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Potent contractile activity of endothelin on the human isolated urinary bladder

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Endothelin (1 nM–0.3 μM) produced a concentration-related contraction of mucosa-free muscle strips excised from the dome of the human urinary bladder. The response to endothelin was unaffected by either atropine (1 μM) or nifedipine (1 μM) at concentrations that abolished the response to carbachol and KCl, respectively. These findings indicate that mechanisms other than activation of dihydropyridine- and voltage-sensitive calcium channels may be involved in the action of endothelin on smooth muscles.

Introduction Endothelin is an endothelium-derived 21-residue peptide recently discovered by Yanagisawa *et al.* (1988). It possesses a very potent vasoconstrictor activity on several isolated blood vessels (Yanagisawa *et al.*, 1988; Tomobe *et al.*, 1988). In vascular smooth muscle, the contractile action of endothelin seems critically dependent upon influx of calcium ions from the extracellular space through dihydropyridine-sensitive calcium channels. On the basis of structural analogies with certain toxins which act directly on membrane ion channels, the hypothesis was advanced that endothelin acts as an endogenous agonist of the dihydropyridine-sensitive calcium channel (Yanagisawa *et al.*, 1988). To date, little information is available about the effects of endothelin on non-vascular smooth muscles. Uchida *et al.* (1988) reported recently that endothelin exerts a potent contractile effect on isolated bronchial tissue from both guinea-pigs and man. Even in this case, the response to endothelin was reported to be markedly sensitive to dihydropyridine calcium blockers. In the course of a more extensive study about the action of endothelin on various non-vascular smooth muscles, we noticed that the contractile responses to this peptide are, in certain instances, markedly or absolutely nifedipine-resistant. Here we describe the potent contractile effect of endothelin on muscle strips from the dome of the human urinary bladder and its modification by atropine and nifedipine.

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Methods Specimens from the dome of human urinary bladder were obtained from 8 (6 males and 2 females) patients (age range, 40–81 years) undergoing total cystectomy for carcinoma of the bladder base. The specimens were from patients showing no signs of urinary retention or detrusor instability. No patient received radio- or chemotherapy before intervention. Pre-anaesthetic medication, induction and maintenance of anaesthesia were as described previously (Maggi *et al.*, 1987; 1988). All specimens appeared macroscopically normal, with no signs of tumour or inflammation. Tissues were placed in ice-cold Krebs solution within 2–3 min of surgical removal. Each sample was freed from mucosa, 4 to 6 small strips of muscular tissue were excised and stored overnight in oxygenated (95% O_2 and 5% CO_2) Krebs solution ($CaCl_2$ 2.5 mM). The strips were mounted in 5 ml baths for isolated organs, maintained at 37°C and isometric tension was recorded by means of a force-displacement transducer connected to a Basile 7050 pen recorder. Concentration-response curves to endothelin or KCl were obtained in a cumulative manner. Each experiment was started after a 120 min equilibration period. At this stage, the strips were challenged three or more times with KCl (80 mM final concentration, added to the bath) at 15 min intervals until reproducible responses were observed. Each value given in the Results section is the mean ± s.e.mean. Statistical analysis of the data was made by Student's *t* test or analysis of variance, followed by Dunnett's test, when applicable.

Drugs used were: endothelin (Peninsula), atropine HCl and isoprenaline HCl (Serva), nifedipine (Sigma) and carbachol (Merck).

Results Endothelin (1 nM–0.3 μM) produced a concentration-dependent, slowly developing contraction of the human isolated bladder (Figure 1b). Concentrations higher than 0.3 μM were not tested

because of the limited availability of the peptide. At 0.3 μM, the contraction reached a plateau and then declined slowly to baseline. Washing with endothelin-free Krebs solution had little influence on this decline but prompt relaxation was observed upon addition of isoprenaline (1–3 μM, $n = 4$). At 0.3 μM, the response to endothelin averaged 90 $\pm$ 9% of that to KCl (80 mM). The action of endothelin was not significantly prevented either by atropine (3 μM) or nifedipine (1 μM) which were added to the bath 30 min before the start of the concentration-response curve (Figure 1c). By contrast, 1 μM nifedipine had a marked inhibitory action on the contractile response produced by addition of increasing concentrations of KCl (10–80 mM) to the bath (Figure 1d). Atropine (1 μM) abolished the contractile response to carbachol (0.3 μM, $n = 3$) which produced a response averaging 75–100% of that to KCl (80 mM).

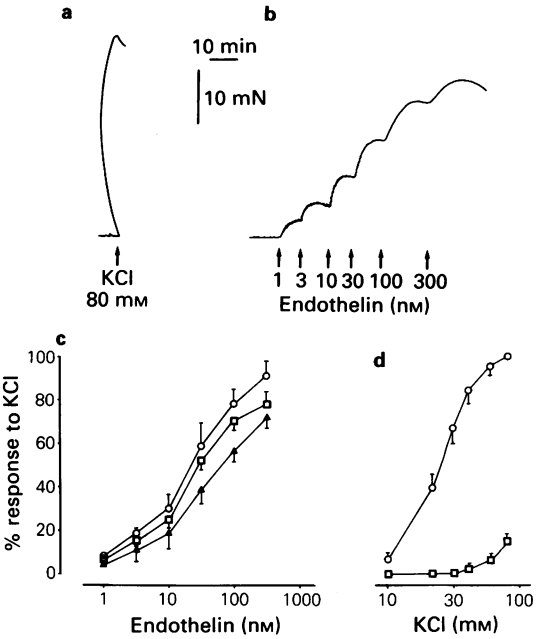

Figure 1 (a and b) Typical tracings showing the contractile response of muscle strips of the human isolated urinary bladder to endothelin as compared to KCl (80 mM). (c) Effect of atropine or nifedipine (1 μM each, 30 min beforehand) on the concentration-response curve to endothelin: (○) control; (□) nifedipine; (△) atropine. (d) Effect of nifedipine (1 μM, 30 min beforehand) on the response to KCl: symbols as in (c). Each point is the mean of at least 4 experiments; s.e.mean shown by vertical bars. All the points of the KCl curve in presence of nifedipine were significantly different from controls: $P < 0.05$.

Discussion Present findings indicate that endothelin exerts a potent contractile effect on the mucosa-free smooth muscle from the human urinary bladder. To date, expression of the pre-proendothelin messenger RNA has been reported only in the vascular endothelium and the potential physiological meaning of these findings is only a matter of speculation. The action of endothelin was substantially atropine-resistant which excludes a significant participation of the cholinergic nerves, the major excitatory innervation of the normal human bladder (Sjogren *et al.*, 1982; Sibley, 1984; Maggi *et al.*, 1988). Further, we found that the action of endothelin is totally resistant to nifedipine at a concentration that abolished the KCl-induced contractions. Therefore the action of endothelin on the human bladder is markedly different from that on isolated blood vessels, this latter being strictly dependent upon extracellular calcium influx through dihydropyridine-sensitive channels (Yanagisawa *et al.*, 1988). The present findings do not necessarily contradict the hypothesis (Yanagisawa *et al.*, 1988) that endothelin might be an endogenous agonist of the dihydropyridine-sensitive calcium channels in vascular smooth muscle but indicate that other mechanisms should also be considered in the action of this peptide. Further studies are needed to determine the calcium sources utilized by endothelin in its action on the human bladder muscle.

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Selective inhibition of adrenaline-induced human platelet aggregation by the structurally related Paf antagonist Ro 19-3704

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1 Two non-lipid antagonists of platelet-activating factor acether (Paf), BN 52021 and WEB 2086, at concentrations which completely blocked Paf-induced platelet aggregation, failed to interfere with aggregation by adrenaline. In contrast, Ro 19-3704, a structurally related antagonist of Paf, inhibited concentration-dependently aggregation induced by adrenaline or by the simultaneous addition of submaximal concentrations of adrenaline and Paf. Reversal of aggregation was obtained when Ro 19-3704 was added to the platelet suspension after adrenaline.

2 Ro 19-3704 was selective for Paf and adrenaline since it failed to interfere with platelet aggregation induced by arachidonic acid or ADP. CV-3988, an antagonist of Paf structurally similar to Ro 19-3704, also inhibited adrenaline-induced aggregation. However, a morpholine analogue (MA) of Paf, which has no anti-Paf activity, failed to interfere with the aggregation induced by adrenaline. This suggests that the effect of Ro 19-3704 and CV-3988 on adrenaline is not simply due to their lipid structure.

3 Experiments on plasma membrane preparations showed that Ro 19-3704 inhibited [^{3}H]-yohimbine binding with an inhibition constant (K_i) of $7 \pm 3\,\mu$M. In contrast, BN 52021 and MA did not interfere with [^{3}H]-yohimbine binding. Equilibrium binding experiments showed that Ro 19-3704 increased the apparent K_D of [^{3}H]-yohimbine binding from 2.02 ± 0.15 to 7.3 ± 0.4 nM. The Paf antagonist Ro 19-3704 interacts specifically with the α_2-adrenoceptor and may thus prevent the early steps involved in the mechanism of adrenaline-induced platelet activation.

Introduction

Platelet aggregation by adrenaline is biphasic and only its secondary wave, which is accounted for by secretion and arachidonic acid metabolites, is inhibited by non-steroidal anti-inflammatory drugs (NSAIDs) (O'Brien, 1963; Charo *et al.*, 1977). In contrast, the whole response (primary and secondary waves) is blocked by α_2-adrenoceptor antagonists, such as yohimbine (Grant & Scrutton, 1980). In fact, the mechanism of adrenaline-induced platelet activation is poorly understood. Despite recent emphasis concerning the importance of calcium mobilization and phosphoinositide hydrolysis, doubts have been raised as to their role in adrenaline-induced platelet activation (Owen *et al.*, 1980; Clare & Scrutton, 1983; Siess *et al.*, 1984; Johnson *et al.*, 1985).

Platelet-activating factor acether (1-O alkyl 2-acetyl sn-glycero 3-phosphocholine) (Paf) is a phospholipid mediator involved in various inflammatory responses. It is released from different cells during activation and may participate in several clinical disorders (Vargaftig *et al.*, 1981; Braquet *et al.*, 1987). Paf may also be important for platelet function because it is formed by platelets themselves (Chignard *et al.*, 1980; Touqui *et al.*, 1985). Adrenaline acts with Paf synergistically to trigger platelet activation (Vargaftig *et al.*, 1982) and accordingly, under pathological conditions the early release of minimal amounts of Paf and adrenaline might interact to amplify intravascular platelet activation.

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This led us to investigate the interactions of recently available Paf antagonists with platelet activation by Paf and adrenaline and to show that one structurally related antagonist, Ro 19-3704 (Hadvary & Baumgartner, 1985), suppresses adrenaline- and Paf-induced human platelet activation completely. We have also evaluated the interaction between adrenaline and two other available Paf antagonists, BN 52021 (Braquet *et al.*, 1985; Nunez *et al.*, 1986) and WEB 2086 (Casals-Stenzel, 1987) which are structurally unrelated to Paf. Our results demonstrate that Ro 19-3704 selectively suppresses the effects of Paf and adrenaline on human platelets, a property not shared by the other antagonists structurally unrelated to Paf.

Methods

Platelet aggregation

Venous blood was obtained from healthy donors who had taken no medication for at least ten days before sampling. Blood (9 vol) was mixed with 3.8% sodium citrate (1 vol) and centrifuged (190 *g*; 25°C for 20 min) to separate the platelet-rich plasma (PRP). Platelet-poor plasma (PPP) was obtained by centrifugation of the remaining blood for 20 min at 2500 *g*. The final concentration of platelets in PRP was adjusted to 3×10^8 ml^{-1} with PPP. Aggregation was measured at 37°C in 0.4 ml aliquots of PRP by the turbidimetric method in a dual-channel Chrono-Log aggregometer. Agonists were added to PRP at concentrations giving 80–90% increase in light transmission. These concentrations varied, according to the donors, between 0.1 to 1 μM and 0.5 to 5 μM for Paf and adrenaline, respectively. Paf antagonists were incubated with PRP for 1 min at 37°C before the addition of the agonists. In some experiments, Ro 19-3704 was added after the agonists, as indicated in Figure 5. Inhibition of platelet aggregation was defined as % decrease in extent of light transmission.

5-Hydroxytryptamine release

PRP was incubated with 1 μM [^{14}C]-5-hydroxytryptamine ([^{14}C]-5-HT, 50 nCi ml^{-1} final concentration) for 60 min at 37°C and then stimulated as indicated above. The reactions were stopped 5 min later by the addition of 0.5 ml of ice-cold saline containing 10 mM EGTA and 2 μM imipramine. Platelet samples were then pelleted by a 3 min centrifugation at 10,000 r.p.m. and the radioactivity from supernatants was measured by liquid scintillation counting. Eighty to 90% of the added 5-HT (40,000–45,000 c.p.m. ml^{-1} platelets) was taken up by platelets.

Binding studies

Platelets were washed according to the method of Mustard *et al.* (1972) and lysed according to Hwang *et al.* (1983). Plasma membranes were isolated by centrifugation (60 min at 100,000 *g*) of platelet lysates and stored at −80°C until use. These membrane preparations (protein concentration 0.4–0.6 mg ml^{-1}) were incubated with [^{3}H]-yohimbine for 30 min at 25°C in a final volume of 250 μl of Tris buffer (50 mM Tris, 1 mM EGTA at pH 7.4). At the end of incubation, samples were diluted with 4 ml aliquots of ice-cold incubation buffer and filtered over glass-fibre filters (Whatman-GF/C). The filters were further washed 3 times with the buffer, dried and transferred to scintillation vials for counting. Specific binding was defined as that inhibited by 5 μM phentolamine and represented 85% of the total binding at a [^{3}H]-yohimbine concentration near the apparent dissociation constant (K_D).

In saturation and competition experiments, Ro 19-3704 was dissolved in ethanol and added to the membrane preparations. The final concentration of ethanol did not exceed 0.25% (at this concentration ethanol has no effect on the binding of [^{3}H]-yohimbine). Data from equilibrium binding and competition studies were analysed by a non-linear least-square curve-fitting procedure (Munson & Rodbard, 1980) adapted to an Apple IIe computer.

Materials

Adrenaline, arachidonic acid (AA), adenosine diphosphate (ADP), yohimbine, phenoxybenzamine and imipramine were obtained from Sigma (St. Louis, U.S.A.). Paf-acether (1-0-octadecyl 2-acetyl sn-glycero 3-phosphocholine) was from Bachem (Switzerland). 5-Hydroxy-[^{14}C]-tryptamine creatinine sulphate ([^{14}C]-5-HT, 50 mCi mmol^{-1}), [^{3}H]-yohimbine (90 Ci mmol^{-1}) and ACS II (Aqueous counting scintillant II) were purchased from Amersham International (U.K.). BN 52021 (9H-1,7 a-(epoxymethanol)-1H-,6 aH-cyclopenta (c)furo (2, 3-b) furo-(3, 2:3, 4) cyclopenta (1,2-d) furan-5,9,12-(4H)-trione,3-tert-butylhexahydro-8-methyl) was a gift from Dr P. Braquet (IHB-IPSEN Research laboratories, Le Plessis-Robinson, France); Ro 19-3704 (3-4(**R**)-2-(methoxycarbonyl) oxy-3-(octadecylcarbamoyl)oxy = propoxy butylthiazolium iodide (Hadvary & Baumgartner, 1985); see Figure 1) was from Hoffman-La Roche (Switzerland). WEB 2086 (3- (4-(2-chlorophenyl)-9-methyl-6H-thieno(3,2-f)(1,2,4)triazolo(4,3-a)(1,4)-thienodiazepine-2-yl)-1-(4-morpholinyl)-1-propanone) (Casals-Stenzel, 1987) was from Boehringer Ingelheim (West Germany). CV-3988 (3-(N-n-octadecylcarbamoyloxy)-2-methoxy)

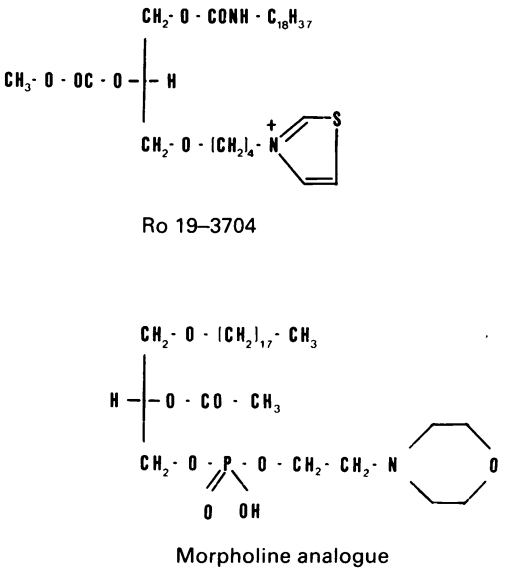

Figure 1 Structures of Ro 19-3704 and of the morpholine analogue (MA) of Paf.

propyl-2-thiazolioethyl phosphate) was from Takeda Chemical Industries (Osaka, Japan). 1-O octadecyl 2-O acetyl sn-glycero 3-phosphoryl-morpholino ethanol (morpholine-analogue, MA; Figure 1) was a gift from Prof J.J. Godfroid (Université Paris VII). Phentolamine was a gift from Ciba-Geigy.

BN 52021, Ro 19-3704 and MA were dissolved in ethanol and dilutions from stock solutions were made with saline. WEB 2086 was first diluted in distilled water with the addition of a few drops of 0.1 N HCl until complete dissolution, further dilutions being made with saline.

Results

Effect of Paf antagonists on adrenaline-induced platelet aggregation

Preincubation of PRP with WEB 2086, BN 52021 and Ro 19-3704 resulted in a concentration-dependent inhibition of aggregation induced by Paf (0.1–1 μM) (Figure 2a). The IC_{50} values were: 0.09 $\pm$ 0.03 μM for WEB 2086, 0.39 $\pm$ 0.05 μM for Ro 19-3704 and 3.2 $\pm$ 1.0 μM for BN 52021. Neither BN 52021 nor WEB 2086 interfered with adrenaline-induced aggregation when used up to concentrations 50 fold above those required to inhibit Paf (Figure 2b). In contrast, Ro 19-3704 suppressed completely the first and secondary waves of aggregation induced

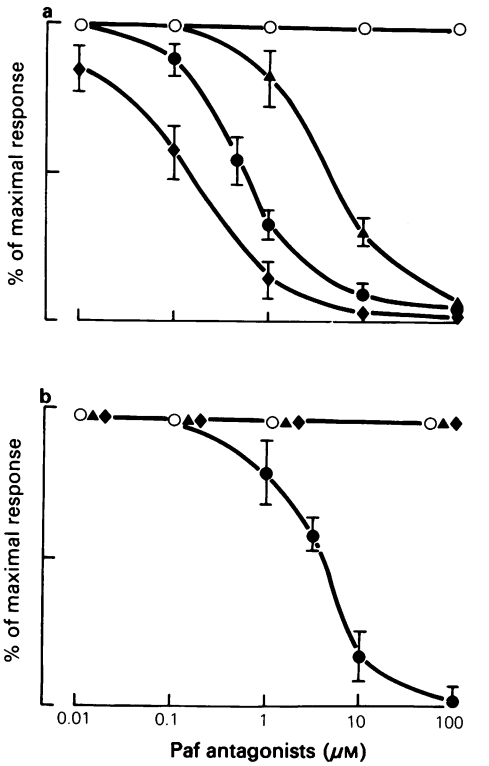

Figure 2 Effects of Paf antagonists on Paf and adrenaline-induced platelet aggregation. WEB 2086 (◆), Ro 19-3704 (●), BN 52021 (▲) and the morpholine analogue (○) or the corresponding vehicles were preincubated with PRP at 37°C for 1 min. Platelet aggregation was then induced by the addition of Paf or adrenaline at concentrations leading to 80 to 90% increase in light transmission. These concentrations varied between 0.1 to 1 μM and 0.5 to 5 μM for Paf and adrenaline, respectively, according to the donors. The figure shows inhibition of aggregation induced by Paf (a) and by adrenaline (b). The results are expressed as a % inhibition as compared to controls and are the mean values of 7–9 experiments; vertical lines indicate s.e.

by adrenaline (0.5–5 μM (Figures 2b and 3). Suppression of platelet aggregation by Ro 19-3704 was accompanied by a complete inhibition of 5-HT release (Figure 3). In contrast, the morpholine analogue (MA) of Paf, which has no anti-Paf activity (Coeffier *et al.*, 1986 and Figure 2b), failed to interfere with adrenaline-induced aggregation (Figure 2b).

In another set of experiments, we compared the effects of Ro 19-3704 with those of CV-3988 (Nunez *et al.*, 1986). The latter is an antagonist of Paf structurally similar to Ro 19-3704. We found that CV-3988 inhibited adrenaline-induced aggregation with a potency similar to that of Ro 19-3704 (Table

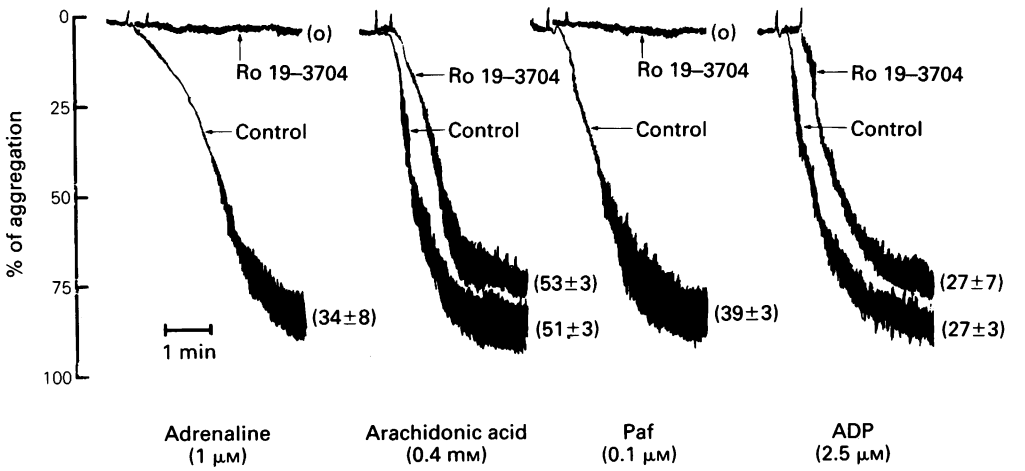

Figure 3 Effects of Ro 19-3704 on Paf, adrenaline, ADP- and arachidonic acid (AA)-induced platelet aggregation and 5-hydroxytryptamine (5-HT) release. Ro 19-3704 (50 μM) or the vehicle (ethanol), were incubated for 1 min with the PRP. Platelet aggregation was then induced by the addition of Paf (0.1 μM), adrenaline (1 μM), ADP (2.5 μM) or AA (0.4 mM). 5-HT release was measured 5 min after the addition of the agonists (see Methods). The figure shows typical tracings of aggregation representative of 3-separate experiments. The values in parentheses represent the release of 5-HT calculated as a % of total radioactivity incorporated into the platelets (45,000 to 50,000 c.p.m. ml^{-1}) and expressed as the mean ± s.e. of 3 separate determinations.

1). Indeed, the IC_{50} ratios indicate that the former is 1.5 to 4.7 times less active than the latter in inhibiting adrenaline-induced aggregation.

Selective inhibition of adrenaline- and Paf-induced platelet aggregation by Ro 19-3704

The effects of Ro 19-3704 on platelet activation induced by Paf, ADP, adrenaline and arachidonic acid were compared. Ro 19-3704 acted as a selective inhibitor of Paf and adrenaline. Indeed, at a concentration 50 fold above those required to suppress the action of adrenaline and Paf, it failed to affect 5-HT release or platelet aggregation induced by either ADP or AA (Figure 3).

Effect of Ro 19-3704 on the concentration-dependent aggregation induced by Paf or adrenaline

The concentration-response curves of platelet aggregation induced by adrenaline or by Paf were progressively displaced to the right when PRP was incubated in the presence of increasing concentrations of Ro 19-3704 (Figure 4). Thus, the EC_{50} of adrenaline was increased from 0.50 ± 0.03 μM in control to 5.2 ± 0.1 μM and 9.6 ± 1.2 μM in the presence of 5 μM or 10 μM Ro 19-3704, respectively.

Table 1 Antagonist concentrations giving 50% inhibition (IC_{50}) of adrenaline and Paf-induced platelet aggregation

	Antagonists IC_{50} (μM)			
	Ro 19-3704 (A)	*CV-3988* (B)	*BN 52021*	*Ratio[a] B/A*
Adrenaline (μM)				
0.5	1.4 ± 0.5	2.1 ± 1.2	NE	1.5
1	4.1 ± 2.5	19.5 ± 9.6	NE	4.7
10	13.5 ± 3.1	46.4 ± 11.0	NE	3.4
Paf (μM)				
0.05	0.27 ± 0.10	2.2 ± 1.4	0.12 ± 0.04	8
0.2	0.45 ± 0.05	7.5 ± 1.5	3.8 ± 1.3	16.6
0.5	1.9 ± 0.20	19.1 ± 2.9	16	10

Aliquots, 0.4 ml, from PRP were incubated for 1 min at 37°C with Ro 19-3704, CV-3988 or BN 52021 at concentrations varying from 0.1 to 50 μM. Then, platelet aggregation was induced by adrenaline or Paf, added at the concentrations indicated in the table. The curves representing inhibition of aggregation as a function of drug concentrations were plotted and the IC_{50} determined graphically.

The table shows the IC_{50} of antagonists towards adrenaline and Paf. The results are expressed in μM and are the mean values ± s.d. of 3–4 experiments.

NE: no effect (up to 100 μM of BN 52021).

[a] The ratio B/A: IC_{50} of CV-3988 to IC_{50} of Ro 19-3704.

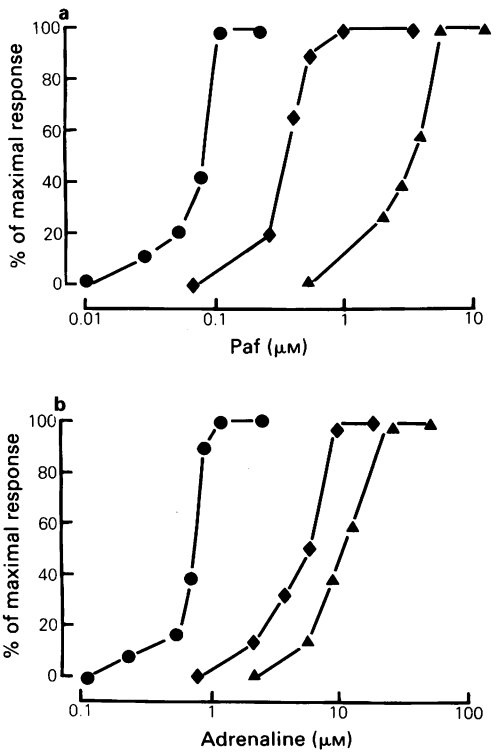

Figure 4 Effect of Ro 19-3704 on concentration-response curves of Paf- and adrenaline-induced aggregation. (a) Concentration-response curves for aggregation induced by Paf were conducted in the presence of 0.5 μM (◆), 5 μM (▲) or in the absence (●) of Ro 19-3704. (b) Concentration-response curves for aggregation induced by adrenaline were conducted in the presence of 5 μM (◆), 10 μM (▲) or in the absence (●) of Ro 19-3704. The figure shows typical curves representative of three separate experiments.

In the case of Paf EC_{50} was increased from 0.07 ± 0.03 μM in control to 0.29 ± 0.02 μM and 2.47 ± 0.43 μM in the presence of 0.5 or 5 μM Ro 19-3704, respectively. This strongly suggests that Ro 19-3704 acted as a competitive inhibitor of both Paf and adrenaline.

Effect of Ro 19-3704 added to platelets after the agonists

Incubation of PRP with Ro 19-3704 1 min before adrenaline or Paf led to the complete inhibition of aggregation. The inhibition was also observed when Ro 19-3704 was added immediately after adrenaline. The inhibitory effect decreased progressively and disappeared completely when this interval was more

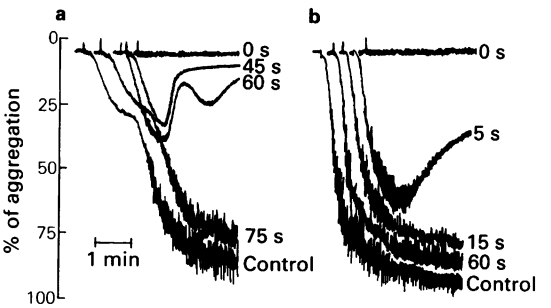

Figure 5 Inhibition of (a) adrenaline- and (b) Paf-induced aggregation by the addition of Ro 19-3704 at different time intervals. Ro 19-3704 (50 μM) was incubated with the PRP immediately before (0 s) or at the indicated intervals after the addition of (b) Paf (0.1 μM) or (a) adrenaline (2.5 μM). The figures next to each trace represent the times of the addition of Ro 19-3704 after the corresponding agonists.

than 1 min (this period corresponds to the lag time of the release reaction). This interval was less than 5 s, if platelets were stimulated by Paf before the addition of Ro 19-3704 (Figure 5).

Inhibition of platelet aggregation induced by the synergistic action of Paf and adrenaline

Submaximal concentrations of Paf (0.25 μM) or adrenaline (10 μM) which induced only marginal aggregation when added separately, acted synergistically to induce maximal aggregation, when added simultaneously. Preincubation of PRP with yohimbine (5 μM) markedly reduced this synergism. The extent of aggregation obtained by Paf alone remained unchanged. This result confirms the previous findings of Fouque & Vargaftig (1984). Synergism was reduced when PRP was pretreated with BN 52021; in this case only the aggregation due to adrenaline alone persisted. However, in the presence of Ro 19-3704 (50 μM) the synergistic response was entirely blocked (Figure 6).

Inhibition of yohimbine binding by Ro 19-3704

Binding of [^{3}H]-yohimbine to the plasma membrane preparation was saturable (206 ± 6 fmol mg^{-1} protein) and of high affinity (2.02 ± 0.15 nM) ($n = 3$). In competition studies, Ro 19-3704 inhibited [^{3}H]-yohimbine binding with an inhibition constant (K_i) of 7 ± 3 μM. In contrast, BN 52021 only marginally affected the [^{3}H]-yohimbine binding (Figure 7a). In order to determine if the interaction of Ro 19-3704 with platelet α_2-adrenoceptors was competitive or non-competitive, equilibrium binding experiments

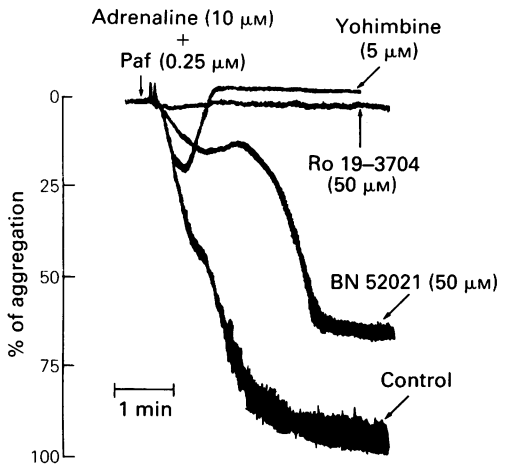

Figure 6 Inhibition of platelet aggregation induced by the simultaneous addition of Paf and adrenaline Platelet aggregation was induced by submaximal concentrations of Paf (0.25 μM) adrenaline (10 μM). Yohimbine, BN 52021 and Ro 19-3704 were added as indicated.

were performed in the presence and in the absence of Ro 19-3704. The addition of 10 μM Ro 19-3704 to the incubation mixture increased the apparent K_D of [^{3}H]-yohimbine binding from 2 ± 0.15 to 7.3 ± 0.4 μM ($n = 3$, $P < 0.0.1$). Moreover, the maximal number of binding sites was reduced from 206 ± 6 to 185 ± 3 fmol mg^{-1} protein ($n = 3$, $P < 0.05$). A similar effect on receptor density was found with the morpholine analogue which nevertheless failed to decrease the apparent K_D of yohimbine binding (data not shown).

Discussion

We have investigated the effect of three Paf antagonists on adrenaline-induced platelet aggregation and secretion. Two structurally different antagonists of Paf, WEB 2086 and BN 52021 failed to interfere with the effects of adrenaline. As expected, we found that WEB 2086 and BN 52021 antagonized the effects of Paf, the former being more potent than the latter, even though this may vary according to species (Agarwal & Townley, 1987). Failure of these Paf-acether antagonists to interfere with the action of adrenaline suggests that adrenaline-induced platelet activation is not accompanied by synthesis of Paf-acether or that the latter does not account for adrenaline-induced platelet activation. In contrast, Ro 19-3704, suppressed adrenaline-induced aggregation and 5-HT release. The inhibitory effect of Ro 19-3704 seems to be selective for Paf-acether and adrenaline since it was not observed when platelets were activated by AA or ADP. This also indicates that the effect of Ro 19-3704 on adrenaline occurs at a step earlier to AA release and ADP secretion.

One would expect that the inhibitory effect of Ro 19-3704 could be due to its lipid structure and thus to a non-specific interaction with platelet membranes. This is unlikely to be the case since the morpholine analogue (MA) of Paf-acether, even at ten fold higher concentrations, failed to interfere

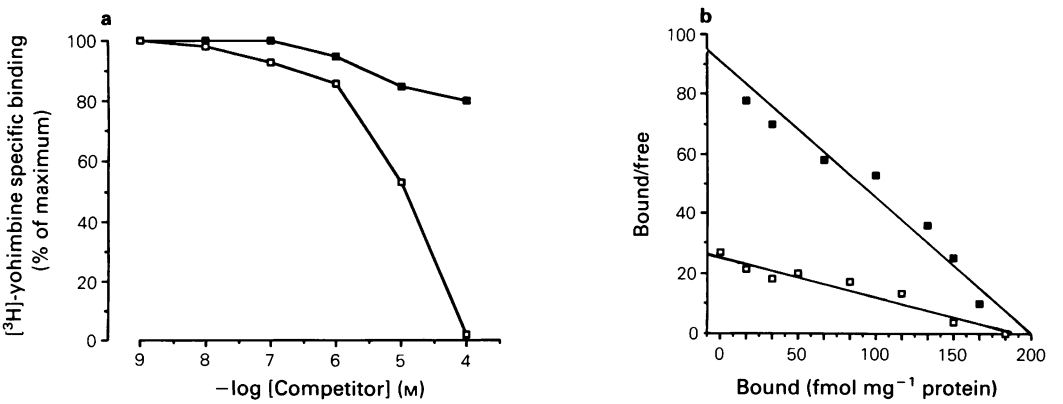

Figure 7 Effect of BN 52021 and Ro 19-3704 on [^{3}H]-yohimbine binding to platelet membranes. (a) Competition of Ro 19-3704 and BN 52021 for [^{3}H]-yohimbine binding: platelet plasma membranes were incubated with increasing concentrations of Ro 19-3704 (□) or BN 52021 (■) in the presence of 2.5 nM [^{3}H]-yohimbine. The values are representative of three separate experiments. For details see Methods. (b) Scatchard analysis of [^{3}H]-yohimbine binding in the presence (□) or in the absence (■) of 10 μM Ro 19-3704. Platelets were incubated with increasing concentrations (0.2–30 nM) of radioligand for 30 min at 25°C.

with the effect of adrenaline. Furthermore, phosphatidylcholine and lyso-Paf (a naturally occurring analogue of Paf-acether) had no effect on adrenaline-induced aggregation (data not shown).

These findings suggest that, besides its anti-Paf-acether properties, Ro 19-3704 acted as an antagonist of α_2-adrenoceptors. In confirmation, binding studies carried out on isolated platelet membranes showed that Ro 19-3704 reduced the specific binding of [^{3}H]-yohimbine with an inhibition constant (K_i) of $7 \pm 3\ \mu M$. This value is near the IC_{50} ($5.4 \pm 1.9\ \mu M$) corresponding to inhibition of adrenaline-induced aggregation by Ro 19-3704. Neither BN 52021 nor MA interfere with the binding of [^{3}H]-yohimbine. Scatchard plots showed that Ro 19-3704 inhibited [^{3}H]-yohimbine binding in a competitive manner. This agrees with the results depicted in Figure 4b which show that Ro 19-3704 shifted the concentration-response curve for adrenaline to the right.

The fact that Ro 19-3704 induced a small decrease of receptor density might be related to its lipid structure and consequently to a non-specific interaction with the plasma membrane. Indeed, the inactive analogue MA, had a similar effect on the receptor density without interfering with the apparent K_D of [^{3}H]-yohimbine binding. Therefore, the change in the apparent K_D induced by Ro 19-3704 is unlikely to be related only to its lipid structure. It rather suggests a specific and competitive interaction between this compound and [^{3}H]-yohimbine at the α_2-adrenoceptor. However, the possibility that the lipid structure of Ro 19-3704 might play a role in facilitating its interaction with α_2-adrenoceptors cannot be ruled out.

Several α_2 agonists and antagonists such as clonidine, UK14304 and idazoxan, possess an imidazole-ring. Therefore, the presence of an imidazole-like moiety in the structure of Ro 19-3704 may explain its ability to interact with the α_2-adrenoceptor. The results of Table 1 are in favour of this hypothesis. We showed that CV-3988, an analogue of Ro 19-3704 containing an imidazole-like moiety, is a potent inhibitor of adrenaline-induced aggregation. Investigations with other analogues of Ro 19-3704 are planned to clarify the importance of the imidazole group and/or lipid structure in the interaction with α_2-adrenoceptors.

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Primary sensory neurones and naloxone-precipitated morphine withdrawal

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1 The C-fibre-evoked depressor reflex following i.a. injection of capsaicin and the wiping movements following chemical irritation of the cornea by capsaicin were both found to be augmented in the naloxone-precipitated morphine withdrawal phase.

2 The *in vitro* capsaicin-evoked release of substance P from central terminals of C-fibre afferents in the spinal cord was decreased in morphine-treated rats. Following naloxone, the release in the morphine-treated group was as large as in the control group.

