●) and 10 (▽) μM) in the rat thoracic aorta preconstricted with methoxamine (3 μM) ($n = 4$). Error bars are ± s.e.mean, and if not visible are contained within the symbol.

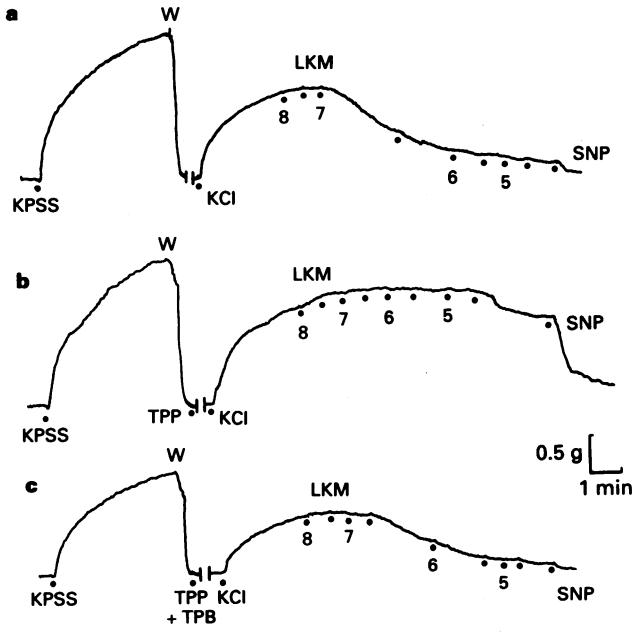


Figure 5 Interaction between tetraphenylboron (TPB) and tetraphenylphosphonium chloride (TPP-Cl) in the rat isolated thoracic aorta. (a) A representative trace showing the vasorelaxation induced by increasing concentrations of levcromakalim (LKM, 0.01–30 μM) in rat thoracic aorta constricted with KCl (25 mM) in the absence of any antagonist. (b) Same as (a) except tetraphenylphosphonium chloride (TPP-Cl) (3 μM, 20 min, incub.) was present. (c) Same as (b) except that tetraphenylboron (TPB) (10 μM) was added in addition to the TPP-Cl (3 μM) (both drugs added 20 min prior to KCl constriction). At the end of each LKM concentration-response curve, sodium nitroprusside (SNP, 100 μM) was added to obtain full tissue relaxation. W = wash.

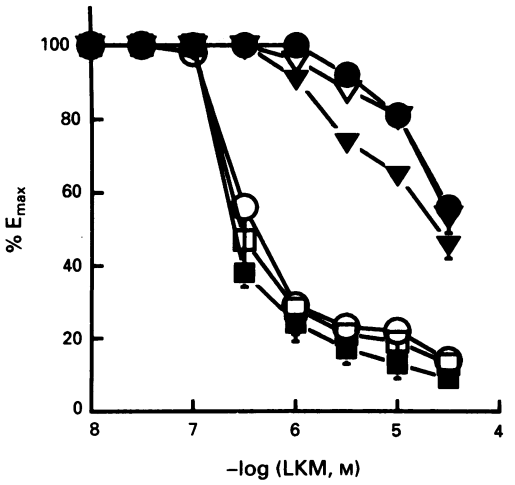


Figure 6 Interaction between tetraphenylphosphonium chloride (TPP) and tetraphenylboron (TPB). Mean levcromakalim (LKM) concentration-response curves obtained in the absence (○) and in the presence (●) of TPP (3 μM) and increasing concentrations of TPB, in the rat isolated thoracic aorta preconstricted with KCl (25 mM). TPB concentrations used = 0 (●), 0.1 (▽), 1 (▼), 10 (□) and 100 (■) μM, $n = 4$. Error bars are ± s.e.mean, and if not visible are contained within the symbol.

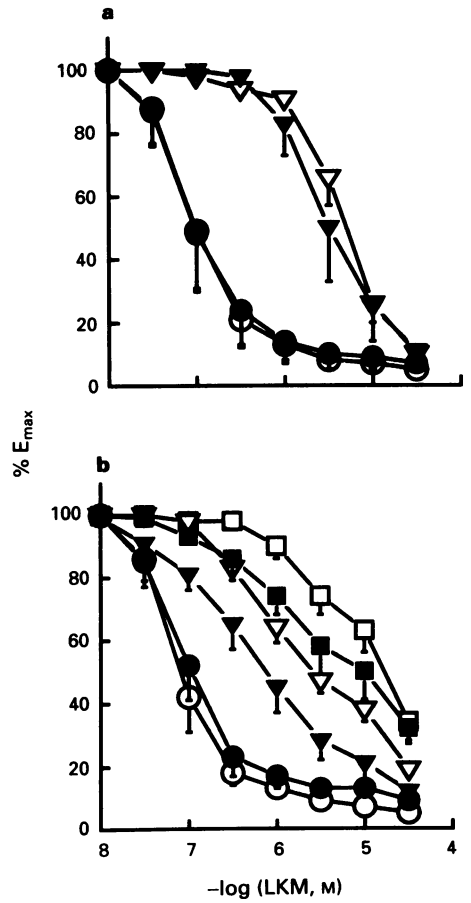


Figure 7 Interaction between tetraphenylboron (TPB) and other levcromakalim (LKM) antagonists, glibenclamide and alinidine. Mean LKM concentration-response curves obtained in KCl (25 mM) constricted rat isolated thoracic aorta in the absence and in the presence of (a) Glibenclamide, control LKM (○); plus glibenclamide (3 μM, ▽); plus TPB (10 μM, ●); glibenclamide and TPB (10 μM, ▼); $n = 5$. (b) Alinidine, control LKM (○); plus TPB (10 μM; ●); plus alinidine (30, ▽ and 100, □ μM); alinidine (30, ▼ and 100, ■ μM) plus TPB (10 μM); $n = 4$. Error bars are ± s.e.mean, and if not visible are contained within the symbol.

Effect of TPB on glibenclamide and alinidine mediated levcromakalim antagonism Following the observation that TPB could reverse TPP-Cl antagonism of LKM responses, we investigated whether the same type of reversal occurred after combining TPB with other antagonists of levcromakalim in particular glibenclamide (sulphonylurea) and alinidine (imidazolidine).

Glibenclamide (3 μM), caused a rightward shift in the LKM concentration-response curve, which was not reversed when the antagonist was combined with TPB (10 μM) (Figure 7a). In the absence of any antagonist the pD_2 for LKM was 7.15 ± 0.17, with glibenclamide (3 μM) pD_2 was 5.38 ± 0.09 and with both glibenclamide and TPB the pD_2 was 5.53 ± 0.2 ($n = 4$). The interaction between alinidine and TPB was studied at two concentrations of alinidine (Figure 7b). Alinidine (30 μM) produced a significant shift in the LKM curve which was partially reversed in the presence of TPB (10 μM). In the absence of any agent LKM had a pD_2 of 7.08 ± 0.09. In the presence of alinidine (30 μM) the pD_2 was 5.57 ± 0.14. When alinidine and TPB were combined together the pD_2 was 6.01 ± 0.23 ($n = 4$) which was significantly more potent (by a factor of 2.7) than in the presence of alinidine alone ($P < 0.05$). A similar phenomenon was also noted when using alinidine (100 μM) (control $pD_2 = 7.08 \pm 0.09$; plus alinidine $pD_2 = 5.27 \pm 0.16$; alinidine plus TPB $pD_2 = 5.56 \pm 0.20$; $n = 4$; $P < 0.05$).

Discussion

The major finding from this study is that some but not all quaternary ions possess the ability to antagonize vasorelaxation responses mediated by levcromakalim in rat isolated thoracic aorta. In addition we have also found that the negatively charged quaternary ion, TPB, is able to interfere with the antagonistic activity of TPP and to a lesser extent, alinidine, on LKM-mediated responses.

The antagonism displayed by the quaternary ions

The nature of the antagonism displayed by the quaternary ions is similar to that displayed by the imidazol(id)ines (Challinor & McPherson, 1993). We have recently shown that imidazol(id)ine based compounds, such as alinidine, are antagonists of K^+ channel openers such as cromakalim, in both vascular and non-vascular smooth muscle (McPherson & Angus, 1989; 1990; Challinor & McPherson, 1993). The antagonism displayed by imidazol(id)ine compounds is characterized by a shift to the right in the cromakalim concentration-effect curve associated with a flattening of the curve at higher concentrations (≥ 10 μM) and a reduction in the maximum response (McPherson & Angus, 1989; 1990; see also Murray *et al.*, 1989; Good *et al.*, 1992). This type of antagonism was also displayed by the active quaternary ions. This contrasts with the results obtained with sulphonylurea compounds such as glibenclamide which cause a parallel shift in the curve with no effect on the maximum response (see Challinor & McPherson, 1993). Other studies showed that the time of onset of the antagonism displayed by TPP was rapid (< 10 min) and was slowly reversible.

The number of compounds tested did not allow extensive structure-activity relationships to be performed. There is no doubt however, that there is a strong correlation between the structure of the ions and their potency as LKM antagonists. Each of the three active quaternary ions: TPP (Br and Cl salts), TPAs and TPeA – shifted the LKM concentration-response curve in what appeared to be a non-parallel fashion. Because of this we did not perform Schild analysis. Instead we calculated an 'apparent pK_B' value to obtain an estimate of compounds' potency. It must be stressed that these values serve only as an approximate indicator of potency and they do not represent the pK_B values normally obtained for true competitive antagonists. By calculating their 'apparent' pK_B

values, we found that the positively charged quaternary ions, containing the phosphonium or arsonium ion at the centre and phenyl residues as side chains, (i.e. TPP-Cl, TPP-Br and TPAs, see Figure 1), were more potent antagonists of LKM action than the ammonium based compounds. This could be due to a combination of factors. For example both phosphonium and arsonium ions can readily delocalize their positive charge onto the phenyl groups, making the whole quaternary molecule very lipophilic, which in turn may allow the ion to interact effectively with the potassium channel (see later). In addition, the mass of the quaternary ion seems to play an important role in determining its potency. Since both TPP and TPAs contain phenyl rings, it is likely that these side chains are important for high potency. This theory is further supported by the observation that molecules such as tetrapentylammonium (TPeA), which carry less compact five carbon pentyl side chains, or tetraethylammonium (TEA) which has only two carbon ethyl groups, displayed little potency (TPeA) or indeed were found to be inactive (TEA).

Quast & Webster (1989) have investigated the effect of several ammonium based quaternary ions on cromakalim-induced $^{86}Rb^+$ efflux in the rat aorta. They also found TEA to be an active blocker of cromakalim albeit at concentrations of approximately 1.0 mM, higher than those used in our experiments. The same researchers also investigated the relationship between quaternary ion structure of ammonium based compounds and their ability to antagonize cromakalim-induced $^{86}Rb^+$ efflux (see also Cook & Quast, 1990). Their findings showed that the potency of these compounds increased with increasing chain length, up to the n-pentyl compound, however the potency was reduced if the chain length was increased further. They suggested that the interaction site of the channel has a strong preference for bulky, but not too bulky, lipophilic cations. In their studies they found the n-pentyl compound had an IC_{50} for inhibiting cromakalim-induced $^{86}Rb^+$ efflux of approximately 0.1 μM. This value indicates a potency for TPeA which is approximately 5–10 times greater than that calculated for the same compound blocking LKM-mediated vasorelaxant responses in this study. At the present time we do not have any explanation for this discrepancy; however, it has been noted that some, but not all, antagonists block cromakalim-mediated $^{86}Rb^+$ efflux at concentrations much lower than those needed to block functional effects in the same tissue (Quast, 1993). In addition, the fact that TPeA was able to activate the smooth muscle directly indicates that the actions of this particular ion are complex and may thus account for some of the discrepancies in the results. However, the potency of TPeA was much less, by a factor of 10 fold, than that displayed by TPP and TPAs.

Mechanism of action of the quaternary ions and their lipophilic properties

It is thought that K^+ channel openers, such as levcromakalim, exert their effect by opening a type of K_{ATP} channel in a variety of tissues (see McPherson, 1993; Edwards & Weston, 1993). This mechanism of action of these compounds has been established primarily through the finding that sulphonylureas, such as glibenclamide, a potent and selective K_{ATP} channel blocker (see Ashford, 1990) in the pancreas, are able to antagonize the actions of the K^+ channel openers in vascular and non-vascular smooth muscle (McPherson & Angus, 1990; see Cook & Quast, 1990; Longman & Hamilton, 1992). The most obvious mechanism of action is that the quaternary ions block K_{ATP} channels to prevent K^+ ion efflux from the cell. What we know at present about the interaction between quaternary ions and the potassium channels, is largely based on studies performed with tetraethylammonium (TEA). Despite its relatively low potency, TEA has been the most commonly used non-selective potassium channel blocker (see Cook & Quast, 1990). The exact mode of interaction between the quaternary compounds and the K_{ATP} channel is still unknown. It has been proposed that the action of TEA, on other types of K^+ channels, is to interact directly with the ion channel to plug it and prevent ion movement, i.e. the so called 'plug' hypothesis (Heginbotham & McKinnon, 1992). While it is possible that the quaternary ions simply act as ion channel plugs of K_{ATP} channels, these compounds are known to have complex actions in the cell and the possibility exists that they exert their effects through another, or perhaps a combination, of mechanisms.

As mentioned previously, some of the quaternary ions, such as tetraphenylphosphonium (TPP) and tetraphenylarsonium (TPAs), are highly lipophilic. This allows them to enter the hydrophobic core of the membrane and delocalize their charge in order to allow passive equilibration with the electrical potential across the membrane. Because of this property, these lipophilic cations have been used in the past to measure the polarity of membrane potentials in biological systems (Grinius *et al.*, 1970; Haydon & Hladky, 1972). Studies examining the mode of action of TPP in this respect have found it to have complex intracellular effects which raises the possibility that these quaternary ions may not solely act as ion channel plugs to antagonize the effects of levcromakalim. For example TPP is known to concentrate in mitochondria (see Saito *et al.*, 1992). It has been also shown to interfere with Na,K-ATPase activity (Klodos & Plesner, 1992). More detailed electrophysiological studies are obviously required to deduce the precise mechanism of action of the quaternary ions such as TPP and TPAs.

Whatever the mechanism of action of TPP and TPAs they represent two of the most potent antagonists of the functional actions of K^+ channel openers identified so far, having a potency similar to that of glibenclamide (see Challinor & McPherson, 1993).

Mechanism of action of a negatively charged tetraphenylboron (TPB)

Although TPB was shown to be inactive as an LKM antagonist, it did reverse the antagonism produced by other quaternary ions such as TPP-Cl and also partially reversed the antagonism of alinidine. The simplest explanation for this phenomenon is that the compounds are displaying chemical antagonism, a situation where the two drugs react chemically to form a product that cannot interact with the receptor (Kenakin, 1987). Thus the negatively charged TPB forms a non-active complex with TPP or alinidine (Timmermans *et al.*, 1980), both of which are positively charged at physiological pH. This would reduce the amount of free antagonist concentration present in the bath and subsequently would diminish the effect of the antagonist. Since the reversal of the antagonism to alinidine was partial, it is possible that alinidine/TPB complex has lesser affinity than the TPP/TPB complex. Although TPB was shown to reverse the antagonism induced both by TPP and alinidine, it had no effect on the actions of glibenclamide. Glibenclamide, and other sulphonylureas, are negatively charged at physiological pH (Zunkler *et al.*, 1989; Findlay, 1992) and consequently would not interact with TPB which is also negatively charged.

Again the situation is likely to be more complicated. In studies using [^{3}H]-TPP to monitor membrane potential it is common practice to include unlabelled TPB. The inclusion of TPB enhances the equilibration of [^{3}H]-TPP between the inside and outside of the cell. This is thought to result from the uptake of TPB into the lipid membrane which has a preference for negatively charged ions such as TPB (Haydon & Hladky, 1972). The uptake of TPB results in a more favourable environment for positively charged ions, such as TPP, to cross. The presence of TPB within the lipid bilayer may compete with TPP for sites present in the K^+ channel, but since TPB cannot occupy the negatively charged environment of the ion channel itself, it does not impede ion move-

ment (see Sunner *et al.*, 1981; Stauffer & Dougherty, 1988; Dougherty & Stauffer, 1990 for additional details). It is an intriguing possibility that TPB may prove to be an 'unblocker' of all K^+ channels (and possibly other ion channels) where the blockade results from channel plugging as may be the case with TEA and TPP. We are currently testing this hypothesis.

In conclusion, we have found that a number of lipophilic quaternary ions antagonize LKM-induced vasorelaxation responses in vascular smooth muscle. The mechanism by which these compounds cause their antagonism is not known. However given the lipophilicity of these compounds it is possible they may act at a number of sites including the K_{ATP} channel itself or possibly via some intracellular mechanism. We have also demonstrated that tetraphenylboron (TPB), a negatively charged quaternary ion, was able to reverse the effect of the antagonism displayed by TPP and, to a lesser extent, the imidazolidine alinidine. The use of these agents alone and in combination should prove valuable tools for the further study of K^+ channel openers and the processes they regulate.

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Strain-dependency of leukotriene C_4 generation from isolated lungs of immunized mice

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1 The antigen-induced leukotriene C_4 (LTC_4)-like-material release from isolated perfused lungs of actively sensitized Swiss, Balb/C and CBA/J mice was compared. The intra-tracheal (i.t.) instillation of 1 and 100 μg ovalbumin to lungs from Swiss mice was followed by a dose-dependent generation of LTC_4-like material into the effluent, as detected by radio-immunoassay and h.p.l.c., followed by an enzyme-immunoassay. In contrast, lungs from sensitized Balb/C and CBA/J mice failed to exhibit LTC_4-like-material release in amounts above the basal values. No histamine secretion was observed when lungs of the three strains of mice were challenged with ovalbumin.

2 The i.t. instillation of 1 or 10 μg platelet-activating factor (PAF) or of 100 μg arachidonic acid to lungs from non-sensitized mice, induced the release of comparable amounts of LTC_4-like-material in the effluent, irrespective to the strain. However, N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP, 0.1, 10 μg), was more effective in inducing the release of LTC_4-like-material from lung from Swiss and CBA, than from Balb/C, mice.

3 The intraplantar injection of 0.01 μg ovalbumin to sensitized Swiss mice induced an intense oedema formation, as measured plethysmographically, while Balb/C mice required a dose of antigen at least 10 fold higher for a similar response. CBA/J mice did not respond to antigen challenge in terms of oedema formation. The intraplantar injection of PAF or fMLP to non-immunized mice induced an oedema of similar intensity in all the strains considered. Accordingly, the different responses to ovalbumin of the three strains of mice is not accounted for by different paw responsiveness to inflammatory mediators.

4 Swiss and CBA/J mice exhibited higher titers of circulating IgG antibodies, as measured by passive cutaneous anaphylaxis (PCA), than Balb/C mice. Conversely, lower IgE titers were measured in the serum of sensitized Swiss and CBA/J mice, as compared to Balb/C.

5 Our results demonstrate a strain-dependency of antigen-induced LTC_4 release from lungs from sensitized mice. This difference is related to the ability of sensitized animals to develop immediate hypersensitivity responses, such as paw oedema formation, but not to the antibody subclass involved in the immunization. Strain-dependent factors may influence the intensity of the response to antigen stimulation. It is thus essential to characterize the different components of the immune response when mouse models for studying hypersensitivity reactions are developed.

Keywords: Platelet-activating factor; histamine; hypersensitivity; mouse paw oedema

Introduction

Increasing evidence suggests that inflammatory allergic reactions display a biphasic pattern with an immediate phase, which depends on the activation of mast cells upon the antigen-antibody recognition, and a late phase, accompanied by the infiltration of inflammatory cells and subsequent tissue damage (Drews, 1990). During the early phase, cells bearing high affinity receptors for immunoglobulins and complement fragments, release a variety of pre-formed mediators, such as histamine and 5-hydroxytryptamine (5-HT) and newly formed lipid mediators, leukotrienes, prostaglandins and platelet-ativating factor (PAF), which account for the amplification and the perpetuation of the inflammatory reaction. Peptido-leukotrienes induce wheal and flare response in human skin (Camp *et al.*, 1983), increase the vascular permeability in the rat mesentery (Leng *et al.*, 1988), induce plasma exudation in the guinea-pig skin (Drazen *et al.*, 1980) and rat skin (Ueno *et al.*, 1981) and in hamster cheek pouch (Dahlen *et al.*, 1982), and are involved in shock states (Lefer, 1986). Since peptido-leukotrienes mimic the manifestations of antigen challenge in different species, including man, a role for them in bronchial asthma and allergic disorders has been proposed (Creticos *et al.*, 1984; O'Byrne, 1988). Indeed, the *in vivo* administration of leukotriene C_4 (LTC_4) induces bronchoconstriction in the guinea-pig (Drazen *et al.*, 1980; Hedqvist *et al.*, 1980) and the *in vitro* preincubation with LTE_4 enhances the contractile response to histamine of guinea-pig tracheal spirals (Lee *et al.*, 1984). Finally, the release of peptido-leukotriene in biological fluid has been demonstrated during allergic reactions in man (Wardlaw *et al.*, 1989; Arm, 1993) and animals (Wei *et al.*, 1986).