3 The C-fibre mediated plasma extravasation in the rat paw after naloxone was found to be the same in morphine-treated and control rats.

4 It is concluded that C-fibre-evoked reflex reactions are augmented during morphine withdrawal, but that the mechanisms responsible are either located postsynaptically to the primary sensory neurone or further centrally.

Introduction

A typical symptom of morphine withdrawal is rebound neuronal hyperactivity. Noradrenergic neurones in the locus coeruleus show increased spontaneous firing rate and metabolic activity (Aghajanian, 1978; Crawley *et al.*, 1979). It has also been suggested that sensory neurones might show augmented responses during the withdrawal phase (Bell & Jaffe, 1986). The latter finding is supported by studies that show a reduction in withdrawal symptoms in rats in which the sensory C-fibres had been destroyed by capsaicin (Sharpe & Jaffe, 1986).

It is generally accepted that chronic opiate administration depresses multiple neuronal systems (for review see Martin, 1984). μ- and δ-receptor agonists such as morphine or enkephalins depress afferent fibres and generally affect sensory transmission in the spinal cord (Yaksh, 1983). The mechanism of opioid suppression of afferent fibres seems to be inhibition of transmitter release in the spinal cord (Jessell & Iversen, 1977; Lembeck & Donnerer, 1985; Mauborgne *et al.*, 1987).

During a prolonged period of reduced sensory input, supersensitivity of receptors on second order neurones can develop (Kuwahara *et al.*, 1987). There are also indications that after chronic morphine administration the transmitters in sensory C-fibres, i.e. substance P, accumulate (Naftchi *et al.*, 1981; Bergström *et al.*, 1984), and that during naloxone-precipitated withdrawal the release of sensory neurotransmitters might be augmented (Ueda *et al.*, 1987).

I wanted to investigate whether primary sensory neurones showed spontaneous or stimulus-evoked hyperactivity in the morphine witdrawal phase of the rat. This was done by studying the C-fibre-evoked depressor reflex resulting from i.a. injection of capsaicin, the wiping movements following irritation of the cornea using capsaicin as a selective stimulus and by measuring the *in vitro* release of an excitatory transmitter of afferent C-fibres, substance P.

Methods

Sprague-Dawley rats of either sex (Himberg, Austria) were used. Tolerance and dependence on morphine was induced by increasing doses of morphine given subcutaneously at intervals of 8 h. The animals received 3 injections of $5\,mg\,kg^{-1}$ morphine the first day, 3 injections of $10\,mg\,kg^{-1}$ the second day and 3 doses of $15\,mg\,kg^{-1}$ the third day. Four hours after another dose of $15\,mg\,kg^{-1}$ on the fourth day, withdrawal was induced by s.c. or i.v. injection of $1\,mg\,kg^{-1}$ naloxone. Control rats received saline injections for 3 days and naloxone on the fourth day.

A three day exposure to morphine was used for all *in vivo* studies; for the *in vitro* studies exposure

periods shorter (15 min or 1 hour) or longer than three days (10 days) were used in addition. For a 1 h exposure the animals received the first s.c. morphine injection of 5 mg kg^{-1}; a 15 min exposure was only performed *in vitro* (see sub-section: '*In vitro* release of substance P'). For the morphine treatment lasting 10 days, the dose, which was always given 3 times a day, was increased by 5 mg kg^{-1} every second day.

Capsaicin-induced depressor reflex

Rats were anaesthetized with sodium pentobarbitone (50 mg kg^{-1} i.p.). The trachea, a carotid artery for blood pressure recording and a jugular vein for i.v. infusions were cannulated. A superficial epigastric artery was cannulated retrogradely for infusion of capsaicin into the hind leg. Thirty ng of capsaicin dissolved in 30 μl 0.9% NaCl was infused for 30 s 5 times at 10 min intervals. The responses to the capsaicin injections were calculated as a decrease in blood pressure (mmHg) from the immediately preceding levels. The duration of depressor reflexes was also assessed by measuring the time it took for the blood pressure to return to the baseline (within a range of -5%).

Neurogenic plasma extravasation

Following sodium pentobarbitone anaesthesia, trachea and jugular vein cannulation, as described above, the saphenous nerve was cut in the thigh, its distal part placed on electrodes and isolated with paraffin oil. Twenty minutes after the i.v. injection of naloxone, at which time the acute morphine withdrawal symptoms are at their peak, the neurogenic plasma extravasation in the hind paw was induced by saphenous nerve stimulation for 5 min with 10 V, 1 ms and 2 Hz and measured by Evans blue exudation as described by Lembeck & Holzer (1977). No spontaneous Evans Blue exudation was observed upon the injection of naloxone in saline- or morphine-treated rats.

Wiping test

As described by Gamse (1982), wiping movements over the forehead were counted for one minute following the instillation of a diluted (10 μg ml^{-1}) capsaicin solution into the eye. This test was also performed 20 min after the s.c. injection of naloxone.

In vitro *release of substance P*

Rats were decapitated, the lumbar spinal cord, approximately 2 cm, was removed and placed on a chilled plate. It was then freed from arachnoidea and pia mater and hemisected longitudinally into dorsal and ventral halves. Next, the dorsal half was cut with a tissue chopper into 200 μm slices which were transferred to a superfusion chamber. Superfusion was performed with an oxygenated Krebs-bicarobonate buffer at 37°C (for composition see Lembeck & Donnerer, 1985) at a rate of 0.75 ml min^{-1}. To prevent premature withdrawal in spinal cord slices from morphine-treated rats, the superfusate contained 10^{-5} M morphine. This was the case for all morphine exposure times from 1 h to 10 days. The 15 min exposure to morphine was performed in the superfusion chamber only.

Two min-fractions were collected in tubes containing 120 μl glacial acetic acid to give a final acid concentration of 2 M. To induce a morphine withdrawal condition in the spinal cord slices, morphine was removed and naloxone (10^{-5} M) was added to the superfusion solution. Release of substance P was evoked by capsaicin (3 μM in the superfusion medium for 6 min). At the end of the experiment the slices were homogenized in 2 ml 2 M acetic acid and centrifuged. The fractions collected during the superfusion experiment and the supernatant of the tissue homogenate were freeze-dried. Substance P (SP)-immunoreactivity in these samples was determined using a C-terminally directed antibody RD_2 (gift of Dr S. Leeman) and [^{125}I]-Tyr^8-SP as tracer. The release rate of SP is expressed as the efflux rate constant, i.e. in % per min according to the formula: pg SP per 2 min fraction/pg SP in the slices at the time of release $\times$ 50%. Capsaicin-evoked release was calculated by subtracting the spontaneous release (immunoreactivity in the 2 fractions immediately before the stimulation) from the release during stimulation (Gamse, 1982).

Statistical analysis

Data were analysed by use of analysis of variance (ANOVA) and differences between treatments were determined by use of Scheffe's multiple comparisons test.

Substances

Capsaicin: Merck (Darmstadt, F.R.G.); morphine hydrochloride: Diosynth (Apeldoorn, Holland); naloxone hydrochloride: DuPont (Geneva, Switzerland).

Results

In pentobarbitone-anaesthetized control rats, each i.a. infusion of capsaicin caused a short drop in blood pressure (depressor reflex, Figure 1), a response which remained constant over a period of 50 min. This depressor reflex was unaltered after nal-

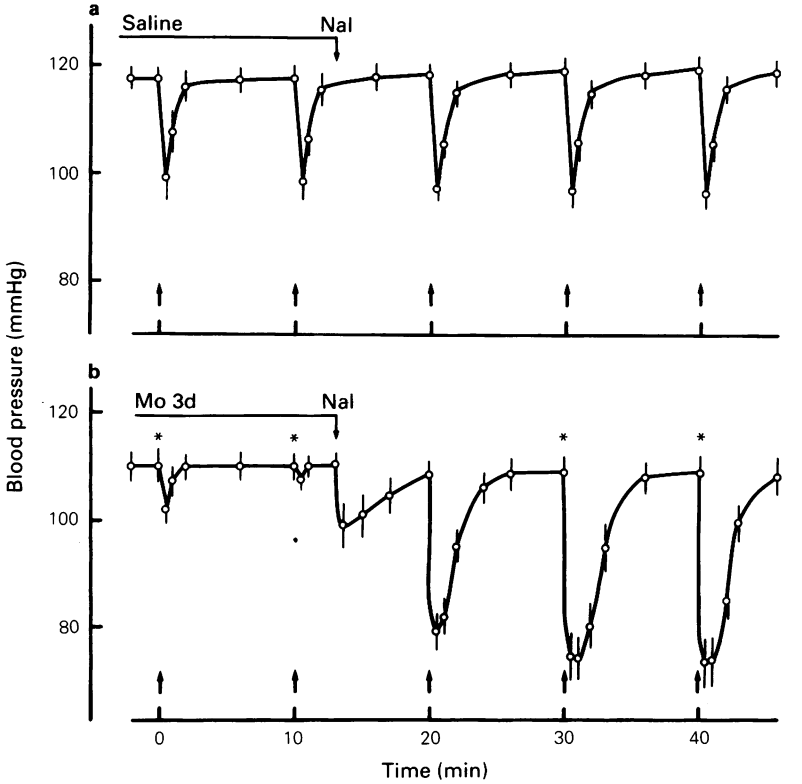

Figure 1 Mean blood pressure values in the rat carotid artery. Effect of naloxone-precipitated morphine withdrawal on the capsaicin-evoked decrease in blood pressure. Capsaicin (30 ng in 30 μl saline) was infused i.a. for 30 s at arrows on abscissa scale. (a) Mean blood pressure values in rats that received saline injections for 3 days before naloxone (Nal, 1 mg kg^{-1}, i.v.); (b) mean blood pressure values in rats that were treated with increasing doses of morphine (Mo) 3 days before naloxone (for morphine treatment schedule see Methods). Mean values are shown (n = 7) and vertical lines indicate s.e.mean. Significance of difference from control values: * $P < 0.05$ (Scheffe's comparison test).

oxone injection in saline-treated rats. After rats were treated with morphine for 3 days, capsaicin evoked only a very weak reflex depressor response. However, after the morphine-dependent rats received 1 mg kg^{-1} naloxone i.v., the capsaicin-evoked depressor reflex was intensified when compared to the controls (35 ± 4 versus 22 ± 2 mmHg, Figure 1). Also the duration of the depressor response was extended. In saline-treated rats the reflex lasted 1.2 ± 0.2 min, whereas in the withdrawal phase it lasted 2.9 ± 0.4 min (n = 7, $P < 0.05$). In a few cases the blood pressure dropped upon the injection of naloxone in the morphine-treated group, but returned to normal levels within 8 min.

The state of morphine withdrawal also exerted a facilitory action on the wiping responses to capsaicin (Table 1). A highly diluted capsaicin solution (10 μg ml^{-1}), which evoked a submaximal number of wiping responses in control rats (maximal response 25–30 wiping movements from a 100 μg ml^{-1} capsaicin solution), was instilled into the eyes of both saline- and morphine-treated rats. The number of wipings was increased by 60% 20 min after inducing

Table 1 Effect of naloxone-precipitated morphine withdrawal on measurements obtained in the wiping test and neurogenic plasma extravasation (Evans Blue content of hind paw skin)

Treatment	*Wiping responses*	*Evans Blue,* (μg 100 mg^{-1} skin)
3 days saline – naloxone	11.8 ± 1.3 (n = 6)	115.7 ± 17.8 (n = 13)
3 days morphine – naloxone	18.7 ± 1.3* (n = 7)	137.4 ± 17.2 (n = 13)

* $P < 0.05$ vs saline – naloxone. Tests were performed 20 min after the injection of naloxone.

withdrawal from morphine by the s.c. injection of 1 mg kg^{-1} of naloxone (Table 1).

On the other hand, the neurogenic plasma extravasation induced by antidromic saphenous nerve stimulation, was found to be in the control range during the withdrawal phase (Table 1). The stimulus parameters were chosen to produce a half-maximal response in the saline-treated rats.

When spinal cord slices were obtained from morphine-treated rats and superfused with a morphine-containing Krebs-bicarbonate solution (10^{-5} M morphine), the capsaicin-evoked substance P release was found to be reduced in comparison to control conditions (Figure 2). Exposure of spinal cord slices from morphine-treated rats (exposure 3 days) to naloxone (10^{-5} M), for 8 min before and during capsaicin stimulus, caused a substance P release that was in the control range. The baseline release of substance P was not affected by changing from a morphine-containing to a naloxone-containing superfusion medium. Exposure to morphine for 15 min, 1 h or 10 days before naloxone gave the same results ($n = 6$; results not shown): the release of substance P was always found to be depressed when morphine was still present in the superfusion medium and upon exposure to naloxone substance P release returned to the control range. Similar results were obtained when a 60 mM K^+ solution was used as a stimulus ($n = 6$, results not shown).

Discussion

The release of substance P from central terminals of sensory fibres in the spinal cord by capsaicin was found to be impaired during exposure to morphine for 15 min to 10 days confirming earlier publications (Jessell & Iversen, 1977; Kuraishi *et al.*, 1983;

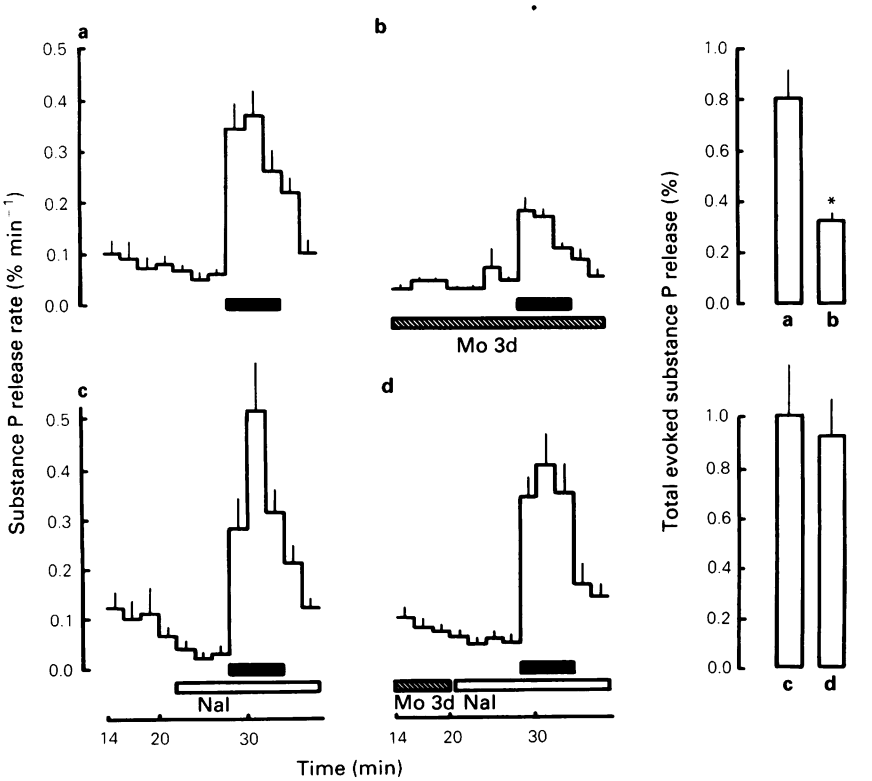

Figure 2 *In vitro* release of substance P from superfused dorsal spinal cord slices of rats. Time course of spontaneous and capsaicin-evoked release (black bar, 3 μM) in the absence and presence of additional drugs. Naloxone (Nal) and morphine (Mo) were present in concentrations of 10^{-5} M. The duration of morphine treatment in (b) and (d) before the capsaicin pulse was 3 days. Columns represent the total capsaicin-evoked substance P release expressed as the efflux rate constant (for calculations see Methods). Mean values are shown ($n = 6$–12) and vertical bars indicate s.e.mean. Significance of difference between (b) and (a) or (c) or (d): * $P < 0.05$ (Scheffe's comparison test).

Lembeck & Donnerer, 1985; Pang & Vasko, 1986). Blockade of the long-term effects of morphine by naloxone re-established the normal response to stimulation of the afferent C-fibres by capsaicin or high extracellular K^+ *in vitro*. It did not, however, induce a spontaneous increased substance P release. There has been one study that showed an augmented substance P release from mouse spinal cord slices, under these conditions, upon K^+ stimulation (Ueda *et al.*, 1987). In that study the basal release of substance P was also already much higher in the morphine-treated group than in controls (which was not the case in the present study), thus species and procedural differences might be responsible for the observation of Ueda *et al.* (1987).

In the morphine withdrawal phase, neurogenic plasma extravasation by antidromic saphenous nerve stimulation, reflecting substance P release from peripheral terminals of sensory fibres, was not larger than in the controls. Both findings, the *in vitro* substance P release and the neurogenic plasma extravasation, do not support the assumption of an overshoot release of substance P in the morphine withdrawal phase.

On the other hand, we clearly found a neuronal reflex hyperreactivity *in vivo* when afferent C-fibres were stimulated during morphine withdrawal. The capsaicin-evoked depressor reflex has its reflex centre in the brainstem, and the efferent pathway consists of a withdrawal of the sympathetic tone (Donnerer & Lembeck, 1983). The wiping response, as tested in our experiments, is a typical defence reaction to chemical irritation of the cornea. This reflex in conscious rats is also initiated by afferent C-fibre stimulation. Both reflexes were augmented in the morphine withdrawal phase.

Several mechanisms could be responsible for an enlarged response during naloxone-precipitated opiate withdrawal: (1) A supersensitivity of receptors postsynaptically to C-fibre afferents in the spinal cord (Zhao & Duggan, 1987) was observed following a period of reduced impulse input due to nerve transection (Kuwahara *et al.*, 1987). Supersensitivity of spinal cord neurones could be due to upregulation of receptors or to altered second messenger mechanisms (Sharma *et al.*, 1975; Nestler & Tallman, 1988); (2) It has also been postulated that morphine has a naloxone-irreversible, potentiating effect on the substance P response to spinal cord neurones; this effect could be unmasked during withdrawal (Piercey *et al.*, 1980); (3) Other neurone systems which may be responsible for the overshooting activity could be in the brainstem where noradrenergic neurones seem to be involved in the capsaicin-evoked depressor reflex (Donnerer *et al.*, 1988). It has been shown that noradrenergic neurones in the locus coeruleus (Aghajanian, 1978) and in the cortex (Pellegrini-Giampietro *et al.*, 1988) are hyperactive during morphine withdrawal. But also many other neurone systems in the CNS have been found to be hyperactive in naloxone precipitated morphine withdrawal as shown by glucose metabolism studies (Geary & Wooten, 1987).

A reason why noradrenergic neurones in the CNS might be more sensitive to morphine withdrawal than primary afferent C-fibres may be a different mechanism of transmitter release or a different distribution of subclasses of opiate receptors (Nestler & Tallman, 1988).

To what extent the activity of C-fibre afferents contributes to the typical unpleasant withdrawal symptoms is not clear. It has been shown in the rat that during acute withdrawal salivation, lacrimation and rhinorrhea (but not wet-dog shakes) are reduced when the C-fibre system is impaired, as in capsaicin-pretreated rats (Sharpe & Jaffe, 1986). Also the release of ACTH was found to be reduced in capsaicin-treated rats under withdrawal conditions (Donnerer & Lembeck, 1988). It is reasonable to assume that even mild stimuli on sensory fibres can induce unpleasant symptoms due to reflex hyperactivity in an individual during withdrawal. From our results it seems that the mechanisms lying behind this hyperexcitability are located centrally to primary afferents.

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Na^+–K^+ pump activity in rat peritoneal mast cells: inhibition by extracellular calcium

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1 Pure populations of rat peritoneal mast cells were used to study cellular potassium uptake. The radioactive potassium analogue, ^{86}rubidium, was used as a tracer for potassium for measurements of the activity of the cellular potassium uptake process.

2 The ouabain-sensitive and the ouabain-resistant potassium (^{86}rubidium) uptake of mast cells incubated in the presence of calcium, 1 mmol l^{-1}, were very low, 52 and 147 pmol per 10^6 cells min^{-1}.

3 Calcium-deprivation of the cells uncovered a large capacity ouabain-sensitive potassium (^{86}rubidium) uptake mechanism. The activity of the uptake mechanism was decreased by reintroduction of calcium into the cell suspension, and it was dependent on cellular energy metabolism, temperature and pH.

4 The potassium (^{86}rubidium) uptake of mast cells incubated in a calcium-free medium occurs through an active and ouabain-sensitive mechanism that has the nature of an enzyme, and it is mediated by the Na^+–K^+ pump located in the plasma membrane. It is demonstrated that the activity of the Na^+–K^+ pump mechanism is inhibited by low concentrations of extracellular calcium (0.1–1.2 mmol l^{-1}). The possibility is discussed that calcium-deprivation may increase the pump activity by increasing the permeability of the plasma membrane for Na^+.

Introduction

The secretory response of a number of tissues is enhanced by digitalis glycosides (see references in Amellal *et al.*, 1985), that inhibit the Na^+–K^+ pump activity (Schatzmann, 1953; Skou, 1957; 1965; 1986; Glynn & Karlish, 1975). However, there are conflicting results concerning the effect of ouabain on the secretion of histamine from rat mast cells and human basophil leucocytes. While ouabain had no effect on histamine secretion from these cells in the presence of calcium (Magro 1977a, b; Fewtrell & Gomperts, 1977), the secretory response of mast cells was enhanced by ouabain when the experiments were performed in a calcium-free medium (Frossard *et al.*, 1983; Amellal *et al.*, 1984; 1985; Binck *et al.*, 1985). These studies did not include measurements of the activity of the Na^+–K^+ pump. Furthermore, there are apparently no data in the literature on the Na^+–K^+ pump activity in intact rat mast cells.

The aim of the present study was to characterize the Na^+–K^+ pump mechanism in the membrane of intact rat mast cells and to study the effect of extracellular calcium on the pump activity. The ouabain-sensitive and ouabain-resistant uptake of potassium into the cells were measured by use of the radioactive potassium analogue, ^{86}rubidium (^{86}Rb), as a tracer for potassium.

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Methods

Isolation of mast cells

Male Sprague–Dawley rats, 320 to 615 g, were used for the experiments. Mast cells were isolated by differential centrifugation in a Percoll gradient. Rats were killed by bleeding from the carotid arteries under light ether anaesthesia. Mixed peritoneal cells were collected by injecting Krebs–Ringer solution containing Tris-HCl buffer through a small incision into the abdominal cavity. The cell suspension was removed and cooled in an ice-chilled waterbath. The remaining part of the cell isolation was carried out at 4°C. After centrifugation, 220 *g* for 10 min, the supernatant was discharged and the cells were resuspended in a small volume (200–300 μl) of Krebs–Ringer solution and transferred to an isotonic

solution of Percoll (density: 1.017 g ml^{-1}) with HEPES buffer, 32.5 mmol l^{-1}. A continuous gradient of Percoll was created by centrifugation at 10,000 *g* for 20 min. The mast cells were concentrated in the lower third part of the gradient, whereas the peritoneal leucocytes and macrophages were located in the upper third part of the gradient. The mast cells were harvested into a syringe and washed three times to remove the remaining Percoll. The cells were then suspended in Krebs–Ringer solution containing bovine serum albumin, 1 mg ml^{-1}, and glucose, 1 mg ml^{-1}, final pH 7.4. The mast cells constitute 93–100% of the cell suspension (mean ± s.d. was 97.8 ± 2.1%).

Incubation procedure

Mast cell suspensions pooled from 2 to 5 rats were divided into samples of the same cell density in a final volume of 0.5 ml, and each sample contained between 1.11×10^5 and 4.66×10^5 cells. The samples were equilibrated at 37°C for 60 min under various experimental conditions (except for the experiments in Figure 1). In some experiments the cells were then preincubated with ouabain or various concentrations of calcium for 15 to 30 min before incubation with $K^+(^{86}Rb^+)$ was carried out in order to determine the Na^+–K^+ pump activity.

In control experiments the number of mast cells in each sample varied between 1.11×10^5 and 4.66×10^5 (Figure 2). Within this range and both in the presence and absence of extracellular calcium neither the ouabain-sensitive nor the ouabain-resistant $K^+(^{86}Rb^+)$-uptake was influenced by the cell density ($P > 0.05$ by the Mann–Whitney U test).

Measurement of Na^+–K^+ pump activity

The cells were incubated in a medium containing potassium in addition to the radioactive tracer (i.e. $K^+(^{86}Rb^+)$). The concentration of $^{86}Rb^+$ was always less than 5 μmol l^{-1}. The incubation of the cells with $K^+(^{86}Rb^+)$ lasted for 2–10 min and it was performed at 37°C, except where otherwise stated. The incubation was terminated by adding 9.5 ml of ice-chilled Krebs–Ringer solution to the cell suspension. In order to remove the extracellular $^{86}Rb^+$, the cell suspensions were washed twice by centrifugation at 600 *g* for 15 min at 4°C and resuspension. Cell lysis was performed by adding an aqueous solution of NaOH (final concentration 47.6 mmol l^{-1}) to the cell suspension, and the whole sample was then transferred to counting vials and mixed with Safe-fluor or Ecoscint. Cellular uptake of $^{86}Rb^+$ was measured in a Mark III Liquid Scintillation Spectrometer (Nuclear Chicago) using the preset window for ^{32}P, and the specific activity of potassium in the extracellular medium was used to calculate the cellular uptake of $K^+(^{86}Rb^+)$. Counting efficiency was 90%. In control experiments the washing procedure was evaluated by prolonged incubation of the cells at 4°C (up to 65 min) after adding the ice-cold Krebs–Ringer solution for the termination of the incubation. This prolonged incubation at 4°C did not change the value of the cellular uptake of $K^+(^{86}Rb^+)$.

Solutions

The calcium-free Krebs–Ringer solution had the following composition (mmol l^{-1}): NaCl 133.8, KCl 4.7, $MgSO_4$ 1.2, Tris-HCl 12.5.

The calcium- and potassium-free Krebs–Ringer solution had the following composition (mmol l^{-1}): NaCl 138.4, $MgSO_4$ 1.2, Tris-HCl 12.5.

The calcium- and potassium-containing Krebs–Ringer solution had the following composition (mmol l^{-1}): NaCl 132.4, KCl 4.7, $CaCl_2$ 1.0, $MgSO_4$ 1.2, Tris-HCl 12.5.

The solutions contained bovine serum albumin, 1 mg ml^{-1}, and glucose, 1 mg ml^{-1}. The pH of the solutions was adjusted to 7.4.

In the experiments in Figure 3 changes in the calcium concentration were balanced by changes in the concentration of NaCl to maintain isotonicity. Phosphate buffer, 12.5 mmol l^{-1}, was used in the experiments in Figure 6.

Materials

Bovine serum albumin was supplied by Sigma Chemical Company (St. Louis, U.S.A.), Percoll by Pharmacia Fine Chemicals (Sweden), Safe-Fluor and Ecoscint by BN Plastic (Helsinge, Denmark) and ^{86}Rb by Amersham (Buckinghamshire, U.K.). The radioactive rubidium was always used within 3 months of manufacture in order to minimize complications resulting from the progressive increase in ^{134}caesium relative to ^{86}Rb. Ouabain and all other chemicals were of analytical grade.

Results

Potassium

After the cell isolation procedure at 4°C, mast cells suspended in a calcium-free medium containing potassium were transferred to a 37°C waterbath for the experiments. This resulted in a high total uptake of $K^+(^{86}Rb^+)$ initially, and that was followed by a time-dependent decrease in the uptake (Figure 1). After 20–30 min incubation a steady state level of cellular $K^+(^{86}Rb^+)$-uptake was observed. This lasted

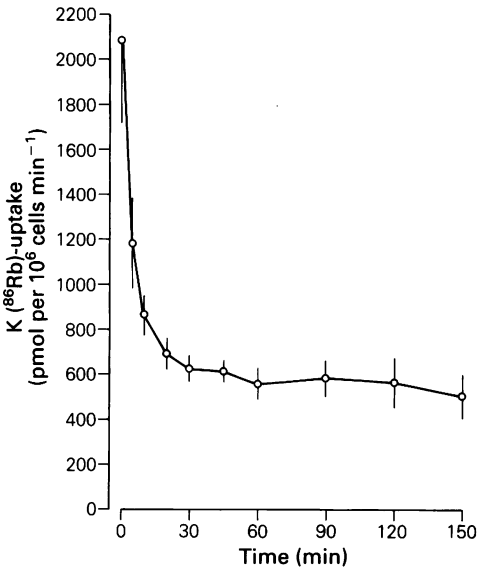

Figure 1 Time course of changes in $K^+(^{86}Rb^+)$-uptake into mast cells after transference from the low temperature (4°C) used during cell isolation to 37°C. The cells were incubated in a calcium-free medium with potassium, 4.7 mmol l^{-1}. Abscissa scale: time of preincubation. Ordinate scale: rate of $K^+(^{86}Rb^+)$-uptake. Mean values from five experiments are shown, vertical lines indicate s.d.

for up to 150 min, which was the longest period of observation. The steady state level of $K^+(^{86}Rb^+)$-uptake was 600 pmol per 10^6 cells min^{-1}, which was about 33% of the initial value.

Calcium

Deprivation of the mast cells of extracellular calcium for 60 min by incubation in calcium-free medium resulted in a large increase in the ouabain-sensitive cellular $K^+(^{86}Rb^+)$-uptake (Figure 2). The ouabain-sensitive uptake into calcium-deprived cells was 381 ± 125 pmol per 10^6 cells min^{-1} (mean ± s.d.) and the ouabain-sensitive control value from cells incubated in presence of calcium, 1 mmol l^{-1}, was 52 ± 23 pmol per 10^6 cells min^{-1} ($P < 0.001$ by two-tailed t test of the two groups of data). In contrast, the ouabain-resistant $K^+(^{86}Rb^+)$-uptake was the same in the absence and presence of calcium (mean values ± s.d. were 147 ± 21 and 147 ± 22 pmol per 10^6 cells min^{-1}, respectively).

Following calcium-deprivation, exposure of the cells to calcium (0.1–10 mmol l^{-1}) for 15 min decreased the ouabain-sensitive $K^+(^{86}Rb^+)$-uptake, but had no effect on the ouabain-resistant uptake. The ouabain-sensitive uptake of $K^+(^{86}Rb^+)$ was

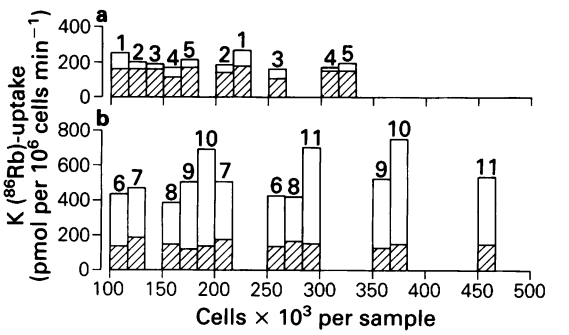

Figure 2 Effect of calcium on cellular potassium uptake. The mast cells were preincubated in the presence and absence of ouabain, 1 mmol l^{-1}, for 60 min at 37°C either in a medium containing calcium, 1 mmol l^{-1}, (a) or in a calcium-free medium (b). In both cases potassium, 4.7 mmol l^{-1}, was present. The incubation with $K^+(^{86}Rb^+)$ lasted 10 min. Abscissa scale: the cell density. Ordinate scale: $K^+(^{86}Rb^+)$-uptake. The hatched areas of the columns indicate the uptake in presence of ouabain and the open areas represent the difference between $K^+(^{86}Rb^+)$-uptake in the absence and presence of ouabain. The numbers indicate results obtained with the same cell suspension.

decreased 43% by calcium, 0.1 mmol l^{-1}, compared to the control value observed in calcium-free medium (Figure 3). Maximal decrease to a value of about 15% of control value (i.e. 85% decrease) was observed in the presence of calcium, 1.2 mmol l^{-1}.

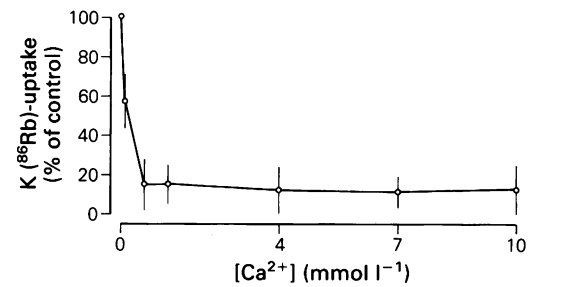

Figure 3 Dose-dependent inhibition of sodium–potassium pump activity by calcium. After 60 min preincubation in the absence of calcium and 15 min in the absence or presence of ouabain, 1 mmol l^{-1}, the cells were exposed to various concentrations of calcium (0.1–10 mmol l^{-1}) for 15 min and then incubated with $K^+(^{86}Rb^+)$ (potassium 4.7 mmol l^{-1}) for 5 min. Control samples were treated as above, but without calcium in the last preincubation of 15 min. Abscissa scale: the calcium concentration. Ordinate scale: ouabain-sensitive $K^+(^{86}Rb^+)$-uptake as % of control samples incubated without calcium: 464 ± 129 pmol per 10^6 cells min^{-1} (mean ± s.d.). Mean values from five experiments are shown; vertical lines indicate s.d.

Higher concentrations of calcium had no further effect on the uptake of $K^+(^{86}Rb^+)$ ($P > 0.1$ by two-tailed t test of the data for $K^+(^{86}Rb^+)$-uptake at 1.2 mmol l^{-1} calcium compared with the uptake at 4.0, 7.0, and 10.0 mmol l^{-1} of calcium). The ouabain-resistant $K^+(^{86}Rb^+)$-uptake of mast cells incubated in the absence of calcium (186 ± 24 pmol per 10^6 cells min^{-1}) was not significantly different from the uptake by cells incubated in presence of calcium, 0.1–10 mmol l^{-1} ($P > 0.05$ or higher by two-tailed t test for unpaired data).

Ouabain and metabolic inibitors

Low concentrations of ouabain, 8 nmol l^{-1} to 8 μmol l^{-1}, inhibited the uptake of $K^+(^{86}Rb^+)$ in calcium-free medium by 5–11%, but there was an increase in the inhibition to 71% when the concentration of ouabain was increased to 0.8 mmol l^{-1} (Figure 4).

While the ouabain-sensitive $K^+(^{86}Rb^+)$-uptake was almost blocked when the cells were pretreated with both 2-deoxyglucose and antimycin A (Figure 5), no significant change in the ouabain-resistant uptake was observed ($P > 0.05$ by two-tailed t-test for two groups of data).

Temperature and pH

The cellular $K^+(^{86}Rb^+)$-uptake in calcium-free medium was dependent on the incubation temperature as shown in Figure 6. The total and the

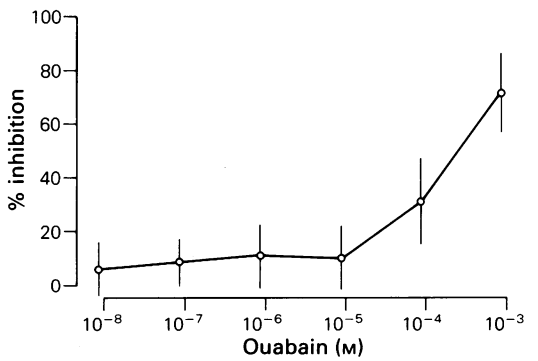

Figure 4 Dose-dependent inhibition of $K^+(^{86}Rb^+)$-uptake by ouabain. The cells were preincubated in a calcium-free medium for 60 min in the presence of potassium, 4.7 mmol l^{-1}, and various concentrations of ouabain (10 nmol l^{-1} to 1.0 mmol l^{-1}) and then incubated with $K^+(^{86}Rb^+)$ for 10 min. Abscissa scale: the concentrations of ouabain during incubation. Ordinate scale: % inhibition of cellular $K^+(^{86}Rb^+)$-uptake determined from comparison with uptake in samples not exposed to ouabain (582 ± 126 pmol per 10^6 cells min^{-1}). Mean values from five experiments are shown; vertical lines indicate s.d.

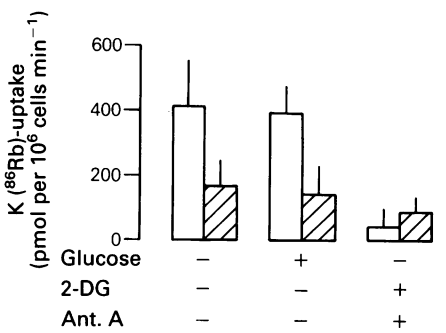

Figure 5 Effect of inhibitors of energy metabolism on the uptake of $K^+(^{86}Rb^+)$. The cells were preincubated for 60 min at 37°C in a calcium-free medium with potassium, 4.7 mmol l^{-1}. 2-Deoxyglucose (2-DG, 5 mmol l^{-1}) was present for the last 20 min of the preincubation period, and antimycin A (Ant. A, 1 μmol l^{-1}) was present for the last 15 s. The ouabain-resistant uptake was determined in samples preincubated similarly and with ouabain, 1.0 mmol l^{-1}, added to the cell suspension. The cells were incubated for 2 min with $K^+(^{86}Rb^+)$. Mean values from five experiments; vertical lines show s.d. Open columns, ouabain-sensitive uptake; hatched columns, ouabain-resistant uptake.

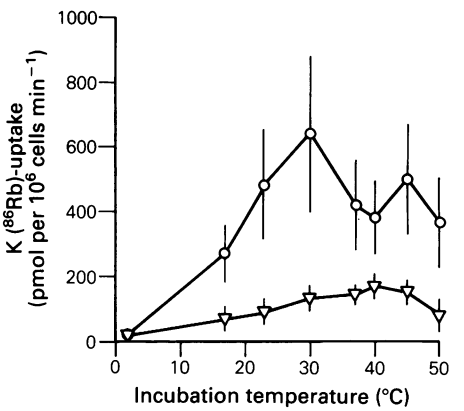

Figure 6 Effect of temperature on ouabain-sensitive and ouabain-resistant uptake of potassium. After preincubation for 60 min at 37°C in a calcium-free medium containing potassium, 4.7 mmol l^{-1}, the cells were equilibrated for 15 min at various temperatures (4–50°C) and then incubated with $K^+(^{86}Rb^+)$ for 5 min. The ouabain-sensitive uptake (○) is the difference between cellular $K^+(^{86}Rb^+)$-uptake in the absence (not shown) and presence (▽) of ouabain. The ouabain-resistant uptake was determined in cells that were exposed to ouabain, 1.0 mmol l^{-1}, during the whole incubation procedure. Abscissa scale: incubation temperature. Ordinate scale: rate of $K^+(^{86}Rb^+)$-uptake. Mean values from five experiments are shown; vertical lines indicate s.d.

ouabain-resistant $K^+(^{86}Rb^+)$-uptake were measured and used to calculate the ouabain-sensitive uptake, which appeared with two apparent peak values at 30°C and at 45°C. The maximum ouabain-sensitive uptake of $K^+(^{86}Rb^+)$ at 30°C and 45°C were (in pmol per 10^6 cells min^{-1}) 645 ± 242 and 498 ± 166, respectively. There was a gradual increase in the ouabain-resistant uptake up to 40°C (159 ± 31 pmol per 10^6 cells min^{-1}).

The ouabain-sensitive $K^+(^{86}Rb^+)$-uptake of the cells was pH-dependent. The maximum uptake was observed at pH 7.3–7.6, and above and below that range the uptake decreased (Figure 7). Changes in pH from 5.8–8.2 had almost no effect on the ouabain-resistant $K^+(^{86}Rb^+)$-uptake.

Discussion

Incubation of rat mast cells for 60 min in a calcium-free medium uncovered a large capacity ouabain-sensitive $K^+(^{86}Rb^+)$-uptake mechanism in the intact cells. The ouabain-sensitive $K^+(^{86}Rb^+)$-uptake was almost completely blocked by incubation of the cells with 2-deoxyglucose and antimycin A, agents which are known to inhibit glycolytic and mitochondrial adenosine 5'-triphosphate (ATP) production in mast cells (Diamant *et al.*, 1974; Garland & Johansen, 1977; Johansen, 1980). In addition, the activity of this uptake mechanism was dependent on temperature and pH. These observations seem to indicate that the ouabain-sensitive uptake of $K^+(^{86}Rb^+)$ into rat mast cells in a calcium-free medium is due to the activity of the Na^+–K^+ pump located in the plasma membrane.

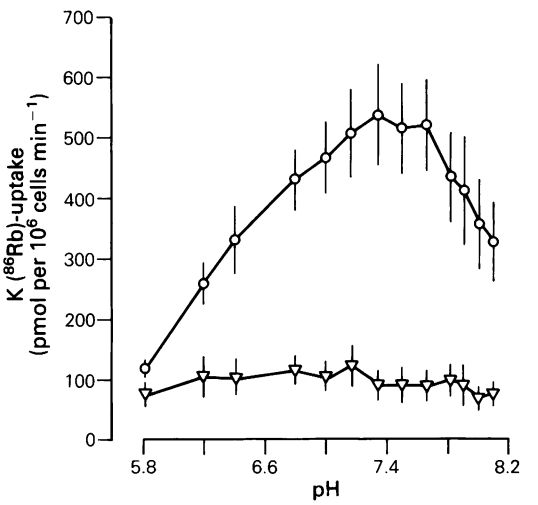

Figure 7 Effect of pH on the ouabain-sensitive (○) and ouabain-resistant (▽) $K^+(^{86}Rb^+)$-uptake of mast cells. These were preincubated for 60 min at 37°C in a calcium-free medium containing potassium, 4.7 mmol l^{-1}. After centrifugation (at room temperature) they were resuspended and equlibrated at 37°C for 15 min at various pH. Then the cells were incubated for 5 min with $K^+(^{86}Rb^+)$. The ouabain-resistant uptake was determined in cells equilibrated at various pH for 15 min in the presence of ouabain, 1.0 mmol l^{-1}. Abscissa scale: the pH of the incubation medium. Ordinate scale: rate of $K^+(^{86}Rb^+)$-uptake. Mean values from five (pH 7.9–8.1) or six (pH 5.8–7.8) experiments; vertical lines show s.d.

During preparation of mast cells the passive transport of Na^+ into the cells is not counteracted by active extrusion of Na^+ by the Na^+–K^+ pump, since that is inhibited by the low temperature used for cell isolation. Consequently, the cellular content of Na^+ may increase during the cell isolation procedure. Due to the enhanced stimulation of the pump from inside the cells by Na^+ a decrease in the cellular content of Na^+ may follow, and this is a likely explanation of the drastic decrease in $K^+(^{86}Rb^+)$-uptake observed after transference of the cells to 37°C (Figure 1). Similarly, a decrease in the cellular content of K^+ during cell isolation and the subsequent increase after incubation at 37°C may contribute to the drastic decrease in cellular $K^+(^{86}Rb^+)$-uptake. By interpolation it seems that the high initial $K^+(^{86}Rb^+)$-uptake was decreased by 50% after 10 min incubation at 37°C. Consequently, for the experiments to be performed with mast cells in a steady state, i.e. in equilibrium with the external medium, measurements were always preceded by 60 min of preincubation at 37°C in the presence of extracellular potassium, 4.7 mmol l^{-1}.

The calcium ion is known to inhibit the activity of the isolated Na^+, K^+-ATPase (Skou, 1957). This occurs by competition for cation binding sites for Mg^{2+} and Na^+ (Tobin *et al.*, 1973; Lindenmayer & Schwartz, 1975; Apell & Marcus, 1986), i.e. at the cytoplasmic face of the plasma membrane. Compared to the intracellular Ca^{2+} concentration, which is below 1 μmol l^{-1}, a large increase in the Ca^{2+} concentration would be necessary for calcium to influence the binding sites for Mg^{2+} and Na^+, since the dissociation constants for calcium at the two sites are 55 and 405 μmol l^{-1}, respectively (Lindenmayer & Schwartz, 1975). Except for active pumping of Ca^{2+} into the cell, there is no transport mechanism that could increase the intracellular Ca^{2+} concentration sufficiently and thus binding at an intracellular site is not a likely mechanism for the inhibition of the pump activity due to reintroduction of Ca^{2+}, 0.1 mmol l^{-1}, into the medium.

In a calcium-free medium, cells lose K^+ and gain Na^+ as a consequence of increased plasma membrane permeability (for references: see Macknight &

Leaf, 1977). Since the activity of the pump in the presence of a fixed concentration of K^+ in the medium is dependent on the intracellular concentration of Na^+, the active extrusion of cell Na^+ by the pump counterbalances the passive transport of Na^+ into the cell. Thus, changes in the permeability of the plasma membrane are reflected by changes in pump activity in the same direction. Our observation of an increased pump activity in mast cells incubated in a calcium-free medium may be explained by an increased membrane permeability due to the lack of calcium.

In conclusion, we have demonstrated a ouabain-sensitive and a ouabain-resistant uptake mechanism for K^+($^{86}Rb^+$) in intact rat mast cells. The ouabain-sensitive uptake, which is energy-dependent and has the characteristics of an enzyme, is mediated by the Na^+–K^+ pump located in the plasma membrane. The activity of the Na^+–K^+ pump but not the ouabain-resistant uptake mechanism is enhanced by calcium-deprivation of the cells, and reintroduction of calcium into the medium, even in a low concentration, inhibits the pump activity. A likely explanation of the calcium-sensitivity of the pump activity is that it is due to changes in the permeability of the plasma membrane. In a calcium-free medium the permeability may increase, and increased entry of Na^+ into the cells may be reflected as increased pump activity.

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Elevation of intracellular cyclic AMP concentration fails to inhibit adrenaline-induced hyperpolarization in amphibian sympathetic neurones

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1 The effect of drugs on the adenosine 3':5'-cyclic monophosphate (cyclic AMP) content of desmethylimipramine (DMI)-treated bullfrog paravertebral sympathetic ganglia was studied by radioimmunoassay. The adrenaline-induced hyperpolarization (Ad_h) in this tissue was recorded by means of the sucrose-gap technique.

2 In the presence of propranolol (1 μM) and DMI (0.5 μM), adrenaline (1 μM) significantly reduced the concentration of cyclic AMP in forskolin-treated ganglia. This effect was prevented by pertussis toxin (5 $\mu g\,ml^{-1}$).

3 The relative potency for drugs which increased ganglionic cyclic AMP content was: 50 μM forskolin $\gg$ 5 mM fluoride > 2 mM fluoride > 2 mM isobutylmethylxanthine (IBMX) > 5 mM caffeine. In contrast, their relative potency for inhibition of the Ad_h was: 2 mM IBMX > 5 mM fluoride > 5 mM caffeine $\gg$ 2 mM fluoride > 50 μM forskolin. The Ad_h was unaffected by pertussis toxin (5 $\mu g\,ml^{-1}$).

4 Although the Ad_h was slightly reduced by the extracellular application of 8-bromo (8-Br) cyclic AMP, the majority of the data suggest that the transduction mechanism underlying the Ad_h is independent of the intracellular cyclic AMP concentration and provide an example of an α_2-adrenoceptor-mediated response that occurs independently of inhibition of adenylate cyclase.

Introduction

The α_2-adrenoceptor is thought to be coupled to adenylate cyclase via the inhibitory guanine nucleotide binding protein (G_i) (Exton, 1985; Gilman, 1987). Binding of GTP induces dissociation of G_i into α- and β-γ subunits. The α-G_i subunit inhibits adenylate cyclase and thereby reduces the rate of synthesis of adenosine 3':5'-cyclic monophosphate (cyclic AMP) (Sabol & Nirenberg, 1979; but see also Gilman, 1987). Andrade & Aghajanian (1985), Aghajanian & Wang (1987) and Wang & Aghajanian (1987) have suggested that the resulting decrease in cyclic AMP levels may be involved in the generation of the physiological response to α_2-adrenoceptor activation. Certain electrophysiological data lend support to this hypothesis. For example, Crain *et al.* (1986) showed that the depressant effects of both opiates and noradrenaline on synaptic transmission in spinal cord cultures could be reduced following treatment with the adenylate cyclase activator, forskolin. Also, Andrade & Aghajanian (1985) demonstrated that membrane-permeable cyclic AMP analogues inhibited α_2-adrenoceptor-induced hyperpolarization in rat locus coeruleus neurones. We have tested the involvement of adenylate cyclase inhibition in the production of an α_2-adrenoceptor-mediated response in a simple and convenient preparation, the amphibian sympathetic ganglion. In this tissue, the hyperpolarization induced by adrenaline (Ad_h, Koketsu & Nakamura, 1976; Smith, 1984; Rafuse & Smith, 1986; Selyanko *et al.*, 1988) has characteristics similar to α_2-adrenoceptor-mediated responses in mammalian neurones (Aghajanian & VanderMaelen, 1982; North *et al.*, 1987). If the transduction mechanism associated with the Ad_h involves adenylate cyclase inhibition, the transduction process would involve a decrease in the concentration of intracellular cyclic AMP. If this were the case, drugs which increase neuronal cyclic AMP content should attenuate the Ad_h and their order of potency should be directly correlated with their ability to increase intracellular cyclic AMP concentration. We therefore compared the effects of various drugs on intracellular cyclic AMP concentration with their ability to attenuate the Ad_h. A combined electrophysiological and biochemical approach has not previously been applied

to the analysis of α_2-adrenoceptor mechanism in any one system (see Aghajanian & Wang, 1987; North *et al.*, 1987).

Methods

Sucrose-gap experiments

The Ad_h was recorded from the IXth or Xth paravertebral sympathetic ganglia of bullfrogs (*Rana catesbeiana*) by means of the sucrose-gap technique (Smith & Weight, 1986). All drugs, except forskolin, were dissolved in Ringer solution of the following composition (mM): NaCl 100, KCl 2, $CaCl_2$ 1.8, tris(hydroxymethyl)-aminomethane-HCl (Tris) (pH 7.2) 16, and (+)-glucose 10. Desmethylimipramine (DMI; 500 nM) was added to the Ringer solution to potentiate the response to adrenaline (Smith, 1984; Rafuse & Smith, 1986). Forskolin was initially dissolved in dimethylsulphoxide (DMSO) and further diluted in Ringer solution such that the final concentration of DMSO was 1% by volume. Ringer solution containing 1% DMSO did not affect the Ad_h. All drugs were administered by superfusion and pertussis toxin was recycled into the recording chamber by means of a mini-perfusion pump. Drug responses were recorded on a d.c. rectilinear pen recorder (Gould-Brush 2400, filters set to −3 db at 5 Hz). Downward deflections on the illustrated records indicate membrane hyperpolarization. Experiments were performed at room temperature (20°C).

Biochemical experiments

The IXth and Xth paravertebral sympathetic ganglia were excised from pithed bullfrogs and dissected free of excess connective tissue. Ganglia were weighed and placed in separate small containers which facilitated their transfer from solution to solution whilst limiting tissue manipulation. To ensure reproducible measurements of basal cyclic AMP concentration, each ganglion was allowed to equilibrate in Ringer solution for at least 2 h (Smith *et al.*, 1979). Each group ($n = 6$) of ganglia were then transferred to various test drug solutions for pre-determined time intervals. To control for possible changes in cyclic AMP levels as a result of tissue manipulation, control ganglia were handled in the same manner as test ganglia, but were exposed only to normal Ringer solution. Following drug exposure, ganglia were blotted to remove excess Ringer solution and immediately frozen in liquid nitrogen. Each ganglion was then fixed in 90% methanol: 0.1 M HCl for 2 h at −20°C. After fixation, individual ganglia were homogenized in 0.5 ml of 6% trichloroacetic acid. Particulate and cell debris were removed by 4 min centrifugation at 12 000 *g* and the supernatant stored at −70°C before cyclic AMP determination. The more sensitive acetylated method, described in the RIANEN™ cAMP[^{125}I] radioimmunoassay kit (Dupont), was followed with one minor modification; the initial sample was diluted to 1:2.5 rather than 1:5 to increase assay sensitivity. Ganglion protein content was determined by the Bradford method (Bradford, 1976) and concentrations of cyclic AMP expressed as pmol mg^{-1} protein.

Biological activity of pertussis toxin samples was verified by monitoring the inhibition of the respiratory burst response in human neutrophils following treatment with the chemotactic peptide, F-Met-Leu-Phe (FMLP) (Bokoch & Gilman, 1984). All data are presented as mean ± s.e.mean and statistical significance determined by Student's two-tailed, unpaired *t* test. All drugs and chemicals were purchased from Sigma (St. Louis MO. U.S.A.), except for desmethylyimipramine (Geigy, Canada), pertussis toxin (List Biologicals, La Jolla, CA. U.S.A.), caffeine, DMSO and trichloroacetic acid (Fisher Scientific, Canada), propranolol (Ayerst) and sodium fluoride (Matheson, Coleman and Bell, Norwood, OH. U.S.A.).

Results

Effect of adrenaline on forskolin-stimulated cyclic AMP accumulation

If α_2-adrenoceptor agonists inhibit adenylate cyclase, they should decrease the intracellular concentration of cyclic AMP. Since it is difficult to demonstrate unequivocally a decrease in low basal cyclic AMP levels (Duman & Enna, 1986), we first stimulated adenylate cyclase with forskolin (Seaman, 1985). As shown in Figure 1, forskolin 50 μM produced a 10 fold increase in basal cyclic AMP levels and a subsequent 30 s exposure to adrenaline (1 μM) reduced cyclic AMP accumulation in forskolin-stimulated ganglia by approximately 35%. Propranolol (1 μM) and DMI (500 nM) were added to the Ringer solution to prevent activation of β-adrenoceptors (Brown *et al.*, 1979) and inactivation of adrenaline by uptake (Rafuse & Smith, 1986).

Effect of pertussis toxin on G_i

If the amphibian α_2-adrenoceptor (Rafuse & Smith, 1986) is coupled to adenylate cyclase via G_i, then pretreatment with pertussis toxin, which ADP-ribosylates certain G-proteins (Katada & Ui, 1982; Gilman, 1987), might be expected to prevent the adrenaline-induced reduction in cyclic AMP concentration in propranolol/forskolin/DMI treated ganglia. Figure 1 shows that the effect of adrenaline was greatly reduced in ganglia which had been

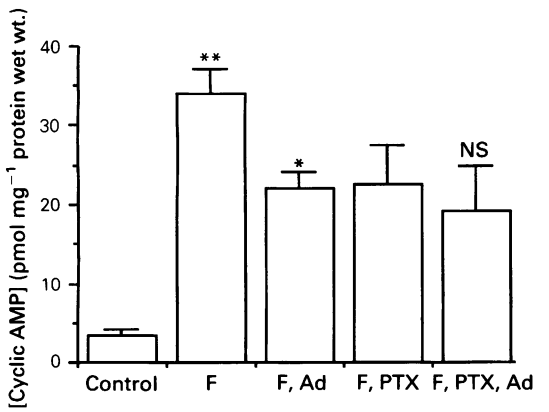

Figure 1 Effects of forskolin (F), adrenaline (Ad) and pertussis toxin (PTX) on cyclic AMP concentration in bullfrog sympathetic ganglia. Ringer solution contained 500 nM desmethylimipramine and 1 μM propranolol. Data obtained from 6 experiments or more. Ganglia were exposed to 50 μM forskolin for 15 min or to forskolin for 14 min 30 s, then to forskolin + 1 μM adrenaline for a further 30 s. For the experiments involving pertussis toxin, ganglia were incubated with 5 μg ml^{-1} of the toxin for 2 h at 20°C before the application of forskolin or forskolin + adrenaline. Error bars indicate s.e.mean. ** $P < 0.001$ compared to control; * $P < 0.005$ compared to forskolin; NS, not significant compared to forskolin + pertussis toxin.

placed for 2 h in Ringer solution containing pertussis toxin 5 μg ml^{-1}.

Effects of phosphodiesterase inhibitors and stimulators of adenylate cyclase on cyclic AMP levels and the adrenaline-induced hyperpolarization

If inhibition of adenylate cyclase is involved in the generation of α_2-adrenoceptor-induced hyperpolarization, the Ad_h should be attenuated by manipulations which increase cytosolic cyclic AMP concentration (Andrade & Aghajanian, 1985). Furthermore, the ability of drugs to elevate cyclic AMP levels should correlate with their ability to attenuate the Ad_h. Table 1 shows the effects of the phosphodiesterase inhibitors, isobutylmethylxanthine (IBMX) and caffeine, and the adenylate cyclase activators, fluoride and forskolin, on concentrations of cyclic AMP in bullfrog sympathetic ganglia. The order of potency for increasing intracellular cyclic AMP concentration was 50 μM forskolin $\gg$ 5 mM fluoride > 2 mM fluoride > 2 mM IBMX > 5 mM caffeine. On the other hand, their order of potency for attenuation of the Ad_h was 2 mM IBMX > 5 mM fluoride > 5 mM caffeine $\gg$ 2 mM fluoride > 50 μM forskolin. Table 1 also shows that most of the drugs tested against the Ad_h produced membrane depolarization. Ganglia were exposed to all drugs for 15 min

Table 1 Effects of various drugs on cyclic AMP concentration, the adrenaline-induced hyperpolarization and apparent membrane potential of bullfrog sympathetic ganglia

Drug	[*Cyclic* AMP] (pmol mg^{-1} protein, wet wt)	*Increase in cyclic AMP* (% of control)[1,5]	P *value for cyclic AMP increase*	*Attenuation*[1] *of* Ad_h (%)	*Depolarization* (mV)
IBMX (2 mM)	5.3 ± 1.3 (6)	155.2	>0.2	63.5 ± 5.5 (6)	+0.96 ± 0.22 (6)
Fluoride (5 mM)	7.2 ± 0.8 (6)	208.7	<0.005	46.2 ± 10.2 (4)	+0.20 ± 0.13 (2)
Fluoride (2 mM)	6.4 ± 1.3 (6)	185.5	<0.05	7.2 ± 4.4 (6)	+0.27 ± 0.09 (3)
Caffeine (5 mM)	4.8 ± 0.8 (6)	139.0	>0.2	38.2 ± 7.3 (5)	+0.91 ± 0.29 (5)
8-Br-cyclic AMP (1 mM)	—	—	—	21.8 ± 7.2 (8)	+0.93 ± 0.35 (3)
Pertussis[2] toxin (5 μg ml^{-1})	—	—	—	2.2 ± 11.3 (4)	—
Forskolin[4] (1–100 μM)	34.0 ± 3.1 (17)	985.5	<0.001	−6.6 ± 11.4 (5[3])	+1.01 ± 0.09 (5)

[1] Ganglia exposed to all drugs (except pertussis toxin) for 15 min.
[2] Ganglia exposed to pertussis toxin for 2 h.
[3] In the presence of forskolin, the Ad_h was potentiated to 106.6 ± 11.4% of control.
[4] For cyclic AMP determinations, all ganglia were exposed to 50 μM forskolin.
[5] Control [cyclic AMP] = 3.44 ± 0.65 pmol mg^{-1} protein wet wt. s.e.mean, $n = 16$.
Number of observations (n) given in parentheses; values are ± s.e.mean.

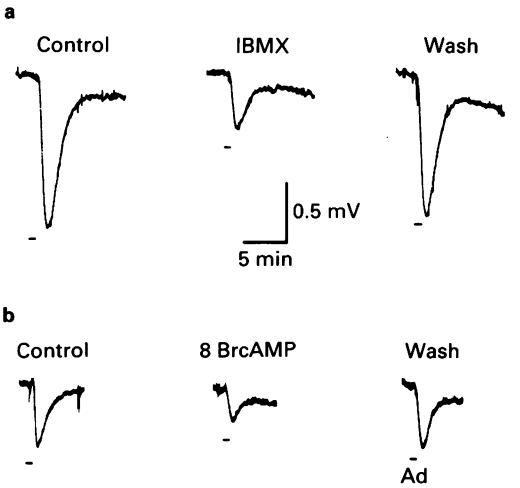

Figure 2 Effects of drugs on the adrenaline-induced hyperpolarization (Ad_h). Sucrose-gap recording from bullfrog sympathetic ganglia in the presence of 500 nM desmethylimipramine. (a) Effects of isobutylmethylxanthine (IBMX); Left hand record, control response to 30 s superfusion of 1 μM adrenaline; centre record, Ad_h recorded after 15 min superfusion of 2 mM IBMX; right hand record, Ad_h recorded 30 min after IBMX was washed out. (b) Effect of 8-Br cyclic AMP (8-BrcAMP); left hand record, control response to 30 s superfusion of 1 μM adrenaline; centre record, Ad_h recorded after 15 min superfusion of 1 mM 8-Br cyclic AMP; right hand record, Ad_h recorded 40 min after 8-Br cyclic AMP was washed out. Traces from rectilinear pen recorder. Black bar under responses indicates period of superfusion of adrenaline (Ad).

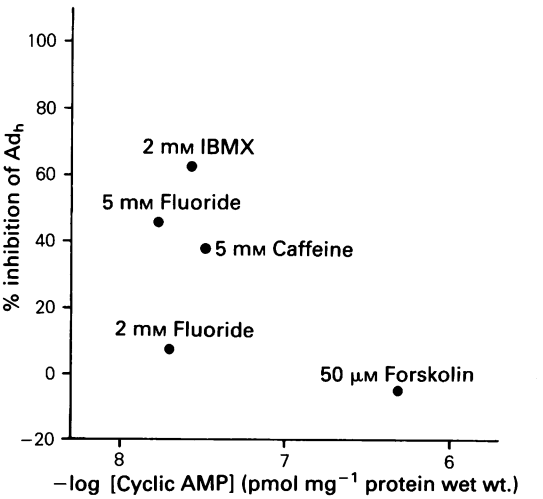

Figure 3 Comparison of the effects of drugs on intracellular cyclic AMP concentration with their effects on the adrenaline-induced hyperpolarization (Ad_h). Abscissa scale: intracellular cyclic AMP concentration determined by radioimmunoassay following 15 min exposure to each drug (log scale, cf. Table 1). Ordinate scale: percentage inhibition of Ad_h following 15 min exposure to the same drugs (cf. Table 1). IBMX = isobutylmethylxanthine.

in both biochemical and electrophysiological experiments. The effect of IBMX on the Ad_h is illustrated in Figure 2a. The lack of correlation between cyclic AMP concentration and attenuation of the Ad_h is further illustrated in Figure 3. The percentage inhibition of the Ad_h is plotted against the logarithm of the cyclic AMP content from Table 1. There is no linear correlation between the log 'agonist' concentration (cyclic AMP) and the observed response (attenuation of the Ad_h).

Effect of pertussis toxin on the adrenaline-induced hyperpolarization

Extracellularly-applied pertussis toxin inhibits the adrenaline-induced reduction in cyclic AMP levels in amphibian sympathetic ganglia (Figure 1). If the Ad_h involves inhibition of adenylate cyclase it should also be antagonized by the toxin. However, when ganglia were superfused with high concentrations of pertussis toxin (5 μg ml^{-1}) for 2 h at room temperature, no significant depression of the Ad_h was observed (Table 1).

Effect of 8-bromo-cyclic AMP on the adrenaline-induced hyperpolarization

Andrade & Aghajanian (1985) reported that membrane permeable analogues of cyclic AMP reduced α_2-adrenoceptor-mediated hyperpolarization in rat locus coeruleus neurones. A similar reduction of the Ad_h was observed in bullfrog sympathetic ganglia (Table 1). The response was reduced to 78.2 $\pm$ 7.2% of control ($n = 8$) after 15–45 min superfusion of 1 mM 8-Br-cyclic AMP and returned to control amplitude following 15–45 min wash out of the nucleotide. A typical experiment is illustrated in Figure 2*b*. 8-Br-cyclic AMP produced a small depolarization in 3 out of 8 experiments (Table 1).

To ensure that the antagonism of Ad_h was not due to stimulation of adenosine receptors by 8-Br-cyclic AMP or its metabolites (Aghajanian & Wang, 1987), we tested the effect of an adenosine receptor agonist on the Ad_h. The response was not antagonized by 2-chloro-adenosine (10 μM–1 mM).

Discussion

The main findings of the present work are that (i) elevation of intracellular cyclic AMP levels fails to

attenuate the Ad_h in amphibian sympathetic ganglia and (ii) the Ad_h is insensitive to pertussis toxin, even though extracellular application of this substance appears to affect G_i. This implies that adenylate cyclase inhibition is independent of the production of this α_2-adrenoceptor-mediated response (Rafuse & Smith, 1986). One possible explanation for the inhibition of the Ad_h by methylxanthines is that these substances cause depolarization associated with an increase in membrane conductance (Kuba & Nishi, 1976) and this would be expected to reduce the amplitude of the Ad_h (Koketsu & Nakamura, 1976). The effect of extracellularly applied 8-Br-cyclic AMP is difficult to explain on this basis because this substance often antagonized the Ad_h without altering membrane potential. This observation may have little significance in the light of some whole-cell patch clamp studies recently performed in our laboratory (Selyanko *et al.*, 1988). We have found that adrenaline-induced outward currents can be recorded in amphibian sympathetic ganglion cells and that the appearance of such responses is favoured by the presence of cyclic AMP in the patch pipette, i.e. the response is clearly not inhibited following elevation of intracellular cyclic AMP concentration.

Several lines of evidence support the hypothesis that a G-protein is involved in the transduction process for α_2-, muscarinic and opiate receptors (Pfaffinger *et al.*, 1985; Aghajanian & Wang, 1986; 1987; Wang & Aghajanian, 1987; Crain *et al.*, 1987; North *et al.*, 1987). Indeed, the inhibition of the Ad_h by 5 mM fluoride may be due to a direct interaction with a G-protein (Pfaffinger, 1987; Gilman, 1987). The lack of effect of pertussis toxin on the Ad_h implies that any G-protein involved in this response is toxin-insensitive. It cannot be argued that the toxin does not have access to the G-protein because extracellularly-applied pertussis toxin prevents the adrenaline-induced depression of cyclic AMP levels in forskolin-treated ganglia (Figure 1). This effect on G_i was unexpected because no known substrate has been reported to exist for pertussis toxin-induced ADP-ribosylation in membrane fractions of bullfrog sympathetic ganglia (Pfaffinger, 1987). The discrepancy between our results and those of Pfaffinger (1987) remains to be resolved.

α_2-Adrenoceptor agonists are thought to share a common effector mechanism with certain opiate agonists (Crain *et al.*, 1986; 1987; North *et al.*, 1987). Also, Aghajanian & Wang (1987) and Wang & Aghajanian (1987) suggested that the relationship between adenylate cyclase inhibition and neuronal membrane hyperpolarization may be more complex than initially reported. They proposed that stimulation of opiate receptors has a dual effect, (1) an opening of K^+ channels which is G-protein-mediated but cyclic AMP-independent and (2) a G-protein-mediated inhibition of adenylate cyclase which turns off a cyclic AMP-dependent inward current. A similar two-part mechanism might therefore underlie hyperpolarization induced by α_2-adrenoceptor agonists in amphibian ganglia. Our data, which demonstrate the complete lack of inhibition of the Ad_h by forskolin provide no support for this possibility.

The present comparison of physiological responses with biochemical changes in cyclic AMP levels in the same tissue has enabled us to dissociate clearly intracellular cyclic AMP concentration from the production of an α_2-adrenoceptor response. In view of data from other experimental systems (Nakaki *et al.*, 1983; Ullrich & Wollheim, 1984; Jones *et al.*, 1987; Musgrave *et al.*, 1987) it is perhaps time to question the general validity of the hypothesis that adenylate cyclase inhibition is involved in the production of α_2-adrenoceptor-mediated responses.

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Noradrenaline (gamma) and ATP responses of innervated and non-innervated rat cerebral arteries

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1 The distribution of sympathetic adrenergic nerves on the rat middle cerebral artery and on the arterioles which originated from it was determined by use of gloxylic histochemistry.

2 Whereas the middle cerebral artery and proximal arterioles arising from this artery received a sympathetic innervation, the distal regions of the same arterioles were devoid of innervation.

3 The arteries and arterioles which were innervated were depolarized by noradrenaline in the combined presence of α- and β-adrenoceptor antagonists. Those which were not innervated were not depolarized by noradrenaline.

4 ATP depolarized all arteries and arterioles examined.

5 These observations are discussed with respect to the similarities and differences between γ-adrenoceptors and P_2 purinoceptors.

Introduction

Sympathetic nerve stimulation evokes relatively brief excitatory junction potentials (e.j.ps) in many systemic arteries (Bolton & Large, 1986). Such e.j.ps are abolished by adrenergic neurone blocking agents such as guanethidine but are resistant to blockade by a wide variety of adrenoceptor antagonists (Neild & Zelcer, 1982). Two hypotheses have been suggested to explain these observations. One is that noradrenaline activates specialized receptors (γ-receptors), which are restricted to regions near sympathetic nerve varicosities (Hirst & Neild, 1981; Luff *et al.*, 1987). The alternative suggestion is that sympathetic nerves release adenosine triphosphate (ATP) as well as noradrenaline and that ATP produces the e.j.p. (Sneddon & Burnstock, 1984). Support for the 'purinergic' hypothesis comes from the observation that α-, β-methylene ATP (mATP), a stable analogue of ATP, desensitizes tissues to ATP and abolishes the e.j.p. (Sneddon & Burnstock, 1984). Recently the selectivity of mATP was questioned, Byrne & Large (1986) showing that this compound abolished the depolarization of rat basilar artery produced either by ATP or by noradrenaline acting on γ-receptors. They suggested that either mATP could not distinguish between P_2 receptors and γ-adrenoceptors or the two receptors were one and the same. The experiments described here were undertaken to see if the two receptor types could be distinguished on the basis of their distributions. Fine arterioles, which originate from the rat middle cerebral artery lack a sympathetic innervation (Hill *et al.*, 1986). These vessels should be devoid of γ-receptors if such receptors are only found close to the points of noradrenaline release. If γ-receptors and P_2 receptors are indeed one and the same, the fine vessels would be unresponsive to both agonists. Alternatively if the two receptors were distinct such a parallel might not be expected.

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Methods

All studies used the rat middle cerebral artery and pial vessels which originated from it. Adult rats (male or female, 180–200 g) were anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, i.p.), exsanguinated and the brain rapidly removed. The brain was transferred to gassed physiological saline and either the left or the right middle cerebral artery along with its associated arterioles dissected free (for further details see Hill *et al.*, 1986). The pattern of sympathetic innervation was determined from preparations incubated in buffered glyoxylic acid solution and processed as described by Furness & Costa (1975). For electrophysiological studies, preparations were pinned out in a small recording chamber (bath volume 0.2 ml). Physiological saline (composition,

mM: NaCl 120, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 2.0, NaH_2PO_4 0.1, $NaHCO_3$ 25 and glucose 11) which had been warmed to 37°C and gassed with $95\%O_2:5\%CO_2$, flowed continuously (flow rate $6\,ml\,min^{-1}$) through the chamber. Intracellular recordings were made from various regions (see Results) of the vascular tree by conventional techniques; in a few experiments responses of short segments of arteriole to agonists were voltage clamped using a single electrode voltage clamp technique (see Finkel *et al.*, 1984). Preparations were superfused with test solution by changing the inflow line from control physiological solution to one containing the appropriate concentration of drug. When the agonists noradrenaline or ATP were applied, the exposure time was 1 min; on most occasions an interval of 10 min was allowed between exposures.

Tissues were superfused with a solution containing the irreversible α-adrenoceptor antagonist benextramine (Melchiorre *et al.*, 1978), (10×10^{-6} M) for 30 min, followed by a 30 min wash period, before the start of each experiment. To prevent the possibility of β-receptor activation, propranolol (1×10^{-6} M) was also included in all physiological saline solutions. Drugs used were noradrenaline bitartrate, adenosine triphosphate, propranolol hydrochloride, cobalt chloride (all obtained from Sigma chemicals) and benextramine hydrochloride (Aldrich Chemicals).

Results

Histological studies

In each preparation, varicose nerve fibres with a characteristic catecholamine fluorescence were detected along the middle cerebral artery until its first major bifurcation (Figure 1a). A few fibres projected for variable distances (1 to 3 mm) along the arterioles which arose from this artery (Figure 1b and c). No fluorescent fibres were ever detected on the surfaces of the pial arterioles at distances greater than 4 mm from their origin on the middle cerebral artery (Figure 1d). In the electrophysiological experiments, cuts were made through the pial arterioles at distances greater than 4 mm from the main artery. Recordings made distal to these cuts were classed as being from 'non-innervated' arterial smooth muscle. Recordings made either from the middle cerebral artery or from arterioles less than 1 mm from their origin were classed as being from 'innervated' arterial smooth muscle.

Electrophysiological studies

Intracellular recordings were made from preparations which had first been incubated in benextramine and in the presence of propranolol (see Methods). The mean resting potential of innervated arteries was −67.0 mV (±0.76 mV, s.e.mean, $n = 17$), that of non-innervated arterioles was −61.7 ± 1.63 mV ($n = 16$). The difference between these two means reflects regional differences in membrane potassium rectifying properties (F.R. Edwards, G.D.S. Hirst and G.D. Silverberg, unpublished observations). Superfusion of noradrenaline (0.5 to 5 mM) invariably caused membrane depolarization of innervated arterioles, as shown in Figure 2a. The mean peak depolarization produced by 5 mM noradrenaline was 16.3 ± 1.7 mV ($n = 6$). The depolarizations persisted after the addition of cobalt chloride (1 mM) to the physiological saline, suggest-

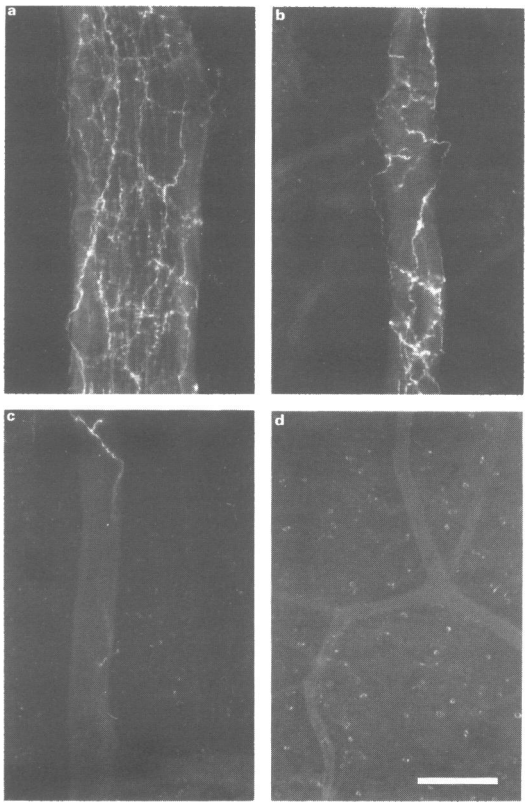

Figure 1 (a–d) Distribution of sympathetic nerve fibres on the rat middle cerebral artery and on the arterioles which originate from this artery. The photomicrograph (a) illustrates a region of the middle cerebral artery; (b) shows the innervation of an arteriole 1 mm from its origin; (c) terminal sympathetic innervation of same arteriole some 2.5 mm from its origin; (d) shows distal arterioles (4 mm from origin) which lack detectable sympathetic innervation. The calibration bar (100 μm) refers to each plate.