Since antigen stimulation of mouse bone marrow-derived mast cells sensitized with monoclonal IgE is followed by the generation of LTB_4 and LTC_4 (Mencia-Huerta *et al.*, 1983), it is likely that, in this species, leukotrienes play a role in immediate hypersensitivity reactions. The aim of the present study was to compare the intensity of the anaphylactic responses of mice from different strains using various *in vitro* and *in vivo* models. Experiments were performed in which isolated perfused lungs from non immunized or ovalbumin-sensitized or Balb/C, CBA/J and Swiss mice were challenged intratracheally with various inflammatory agonists, or with the antigen, respectively, and the release of histamine and LTC_4 in the effluent was evaluated. In order to characterize further the difference in the sensitivity of the isolated lung tissues from the three strains to antigen stimulation, anaphylactic paw oedema and IgG and IgE antibody titers in the serum from the three strains of mice were determined.

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Methods

Animals and sensitization procedure

Male Swiss, Balb/C and CBA/J mice aged 8-weeks, weighing approximately 25–30 g raised at the Pasteur Institute (Paris, France) were actively sensitized by a subcutaneous (s.c.) injection of 0.4 ml 0.9% w/v NaCl (saline) containing 100 μg ovalbumin adsorbed in 1.6 mg $Al(OH)_3$ (Andersson & Brattsand, 1982). Seven days later, the animals received the same dose of ovalbumin with $Al(OH)_3$ and were used 7 days thereafter.

Isolated perfused lungs

Sensitized mice were anaesthetized with sodium pentobarbitone (3 mg 10 g^{-1} body weight, i.p.), the lungs were dissected, rinsed via the right ventricle with 5 ml Krebs solution supplemented with 0.25% bovine serum albumin (Krebs-BSA) and placed in a glass chamber where they were perfused via the trachea with a gassed (95% O_2/5% CO_2) Krebs-BSA solution at 0.35 ml min^{-1}. After a 10 min period for equilibration, lungs from non-immunized mice were challenged intra-tracheally (i.t.) either with 1 or 10 μg PAF, 0.1 or 10 μg N-formyl -L-methionyl -L-leucyl-L-phenylalanine (fMLP) or 100 μg arachidonic acid. A single dose of agonist was injected to each lung preparation in a volume of 100 μl of saline or distilled water, except for ovalbumin, which was administered to lungs from sensitized mice twice, at 1 and 100 μg, at 20 min intervals between each dose. Fractions of lung effluent were collected during 5 min before and 5 min after the administration of the agonists for the determination of LTC_4-like material and histamine, as described below.

Measurement of immunoreactive LTC_4-like material in the lung effluent

Immunoreactive LTC_4-like material was measured in the lung effluent by radioimmunoassay according to Aehringhaus *et al.* (1982) and to Young *et al.* (1991). Dextran-coated charcoal was used to separate unbound ligand by centrifugation at 1,200 r.p.m. for 10 min at 4°C. The monoclonal anti-LTC_4 antibody employed (kindly provided by Dr U. Zor and Dr F. Kohen, Weizmann Institut, Rehovot, Israel) was 10% cross-reactive with LTD_4 and less than 0.1% with LTA_4 and LTB_4. The sensitivity of the assay was approximately 15 pg of immunoreactive LTC_4 in 0.1 ml sample.

Purification of LTC_4 in the lung perfusates by h.p.l.c. and measurement by enzyme-immunoassay

The purification of LTC_4 and enzyme-immunoassay determination were performed according to Antoine *et al.* (1991). Briefly, 1.5 ml of lung perfusates from Balb/C and Swiss mice, collected during 5 min after antigen challenge, were mixed with 2.5 ml methanol and centrifuged at 1,500 *g* during 15 min. Radiolabelled LTC_4 (0.1 μCi) was added to each sample to account for recovery losses during the purification procedure. The supernatant was adjusted to 10% methanol and passed through a Sep-Pak cartridge (Waters/Millipore, Milford), which had been preconditioned with 5 ml of 0.5% EDTA solution of methanol/water (1:1, v/v), followed by 8 ml methanol and 10 ml water. The biological material retained was washed with 10 ml water and leukotrienes were eluted with 3 ml of methanol. The eluate was dried under reduced pressure and dissolved in the high performance liquid chromatography (h.p.l.c.) solvent together with 30 ng of prostaglandin B_2 (PGB_2). Purification of LTC_4 was carried out using a LDC h.p.l.c. system (Riviera Beach, FL, U.S.A.). The samples were injected onto an Ultrasphere column (5 μm, 25 cm × 4.6 mm, Beckman, San Ramon, CA, U.S.A.). The samples were eluted with methanol/water/acetic acid (70:30:0.05%, v/v) adjusted to pH 5.7 with ammonium hydroxide at 1 ml min^{-1}. With this system, PGB_2 and LTC_4 were eluted at 10.4 and 7.20 min, respectively (Antoine *et al.*, 1991). Between each sample, the system was run 5 min using 100% methanol and re-equilibrated in initial solvent. PGB_2 served to assess the chromatographic reproducibility of the system. Fractions corresponding to the elution of radioactive LTC_4 were pooled, dried in vacuo and assayed directly by enzyme-immunoassay. The values were then corrected for recovery losses.

The enzyme-immunoassay for LTC_4 consisted of a solid phase immunoassay by competition which used acetylcholinesterase coupled to LTC_4. All assays were performed in enzyme-immunoassay buffer (0.1 M phosphate buffer, pH 7.4, containing 0.4 M NaCl, 1 mM EDTA, 0.1% BSA and 0.01% sodium azide). Standards or samples (50 μl) were added to each well of a 96-well microtiter plate (Nunc 96 F with certificate, Denmark), which was previously coated with mouse anti-rabbit IgG antibody solution (2 μg/well) to separate bound from free. Tracers and specific antisera, were added (50 μl each) at appropriate dilutions. After incubation of 4°C during 18 h, the plates were washed with a 0.05 M phosphate buffer (pH 7.4) containing 0.05% Tween 20, and an automatic washer (Washer 120, Labsystem Oy, Helsinki, Finland). Each well was filled with 200 μl of the Ellman's reagent of enzymatic substrate (acetylthiocholine, 0.75 mM and chromagen (5,5'-dithiobis(2-nitro-benzoic acid), 0.5 mM) in 0.01 M phosphate buffer. After shaking 1–3 h in the dark at 22°C, the absorbance of a yellow coloured product in each well was measured at 414 nm using Multiskan MC spectrophotometer (Lab System, Les Ulis, France). Results were expressed in terms of $B/B_0 \times 100$, where B and B_0 represented the absorbance measured on the bound enzyme fraction in the presence or in the absence of LTC_4, respectively. Fitting of the standard curves and calculations were done with a microcomputer (IBM) using a linear log-logit transformation. Non-specific binding (less than 0.1% of the total enzyme activity) was determined, replacing specific antibody by 50 μl of enzyme-immunoassay buffer. The monoclonal anti-LTC_4 antibody employed was 10% crossreactive with LTE_4, 80% with LTD_4, and less than 0.1% and 0.01% with LTB_4 and with GSH, respectively. The sensitivity of the assay for LTC_4 was approximately 4.3 pg/well.

Measurement of histamine in lung effluent and tissue

Aliquots of lung effluent (500 μl) were mixed with 0.5 ml of a 0.8 N solution of perchloric acid in distilled water. After centrifugation for 10 min at 1,200 *g* and at 4°C, the supernatants were stored at 4°C. An automatic spectrofluorometric assay for histamine was performed, according to a previously published method (Lebel, 1983).

Lungs from sensitized Swiss, Balb/C and CBA/J mice were prepared as described above, chopped to approximately 3 to 5 mm^3 fragments and suspended in 2 ml of 0.8 N perchloric acid in water. After 24 h at 4°C, the samples were centrifuged at 1,200 *g* for 10 min at 4°C and the supernatants were collected and stored at 4°C prior to histamine determination, as described above.

Paw oedema in mice

Mice paw oedema was induced by the intra-plantar injection of a 50 μl solution containing 0.01–1 μg PAF or 0.1–1 μg fMLP, or 0.001–0.1 μg of ovalbumin, in the case of sensitized mice. Each agonist was injected into one of the hind paws and 50 μl of saline was injected into the controlateral paw, as the control. Oedema formation was measured plethysmographically (Ferreira, 1979) 30 min after the administration of the appropriate agonist. Results are expressed as the increase in volume (in μl) of the challenged paws, as compared to vehicle-injected paws.

Determination of IgE and IgG titers

Determination of the IgE and IgG titers was performed by passive cutaneous anaphylaxis (PCA), (Braga & Mota, 1976). Briefly, blood was obtained from the orbital plexus of sensitized mice killed 14 days after the first day of immunization and serum was stored at −20°C. IgE-dependent PCA was induced in Wistar rats (IFFA CREDO, L'Arbresle, France), weighing 200–250 g, by the technique of Ovary (1964). IgG titers in serum heated at 56°C for 2 h were determined in Swiss mice (Braga & Mota, 1976). Briefly, the dorsal region of Wistar rats or Swiss mice was shaved and 50 µl of diluted serum (1:2 or 1:5, 1:10, 1:50 in saline) were injected intradermally into four sites. Four or 24 h later (for IgG and IgE determination, respectively), 1 mg/animal ovalbumin and 25 mg kg^{-1} of Evans blue dye were injected i.v. simultaneously into the penial plexus (rats) or orbital plexus (mice), under light anaesthesia with ether. Thirty minutes later, the animals were killed by ether inhalation and the same diameter (15 mm) for each wheal of skin was removed. The Evans blue dye was extracted by incubating the tissues in 5 ml of 100% formamide for 24 h at 56°C and its concentration determined by light absorbance at 650 nm wavelength (Tarayre & Lauressergues, 1979). Evans blue dye content of each sample was expressed as µg/site.

Materials

fMLP (N-formyl-L-methionyl-L-leucyl-L-phenylalanine) and arachidonic acid were purchased from Sigma Chemical Co (St. Louis, MO, U.S.A.); PAF (1-O-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine) was from Bachem, (Bubendorf, Switzerland). $Al(OH)_3$, formamide and Evans blue dye were from Merck (Darmstadt, Germany). Ovalbumin (5 × crystallised) was from Immunobiological (Costa Mesa, CA, U.S.A.). Pentobarbitone sodium, was from Sanofi Santé Animale (Libourne, France). Radiolabelled LTC_4 was from Amersham (Buckinghamshire, UK) and LTC_4 was from PIRMED-CNRS (Paris, France). Methanol was from Merck, Darmstadt, Germany. HCl and BSA (fraction V) were from Sigma Chemicals (St. Louis, MO, U.S.A.). Acetylcholinesterase was purified by a one-step affinity chromatography, as described by Massoulié & Bon (1976).

Statistical analysis

Data were analysed with a Macintosh computer using standard statistical packages for one way analysis of variance (ANOVA) followed by Student's *t* test for unpaired values. *P* values of 0.05 or less were considered significant. Results are expressed as means ± standard error of the mean (s.e.mean).

Results

Effect of antigen challenge on histamine and LTC_4-like-material release from isolated lungs from sensitized mice

The i.t. instillation of 1 µg ovalbumin to lungs from sensitized Swiss mice induced the release of amounts of LTC_4-like-material significantly above the basal levels, as detected by radioimmunoassay (Figure 1). A further increase in LTC_4-like-material release was observed after the administration of 100 µg ovalbumin, but the difference was not statistically significant (Figure 1). The administration of the same doses of ovalbumin to isolated lungs from sensitized Balb/C or CBA/J mice failed to trigger LTC_4-like-material generation in the effluent at any time-point considered (Figure 1).

In order to confirm the presence of authentic LTC_4 in the effluent from antigen-challenged lungs, h.p.l.c. analysis followed by enzyme-immunoassay were performed. Higher amounts of LTC_4 were found in the fractions from lung effluent from Swiss than from Balb/C mice, 1.83 ± 0.25 ng ml^{-1} and 0.94 ± 0.24 ng ml^{-1} LTC_4 being measured in the effluent from antigen-challenged Swiss and Balb/C mice lungs, respectively ($P<0.001$; $n=5-6$).

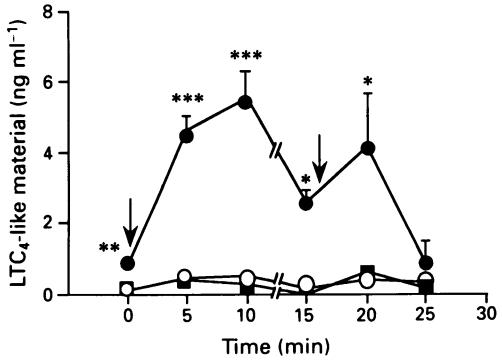


Figure 1 Kinetics of ovalbumin (1 and 100 µg, i.t. at 20 min intervals)-induced leukotriene C_4 (LTC_4)-like material release from isolated lungs from sensitized Balb/C (○), Swiss (●) or CBA/J (■) mice. Immunoreactive LTC_4-like material was determined by radioimmunoassay in fractions of lung effluent collected 5 min before and between 1 and 10 min after antigen challenge. Results are expressed as ng ml^{-1} and they represent the mean ± s.e.mean of 3–7 experiments. The two injections of ovalbumin (1 and 100 µg) are indicated by the arrows. Basal levels of LTC_4-like material in the lung effluent were 0.12 ± 0.06, 0.83 ± 0.11 and 0.10 ± 0.10 ng ml^{-1} for Balb/C, Swiss and CBA/J mice, respectively. $^*P<0.05$; $^{**}P<0.01$ and $^{***}P<0.001$, as compared to CBA/J and Balb/C mice.

No histamine release was measured in the effluent from lungs from the three strains of mice in response to either 1 or 100 µg ovalbumin (data not shown). However, detectable amounts of histamine were found in their lung tissues, since 0.28 ± 0.03, 0.33 ± 0.01 and 0.40 ± 0.03 ng histamine mg^{-1} of tissue were measured in Swiss, Balb/C and CBA/J mice, respectively ($n=3$, difference non statistically significant). It is noteworthy that the contents of histamine in the lungs from non-immunized mice were not statistically different from those from immunized mice (data not shown).

Effect of PAF, fMLP and arachidonic acid on the release of LTC_4-like-material from lungs from non immunized mice

The i.t. instillation of 1 or 10 µg PAF to lungs from non-sensitized mice, induced the release of amounts of LTC_4-like-material above the background values (Figure 2a). The responses to PAF were not dose-dependent and reached an intensity similar for the three strains of mice (Figure 2a). In contrast, the i.t. injection of fMLP (0.1–10 µg) induced a dose-dependent release of LTC_4-like-material from lungs from Balb/C and CBA/J, but not from Swiss mice. In particular, lungs from Balb/C mice were more responsive to 10 µg fMLP in terms of LTC_4-like-material generation than those from Swiss and CBA/J mice, even though this was not statistically significant ($n=5-6$) (Figure 2b).

Similar amounts of LTC_4-like material were measured in the effluent of lungs from non-sensitized Swiss, Balb/C or CBA/J mice following the i.t. instillation of 100 µg arachidonic acid. Indeed, 6.63 ± 0.39, 7.44 ± 0.49 and 7.25 ± 0.21 ng ml^{-1} LTC_4-like material were released by lungs from Swiss, Balb/C or CBA/J mice, respectively in response to arachidonic acid ($n=4-9$, differences not statistically significant). The possibility that arachidonic acid present in the effluent from stimulated lungs might be detected as LTC_4-like material was ruled out. Indeed, when concentrations up to 10 mg ml^{-1} of arachidonic acid were measured by the radioimmunoassay, amounts of LTC_4-like material below 1 ng ml^{-1} were detected (data not shown).

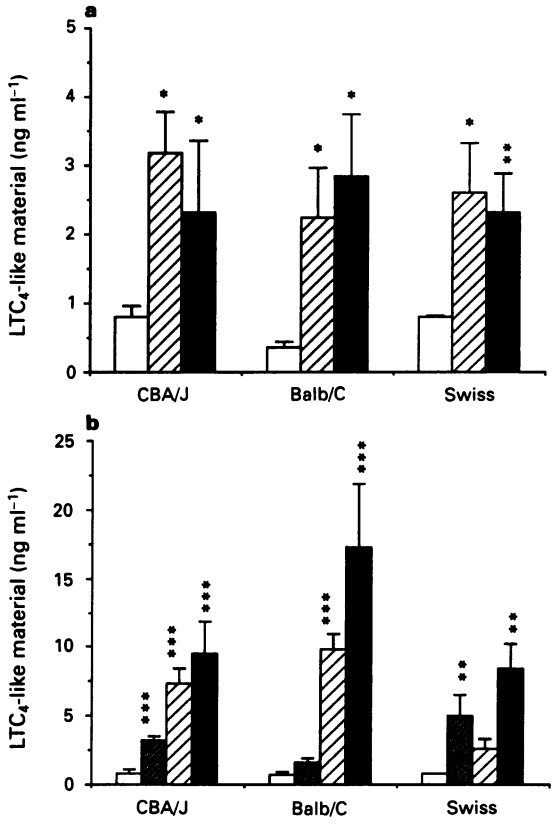


Figure 2 Determination of immunoreactive leukotriene C_4 (LTC_4)-like material in fractions of effluent collected from lungs from non-immunized Balb/C, Swiss or CBA/J mice challenged i.t. with 0.1 μg (widely hatched columns), or 1 μg (solid columns) PAF (a), or with 0.1 μg (closely hatched columns); or 1 μg (widely hatched columns) or 10 μg (solid columns) fMLP (b). Open columns represent the concentrations (in ng ml^{-1}) of immunoreactive LTC_4-like material determined during 5 min before the injection of the agonist. Amounts of LTC_4-like material following the administration of PAF or fMLP are expressed at the peak release, i.e., between the 1st and 5th min after stimulation. The results represent the mean ± s.e.mean of 4–9 experiments. $^*P<0.05$; $^{**}P<0.01$ and $^{***}P<0.001$, as compared to basal values.

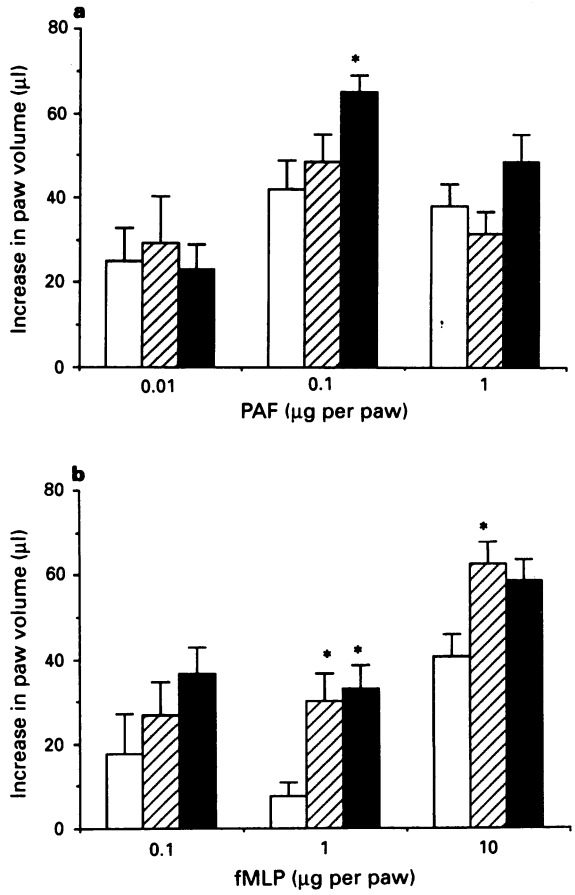


Figure 3 Increase in mouse paw volume, as measured plethysmographically 30 min after the intraplantar injection of platelet activating factor (PAF, 0.01–0.1 μg, a) or of N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP, 0.1–10 μg, b) to non-sensitized CBA/J (open columns), Balb/C (hatched columns) and Swiss mice (solid columns). Values represent the increase in the volume (in μl) of the challenged paws, as compared to vehicle-injected paws. Results are expressed as the mean ± s.e.mean of 4–6 experiments. $^*P<0.05$, as compared to CBA/J mice.

PAF- and fMLP-induced paw oedema in non sensitized mice

The intraplantar injection of 0.01–1 μg PAF to non sensitized mice induced a dose-dependent increase in the paw volume 30 min after its administration. The intensity of the oedema formation was similar in all the strains studied, except for Swiss mice which exhibited a significantly higher response to 0.1 μg PAF than Balb/C and CBA/J mice ($P<0.05$; $n=6$) (Figure 3a). The intraplantar administration of 0.1–10 μg fMLP was also followed by a dose-dependent increase in the paw volume (Figure 3b). The response to 1 or 10 μg fMLP was significantly higher in Balb/C and Swiss, as compared to CBA/J mice ($P<0.05$; $n=5$–6) (Figure 3b).