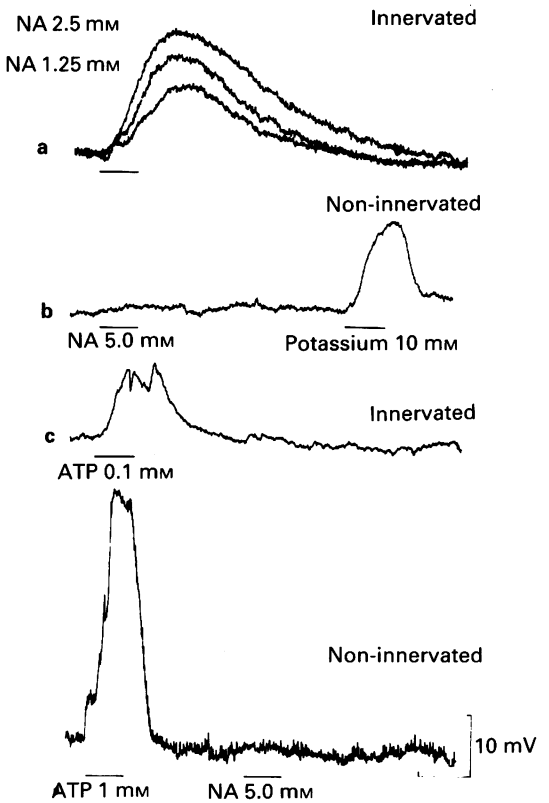

Figure 2 (a–d) Responses of 'innervated' and 'non-innervated' cerebral arteries and arterioles to noradrenaline and ATP. Each recording was made from a preparation which had been previously incubated in benextramine (1×10^{-5} M) to prevent α-adrenoceptor activation: propranolol (1×10^{-6} M) was also present throughout. (a) Shows the membrane potential depolarizations of rat middle cerebral artery produced by perfusing noradrenaline (2.5, 1.25 and 0.625 mM) for 1 min; (b) shows that noradrenaline (5 mM, applied for 1 min) failed to produce a detectable depolarization of a non-innervated segment of distal arteriole (same preparation as in a). Note that increasing the extracellular potassium concentration to 10 mM did cause a depolarization. In contrast both innervated (c) and non-innervated (d) arterial muscles were depolarized by ATP (1 mM and 0.1 mM, respectively). In (d), after the response to ATP, noradrenaline (5 mM) was applied and it again failed to depolarize the non-innervated arteriole. Calibration bars apply to each trace.

ing that noradrenaline was acting directly rather than causing release of a transmitter from sympathetic nerves.

In contrast, 5 mM noradrenaline, never depolarized the non-innervated arterioles of the same six preparations from which the 'innervated' data were obtained. Examples are shown in Figures 2b and d. In Figure 2b, after the application of noradrenaline (5 mM), the external concentration of potassium was doubled to confirm that the arteriole would respond to other depolarizing agents. In Figure 2d, noradrenaline (5 mM) was applied after a response to 0.1 mM ATP had been detected. ATP, applied for 1 min in a concentration of 1 mM or less, caused membrane depolarization of both innervated and non-innervated regions of the vascular tree (Figure 2c and d). ATP was more potent than noradrenaline in causing membrane depolarization (see also Byrne & Large, 1986). The mean depolarization produced by ATP (1 mM) when applied to innervated arteries was 14.0 ± 2.9 mV ($n = 5$). With non-innervated arterioles concentrations of ATP much lower than 1 mM also produced marked depolarizations, the threshold for a detectable depolarization being about 0.05 mM.

The responses of arterial smooth muscle to noradrenaline and ATP could be further distinguished. Visual observation of the preparations indicated that ATP constricted arterioles before the membrane potential change was fully developed, i.e. after some 5 mV depolarization. ATP, when applied to short segments of arteriole whose membrane potentials were being held constant at −65 mV with a single electrode voltage clamp, generated an inward current. Although the membrane potential remained constant the arteriole constricted. When noradrenaline was used as the agonist on innervated arteries, constriction was only detected when a substantial membrane depolarization (>20 mV) had been produced.

Discussion

These experiments indicate that γ-adrenoceptors and excitatory purine receptors have different distributions on rat pial arteries and arterioles. It is unlikely that noradrenaline and ATP activate a common receptor. ATP directly increases the calcium permeability of isolated arterial cells (Benham *et al.*, 1987) and hence may produce constriction with only a small membrane potential change. Noradrenaline caused constriction only after producing a substantial depolarization. Thus the two agonists do not act on a common channel. The observations indicate the failure of mATP to distinguish between responses produced by either γ-adrenoceptor activation or by P_2 purinoceptor activation (Byrne & Large, 1986) reflects a lack of selectivity of mATP as an antagonist.

The distribution of γ-adrenoceptor responses in cerebral arterioles suggests that the receptors lie near sympathetic nerves. If their location is as precise on

cerebral arteries as it is on submucosal arterioles (Hirst & Neild, 1981; Luff *et al.*, 1987), it is difficult to see how they could fail to be activated by the locally high concentrations of noradrenaline that result from release of single sympathetic vesicles (Bevan *et al.*, 1980). Similarly, if ATP were released from sympathetic nerves by nerve action potentials (see however White *et al.*, 1981) it too would produce an α-adrenoceptor-resistant depolarization. However, if the estimates of the amounts of ATP in single peripheral sympathetic vesicles, that is some 20 to 50 molecules per vesicle (Fried *et al.*, 1984), reflect the amount released it is difficult to see how ATP could produce a sufficient local concentration to produce an e.j.p. by itself.

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An evaluation of the structure-activity relationships of a series of analogues of mephenesin and strychnine on the response to pressure in mice

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1 A range of compounds structurally related to the centrally acting muscle relaxant mephenesin and to the chemical convulsant strychnine were synthesized and tested for their ability to alter the threshold pressures for the onset of high pressure convulsions in mice.

2 The ability of both groups of compounds to alter the threshold pressures for convulsions was found to be dependent on the nature of a simple molecular skeleton. Thus, compounds that possessed a negatively polarized group located both in the same plane as and some 4.5 A from an aromatic nucleus increased the thresholds whereas compounds with a positively polarized group at the same location reduced the thresholds.

3 These findings support the suggestion that pressure elicits convulsions via a selective action on a receptor protein complex rather than via some general perturbation of the lipid regions of cellular membranes.

Introduction

Exposure to pressures above 30 bar (equivalent to 300 metres of sea water) leads to excitatory responses in the central nervous system (CNS), collectively referred to as the high pressure neurological syndrome (HPNS), which in man is observed as tremors and myoclonus (Bennett, 1982). Animals similarly exhibit tremors and involuntary muscular spasms and, at pressures above 70 bar, convulsions (Brauer, 1975). These effects not only represent a serious threat to the safety of divers presently operating at pressures up to 45 bar but also provide a barrier to deeper human diving.

Mephensin (3[2-methylphenoxy]propan-1,2-diol), a centrally acting muscle relaxant, has been shown to be a most potent anti-HPNS drug in mice: it raised the onset pressure for tremors by a factor of 2.5 and that for convulsions by a factor of 1.5 (Bowser-Riley, 1984; Bowser-Riley *et al.*, 1988). Experiments with simple structural isomers of mephenesin also suggested a possible mechanism for the convulsive action of pressure. Aromatic propandiols conferred a substantial protection against HPNS whereas aliphatic propandiols were ineffective. This division in action was related to their anticonvulsive properties against strychnine, picrotoxin and metrazol (Bowser-Riley, 1984), with the aromatic propandiols being effective only against strychnine and ineffective against metrazol or picrotoxin and the reverse being the case for the aliphatic compounds. In addition, it was shown that the *ortho*, *meta* and *para* isomers of mephensin and the *ortho* and *para* isomers of chlorphenesin (3[2-chlorphenoxy]propan-1,2-diol) exhibited an identical order of potency against both the convulsive action of pressure and that of strychnine (Bowser-Riley *et al.*, 1988). The apparent link between the action of pressure and strychnine and their mutual antagonism by mephenesin was strengthened by the finding that strychnine and pressure act in a strictly additive manner in the production of convulsions and that this relationship was maintained in the presence of mephenesin (Bowser-Riley *et al.*, 1988). That such strict additivity of action is not a feature common to the combination of pressure and all analeptics was shown by experiments with picrotoxin where gross deviations from additivity were observed (Bowser-Riley *et al.*, 1984; 1988).

To further the molecular interpretation of the pharmacology of pressure we have synthesized a series of compounds with molecular structures related to both mephenesin and strychnine and determined the degree of protection they afford against the convulsions elicited by pressure.

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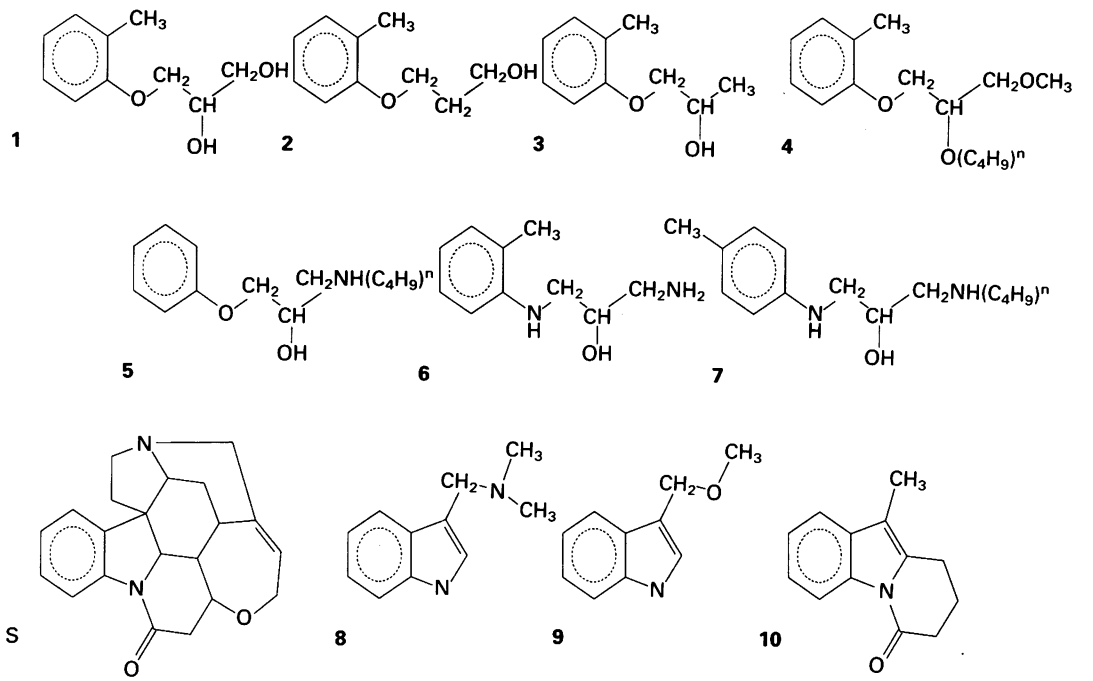

Figure 1 Structures of the mephenesin- and strychnine-related compounds used in this study. (1) 3[2-methylphenoxy]propan-1,2-diol (mephenesin); (2) 3[2-methylphenoxy]propan-1-ol; (3) 3[2-methylphenoxy]propan-2-ol; (4) 3[2-methylphenoxy]2-n-butyloxy-1-methoxypropane; (5) N-n-butyl,2-hydroxyl,3-phenoxypropylamine; (6) N-2-methylphenyl,2-hydroxy-propane 1,3 diamine; (7) N-n-butyl,N-4-methylphenyl,2-hydroxypropane 1,3 diamine; (8) 3[dimethylaminomethyl]indole (gramine); (9) 3-methoxy-methyl-indole; (10) 3-methylindole-2-butyrolactam; (S) strychnine.

Synthesis

Mephenesin analogues (see Figure 1) were produced by the reaction of the corresponding phenol and epichlorohydrin followed by the opening of the epoxide with a suitable base (Chizhevskaya *et al.*, 1958). The 2-methylphenoxy component of mephenesin was retained in all these compounds, since previous examination of this group, employing single and multiple substituents on the phenoxy ring, had shown that the 2-methylphenoxy structure produced compounds with the greatest potency (Bowser-Riley *et al.*, 1988).

Three mephenesin-like amine compounds, 2-hydroxy-3-[2-methylanilino]propylamine, N-n-butyl, N-4-methylphenyl,2-hydroxypropane 1,3 diamine and N-2-methylphenyl, 2,hydroxypropane 1,3 diamine (Figure 1) were prepared using the appropriate aniline and epichlorohydrin followed by ring opening of the epoxide with the relevant amine (Davies & Savage, 1950; Petrow *et al.*, 1956, McKelvey *et al.*, 1960).

One of the three compounds with structures related to strychnine (Figure 1), 3[dimethylaminomethyl]indole (gramine) was obtained from Aldrich. The other two were synthesized as follows: 3-methylindole,2-butyrolactam, by acid catalysed rearrangement of the monophenylhydrazone of 2-methyl,1,3-cylcohexanedione (Merling, 1894; Teuber *et al.*, 1964) and 3-methyoxy-methyl-indole from gramine methiodide by methoxide substitution (Jacob & Madinaveitia, 1937; Schram, 1951).

The structure of each new compound was confirmed using nuclear magnetic resonance and infrared spectrometry and the purity tested using thin layer chromatography, and either melting point or refractive index determinations. The structures of all the compounds employed in this study are shown in Figure 1.

Experimental

The pharmacological properties of the synthesized compounds were established following intraperitoneal (i.p.) injection into mice (male, C57/BL6; Oxford University, Sir William Dunn School of Pathology; body weight 25–30 g). The drug vehicle comprised 10% v/v ethanol, 20% w/v chremaphor EL in

aqueous solution with a maximum injection volume of 0.1 cm^3. Compounds were screened in groups of 8 mice at four dose levels above and below a reference test dose of 1 mmol kg^{-1}. Gross behavioural changes compared with untreated and vehicle-treated controls were noted. For compounds that exhibited either significant muscle relaxant or convulsant activity the dose-response relationship was established (minimum 8 mice per dose) and the ED_{50} for loss of righting reflex or the onset of convulsions in each case derived by Probit Analysis (Finney, 1964).

For experiments at pressure, groups of 4 mice were compressed within a 25 litre pressure vessel equipped with full environmental control. They were housed within the chamber in a specially designed holder which allowed for the injection of drugs at pressure (Bowser-Riley & Price, 1986). A rectal probe, in one mouse, was used to monitor body temperature which was maintained at 37°C by adjusting the chamber temperature. Before compression, the chamber was flushed with oxygen for 10 min. Pressure was applied with helium at 3 bar min^{-1}. Drugs were injected, as described previously (Bowser-Riley *et al.*, 1988), via an indwelling subcutaneous cannula at a pressure of 65 bar. Continuous video recordings of animal behaviour were made throughout compression and the ability of the drugs to alter the onset pressure for convulsions was subsequently assessed by two independent observers. Previous experiments (Bowser-Riley, 1984) to study the effects of pressure employed a number of behavioural endpoints, comprising fine tremor, coarse tremor and convulsions. In the present study because of the short duration of action of the drugs employed they were injected s.c. at 65 bar. In consequence the animals had already begun to exhibit tremors (mean onset pressure 45 ± 2 bar) therefore the only behavioural endpoint reported was the onset pressure for convulsions. The convulsive endpoint was defined as a sequence of uncoordinated, violent involuntary movements of sufficient severity to cause total loss of balance. The mean convulsive threshold was the average derived from at least two groups of mice for each drug treatment. A randomised test sequence was used and control values obtained throughout the course of the experiments.

Results

General pharmacological profile

The centrally acting muscle relaxant mephenesin (compound 1, Figure 1) when tested at doses up to 1 mmol kg^{-1} in mice produced the well defined, reversible, ascending flaccid paralysis characteristic of this class of compound (see Berger, 1952). Its ED_{50} for loss of righting reflex was found to be 0.9 ± 0.05 mmol kg^{-1}. The analogues of mephenesin, with the notable exception of 3[2-methylphenoxy] 2-n-butyloxy-1-methoxypropane (compound 4) which was ineffective over the dose range 0.5–1.5 mmol kg^{-1}, exhibited identical actions. However, whilst 3[2-methylphenoxy] propan-1-ol (compound 2) was almost equipotent with mephenesin (ED_{50} for loss of righting reflex 1.1 ± 0.06 mmol kg^{-1}) as a muscle relaxant, 3[2-methylphenoxy] propan-2-ol (compound 3) was much more potent than either, with an ED_{50} of 0.15 ± 0.004 mmol kg^{-1}.

The mephenesin-like amine compounds (compounds 5–7) elicited a complex spectrum of activity. Two of these compounds, N-n-butyl, 2-hydroxyl, 3-phenoxypropylamine (compound 5) and N-2-methylphenyl, 2,hydroxypropane 1,3 diamine (compound 6), exhibited muscle relaxant activity with ED_{50}s for loss of righting reflex of 0.13 ± 0.005 mmol kg^{-1} and 1.2 ± 0.07 mmol kg^{-1}, respectively. However, compound 5 had a low safety margin of action since doses above the ED_{50} induced a marked respiratory depression which proved to be lethal. When compound 6 was tested at higher doses the animals passed from a flaccid into a convulsive state followed by respiratory depression and death. The remaining compound in this series, N-n-butyl, N-4-methylphenyl, 2,hydroxypropane 1,3 diamine (compound 7) exhibited no muscle relaxant properties, but proved to be a potent strychnine-like agent with an ED_{50} for convulsions of 20 ± 0.5 μmol kg^{-1}.

The compounds structurally related to strychnine (compounds 8–10) also exhibited contrasting pharmacological properties. Gramine (compound 8) when tested over the dose range 0.5–1.5 mmol kg^{-1} was neither a convulsant nor muscle relaxant; however, it did induce a hyperactive state at a threshold dose of 1 mmol kg^{-1}. Over a similar dose range compound 9, 3-methoxy-methyl-indole, was also inactive, although for this compound the animals were observed to be quiescent at doses in excess of 1 mmol kg^{-1}. Compound 10, 3-methylindole-2-butyrolactam induced a marked degree of muscle relaxation at doses between 0.2 and 0.4 mmol kg^{-1}. Due to its extremely poor solubility the upper range of doses was not explored and its ED_{50} could not be estimated with precision.

Experiments at pressure

The mean onset pressures for convulsions for control (vehicle-treated) and drug-treated C57/BL6 mice are given in Table 1.

The most potent compound tested against high pressure convulsions was mephenesin (compound 1). At doses of 0.5 and 1.0 mmol kg^{-1}, administered subcutaneously at 65 bar, the onset pressures for convulsions were raised by 19% ($P < 0.001$) and

Table 1 The effects of structural derivatives of mephenesin and strychnine on the onset pressure for convulsions in C57/BL6 mice

		Dose (mmol kg^{-1})	*Onset pressure for convulsions* (bar)	n
	Control (vehicle treated)		104 (1.4)	20
(1)	3[2-methylphenoxy] propan-1,2-diol	0.5	124 (2.0)[d]	8
	(Mephenesin)	1.0	154 (1.0)[d]	8
(2)	3[2-methylphenoxy] propan-1-ol	1.0	127 (1.5)[d]	8
(3)	3[2-methylphenoxy] propan-2-ol	1.0	126 (2.5)[d]	8
(4)	3[2-methylphenoxy] 2-n-butyloxy-1-methoxypropane	1.0	100 (1.6)[a]	8
(5)	N-n-butyl,2-hydroxyl, 3-phenoxypropylamine	0.08	99 (1.6)[b]	8
(6)	N-2-methylphenyl,2-hydroxy-propane 1,3 diamine	0.80	95 (2.0)[c]	8
(7)	N-n-butyl,N-4-methylphenyl 2-hydroxypropane 1,3 diamine	0.008	77 (1.0)[d]	12
(8)	3[dimethylaminomethyl] indole (Gramine)	1.0	94 (1.6)[d]	8
(9)	3-methoxy-methyl-indole	1.0	128 (2.1)[d]	8
(10)	3-methylindole-2-butyrolactam	0.2	104 (2.5)[a]	8

Results shown are means with s.e.mean in parentheses.
Statistical significance of changes in onset pressures for convulsions compared with control values by use of Student's *t* test: [a] $P > 0.05$ (not significant); [b] $0.05 > P > 0.01$; [c] $0.01 > P > 0.002$; [d] $P < 0.001$.

48% ($P < 0.001$), respectively. Before injection all animals exhibited the characteristic tremors associated with the action of high pressure, with threshold pressure of onset of 45 ± 2 bar. The tremors disappeared within two minutes of injection of mephenesin and were only observed again immediately before convulsions. Removing the terminal hydroxyl group from mephenesin, to give 3[2-methylphenoxy]-propan-2-ol (compound 3), markedly reduced the potency and no protection at doses between 0.1 and 0.5 mmol kg^{-1} was observed. However, at a dose of 1 mmol kg^{-1} the onset pressure was raised by 22% ($P < 0.001$). Removing the secondary hydroxyl group to give 3[2-methylphenoxy]propan-1-ol (compound 2) also reduced the potency against high pressure convulsions. At a dose of 1 mmol kg^{-1}, the onset pressures were increased by 21% ($P < 0.001$). When both hydroxyl groups were removed to give 3[2-methylphenoxy]2-n-butyloxy-1-methoxypropane (compound 4) no significant activity against pressure was observed within the dose range 0.5–1.5 mmol kg^{-1}.

The mephenein-like amine compounds (5, 6 and 7) all acted to reduce the onset pressures for convulsions. For compound 5, where the terminal hydroxyl group of mephenesin was replaced by a secondary amine (capable of being positively charged at body pH) to give N-n-butyl,2-hydroxyl,3-phenoxypropylamine, a small (5%; $0.1 > P > 0.05$) reduction in onset pressure was observed. This represents the maximum effect obtained with this compound since at higher doses its combined action with pressure proved to be lethal. The two diamine derivatives of mephenesin, N-2-methylphenyl, 2,hydroxypropane 1,3 diamine (compound 6) and N-n-butyl, N-4-methylphenyl, 2,hydroxypropane 1,3 diamine (compound 7) were both more effective and reduced the onset pressure for convulsions by 9% ($0.01 > P > 0.002$) and 26% ($P < 0.001$), respectively. These effects were seen at threshold doses of 0.8 mmol kg^{-1} for compound 6 and 0.008 mmol kg^{-1} for compound 7 which were defined as the highest compatible with the failure to elicit convulsions within a 1 h period at atmospheric pressure.

The compounds with structures relating to that of strychnine elicited different effects when tested on the response to pressure. Gramine, 3[dimethylaminomethyl]indole (compound 8), potentiated the effects of pressure, reducing the onset pressure for convulsions by 10% ($P < 0.001$) at a threshold dose of 1 mmol kg^{-1}. However, when the amino group in gramine was replaced by an ether to give 3-methoxymethyl-indole (compound 9), a substantial protection was obtained at a threshold dose of 1 mmol kg^{-1}

and the onset pressure for convulsions was increased by 23% ($P < 0.001$). Finally, 3-methylindole-2-butyrolactam (compound 10) which contains much of the structure of strychnine but not the region between the amino nitrogen and the aliphatic double bond, afforded no protection against pressure nor did it potentiate its effect.

Discussion

In agreement with previous findings (Bowser-Riley, 1984; Bowser-Riley *et al.*, 1988) the results of the present study show that mephenesin, the parent compound of a wide range of propandiol centrally acting muscle relaxants, is the most potent when tested for its anticonvulsant properties against pressure. The ability of mephenesin to increase the onset pressures for convulsions by a factor of 1.2 and 1.5 at doses of 0.5 and 1.0 mmol kg^{-1}, respectively, also demonstrates that it is the most effective non-anaesthetic agent so far tested against pressure. Comparison of the present findings, in which mephenesin was injected at 65 bar, with our previous results following injection at 1 bar, clearly supports the suggestion (Bowser-Riley, 1984) that the short duration of action of mephenesin limits its effectiveness against the convulsive action of pressure. The earlier studies also demonstrated that the ability of this class of compound to protect against high pressure convulsions was independent of their ability to induce muscle relaxation. This is also clearly illustrated in the results with the present series of compounds. Thus, the mephenesin analogue, 3[2-methylphenoxy]propan-2-ol (compound 3), was 6 times more potent as a muscle relaxant, as judged by loss of righting reflex, but only half as effective against pressure when compared with mephenesin. This marked separation between anticonvulsant and muscle relaxant ability is also apparent with the two indole related compounds, where 3-methylindole-2-butyrolactam (compound 10) which proved to be a potent muscle relaxant was ineffective against pressure and 3-methoxy-methyl-indole (compound 9) which was ineffective as a muscle relaxant but highly effective against pressure. It is therefore apparent that the mechanism by which these agents protect against high pressure convulsions is quite different from that which gives rise to muscle relaxation.

When the pressure protection conferred by this series of compounds is considered, a consistent pattern of structure-activity relationships is revealed. Comparison of the activity of mephenesin (compound 1) with either of the two alcohols (compounds 2 and 3) demonstrates that both the primary and secondary hydroxy groups are required for optimum anti-pressure activity. The importance of the secondary hydroxyl group is emphasized by the finding that when it is replaced, as in 3[2-methylphenoxy]2-n-butyloxy-1-propane (compound 4) with an n-butyl group, which is large enough to provide a steric hindrance to binding, the anti-pressure activity is abolished. When the polarity of the terminal hydroxyl is reversed by substitution with an amine group as in N-n-butyl, 2hydroxy, 3-phenoxypropylamine (compound 5) and the two 1,3 diamines (compounds 6 and 7) the activity is changed from that which protects against pressure to that which is synergistic with pressure. Not only does this emphasize the importance of the primary hydroxyl group but also suggests that it is the nature of the terminal region that is vital in determining the overall outcome.

Analysis of the structures of these compounds with the aid of computerised molecular modelling techniques (Ricketts, 1987) revealed that, at the lowest energy conformation, the terminal oxygen of mephenesin and its isomers was located 4.5 Å from the phenoxy oxygen and lay in the same plane as the aromatic ring. A similar arrangement was found for N-n-butyl, 2hydroxy, 3-phenoxypropylamine (compound 5) and the two 1,3 diamines (compounds 6 and 7) where the terminal nitrogen also lay in the same plane at a distance 4.5 Å from the phenoxy oxygen or anilino nitrogen, in each case. This common structural feature was also apparent when the molecular structure of strychnine was analysed. Thus the indole and amino nitrogen atoms lie in the same plane with a linear separation of 4.5 Å. The series of strychnine related compounds (8, 9 and 10), which were synthesized to test the importance of this molecular arrangement on the response to pressure, exhibited the following features. For 3[dimethylaminomethyl]-indole (compound 8) the amino nitrogen was located 4.5 Å from the indole nitrogen, for 3-methoxy-methyl-indole (compound 9) the amino nitrogen was substituted with an oxygen at the same position and 3-methylindole-2-butyrolactam (compound 10) retained much of the structure of strychnine but not the region between the amino nitrogen and the aliphatic double bond. When these compounds were tested on the response to pressure it was shown that the presence of a terminal amino nitrogen (compound 8) reduced the threshold for high pressure convulsions, replacing the amino nitrogen with an ether oxygen (compound 9) increased the thresholds and with no group in this region no change was observed. Thus for all the compounds tested a negatively polarized group located in the same plane and some 4.5 Å from an aromatic nucleus is essential for anticonvulsive activity against pressure. Conversely, a positively polarized group in that region leads to enhanced convulsive activity and with no group in that region no effect on high pressure convulsions is observed.

The finding that the active region of these molecules has a consistent molecular arrangement similar to that of strychnine, when taken together with the findings that pressure and strychnine act in a strictly additive manner in the production of convulsions (Bowser-Riley *et al.*, 1988), support the assumption that one of the principal sites of action of pressure is restricted to a discrete area of the strychnine receptor complex within the central nervous system. Although more detailed investigations of the molecular and synaptic action of these compounds are required, an analogy with the well established action of strychnine strongly suggests that the convulsive effects of high pressure result from some action on glycine mediated inhibition. Whilst such an action may represent one of the more sensitive rate limiting factors in the production of high pressure convulsions, it is clear that pressure may well exert additional actions on the CNS since a wide range of compounds, unrelated to the present series, can modify the response to pressure (e.g. Bichard & Little, 1982; Bowser-Riley, 1984; Wardley-Smith & Meldrum, 1984). Nevertheless, it may be concluded that the structural specificity indicated by the present findings rules out the possibility that pressure acts via a general perturbation of the lipid regions of the cell membrane (Miller, 1985), but rather that the convulsive action of pressure may arise principally through a perturbation of the glycine receptor protein.

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Potentiation of atrial natriuretic peptide-stimulated cyclic guanosine monophosphate formation by glucocorticoids in cultured rat renal cells

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1 The effect of steroid hormones on atrial natriuretic peptide (ANP)-stimulated cyclic guanosine monophosphate (cyclic GMP) formation was studied in cultured rat renal cells.

2 ANP increased cyclic GMP formation in a dose-dependent manner, while cyclic AMP was not changed by ANP.

3 Steroid hormones did not affect basal cyclic GMP levels in cultured rat renal cells.

4 Dexamethasone at 10^{-8} M increased ANP (human and rat ANP)-stimulated cyclic GMP dose-dependently in cultured rat renal cells. Cortisol, corticosterone and aldosterone at a concentration of 10^{-7} M also potentiated ANP-stimulated cyclic GMP formation, although triiodothyronine, oestradiol and testosterone were ineffective. Potentiation of ANP action by these steroids seems to parallel glucocorticoid activity.

5 Dexamethasone did not affect cyclic GMP formation stimulated by sodium nitroprusside which stimulates soluble guanylate cyclase in the cytosol. Therefore, the potentiating action of dexamethasone may be mediated through the action on particulate guanylate cyclase at the plasma membrane.

6 It is suggested that the diuretic action of glucocorticoids may, at least in part, be mediated through the potentiating effect of glucocorticoids on cyclic GMP response to ANP.

Introduction

A previous study (Hatano *et al.*, 1988) from our laboratory showed that ovarian steroid hormones but not glucocorticoids, inhibited arginine vasopressin(AVP)-stimulated adenosine 3',5'-cyclic monophosphate (cyclic AMP) formation in rat renal cells. The diuretic action of atrial natriuretic polypeptide (ANP) seems to be mediated through an increase of cyclic guanosine monophosphate (cyclic GMP) formation (Gerzer *et al.*, 1984; Higuchi *et al.*, 1986). Since glucocorticoids are known to have a diuretic action, it is interesting to determine whether the diuretic action induced by glucocorticoids is mediated through modification of cyclic nucleotide response to water-regulating hormones. In the present study, the effect of various hormones on ANP-stimulated cyclic GMP formation has been examined in cultured rat renal cells.

As hormones, dexamethasone (Dex), cortisol, corticosterone, aldosterone, triiodothyronine (T_3), oestradiol and testosterone were used.

Methods

Cell culture

The method for primary culture of rat renal cell was described previously (Hatano *et al.*, 1985). In brief, kidneys from male Wistar rats weighing 100–200 g were removed under pentobarbitone anaesthesia. Kidneys were sliced, chopped, and digested with collagenase at 37°C for 30 min. After centrifugation at 1,000 r.p.m. for 10 min, the cell pellet was resuspended in DME supplemented with 10% FCS. Aliquots of 2 ml cell suspension were plated into 35 × 10 mm tissue culture dishes (Corning Glass Works, Corning, N.Y., U.S.A.). The cell concentration was adjusted so that 10–15 dishes would be prepared from one kidney. The dishes were placed in a CO_2 incubator, and the medium was changed 24 h after plating. In this experiment, whole kidney was cultured.

Experimental procedure

Effects of steroid hormones were studied as follows. After 24 h of culture, the medium was replaced with Dulbecco's modified Eagle medium plus foetal calf serum containing steroid hormones and culture was continued for a further 48 h, unless otherwise mentioned. Then, ANP stimulation was performed in the presence of 2 mM 3-isobutyl(1-methyl)-xanthine (IBMX) at 37°C under constant shaking for 10 min unless otherwise mentioned. In a preliminary experiment, it was found that addition of 2 mM IBMX resulted in maximal response of cyclic GMP and cyclic AMP to ANP and AVP, respectively. Thus, this concentration was used throughout the experiments. The incubations were terminated by removal of medium and addition of 600 μl of 6% trichloroacetic acid (TCA). The dishes were kept at room temperature for at least 30 min to allow intracellular cyclic nucleotide to be extracted by the TCA solution. TCA was removed by washing with water-saturated diethylether 3 times and discarded. The aqueous phase containing cyclic nucleotides was evaporated under nitrogen at 50°C and dried samples were dissolved in water. Cyclic AMP and cyclic GMP were assayed by radioimmunoassay with kits obtained from Yamasa Shoyu Co. Ltd., Choshi, Japan.

It is known that nitrosocompounds stimulate soluble guanylate cyclase in the cytoplasm, while ANP stimulates particulate guanylate cyclase in the plasma membrane. Therefore, the effect of dexamethasone on sodium nitroprusside-stimulated cyclic GMP formation was studied. In this experiment, ANP was replaced by 1 mM and 10 mM sodium nitroprusside (SNP) unless otherwise mentioned.

All cultures were prepared in triplicate. A control experiment was performed in the same manner with the vehicle. Each experiment was performed at least twice as explained in the figure legends.

Cell count and protein determination

As indicated in the figure legends, protein content was determined in control and experimental dishes. A count of cell numbers was made on separate dishes. The number of cultured cells in the dish was counted with a haemocytometer after the cells had been dispersed with trypsin. In this study, the number of cells varied from one experiment to the other and ranged between 2×10^5 and 5×10^5 cells/dish. Protein concentration was determined according to the procedure of Lowry *et al.* (1951) following cell lysis by 0.1 M NaOH. Protein content ranged between 100 and 300 μg/dish. Variation of cell number and protein content within one experiment was less than 7.5%. Various hormones did not significantly affect cell number or protein content.

Calculation of % stimulation and statistical analysis

Percentage stimulation of cyclic GMP formation was calculated as follows,

$$\left(1 - \frac{\text{amounts of cyclic GMP of experimental dish}}{\text{amounts of cyclic GMP of control dish}} \right.$$

$$\left. \frac{- \text{amounts of cyclic GMP of blank dish}}{- \text{amounts of cyclic GMP of blank dish}}\right) \times 100$$

'Experimental dish' indicates ANP-stimulation with steroid hormones. 'Control dish' indicates ANP-stimulation alone. 'Blank dish' indicates without ANP-stimulation.

Statistical analysis was performed by Student's t test (Sakuma, 1964) and P values less than 0.05 were considered significant.

Reagents

Collagenase (type II) was purchased from Worthington Biochemicals Corp., N.J., U.S.A. Dulbecco's modified Eagle medium (DME) was from GIBCO Laboratories, Grand Island, N.Y., U.S.A. Foetal calf serum (FCS) was purchased from M.A. Bioproducts, Md., U.S.A., and was inactivated by heating at 56°C for 30 min before use. 3-Isobutyl(1-methyl)-xanthine (IBMX), N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), sodium pentacyanonitrosylferrate (III) (SNP), Dex, cortisol, corticosterone, T_3, aldosterone, oestradiol and testosterone were purchased from Sigma Chemical Company, St. Louis, Mo., U.S.A. Human atrial natriuretic polypeptide (h-ANP) and rat atrial natriuretic polypeptide (r-ANP) were purchased from Peptide Laboratories, Osaka, Japan. All other reagents used were of analytical grade.

Results

Effect of human atrial natriuretic peptide on formation of cyclic AMP and cyclic GMP in cultured rat renal cells

When cultured rat renal cells were exposed to 3×10^{-8} M h-ANP, a rapid increase in intracellular cyclic GMP occurred at 5–10 min, which was followed by a gradual increase during 60 min of incubation. Therefore 10 min incubation was employed to examine the effect of h-ANP.

The effect of various concentrations of h-ANP on the intracellular accumulation of cyclic AMP and cyclic GMP are shown in Figure 1. Within a concentration of h-ANP between 3×10^{-10} M and

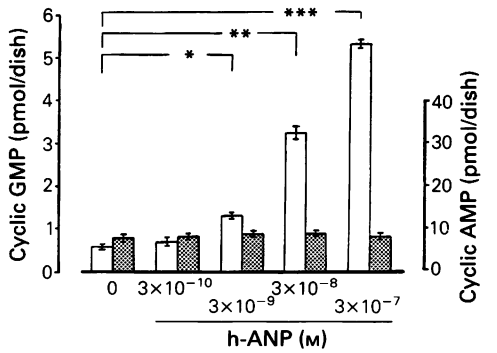

Figure 1 Effect of human atrial natriuretic peptide (h-ANP) on cyclic AMP (stippled column) and cyclic GMP (open column) amount in cultured rat renal cells. After 2 days of culture, renal cells were incubated with 3×10^{-10}–3×10^{-7} M h-ANP for 10 min in the presence of 2 mM 1-isobutyl methyl xanthine. Intracellular cyclic AMP and cyclic GMP were determined and results are shown as mean of triplicate determinations; vertical bars show s.e.mean. Cyclic AMP did not change, whereas cyclic GMP reached a significant increase at 3×10^{-9} M h-ANP. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

3×10^{-7} M, cyclic AMP did not change, whereas cyclic GMP increased in a dose-dependent manner, reaching a significant increase at 3×10^{-9} M.

Effect of dexamethasone on human atrial natriuretic peptide-stimulated cyclic GMP formation

As shown in Figure 2, Dex within a concentration range between 10^{-9} M and 10^{-7} M enhanced cyclic GMP formation stimulated by 3×10^{-8} M h-ANP in a dose-dependent manner. The increase was significant at 10^{-8} M Dex. The amount of cyclic AMP determined in the same sample was not changed by either h-ANP or Dex.

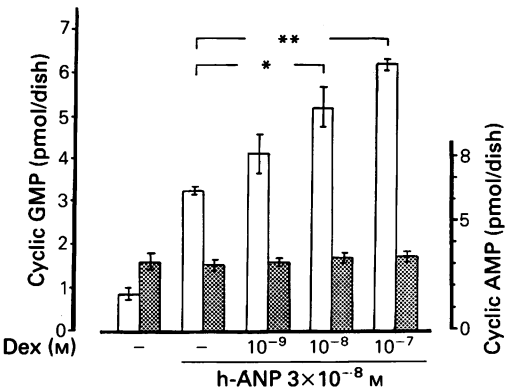

Figure 2 Dose-dependent effect of dexamethasone (Dex) on cyclc GMP formation stimulated by 3×10^{-8} M human atrial natriuretic peptide (h-ANP). After 1 day of culture, renal cells were incubated with 10^{-9} M–10^{-7} M Dex for a further 2 days. Thereafter, the experiment was performed as described in the legend of Figure 1. Intracellular cyclic GMP (open column) and cyclic AMP (stippled column) were determined and results are shown as mean of triplicate determinations; vertical bars show s.e.mean. Dex potentiated h-ANP-stimulated cyclic GMP formation in a dose-dependent manner and reached a significant increase at 10^{-8} M Dex(* $P < 0.05$) and at 10^{-7} M Dex(** $P < 0.01$). The amount of cyclic AMP in the same sample did not change with either h-ANP or Dex. Protein content was $281 \pm 10\,\mu g$ protein/dish (mean ± s.e. of 3 dishes) at zero Dex, $278 \pm 12\,\mu g$ protein/dish (3 dishes) at 10^{-7} M Dex, and Dex did not change protein content. The experiments were repeated 2 times, and similar results were obtained.