Antigen-induced paw oedema in sensitized mice

Figure 4 represents ovalbumin (0.001–0.1 μg)-induced paw swelling in sensitized mice. Increase in paw volume following the intra-plantar administration of ovalbumin to sensitized Swiss and Balb/C mice was significantly higher than that measured in CBA/J mice. Swiss mice were more sensitive in terms of oedema formation than the other strains studied (Figure 4).

The intraplantar injection of 10 μg ovalbumin to non-sensitized mice did not significantly modify the paw volume (data not shown).

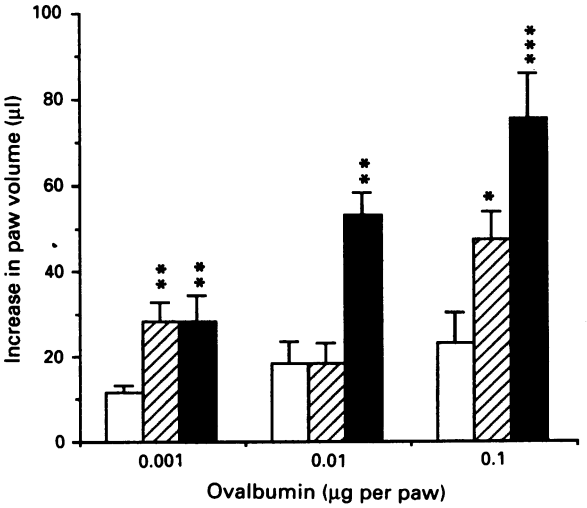


Figure 4 Increase in mouse paw volume, as measured plethysmographically 30 min after the intraplantar injection of ovalbumin (0.001–0.1 μg) to sensitized CBA/J (open columns), Balb/C (hatched columns) and Swiss mice (solid columns). Values represent the increase in the volume (in μl) of the challenged paws, as compared to vehicle-injected paws. Results are the mean ± s.e.mean of 4–6 experiments. $^*P<0.05$; $^{**}P<0.01$ and $^{***}P<0.001$, as compared to CBA/J mice.

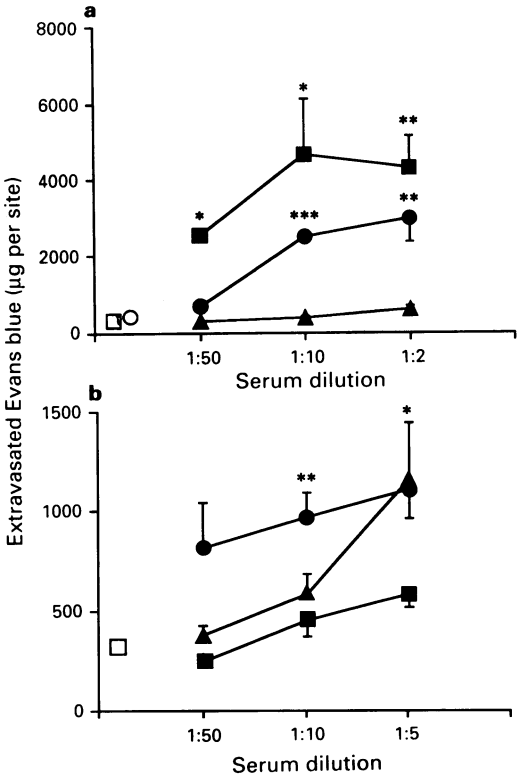


Figure 5 Titers of ovalbumin anti-IgE (a) and anti-IgG (b) antibodies in serum collected from sensitized CBA/J (●); Balb/C (■) and Swiss mice (▲). Levels of anaphylactic antibodies were determined by PCA in recipient Wistar rats (for IgE) and in Swiss mice (for IgG). The results are expressed as means ± s.e.mean of 3–5 experiments; (□) and (○) correspond to Evans blue dye extravasation observed after the intra-dermal injection of saline or heated serum at 56°C for 2 h, respectively. $*P<0.05$; $**P<0.01$; $***P<0.001$, as compared to Swiss mice (a). $*P<0.05$ and $**P<0.01$, as compared to Balb/C mice (b).

Determination of circulating antibody titers in sensitized mice

The i.v. injection of ovalbumin to Wistar rats that had received, 24 h beforehand, intra-dermal injections of diluted serum from Swiss mice, did not induce significant Evans blue extravasation, even at the low dilution (1:2) (Figure 5a). In contrast, ovalbumin administration was followed by marked vascular leakage at the site of intra-dermal injections of serum from Balb/C and CBA/J mice. This effect was observed particularly at the dilution of 1:10. Heated serum (56°C for 1 h) from all mice strains did not elicit Evans blue dye extravasation (Figure 5a).

The i.v. administration of ovalbumin to recipient Swiss mice treated intra-dermally, 2 h beforehand, with diluted serum from Swiss and, particularly, from CBA/J animals, induced Evans blue extravasation, more intense than that of Balb/C mice, indicating the presence of higher titers of IgG antibodies (Figure 5b). These results suggest that IgE antibodies are primarily involved in the immune response of Balb/C and CBA/J, but not, Swiss mice.

Discussion

Unlike the rat or guinea-pig lung for which the pattern of airway responsiveness, particularly in relation to mediator release, has been widely described, little is known concerning the capacity of mouse lung tissue to generate pro-inflammatory mediators in response to different stimuli. In the present study, we have developed a model of isolated and perfused mouse lungs and compared the responsiveness to immunological and non-immunological stimuli in different strains. The advantage of characterizing a model of isolated lung in the mouse is linked to the availability in this species of well-defined strains or mutants with deficiencies in immunological and/or inflammatory cells. This allows the identification of the precise role of these cell types in the development of hypersensitivity reactions, with a particular emphasis to bronchopulmonary anaphylaxis.

Our results demonstrate that antigen challenge induces the release of higher amounts of LTC_4-like material from isolated lungs from sensitized Swiss, than from Balb/C or CBA/J mice, when measured by radioimmunoassay. LTC_4 in the lung effluent was resolved by h.p.l.c. and quantified by enzyme-immunoassay in the eluted fractions. LTC_4 formation from a murine tissue extends the data from De Andres *et al.* (1990), who showed the ability of mouse peritoneal eosinophils to release this metabolite upon *in vitro* stimulation.

Lower amounts of LTC_4 in the lung effluent from antigen-challenged Swiss mice were detected by enzyme-immunoassay coupled to h.p.l.c. compared to the levels measured by radioimmunoassay by direct analysis. Such a discrepancy between direct assay and assays performed after h.p.l.c. was observed earlier by Wescott *et al.* (1986). Under our conditions, this may result from the fact that the antibody used for radioimmunoassay shows 10% cross-reactivity with LTD_4 (see Methods section). It may thus be hypothesized that, besides LTC_4, antigen challenge also induced the release of LTD_4 from mouse lung tissue in amounts detectable by the radioimmunoassay.

LTC_4-like material production was not accompanied by histamine release into the effluent from lungs of Swiss mice, even though histamine was found in similar amounts of the lung tissue from the three strains considered. The amounts of histamine/mg tissue were, however, much lower, when compared to the lungs from sensitized guinea-pigs (Pretolani *et al.*, 1988), a species for which the ability to secrete large amounts of this vasoactive amine in response to antigen challenge is widely described.

The lack of correlation between LTC_4 like-material and histamine release may reflect the complexity of the ongoing allergic reaction. Thus, at the present time, the cell origin of the LTC_4 generated upon antigen challenge remains to be determined. Jakschik *et al.* (1987) demonstrated that murine mast cells release LTC_4 during immediate hypersensitivity reactions. Consequently, if mast cells are the cell source of LTC_4 in the lung, histamine secretion and LTC_4 production are independent phenomena. In confirmation, antigen injection into the peritoneal cavity of sensitized Balb/C mice induces histamine and LTC_4 release which followed different kinetics (Zuany-Amorim *et al.*, 1993). This suggests that these mediators are generated by different cell types and that mast cell activation by antibody-dependent mechanisms may trigger the release of pre-formed mediators without leading to the synthesis of the newly generated lipid mediators. However, the possibility that antigen-induced mediator release does not necessarily involve mast cell activation may not be ruled out. Indeed, Pellon *et al.* (1993) reported that PAF production by rat peritoneal cells stimulated with either monoclonal IgE, IgG1 or IgG2b anti-DNP-BSA was unrelated to mast cell activation, since similar amounts of PAF were measured in populations depleted of mast cells. Moreover, peritoneal cells sensitized with IgG1 and IgG2b failed to release 5-HT under conditions where PAF generation was observed (Pellon *et al.*, 1993), suggesting once again that different mechanisms account for pre-formed and newly-formed mediator release. In contrast, Ramos *et al.* (1991) demonstrated that mast cells are required for the production of leukotrienes in immune complex-induced peritonitis in mice. Macrophages may also be considered as a source for eicosanoids, since it has been demonstrated that human alveolar macrophages release high amounts of arachidonic acid metabolites upon stimulation (Damon *et al.*, 1986). Fur-

thermore, as these cells express low and high affinity receptors for IgE (Capron *et al.*, 1986; Soussi Gounni *et al.*, 1994) and IgG1 and IgG2b (Unkeless & Eisen, 1975; Diamond & Scharff, 1980), their ability to respond to antigen challenge with LTC_4 production appears likely (M'Rini-Puel *et al.*, 1993). Finally, the eosinophil is also capable of producing LTC_4, as shown in man (Brujinzeel *et al.*, 1985) and mice (De Andres *et al.*, 1990). In our experiments, however, the difference in the ability of lungs from the three strains of mice to generate LTC_4 upon antigen challenge was not accounted for by a pre-established airway inflammation. Indeed, no eosinophils or neutrophils were observed in the bronchoalveolar lavage fluid or lung tissues of sensitized mice, irrespective of the strain (unpublished results).

In order to verify whether the different responsiveness of lungs from Balb/C, Swiss and CBA/J mice involves specific antigen stimulation, the ability of three non-immunological secretagogues, arachidonic acid, PAF and fMLP, to generate LTC_4-like material was investigated. The i.t. injection of PAF or arachidonic acid induced the release of comparable amounts of LTC_4-like material in the effluent of lungs from the three mice strains, whereas fMLP administration triggered the release of larger amounts of LTC_4-like material from lungs from Balb/C, as compared to CBA/J and Swiss mice. This difference, however, did not parallel that observed for lung responses to antigen challenge, since Swiss mice were unique in releasing LTC_4 when stimulated with ovalbumin. Thus, even though lungs from Balb/C and CBA/J mice do not respond to antigen provocation in terms of LTC_4 release, they are capable of doing so when challenged with non-immunological stimuli.

In an attempt to verify whether the increased response to antigen challenge observed in Swiss mice reflects their capability to develop an anaphylactic response more intense than that of mice from other strains, anaphylactic paw oedema was evaluated. The intra-plantar administration of 0.01 μg ovalbumin to Swiss mice induced a significant swelling under conditions where Balb/C mice required a ten fold higher dose of antigen to induce a similar effect and CBA/J mice were unresponsive. This suggests that Swiss mice are more sensitive to antigen stimulation and that this phenomenon is not restricted to the lung. In contrast, paw oedema induced by PAF showed similar intensities in all strains of mice. Finally, Swiss and Balb/C mice were more responsive to fMLP than CBA/J mice, suggesting again different intrinsic capacities of the various mice strains to develop inflammatory reactions in response to non-immunological stimuli.

Since a clear-cut difference between the three strains of mice analysed was observed only for the anaphylactic response, the first hypothesis that arose was whether different immunoglobulin subclasses were involved in the response of the animals to immunisation. Indeed, Swiss and CBA/J mice showed higher titers of IgG than Balb/C, whereas Balb/C mice exhibited higher levels of IgE, when compared to CBA/J and Swiss mice. These findings rule out the hypothesis that the different responses to antigen provocation result from different intensities of the immune response or to the antibody subclass involved.

These results suggest that antigen-antibody dependent activation of inflammatory cells is regulated by different mechanisms depending on the mouse strain considered. Furthermore, since lungs from Swiss mice failed to secrete histamine in response to antigen challenge under conditions where LTC_4 was released, it may be suggested that, during immediate hypersensitivity reactions, histamine release is not a fundamental event.

Finally, our data emphasize that the characterization of the immune and inflammatory response is an important step in the development of a model for studying hypersensitivity reactions in the mouse.

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Contractions mediated by α_1-adrenoceptors and P_2-purinoceptors in a cat colon circular muscle

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1 The postjunctional excitatory and inhibitory effects of adrenoceptor and purinoceptor agonists and antagonists were studied in circular smooth muscle strips of cat colon.

2 In the presence of tetrodotoxin (0.5 μM), noradrenaline caused contraction or relaxation of circular smooth muscle at resting tension or with raised tone, respectively. The noradrenaline-evoked contractions were potentiated and the noradrenaline-evoked relaxations were antagonized by propranolol (1 μM), suggesting β-adrenoceptor involvement.

3 At resting tension, noradrenaline, adrenaline and the selective α_1-adrenoceptor agonist, phenylephrine, caused concentration-dependent contractile responses, with EC_{50} values of 1.8 ± 0.2 μM, 1.9 ± 0.4 μM and 4.3 ± 1.7 μM, respectively. The EC_{50} values and the amplitude of maximal responses were not significantly different from one another. Clonidine (0.1–500 μM), a selective α_2-adrenoceptor agonist, was not effective.

4 Prazosin (0.1–9 μM), competitively antagonized the contractile effects of noradrenaline with an estimated pA_2 value of 6.93 and a slope of 1.07 ± 0.03. The K_b values, estimated from a single shift (0.1 μM prazosin) of the concentration-response curves to noradrenaline, adrenaline and phenylephrine were 92.8 ± 9.3 nM, 108.7 ± 6.4 nM and 18.4 ± 3.1 nM, respectively.

5 At resting tension, adenosine 5′ triphosphate (ATP, 5–1000 μM), α,β-methylene adenosine 5′-triphosphate (α,β-MeATP, 0.05–50 μM), β,γ-methylene adenosine 5′-triphosphate (β,γ-MeATP, 0.5–100 μM), and 2-methylthioadenosine 5′-triphosphate (2-MeSATP, 1–500 μM) caused concentration-dependent contractions with EC_{50} values of 60.5 ± 15.9 μM, 0.7 ± 0.1 μM, 7.6 ± 0.1 μM and 25.3 ± 12.8 μM, respectively. The maximal responses to α,β-MeATP and β,γ-MeATP were greater than maximal responses to 2-MeSATP and ATP.

6 Suramin (50–500 μM), competitively antagonized the contractile responses of α,β-MeATP with an estimated pA_2 value of 4.92 and a slope of 1.08 ± 0.04. The K_b values, estimated from a single shift (100 μM suramin) of the concentration-response curves to ATP, α,β-MeATP, β,γ-MeATP and 2MeS-ATP were 52.3 ± 20.2 μM, 25.2 ± 4.5 μM, 21.7 ± 11.0 μM and 11.6 ± 2.7 μM, respectively.

7 At resting tension, reactive blue 2 (100 μM), a selective antagonist of the P_{2Y}-purinoceptor, and 8-(*p*-sulphophenyl)-theophylline (8-SPT) (1 μM), a selective antagonist of the P_1-purinoceptor, did not antagonize the contractile responses to α,β-MeATP (0.5–5 μM). Contractile responses to ATP (50–500 μM) were not altered by 8-SPT (1 μM) but were potentiated by reactive blue 2 (100 μM).

8 With raised tone, ATP and 2-MeSATP caused a relaxant effect. This effect of ATP was not altered by either tetrodotoxin (TTX) (0.5 μM) or suramin (100 μM), but was antagonized by reactive blue 2 (100 μM) and 8-SPT (1 μM), suggesting that inhibitory P_{2Y}- and P_1-purinoceptors are involved. In contrast, α,β-MeATP and β,γ-MeATP caused only contractions. This contractile effect of α,β-MeATP was resistant to TTX (0.5 μM) and antagonized by suramin (100 μM).

9 In summary, cat colon circular muscle contains postjunctional α_1-adrenoceptors and P_{2X}-purinoceptors which mediate contractions and P_{2Y}- and P_1-purinoceptors which mediate relaxation. Postjunctional α_2-adrenoceptors appear not to be present.

Keywords: α_1-Adrenoceptors; P_{2X}-purinoceptors; contractile responses; cat colon

Introduction

Electrical stimulation of lumbar sympathetic fibres contracts circular muscle of sphincter regions of the colon via postjunctional α-adrenoceptors (Pahlin & Kewenter, 1976; Bouvier & Gonella, 1981) and inhibits neurogenic contractions in non-sphincter regions of colon circular and longitudinal muscle via prejunctional α-adrenoceptors (Gillespie & McKenna, 1961; de Groat & Krier, 1976; Gillespie & Khoyi, 1977) and pre- and postjunctional β-adrenoceptors (Ek, 1985).

Electrical stimulation of lumbar sympathetic fibres (lumbar colonic nerves) also cause contractions of circular muscle, in non-sphincter regions of cat colon. The contractions are partially reduced by either prazosin or by desensitization with α,β-methylene ATP, but are almost abolished by application of both compounds. This suggests that the neurogenic contractions are mediated by the co-release of noradrenaline and ATP or a related nucleotide through postjunctional α_1-adrenoceptors and P_{2X}-purinoceptors (Venkova & Krier, 1993).

The circular muscle layer of cat, dog, rat and human colon contains excitatory α-adrenoceptors (Gagnon & Belisle, 1970; Gagnon *et al.*, 1972; Anurus & Christensen, 1981; Zang *et al.*, 1992; Venkova & Krier, 1993) and P_2-purinoceptors (Hedlung *et al.*, 1986; Venkova & Krier, 1993). In cat colon circular muscle, postjunctional adrenoceptors and purinoceptors have not been pharmacologically characterized. Our aim was to study the excitatory effects of adrenoceptor agonists and purine nucleotides in circular muscle strips of cat colon *in vitro*. Agonist potency and the effects of selective antagonists were used to characterize the α-adrenoceptors and purinoceptors involved. Prazosin, a selective antagonist of the α_1-adrenoceptors was used to study agonist responses

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mediated by α_1-adrenoceptors. Suramin, an antagonist of the P_{2X}-purinoceptor (Dunn & Blackley, 1988; Hoyle *et al.*, 1990; Leff *et al.*, 1990; Kennedy, 1990) and reactive blue, an antagonist of the P_{2Y}-purinoceptor (Kerr & Krantis, 1979) were used to study agonist responses mediated by P_{2X}- and P_{2Y}-purinoceptors. 8-(*p*-sulphophenyl)-theophylline (8-SPT), a putative selective antagonist of the P_1-purinoceptor (Collis *et al.*, 1987), was used to distinguish agonist responses mediated by P_1- and P_2-purinoceptors.

Methods

Male or female cats, weighing 2.5–4.5 kg were anaesthetized with sodium pentobarbitone (50–70 mg kg^{-1}, i.p.). After exsanguination, a segment (4–5 cm) of the mid colon, 4 cm caudad to the ileocecal sphincter, was excised and placed in ice-cold Krebs solution. It was cut along the antimesenteric border and the mucosa removed. Muscle strips (about 10 mm long and 3 mm wide) were cut in the long axis of circular muscle fibres. They contained both muscle layers and the myenteric plexus. Isolated preparations were mounted vertically in the circular axis in 10 ml organ baths containing a modified Krebs solution [(mM): NaCl 117, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11] maintained at 37°C and continuously aerated with 95% O_2 and 5% CO_2.

Spontaneous isometric contractions of the circular smooth muscle layer were recorded. Muscle strips were equilibrated for 60–90 min under a load of 5 g. Optimal tension was determined previously by repeatedly stretching the muscle strips and adding acetylcholine (10 μM). When contractions to acetylcholine ceased to increase with increasing stretch, that tension was considered optimal. This corresponded to a load of approximately 5 g. Muscle strips which changed their tone after equilibration were discarded.

Experimental protocol

The mechanical responses of cat colon circular muscle strips to exogenous adrenoceptor agonists were studied at resting tension and with raised tone. At resting tension, concentration-response curves to noradrenaline, adrenaline and phenylephrine were constructed. Peak contractions to adrenoceptor agonists occurred within 3–4 min. Increasing concentrations of adrenoceptor agonists were added cumulatively during the plateau phase of each agonist contraction. After completion of each concentration-response curve, drugs were 'washed out' and tension returned to the resting level. Also, concentration-response curves to noradrenaline were constructed in the absence and presence of either prazosin or propranolol. Muscle strips were pretreated with antagonists for 20–25 min before construction of a concentration-response curve. Each muscle strip served as its own control. Only one agonist and one antagonist was applied per muscle strip.