In a separate experiment, the time course effect of Dex on h-ANP-stimulated cyclic GMP formation was examined. Dex was added 1, 3, 12, 24, 48 h before h-ANP stimulation. Formation of cyclic

Table 1 Effect of dexamethasone (Dex) on soluble guanylate cyclase (sodium nitroprusside-stimulated cyclic GMP) and particulate guanylate cyclase (human atrial natriuretic protein (h-ANP)-stimulated cyclic GMP)

Stimulant	–	*h-ANP*: 3×10^{-8} M		*Sodium nitroprusside (SNP)* 1 mM		10 mM	
Dex (M)	–	–	10^{-7}	–	10^{-7}	–	10^{-7}
Cyclic GMP(pmol/dish)	0.268 ±0.044	1.078 ±0.074	1.832** ±0.081	2.984 ±0.617	3.016 ±0.092	4.500 ±0.492	4.140 ±0.295

After preincubation with Dex for 2 days, cultured renal cells were incubated with h-ANP or SNP for 10 min, and results are shown as mean ± s.e. of triplicate determinations. Dex increased h-ANP-stimulated cyclic GMP whereas Dex did not affect SNP-stimulated cyclic GMP formation. The experiment was repeated twice and similar results were obtained.
Significant difference from without Dex: ** $P < 0.01$.

GMP slightly increased following preincubation with Dex for 1 h, and the potentiating effect of Dex progressively increased with time interval of preincubation: 3 h, 16%; 12 h, 45%; 24 h, 81%; 48 h, 108%. The effect of Dex was significant when the preincubation time was more than 12 h.

Effect of dexamethosone on sodium pentacyanonitrosylferrate (III)-stimulated cyclic GMP formation

As shown in Table 1, incubation with SNP for 10 min at concentrations of 1 mM and 10 mM significantly increased intracellular cyclic GMP of rat renal cells. Dex at a concentration of 10^{-7} M did not affect SNP-stimulated cyclic GMP formation, while it significantly increased cyclic GMP formation stimulated by h-ANP in the same experiment.

In a separate experiment, the duration of h-ANP or SNP treatment was examined. One hour incubation with h-ANP (3×10^{-8} M) or SNP (100 μM) after preincubation with Dex for 48 h was carried out. Dex at a concentration of 10^{-7} M significantly increased h-ANP-stimulated cyclic GMP formation (% increase: 116%), however, 10^{-7} M Dex did not affect SNP-stimulated cyclic GMP formation (% increase: 4%).

Effect of various hormones on human atrial natriuretic peptide-stimulated cyclic GMP formation

The effect of Dex, cortisol, corticosterone, aldosterone, T_3, oestradiol and testosterone at a concentration of 10^{-7} M on h-ANP stimulated cyclic GMP formation was examined and results are shown in Figure 3. Although none of these hormones altered basal cyclic GMP levels, Dex, cortisol, corticosterone and aldosterone significantly increased cyclic GMP formation stimulated by h-ANP, whereas T_3, oestradiol and testosterone did not change cyclic GMP formation significantly. The percentage increase by these hormones was as follows: Dex 173%; cortisol, 123%; corticosterone, 32%; aldosterone, 29%; T_3, −6%; oestradiol, 18%; and testosterone, 13%, respectively.

Effect of dexamethasone on rat atrial natriuretic peptide-stimulated cyclic GMP formation

In order to evaluate species difference, h-ANP was replaced with r-ANP. The effect of 10^{-7} M Dex on cyclic GMP formation stimulated by various concentrations of r-ANP is shown in Figure 4. r-ANP at concentrations of 3×10^{-9} M to 3×10^{-7} M increased cyclic GMP formation in a dose-dependent manner like h-ANP. Dex at a concentration of 10^{-7} M significantly potentiated cyclic GMP formation stimulated by every concentration of r-ANP. In a separate experiment, the effect of various hormones on cyclic GMP formation stimulated by r-ANP was examined; the effects were similar to those observed by h-ANP.

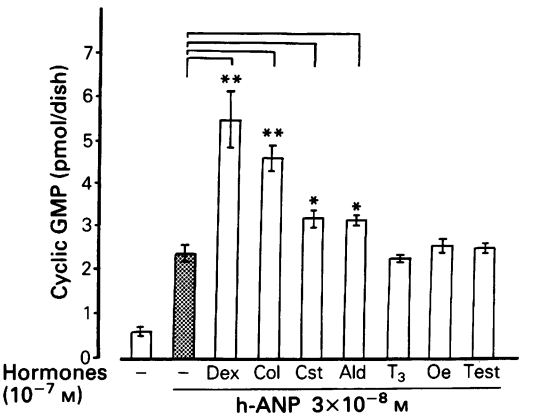

Figure 3 Effect of various hormones on cyclic GMP formation stimulated by 3×10^{-8} M human atrial natriuretic peptide (h-ANP) in cultured rat renal cells. The experiments were performed as indicated in the legend of Figure 2. Results are shown as mean of triplicate determinations with s.e.mean shown by vertical bars. Ald = aldosterone, Test = testosterone; Dex = dexamethasone; Col = cortisol; Cst = corticosterone. Control (hormone not added during preincubation): stippled column. h-ANP-stimulated cyclic GMP was significantly increased by Dex, Col (** $P < 0.01$), Cst and Ald (* $P < 0.05$). T_3, Oe and Test did not change cyclic GMP formation. None of these hormones changed protein content. The experiments were repeated 3 times, and similar results were obtained.

Discussion

In cultured rat renal cells, ANP increased cyclic GMP in a dose-dependent manner, and a significant increase was observed at a concentration of 3×10^{-9} M. We cannot specify the cell types in the present experiment. Tremblay *et al.* (1985) reported that, in different fractions of dog nephron, the most striking action of ANP on cyclic GMP occurred in the glomeruli, which was not significantly modified by the addition of 0.5 mM IBMX. In contrast, ANP increased cyclic GMP formation in the thick loop of Henle and in the collecting duct were demonstrable only in the presence of IBMX. In our experiment, the cells obtained from the cortex and those from the

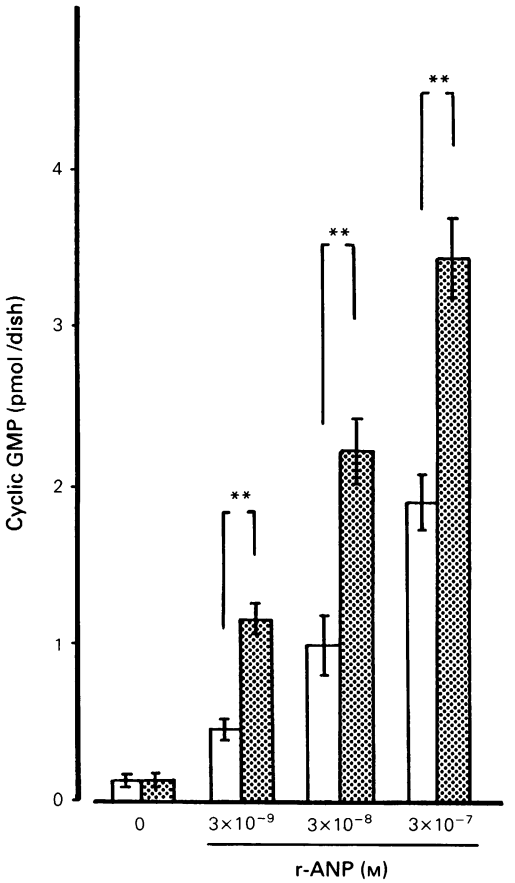

Figure 4 Effect of dexamethasone (Dex) on rat atrial natriuretic peptide (r-ANP)-stimulated cyclic GMP formation. Cells were preincubated with 10^{-7} M Dex for 2 days; thereafter, cells were stimulated by 3×10^{-9} M–3×10^{-7} M r-ANP for 10 min, and results were shown as mean of triplicate determinations; s.e. shown by vertical bars. Dex (stippled column) significantly increased cyclic GMP formation (** $P < 0.01$) at every concentration of r-ANP (open columns: without Dex). Number of cells were counted in 6 separate dishes, $2.15 \pm 0.11 \times 10^5$ cells/dish without Dex (mean ± s.e. of 3 dishes) and $2.20 \pm 0.15 \times 10^5$ cells/dish with Dex (3 dishes). The experiments were repeated twice, and similar results were obtained.

medulla exhibited a similar cyclic GMP response to ANP in the presence of IBMX; in the absence of IBMX, little increase of h-ANP-stimulated cyclic GMP formation was observed. Thus, in this experiment, ANP-stimulated cyclic GMP formation might also reflect the tubular reaction to ANP. Hamet *et al.* (1984) also reported that primary culture of renal tubular cells increased cyclic GMP formation stimulated by ANP and suggested the relationship of ANP-stimulated cyclic GMP formation with natriuresis.

In the present study, Dex, cortisol, corticosterone and aldosterone at a concentration of 10^{-7} M significantly increased h-ANP-stimulated cyclic GMP formation. The potentiation by Dex, the most potent synthetic glucocorticoid, was strongest among these steroids. The effect of cortisol, a physiological glucocorticoid of humans, was less potent than Dex but stronger than corticosterone, a physiological glucocorticoid of rats with less glucocorticoid activity. Thus, potentiation of ANP action by these steroids seems to parallel their glucocorticoid activity. As far as concentration is concerned, the concentration of 10^{-7} M can be achieved *in vivo* during steroid therapy by Dex, and the concentration is considered well within physiological range for cortisol and corticosterone, although it is outside the physiological range for aldosterone. Since the potentiation of h-ANP action by glucocorticoids was reproduced when h-ANP was replaced by r-ANP, this effect of glucocorticoids may not be species-specific and a similar mechanism seems to operate in human beings. Therefore, these glucocorticoids may exert the potentiation of ANP action *in vivo* under physiological conditions as well as during steroid therapy.

It is well-known that glucocorticoids increase urine volume in various pathological conditions (Miwa & Tojo, 1962; Kato, 1962; Miller *et al.*, 1969). Increased glomerular filtration rate has been suggested as the mechanism of glucocorticoid-induced diuresis (Kleeman, 1958). Ahmed *et al.* (1967) reported that plasma AVP increases in adrenocortical insufficiency and glucocorticoid inhibits vasopressin increase. Stillman *et al.* (1977) and Seif *et al.* (1978) found that Dex treatment suppresses the increase in neurophysin-II and AVP seen after adrenalectomy in the rat. Thus, the suppression of AVP secretion by glucocorticoid seems to be one of the mechanisms of glucocorticoid-induced diuresis. In addition, it was also suggested that a direct effect of glucocorticoid on the distal tubule and collecting ducts might explain the mechanism of diuretic action of glucocorticoid (Schwartz & Kokko, 1980). Our previous study demonstrated that Dex has no effect on AVP-stimulated cyclic AMP formation (Hatano *et al.*, 1988). In the present study, we found that glucocorticoid potentiates ANP action in renal cells. Potentiation of ANP action by glucocorticoid may be the basis of the direct effect of glucocorticoid on renal tubles and may represent one of the mechanisms of glucocorticoid-induced diuresis.

Kuno *et al.* (1986) reported that guanylate cyclase is coupled with ANP receptor (particulate guanylate cyclase) in the membrane and binding of ANP to membrane receptor activates particulate guanylate

cyclase. On the other hand, it was found that SNP stimulates soluble guanylate cyclase in the cytosol (Smith & Lincoln, 1987). In order to eliminate the possibility that glucocorticoid activates soluble guanylate cyclase, the effect of glucocorticoid on SNP-stimulated cyclic GMP was tested. Dex did not affect SNP-stimulated cyclic GMP formation, while Dex increased ANP-stimulated cyclic GMP formation. The results clearly indicate that Dex affects guanylate cyclase activity coupled with ANP receptor in the membrane.

Thus, it may be postulated that glucotocorticoids produce diuresis at least in part, through potentiation of ANP action in the kidney.

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Concurrent determination of effects of *p*-chloroamphetamine on central extracellular 5-hydroxytryptamine concentration and behaviour

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1 The effects of *p*-chloroamphetamine (PCA) (5 mg kg^{-1}, i.p.) on extracellular concentrations of 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) were monitored in the frontal cortex of conscious, freely moving rats by means of intracerebral dialysis. Concomitant behaviour comprising the 5-HT behavioural syndrome (reciprocal forepaw treading, headweaving, wet dog shakes) and locomotion and grooming was also recorded.

2 PCA markedly and transiently increased extracellular 5-HT but extracellular 5-HIAA values decreased to a moderate extent.

3 The time-course of 5-HT changes correlated positively ($P < 0.001$) with those of the three components of the 5-HT syndrome but did not correlate significantly with locomotion and correlated negatively ($P < 0.01$) with grooming.

4 As previous work using conventional methodology did not reveal clear relationships between the effects of PCA on 5-HT metabolism and behaviour, the present results emphasise the value of *in vivo* dialysis in the study of the neurochemical mediation of the behavioural effects of drugs.

Introduction

Acute administration of *p*-chloroamphetamine (PCA) has numerous behavioural effects which appear to be due to the release of 5-hydroxytryptamine (5-HT) as indicated by their prevention by inhibition of 5-HT synthesis or by 5-HT receptor antagonists. Examples are the impairment of avoidance learning and increased threshold to nociceptive stimuli (Ogren, 1985; Curzon *et al.*, 1986), the stimulation of ejaculation (Renyi, 1985) and the 5-HT behavioural syndrome (Trulson & Jacobs, 1976; Lucki *et al.*, 1984) which includes headweaving, forepaw treading and wet dog shakes.

Using *in vivo* dialysis, Sharp *et al.* (1986) showed that PCA transiently increased both 5-HT and dopamine in the extracellular fluid of the frontal cortex and the striatum, thus providing direct evidence for monomine release by the drug. The above work was done on anaesthetized rats. Using a novel power-assisted liquid swivel (Beastall *et al.*, 1987), which facilitates the application of *in vivo* dialysis to highly active animals, we have used intracerebral dialysis to determine the relation between the time-courses of release of 5-HT by PCA and of components of the 5-HT syndrome and other aspects of motor behaviour in the same animals. A brief abstract of some of the work described in this paper has been published (Hutson & Curzon, 1987).

Methods

Animals

Male Sprague-Dawley rats (200–250 g, Charles River, U.K.) were housed individually in a room with a 12 h light-dark cycle (lights on 06 h 00 min). Food (diet 22F, Labsure, Poole, Dorset) and water were freely available. Ambient temperature was maintained at 20°C ± 1°C. Each animal was used only once.

Surgery

Rats were anaesthetized with pentabarbitone (Sagatal, May and Baker, 60 mg kg^{-1}, i.p.) and

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Table 1 Definition of behavioural components monitored

Behavioural component		*Definition*
Locomotion	Frequency	Each cage cross (in any direction) is one episode of locomotion
	Duration	Total time spent in locomotion in any 20 min period
Grooming	Frequency	Number of episodes of body washing and cleaning with paws
	Duration	Total time spent grooming in any 20 min period
Forepaw treading*	Frequency	Number of episodes of up and down movements of the forepaws
	Duration	Total time spent forepaw treading in any 20 min period
Head weaving*	Frequency	Number of episodes of repetitive up and down or side to side head movements
	Duration	Total time spent headweaving in 20 min
Wet dog shakes*	Frequency	Number of episodes of head and body shakes

* Components of the 5-HT behavioural syndrome.

implanted with a guide cannula and stylet (Hutson *et al.*, 1985) in the frontal cortex using co-ordinates according to Paxinos & Watson (1982): A 3.5 mm from bregma, L 3.0 mm, H 1 mm below dura.

Dialysis

Approximately five days later, when animals had recovered from surgery and the effects of anaesthesia, the stylet was removed from the guide cannula and a dialysis probe inserted through it into the frontal cortex so that its tip projected 3 mm past the end of the guide cannula. The probe was constructed essentially as described by Hutson *et al.* (1985), except that the two inner glass capillaries were replaced with stainless steel tubes (0.005 in o.d.; 0.003 in i.d.) which made the probe more robust. The probe was perfused with artificial CSF containing 0.01% L-cysteine (Hutson *et al.*, 1985) at $1\,\mu l\,min^{-1}$ via a power-assisted liquid swivel (Beastall *et al.*, 1987), which allowed the rat to rotate and rear without entangling the fluid lines to and from the dialysis probe. Dialysate samples were collected every 20 min for about 3 h before injecting the animals with either 0.9% NaCl or PCA and then for a subsequent 4 h.

Biochemical analysis

Dialysate samples were analysed immediately on collection for 5-hydroxytryptamine (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA), by high performance liquid chromatography (h.p.l.c.) with electrochemical detection. Values were not corrected for recovery *in vitro*. The h.p.l.c. system used an Altex ultrasphere 3 μm ODS column (4.6 mm × 7.5 cm) (Beckman Limited). The mobile phase consisted of 0.1 M phosphate buffer pH 2.75, 18% v/v methanol, 0.025% sodium octyl sulphate and 0.0035% disodium edetate (EDTA). This was passed through a 0.2 μm filter (Millipore, London) and degassed with helium before use. The electrochemical detector (Coulochem model 5100A, Severn Analytical Limited) used a porous carbon analytical cell (model 5011) with electrode 1 set at −0.04 V and electrode 2 at +0.38 V with respect to the reference electrodes.

Behavioural analysis

Following injection with PCA ($5\,mg\,kg^{-1}$, i.p.) behaviour was recorded on videotape and subsequently analysed using a Sharp MZ80B microcomputer with a modified BASIC Ethol programme as described by Hendrie & Bennett (1983). The analysis was performed by depressing the two letter codes on the computer which corresponded to specific behavioural components (Table 1). When an episode of a particular component ended, the 'RETURN' key was pressed which caused the programme to calculate the duration of the behaviour. At the end of each 20 min period the command 'END' caused the computer to print out frequency and duration for each component during that period.

Drugs

(±)-*p*-Chloroamphetamine HCl (PCA, Sigma, U.K.) was dissolved ($5\,mg\,kg^{-1}$) in 0.9% NaCl and injected i.p. in a volume of $1\,ml\,kg^{-1}$. Control animals received an equivalent volume of 0.9% NaCl by the same route.

Statistics

Mean 5-HT and 5-HIAA concentrations following PCA injection were correlated with mean behavioural scores over the same 20 min periods using Pearson's product moment correlation coefficient. The Wilcoxon signed rank test was used to deter-

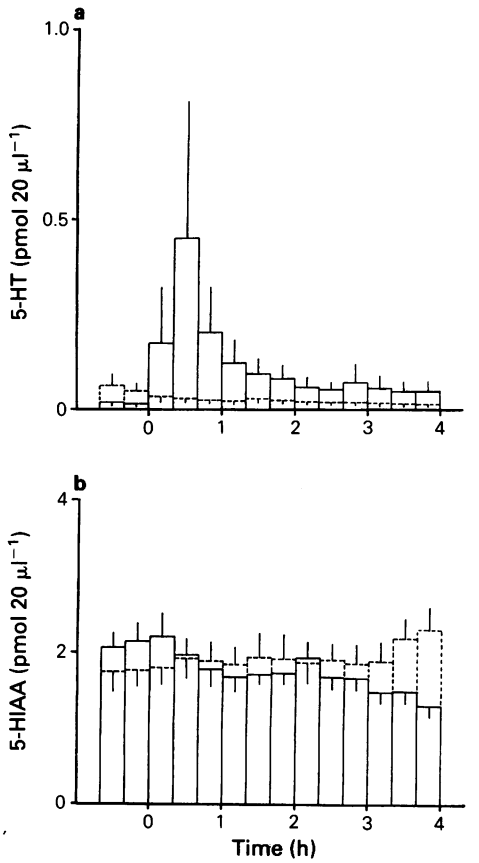

Figure 1 5-Hydroxyindoles in serial dialysates collected from frontal cortex of freely moving rats given *p*-chloroamphetamine (PCA) (5 mg kg^{-1} i.p., complete lines) or 0.9% NaCl (broken lines) at 0 time: (a) 5-HT: (b) 5-HIAA. Results are shown as means with s.d. shown by vertical lines, $n = 6$.

Areas under the curve pre- and post-injection values were calculated over 1 and 4 h, respectively, and are given as pmol h^{-1}, means ± s.d. 0.9% NaCl: 5-HT pre 0.129 ± 0.137, post 0.078 ± 0.029 (NS); 5-HIAA pre 5.27 ± 1.64, post 5.93 ± 1.85 (NS). PCA: 5-HT pre 0.071 ± 0.052, post 0.337 ± 0.519 ($P < 0.05$); 5-HIAA pre 6.39 ± 2.49, post 4.94 ± 1.23($P < 0.05$).

mine the significance of the effects of PCA on the areas under the curve for 5-HT and 5-HIAA.

Results

Dialysates

Figures 1a and b show 5-HT and 5-HIAA concentrations in dialysates collected over a period starting

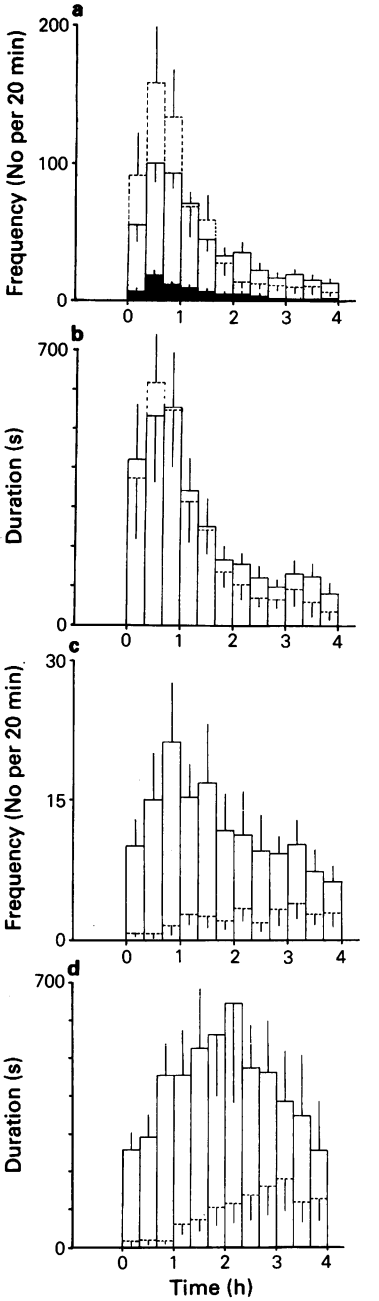

Figure 2 Behavioural effects of *p*-chloroamphetamine (PCA) (5 mg kg^{-1}, i.p.). (a) Frequency of reciprocal forepaw treading (broken lines), head weaving (complete lines) and wet dog shakes (solid areas). (b) Duration of reciprocal forepaw treading (broken lines) and head weaving (complete lines). (c) Frequency of grooming (broken lines) and locomotion (complete lines). (d) Duration of grooming (broken lines) and locomotion (complete lines). All results are shown as means with s.d. shown by vertical lines, $n = 6$.

Table 2 Correlation coefficients between values for mean extracellular 5-hydroxytryptamine (5-HT), 5-hydroxyindoleacetic acid (5-HIAA) concentrations and various behavioural components

Behavioural component		*Extracellular conc* (pmol 20 μl^{-1}) *5-HT*	P	*5-HIAA*	P
Wet dog shakes*	(Frq)	+0.92	<0.001	+0.53	NS
Head weaving*	(Frq)	+0.85	<0.001	−0.38	NS
	(Dur)	+0.83	<0.001	+0.53	NS
Forepaw padding*	(Frq)	+0.90	<0.001	+0.51	NS
	(Dur)	+0.89	<0.001	+0.51	NS
Locomotion	(Frq)	+0.50	NS	+0.42	NS
	(Dur)	−0.37	NS	+0.17	NS
Grooming	(Frq)	−0.71	<0.01	−0.65	<0.05
	(Dur)	−0.72	<0.01	−0.62	<0.05

* Components of the 5-HT behavioural syndrome.
Frq = frequency, Dur = duration.

40 min before injection of PCA or vehicle. Earlier 5-HT values (not shown) declined from initial high values (mean of 0.2 pmol 20 μl^{-1}), which may have resulted from tissue damage on insertion of the dialysis probe, to plateau values of about 0.05 pmol 20 μl^{-1}. 5-HIAA tended to rise over the first 80 min after insertion of the dialysis probe from about 1.2 pmol 20 μl^{-1} to mean values of 1.7–2.0 pmol 20 μl^{-1} and then remained essentially constant. Thus, when PCA or vehicle was injected (0 time, Figure 1), 5-HT values were about 1/100th of those for 5-HIAA.

Injection of vehicle was followed by slight decreases and increases of 5-HT and 5-HIAA respectively, over the next 4 h which were not statistically significant (areas under curve). Injection of PCA was followed by a rapid rise of 5-HT to a mean value of 0.4 pmol 20 μl^{-1} 20–40 min after injection. Concentrations then declined sharply but tended to be slightly above baseline values 4 h after drug injection. Corresponding 5-HIAA values gradually decreased to below baseline values. Considerable rat-to-rat variation was apparent. Thus, peak 5-HT concentrations varied between 0.04 and 1.9 pmol 20 μl^{-1} with peaks at 20–40 min after PCA injection. Areas under the curve for 5-HT and 5-HIAA were substantially and significantly increased and moderately and significantly decreased, respectively (see legend to Figure 1).

Behaviour

Reciprocal forepaw treading, head weaving and wet dog shakes occurred within the first 20 min after PCA injection (Figure 2). These components were not seen before drug injection or after injection of 0.9% NaCl solution. They attained maximal frequency and/or duration 20–40 min after PCA injection and then declined but were still evident 4 h after injection. Locomotion and grooming also increased but more slowly. Thus locomotor episodes were most frequent 40–60 min after injection and then declined, while their total duration was greatest 120–140 min after injection and then declined. Grooming did not attain maximal frequency and duration until 180–200 min after injection. Hind limb abduction was not clearly apparent in the above experiments.

Correlations between neurochemical and behavioural effects of p-*chloroamphetamine*

Figures 1 and 2 reveal the overall similarity between the time courses of extracellular 5-HT concentration and the components of the 5-HT syndrome. Table 2 shows that both the frequencies and durations of these components correlate positively and highly significantly with 5-HT, but not with 5-HIAA values. Unlike the components of the 5-HT syndrome, locomotion and grooming did not correlate positively and significantly with extracellular 5-HT concentrations. Indeed, both the frequency and duration of grooming correlated negatively and significantly with levels of both hydroxyindoles.

Discussion

Basal 5-HT levels and the time-course of its increase in frontal cortex dialysates following injection of PCA (5 mg kg^{-1}, i.p.) into freely moving rats, were very similar to results obtained by Sharp *et al.* (1986) for anaesthetized animals. Comparison of the neurochemical findings with the components of the 5-HT syndrome revealed largely parallel time-courses. The

effects of 5-HT synthesis inhibitors (Dickinson & Curzon, 1983) and of 5-HT receptor antagonists (Lucki *et al.*, 1984) on the 5-HT syndrome induced by PCA clearly show that it is mediated by release of 5-HT which interacts with 5-HT receptors. Therefore, the present findings indicate that the dialysis technique is able to monitor changes of availability of 5-HT to postsynaptic receptors. Parallelism between behaviour and extracellular 5-HT was marked, even though the transmitter was measured in a diffuse extracellular pool and not specifically in the synaptic cleft which is not directly accessible to the relatively large dialysis probe.

The frontal cortex was a convenient site for *in vivo* dialysis and 5-HT measurement and was used on the assumption that the time-course of release of 5-HT following systemic injection of PCA would be similar throughout the brain. Thus, the results obtained on the frontal cortex do not provide evidence of its role in the 5-HT syndrome. Indeed, apart from indirect evidence (Dickinson *et al.*, 1984) that striatal 5-HT may be required for the production of 'wet dog' shakes by PCA, little is known about the location of the 5-HT terminals at which release of 5-HT by PCA leads to the behavioural components measured in the present study, although it seems likely that spinal 5-HT is required for minor components that were not measured, i.e. Straub tail and tremor (reviewed Curzon, 1988).

The 5-HT syndrome does not occur without drug treatment, which suggests that sufficient 5-HT is not released to the mediating receptors under physiological circumstances. It is apparent from Figure 1 that dialysate 5-HT concentrations over the 1 h 40 min period after PCA injection during which the syndrome is most intense, were at least 2–10 times greater than before drug injection. Data obtained at later times are less readily interpreted, since the 5-HT syndrome in an attenuated form persisted when dialysate 5-HT values were rather above those seen before drug injection but similar to the values of another group of rats before they were injected with 0.9% NaCl. However, 5-HT values for the latter group throughout the period of behavioural study were well below those of the animals given PCA. Also, PCA may well release 5-HT by a non-physiological mechanism (i.e. from the neuronal cytoplasm), as it induces the 5-HT syndrome in rats with (vesicular) 5-HT stores largely depleted by reserpine treatment (Kuhn *et al.*, 1985).

The overall relationship between the neurochemical and behavioural responses to PCA was close. Comparable results were obtained by Sharp *et al.* (1987) for relationships between dopamine release and behaviour induced by amphetamine. However, parallelism between dialysate 5-HT and the 5-HT syndrome was not seen when Sleight *et al.* (1988) compared the effects of various monomine oxidase inhibitors on responses to L-tryptophan; while the syndrome did not occur without a rise of hypothalamic extracellular 5-HT, considerable rises could occur in its absence. Sleight *et al.* suggested that the syndrome may also require factors such as tryptamine or dopamine. It is relevant that tryptamine plays a large part in the 5-HT syndrome caused by tranylcypromine and L-tryptophan (Marsden & Curzon, 1979) and that PCA releases not only 5-HT but also dopamine from the striatum (Sharp *et al.*, 1986).

Differences between the time-dependencies of dialysate 5-HT and those of locomotion and grooming after PCA injection suggest that these behavioural components, which are not usually reported as characteristic of the 5-HT syndrome, are not directly mediated by released 5-HT. Indeed, locomotion may be inhibited by 5-HT release so that it becomes more prominent when dialysate 5-HT declines, especially if there is an associated release of catecholamines (Warbritton *et al.*, 1978; Lees *et al.*, 1979). It is relevant that after PCA treatment, striatal dialysate dopamine concentration returns to normal more slowly than does that of 5-HT (Sharp *et al.*, 1986).

The present results as a whole illustrate the power of the *in vivo* dialysis method when applied to the study of neurochemical-behavioural relationships. Conventional neurochemical methods merely show that both brain 5-HT and 5-HIAA concentrations slowly decline after PCA administration (Leonard, 1976; Fuller *et al.*, 1981) and (unlike dialysis) these methods do not detect the minute fraction of brain 5-HT released from neurones by PCA and responsible for the behavioural effects of the drug.

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Reversal of the cardiotonic and action-potential prolonging effects of DPI 201-106 by BDF 8784, a methyl-indol derivative

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1 We studied the interaction of the cardiotonic compound DPI 201-106 (4-[3'-(4''-benzhydryl-1''-piperazinyl)-2'-hydroxypropoxy]-1H-indole-2-carbonitrile; DPI) and its derivative BDF 8784 (2-methyl-4-[3'-(4''-benzhydryl-1''-piperazinyl)-2'-hydroxypropoxy]-1H-indole; BDF) in isolated right ventricular papillary muscles of guinea-pig heart.

2 In contrast to the cardiotonic DPI, the methyl-indole derivative lacked a positive inotropic effect and even caused negative inotropic effects in concentrations above 1 μM. At 10 μM BDF significantly reduced the force of contraction and dV/dt_{max}, but did not affect action potential duration (APD).

3 Pretreatment of papillary muscles with BDF prevented the positive inotropic action of DPI in a concentration-dependent, but non-competitive fashion. At 10 μM, BDF prevented the inotropic effect of racemic DPI and shortened the DPI-induced prolongation of action potential duration. BDF similarly affected the inotropic and APD-prolonging effects of the sea anemone polypeptide ATX II.

4 In cardiac myocytes, DPI induced a tetrodotoxin (TTX)-sensitive, slowly inactivating inward current. The slow decay of this current was enhanced by BDF. In cells pretreated with BDF, DPI was not effective. BDF alone depressed the sodium and the calcium current.

5 In conclusion, the non-inotropic methyl-indole derivative BDF interacts with DPI non-competitively at the sodium channels to abolish the inotropic and APD-prolonging effects of DPI, emphasizing the importance of the substituent in position 2 of the indole moiety for this effect.

Introduction

DPI 201-106 (4-[3'-(4''-benzhydryl-1''- piperazinyl)-2'-hydroxypropoxy]-1H-indole-2-carbonitrile; DPI) is a synthetic cardiotonic agent developed for treating congestive heart failure in man (Thormann *et al.*, 1986). The compound has been described as exerting an inotropic effect and prolonging action potential duration by slowing down the inactivation of the sodium channels (Scholtysik *et al.*, 1985; Buggisch *et al.*, 1985; Kohlhardt *et al.*, 1986). Because of the stereoselectivity of the effects of DPI (only the S-enantiomer is effective), Scholtysik and coworkers (1986) have proposed a specific receptor site for DPI on cardiac sodium channels. The proposal was strengthened by recent studies in which DPI was found to interfere with the binding of [^{3}H]-batrachotoxinin A 20-α-benzoate to specific binding sites in guinea-pig brain membranes (Romey *et al.*, 1987; Armah *et al.*, 1988b).

The sodium channel is the target for various neurotoxins like tetrodotoxin, batrachotoxin, veratridine and the sea anemone toxin ATX II. For these toxins specific binding sites on the sodium channel have been identified, but the binding site for DPI has yet to be specified. A direct approach to identify such a specific binding site is not possible, partly because a radiolabelled DPI suited for such experiments is not as yet available. The development of a specific antagonist of DPI would, however, offer a greater access to characterizing the putative receptor site mediating the inotropic effects of DPI. In a search for such a compound we studied the actions of the methyl-indole analogue of DPI, 2-methyl-4-[3'-(4''-benzhydryl-1''-piperazinyl)-2'-hydroxypropoxy]-1H-indole; code name BDF 8784 (BDF; see Figure 1 for

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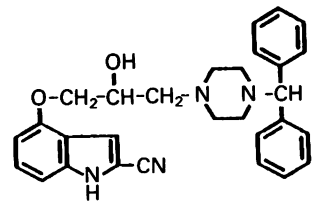
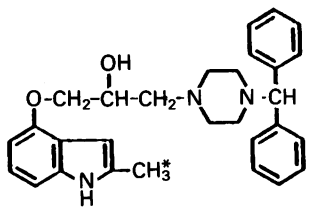


DPI 201 106



BDF 8784

Figure 1 The structural formulae of DPI 201-106 and BDF 8784.

structural formula) in isolated electrically driven papillary muscles of the guinea-pig.

As already found in preliminary experiments (Armah *et al.*, 1988a), BDF not only lacked the positive inotropic effects of DPI, but actually counteracted the positive inotropic effects of DPI in a concentration-dependent manner. Poor solubility of both agents prevented a meaningful study of their interaction at high concentrations.

To assess the specificity of the interaction of the two compounds, we have studied the combined effects of DPI and BDF on force of contraction and on the transmembrane action potentials in the same preparations. Furthermore, the effects of DPI and BDF on membrane currents in isolated myocytes were studied. In addition we investigated the interactions of BDF with ATX II, the polypeptide considered to possess a similar though not identical mechanism of action to DPI (Buggisch *et al.*, 1985; Scholtysik *et al.*, 1986). Our results suggest that an interaction with binding sites at the sodium channels could account for the interaction of BDF with the effects of DPI.

Methods

Right ventricular papillary muscles were isolated from the hearts of guinea-pigs and mounted vertically in 50 ml organ baths between two parallel platinum electrodes for field stimulation. For electrophysiological studies, the preparations were mounted horizontally in an organ bath which was continuously perfused with prewarmed, oxygenated Tyrode solution. The preparations were stimulated electrically via two platinum wires glued into the floor of the muscle chamber. Stimulation parameters were: voltage 4–8 V (20% above threshold), impulse duration 2 ms, frequency 1 Hz.

The force of contraction was registered isometrically by a strain gauge (Statham UC-2 cell) and recorded on an oscilloscope (Tektronix 502A). Action potentials were measured with conventional glass microelectrodes filled with KCl (3 M). The bioelectrical signals were also recorded on the oscilloscope. For documentation and later analysis of the action potentials and contractions, the screen of the oscilloscope was photographed at regular time intervals in the course of an experiment.

After an equilibration period of 90–120 min the preparations were exposed to the drug under investigation or to a combination of two drugs. The continuous flow of Tyrode solution through the organ bath was controlled by a roller pump and drugs were added to the silicone tubing close to the inflow by an infusion pump. In this way contamination of the tubing between the reservoir of Tyrode solution and the organ bath by drugs was minimized and the final (nominal) drug concentration in the bath could be calculated from the respective flow rates. The concentrations of DPI and BDF in the bath were limited to 10 μM because of their poor solubility in water. After each experiment, the apparatus had to be cleaned carefully in order to obtain correct values in the next preparation (see Buggisch *et al.*, 1985).

The Tyrode solution had the following composition (in mM): NaCl 137, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.1, $NaHCO_3$ 11.9, NaH_2PO_4 0.21, glucose 5.5. The pH was adjusted to 7.4 by gassing the solution with carbogen (95% O_2 and 5% CO_2). The temperature was maintained at 35 ± 0.5°C. DPI was dissolved with an equimolar amount of tartaric acid in 1 part of N-methyl-pyrolidin and 9 parts of warm, distilled water were then added slowly. BDF was dissolved in DMSO. The *Anemonia sulcata* polypeptide was dissolved in distilled water as described previously (Isenberg & Ravens, 1984).

Single myocytes from guinea-pig hearts were prepared as described previously (Borchard & Ravens, 1986). A single patch electrode voltage clamp circuit was used to study membrane currents (for details see Cragoe *et al.*, 1987). Patch electrodes were filled with a solution containing KCl 150 mM, $MgCl_2$ 5 mM, EGTA (ethylene-glycol-*bis*(β-aminoethyl-ether)-N, N,N',N'-tetraacetic acid) 20 μM, HEPES (hydroxyethylpiperazinyl-ethane-sulphonic acid) 10 mM adjusted with 1 N KOH to a pH of 7.2. The solution for superfusing the cells contained (in mM): NaCl 150, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 2, glucose 10, HEPES 10 adjusted to pH 7.4 with 1 N NaOH.

DPI 201-106 was a kind gift from Sandoz, Basel. BDF 8784 was provided by one of us (B.I.A.). ATX

II was provided by Dr L. Beress, Kiel. Tetrodotoxin (TTX) was obtained from Boehringer, Mannheim. All chemicals were of analytical grade and purchased from Merck, Darmstadt, F.R.G.

Results

Concentration-response curves for DPI and BDF

In guinea-pig papillary muscles, racemic DPI increased force of contraction by a maximum of 150% of pre-drug levels, when applied in a cumulative manner. The complete concentration-response curve for the positive inotropic effect of DPI is depicted in Figure 2a. In contrast to DPI, cumulative addition of BDF to a separate group of isolated papillary muscles did not exert a positive inotropic effect (Figure 2b). BDF concentrations of up to 1 μM did not affect the force of contraction. When untreated organs were preincubated for 90 min with BDF, the inotropic effect of DPI was abolished in a concentration-dependent fashion. As shown in Figure 1a, 0.1 μM BDF caused a rightward shift of the concentration-response curve of DPI without affecting the maximum effect of DPI. At 10 μM, BDF caused a rightward shift of the DPI curve and also suppressed the maximum effect of DPI.

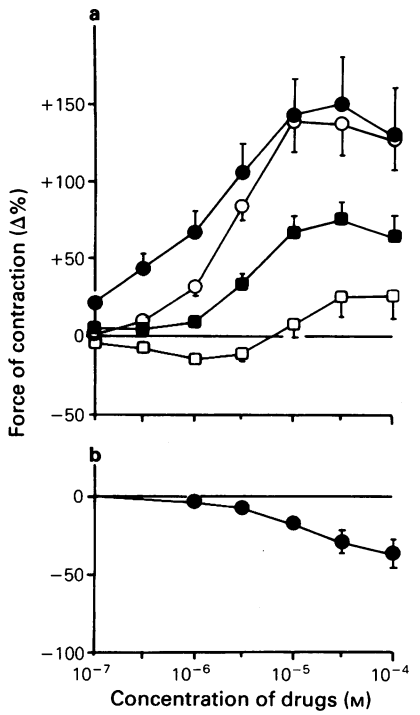


Figure 2 Concentration-response curves for the effects of DPI 201-106 and BDF 8784 on force of contraction of guinea-pig papillary muscles. (a) Effects of increasing concentrations of DPI after pre-exposure to various concentrations of BDF 0.1 μM (○) 1 μM (■), 10 μM(□). (●) Control response to DPI in the absence of BDF. (b) Concentration-response curve for BDF alone. Note that the threshold concentration of BDF (0.1 μM) for depression of the effect of DPI has no effect of its own on the force of contraction.

Effects of DPI and BDF on electrical and mechanical parameters of guinea-pig papillary muscles

The combined action of BDF and DPI was studied in different electrophysiological experiments (Figures 3 and 4). We chose 1 μM DPI as a standard concentration that prolonged the action potential duration (APD) and enhanced force of contraction as described previously (Buggisch *et al.*, 1985; Scholtysik *et al.*, 1985). When the inotropic effect of DPI had attained a steady state after 90 min of incubation, the addition of BDF (10 μM) reversed the inotropic and APD prolonging effects of DPI (Figure 3a). The maximum rate of depolarization, dV/dt_{max}, remained unaffected. When papillary muscles were pretreated with 10 μM BDF, the APD-prolonging and positive inotropic effect of DPI were prevented (Figure 3b). The first treatment with BDF depressed dV/dt_{max} from 160 $V s^{-1}$ to 100 $V s^{-1}$ within 90 min, and to 75 $V s^{-1}$ after a further 90 min during which DPI had been added.

The respective time courses of the effects of DPI and a combination of DPI and BDF on action potential duration and force of contraction are depicted in Figure 4. The lower concentration of BDF (1 μM) reduced the positive inotropic effect of DPI but hardly affected the DPI-induced prolongation of action potential duration. The higher concentration of BDF (10 μM) reversed both effects of DPI; however, the APD-prolongation disappeared with a slower time course than the positive inotropic effect.

When the preparations were first exposed to BDF (Figure 5), 1 μM and 10 μM of the compound had no effect on APD but reduced force of contraction to 51% and 32%, respectively, of the pre-drug control after 90 min of equilibration. This compares to a decline of force to 71 ± 7% ($n = 4$) during a similar experimental period in control preparations. One μM BDF did not influence dV/dt_{max} in 2 experiments with constant impalements, but 10 μM BDF depressed dV/dt_{max} from 215 to 170 $V s^{-1}$ and from 225 to 135 $V s^{-1}$ in 2 other experiments (see also Figure 3).

In the presence of 1 μM BDF, DPI (1 μM) still prolonged the APD from 179 ± 12 ms to 208 ± 8 ms

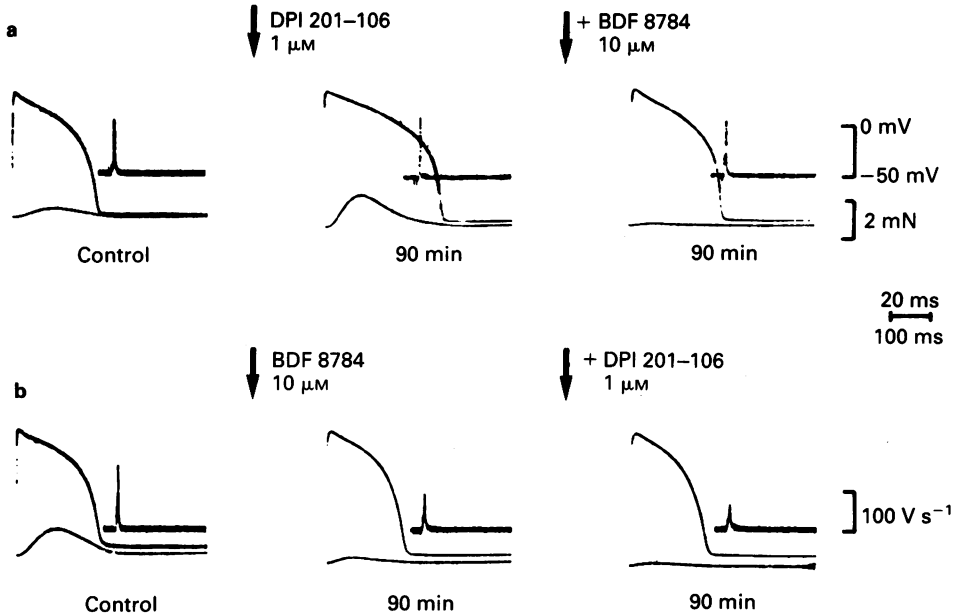


Figure 3 Original recordings of action potentials (middle tracing) and force of contraction (lower tracing) of guinea-pig papillary muscles exposed to DPI 201-106 and BDF 8784. The upper tracing in each frame is the 1st derivative of potential against time (dV/dt). (a) From left to right: control recording at the end of an equilibration period of 90 min, after 90 min of exposure to 1 μM DPI, after 90 min of exposure to DPI and 10 μM BDF. (b) From left to right: control recording at the end of an equilibration period of 90 min, after 90 min of exposure to 10 μM BDF, after 90 min of exposure to BDF and 1 μM DPI. Stimulation frequency 1 Hz, calibration bars are valid for all traces.

($n = 4$) compared to a prolongation from 180 ± 15 ms to 253 ± 9 ms ($n = 16$) without BDF, and prevented the further decline in force. Ninety min of pretreatment with the higher concentration of BDF (10 μM) also did not affect the action potential duration but rendered DPI ineffective in enhancing either force or APD.

Figure 6 shows the corresponding time courses of the time-to-peak tension and of the duration of contraction after the respective exposures to BDF and DPI. Time-to-peak tension was not influenced either by DPI or BDF, whereas the duration of contraction closely followed the changes described for the APD.

Combined effects of BDF and ATX II

The antagonism between BDF and DPI could involve some kind of competition for the DPI-binding sites related to the Na channels. We therefore tested whether BDF could also modify the effects of the sea anemone toxin ATX II, since this polypeptide mediates its positive inotropic and APD prolonging effect via the sodium channels. (Ravens, 1976; Isenberg & Ravens, 1984; Schreibmayer *et al.*, 1987), although it is thought to bind to a different site from DPI (Scholtysik *et al.*, 1986; Romey *et al.*, 1987). Figure 7 summarizes the results of these experiments.

When added before exposure to the polypeptide, BDF (10 μM) prevented the electrical and mechanical effects of ATX II. Similar to the combined exposure of DPI and BDF, BDF also reversed the prolonged action potential and the accompanying positive inotropic effect of ATX II once the full effect of the polypeptide had been established. We measured a significant reduction in dV/dt_{max} when BDF was added after ATX II (the values from 1 experiment with a constant impalement were: pre-drug control 225 $V s^{-1}$, 90 min of exposure to ATX II (20 nM) 230 $V s^{-1}$, and 90 min of exposure to ATX II and BDF (10 μM) 185 $V s^{-1}$). Neither BDF nor ATX II influenced the time-to-peak tension. The changes in duration of contraction followed the same direction and time course as the changes in APD (data not shown).

Effects of DPI and BDF on membrane currents

The above experiments have hitherto demonstrated that BDF may interfere with the action of com-

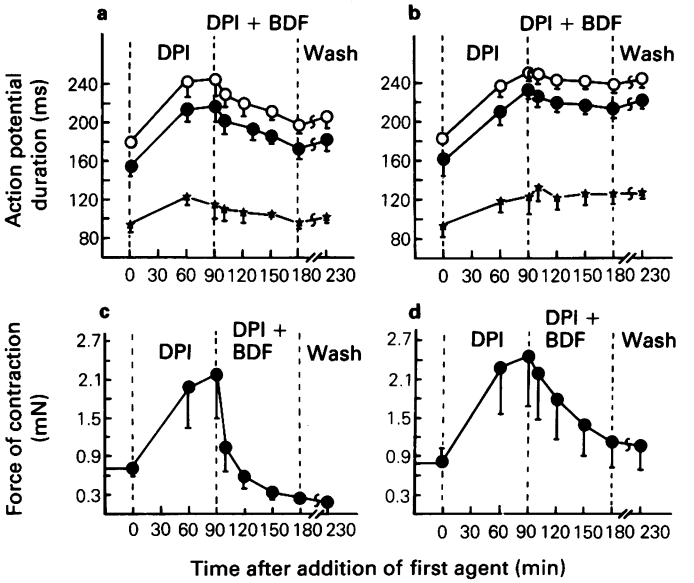


Figure 4 The influence of BDF 8784 on the effects of DPI 201-106 (1 μM) on action potential duration (a, b) and on force of contraction (c, d) in guinea-pig papillary muscles. (a and c) 1 μM BDF added after pretreatment with 1 μM DPI; (b and d) 10 μM BDF added after pretreatment with 1 μM DPI. Ordinates, (a and b): action potential duration in ms at 90% (APD_{90}, ○), 50% (APD_{50}, ●), and 20% of repolarization (APD_{20}, ★); (c and d): force of contraction in mN Abscissae: time in min after addition of first agent. The vertical dashed lines indicate the addition and the removal (wash) of agents, respectively. Mean values from 4 experiments in each group are shown; vertical lines indicate s.e.mean.

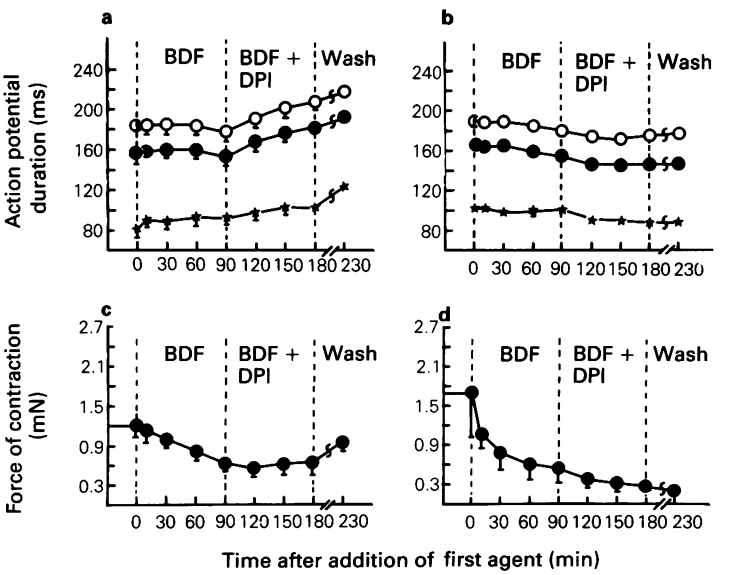


Figure 5 The influence of pretreatment with BDF 8784 (1 μM in a and c; 10 μM in b and d) on the effects of DPI 201-106 (1 μM) on action potential duration and force of contraction in guinea-pig papillary muscles. For key to symbols see Figure 4.

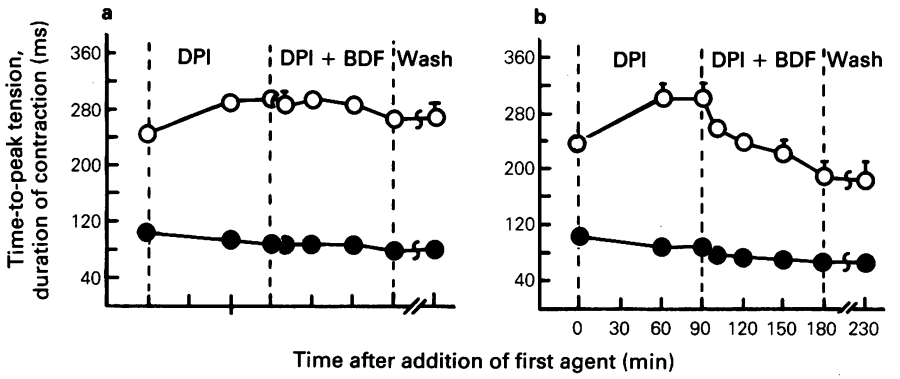


Figure 6 The combined action of BDF 8784 (1 μM, a; 10 μM, b) and DPI 201-106 (1 μM) on time-to-peak tension (●) and duration of contraction (○) of guinea-pig papillary muscles. Ordinates: time-to-peak tension or duration of contraction in ms, otherwise lay-out as in Figure 4.

pounds which previously have been shown to interact with the sodium channels. In order to obtain further evidence for the site of action of BDF we have investigated its effect on net membrane currents (Figure 8). As described previously by Buggisch *et al.* (1985), DPI (1 μM, Figure 8a) induced a slowly decaying inward current which was blocked completely by tetrodotoxin (TTX, 30 μM) and is therefore most likely to flow through sodium channels of inhibited inactivation. Since voltage control of the membrane potential was not possible during peak sodium current (I_{Na}) flow (for discussion see Isenberg & Ravens, 1984) we have determined the amplitude of I_{Na} 10 ms after the depolarization step. Under

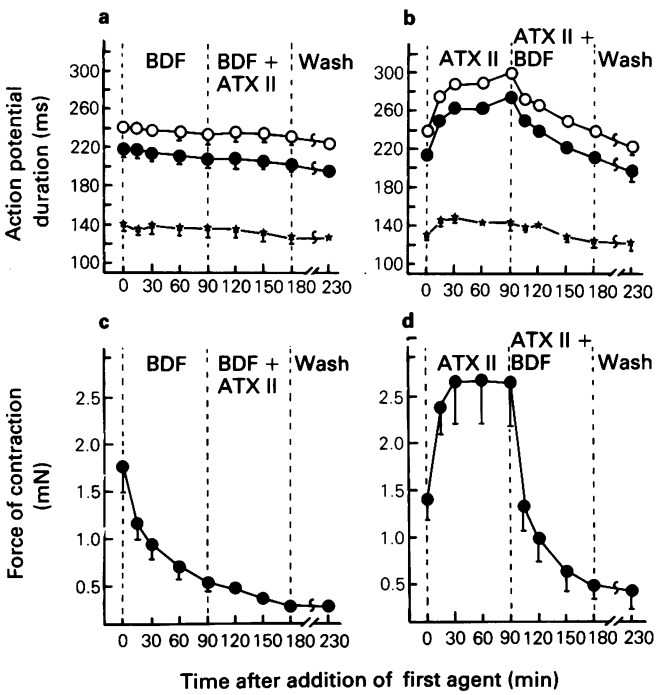


Figure 7 The effect of the combined exposure to BDF 8784 (10 μM) and to the sea anemone polypeptide ATX II (20 nM) on action potential duration and on force of contraction of guinea-pig papillary muscles. Layout and symbols as in Figure 4.

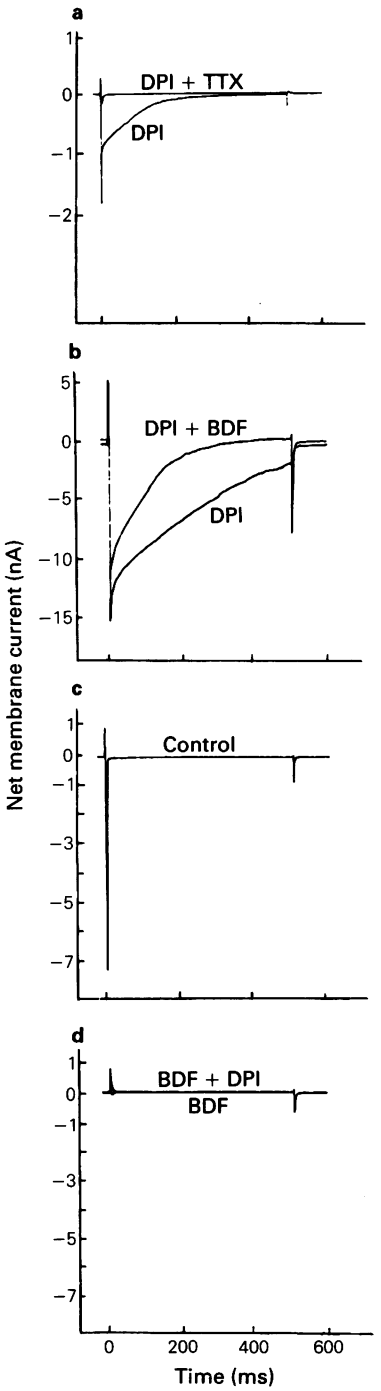


Figure 8 Modification of net membrane currents in 3 different cardiac myocytes from guinea-pig hearts by DPI 201-106 (1 μM), tetrodotoxin (TTX, 30 μM), BDF 8784 and combinations of two of each compounds. Clamp pulses were applied at a frequency of $0.2\,s^{-1}$, holding potential −80 mV, clamp potential −35 mV, duration of depolarization 500 ms; temperature of the superfusing solution 35°C. (a) Four min after addition of DPI (DPI) and 1 min after addition of TTX (DPI + TTX) to the superperfusing solution; (b) 4 min after addition of DPI (DPI) and 15 min after addition of BDF (1 μM; DPI + BDF); (c) control recording without drug addition; (d) (same cell as in c) 7 min after addition of BDF (10 μM; BDF) and 5 min after addition of DPI (DPI + BDF).

control conditions, I_{Na} had inactivated after 10 ms (Figure 8c). When BDF (1 μM) was added (Figure 8b, different cell), the DPI-induced current was reduced by 25% as determined 10 ms after the depolarization. The decay of the DPI-induced current was enhanced. When the same cell as in (c) was superfused with BDF-containing solution (10 μM, d) for 7 min the rapid inward current was completely abolished, and no recovery or enhancement was observed after further addition of DPI (1 μM).

Since the plateau phase of single-cell action potentials was abbreviated upon exposure to BDF (Figure 9) we studied whether BDF also influenced the calcium current. Calcium currents were activated by 500 ms long clamp pulses from a holding potential of −40 mV to 0 mV (lower part of Figure 9). Addition of BDF alone reduced the amplitude of the net membrane currents in response to these pulses, suggesting that it not only interacted with the sodium channels but with calcium channels as well.

Discussion

The pharmacological profile of DPI 201-106 in isolated myocardial preparations includes enhancement of force of contraction and prolongation of the action potential duration. Since both effects are abolished in the presence of tetrodotoxin they are mediated by an interaction of DPI with the voltage-dependent sodium channels (Buggisch *et al.*, 1985). DPI modifies the sodium channels by prolonging their mean open time (Kohlhardt *et al.*, 1986; 1987). Therefore, extra sodium ions enter the cell during an action potential and increase the intracellular Na^+ concentration. The subsequent decrease in the transmembrane Na^+ concentration gradient will increase the concentration of free cellular Ca^{2+} because it reduces the driving force for the outward transport of Ca^{2+} via the Na/Ca-exchange (Reuter & Seitz, 1968). Alternatively, Na^+ entering the cells during the prolonged action potential could displace calcium from cellular binding sites (Lüllmann *et al.*, 1983; Frankis & Lindenmayer, 1984). In any case, calcium not removed from the cell will be taken up

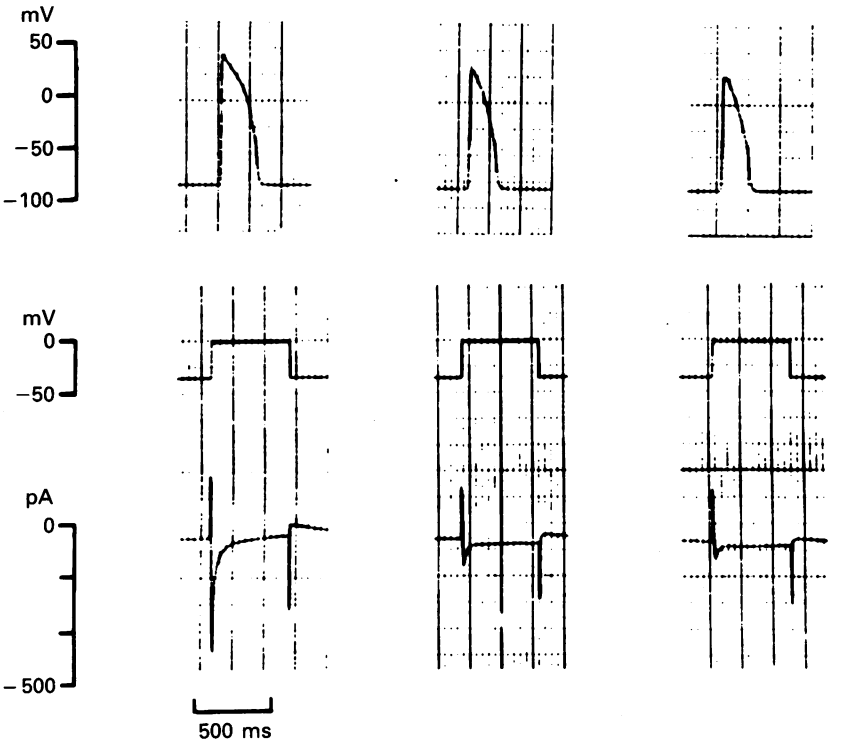


Figure 9 The effects of BDF 8784 (10 μM) in a cardiac myocyte on action potentials (upper row of records) and net membrane currents (lower row) in response to 500 ms long clamp pulses from −40 mV to 0 mV (middle row). Columns of records from left to right: control recording 5 min after establishing a seal of the patch electrode, 7 and 12 min, respectively, after exposure to BDF.

by cellular stores from which it can be released for contractile activation of subsequent contractions.

It has been proposed that the voltage-dependent sodium channel possesses several different sites for neurotoxins and/or drug binding (for review, see Catterall, 1980; Hille, 1984; Ravens & Wettwer, 1989). From their studies of the combined effects of DPI with the ceveratrum alkaloid veratridine or with the sea anemone toxin ATX II on the action potential duration, Scholtysik and coworkers (1986) concluded that the binding site for DPI should be different from, though allosterically coupled to sites 2 and 3 for the lipophilic and the polypeptide toxins, respectively (Catterall, 1980). A distinct binding site at the sodium channel for DPI was also proposed by Romey *et al.* (1987), because the binding of [^{3}H]-batrachotoxinin A 20-α-benzoate was inhibited by DPI but promoted by a scorpion toxin, although DPI and the scorpion toxin both inhibited the inactivation of the sodium current. Furthermore, the existence of a specific binding site for DPI is supported by the stereoselectivity of the action of the S- over the R-enantiomer of DPI (Scholtysik *et al.*, 1985; 1986).

If DPI binds primarily to a proposed site at the sodium channels, the simultaneous inhibition of both action potential prolongation and positive inotropic effect by BDF could indeed be explained by a BDF-induced displacement of DPI from these sites. However, BDF also prevents and inhibits the effects of ATX II (see Figure 7). Since this polypeptide is thought to bind to a site different from that for DPI (Scholtysik *et al.*, 1986), it is unlikely that BDF would displace both at the same site. Furthermore, BDF does not shift the concentration-response curve for the positive inotropic effect of DPI to the right in a competitive fashion but depresses the maximum efficacy of DPI (Figure 2). Therefore, a non-competitive antagonism is anticipated, the nature of which cannot be determined on the basis of our present results.

Scholtysik & Williams (1986) showed that DPI possesses local anaesthetic activity in cat vagal nerve. In heart preparations, high concentrations of DPI

inhibit dV/dt_{max} (Buggisch *et al.*, 1985; Scholtysik *et al.*, 1985), the effect being particularly evident at potentials positive to the resting membrane potential (Kohlhardt *et al.*, 1987). In the present study we did not find a significant depression of dV/dt_{max} by DPI because the concentration (1 μM) was too low. BDF decreased the maximum rate of depolarization suggesting some local anaesthetic activity. However, a reduced dV/dt_{max} cannot explain the reversal of the DPI-induced positive inotropism and APD-prolonging effect, because BDF did not decrease dV/dt_{max} when given after the effects of DPI had already been established (see Figure 2). Although a strict quantitative comparison is not possible, the changes in membrane currents measured in single cells reflect the findings in multicellular preparations: a strong depression of the sodium current is induced if the cells are first exposed to BDF, whereas the peak amplitude of the DPI-modified sodium current is only moderately decreased by BDF.

During the first few ms after depolarizing the membrane, potential is not expected to be well controlled because of the size and the rapid kinetics of the sodium current. However, measurement of the sodium current becomes reliable when it changes slowly because of the DPI-induced inhibition of inactivation (see also Buggisch *et al.*, 1985).

The negative inotropic effect of BDF can be explained by a decrease in the calcium current, although the depression of dV/dt_{max} may also contribute, since blockade of sodium channels by several antiarrhythmic drugs was shown recently to be responsible for part of their negative inotropic effect (Honerjäger *et al.*, 1986). The extent of the negative inotropic action of BDF was much larger in the electrophysiological experiments than in the ones where only force of contraction was measured (compare Figures 2 and 5). The reason for this quantitative difference must be due to a variation in experimental procedure (e.g. 30 min of exposure in Figure 2 versus 90 min in Figure 5).

In summary, methyl replacement of the CN-substituent in DPI not only renders DPI ineffective with regard to its ability to slow down the inactivation of sodium channels, but also converts DPI from an agonist at the sodium channels to an antagonist. BDF blocks the sodium current (as evidenced by a reduction of dV/dt_{max}) without affecting APD on its own. Despite its inhibition of calcium currents, the inhibitory effects of BDF against DPI and ATX II are a consequence of sodium channel blockade. The non-competitive nature of the antagonism by BDF of DPI suggests that both compounds—in spite of their chemical similarity—bind to two different sites on the sodium channel. BDF thus resembles R(+)-DPI, which has been found by Romey *et al.* (1987) to block the sodium channels and to lack the inotropic effects of DPI. Thus whereas the binding to sodium channels is a property common to racemic DPI and BDF, the ability to bind to the inactivated channel is a property reserved for the stereospecific S(−)-enantiomer of DPI combined with the electron-capturing properties of the CN-group. Replacement of the CN-group with a CH_3-group converts DPI from an activator to a blocker of sodium channels.

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Evidence that the hypertrophic action of clenbuterol on denervated rat muscle is not propranolol-sensitive

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1 The effect of propranolol on the clenbuterol-induced protein anabolism in innervated and denervated soleus and plantaris muscles of the rat was studied.

2 The response to the β-agonist, clenbuterol, in both innervated and denervated muscles, was not significantly inhibited by the β-antagonist, propranolol.

3 The results provide further evidence to suggest that the action of clenbuterol on skeletal muscle protein accretion may not be directly mediated by β-adrenoceptors.

Introduction

The novel protein anabolic action (Ricks *et al.*, 1984; Emery *et al.* 1984; Baker *et al.*, 1984) of the β-adrenoceptor agonist clenbuterol has aroused considerable debate as to whether the actions of the drug are directly mediated by β-receptors. The changes in muscle glycogen, body fat, and energy expenditure and the effects of the drug on smooth muscle are all fairly typical responses to a classical β-adrenoceptor agonist. Furthermore, Rothwell *et al.* (1987) suggested that the observed effect on blood flow as well as the apparent down regulation of receptors can be accounted for by direct effects of the drug on β-adrenoceptors.

In contrast, the use of a variety of adrenoceptor antagonists demonstrated that the typical β effects of clenbuterol (lipolysis, increased energy expenditure, and increased heart mass) were separable from the skeletal muscle growth response (Reeds *et al.*, 1988). In particular, propranolol blocked the clenbuterol-induced rise in cardiac muscle mass and significantly limited the reduction in body fat. However, propranolol did not reverse the protein anabolic effect of clenbuterol in skeletal muscle (Reeds *et al.*, 1988), although fibre hypertrophy was reduced (Maltin *et al.*, 1987a). These data therefore suggest that at least the protein anabolic effects of clenbuterol might not be β-receptor mediated.

We have already shown that clenbuterol can reverse or ameliorate the effects of denervation-induced atrophy and that innervation status influences the sensitivity of muscle to clenbuterol treatment (Maltin *et al.*, 1986a; 1987b). Denervation is associated with an up-regulation of β-receptors (Banerjee *et al.*, 1977), and this might be considered to be the basis for the apparent greater sensitivity of denervated muscle to clenbuterol. Thus the present study was designed to examine the effect of a mixed β-receptor antagonist, propranolol, on the amelioration of denervation-induced atrophy by clenbuterol.

Methods

Male Hooded Lister rats of the Rowett Research Institute strain were used throughout. The rats were weaned at 19 days of age and were divided into four groups of 12 animals of equal mean body weight. The animals were fed to appetite a standard laboratory rat chow (Labsure CRM nuts, K and K Greff, Croydon, U.K.) and water was freely available at all times. After 4 days the animals were re-weighed and, if necessary, re-grouped to ensure that all groups had the same mean body weight and mean growth rate over the preliminary period. The animals were then housed singly in plastic, flat bottomed cages and fed to appetite a semi-synthetic diet, PW3 (Pullar & Webster, 1977). After 4 days all the animals were anaesthetized with ether and a short (1 cm) piece of the left sciatic nerve was removed under aseptic conditions. The 4 groups were subsequently treated as follows:– control group – fed control diet PW3 for 7 days post-denervation; clenbuterol group – fed control diet PW3 for 4 days followed by PW3 containing clenbuterol ($2\,mg\,kg^{-1}$) for 3 days; propranolol group – fed control diet PW3 for 3 days followed by PW3 containing propranolol ($200\,mg\,kg^{-1}$) for 4 days; clenbuterol + propanolol group – fed control diet PW3 for 3 days, followed by PW3 containing propranolol ($200\,mg\,kg^{-1}$) for 24 h

Table 1 Fibre size and frequency in innervated and denervated soleus muscles of rats from the four treatments groups

	Control	*Prop.*	*Clen.*	*Cl + P*	*Control*	*Prop.*	*Clen.*	*Cl + P*	
		Innervated limb				*Denervated limb*			
				Mean fibre cross-sectional area (μm^2)					
FOG	1001	980	1294*	1356*	239	280	504*	403	LSD 222.1
SO	1209	1205	1226	1354	404	485	932***	803***	LSD 194.0
				Mean percentage frequency					
FOG	44.5	43.9	47.4	45.0	48.1	51.4	49.5	48.4	LSD 5.8
SO	55.5	56.1	52.6	55.0	52.0	48.6	50.5	51.6	LSD 5.8
				Mean percentage area					
FOG	39.8	38,8	48.6**	44.8*	35.5	38.4	34.4	32.1	LSD 4.9
SO	60.2	61.2	51.4**	55.2*	64.5	61.6	65.6	67.9	LSD 4.9

Values represent means of groups. Least significant difference (LSD) values were derived from the standard error of the difference of the means and the t statistic. Using the LSD, comparison between treated groups and the control group is made for innervated and denervated limbs. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Prop = propranolol, Clen = clenbuterol, Cl + P clenbuterol in combination with propranolol. FOG = fast twitch oxidative glycolytic, SO = slow twitch oxidative.

and then PW3 containing propranolol (200 mg kg^{-1}) and clenbuterol (2 mg kg^{-1}) for 3 days. The dose chosen for propranolol was a 100 × weight excess to that of clenbuterol since this dose had been shown to inhibit the typical β-agonist effects of clenbuterol (Reeds *et al.*, 1988). From each group of 12 animals, 6 were assigned at random for biochemical analysis, and 6 for histochemical analysis. Body weights of all animals were recorded daily.

At the end of the experimental period the animals were killed by cervical dislocation and the muscles removed. Only the soleus muscles were taken for histochemical analysis and treated as described previously (Maltin *et al.*, 1986b). Fibre type composition was assessed from the staining reactions for Ca^{2+} activated myofibrillar ATPase at pH 9.4 after methanol-free formalin fixation (Hayashi & Frieman, 1966). Those fibres giving the most dense reaction product were identified as fast twitch oxidative glycolytic (FOG) fibres, those which gave no reaction product were designated slow twitch oxidative (SO) fibres, while those given an intermediate reaction product were characterized as fast twitch glycolytic (FG) fibres. However, FG fibres represented less than 2% and were omitted from measurements and calculations. Quantitative assessments were made directly from the stained muscle preparations using a computer based (Torch Computers Ltd, Cambridge, U.K.) image analysis system (Vision Dynamics, Hemel Hempstead, Herts, U.K.). The use of this system required slight modifications of the ATPase stain to enhance the contrast for imaging (M.I. Delday, personal communication). A minimum of 200 fibres per muscle was measured. Soleus and plantaris muscles taken for biochemical analysis were frozen and stored at −20°C until estimations of protein, RNA and DNA were made. The methods used for determinations of protein and RNA were the same as outlined previously (Maltin *et al.*, 1986a). DNA was determined following the method of Burton (1956).

Statistical analysis

A three way analysis of variance was carried out on the data using Genstat (Rothampstead Experimental Station, 1977) on a Prime 550 computer to examine the effects of innervation, clenbuterol and propranolol. The interactions were examined in the general linear model, followed by means comparison of either main effects (no interaction) or significant interactions.

Results

As in previous studies all the animals grew well on their respective diets despite the trauma of denervation. At the end of the experiment the body weights for the groups were as follows: control 116 ± 1, clenbuterol 120 ± 3, propranolol 114 ± 3, clenbuterol + propranolol 119 ± 2 (mean values (g) ± s.e.mean, $n = 12$ for each group).

Histochemical analysis

The results of the histochemical analysis of soleus muscle are presented in Table 1. The soleus comprised two main fibre types FOG and SO.

The results from this study were broadly in line with those from previous studies. Briefly, analysis of variance for the variate fibre area showed that there were two significant main effects, those of innerva-

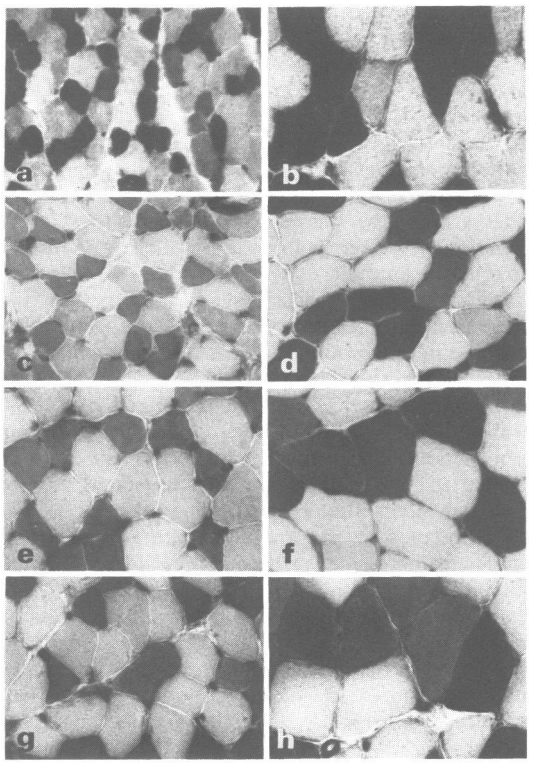

Figure 1 Transverse cryosections of soleus muscle from innervated and denervated limbs of rats from each of the four treatment groups. The tissue has been reacted to demonstrate the activity of Ca^{2+} myofibrillar ATPase. (a) Denervated muscle from control fed rat. (b) Innervated muscle from control fed rat. (c) Denervated muscle from propranolol fed rat. (d) Innervated muscle from propranolol fed rat. (e) Denervated muscle from clenbuterol fed rat. (f) Innervated muscle from clenbuterol fed rat. (g) Denervated muscle from clenbuterol + propranolol fed rat. (h) Innervated muscle from clenbuterol + propranolol fed rat. Bar = 50 μm.

tion and clenbuterol. Denervation resulted in a significant reduction in fibre size for both fibre types (Table 1). As found previously (Maltin *et al.*, 1986b) clenbuterol had an anabolic effect which was expressed as fibre hypertrophy. The effect was statistically significant in both fibre types in denervated muscles (Figure 1, Table 1) but was significant only in FOG fibres of innervated muscles (Figure 1, Table 1). Denervation, also significantly increased the frequency of FOG and decreased SO fibres as reflected in the grand means across the four groups ($P < 0.01$). There was also an interaction effect on percentage area ($P < 0.01$) between innervation status and the effect of clenbuterol. This changed in innervated muscles (Table 1), but not in denervated muscles. Hence this result provided further support for the contention that the innervation status of the muscle (for soleus at least) was important in determining the effect of clenbuterol (see also Maltin *et al.*, 1987b).