The relaxation responses to noradrenaline were studied at raised tone. To study relaxation, carbamylcholine (carbachol, 1 μM) was added to the Krebs solution. This concentration of carbachol caused a submaximal, sustained rise in tone. Tetrodotoxin (TTX) (0.5 μM) was used to abolish neuronal input to smooth muscle. The effects of noradrenaline (10 μM) were tested during the 'plateau phase' of the evoked contraction. The involvement of β-adrenoceptors was studied by examining relaxant responses to noradrenaline in the absence and presence of propranolol (1 μM).

The mechanical responses of cat colon circular muscle strips to ATP, 2-MeSATP, α,β-MeATP and β,γ-MeATP were studied at resting tension. At resting tension, purinoceptor agonists caused contractions of circular smooth muscle. Concentration-response curves to ATP, 2-MeSATP, α,β-MeATP and β,γ-MeATP were constructed. Purine nucleotides were consecutively applied at intervals of 20 min to avoid densensitization. Concentration-response curves to ATP, 2MeSATP, α,β-MeATP and β,γ-MeATP were constructed in the absence and presence of suramin, a P_{2X}-purinoceptor antagonist. Contractile responses to ATP and α,β-MeATP were studied in the absence and presence of reactive blue 2, a P_{2Y}-purinoceptor antagonist and 8-(*p*-sulphophenyl)-theophylline (8-SPT), a P_1-purinoceptor antagonist. Each muscle strip served as its own control. Only one agonist was applied per muscle strip.

Some experiments examined the effects of ATP (10 μM), α,β-MeATP (5 μM), 2-MeSATP (5 μM) and β,γ-MeATP (10 μM) in muscle strips with raised tone. Noradrenaline (10–50 μM) was added to the bathing solution to raise tone. The involvement of P_{2Y}-purinoceptors and P_1-purinoceptors in relaxation was studied by examining responses to ATP (10 μM) in the absence and presence of reactive blue 2 (100 μM) and 8-SPT (50 μM).

Data analysis

The amplitude of contractile responses to agonists for each muscle strip was measured and expressed either as a percentage of the maximal response or as percentage of the carbachol (10 μM)-evoked contractile response. The concentration of agonists required to cause 50% of the maximal effect (EC_{50} values) was calculated by linear regression of the relation between negative $\log_{10}$ of the concentration of agonist and the percentage maximum response. Data obtained from responses between 20% and 80% of the maximum were used to fit linear regression lines for each concentration-response curve. Linear equations were used to calculate the slope and EC_{50} value for each concentration-response curve. The correlation coefficients of the linear regression lines ranged between 0.91 and 0.99.

The antagonistic potency and the type of antagonism of prazosin (0.1–9 μM) against noradrenaline and suramin (50–500 μM) against α,β-MeATP were defined by Schild plot analysis (Arunlakashana & Schild, 1959). Concentration-response curves were constructed in the absence and in the presence of multiple concentrations of antagonists. The pA_2 values and slopes were calculated from regression lines of the relation between logarithmic concentration of antagonist and the logarithmic concentration ratio (DR-1).

The antagonistic actions of single concentrations of prazosin (0.1 μM) against noradrenaline, adrenaline and phenylephrine and suramin (100 μM) against α,β-MeATP, β,γ-MeATP, 2-MeSATP and ATP were also studied. Antagonist potency was expressed as the dissociation constant K_b, which was estimated from a single shift in the agonist concentration-response curves. The K_b values were calculated by the equation:

$$K_b = a/(DR-1)$$

where a is the antagonist concentration and DR is the ratio of the equieffective agonist concentration (at the EC_{50} level) determined in the absence and presence of the antagonist (Kenakin, 1993).

The EC_{50} slopes and K_b values were calculated for each muscle strip and data are presented as mean ± s.e. mean. The significance of differences was assessed by a Student's *t* test for paired and unpaired observations and *P* values of less than 0.05 were considered significant. The number of observations (*n*) are the number of muscle strips.

Drugs

Drugs used were: tetrodotoxin, acetylcholine chloride, carbamylcholine chloride (carbachol), adenosine hemisulphate, (±)-propranolol hydrochloride, prazosin hydrochloride, suramin hexasodium salt, (−)-noradrenaline bitartrate, (−)-phenylephrine hydrochloride, adenosine 5′-triphosphate disodium salt (ATP), α,β-methylene adenosine 5′-triphosphate lithium salt (α,β-methylene ATP), 2-methylthioadenosine 5′ triphosphate tetrasodium salt (2-MeSATP), β,γ-methylene

adenosine 5'-triphosphate sodium salt (β,γ-methylene ATP), adenosine hemisulphate salt and reactive blue 2 (cibacron blue 3GA). All drugs except 8-(*p*-sulphophenyl)-theophylline (Research Biochem. Inc.), 2-methylthioadenosine 5' triphosphate tetrasodium salt (Research Biochem. Inc.) and suramin hexasodium salt (a gift from Center for Disease Control; Atlanta, Georgia, U.S.A.) were obtained from the Sigma Chemical Co. Drugs were dissolved in distilled water and diluted to final concentrations in Krebs solution. Drug concentrations reported refer to final bath values.

Results

Responses of circular muscle to adrenoceptor agonists

Effects of noradrenaline at different tone levels At resting tension, noradrenaline (10–100 μM) caused concentration-dependent contractions of circular muscle (Figure 1b). The noradrenaline-evoked contractions were potentiated by tetrodotoxin (0.5 μM) and blocked by the α_1-adrenoceptor agonist, prazosin (Figure 1a). Propranolol (3 μM) significantly ($P<0.05$) potentiated the amplitude of noradrenaline-evoked contractile responses (Figure 1b). When tone was raised by carbachol (1 μM), noradrenaline (0.1–10 μM) caused relaxation (Figure 2, for 10 μM noradrenaline). The relaxant responses to noradrenaline occurred in muscle strips pretreated with tetrodotoxin (0.5 μM) and were reduced by the β-adrenoceptor antagonist, propranolol (1 μM, $n=6$). At resting tension, the inhibitory effect of noradrenaline is masked by the contraction caused by excitatory α_1-adrenoceptors.

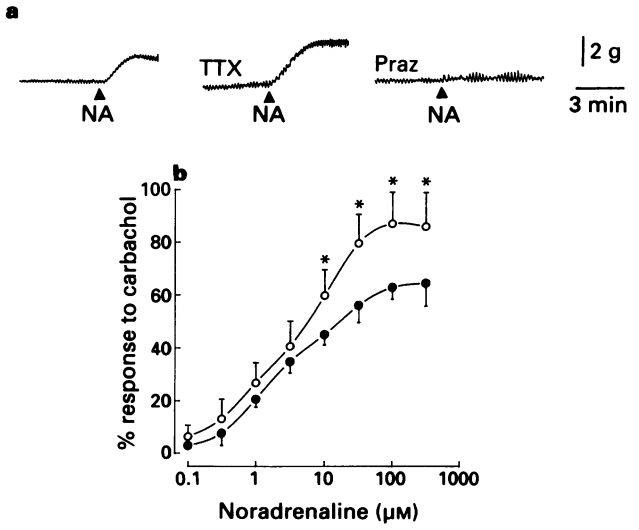


Figure 1 Cat mid-colon circular muscle strip *in vitro*. (a) Mechanical responses to noradrenaline (NA, 10 μM), at resting tension. Responses of one muscle strip in the absence and presence of tetrodotoxin (TTX, 0.5 μM) or TTX and prazosin (Praz, 1 μM). (b) Effect of propranolol on the contractile responses to noradrenaline, at resting tension. Abscissa scale, concentration of noradrenaline (μM). Ordinate scale, contractile responses expressed as a percentage relative to the contraction caused by carbachol (10 μM). Contractile responses to noradrenaline in the absence (●, $n=10$) and presence of propranolol (1 μM, ○, $n=6$). Noradrenaline was applied in cumulatively increasing concentrations. Effects of propranolol were significant (*$P<0.05$, paired test). Mean values with s.e. mean are shown.



Figure 2 Cat mid-colon circular muscle strip *in vitro*. Mechanical responses to noradrenaline (NA, 10 μM) when tone was raised by carbachol (CCh, 10 μM). Responses of single muscle strip in the absence and in the presence of tetrodotoxin (TTX, 0.5 μM) or TTX and propranolol (Prop, 1 μM).

Potency of α-adrenoceptor agonists At resting tension, phenylephrine, noradrenaline and adrenaline (each at 0.1–1000 μM) caused concentration-dependent contractions in colon circular muscle strips whereas clonidine (1–1000 μM) did not. The amplitude of each agonist-evoked contraction was expressed as the percentage of the response to carbachol (10 μM). With low concentrations of agonists (0.1–10 μM), the amplitude of contractile responses to adrenaline were greater than those to noradrenaline and phenylephrine. This resulted in significant differences ($P<0.05$) in the slopes of the linear regression lines between adrenaline and noradrenaline and between adrenaline and phenylephrine but not between noradrenaline and phenylephrine (Table 1). In contrast, EC_{50} values for phenylephrine, noradrenaline and adrenaline and the amplitude of the maximal responses were not significantly different (Table 1). This suggests that each adrenoceptor agonist has a similar potency for contracting circular smooth muscle.

Antagonist potency of prazosin The contractile responses to noradrenaline, adrenaline and phenylephrine were antagonized by prazosin. Increasing concentrations of prazosin (0.1, 1, 3, 9 μM) caused parallel shifts to the right of the concentration-response curves to noradrenaline (Figure 3a). The Schild plot analysis of the rightward shifts yielded a pA_2 value of 6.93 and a slope of 1.07 ± 0.03 (Figure 3b). The K_b values for prazosin, estimated from a single shift (0.1 μM prazosin) of the concentration-response curves to noradrenaline, adrenaline and phenylephrine were 92.8 ± 9.3 nM ($n=8$), 108.7 ± 6.4 nM ($n=6$) and 18.4 ± 3.1 nM ($n=5$), respectively. The K_b value against phenylphrine was significantly lower than the K_b values against the other two adrenoceptor agonists. Also, there was no difference between the K_b values estimated against noradrenaline by Schild plot analysis and by the single shift of the concentration-response curves.

Responses of circular muscle to purinoceptor agonists

Effects of purinoceptor agonists at different tone levels The effects of ATP and ATP analogues were tested on the mechanical activity of colon circular muscle at resting tension and when tone was raised. At resting tension, the purinoceptor agonists evoked contractions that were resistant to tetrodotoxin (0.5 μM). They were reduced by the P_{2X}-purinoceptor antagonist, suramin (100 μM, $n=3$ for each agonist) (Figure 4a,b, for ATP and α,β-MeATP).

Table 1 Effects of adrenoceptor agonists on cat colon circular smooth muscle

	Maximal contraction (% of carbachol)	EC_{50} (μM)	*Slope*	r
Noradrenaline ($n=14$)	66.0 ± 3.0	1.8 ± 0.2	46.0 ± 2.4	0.92–0.99
Adrenaline ($n=10$)	72.2 ± 10.5	1.9 ± 0.4	33.0 ± 2.3*	0.91–0.92
Phenylephrine ($n=12$)	56.1 ± 8.3	4.3 ± 1.7	46.1 ± 3.3	0.92–0.99

Values are means ± s.e., n = number of preparations. r = correlation coefficient of regression line.
*Significantly different from all others at $P<0.05$.

Contractile responses to ATP (50–500 μM) and α,β-MeATP (0.5–5 μM) were also studied in the absence and presence of reactive blue 2 (100 μM) or 8-(*p*-sulphophenyl)-theophylline (8-SPT) (50 μM). In other visceral and vascular smooth muscle, similar concentrations of reactive blue 2 and 8-SPT antagonized the effect of ATP at P_{2Y}-purinoceptors (Burnstock & Warland, 1987; Hopwood & Burnstock, 1987; Reilly *et al.*, 1987) and the effect of adenosine at P_1-purinoceptors (Kennedy, 1990; Matharu & Hollingsworth, 1992), respectively. In muscle strips, where tone was raised with noradrenaline, 8-SPT antagonized relaxations evoked by adenosine (1 μM, $n = 5$) (data not shown) but had no significant effect on contractions evoked by ATP (Figure 4c) or α,β-MeATP (Figure 4d). Reactive blue 2 significantly ($P < 0.05$) potentiated the responses to ATP but did not alter contractions to α,β-MeATP (Figure 4c,d).

When tone was raised by noradrenaline, ATP and 2-MeSATP caused relaxation. In the presence of TTX (0.5 μM), the ATP-evoked relaxation was reduced by reactive blue 2 (100 μM) and abolished by both reactive blue and 8-SPT (50 μM) (Figure 5a). Also, the ATP-evoked relaxant response was resistant to suramin (100 μM, $n = 4$, data not shown). In contrast, α,β-MeATP and β,γ-MeATP induced low-amplitude contractile responses that were superimposed on the plateau of the noradrenaline-evoked contraction. The contractile responses to α,β-MeATP were resistant to TTX but were blocked by suramin (100 μM, $n = 5$) (Figure 5b).

Potency of P_2-purinoceptor agonists At resting tension ATP (1–1000 μM), α,β-MeATP (0.05–10 μM), β,γ-MeATP (0.1–

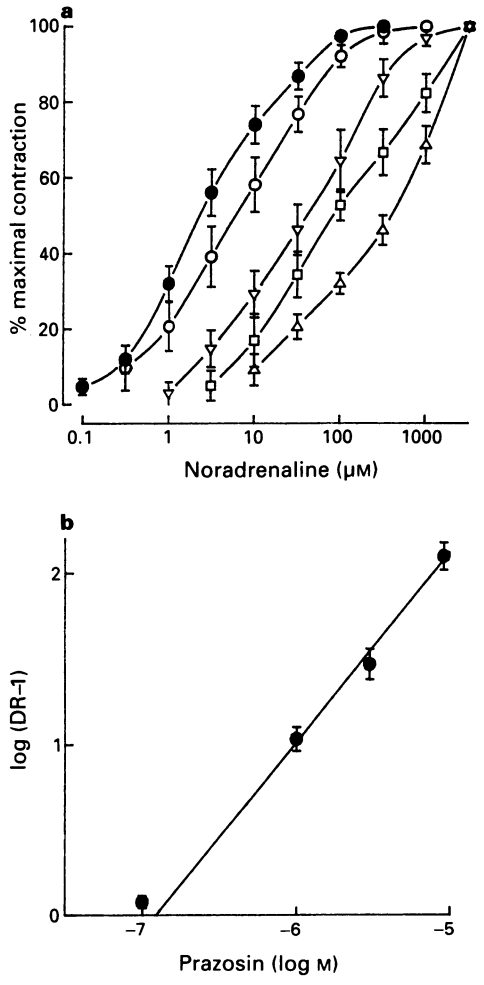


Figure 3 Cat mid-colon circular muscle strip *in vitro*. (a) Effects of prazosin on the contractile responses to noradrenaline, at resting tension. Ordinate scale, responses expressed as a percentage relative to the maximum contraction. Abscissa scale, concentration of noradrenaline (μM). Contractile responses to noradrenaline in the absence (●, $n = 22$) and presence of prazosin (0.1 μM, ○, $n = 7$), (1 μM, ▽, $n = 6$), (3 μM, □, $n = 6$), (9 μM, △, $n = 5$). (b) Schild plot of the relation between log (agonist dose ratio −1) and log molar concentration of the antagonist, prazosin. Noradrenaline was applied in cumulatively increasing concentrations. Mean values with s.e. mean are shown. The values of pA_2 and slope of the Schild plot are 6.9 and 1.07 ± 0.03, respectively. The correlation coefficient of the regression line is 0.97.

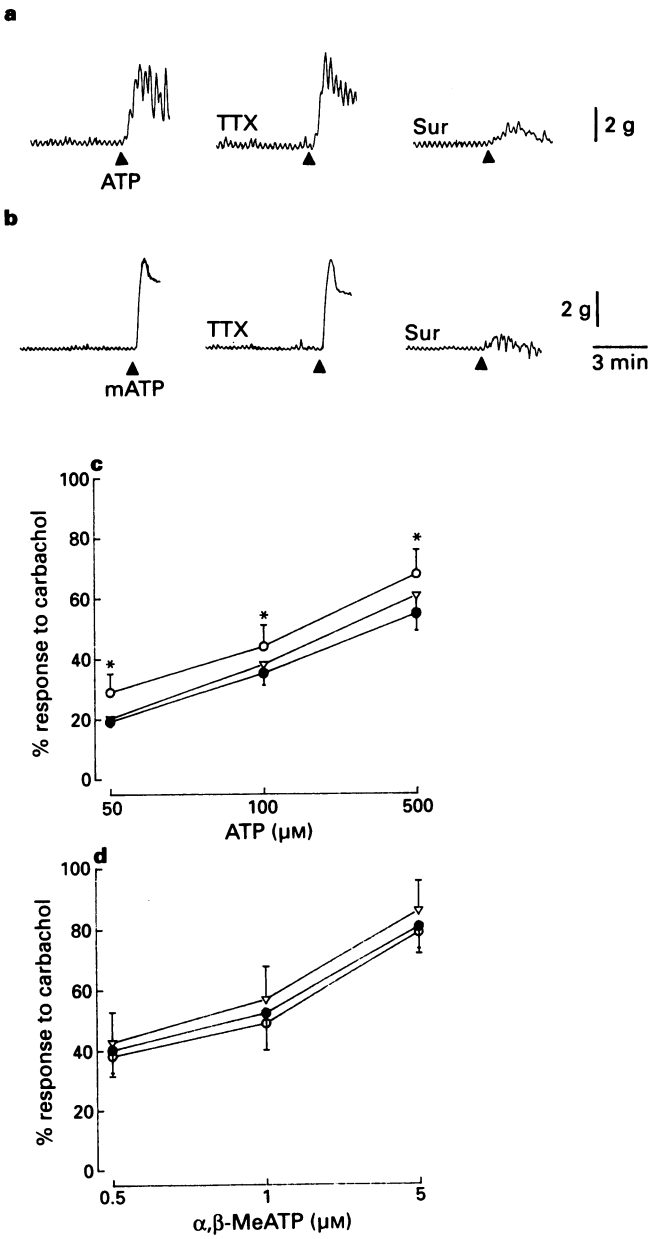


Figure 4 Cat mid-colon circular muscle strip *in vitro*. Contractile responses to ATP and α,β-MeATP at resting tension. (a,b) Responses to ATP (10 μM) and α,β-MeATP (mATP, 5 μM) were obtained in two different muscle strips. Contractile responses in the absence and presence of tetrodotoxin (TTX, 0.5 μM) or suramin (Sur, 100 μM). (c,d) Effects of 8-sulphophenyl-theophylline (8-SPT, 50 μM) and reactive blue 2 (100 μM) on the contractile responses to ATP and α,β-MeATP. Abscissa scale, agonist concentration, (μM). Ordinate scale, contractile responses expressed as a percentage of the contraction caused by carbachol (10 μM). (c) Responses to ATP in the absence (●, $n = 8$) and presence of 8-SPT (▽, $n = 4$) or reactive blue 2 (○, $n = 4$). (d) Responses to α,β-MeATP in the absence (●, $n = 8$) and presence of 8-SPT (▽, $n = 4$) or reactive blue 2 (○, $n = 4$). Mean values with s.e. mean are shown. Effects of reactive blue 2 were significant (*$P < 0.05$, paired test).

100 μM) and 2-MeSATP (1–500 μM) caused concentration-dependent contractions of circular muscle. The concentration-response curves for each agonist were sigmoidal with different maxima. The maximal responses to α,β-MeATP and β,γ-MeATP did not differ from one another but were significantly greater than maximal responses evoked by ATP and 2-MeSATP (Table 2). The order of potency for contractile responses was α,β-MeATP > β,γ-MeATP > 2-MeSATP > ATP. There were no significant differences in the slopes of the linear regression lines (Table 2).

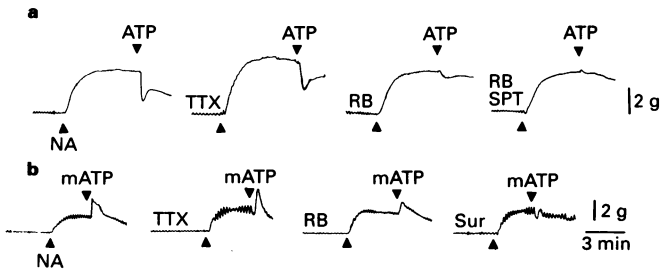


Figure 5 Cat mid-colon circular muscle strip *in vitro*. Mechanical responses to ATP (10 μM) and α,β-MeATP (MeATP, 5 μM) when tone was raised by noradrenaline (NA, 10 μM). Responses to ATP (a) and α,β-MeATP (b) were recorded in two different muscle strips in the absence and in the presence of tetrodotoxin (TTX, 0.5 μM), suramin (Sur, 100 μM), reactive blue 2 (RB, 100 μM) or reactive blue 2 and 8-sulphophenyl-theophylline (SPT, 50 μM).

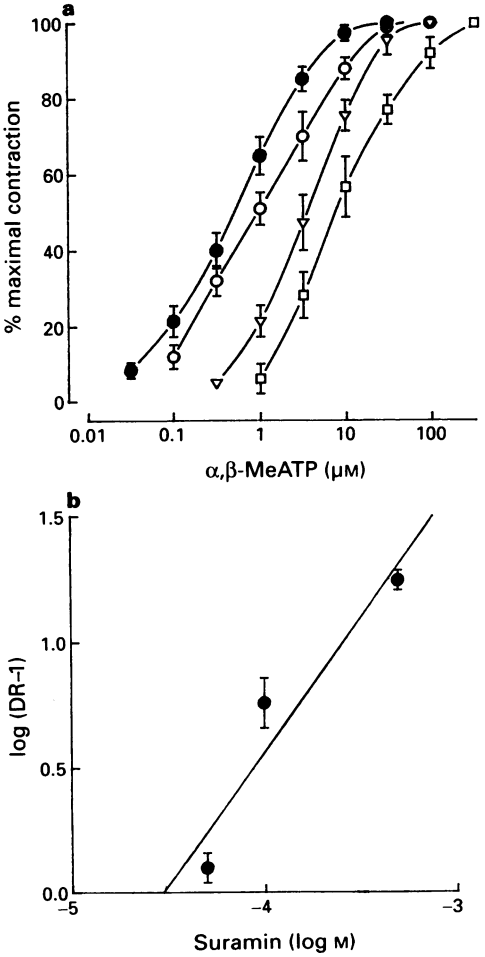


Figure 6 Cat mid-colon circular muscle strip *in vitro*. (a) Effect of suramin on the contractile responses to α,β-MeATP. (a) Abscissa scale, concentration of α,β-MeATP (μM). Ordinate scale, responses expressed as a percentage relative to the maximum contraction. Contractile responses in the absence of suramin (●, $n = 16$), and in the presence of suramin (50 μM, ○, $n = 5$), (100 μM, ▽, $n = 6$), (500 μM, □, $n = 5$). (b) Schild plot of the relation between log (agonist dose ratio −1) and log molar concentration of the antagonist, suramin. α,β-MeATP was applied non-cumulatively. The values of pA_2 and slope of the Schild plot are 4.52 and 1.06 ± 0.34, respectively. The correlation coefficient of the regression line is 0.95. Mean values with s.e. mean are shown.

Antagonist potency of suramin The contractile responses to ATP and the ATP analogues were antagonized by suramin (100 μM). At this concentration, suramin is a selective P_{2X}-purinoceptor antagonist in guinea-pig vas deferens (Dunn & Blakely, 1988), submocosal arterioles (Evan & Surprenant, 1992) and urinary bladder (Hoyle *et al.*, 1990). The concentration-effect curves to α,β-MeATP were shifted to the right by increasing concentrations of suramin (50–500 μM) (Figure 6a). The Schild plot analysis of the rightward shifts yielded a slope of 1.06 ± 0.3 and a pA_2 value of 4.45 (K_b = 30.6 μM) (Figure 6b). The K_b values for suramin, estimated from single shifts (100 μM suramin) of the concentration-effect curves for α,β-MeATP, β,γ-MeATP, 2-MeSATP and ATP, were 25.2 ± 4.5 μM ($n = 5$), 21.7 ± 9.9 μM ($n = 6$), 11.5 ± 2.7 μM ($n = 3$) and 52.3 ± 10.2 μM ($n = 5$), respectively. The K_b value against ATP was significantly ($P < 0.05$) higher when compared to the K_b values for suramin against the ATP analogues. There were no significant differences between K_b values for suramin calculated against α,β-MeATP and β,γ-MeATP. Also, there were no significant differences between K_b values for suramin against α,β-MeATP calculated by either Schild plot analysis or by a single-shift of the concentration-response curves.

Discussion

The data show that the mechanical responses of cat colon circular muscle to noradrenaline and ATP involve postjunctional α_1-adrenoceptors, β-adrenoceptors, P_{2X}-purinoceptors and P_{2Y}-purinoceptors. Postjunctional adrenoceptors and purinoceptors in cat colon circular muscle were characterized by agonist potency and the effects of antagonists.

Effects of noradrenaline at different tone levels

The responses of colon circular smooth muscle to noradrenaline were dependent on tone. At resting tension, noradrenaline caused contractions that were potentiated by propranolol, a selective β-adrenoceptor antagonist and blocked by prazosin, a selective α_1-adrenoceptor antagonist whereas with raised tone, noradrenaline caused relaxation blocked by propranolol. The α- and β-adrenoceptors were considered postjunctional, because contractions or relaxations evoked by noradrenaline occurred in the presence of tetrodotoxin. The

Table 2 Effects of purinoceptor agonists on cat colon circular smooth muscle

	Maximal contraction (% of carbachol)	EC_{50} (μM)	*Slope*	r
α,β-MeATP ($n = 9$)	94.3 ± 8.0	0.7 ± 0.1*	39.5 ± 0.9*	0.91–0.99
β,γ-MeATP ($n = 9$)	98.5 ± 9.5	7.6 ± 0.1*	46.2 ± 8.4	0.93–0.99
2-MeSATP ($n = 6$)	65.3 ± 10.8	25.3 ± 12.8*	50.4 ± 2.4	0.94–0.99
ATP ($n = 14$)	70.8 ± 8.6	60.5 ± 15.9*	48.6 ± 3.9	0.93–0.99

Values are means ± s.e., n = number of preparations. r = correlation coefficient of regression lines.
*Significantly different from all others at $P < 0.05$.

inhibitory β-adrenoceptors present on cat colon circular muscle are the β_2-subtype (Ek & Lundgren, 1982; Ek *et al.*, 1987). In rat and human isolated colon smooth muscle strips, β-adrenoceptor blocking agents inhibit relaxations to adrenaline and unmask contractile responses (Gagnon & Belisle, 1970; Gagnon *et al.*, 1972).

Characteristics of postjunctional α_1-adrenoceptors

At resting tension, postjunctional α_1-adrenoceptors that mediate contractions were characterized in cat colon circular muscle by the potency of adrenoceptor agonists and the antagonist effects of prazosin. There were no significant differences among the EC_{50} values for the contractile effects of noradrenaline, adrenaline and the selective α_1-adrenoceptor agonist phenylephrine. In guinea-pig terminal ileum (Bauer, 1981), the EC_{50} values, calculated for the contractile effects of noradrenaline (7.32 μM; 5.24–10.47 μM), adrenaline (2.56 μM; 1.91–3.40 μM) and phenylephrine (5.23 μM; 3.02–9.13 μM), were similar to the EC_{50} values we obtained in the present study. Also, there were no differences between the slopes of the regression lines for noradrenaline and phenylephrine, while the slope of the regression line for adrenaline was significantly different. At low concentrations, adrenaline evoked contractile responses which are greater than those evoked by either phenylephrine or noradrenaline. This higher sensitivity to low concentrations of adrenaline may represent its interaction with adrenoceptors that do not have an innervation (extrajunctional location) and are activated by circulating catecholamines (Illes, 1983).

Prazosin antagonized the contractile effects of adrenoceptor agonists. The Schild analysis of the rightward shifts of the concentration-effect curves to noradrenaline yielded a slope that was not different from unity and a pA_2 value of 6.93. This pA_2 value is similar to that obtained against noradrenaline in human saphenous vein (pA_2 value = 6.45, Connaughton & Docherty, 1989). Also, the K_b value, against phenylephrine, was significantly lower than the K_b values against noradrenaline and adrenaline. This suggests that noradrenaline and adrenaline activate adrenoceptors less sensitive to prazosin. In rat tail artery, two subtypes of smooth muscle α_1-adrenoceptors have been proposed. One type was neurally innervated and exhibited a high affinity (low K_b value) for prazosin while the other was not neurally innervated (extrajunctional location) and exhibited a lower affinity (high K_b value) for prazosin (Medgett & Langer, 1984).

Effects of ATP and analogues of ATP at different tone levels

At resting tension, all purinoceptor agonists caused only contractions of circular smooth muscle, mediated by suramin-sensitive P_{2X}-purinoceptors. At raised tone ATP and 2-MeSATP caused only relaxations whereas α,β-MeATP and β,γ-MeATP, specific P_{2X}-purinoceptor agonists (Kennedy, 1990), caused only contractions. Both the contractions and relaxations to exogenous ATP are due to activation of postjunctional purinoceptors, since they were resistant to tetrodotoxin.

Characteristics of postjunctional P_{2X}-purinoceptors

The data show that cat colon circular muscle has P_{2X}-purinoceptors which mediate contractions. At resting tension, these receptors were characterized by the potency of purinoceptor agonists and the antagonist effects of suramin. The potency order was: α,β-methylene ATP > β,γ-methylene ATP > 2-methylthio ATP > ATP. This suggests the presence of P_{2X}-purinoceptors. A similar potency order of purinoceptor agonists to contract smooth muscle via P_{2X}-purinoceptors occurs in urinary bladder (Hoyle *et al.*, 1990) and in a number of blood vessels (see Burnstock, 1991). However, in the present study, receptor classification based solely on agonist potency may be limiting. For example, the differences in potency as well as the maximal responses could occur because α,β-MeATP and β,γ-MeATP caused only contractions at resting tension and at raised tone whereas 2-MeSATP and ATP causes contractions and relaxation at resting tension and raised tone, respectively. Also differences in potency we report could reflect different rates of the metabolism of ATP and its analogues. Similar data were reported in the rat mesenteric artery (Burnstock & Warland, 1987).

Suramin antagonized the contractile responses to all the purinoceptor agonists. The Schild analysis of the rightward shifts of the concentration-effect curves to α,β-MeATP indicated that suramin is a competitive antagonist of α,β-MeATP-evoked contractile responses in colon circular muscle. Suramin is also a competitive antagonist of contractions to α,β-methylene ATP and ATP in mouse vas deferens (von Kugelgen *et al.*, 1990) and in rabbit ear artery (Leff *et al.*, 1990).

The P_2-purinoceptor agonists showed different sensitivites to suramin. ATP had a lower sensitivity (high K_b value) to suramin than did the analogues of ATP, suggesting that ATP activates a suramin-insensitive component of the P_{2X}-purinoceptor (von Kugelgen *et al.*, 1990) and/or another nucleotide receptor (O'Conner *et al.*, 1991). The higher sensitivity of 2-MeSATP evoked contractions to suramin may reflect a predominant effect of 2-MeSATP on the P_{2Y}-purinoceptor. P_2-purinoceptors mediating biphasic muscle effects that depend on smooth muscle tone occur in rat gastric fundus (Lefebvre & Burnstock, 1990). In contrast, in cat colon, ATP and 2-MeSATP evoked either contraction at resting tension or relaxation at high tone.

Postjunctional P_{2Y}- and P_1-purinoceptors

The relaxation responses to ATP were mediated by reactive blue-sensitive P_{2Y}-purinoceptors since they were resistant to suramin and reduced approximately 80–90 percent by reactive blue 2 (100 μM), a selective inhibitor of relaxation responses mediated by P_{2Y}-purinoceptors. Also, we surmise that a small component of the ATP-evoked relaxation is due to activation of P_1-purinoceptors by the hydrolytic breakdown of ATP to adenosine because it was inhibited by the addition of 8-SPT. A similar antagonism of ATP-evoked inhibitory responses by reactive blue 2 (100 μM) occurs in rat gastric fundus (Lefebvre & Burnstock, 1991), duodenum (Manzini *et al.*, 1985) and caecum (Manzini *et al.*, 1986) and in rabbit mesenteric and ear arteries (Kennedy & Burnstock, 1985; Burnstock & Warland, 1987). Furthermore, the antagonism of ATP-evoked relaxations by reactive blue 2 and 8-SPT in the cat colon appears specific, because neither reactive blue 2 nor 8-SPT altered contractile responses to α,β-MeATP at resting tension, whereas the contractile responses to ATP were significantly potentiated by reactive blue 2 and slightly increased by 8-SPT. These results suggest that the contractile responses to α,β-MeATP do not involve the P_{2Y}-purinoceptor whereas ATP causes contractile and relaxant responses via P_{2X}- and P_{2Y}-purinoceptors, respectively. In mouse urinary bladder (Boland *et al.*, 1993) and guinea-pig coronary vessels (Hopwood & Burnstock, 1987), ATP causes contractile and relaxant responses mediated by P_{2X}- and P_{2Y}-purinoceptors, respectively.

In summary, we have characterized postjunctional α_1-adrenoceptors and P_{2X}-purinoceptors that mediate colonic contractions. In addition, postjunctional β-adrenoceptors and P_{2Y}-purinoceptors mediate muscle relaxation. It is likely that all the post-junctional receptors are activated by the co-release of ATP and noradrenaline from lumbar sympathetic nerves (Venkova & Krier, 1993). We speculate that the lumbar sympathetic nerves regulate colonic tone and phasic contractions *in vivo* by activating both postjunctional receptors

and the prejunctional receptors (Vizi *et al.*, 1991) which modulate cholinergic and noradrenergic, noncholinergic neurotransmission.

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Characterization of endothelin (ET) receptors in the isolated gall bladder of the guinea-pig: evidence for an additional ET receptor subtype

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1 We have characterized the receptors mediating contractions induced by endothelin-1 (ET-1), ET-2, ET-3 and the ET_B-selective receptor agonists, sarafotoxin 6c (SX6c), IRL 1620, BQ-3020, [$Ala^{1,3,11,15}$]ET-1 and ET (16-21) in strips of the isolated gall bladder of the guinea-pig (GPGB). We used as antagonists BQ-123 (ET_A receptor selective) and PD 145065 (ET_A/ET_B receptor non-selective).

2 ET-1, ET-2 and ET-3 (10^{-10} M to 3×10^{-7} M) caused similar slowly-developing concentration-dependent contractions of the GPGB. Contractile effects induced by ET-1, ET-2 or ET-3 (at 3×10^{-7} M) were also similar (230 ± 25, 241 ± 7 and $287 \pm 37\%$ of that to histamine at 5×10^{-6} M, $n = 7$, 6, 12, respectively). However, the threshold concentration for ET-1 or ET-2 was 10^{-10} M whereas it was 3×10^{-9} M for ET-3.

3 SX6c (10^{-10} M to 3×10^{-7} M) also caused slowly-developing concentration-dependent contractions at a threshold concentration of 10^{-10} M ($n = 16$). However, the contraction caused by SX6c at 3×10^{-7} M was $116 \pm 9\%$ of that to histamine at 5×10^{-6} M, which was half of that induced by the same concentration of the ET isopeptides. The contraction induced by IRL 1620 at 3×10^{-7} M ($n = 9$) was $43 \pm 9\%$ of that to histamine at 5×10^{-6} M, which was one fifth of that produced by the same concentration of ET-1. Contractions induced by BQ-3020 or [$Ala^{1,3,11,15}$]ET-1 at 3×10^{-7} M were even less than those produced by IRL 1620. ET (16-21) was inactive up to 10^{-5} M. Addition of a concentration of 3×10^{-7} M of ET-1 to tissues with developed contractions induced by the bolus addition of 3×10^{-7} M SX6c caused a further contraction of the GPGB to the level observed with ET-1 alone at 3×10^{-7} M ($n = 8$).

4 BQ-123 (10^{-5} M) did not affect the concentration-response curve to ET-1 and the contraction induced by 3×10^{-7} M was also not affected ($n = 5$; $239 \pm 19\%$ of histamine at 5×10^{-6} M). PD 145065 (10^{-5} M) shifted the ET-1 concentration-response curve to the right and the contraction induced by ET-1 at 3×10^{-7} M was inhibited by 15% ($n = 6$; NS). A higher concentration of BQ-123 (10^{-4} M) caused a significant shift to the right of the ET-1 concentration-response curve similar to that caused by PD 145065 (10^{-5} M) and caused a 24% ($n = 6$; NS) inhibition of the contractions induced by ET-1 at 3×10^{-7} M. PD 145065 (10^{-4} M) abolished contractions induced by ET-1 (up to 10^{-7} M) and inhibited the response to ET-1 at 3×10^{-7} M by 52% ($n = 4$; $P < 0.05$).

5 Contractions induced by ET-3 were more sensitive to inhibition by the antagonists. BQ-123 (10^{-6}, 10^{-5} or 10^{-4} M) inhibited responses to 3×10^{-7} M ET-3 by 66, 71 and 83%, respectively ($n = 5$, 5, 3; $P < 0.05$). PD 145065 (10^{-6}, 10^{-5} or 10^{-4} M) attenuated more strongly than did BQ-123 the contractions induced by ET-3. For instance, the contractions caused by ET-3 at 3×10^{-7} M were decreased by 73 and 80% ($n = 5$, 5; $P < 0.05$) in the presence of PD 145065 (10^{-6} or 10^{-5} M, respectively). PD 145065 (10^{-4} M) completely abolished contractions to ET-3 ($n = 4$; up to 3×10^{-7} M).

6 Contractions induced by SX6c, especially those observed at concentrations lower than 10^{-8} M, were attenuated by BQ-123 (up to 10^{-4} M). PD 145065 (10^{-5} M) shifted to the right the concentration-response curve to SX6c and inhibited by 38% ($P < 0.05$) the contractions induced by 3×10^{-7} M. However, the contractions induced by a bolus addition of a high concentration of SX6c (3×10^{-7} M) and the subsequent addition of an identical concentration of ET-1 on top of SX6c were not affected by BQ-123 (10^{-6} or 10^{-5} M).

7 These results suggest that ET_B receptors are involved in the contractions induced by endothelins in the GPGB. However, SX6c and other selective ET_B agonists produced only half or less than half of the contractile response induced by non-selective agonists. In addition, the responses to ET-1 but not to ET-3, were insensitive to the antagonist action of BQ-123 at 10^{-5} M whereas BQ-123 or PD 145065 at 10^{-5} M strongly antagonized contractions induced by ET-3. Finally, BQ-123 at 10^{-4} M inhibited contractions to ET-1 and SX6c. Thus, within the GPGB there may well be additional ET receptor(s) not conforming to the established ET_A/ET_B receptor subtype classification, as well as ET_B receptors.

Keywords: Endothelin; sarafotoxin; receptors; antagonists; gall bladder; guinea-pig

Introduction

Endothelin-1 (ET-1) is a 21 amino acid peptide, first isolated from the culture medium of aortic endothelial cells (Yanagisawa *et al.*, 1988). ET-1 and the related peptides ET-2, ET-3 and the sarafotoxins (SX6a, SX6b, SX6c, SX6d) cause vasoconstriction, vasodilatation, mitogenesis and various renal, cardiac and endocrine effects (see Huggins *et al.*, 1993).

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ET/SX peptides are also potent contractors of non-vascular smooth muscle preparations such as airways (Battistini *et al.*, 1992; Hay *et al.*, 1993), the urinary bladder (Maggi *et al.*, 1989b; Saenz de Tejada *et al.*, 1992; Traish *et al.*, 1992), the uterus (Bousso-Mittler *et al.*, 1989; Svane *et al.*, 1993; Rae *et al.*, 1993), the stomach fundus (Wood *et al.*, 1992; Warner 1993a), the ileum (Maggi *et al.*, 1989a; Wollberg *et al.*, 1991; Guimaraes & Rae, 1992; Miasiro *et al.*, 1993; Warner *et al.*, 1993b), the colon (Lin & Lee, 1990; Takahashi *et al.*, 1990) and the gall bladder (Maggi *et al.*, 1990; Moummi *et al.*, 1992). To date, two distinct ET receptors (ET_A and ET_B; Sakurai *et al.*, 1992) have been cloned from mammalian species. A third ET receptor, ET_C, has been cloned from dermal melanophores of *Xenopus laevis* (Karne *et al.*, 1993). The ET_A receptor displays greater affinity for ET-1 than ET-3 whereas the ET_B receptor has equal affinities to the two peptides. The ET_C receptor displays greater affinity for ET-3 than ET-1. In non-vascular tissues, either ET_A or ET_B receptors may mediate contractions to the endothelins. For instance, ET_A receptors mediate contractions of the rat uterus (Rae *et al.*, 1993) and the human urinary bladder (Maggi *et al.*, 1990; Sakurai *et al.*, 1992) while ET_B receptors mediate contractions of the guinea-pig bronchus (Battistini *et al.*, 1994b) and the rat stomach fundus (Warner *et al.*, 1993a). Here, using ET-1, ET-2, ET-3 and the ET_B selective agonists sarafotoxin 6c (Williams *et al.*, 1991), IRL 1620 (Takai *et al.*, 1992; Watakabe *et al.*, 1992), BQ-3020 (Ihara *et al.*, 1992c), [$Ala^{1,3,11,15}$]-ET-1 (Saeki *et al.*, 1991) and ET (16-21) (Maggi *et al.*, 1989a) together with the antagonists, BQ-123 (ET_A receptor selective; Ihara *et al.*, 1992a,b) and PD 145065 (ET_A/ET_B receptor non-selective; Cody *et al.*, 1993), we have attempted to characterize the receptors mediating the contractions of the endothelins in the isolated gall bladder of the guinea-pig.