There were no significant main effects or interactions for the effects of propranolol alone or in combination with clenbuterol in either innervated or denervated muscles with respect to area, percentage frequency or percentage area.

Biochemical analysis

The results of the biochemical analysis of soleus and plantaris muscles are presented in Tables 2 and 3.

Soleus muscle (*Table 2*) Statistical analysis highlighted the significant ($P < 0.001$) main effects of innervation and clenbuterol treatment on muscle weight, protein, RNA and DNA contents. As described elsewhere (Maltin *et al.*, 1986a), denervation lead to the typical loss of muscle weight and protein and RNA content ($P < 0.001$). The presence of clenbuterol in the diet caused the typical increase ($P < 0.01$) in muscle weight irrespective of innervation status. The response in weight was accompanied by a simultaneous increase in protein and RNA

Table 2 Compositional changes in innervated and denervated soleus muscles of rats from the four treatment groups

	Control	*Prop.*	*Clen.*	*Cl + P*	
		Mean weight (mg)			
Inn.	57.9	56.1	66.2**	66.2**	
Den.	21.1	22.0	33.2***	35.8***	LSD 5.9
		Mean total protein content (mg)			
Inn.	8.6	8.7	9.9*	10.3**	
Den.	2.4	2.7	4.4**	4.8***	LSD 1.1
		Mean total RNA content (μg)			
Inn.	104.6	103.1	129.3***	126.9***	
Den.	40.2	49.9	70.9***	75.3***	LSD 10.7
		RNA/protein ($\mu g\,mg^{-1}$)			
Inn.	15.1	14.9	15.7	14.6	
Den.	24.9	23.9	20.3**	21.1*	LSD 3.0
		Mean total DNA content (μg)			
Inn.	73.7	76.3	72.7	81.0	
Den.	46.3	44.9	60.7*	64.0**	LSD 11.7
		RNA/DNA			
Inn.	1.7	1.7	2·2**	1.9	
Den.	1.2	1.5*	1.5	1.6*	LSD 0.3

Values represent means of groups. Least significant difference (LSD) values were derived as for Table 1. Using the LSD, comparison between treated groups and the control group is made for the innervated (Inn.) and denervated (Den.) limbs. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. For key to abbreviations used see legend of Table 1.

Table 3 Compositional changes in innervated and denervated plantaris muscles of rats from the four treatment groups

	Control	*Prop.*	*Clen.*	*Cl + P*	
		Mean weight (mg)			
Inn.	95.7	97.7	101.6	101.3	
Den.	37.7	41.2	52.7**	49.2*	LSD 10.6
		Mean total protein content (mg)			
Inn.	18.1	21.3*	23.0**	21.7**	
Den.	7.4	10.2*	11.4**	11.3**	LSD 2.3
		Mean total RNA content (μg)			
Inn.	171.6	164.7	201.7**	185.3	
Den.	94.2	108.8	140.7***	130.8***	LSD 17.9
		RNA/protein ($\mu g\,mg^{-1}$)			
Inn.	8.8	7.7	8.8	8.6	
Den.	10.1	10.8	12.4**	11.7*	LSD 1.3
		Mean total DNA content (μg)			
Inn.	120.2	116.0	106.8	104.3	
Den.	79.2	91.0	92.8	94.8	LSD 18.7
		RNA/DNA			
Inn.	1.4	1.4	1.9***	1.8***	
Den.	1.2	1.2	1.5***	1.4*	LSD 0.2

Values represent means of groups. Least significant difference (LSD) values and comparisons are as in Table 2. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. For key to abbreviations used see legend of Table 1.

content, which could be demonstrated in both denervated and innervated muscles. This response in denervated muscles correlated with the amelioration of atrophy as observed from the fibre sizes (Figure 1).

The sensitivity of the muscle to clenbuterol treatment was dependent on the innervation status, particularly with respect to the RNA/protein ratio and DNA content. The RNA/protein ratio was unchanged by clenbuterol in innervated muscles but was significantly ($P < 0.01$) reduced in denervated muscles and the DNA content was also largely unchanged in innervated muscles (although slightly increased in the presence of propranolol) but was significantly ($P < 0.05$) increased in denervated muscles.

Different sensitivities of innervated and denervated muscles were also evident in the effect of propranolol. Whilst for the majority of independent parameters measured propranolol could be found to have no significant effect alone or in combination with clenbuterol, with respect to RNA/DNA ratios this was not true. In denervated muscles, propranolol treatment alone was associated with an increase in RNA/DNA which was significantly ($P < 0.05$) augmented in the presence of clenbuterol. In contrast, propranolol alone had no effect on innervated muscles, whereas in combination with clenbuterol, propranolol reduced the clenbuterol-induced increase in RNA/DNA.

Plantaris muscle (*Table 3*) As described for soleus muscle, the effects of innervation and clenbuterol comprised the main significant influences on the parameters measured. Clenbuterol produced an anabolic effect in innervated muscle, and although the increase in weight was not statistically significant the anabolic effect was clearly evident as a significant ($P < 0.01$) increase in protein content. Typically, denervation gave rise to a significant ($P < 0.001$) reduction of protein and weight which was ameliorated by the anabolic effect of clenbuterol.

The difference in sensitivity of denervated and innervated muscles to treatment with clenbuterol was apparently confined to DNA for which the grand means showed that there was a significant ($P < 0.05$) interaction between the effects of clenbuterol and innervation. In innervated muscle clenbuterol produced a decrease in DNA whereas in denervated muscle the effects appeared to be opposite with clenbuterol increasing DNA.

Treatment with propranolol alone gave rise to a significant increase in protein content of both denervated and innervated muscles. In combination with clenbuterol there was significant interaction ($P < 0.05$) which was evident as a reduction in the clenbuterol-induced anabolic response in innervated but not denervated muscles due to propranolol. With regard to RNA a similar phenomenon was evident. There was the suggestion that propranolol also reduced the effect of clenbuterol but in this case independent of the innervation status of the muscle.

Discussion

The aim of this study was to assess whether the ameliorative effects of clenbuterol on denervation atrophy were inhibited by β-receptor antagonism and thereby to gain further insight into the mode of action of the drug. The results suggest that the anabolic effect of clenbuterol in denervated muscles is not significantly impaired by the administration of propranolol. In addition, the study has also produced some other important observations. First, the differences between the responses of the two muscles, and second the importance of innervation status in the response of the muscle.

The responses of both muscles to treatment with either drug alone or in combination were broadly similar to those described elsewhere for innervated soleus alone (Maltin *et al.*, 1987a). However, it must be noted that in the present experiment, the apparent separation of hypertrophic and compositional responses in innervated soleus muscles treated with the combination of drugs seen in the previous experiment was not observed. The reason for this is unclear, the only differences between this and the previous study (Maltin *et al.*, 1987a) were that in the

present study the animals were 4 days older and had been unilaterally denervated at the start of drug treatment. It is possible that denervation influenced the response of the innervated contralateral muscle by imposing an increased work load on the innervated limb. The anabolic effect of an increased work load has been shown to be additive to that of clenbuterol (Maltin *et al.*, 1987c).

Although overall the responses of plantaris and soleus were similar there were some important differences. Specifically, in plantaris muscle (but not soleus) there was a stimulation of protein accretion by propranolol alone. Furthermore in the innervated muscles, propranolol appeared to reduce the clenbuterol-induced protein anabolism. The explanation for this result is unclear. However, in other situations, such as in the myocardium (Maisel *et al.*, 1986) propranolol has been shown to up regulate β-adrenoceptors and it is possible that propranolol may do the same in skeletal muscle. Moreover in denervated muscle the 'up regulation' of receptors (Banerjee *et al.*, 1977) would also contribute to the response and might account for the greater increase in protein content in denervated plantaris muscles exposed to propranolol (38% for denervated cf 18% for innervated). Thus if the contention of Garber *et al.* (1976) that adrenaline increases amino acid retention was correct, then an up regulation of receptors available for endogenous catecholamine binding could lead to an increase in protein content of these muscles. However, this explanation is probably not satisfactory. Soleus muscle (which is often compared to cardiac muscle) does not exhibit this response, nor does this reasoning explain the observed reduction by propranolol of the clenbuterol-induced protein accretion in innervated muscle.

The changes in RNA, DNA and RNA/DNA were largely consistent with the clenbuterol-induced protein anabolic response and previous observations (Reeds *et al.*, 1986; 1988; Maltin *et al.*, 1987b). In the innervated muscles the increase in RNA and protein were not accompanied by increases in DNA, indicating that growth was expressed as hypertrophy rather than hyperplasia (see Maltin *et al.*, 1986b). In contrast, in the denervated muscles there was a tendency for increases in RNA and protein to be accompanied by increases in DNA content, implying some increase in cellularity or nucleation. The most likely source of such DNA would be satellite cells. In this context the greater increase of DNA content in denervated soleus is of interest since, in man at least, this muscle has been shown to have a large complement of satellite cells (Schmalbruch & Hellhammer, 1976). Preliminary morphological evidence suggests that these cells may contribute to nucleation of the regrowing fibres rather than to new fibres (Maltin, unpublished data).

Much of the data from the literature (see Stock & Rothwell, 1986 for review) would support the contention that the anabolic effect of clenbuterol is mediated through an action on the β-adrenoceptors. For example, Garber *et al.* (1976) demonstrated a reduction in amino acid release in incubated muscles treated with physiological levels of naturally occurring catecholamines. Interestingly this effect appeared to be mediated by the β-receptors and could be accounted for by a depression in muscle protein degradation. This might be considered analogous to the anabolic effects of clenbuterol, the action of which appeared to be mediated through a depression in protein degradation (Reeds *et al.*, 1986). Furthermore, the response of muscles to clenbuterol (slow muscles being more responsive than fast muscles (Festoff *et al.*, 1977; Maltin *et al.*, 1986a) and denervated muscles being more sensitive than innervated muscles (Maltin *et al.*, 1987b, and the present study)) is apparently consistent with the receptor densities in these muscles (Banerjee *et al.*, 1977; Williams *et al.*, 1984) and the β-receptor-mediated responses seen in innervated (Bowman & Nott, 1969) and denervated (Festoff *et al.*, 1977) muscles exposed to catecholamines.

However, there are several pieces of evidence to suggest that while the non-anabolic effects of clenbuterol are indeed mediated through β-adrenoceptors, the protein anabolic action is not. The work of Reeds *et al.* (1988) revealed that while the effects of clenbuterol on cardiac and fat mass and energy expenditure were inhibited by propranolol, the anabolic effects on muscle were unaltered. They concluded that the 'anabolic and anti-lipogenic actions' of clenbuterol were 'mechanistically distinct'. While this conclusion might be applied to the present study some caution should be used since in innervated plantaris, propranolol limited the effects of clenbuterol on protein and RNA, and furthermore in innervated soleus the effect of the drug on the RNA/DNA ratio was blocked. However, the remaining observations from the present study might lend some support to the contention of Reeds *et al.* (1988). It might be argued that the apparent inability of propranolol to block the protein response (in all muscles except innervated plantaris) was due to a variety of possibilities including different affinities of the appropriate receptors, an insufficient dose of propranolol or the relative pharmacokinetics of the two drugs. The animals were pre-fed with propranolol, (see Reeds *et al.* 1988) and the large dose of propranolol used was similar to that shown by Garber *et al.* (1976), to block the effects of adrenaline on amino acid release and was in excess of that required for 50% inhibition of isoprenaline stimulated adenylate cyclase activity (Maillet & Garber 1986). Concerning the pharmacokinetics of the two drugs, propranolol

is rather short acting (circa 2–4 h (Shand, 1974)) whereas clenbuterol has a half-life of 20–30 h in the rat. It is therefore possible that the effect of propranolol was wearing off during the periods when the rats were not feeding. If this were the case, however, it is difficult to explain the inhibition by propranolol of the clenbuterol-induced lipolysis which has been consistently observed in these experiments.

Consequently, the present observations that, in general, the anabolic response in both innervated and denervated muscles is not inhibited by propranolol may provide further evidence that the action of clenbuterol (or a metabolite) on protein accretion is not directly mediated by typical propranolol-sensitive β-adrenoceptors. This is in contrast to the typical β-receptor-mediated effects of the drug on fat, glycogen and energy expenditure.

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The relaxant properties of human calcitonin gene-related peptide on vascular and extravascular (capsular) smooth muscle of the isolated blood-perfused spleen of the anaesthetized dog

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1 The 37 amino acid human calcitonin gene-related peptide (CGRP), was injected intra-arterially into the isolated, blood perfused spleen of the dog.

2 The only vascular response observed to CGRP, once threshold had been reached (10–20 fmol), was a dose-dependent splenic arterial vasodilatation.

3 The mean intra-arterial bolus dose of CGRP to reduce the splenic arterial vascular resistance by 50% of maximum response was 0.52 ± 0.12 pmol. This value was significantly lower than the ED_{50} for the non-selective β-adrenoceptor agonist isoprenaline ($P < 0.01$) in the same experiments. CGRP is the most potent splenic vasodilator yet tested.

4 The mean maximum vasodilator response to CGRP was significantly less ($P < 0.01$) than that achieved with isoprenaline.

5 The time course of the splenic arterial vascular response to CGRP was substantially longer than that to isoprenaline.

6 The splenic vasodilator response to CGRP was not altered by the prior administration of the selective β_2-adrenoceptor antagonist, ICI 118,551.

7 At all doses of CGRP that caused splenic vasodilatation there were substantial increases in spleen volume. The time course of the response and slope of the regression line suggested an active capsular relaxation component.

8 In view of its location within the spleen and high molar potency, CGRP may be considered as a potential factor in the local control of the circulation through the spleen.

Introduction

A consequence of the alternative processing of RNA from the calcitonin gene within neural tissue is a mRNA which codes for a 37 amino acid peptide termed calcitonin gene-related peptide (CGRP). CGRP-like immunoreactivity has been identified in nerves supplying many tissues including the heart, systemic blood vessels and gastrointestinal tract (Mulderry *et al.*, 1985a,b). CGRP is a potent relaxant of isolated preparations of vascular smooth muscle (Hanko *et al.*, 1985; Uddman *et al.*, 1986) and causes peripheral vasodilatation and hypotension when infused intravenously into man (Franco-Cereceda *et al.*, 1987a) or into spontaneously hypertensive rats (Lappe *et al.*, 1987). In many vascular preparations its vasodilator properties have been shown to be dependent upon the presence of an intact endothelial lining (Brain *et al.*, 1985; Grace *et al.*, 1987). However, the actions of CGRP on extravascular smooth muscle are less well defined.

The aim of the present series of experiments was to establish the primary actions of human CGRP on the vascular and extravascular (capsular) smooth muscle of a single blood perfused organ, the spleen of the dog. This is particularly relevant since CGRP binding sites have been reported in the red pulp of the rat spleen (Sigrist *et al.*, 1986) although the precise histological location has not been established. In addition, the molar potency of CGRP was

assessed and its relative potency to other peptides was examined so that any potential physiological role could be evaluated.

A preliminary account of these results has been published (Withrington, 1986).

Methods

The experiments were performed on 4 dogs (mean weight 25.5 ± 0.54 kg; range 24.5–27.0 kg) anaesthetized with an intravenous mixture of chloralose and urethane (50 and 500 mg kg^{-1} respectively) after induction with methohexitone sodium (Brietal, 6.0 mg kg^{-1}).

The surgical procedures for isolation and perfusion of the spleen and continuous recording of splenic arterial blood, perfusion pressure and changes in spleen volume were as described previously (Corder *et al.*, 1987). Essentially, after careful preparation and isolation of the major splenic blood vessels the spleen was removed from the donor and, after the splenic artery and vein had been cannulated, placed in a perspex plethysmograph. The spleen was then perfused with arterial blood derived from the femoral circuit whilst the splenic venous blood drained passively into the femoral vein. An electromagnetic flow probe and strain gauge transducer was incorporated into the splenic arterial circuit to measure splenic arterial mean blood flow (SABF) and splenic arterial mean perfusion pressure (SAPP) respectively. These averaged signals were fed into an IBM personal computer programmed to calculate absolute values and changes in splenic arterial vascular resistance (SAVR). The plethysmograph was filled with liquid paraffin and sealed; the displacement of liquid paraffin from a weighed reservoir, connected to the plethysmograph contents, by changes in spleen volume allowed an accurate measurement of alterations in organ size (dSV) to be evaluated. A 'T' piece inserted into the arterial circuit permitted the close arterial administration of low doses of vasoactive substances. The majority of the dose-response relationship of the splenic smooth muscle systems could therefore be constructed without evoking general cardiovascular responses in the donor animal and altering the conditions of the perfusion. The temperature of the spleen and of the donor dog was maintained at approx 37°C. Hourly arterial blood samples allowed the monitoring of arterial blood P_{CO_2},P_{O_2} and pH and, if appropriate, correction to normal values was made by the intravenous infusion of 1 mmol $NaHCO_3$. The flow probe was calibrated with whole blood at the end of each experiment at which stage the spleen was also weighed after clamping of the artery and vein.

Drugs and vehicles

Isoprenaline and CGRP were injected directly into the splenic arterial line through a 'T' piece and were then washed in with saline (0.9% w/v NaCl solution) to give a constant injection volume of 2.0 ml. Human α-CGRP was purchased from Bachem and made up in sterile saline which contained human serum albumin (10 mg ml^{-1}; Elstree) and Polypep (2.5 mg ml^{-1}; low viscosity; Sigma). Close-arterial injection of this vehicle produced no change in either splenic arterial blood flow or spleen volume. The human serum albumin and Polypep were used to reduce non-specific binding of CGRP on to plastic surfaces. Isoprenaline hydrochloride (Pharmax Limited) was diluted immediately prior to injection in normal saline. All solutions were stored in ice. ICI 118,551 (erythro-DL-1-(7-methylindan-4-yloxyl) 3-isopropylamino-butan-2-ol) was prepared in saline at a concentration of 1.0 mg ml^{-1}.

Statistics

Results are presented as means ± standard errors of mean (s.e.mean). Tests for significance refer to Student's *t* test.

Results

Control values

The spleen weight was 288 ± 50.3 g representing 1.12 ± 0.18% of the body weight. The initial splenic arterial mean blood flow was 153 ± 16 ml min^{-1}. The initial splenic arterial mean perfusion pressure was 140 ± 11 mmHg giving a calculated splenic arterial mean vascular resistance of 0.95 ± 0.10 mmHg ml^{-1} min. These initial control values approximate to those recently reported from this laboratory for similar perfused spleen preparations (Corder *et al.*, 1987).

Splenic vascular smooth muscle responses

Isoprenaline Isoprenaline (Iso) has been shown previously to produce, following close arterial bolus injection, a characteristic splenic vascular response consisting of an increase in blood flow of rapid onset and short duration (Figure 1). This increase in splenic arterial blood flow represents, at constant arterial perfusion pressure, a reduction in splenic arterial vascular resistance and vasodilatation. This splenic vasodilatation is predominantly due to β_2-adrenoceptor activation since it is diminished by prior administration of the selective β_2-adrenoceptor antagonist ICI 118,551 (Corder & Withrington,

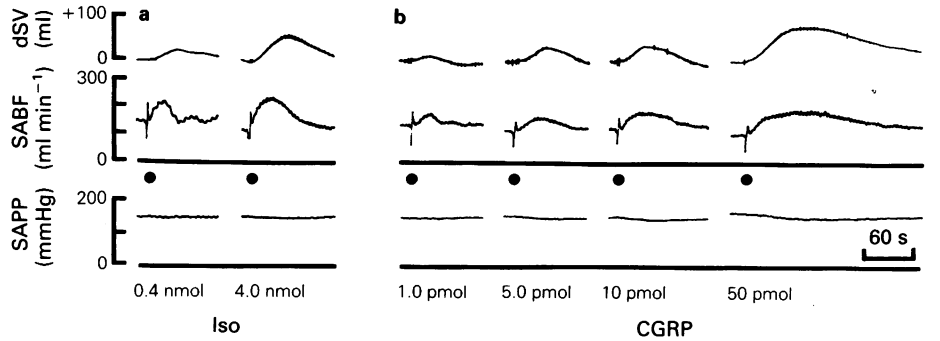

Figure 1 Responses of splenic vascular and capsular smooth muscle to intra-arterial bolus injections of isoprenaline and calcitonin gene-related peptide (CGRP). Records are from the top; dSV, increase in spleen volume; SABF, splenic arterial blood flow; SAPP, splenic arterial perfusion pressure. The two panels (a) and (b) illustrate the changes in response to intra-arterial injections of 2 doses of isoprenaline (Iso, 0.4 and 4.0 nmol) and 4 doses of CGRP (1.0, 5.0, 10 and 50 pmol). Spleen 152 g. Note the increased duration of the CGRP responses and also the greater increases in spleen volume to CGRP for approximately the same maximum vasodilator response (compare 0.4 nmol isoprenaline and 10 pmol CGRP).

1988). In the present series of experiments isoprenaline was injected over the dose-range 0.5 pmol–10 nmol and the only vascular response observed, once the threshold dose (usually 5–10 pmol) had been reached, was splenic vasodilatation. The mean maximum vasodilator effect of isoprenaline was to increase splenic arterial mean blood flow by 82.3 ± 7.4% of the control flow before the injection. The mean ED_{50}, the mean molar dose of isoprenaline to reduce the splenic arterial vascular resistance by 50% of the maximum in each experiment, was 109 ± 26.8 pmol.

Calcitonin gene-related peptide The peptide CGRP was injected as a bolus directly into the splenic artery over the dose range 1.0 fmol to 100 pmol. The only vascular response to CGRP, once the threshold (10–20 fmol) had been reached, was an increase in splenic arterial blood flow in the absence of any changes in the systemic perfusion pressure. This vasodilator response was graded with dose (Figure 1) and, characteristically, had a longer duration of action than isoprenaline when responses were compared which reached the same maximum change. Vasoconstriction was never observed. However, in any individual experiment, the maximum vasodilator effect to CGRP was less than that to isoprenaline. In this series the mean maximum increase in blood flow to CGRP was 53.8 ± 3.01% of control; this was significantly less ($P < 0.01$) than the mean maximum, in the same experiments to isoprenaline. The mean maximum increase in blood flow to CGRP was 67.3 ± 8.4% of the maximum observed to isoprenaline in the same experiments. In this series the mean molar dose-response curve relating the vasodilator activity, i.e. the reduction in splenic arterial vascular resistance, to the molar intra-arterial bolus dose of CGRP lay well to the left of the dose-response curve for isoprenaline (Figure 2). The mean ED_{50} for CGRP was 0.52 ± 0.12 pmol; a value highly significantly less ($P < 0.01$) than the ED_{50} for isoprenaline.

There was no obvious change in the splenic vasodilator response to CGRP or in the position of its dose-response curve following the i.v. administration

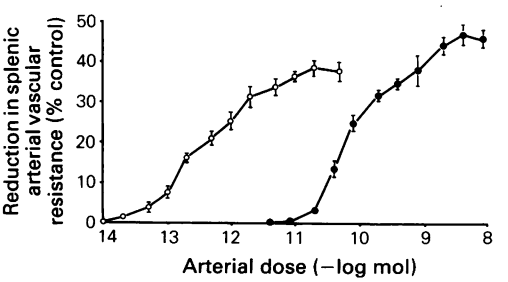

Figure 2 The relationship obtained in 4 separate isolated blood-perfused preparations, between the vasodilator activity (reduction in splenic arterial vascular resistance as a percentage of the preinjection control) and the intra-arterial molar dose of isoprenaline (●) and calcitonin gene-related peptide (CGRP, ○). The points represent the means of between 4 and 6 observations with the bars indicating the s.e.mean. There is a significant difference in the position of the curves within the co-ordinates and between the maximum change induced by the two substances.

of a dose of ICI 118,551, a selective β_2-adrenoceptor antagonist, which previous observations (Corder & Withrington, 1988) have shown to reduce significantly the splenic vasodilator response to intra-arterial injections of isoprenaline.

Splenic extravascular (capsular) smooth muscle responses

Accompanying the graded splenic arterial vasodilator responses to both intra-arterial isoprenaline and CGRP were increases in spleen volume (Figure 1). These were analysed to assess the quantitative relationship to the concomitant increases in splenic arterial blood flow. A distinction may be drawn between an active relaxation of the splenic capsule and an increase in volume that results passively from the primary relaxation of vascular smooth muscle leading to vasodilatation.

Isoprenaline There was a high correlation (0.94) between the maximum vasodilator action of isoprenaline and the maximum increase in spleen volume to any bolus dose. The slope of the regression line (Figure 3) was significantly different from zero (0.50; $P < 0.05$) although there was no significant intercept on either axis. That is, no increase in spleen volume occurred in the absence of an increase in splenic arterial blood flow. This result confirms, in the present series of experiments, previous observations (Corder & Withrington, 1988), that β-adrenoceptors are not present in the splenic capsule to induce active relaxation.

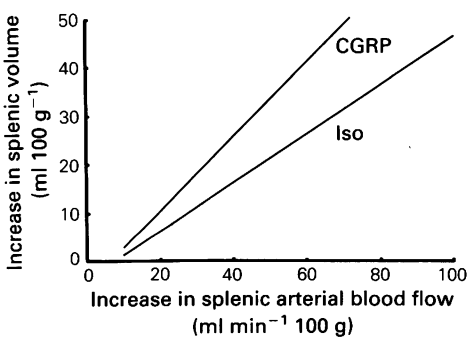

Figure 3 Computer plotted regression lines relating the increase in spleen volume (per 100 g) to the increase in splenic arterial blood flow (per 100 g) for close arterial bolus injections of isoprenaline (Iso, 45 points) and calcitonin gene-related peptide (CGRP, 40 points) in the 4 isolated, blood-perfused spleen preparations. The slopes of both lines are significantly different from zero and from each other. None of the intercepts are significantly different from zero. Individual points not included for clarity.

Calcitonin gene-related peptide Again there was a high positive correlation (0.90) between the increase in spleen volume/100 g weight and the increase in splenic arterial blood flow/100 g weight due to CGRP injections. However the slope of the regression line (0.75) was significantly greater than zero and also significantly greater than that for isoprenaline ($P < 0.01$; 0.05 respectively). These results suggest a direct relaxant action of CGRP on splenic capsular smooth muscle. There was no significant intercept on either axis, indicating the close relationship between the two parameters flow and volume for the peptide (Figure 3).

Discussion

The present experiments on the isolated, blood-perfused spleen of the anaesthetized dog reveal that the 37 amino acid calcitonin gene-related peptide (CGRP) is one of the most potent vasodilator substances yet examined in this particular organ. The mean intra-arterial bolus dose required to reduce splenic arterial vascular resistance to 50% of the control value was found to be 0.52 pmol; in similar experiments the ED_{50} for vasoactive intestinal peptide (VIP), an established vasodilator neuropeptide, was 9.9 pmol (Corder & Withrington, 1988). The two values are significantly different ($P < 0.05$). These observations in the spleen confirm the high vasodilator potency of CGRP reported for other vascular preparations either blood-perfused (dog liver, Withrington, 1987; rat renal, mesenteric and hindquarter vascular, Lappe *et al.*, 1987), *in vivo* (skin, Brain *et al.*, 1985) or isolated preparations (rat and rabbit mesenteric vasculature perfused with Krebs solution containing noradrenaline, Marshall *et al.*, 1986). In some isolated preparations e.g. rat aortic rings, the relaxant potency of the peptide has been found to be dependent upon an intact endothelium (Brain *et al.*, 1985; Grace *et al.*, 1987) the removal of which significantly lowers the sensitivity of the preparation to CGRP. However, this is not true of bovine isolated coronary vessels (Greenberg *et al.*, 1987), suggesting the peptide acts directly, at this site, on the vascular smooth muscle.

There is little available information on the histochemical distribution of CGRP-like immunoreactivity within the spleen. In the guinea-pig CGRP-LI was found in the gastroepiloic and splenic arteries (Uddman *et al.*, 1986) although very few fibres were found in the small arteries within the splenic parenchyma. Autoradiography of the rat spleen has revealed [^{125}I]-iodo-CGRP binding associated with the red pulp, but not white pulp (Sigrist *et al.*, 1986). The distribution within other tissues of the cardiovascular system (Mulderry *et al.*, 1985b) and gastro-

intestinal tract (Mulderry *et al.*, 1985a) would suggest that the peptide is located within the rich sensory innervation of the spleen. The role of the sensory innervation of visceral structures and the major blood vessels has been the subject of much constructive discussion recently since many potent vasoactive peptides, in addition to CGRP, appear to be located within defined afferent terminals. Substance P (SP), a very potent vasodilator is, in many tissues including the spleen (Franco-Cereceda *et al.*, 1987b), co-located with CGRP within terminal afferent neurones. It is necessary to reconsider the role of the sensory innervation to provide a more local function for afferent terminals in terms of a fine sensing of the environment within an organ or tissue. If appropriate, regulation of local blood flow could be achieved by the release of vasoactive peptides within the same neurone by a mechanism similar to the 'axon-reflex'. Such a local mechanism would operate in addition to the conventional projection of afferent information to the CNS.

In the present experiments the vasodilator activity of CGRP was, unlike that of isoprenaline, unaffected by the prior administration of the selective β_2-adrenoceptor antagonist ICI 118,551. This confirms, in the spleen vasculature, previous observations in other vascular beds that activation of smooth muscle β-adrenoceptors is not involved in the relaxant properties of CGRP.

There is conflicting information available about the actions of CGRP on extravascular smooth muscle. In human isolated bronchi CGRP causes dose-dependent contraction of smooth muscle (Palmer *et al.*, 1987). The extravascular smooth muscle of the dog's spleen forms the capsular and trabecular system by means of which the spleen exerts a significant capacitative function (Davies & Withrington, 1973). In the present experiments substantial increases in spleen volume accompanied the vasodilator responses to intra-arterial CGRP and there was a close quantitative relationship between the maximum increase in splenic arterial blood flow and the maximum increase in spleen volume. The similarity and interdependence of these two splenic responses may suggest a causal relationship in that the increase in volume to CGRP is the passive result of a primary active increase in arterial blood flow similar to the mechanisms proposed to occur to isoprenaline. However the increased slope of the regression line of CGRP compared to isoprenaline and vasoactive intestinal peptide (Corder & Withrington, 1988) indicates the involvement of another mechanism. This may be the active relaxation of the splenic capsule following activation of CGRP receptors on the smooth muscle cells forming the enveloping structure. It is difficult to be more precise in analysing this component since any spleen volume change represents the integral of the instantaneous arterial-venous flow difference. In the present experiments, splenic venous flow was not continuously measured and, with electromagnetic flow probes, would be difficult since the calibrations of these instruments are haematocrit-sensitive. In the dog the splenic venous haemotocrit may vary considerably from over 80% during contraction to below 30% during splenic enlargement, for example, as the result of splenic venous pressure elevation which results in selective erythrocyte sequestration (Withrington *et al.*, 1980). Nevertheless these observations confirm in the whole organ the previous accounts (Sigrist *et al.*, 1986) of an active relaxation of rat spleen strips to CGRP although only when contracted by prior addition of noradrenaline.

CGRP appears to possess unique properties of a potent vasodilator together with active relaxation of splenic capsular smooth muscle. This profile of vasodilatation and active increase in splenic capacitance is in contrast to other neuropeptides such as NPY and VIP. Its presence within the sensory innervation to the spleen indicates that its function, in contrast to the other peptides, may be to modify splenic microcirculation and cell sequestration functions as the results of local factors.

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Evidence that central 5-hydroxytryptaminergic neurones are involved in the anxiolytic activity of buspirone

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1 A two-compartment exploratory test was used to assess the role of central 5-hydroxytryptaminergic neurones in the anxiolytic activity of buspirone in rats.

2 Buspirone 0.1 mg kg^{-1}, administered subcutaneously 15 min before testing, significantly increased black-white transitions (BWT) in control rats but had no effect in animals injected intracerebroventricularly one week before with 150 μg 5,7-dihydroxytryptamine (in 20 μl).

3 Infusion of buspirone in the median raphe (but not in the dorsal raphe) significantly enhanced BWT, at doses from 1 μg to 10 μg (in 0.5 μl). Buspirone 5 and 10 μg, but not 1 μg, administered in the median raphe, significantly enhanced motor activity of rats during the first 10 min of testing in the activity cages.

4 The effect on BWT of 5 μg buspirone in the median raphe was completely antagonized in animals which had received either 5,7-dihydroxytryptamine intraventricularly, 150 μg (in 20 μl), one week before or an infusion of 0.1 μg (in 0.5 μl) (−)-propranolol in the same area 5 min before. (−)-Propranolol infused in the median raphe did not modify the effect of buspirone on locomotion.

5 Infusion of 5 μg buspirone (in 0.5 μl) in the median raphe significantly enhanced punished responses in a conflict test with no effect on unpunished responding. Buspirone infused in the dorsal raphe had no effect on punished or unpunished responding over a wide dose range.

6 The results indicate that at the relatively low dose used in the present study buspirone produces an anxiolytic effect by acting on central 5-hydroxytryptaminergic neurones. It is likely that activation of 5-hydroxytryptamine$_{1A}$-receptors in the median raphe is involved.

Introduction

It has been suggested that the ability of buspirone, a non-benzodiazepine anxiolytic compound (Riblet *et al.*, 1982; Rickels *et al.*, 1982; Garattini *et al.*, 1982), to act as an agonist at central 5-hydroxytryptamine$_{1A}$ (5-HT$_{1A}$) receptors is important for its anxiolytic activity (Peroutka, 1985; Eison *et al.*, 1986). The unavailability of selective antagonists at these receptors and the fact that the anxiolytic activity of buspirone is not identified in some models of anxiety (Pellow *et al.*, 1987; Critchley & Handley, 1987) have made it difficult to support this suggestion more directly.

Two studies recently examined the role of central 5-hydroxytryptaminergic neurones in the anxiolytic effect of buspirone by using intraventricular injections of 5,7-dihydroxytryptamine (5,7-DHT) or electrolytic lesions of the dorsal (DR) and median (MR) raphe nuclei (Eison *et al.*, 1986; Davis *et al.*, 1988). Eison *et al.* (1986) showed that 5,7-DHT reduced the anticonflict effect of buspirone, whereas Davis *et al.* (1988) found that the ability of buspirone to block fear-potentiated startle was not modified in raphe lesioned rats. Apart from the differences in anxiety models and the means used to destroy central 5-HT-ergic neurones, a possible explanation of these findings is that pre- and postsynaptic 5-HT$_{1A}$ receptors respectively are involved in the experiments of Eison *et al.* (1986) and Davis *et al.* (1988).

A difficulty with these studies is that both authors used 10 mg kg^{-1} buspirone, a dose causing profound changes in other transmitter systems such as catecholamines and acetylcholine (Kolasa *et al.*, 1982; Cimino *et al.*, 1983). In fact, even 1 mg kg^{-1} buspirone i.p. was shown, using *in vivo* voltametry, to increase striatal dopamine metabolism in rats (Louilot *et al.*, 1986). Doses of buspirone higher than

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1 mg kg^{-1} may cause sedative effects (Merlo Pich & Samanin, 1986; Critchley & Handley, 1987) which further complicate the picture.

It has been recently found that the ED_{50} of buspirone for inhibiting raphe 5-HT neuronal firing is 0.088 mg kg^{-1} (Vander Maelen *et al.*, 1986). Interestingly, 0.1 mg kg^{-1} buspirone was demonstrated to have anxiolytic activity in a two-compartment exploratory test in rats (Merlo Pich & Samanin, 1986). Even though apomorphine (0.2 mg kg^{-1} s.c.) counteracted the effect of 0.1 mg kg^{-1} buspirone s.c. and this dose of buspirone may enhance dopamine metabolism in the nucleus accumbens of freely moving rats (Louilot *et al.*, 1986), this does not exclude the possibility that the primary mechanism of action of buspirone at doses lower than 1 mg kg^{-1} is on central5-HTergic neurones.

The present study addressed this issue by examining the effects of 0.1 mg kg^{-1} buspirone in the two-compartment exploratory test in rats injected intraventricularly (i.v.t.) with 5,7-DHT. Since in a previous study it was suggested that the MR may be specifically involved in the anxiolytic activity of 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT), another 5-HT_{1A} receptor agonist (Carli & Samanin, 1988), we administered various doses of buspirone into the MR or DR and studied its effect in a two-compartment exploratory test and in a conflict test previously shown to be sensitive to systemically administered buspirone (Merlo Pich & Samanin, 1986).

Methods

Male CD-COBS rats (Charles River, Italy), weighing 200–300 g were housed at constant room temperature (21 ± 1°C) and relative humidity (60%) with a regular light/dark schedule (light: 07 h 00 min–19 h 00 min). Food (Altromin pellets for rats) and water were freely available in the two-compartment exploratory test, whereas rats used in the test for conditioned suppression of drinking had limited access to water (45 min per day after testing).