Methods

Experimental protocols

Male Hartley guinea-pigs (300–400 g) were killed by cervical dislocation. The gall bladder was rapidly excised, placed in warmed (37°C) and oxygenated (95% O_2:5% CO_2) Krebs-Henseleit buffer solution (composition, mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.18, $NaHCO_3$ 25, $MgSO_4.7H_2O$ 1.18 and glucose 5.6, pH 7.4) and trimmed free of connective tissue and fat. Each gall bladder was opened along its longitudinal axis and two muscle strips prepared by cutting parallel to the long axis. The strips were mounted in 10 ml organ baths under tensions of 1 g. They were connected to Grass FT.03 force-displacement isometric transducers coupled to a Grass 79D pen recorder (Grass Instrument Co., Quincy, MA, U.S.A.). After equilibration for 60 min, tissues were contracted three times with 5×10^{-6} M histamine, with a 30 min washout period between each contraction. Tissues were then incubated with or without BQ-123 (10^{-5} or 10^{-4} M), PD 145065 (10^{-6}, 10^{-5} or 10^{-4} M) or vehicle for 20 min before addition of cumulative concentrations of the various agonists. The effects of each concentration of agonist were allowed to reach a plateau before addition of the next concentration. Only one cumulative concentration-response curve with or without antagonist was constructed with each preparation. Responses in the presence of antagonists and their respective vehicle controls were not compared in paired tissues. In a second set of experiments, one bolus addition of either ET-1, SX6c or SX6c followed by ET-1 at 3×10^{-7} M, with or without BQ-123 (10^{-6} or 10^{-5} M) given 20 min before, was given to equilibrated preparations. The responses in both sets of experiments are expressed as the percentage of the mean contraction evoked by histamine at 5×10^{-6} M.

Agonists and antagonists

Endothelin-1 (ET-1), ET (16-21) and IRL 1620 (Suc-[Glu^9, $Ala^{11,15}$]-ET-1 (8-21)) were synthesized at the INRS-Santé (Montréal, P.Q., Canada). Endothelin-3 (ET-3) and sarafotoxin 6c (SX6c) were obtained from Peptide Institute Inc. (Scientific Marketing Associates, Barnet, Herts.). BQ-3020 (as a non-acetylated analogue of [$Ala^{11,15}$]ET-1 (6-21)) and [$Ala^{1,3,11,15}$]ET-1 were obtained from Peninsula Laboratories Europe Ltd. (St. Helens, Merseyside). BQ-123 (cyclo[-D-Trp-D-Asp-L-Pro-D-Val-L-Leu-]), PD 145065 (Ac-D-Bhg-L-Leu-L-Asp-L-Ile-L-Ile-L-Trp; Bhg = 5H-dibenzyl[a,d]cycloheptene-10, 11-dihydroglycine) and additional acetylated ET (16-21) were obtained as sodium salts from Parke-Davis Pharmaceuticals (Ann Arbor, MI, U.S.A.). All agonists and antagonists were dissolved in distilled water and stored at −20°C in aliquots of 10^{-3} to 5×10^{-5} M. Histamine hydrochloride was obtained from Sigma Chemical Co. (Poole, Dorset).

Statistical analysis

All values are expressed as mean ± s.e.mean. Statistical analysis of the data was performed by an unpaired, two-tailed, Mann-Whitney U test and $P<0.05$ was considered significant.

Results

Effect of ET/SX peptides and other related analogues on the GPGB

ET-1, ET-2 and ET-3 caused slowly developing and long-lasting concentration-dependent contractions of the GPGB (Figure 1, 2). However, the threshold for ET-1 (10^{-10} M) was lower than that for ET-3 (3×10^{-9} M) (Figures 1, 2). SX6c or IRL 1620 also induced concentration-dependent contractions but caused only 50% and 19%, respectively, of the contractile responses induced by ET-1 at 3×10^{-7} M (Figures 1, 2).

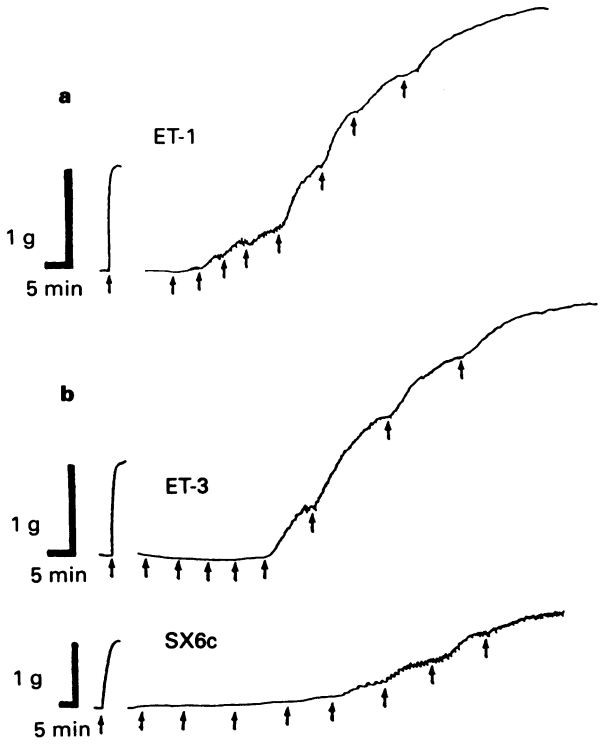


Figure 1 Representative tracings of contractions of strips of the isolated gall bladder of the guinea-pig induced by endothelin-1 (ET-1, a), ET-3 (b) and sarafotoxin 6c (SX6c, c). The first arrow indicates the additions of histamine (5×10^{-6} M). The second arrow indicates addition of the first concentration (1×10^{-10} M) of the peptide followed by cumulative concentrations of 0.3, 1, 3, 10, 30, 100 and 300×10^{-9} M.

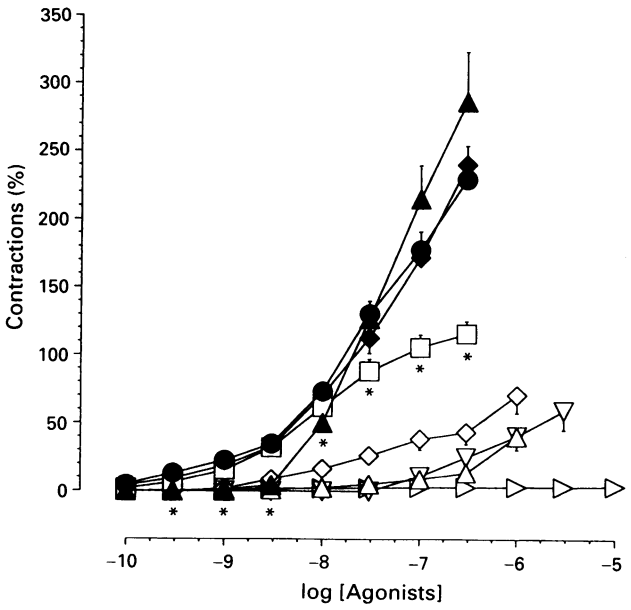


Figure 2 Contraction of strips of the isolated gall bladder of the guinea-pig induced by endothelin-1 (ET-1) (●), ET-2 (◆), ET-3 (▲), sarafotoxin 6c (SX6c) (□), IRL 1620 (◇), BQ-3020 (△), [Ala^{1,3,11,15}]ET-1 (▽) or ET (16-21) (▷); $n = 5-16$. Results are expressed as the percentage of the response to histamine (5×10^{-6} M), and are means with s.e.mean. $^{*}P<0.05$ between ET-1 and other agonists as compared by an unpaired, two-tailed, Mann-Whitney U test.

BQ-3020 and [Ala^{1,3,11,15}]ET-1 were much less active than SX6c and caused very weak contractions ($n = 14 \pm 3$ and $25 \pm 8\%$ of histamine at 5×10^{-6} M; Figure 2). ET (16-21) was without effect up to 10^{-5} M (Figure 2). Bolus addition of ET-1, ET-2, ET-3 or SX6c at (3×10^{-7} M) caused similar contractions to those following cumulative addition of agonist (data not shown; Figure 3). The contractions produced by the ET isopeptides were stable and long lasting while the contraction induced by SX6c (3×10^{-7} M) was not sustained. Addition of ET-1 (3×10^{-7} M) to tissues already precontracted by a bolus addition of SX6c (3×10^{-7} M) caused further contractions to the level observed with ET-1 alone at 3×10^{-7} M (Figure 3).

Effect of BQ-123 or PD 145065 on ET-1-induced contractions of the GPGB

BQ-123 (10^{-5} M) had no effect on contractions induced by ET-1 (Figure 4a) whereas BQ-123 (10^{-4} M) shifted the concentration-response curve 6 fold to the right and inhibited the contractions at 3×10^{-7} M by 24% (NS; Figure 4a). PD 145065 (10^{-5} M) caused a shift to the right of the ET-1 concentration-response curve and inhibited by 15% (NS) the contraction induced by ET-1 (3×10^{-7} M) (Figure 4b). PD 145065 (10^{-4} M) abolished contractions induced by ET-1 at concentrations $\leqslant 10^{-7}$ M and inhibited by 52% the response induced by ET-1 (3×10^{-7} M) ($P<0.05$; Figure 4b). BQ-123 (10^{-6} M or 10^{-5} M) did not affect the contractions induced by a bolus addition of a high concentration of SX6c (3×10^{-7} M), nor the contractions caused by the subsequent addition of ET-1 (at 3×10^{-7} M; Figures 3, 5).

Effect of BQ-123 or PD 145065 on ET-3-induced contractions of the GPGB

BQ-123 (10^{-6} M, 10^{-5} M or 10^{-4} M) significantly inhibited contractions induced by ET-3 (Figure 6a). For instance, the

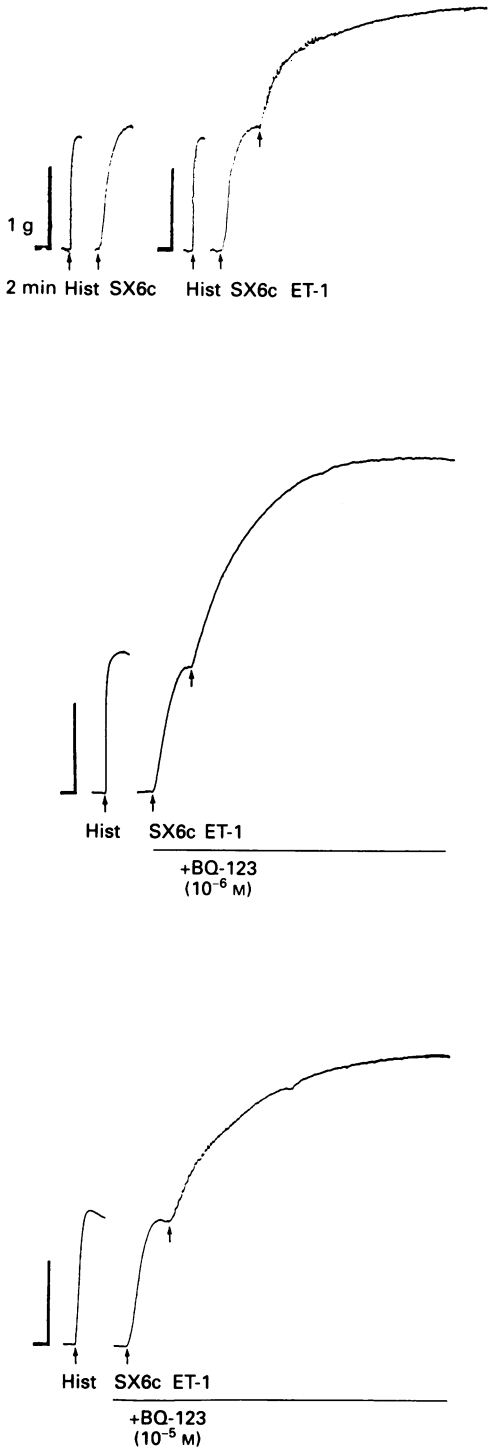


Figure 3 Representative tracings of contractions of strips of the isolated gall bladder of the guinea-pig induced by: a bolus addition of histamine (5×10^{-6} M); sarafotoxin 6c (SX6c) (3×10^{-7} M); ET-1 (3×10^{-7} M); SX6c + ET-1 (3×10^{-7} M); SX6c + ET-1 (3×10^{-7} M) in the presence of BQ-123 (10^{-6} M) and SX6c + ET-1 (3×10^{-7} M) in the presence of BQ-123 (10^{-5} M).

contractions induced by ET-3 (3×10^{-7} M) were reduced by 66, 71, and 83%, respectively ($P<0.05$). Similarly, PD 145065 (10^{-6} M or 10^{-5} M) inhibited by 73 and 80%, respectively ($P<0.05$), the contractions induced by ET-3 (3×10^{-7} M) (Figure 6b). PD 145065 (10^{-4} M) abolished contractions induced by ET-3 up to 3×10^{-7} M (Figure 6b).

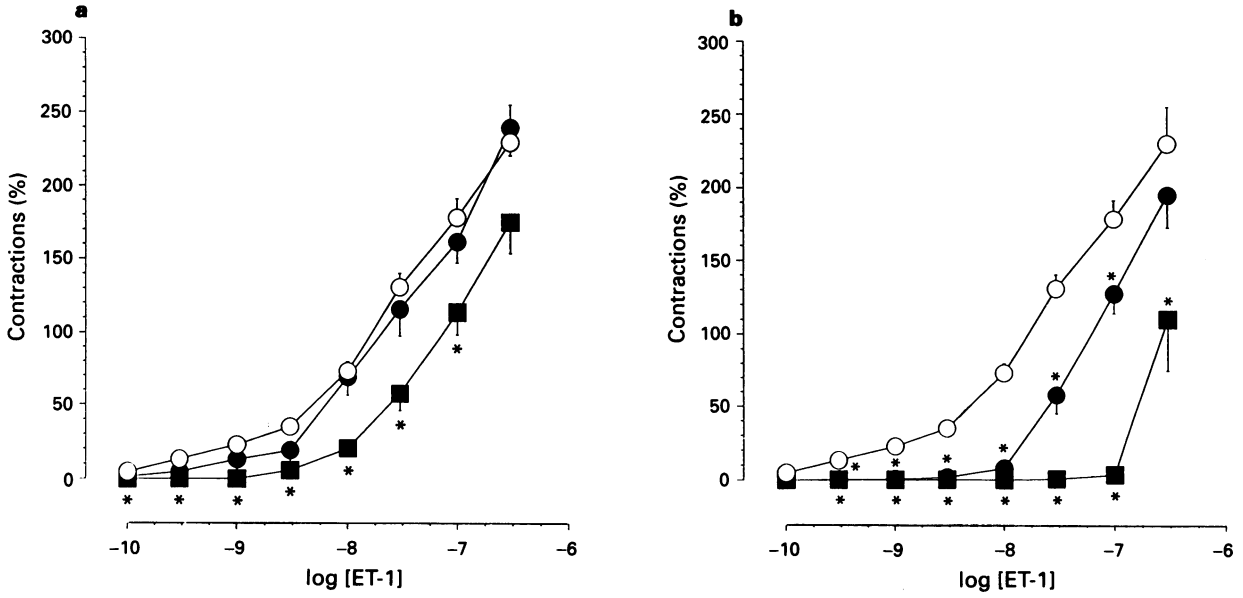


Figure 4 Effects of (a) BQ-123 (10^{-5} M, ●; 10^{-4} M, ■) or (b) PD 145065 (10^{-5} M, ●; 10^{-4} M, ■) on contractions induced by endothelin-1 (ET-1, ○) of strips of the isolated gall bladder of the guinea-pig ($n = 4-7$). Results are expressed as the percentage of the response to histamine (5×10^{-6} M), and are the means with s.e.mean. $^*P < 0.05$ as compared to ET-1 by an unpaired, two-tailed, Mann-Whitney U test.

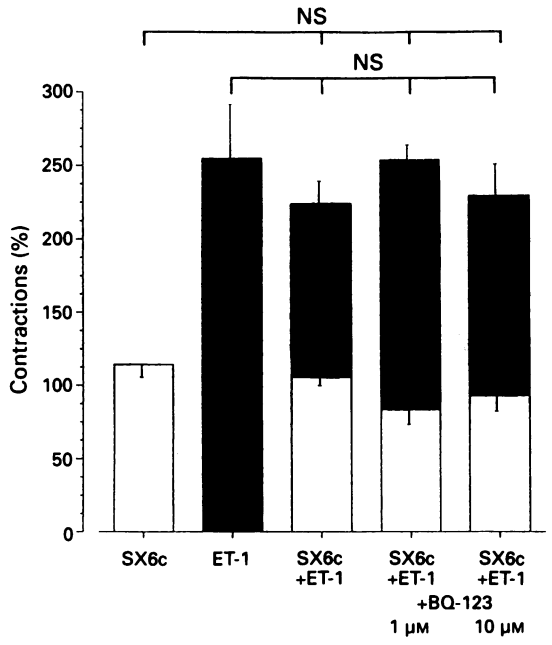


Figure 5 Contractions of strips of the isolated gall bladder of the guinea-pig induced by bolus concentrations of sarafotoxin 6c (SX6c, $n = 14$), endothelin-1 (ET-1, $n = 5$) or SX6c + ET-1 ($n = 7$) at 3×10^{-7} M and the effects of BQ-123 (10^{-6} M or 10^{-5} M) on contractions induced by SX6c + ET-1 ($n = 6$). Results are expressed as the percentage of the response to histamine (5×10^{-6} M), and are means with s.e.mean. NS, non-significant, as compared to ET-1 or SX6c by an unpaired, two-tailed Mann-Whitney U test.

Effect of BQ-123 or PD 145065 on SX6c-induced contractions of the GPGB

BQ-123 (10^{-5} M or 10^{-4} M) attenuated contractions induced by low concentrations ($< 10^{-8}$ M) of SX6c in the isolated GPGB without affecting the responses induced by SX6c at concentrations $> 3 \times 10^{-8}$ M (Figure 7). PD 145065 (10^{-5} M) concentrations $> 3 \times 10^{-8}$ M (Figure 7). PD 145065 (10^{-5} M) shifted the concentration-response curve to the right 11 fold and caused a 38% ($P < 0.05$) inhibition of the response to SX6c at 3×10^{-7} M (Figure 7).

The two antagonists used (up to 10^{-4} M) affected neither the basal tone of the GPGB, nor the contractions induced by histamine (5×10^{-6} M). The latter concentration of histamine induced an increase in tension of 1.58 ± 0.09 g ($n = 116$).

Discussion

The present study illustrates that all the members of the ET family of peptides potently contract the GPGB as has been shown for ET-1 (Moummi *et al.*, 1992). ET-1, ET-2 or ET-3 produced clearly similar effects, although financial restraints prevented the construction of complete concentration-response curves. It suggests that these contractile effects are mediated by an ET_B receptor subtype. This conclusion may be supported by the observation that PD 145065, a mixed ET_A/ET_B receptor antagonist, inhibited contractions induced by either ET-1, ET-3 or SX6c whereas 10 μM BQ-123 which strongly inhibits contractions to ET-1 in the rat thoracic aorta (Sumner *et al.*, 1992), was inactive against ET-1 in the GPGB. However, this conclusion is not fully supported by our other data. Firstly, SX6c and the other ET_B selective agonists did not cause contractile responses as great as those produced by the non-selective ET receptor agonists. Secondly, addition of ET-1 on top of the constriction induced by SX6c further contracted the tissue, a response which was unaffected by BQ-123 (up to 10^{-5} M). Thirdly, both ET receptor antagonists were more active against ET-3 than against ET-1. Fourthly, ET-1 and ET-2 produced contractions at lower concentrations ($\leqslant 10^{-8}$ M) than did ET-3. These observations suggest mediation by an additional non-ET_B non-ET_A receptor.

Interestingly, 10^{-5} M of either BQ-123 or PD 145065 was more effective in antagonizing contractions induced by ET-3 than by ET-1. Similar differences in the effectiveness of antagonists against various agonists have been reported previously in experiments on the human umbilical artery (Bodelsson & Stjernquist, 1993), the human saphenous vein (Bax *et al.*, 1993), the goat cerebral artery (Salom *et al.*,

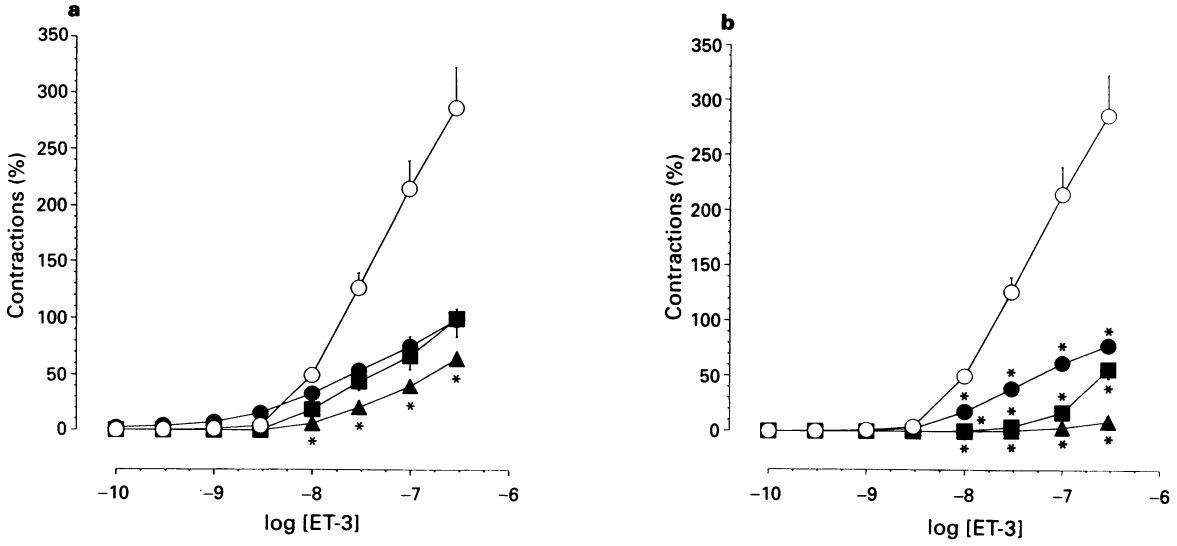


Figure 6 Effects of (a) BQ-123 (10^{-6} M, ●; 10^{-5} M, ■; or 10^{-4} M, ▲) or (b) PD 145065 (10^{-6} M, ●; 10^{-5} M, ■; or 10^{-4} M, ▲) on contractions induced by endothelin-3 (ET-3, ○) of strips of the isolated gall bladder of the guinea-pig ($n = 3-12$). Results are expressed as the percentage of the response to histamine (5×10^{-6} M), and are the means with s.e.mean. $^*P < 0.05$ as compared to ET-1 by an unpaired, two-tailed, Mann-Whitney U test.

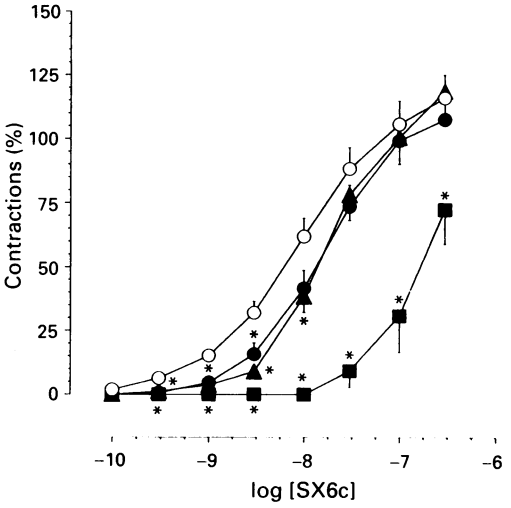


Figure 7 Effects of BQ-123 (10^{-5} M, ●; 10^{-4} M, ▲) or PD 145065 (10^{-5} M, ■) on contractions induced by sarafotoxin 6c (SX6c, ○; $n = 3-16$) of strips of the isolated gall bladder of the guinea-pig. Results are expressed as the percentage of the response to histamine (5×10^{-6} M), and are the means with s.e.mean. $^*P < 0.05$ as compared to ET-1 by an unpaired, two-tailed, Mann-Whitney U test.

1993), the rat vas deferens (Eglezos *et al.*, 1993) and the rat aorta (Sumner *et al.*, 1992). These data have been taken by some to suggest that different receptors are being activated by the agonists, although this may be incorrect. It may rather be that the effectiveness of e.g. BQ-123, as an antagonist, varies with different agonists at the same receptor, possibly because ET-3 does not bind to ET receptors in the same irreversible manner as ET-1 (Hirata *et al.*, 1988; Waggoner *et al.*, 1992; Marsault *et al.*, 1993). For example, we have recently reported that the effectiveness of two ET_A/ET_B non-selective antagonists, Ro 46-2005 and PD 145065, against contractions mediated by ET_B receptors in the guinea-pig upper bronchus vary with the agonist used (Battistini *et al.*, 1994c). In this tissue, both antagonists produced greater inhibition of contractions induced by IRL 1620 than they did of contractions induced by ET-1. This variability in antagonist potency is a very important consideration since many of the pA_2 values reported for antagonists of the ET_B receptor have relied upon other ET-related peptides as agonists (Clozel *et al.*, 1993). We also found that a high concentration of BQ-123 (10^{-4} M) reduced contractions induced by either ET-1 or SX6c. However, at this concentration, BQ-123 has been reported to inhibit the binding of ET-1 to ET_B receptors (Williams *et al.*, 1993) and may no longer be a selective ET_A receptor antagonist.

In conclusion, the data presented in this study suggests that ET-1 activates at least two ET receptor subtypes, an ET_B receptor and an additional, atypical, ET receptor subtype. Until recently, the classification of endothelin receptor subtypes was based on the relative potency of two non-selective ET receptor agonists: ET-1 and ET-3 (Masaki *et al.*, 1994). The present study, like several others (see Battistini *et al.*, 1994a), suggests the existence of additional types of ET receptors and also that the classification of endothelin receptors needs reconsideration. Functionally distinct receptors cannot readily be differentiated on the basis of the relative potencies of the ET-1 and ET-3 isopeptides alone but requires the use of additional ligands such as SX6c or IRL 1620 and antagonists of ET_A and/or ET_A/ET_B receptors. Finally, as ET-1 may be an endogenous regulator of the gall bladder tone and is secreted from the epithelial cells (Housset *et al.*, 1993), endothelins may regulate gall bladder motility via these receptor subtypes.

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Characterization of three non-peptide endothelin receptor ligands using human cloned ET_A and ET_B receptors

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1 A number of putative endothelin (ET) receptor ligands were synthesized with a view to assessing their relative affinity for human recombinant ET receptors.

2 Human (h) and endothelin ET_A and ET_B receptor open reading frames were cloned by reverse transcription-polymerase chain reaction into the mammalian expression vector pcDNA1 and stable cell lines were created by transfection of Chinese hamster ovary cells.

3 Scatchard analyses of saturation isotherms for the specific binding of [^{125}I]-endothelin-1 ([^{125}I]-ET-1) to membranes, prepared from Chinese hamster ovary cells transfected with hET_A or hET_B receptors, yielded values for equilibrium dissociation constants (K_d) of 20.5 ± 1.8 pM and 25.5 ± 5.5 pM, respectively. Hill coefficients did not differ significantly from unity, suggesting binding to homogeneous, non-interacting receptor populations.

4 Pharmacological characterization of the transfected hET_A and hET_B receptors was undertaken by measuring the relative abilities of ET_A and ET_B receptor-selective peptide ligands to inhibit binding of [^{125}I]-ET-1. For interaction with hET_A receptors, the relative order of potency was ET-1 > ET-3 = FR139317 = BQ123 > [$Ala^{1,3,11,15}$]-ET-1 = sarafotoxin S6c (S6c). In contrast, the relative order of potency, at hET_B receptors, was ET-1 = ET-3 = [$Ala^{1,3,11,15}$]-ET-1 = S6c >> FR139317 = BQ123.

5 The novel non-peptide ligands, Ro 46-2005, SB 209670 and BMS 182874, were found to inhibit [^{125}I]-ET-1 binding to human recombinant ET_A and ET_B receptors. At hET_A receptors, the calculated pIC_{50} values were 6.7 (Ro 46-2005), 8.7 (SB 209670) and 5.8 (BMS 182874), while at hET_B receptors, the corresponding pIC_{50} values were 6.8, 7.5 and < 5, respectively.

6 In conclusion, we have characterized the pharmacology of human cloned ET_A and ET_B receptors and used these in membrane binding assays to determine the affinity and selectivity of three structurally-diverse non-peptide ET receptor ligands. SB 209670 is, to date, the highest affinity non-peptide ligand to be described for ET receptors. As such, it may prove to be a valuable tool in further examination of the physiological and pathophysiological roles of endothelins.

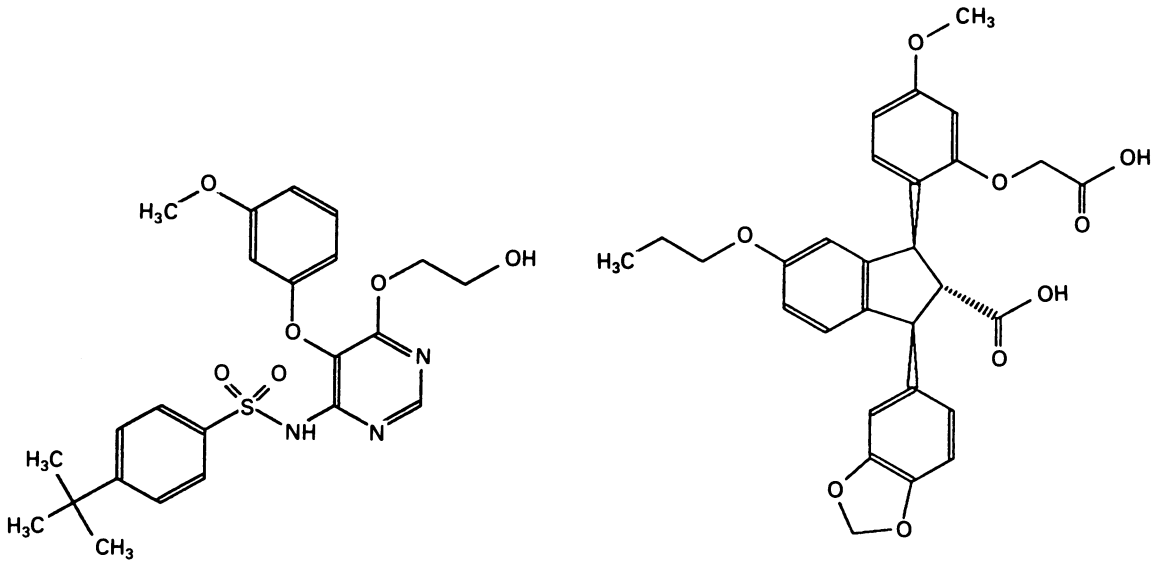
Keywords: BMS 182874; BQ123; endothelin; ET_A receptor; ET_B receptor; FR139317; human recombinant receptors; Ro 46-2005; SB 209670

Introduction

The endothelins (ET-1, ET-2 and ET-3) are 21-amino acid isopeptides which have multiple biological actions, including contraction of both vascular and non-vascular smooth muscle, positive inotropic effects in cardiac muscle, mitogenic actions in smooth muscle and induction of the release of endothelium-derived relaxing factor and prostacyclin from the vascular endothelium (reviewed by Yanagisawa & Masaki, 1989). The ETs are thought to act predominantly through two receptor subtypes, denoted ET_A and ET_B (IUPHAR classification). At ET_A receptors, ET-1 and ET-2 are some 30–100 fold more potent than ET-3. In contrast, ET-1, ET-2 and ET-3 are equipotent at ET_B receptors (reviewed by Sakurai *et al.*, 1992). The recent identification of novel peptide ligands selective for either ET_A (e.g. BQ123 [cyclo D-Asp-L-Pro-D-Val-L-Leu-L-Trp] and FR139317 ((**R**)2-[(**R**)-2-[S-2-[[1-hexahydro-1*H*-azepinyl)] carbonyl] amino-4-methyl pentanoyl] amino-3- [3-(1-methyl-1*H*-indolyl)] propionyl amino-3-(2-pyridyl) proprionic acid]; Ihara *et al.*, 1992; Cardell *et al.*, 1993) or ET_B receptors (e.g. [$Ala^{1,3,11,15}$]-ET-1 and sarafotoxin S6c; Saeki *et al.*, 1991; Williams *et al.*, 1991) has supported this classification.

Elevated plasma concentrations of immunoreactive endothelin have been measured in a number of pathophysiological conditions, including congestive heart failure, renal dysfunction, myocardial infarction, septic shock, Raynaud's phenomenon, pre-eclampsia and sub-arachnoid haemorrhage (Haynes & Webb, 1993). However, a cause and effect relationship for endothelin in disease has not been established and is likely to require the develoment of non-peptide endothelin receptor blocking drugs. In this respect, Clozel and co-workers (1993) have recently reported the pharmacology of Ro 46-2005 (4-tert-butyl-N-[6-(2-hydroxyethoxy)-5-(3-methoxphenoxy) pyrimidin-4-yl]-benzene sulphonamide; Figure 1), a non-peptide ET antagonist. Furthermore, a recent patent from SmithKline Beecham (Cousins *et al.*, 1993; WO/93/08799) has described a series of structurally-diverse, putative ET receptor ligands, an example of which is referred to as SB 209670 ((**1RS**, **2SR**, **3RS**)-3-[2-(1-carboxy-eth-2-yloxy)-4-methoxyphenyl]-1-(3, 4-methylenedioxyphenyl) -5-(prop-1-yloxy)-indane-2-carboxylic acid; Figure 1) in this manuscript. Similarly, BMS 182874 (5-(dimethylamino)-*N*-(3,4-dimethyl-5-isoxazolyl)-1-napthalenesulphonamide; Figure 1) was also described recently by Stein and coworkers (1994) and this ET receptor-blocking drug has been reported to lower blood pressure in hypertensive rats (Stein *et al.*, 1994). Characterization of the pharmacology of such agents, at human receptors, requires a specific, reproducible assay system. Unfortunately, obtaining a consistent source of human tissue is often difficult. However, the cloning of ET_A and ET_B receptors (Arai *et al.*, 1990; Sakurai *et al.*, 1990; Adachi *et al.*, 1991; Sakamoto *et al.*, 1991; Haendler *et al.*,

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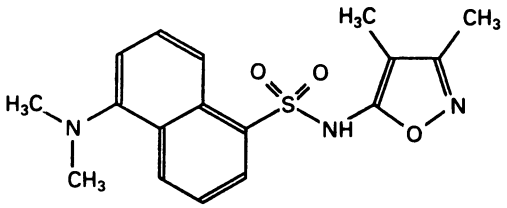


Figure 1 Structure of Ro 46-2005, SB 209670 and BMS 182874.

1992) and the expression of such receptors in cell lines may provide a useful assay system for characterization of such ligands. In this study, we describe the stable expression and pharmacological characterization of human ET_A and ET_B receptors in Chinese hamster ovary (CHO) cells. Furthermore, we have used these assay systems to characterize and compare the affinity and selectivity of three non-peptide ET receptor ligands. A preliminary account of these findings has been presented to the British Pharmacological Society (Buchan *et al.*, 1993a).

Methods

Culture of CHO cells

CHO cells were routinely cultured in MEM alpha medium, supplemented with 10% foetal calf serum, 2 mM L-glutamine, and penicillin (100 u ml^{-1})/streptomycin (100 $\mu g\ ml^{-1}$) at 37°C and in an atmosphere of 5% CO_2. The cells were passaged twice weekly by use of trypsin-EDTA.

Cloning and sequencing of cDNA for human endothelin receptors

Human ET_A and ET_B receptor open reading frames (1284 and 1329 base pairs, respectively) were cloned by reverse transcription-polymerase chain reaction (RT-PCR), using total RNA extracted from human lung (Clontech Laboratories Inc). Reverse transcription was performed according to the procedure described in the GeneAmp RNA PCR kit (Perkin Elmer Cetus). PCR amplifications contained the following: 10 μl Promega 10 × Taq buffer; 50 pmol each oligonucleotide primer; dATP, dCTP, dTTP, dGTP each at 50 μM; 2 μl of cDNA obtained above; 4.5 u Taq polymerase and distilled water to final volume of 100 μl. After an initial denaturing (95°C, 10 min) and a subsequent annealing step (50°C, 5–20 min), 30 cycles of PCR were performed (70°C for 2 min, 92°C for 1 min, 50°C for 1 min). A final polymerisation step was performed at 70°C for 5 min.

In order to facilitate cloning, the PCR primers for the hET_A and hET_B genes were designed to incorporate PstI and a XhoI restriction sites at the 5′ and 3′ ends of the gene, respectively. The sequences of the primers were as follows: hET_A (forward, ACGTCACTGCAGATGGAAACCCTTTGCCTCAGGGCA: reverse, CGTATCCTCGAGTCAGTTCATGCTGTCCTTATGGCT) and hET_B (forward, ACGTCACTGCAGATGCAGCCGCCTCCAAGTCTGTGC; reverse, CGTATCCTCGAGTCAAGATGAGCTGTATTTATTACTG. The PCR products were digested with PstI and XhoI, purified by agarose gel electrophoresis and cloned into the mammalian expression vector pcDNA1 (Invitrogen Inc). The DNA sequence of individual clones was verified by dideoxy DNA sequencing.

Expression of hET_A and hET_B receptors in CHO cells

Prior to transfection, CHO cells were seeded in 10 cm dishes at 1 × 10^5 cells per dish, and left to adhere overnight. DNA was then transfected into the cells by the calcium phosphate

precipitation method, involving the addition of 40 μg of the endothelin receptor expression plasmid and 10 μg of pRSV-neo (a constitutive neomycin-resistance gene expression vector) to 0.75 ml of 0.25 M $CaCl_2$. The DNA/$CaCl_2$ mixture was then added dropwise, with gentle agitation, to an equal volume of 2 × HEPES phosphate-buffered saline (PBS: NaCl 0.28 M, HEPES 50 mM, $Na_2HPO_4.2H_2O$ 2.25 mM, pH 7.12) to form a precipitate which was added to a 10 cm dish of CHO cells. The precipitates were left on the cells overnight and then washed off using PBS. The transfected cells were allowed to recover in standard growth medium for 48 h, before being selected for expression of neomycin resistance using G418, a neomycin analogue (1 mg ml^{-1}).

Colonies of G418-resistant cells were visible after 2–3 weeks of selection and lifted into wells of 96 well plates using cloning rings. Although selection of resistant cells was now complete, the cells were subsequently maintained at 0.25 mg ml^{-1} G418. The G418 resistant clones were expanded and analysed for endothelin receptor expression by binding assay (data not shown).

To overcome problems of unstable receptor expression with increasing passage number, the hET_A and hET_B transfected cells were subjected to limiting dilution cloning, i.e., cells were plated in 96 well plates at a theoretical density of 1 cell per well. After approximately 2 weeks, wells containing single colonies, and therefore clonally pure cells, were subcultured and assayed for receptor expression by binding assay.

Preparation of membranes from CHO cells

The medium was removed from each culture flask and the CHO cell confluent monolayer carefully rinsed with 2 × 25 ml calcium- and magnesium-free PBS, followed by the addition of 10 ml PBS (+ 5 mM EDTA). Following incubation for 5–10 min at 37°C, the cell suspension was then centrifuged (170 *g*, 5 min, 20°C) and the pellet obtained was resuspended in ice-cold lysis buffer (Tris HCl 15 mM, EDTA 0.3 mM, pH 7.4). The cell lysate preparation was homogenized, on ice, for 10 s. Following centrifugation (170 *g*, 15 min, 4°C), the supernatant was re-centrifuged (40000 *g*, 10 min, 4°C). Each pellet was then resuspended in assay buffer (Tris 50 mM, bacitracin 100 μg ml^{-1}, $CaCl_2$ 1 mM, BSA 0.1% w/v) and stored frozen until needed or, alternatively, resuspended in assay buffer to a protein concentration of 30–100 μg ml^{-1} and used immediately.

Binding studies

In competition-inhibition experiments, CHO cell membranes (30–100 μg protein ml^{-1}) were incubated for 3 h (time at which [^{125}I]-ET-1 binding to hET receptors approached equilibration) at 25°C in assay buffer containing Tris 50 mM, bacitracin 100 μg ml^{-1}, $CaCl_2$ 1 mM, BSA 0.1% w/v and a single concentration of [^{125}I]-ET-1 (15–25 pM). However, when undertaking saturation analysis of the binding of [^{125}I]-ET-1 to hET receptors, a range of concentrations of [^{125}I]-ET-1 was used (2–150 pM). At the end of the incubation period, the assay was terminated by washing with 3 × 3 ml ice-cold Krebs followed by rapid filtration in either a Tomtec or a Brandel cell harvester. The filter-bound radioactivity was then determined in either a gamma counter or a Wallac 1204 β-plate counter. In all experiments, specific binding of [^{125}I]-ET-1 was defined as that inhibited by unlabelled ET-1 (1 μM).

Materials

Endothelin-1, endothelin-3 and sarafotoxin S6c were obtained from the Peptide Institute, Japan. BQ123 and FR139317 were obtained from Neosystem Laboratories, France. [$Ala^{1,3,11,15}$]-ET-1 ([Ala_4]-ET-1), Ro 46-2005 (4-tert-butyl-*N*-[6-(2-hydroxyethoxy)-5- (3-methoxphenoxy) pyrimidin-4-yl]-benzene sulphonamide), SB 209670 ((1**RS**, 2**SR**, 3**RS**)-3-[2-(1-carboxyeth-2-yloxy)-4-methoxphenyl]-1-(3, 4-methylenedioxyphenyl)-5-(prop-1-yloxy)-indane-2-carboxylic acid), BMS182-874 (5-(dimethylamino)-*N*-(3,4-dimethyl-5-isoxazolyl)-1-naphthalenesulphonamide) and ET(16–21) were synthesized by Glaxo Research and Development Ltd. [^{125}I]-ET-1 (2200 Ci $mmol^{-1}$) was obtained from New England Nuclear, U.K. Bacitracin and bovine serum albumin (BSA) were obtained from Sigma, U.K. MEM alpha medium, trypsin-EDTA, and G418 were obtained from Life Technologies, U.K. Glutamine and penicillin/streptomycin were obtained from Northumbria Biologicals, U.K. Foetal calf serum was obtained from Tissue Culture Services, U.K. All other reagents were of analytical grade.

Stock solutions (10 mM) of BQ123 and FR139317 were prepared in distilled water. Stock solutions (1 and 10 mM) of ET-1, ET-3, ET(16–21), [Ala_4]-ET-1 and S6c were prepared in ammonium acetate (0.1 M; pH 9.0). Subsequent dilutions of all peptides were made in assay buffer. Stock solutions of Ro 46-2005, SB 209670 and BMS 182874 were prepared in dimethylsulphoxide as a stock solution of 10 mM and diluted in assay buffer.

Binding and statistical analyses

Specific binding data (c.p.m.) were analyzed using methods described by Weiland & Molinoff (1981) and using the computer programme, ALLFIT (DeLean *et al.*, 1978). The ALLFIT programme was used to estimate slope and pIC_{50} (negative log of the concentration of inhibitor giving 50% inhibition of specific [^{125}I]-ET-1 binding) values from competition-inhibition experiments. The pIC_{50}/IC_{50} values were not converted to inhibitor equilibrium dissociation constants (K_i) as this transformation is only valid for reversible, competitive binding (Cheng & Prusoff, 1973). This is not the case with [^{125}I]-ET-1 binding which has been found to dissociate very slowly (Hiley *et al.*, 1990; Waggoner *et al.*, 1993; this study, data not shown).

[^{125}I]-ET-1 saturation experiments were analysed by Scatchard and Hill methods (Weiland & Molinoff, 1981) to determine the radioligand equilibrium dissociation constant (K_d), the binding site density (B_{max}) and the Hill coefficient (n_H).

Analysis of variance was used to determine whether the slopes of inhibition curves differed from unity. A probability value of less than 0.05 was taken to represent a significant difference.

Results

Saturability of [^{125}I]-ET-1 binding sites

Membranes, prepared from CHO cells transfected with either hET_A or hET_B receptors, were incubated with a range of concentrations of [^{125}I]-ET-1 (2–150 pM) for 3 h at 25°C. Scatchard analysis of the saturation isotherms demonstrated that [^{125}I]-ET-1 bound to hET_A and hET_B receptors with K_d's of 20.5 ± 1.8 pM ($n = 3$) and 25.5 ± 5.5 pM ($n = 3$), respectively (Figure 2). The calculated B_{max} values for hET_A and hET_B receptors were 71 ± 5.5 fmol mg^{-1} protein and 170 ± 9.5 fmol mg^{-1} protein, respectively. Hill coefficients (n_H) for binding of [^{125}I]-ET-1 to hET_A (0.997 ± 0.003) or hET_B (0.963 ± 0.050) receptors were not significantly different from unity.

Characterization of human cloned ET receptors

The inhibition of [^{125}I]-ET-1 binding to membranes, prepared from CHO cells transfected with hET receptors, was examined. ET-1 produced a concentration-dependent inhibition of [^{125}I]-ET-1 binding to hET_A and hET_B receptors, with IC_{50} values of 0.15 nM and 0.06 nM, respectively (Table 1; Figure 3). ET-3 was 30 fold less potent than ET-1 at hET_A receptors, but equipotent with ET-1 at hET_B receptors (Table 1;

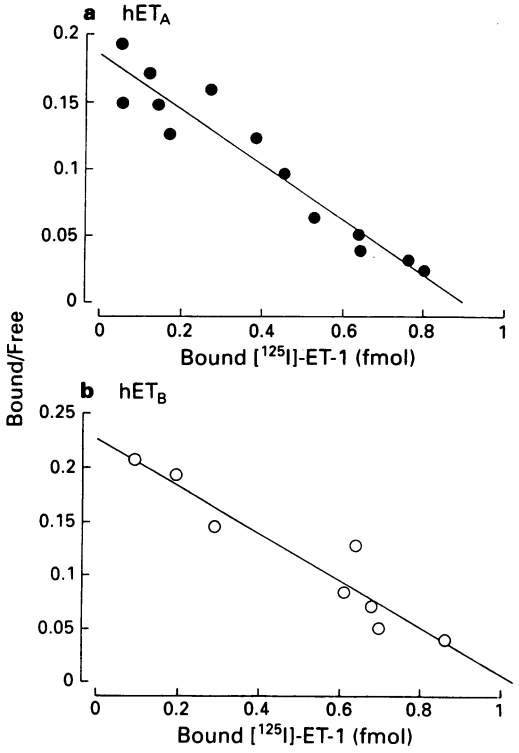


Figure 2 Data from single experiments in which membranes, prepared from CHO cells transfected with hET$_A$ (a) or hET$_B$ (b) receptors, were incubated with 2-150 pM [^{125}I]-endothelin-1 ([^{125}I]-ET-1) for 3 h. Scatchard plots of the data show the linear least squares regression lines. Each point is the mean of triplicate determinations and each experiment was repeated on three separate occasions.

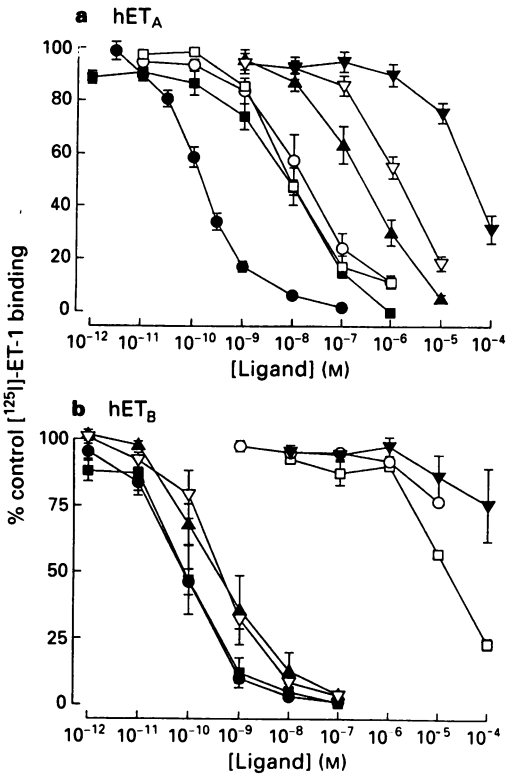


Figure 3 Inhibition of the specific binding of [^{125}I]-endothelin-1 ([^{125}I]-ET-1) to membranes, prepared from CHO cells transfected with either human ET$_A$ (a) or human ET$_B$ (b) receptors, by ET-1 (●), ET-3 (■), BQ123 (○), FR139317 (□), [Ala$_4$]-ET-1 (▲), sarafotoxin S6c (▽) and ET(16–21) (▼). Data are expressed as the mean ± s.e.mean of 3–14 experiments. Where error bars are not shown, they are encompassed within the size of the symbol.

Figure 3). The ET$_A$ receptor selective-blocking drugs, BQ123 and FR139317 were at least 1000 fold more selective for hET$_A$ receptors than for hET$_B$ receptors (Table 1; Figure 3). In contrast, the ET$_B$ receptor-selective agonists, sarafotoxin S6c and [Ala$_4$]-ET-1, were 7000 and 2500 fold less potent than ET-1 at ET$_A$ receptors,but approximately equipotent with ET-1 at hET$_B$ receptors (Table 1; Figure 3). The C-terminal hexapeptide, ET(16–21), was found to have only weak affinity for both hET$_A$ and hET$_B$ receptors (Table 1; Figure 3).

The slopes of the inhibition curves did not differ from unity, with the exception of ET-3 and [Ala$_4$]-ET-1 at hET$_A$ receptors, and sarafotoxin S6c at hET$_B$ receptors (Table 1).

Characterization of Ro 46-2005, SB 209670 and BMS 182874

The structurally diverse non-peptides, Ro 46-2005, SB 209670 and BMS 182874 (Figure 1) produced concentration-dependent inhibition of [^{125}I]-ET-1 binding to hET$_A$ and hET$_B$ receptors (Figure 4). Ro 46-2005 had a similar affinity for each receptor subtype (Figure 4; Table 1). SB 209670 exhibited approximately 100 fold and 5 fold greater affinity than Ro 46-2005 for ET$_A$ and ET$_B$ receptors, respectively (Figure 4; Table 1). BMS 182874 was some 10 fold and 1000 fold less potent than Ro 46-2005 and SB 209670, respectively

Table 1 Inhibition of [^{125}I]-endothelin-1 ([^{125}I]-ET-1) binding to membranes prepared from CHO cells transfected with either the hET$_A$ or hET$_B$ receptor

	hET$_A$			*hET$_B$*		
Ligand	*pIC$_{50}$*	*Slope*	n	*pIC$_{50}$*	*Slope*	n
ET-1	9.8 ± 0.1	0.93 ± 0.05	15	10.2 ± 0.1	0.97 ± 0.06	12
ET-3	8.3 ± 0.2	0.71 ± 0.04*	3	10.2 ± 0.2	1.05 ± 0.09	5
[Ala$_4$]-ET-1	6.4 ± 0.2	0.62 ± 0.06*	4	9.6 ± 0.3	0.93 ± 0.08	6
S6c	5.9 ± 0.1	0.92 ± 0.08	4	9.5 ± 0.1	0.83 ± 0.06*	6
BQ 123	7.9 ± 0.2	0.88 ± 0.03	8	<5	–	4
FR 139317	8.2 ± 0.1	0.95 ± 0.04	5	4.7 ± 0.1	0.92 ± 0.20	3
ET (16–21)	4.4 ± 0.1	–	3	<4	–	5
Ro 46-2005	6.7 ± 0.1	1.03 ± 0.11	5	6.8 ± 0.1	0.94 ± 0.06	5
SB 209670	8.7 ± 0.3	1.02 ± 0.17	6	7.5 ± 0.1	0.98 ± 0.12	9
BMS 182874	5.8 ± 0.2	1.03 ± 0.24	6	<5	–	7

Values are arithmetic means ± s.e.mean from *n* experiments. *$P<0.05$ indicates a significant difference from unity.

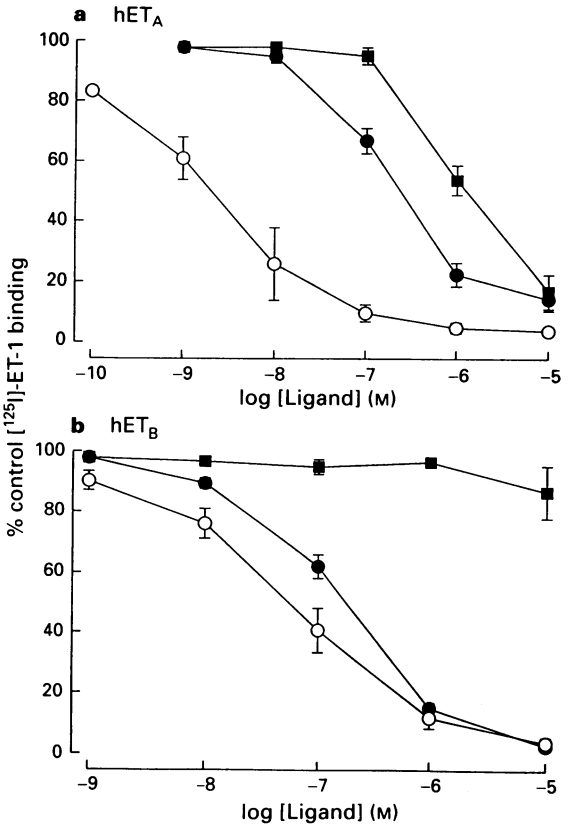


Figure 4 Inhibition of specific binding of [^{125}I]-endothelin-1 ([^{125}I]-ET-1) to membranes, prepared from CHO cells transfected with either human ET_A (a) or human ET_B (b) receptors, by Ro 46-2005 (●), SB 209670 (○) and BMS 182874 (■). Data are expressed as the mean ± s.e.mean of 5–9 experiments. Where error bars are not shown, they are encompassed within the size of the symbol.

for the hET_A receptor and had negligible affinity for the hET_B receptor (Figure 4; Table 1).

The slopes of the inhibition curves for Ro 46-2005, SB 209670 and BMS 182874 did not differ significantly from unity (Table 1).

Discussion

In this study, we have used a receptor binding assay to characterize human cloned ET_A and ET_B receptors, stably expressed in a cell line, and compared the profile of three non-peptide ET receptor ligands. The stable expression of human endothelin receptors, using limiting dilution cloning, also demonstrated previously for human ET_A receptors (Elshourbagy *et al.*, 1993) and for bovine ET_A and rat ET_B receptors (Aramori & Nakanishi, 1992), obviates the need for repeated transient transfections of cell lines in such endothelin receptor studies.

By using peptide ligands selective for ET_A and ET_B receptors, we were able to confirm, in competition experiments, that the two subtypes of human recombinant ET receptor corresponded to ET_A and ET_B receptors, respectively. Thus, ET-1 was 30 fold more potent than ET-3 at inhibiting binding of [^{125}I]-ET-1 to hET_A receptors, whilst ET-1 and ET-3 were equipotent at hET_B receptors. In addition, the ET_A receptor-selective antagonists, BQ123 and FR139317 (Ihara *et al.*, 1992; Cardell *et al.*, 1993), also inhibited binding of [^{125}I]-ET-1 to hET_A receptors, with very weak affinity for hET_B receptors. In contrast, the ET_B receptor-selective agonists, sarafotoxin S6c and [Ala_4]-ET-1 (Saeki *et al.*, 1991; Williams *et al.*, 1991), proved to be very weak inhibitors of [^{125}I]-ET-1 binding to ET_A receptors, but recognised hET_B receptors with high affinity. Surprisingly, certain competing ligands produced inhibition curves with slopes which were significantly less than unity. For example, this was observed with ET-3 and [Ala_4]-ET-1 in the hET_A assay and S6c in the hET_B assay. The reasons for this are unclear, but may reflect differences in receptor coupling which may result from the use of an agonist as a radioligand. Nevertheless, the overall inhibitory ligand profiles and potencies, observed in this study, using cloned hET_A and hET_B receptors, correlate closely with studies undertaken using the same ligands in non-recombinant ET_A and ET_B receptor systems (Hiley *et al.*, 1990; Williams *et al.*, 1991; Cioffi *et al.*, 1992; Ihara *et al.*, 1992; Panek *et al.*, 1992; Buchan *et al.*, 1993b), suggesting that the binding characteristics of these human recombinant ET receptors are virtually identical to those of ET receptors found in non-transfected systems.

The C-terminal hexapeptide of ET-1, ET(16–21), has been reported to be a full agonist, at sub-micromolar concentrations, in a number of ET_B receptor-containing isolated tissue preparations, while having little or no effect at ET_A receptors (Maggi *et al.*, 1989). In addition, similar agonist activity of ET(16–21) has been demonstrated in the rat perfused mesenteric bed (Douglas & Hiley, 1990). However, in the present study ET(16–21) displayed extremely weak affinity for human cloned ET_A and ET_B receptors. Our data are in accordance with the findings of Cody and co-workers (1991), who found that ET(16–21) had only weak binding affinity in both ET_A (rabbit aorta) and ET_B (rabbit pulmonary artery) receptor-containing systems and, furthermore, had no functional activity in these tissues. The reasons for the differences in the pharmacological activity of ET(16–21) are not apparent. However, a recent study undertaken by Warner and co-workers (1993) describes differences in the pharmacology of the ET_B receptors mediating contraction and relaxation of vascular smooth muscle. It is possible, therefore, that ET(16–21) may discriminate between ET_B receptor subtypes. However, to date, the isolation of different human genomic DNA fragments, encoding for receptor subtypes other than ET_A and ET_B, has proved elusive, even under conditions of low stringency (Sakamoto *et al.*, 1991). Hence, the possibility that differences in ET_B receptor pharmacology occur as a result of differential post-translational modification merits further investigation.

Although plasma levels of immunoreactive endothelin are raised in a number of disease states (Haynes & Webb, 1993), establishing a role for endothelin in these conditions is likely to require the use of non-peptide ET receptor blocking drugs. Using human cloned ET receptors, we have determined the affinity and selectivity of three putative non-peptide ET receptor antagonists. Specifically, we have compared the properties of Ro 46-2005 (Breu *et al.*, 1993; Clozel *et al.*, 1993) with those of BMS 182874 (Stein *et al.*, 1994) and SB 209670, a putative ET receptor-blocking drug described in a recent patent from SmithKline Beecham (WO/93/08799). In this study, the pyrimidinyl sulphonamide, Ro 46-2005, inhibited binding of [^{125}I]-ET-1 to human recombinant ET receptors with a potency comparable to that described previously (Breu *et al.*, 1993). SB 209670, a putative ET receptor ligand, was almost 100 fold more potent than Ro 46-2005 at inhibiting binding of [^{125}I]-ET-1 to human recombinant ET_A receptors, but possessed only 5 fold greater affinity than Ro 46-2005 for hET_B receptors. Thus, SB 209670 is the most potent non-peptide ET ligand, yet described, and appears to display a degree of selectivity for the ET_A receptor. In preliminary functional studies (unpublished observations), we have found SB 209670 to behave as a potent, competitive antagonist in both ET_A (rat aorta; Sumner *et al.*, 1992) and ET_B (rabbit pulmonary artery; White *et al.*, 1993) receptor-containing preparations.

For comparison, we also examined the affinity of BMS 182874 for hET receptors. This benzene sulphonamide has been reported to be an ET_A receptor-selective antagonist

(Stein et al., 1994). Using human cloned ET receptors, we were able to confirm the ET_A selectivity of this ligand. However, in this study using hET_A receptors, BMS 182874 was approximately 10 fold less potent than described previously (Stein et al., 1994). Rat A10 vascular smooth muscle cells were used in the latter study (Stein et al., 1994) and, as differences in the pharmacology of rat and human ET receptors have been proposed (Elshourbagy et al., 1993), this may account for the difference in potency estimates obtained. Nonetheless, it is clear that BMS 182874 is selective for ET_A receptors and, thus, may serve as a useful pharmacological tool to examine the role of this receptor subtype.

In conclusion, using radioligand binding experiments we have pharmacologically characterized human ET_A and ET_B receptors stably expressed in CHO cells. In this respect, we have determined the affinity and selectivity of three structurally-diverse, non-peptide ET ligands. Of these, SB 209670 is, to our knowledge, the highest affinity non-peptide ligand to be described for human ET receptors. Endothelin receptor-blocking drugs of this type should prove invaluable in the determination of the physiological and pathophysiological role(s) of endothelins.

Note added in proof

Following submission of this manuscript, Wu and coworkers (1994) (*Faseb J.*, **8**, 592) have described results regarding SB 209670, which are in accordance with the findings of our study.

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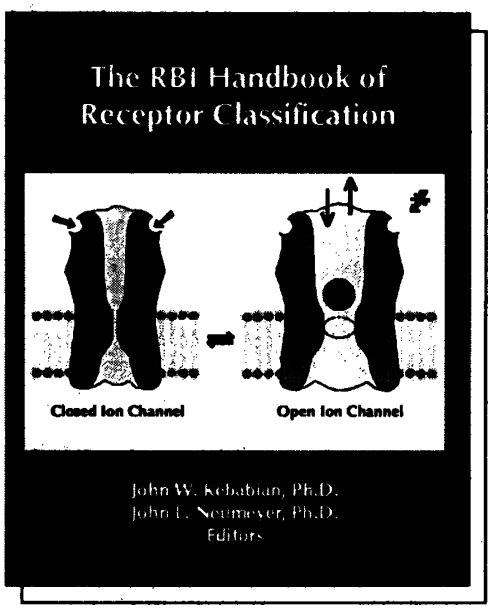
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