Two-compartment exploratory test

The apparatus employed to test 'approach-avoidance behaviour' has been described previously (Merlo Pich & Samanin, 1986; Carli & Samanin, 1988). It consists of a conventional open field (100 × 100 cm), divided into two compartments: a square area (40 × 40 cm) in one corner of the open field with all surfaces blackened and a roof, fitted 35 cm from the floor, to prevent light entering from above (black compartment); the rest of the open field uniformly lit by a fluorescent lamp (white compartment). Each rat was placed gently in the same peripheral lighted square close to the 'black' compartment to start the test session. The number of black-white transitions (BWT) between the two compartments was counted separately for 5 min by an observer sitting quietly at a distance of 2 m, unaware of the treatment. Whenever a rat entered a new square with all four legs an event was recorded. After testing each rat the floor was thoroughly cleaned. Testing was done between 14 h 00 min and 18 h 00 min. Animals were assigned randomly to different experimental groups.

Conflict test

The experimental chamber was a rectangular box (30 × 30 × 40 cm) with plastic sides and top and a metal grid floor. Through a hole in the middle of one wall, 4 cm in diameter, 8 cm above the grid floor, the rat had access to a stainless steel drinking tube, 7 mm in diameter, recessed 2 cm from the plane of the cage wall and connected to a plastic bottle containing approximately 100 ml of water. The support of the drinking tube contained a metal clip and leads connected to a shock device adjustable from 90 to 440 μA a.c. at 220 V. The grid floor served as the other pole to complete the shock circuit through the animal's paws. Thus only animals licking the tube received a shock under 'punished' condition. A tone signal was presented through a speaker mounted in the cage ceiling. The sensing device for the rat's contacts with the drinking tube was a photocell set horizontally in the tube support, its beam just clearing the end of the tube. The photocell beam was narrow (less than 2 mm) making it unlikely that the animal could break it without contacting the drinking tube. Direct observation indicated that in fact the beam was broken only when rats actually touched the tube. A digital programmer was used to control experimental contingencies and record responses. Three separate timers controlled cycles of tone, tone-plus-shock and silent period (no shock). Digital clocks recorded the total time of interruption of the photocell beam for each period.

During training, water-deprived rats were placed in the box without shock delivery, to locate the drinking tube and become accustomed to drinking from it. After drinking behaviour had stabilized (about 10 days of training), a schedule comprising cycles of 3 s (tone), 10 s (tone-plus-shock) and 25 s (silent period) was presented with the shock level set at 250 μA. Daily sessions lasted 10 min, yielding about 16 cycles of the sequence tone alone, tone-plus-shock and silent period. The animals were tested for drug effect when they had reached a fairly stable rate of responding during 10 min sessions (about 15 sessions). Each animal was assigned to

receive a series of counterbalanced single drug or saline injections into the MR or DR.

Motor activity

Motor activity was measured using eight wire grid cages (20.5 × 28 × 21 cm) each with two horizontal infrared photocell beams 3 cm above the floor along the long axis. Beam interruptions were recorded by an on-line microcomputer programmed in ONLI-BASIC (EuroCUBE System, Control Universal, U.K.). After 30 min habituation to the new environment, rats were injected with various doses of drug or saline and left in their home cage for 10 min. At the end of this period, they were placed in the activity cages and their motor activity recorded at 10 min intervals for 90 min.

Cannulae implantation, microinjection procedure and histological verification of guides

Rats were stereotaxically implanted under sodium pentobarbitone/chloral hydrate (equitensin) anaesthesia. One stainless steel guide cannula (23 gauge) was implanted to give access to the MR, AP +0.35, L 0.0 and V −2.6 or to the DR, AP +0.35, L 0.0 and V −0.6 (Konig & Klippel, 1963). In order to prevent clogging, 30 gauge stainless steel stylets were placed in the guide cannulae until the animals were given intracerebral injections.

On the day of the test the stylets were withdrawn and replaced by injection units (30 gauge stainless steel tubing) terminating 2 mm below the tip of the guides. Intracerebral injections of drugs or saline were made using a Harvard Apparatus compact infusion pump. Rats were hand-held while a 30 gauge injection needle was placed in the surgically implanted guide cannula. The injection needle was attached to the pump syringe by polyethylene tubing filled with drug or vehicle solution. Buspirone or (−)-propranolol HCl at the dose to be administered was dissolved in 0.5 μl saline and this volume was infused over a 1 min period. The injection cannula was withdrawn 30 s after the end of the infusion and the 30 gauge stylet replaced into the guide cannula.

Animals were returned to their home cages for 10 min before the test session commenced. Animals were tested only once in the two-compartment exploratory test and 4–5 times, each separated by two training sessions, in the conflict test.

In the experiments studying interactions between (−)-propranolol and buspirone, rats were infused with saline 0.5 μl or (−)-propranolol 0.1 μl in 0.5 μl into the MR. Five min later they were infused with buspirone 5 μg in 0.5 μl or saline 0.5 μl into the MR and left in their home cages 10 min before the test session began.

The locations of the cannulae were determined histologically after the experiments. Only data from rats in which the cannulae were located within the MR or DR nucleus were included in the results.

Injection of 5,7-dihydroxytryptamine

The rats, anaesthetized with equitensin, were immobilized in a Kopf stereotaxic instrument. 5,7-DHT creatinine sulphate 150 μg (calculated as free base) was dissolved in 20 μl of ascorbic acid solution (1 mg ml^{-1}) and administered into the right lateral ventricle. Control animals received only the ascorbic acid solution (vehicle). To protect noradrenaline containing neurones from the action of 5,7-DHT (Baumgarten *et al.*, 1973), 30 min before the injection of 5,7-DHT the rats were given desipramine (DMI) 25 mg kg^{-1} i.p., an inhibitor of noradrenaline uptake into nerve endings (Samanin *et al.*, 1975).

In one experiment, animals were treated i.v.t. with 5,7-DHT or ascorbic acid solution and implanted with guide cannulae to give access to the MR. Animals treated with 5,7-DHT and their controls were caged singly until testing. After surgery, animals were allowed to recover for 7 days during which time they were accustomed to being handled.

At the end of the experiments rats treated with 5,7-DHT and their controls were killed by decapitation and their brains were rapidly removed for assay of 5-HT according to Achilli *et al.* (1985). When buspirone was infused in the MR, 5-HT was assayed in the forebrain and the remaining brainstem was used for histological examination of the cannulae locations.

Statistics

Data were analysed by one-way analysis of variance followed by Dunnett's *t* test. Two-way analysis of variance (ANOVA 2 × 2) (experiments with 5,7-DHT or (−)-propranolol) was followed by Tukey's test. Biochemical data were analysed by Student's *t* test. The motor activity data were processed by analysis of variance with split-plot design with factors 'time' within subjects and factor 'treatment' between subjects. In the experiment in which (−)-propranolol and buspirone were infused into the MR and activity measured in activity cages, each interval was analysed by ANOVA 2 × 2 followed by Tukey's test.

Drugs

5,7-DHT creatinine sulphate was purchased from SERVA, Feinbiochemical (Heidelberg F.R.G.). The various drugs were kindly provided by Bristol Myers (Evansville, Indiana, U.S.A.) (buspirone); ICI

Pharma. (Macclesfield, Cheshire, U.K.) ((−)-propranolol HCl); Ciba-Geigy (Origgio VA, Italy) (DMI) and Abbott (Saint-Remy-sur Avre, France) (sodium pentobarbitone). All drugs were dissolved in saline or distilled water.

Results

Two-compartment test

In agreement with previous findings (Merlo Pich & Samanin, 1986) in preliminary experiments buspirone showed anxiolytic activity in the two-compartment exploratory test only in a narrow dose range, 0.1 mg kg^{-1} being the most effective.

Figure 1 shows that buspirone 0.1 mg kg^{-1} s.c. administered 15 min before testing significantly increased BWT ($P < 0.01$, Tukey's test) in rats treated i.v.t. with vehicle but had no effect on BWT of 5,7-DHT-treated rats (ANOVA 2 × 2; Fint(1,33) = 11.2, $P < 0.01$). Rats receiving buspirone or saline had comparable 5-HT depletion in the brain. 5,7-DHT depleted brain 5-HT to about 20% of controls. Levels of 5-HT (±s.e.mean) were (in ng g^{-1}): sham + saline 332.5 ± 11.9; sham + buspirone 303.5 ± 15.6; 5,7-DHT + saline 70.1 ± 6.9** and 5,7-DHT + buspirone 54.8 ± 5.1** (**$P < 0.01$ vs sham, Student's t test). Three animals injected with buspirone but which responded to 5,7-DHT treatment with only a slight decrease in brain 5-HT levels (less than 40%) showed a high number of BWT and were not included in the results.

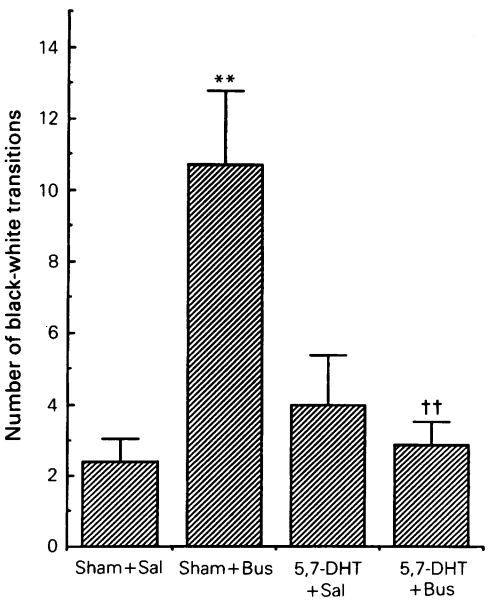

Figure 1 Effects of buspirone 0.1 mg kg^{-1} s.c. (Bus) or saline 2 ml kg^{-1} (Sal) on black-white transitions by vehicle (Sham) or 5,7-dihydroxytryptamine (5,7-DHT, 150 μg in 20 μl) intraventricularly-treated rats. Buspirone was injected 15 min before the test session. ANOVA 2 × 2, Fint(1,33) = 11.2 ($P < 0.01$). ** $P < 0.01$ vs Sham + saline (Tukey's test). †† $P < 0.01$ vs Sham + Bus (Tukey's test).

Table 1 Effects of buspirone infused into the rat median raphe nucleus on black-white transitions (BWT) and motor activity

Drug	(μg in 0.5 μl)	*BWT*	*Activity counts*
Saline		1.4 ± 0.4	27.7 ± 15.8
Buspirone	1.0	6.7 ± 1.1*	42.8 ± 6.6
Buspirone	5.0	16.0 ± 2.5**	69.3 ± 9.4**
Buspirone	10.0	18.0 ± 2.0**	71.5 ± 13.4**

The values are means ± s.e.mean of 6 rats per group. Separate groups of animals were used.
BWT: * $P < 0.05$; ** $P < 0.01$ compared to saline; Dunnett's t test.
Activity counts: ** $P < 0.01$ compared to saline; Tukey's test.

Table 1 shows the effect of buspirone administered in the MR on BWT and activity counts. Control rats (saline) showed a clear preference for the dark area (very few BWT) but, when infused with buspirone into the MR, frequently moved towards the illuminated area, with a significant increase in BWT (ANOVA F(3,20) = 21.0, $P < 0.001$). The effect of buspirone infused into the MR on BWT was dose-dependent between 1 μg ($P < 0.05$, Dunnett's t test) and 5 μg ($P < 0.01$, Dunnett's t test). Buspirone 10 μg ($P < 0.01$, Dunnett's t test) did not raise the number of BWT further. Infusions of buspirone (1 and 5 μg in 0.5 μl) into the DR did not increase BWT at any dose tested (F(2,16) = 0.35, NS) (data not shown).

Motor activity

To examine whether increased motor activity might account for the effect of buspirone on BWT, doses of 1.0, 5.0 and 10 μg buspirone were infused into the MR and motor activity was measured in the activity cages. As shown in Table 1, 5 μg and 10 μg but not 1 μg significantly enhanced motor activity (measured as increase in photocell interruptions) during the first 10 min of testing ($P < 0.01$, Tukey's test).

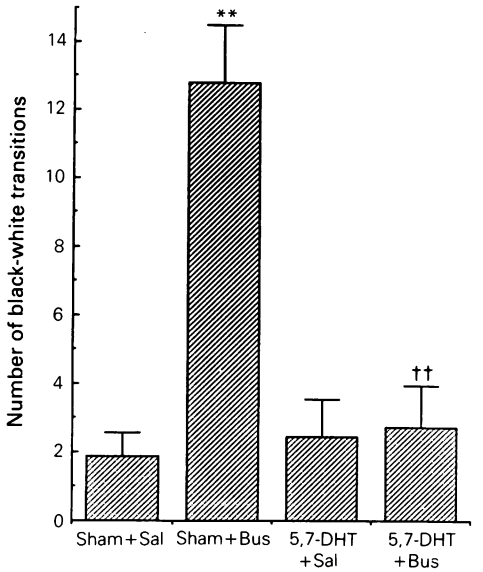

Figure 2 Effects of buspirone 5 μg in 0.5 μl (Bus) or saline 0.5 μl (Sal) injected into the median raphe nucleus on black-white transitions by vehicle (Sham) or 5,7-dihydroxytryptamine (5,7-DHT, 150 μg in 20 μl) intraventricularly treated rats. Buspirone was injected 10 min before the test session. ANOVA 2 × 2, Fint(1,34) = 13.3 ($P < 0.001$). ** $P < 0.01$ vs Sham + saline (Tukey's test). †† $P < 0.01$ vs Sham + Bus (Tukey's test).

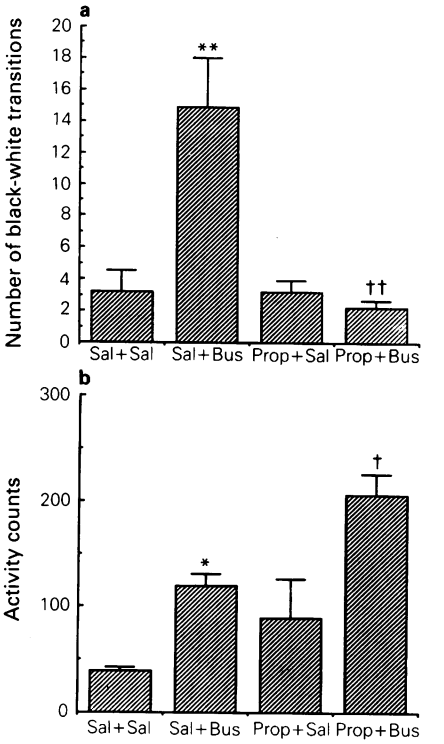

Figure 3 Effects of (−)-propranolol 0.1 μg in 0.5 μl (Prop), saline 0.5 μl (Sal) and buspirone 5 μg in 0.5 μl (Bus) injected alone or in combination into the median raphe nucleus on black-white transition (a) and activity counts (b). (a) Black-white transitions ANOVA 2 × 2, Fint(1,19) = 10.3 ($P < 0.01$). * $P < 0.05$, ** $P < 0.01$ vs Sal + Sal (Tukey's test). † $P < 0.05$, †† $P < 0.01$ vs Sal + Bus (Tukey's test). (b) Activity counts ANOVA 2 × 2, Fint = 0.69 (NS).

Effects of buspirone in animals pretreated with 5,7-DHT or (−)-propranolol

Figure 2 presents the effects on BWT of buspirone infused into the MR of rats which had received 5,7-DHT 150 μg i.v.t. or vehicle 7 days before. 5,7-DHT did not modify BWT but completely antagonized the effect of buspirone 5 μg in 0.5 μl infused into the MR (ANOVA 2 × 2; Fint(1,34) = 13.3, $P < 0.001$). Forebrain levels of 5-HT were markedly reduced in 5,7-DHT-treated animals. Levels of 5-HT (± s.e.mean) were in $ng\,g^{-1}$: sham + saline 258 ± 9.4; sham + buspirone 258.8 ± 6.1; 5,7-DHT + saline 34.9 ± 2.3**; 5,7-DHT + buspirone 32.0 ± 2.4** (** $P < 0.01$ vs sham, Student's t test).

The effects on BWT of (−)-propranolol and buspirone infused into the MR, are shown in Figure 3a. A dose of 0.1 μg (−)-propranolol in 0.5 μl infused into the MR had no effect on BWT but completely antagonized the increase induced by 5 μg (in 0.5 μl) buspirone infused into the MR (ANOVA 2 × 2; Fint(1,19) = 10.32, $P < 0.01$). (−)-Propranolol 0.1 μg (in 0.5 μl) infused into the MR tended to enhance motor activity (Figure 3b), and had an additive effect with 5 μg buspirone (ANOVA 2 × 2; Fint(1,24) = 0.69, NS; $P < 0.05$, Tukey's test).

Conflict test

Figure 4 shows the effects of buspirone infused into the MR on punished and unpunished responding in a conflict test. After about 20 days of training the animals showed low, stable levels of responding during shock presentation periods and a high level of responding during unpunished periods. Infusions of 5 μg buspirone into the MR increased the drinking time during the punished period of the schedule to 204% of controls (ANOVA; F(2,16) = 20.5, $P < 0.01$) but had no effect on unpunished responding (ANOVA; F(2,16) = 1.15, NS). Buspirone 1 μg administered into the MR had no effect on

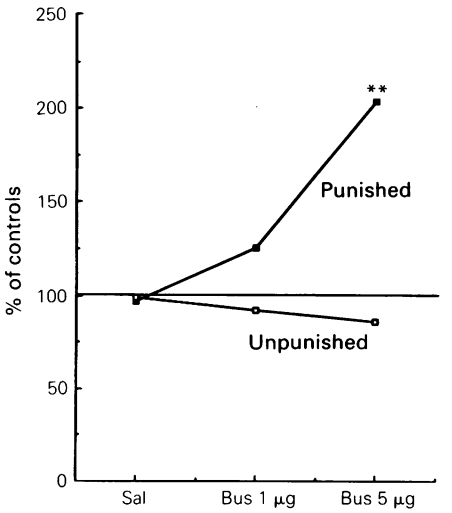

Figure 4 Effects of buspirone (Bus, 1 and 5 μg in 0.5 μl) and saline injected into the nucleus raphe medianus on punished (■) and unpunished (□) responding in a conflict test. Control values (mean ± s.e.mean) for punished and unpunished responses were 1.8 ± 0.4 and 260 ± 19.1 (s), respectively. ** $P < 0.01$ compared to saline (Dunnett's t test).

either component. Infusions of buspirone into the DR did not release shock-suppressed drinking (ANOVA; $F(4,32) = 0.17$, NS) or modify unpunished responding (ANOVA; $F(3,32) = 0.18$, NS) over a wide dose range (0.1–10 μg) (data not shown).

Discussion

An intracerebroventricular injection of 5,7-DHT which depleted brain 5-HT by about 80% completely antagonized the increase of BWT caused by 0.1 mg kg^{-1} buspirone. This suggests that under the present experimental conditions buspirone acts on brain 5-HTergic neurones to exert its anxiolytic activity. This agrees with the results of Eison *et al.* (1986) who found that the anticonflict effect of 10 mg kg^{-1} buspirone was reduced in 5,7-DHT-treated rats. In a previous study we were unable to find an anticonflict effect, in a procedure using conditioned suppression of drinking, with doses of buspirone higher than 1.2 mg kg^{-1}. A possible explanation for these differences is that Eison *et al.* (1986) used a modified version of Vogel's test in which *untrained* rats were required to lick a drinking tube during punishment with electric shocks. Since 8-OH-DPAT, another 5-HT$_{1A}$ receptor agonist (Middlemiss & Fozard, 1983), was recently shown to enhance drinking in naïve but not in habituated rats (Carli & Samanin, 1988), effects of buspirone on primary drives may have influenced the punished responses in the Vogel test. Eating caused by 5-HT$_{1A}$ agonists in sated rats has been found to be prevented by intraventricular injection of 5,7-DHT (Bendotti & Samanin, 1986).

As regards the failure of Davis *et al.* (1988) to antagonize the effect of 10 mg kg^{-1} buspirone on the fear-potentiated startle response with raphe lesions or 5-HT antagonists, it is possible that non-5-HT mechanisms are involved in the effect of this dose of buspirone in this particular model. Buspirone can have different effects on different populations of dopaminergic pathways (Louilot *et al.*, 1986) and the central nucleus of the amygdala has been shown to be critical for the potentiated startle response (Hitchcock & Davis, 1986). Therefore, as suggested by Davis *et al.* (1988), at a dose of 10 mg kg^{-1} some action of buspirone on dopamine receptors in this limbic area may be involved in its effect on the potentiated startle response.

As stated in the introduction, the dose of buspirone used in the present study is closely related to that inhibiting the firing of 5-HTergic neurones in raphe nuclei (Vander Maelen *et al.*, 1986) and in a previous study administration of 8-OH-DPAT into the nucleus raphe medianus (but not the dorsalis) enhanced BWT in the two-compartment exploratory test and punished responding in conditioned suppression of drinking (Carli & Samanin, 1988). It is likely therefore that the anxiolytic effect of buspirone found in the present study depends on its ability to activate 5-HT$_{1A}$ receptors in midbrain raphe nuclei.

To prove this more directly, we administered various doses of buspirone in the MR or DR and studied its effect in the two-compartment exploratory test. At doses ranging from 1 to 10 μg buspirone enhanced BWT of rats when administered in the MR but not in the DR. Although 5 and 10 μg buspirone in the MR markedly enhanced motor activity in the activity cages, 1 μg had no significant effect, making it unlikely that the anxiolytic-like activity is due to increased general activity of the animals.

That an increase in activity does not necessarily influence the effect of buspirone on BWT is shown by the fact that administration of 0.1 μg (−)-propranolol into the MR completely antagonized the anxiolytic-like effect of buspirone without changing its effect on motor activity. (−)-Propranolol has affinity for 5-HT$_1$ receptors (Nahorski & Willcocks, 1983) and was demonstrated to block the inhibitory effects of 5-HT$_{1A}$ agonists on the firing of 5-HTergic neurones in the raphe area (Sprouse & Aghajanian,

1986). Although a possible contribution of (−)-propranolol effects on β-adrenoceptors cannot be excluded with certainty, it is likely that 5 μg buspirone in the MR produces an anxiolytic effect by acting on 5-HTergic neurones. Involvement of 5-HT in the effect of buspirone is further supported by the finding that this dose of buspirone in the MR had no effect in 5,7-DHT-treated animals.

To confirm that buspirone administered in the MR has anxiolytic activity, we studied its effect in a conflict test where punished and unpunished responses may provide information, respectively, on anxiolytic activity and effects on sensorimotor performance (Merlo Pich & Samanin, 1986; Carli & Samanin, 1988). Five μg buspirone significantly enhanced punished responses while 1 μg caused only an insignificant increase. No significant effects on unpunished responding were found with any dose. These results can be taken to indicate that the anxiolytic effect of buspirone is not secondary to changes in sensorimotor performance. Over a wide dose range, buspirone administered into the DR did not significantly modify punished or unpunished responses. These findings confirm a previous study (Carli & Samanin, 1988) suggesting that activation of 5-HT_{1A} receptors, specifically in the MR, produces anxiolytic-like effects in rats.

In conclusion, in this study systemic administration of a relatively low dose of buspirone caused anxiolytic-like effects which require the integrity of central 5-HTergic neurones. Anxiolytic effects were also found when the drug was administered into the MR (but not the DR) and the effects were prevented by selective destruction of 5-HTergic neurones or administration of (−)-propranolol in the same brain region. Buspirone administered in the MR increased locomotion but this effect could be separated from the anxiolytic activity. It thus appears that activation of 5-HT_{1A} receptors in one brain region rich in 5-HT cells (the MR nucleus) causes anxiolytic activity. Since activation of these receptors results in reduced 5-HT transmission (Vander Maelen *et al.*, 1986), the findings are compatible with the hypothesis that 5-HT in the brain acts by favouring responses to signals of punishment and anxiety.

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Differential susceptibility of cholinergic and noncholinergic neurogenic responses to calcium channel blockers and low Ca^{2+} medium in rat urinary bladder

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1 The influence of calcium channel blockers and low Ca^{2+} medium on the neurogenic responses to single pulse electric field stimulation in rat urinary bladder has been examined.

2 Single pulse stimulation evoked a biphasic contractile response consisting of a fast component with a time to peak of 0.72 ± 0.05 s and a slow component that reached a maximal tension at 2.8 ± 0.21 s, possibly mediated by two different neurotransmitters.

3 Atropine (3×10^{-6} M) selectively inhibited the slow component without altering the fast component, suggesting the involvement of cholinergic and non-cholinergic neurotransmitters, respectively.

4 Reducing Ca^{2+} in the medium to 1/4 of the normal, abolished the slow component of the neurogenic response while the fast contractile response was not altered which may indicate a relatively greater dependence of the cholinergic component on extracellular Ca^{2+} than the non-cholinergic one.

5 The IC_{50} values for the fast component with respect to verapamil and diltiazem were 1.08 μM and 1.76 μM, respectively. The greater susceptibility of the slow component to calcium channel blockers (IC_{50} values of verapamil: 0.07 μM and of diltiazem: 0.25 μM) indicates the differential activation of slow calcium channels by the endogenously released substances.

6 Calcium channel blockers inhibited the ATP-induced contraction which was comparable to that of the non-cholinergic component of the neurogenic response suggesting the involvement of ATP as a possible neurotransmitter.

7 ACh-induced contractions were relatively less susceptible to calcium channel blockers and low Ca^{2+} medium than was the atropine-sensitive cholinergic component of the neurogenic response.

Introduction

A rise in the intracellular Ca^{2+} is critical in triggering smooth muscle contraction and this is achieved by various agonists through an influx of extracellular Ca^{2+} into the cell or through the release of intracellular Ca^{2+} from the subcellular organelles. The agonists' selectivity of utilizing cellular Ca^{2+} from a particular source(s) is evident from their differential susceptibility to calcium channel blockers/Ca^{2+} withdrawal (Godfraind *et al.*, 1986). For instance, methoxyverapamil (D-600) inhibited K^+-induced contractions significantly more than the carbachol-induced responses in rabbit urinary bladder (Batra *et al.*, 1987). A similar phenomenon of different susceptibility of various components of the neurogenic contractile responses to calcium channel blockers is known to occur in some smooth muscles (rat vas deferens: French & Scott, 1981; Raviprakash *et al.*, 1985, rabbit urinary bladder: Andersson *et al.*, 1986). The endogenous neurotransmitters are known to play a significant role in the physiological regulation of smooth muscle contractility. Although electrically field-stimulated myogenic contractile responses in rat urinary bladder have been reported to occur as a result of $[Ca^{2+}]_o$ influx through voltage-dependent calcium channels (Huddart & Butler, 1986), no such information with respect to neurogenic responses is available. Hence, the present study was undertaken to elucidate the sources of activator Ca^{2+} and the

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pathways responsible in the translocation of Ca^{2+} in the field-stimulated neurogenic responses of urinary bladder.

Methods

Male albino rats, obtained from the Laboratory Animal Resource Section of this Institute, were used in the present study. Animals were killed with a blow to the head followed by cervical dislocation. Smooth muscle strips of about 3 × 10 mm were dissected from the urinary bladder. The strips were attached to the holder assembled with a pair of platinum plate electrodes (8 mm apart), and were suspended in Tyrode solution of the following composition (mM): NaCl 138, KCl 5.9, $NaHCO_3$ 11.9, glucose 5.5, $NaH_2PO_4 \cdot 2H_2O$ 0.5, $MgCl_2 \cdot 6H_2O$ 0.5 and $CaCl_2 \cdot 2H_2O$ 1.9. In experiments where the effects of low Ca^{2+} were studied, the $CaCl_2$ in the Tyrode solution was 1/4 (0.47 mM) of the normal. The solution was bubbled with oxygen (pH 8.0) and maintained at 37 ± 0.5°C. The preparations were allowed to equilibrate for a period of 1 h under a resting tension of 0.5 g.

Isometric contractions were recorded with a force displacement transducer connected to a polygraph (M/S Medicare, India). The urinary bladder strip was stimulated with a single square wave pulse (1 ms pulse duration, 80 V), every 15 min. This stimulation produced a biphasic contraction consisting of an early fast phase and a late slow phase. These responses were abolished by tetrodotoxin (0.5×10^{-7} M) indicating that the responses were entirely of neural origin. Calcium channel blockers were added cumulatively, with each concentration being left in contact for a period of 15 min.

Contractions were elicited with either acetylcholine (ACh) or adenosine triphosphate (ATP) at doses that produced submaximal responses of the urinary bladder, every 30 min. The contact periods for ACh and ATP were 10 min and 20 s, respectively. To evaluate the effect of calcium channel blockers on the agonist-induced contractions, a single dose (10^{-6} M) of verapamil or diltiazem was added 15 min before eliciting the response. The dose selected was based on the preferential inhibition of the slow component of the neurogenic response.

To study the dependence of the contractile responses to electrical field stimulation, to ACh and to ATP on extracellular Ca^{2+}, the tissues were incubated for 15 min in low Ca^{2+} solution containing 1/4 of the normal Ca^{2+}.

The following drugs were used: acetylcholine chloride (Sigma), adenosine triphosphate disodium salt (Sigma), atropine sulphate (Sigma), diltiazem HCl (Gift; Marion Laboratories), verapamil HCl (Gift; German Remedies) and tetrodotoxin (Sigma).

Results are given as mean ± s.e.mean. Student's t test was used to test for significance and regression analysis was used to calculate IC_{50} values and their 95% confidence limits.

Results

Single pulse electrical field stimulation at 15 min intervals, produced a biphasic contractile response consisting of a fast component, with an average time to peak of 0.72 ± 0.05 s and a slow component which reached the maximum tension at 2.8 ± 0.21 s in rat

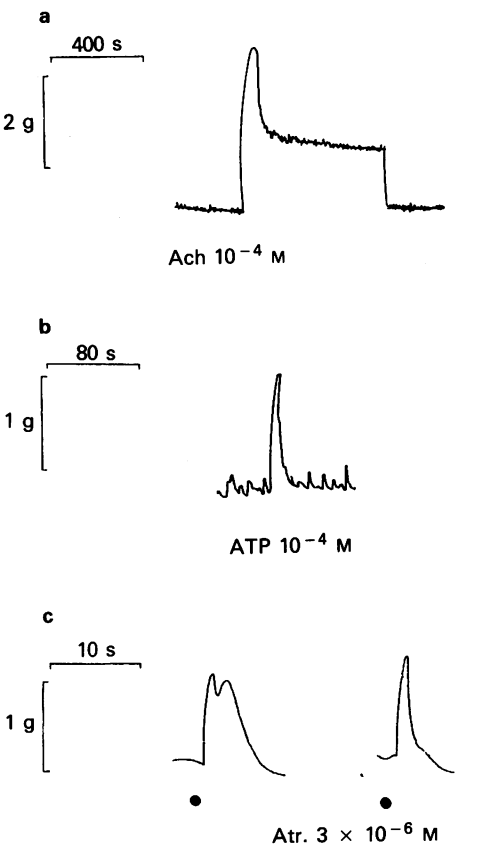

Figure 1 Isometric recordings made from rat urinary bladder strip preparation to illustrate (a) the biphasic contractile response induced by acetylcholine (ACh, 10^{-4} M); (b) the monophasic contractile response induced by ATP (10^{-4} M) and (c) the effect of atropine (3×10^{-6} M) on the fast and slow components of the single pulse (1 ms; supramaximal voltage)-induced neurogenic response (●; n = 4–8).

urinary bladder strip ($n = 8$; Figure 1c). Incubation of the tissue for 15 min with atropine (3×10^{-6} M) selectively inhibited the slow component without affecting the fast non-cholinergic component (Figure 1c).

Verapamil (3×10^{-7} M) and diltiazem (3×10^{-7} M) preferentially abolished the slow component while the fast component was not significantly affected (Figure 2a,b). The differential susceptibility of the fast and slow components to calcium channel blockers is evident from the concentration-related inhibition of the neurogenic responses (Figure 3a,b) by verapamil (10^{-8} M to 10^{-5} M) and diltiazem (10^{-8} M to 10^{-5} M). The IC_{50} values for verapamil and diltiazem and the confidence limits are presented in Table 1.

Incubation of the tissue in 1/4 Ca^{2+} medium selectively abolished the slow component while the fast component of the neurogenic response was not affected (Figures 2c and 5). Restoration of full Ca^{2+} to tissues exposed to low Ca^{2+} completely restored the responses (Figure 2c).

ATP (10^{-4} M) produced reproducible monophasic contractions in rat urinary bladder with a maximal tension of 0.98 ± 0.12 g ($n = 12$; Figure 1b). Verapamil (10^{-6} M) and diltiazem (10^{-6} M) significantly ($P < 0.05$) inhibited ATP-induced contractions

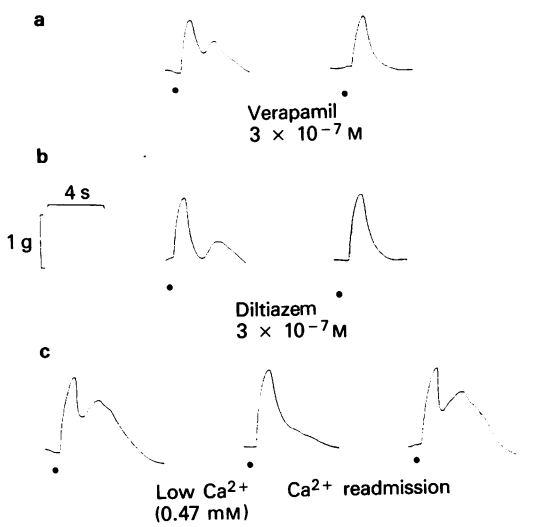

Figure 2 Effect of calcium channel blockers and low Ca^{2+} medium (0.47 mM) on the single pulse-induced neurogenic responses (●) in rat urinary bladder. (a) Verapamil (3×10^{-7} M) selectively inhibited the slow component ($n = 4$). (b) Diltiazem (3×10^{-7} M) selectively inhibited the slow component ($n = 4$). (c) Preferential inhibition of the slow component in low Ca^{2+} medium and its restoration on readmission of full Ca^{2+} ($n = 4$).

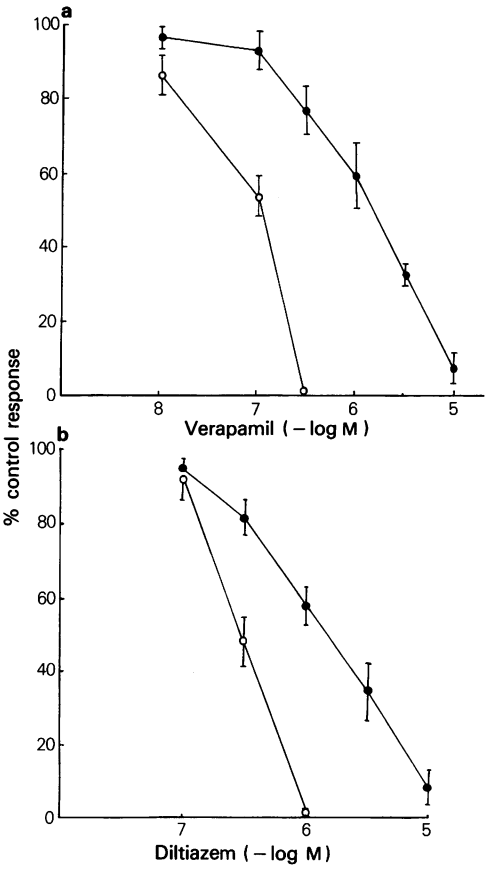

Figure 3 (a) Effect of cumulative concentrations of verapamil on the fast (●) and slow (○) components of single pulse-induced neurogenic response in rat urinary bladder. (b) Effect of cumulative concentrations of diltiazem on the fast (●) and slow (○) components of single pulse-induced neurogenic response. Each point is the mean of four determinations; s.e.mean shown by vertical bars.

(Figure 4); the mean percentage inhibition was 46.47 and 28.8, respectively. Exposure of the tissues to low Ca^{2+} medium resulted in significant ($P < 0.01$) inhibition of the ATP-induced contractions (Figure 5). These contractions were restored in medium containing normal Ca^{2+}.

ACh (10^{-7} M to 10^{-4} M) caused dose-related biphasic contractile response in rat urinary bladder consisting of an initial phasic component followed by a slow tonic component. The mean maximal tension of the phasic and tonic components recorded with ACh (10^{-4} M) was 3.03 ± 0.38 g and 0.8 ± 0.15 g, respectively ($n = 12$; Figure 1a). Verapamil (10^{-6} M) and diltiazem (10^{-6} M) significantly

Table 1 Effect of verapamil and diltiazem on fast and slow components of electrically field-stimulated (1 ms supramaximal voltage) rat urinary bladder

	IC_{50} (M)	
	Fast component	*Slow component*
Verapamil $n = 4$	1.081×10^{-6} (0.816×10^{-6}–1.425×10^{-6})	0.697×10^{-7} (0.429×10^{-7}–1.13×10^{-7})*
Diltiazem $n = 4$	1.758×10^{-6} (1.303×10^{-6}–2.371×10^{-6})	2.552×10^{-7} (1.999×10^{-7}–3.258×10^{-7})*

* $P < 0.05$ when the IC_{50} values of fast and slow components are compared.
Values in parentheses indicate 95% confidence limits.

($P < 0.01$) inhibited both the components of ACh (10^{-4} M)-induced contractions, the tonic component being relatively more susceptible than the phasic one (Figure 4). The differential potency of equimolar concentrations of verapamil and diltiazem is evident from the percentage inhibition which was 56.7 and 30.1, respectively for the phasic component, and 100 and 63.5, respectively for the tonic one. Incubation of the tissues in a low Ca^{2+} medium significantly ($P < 0.01$) inhibited the ACh responses (Figure 5). The inhibition was reversible and inversely proportional to the concentration of exogenously applied ACh. For instance, low Ca^{2+} medium inhibited the phasic and the tonic components of ACh (10^{-4} M) induced-response by about 27.1 ± 5.5% and 27.6 ± 4.9%, respectively ($n = 5$), while at 10^{-6} M ACh the inhibition of phasic and tonic components was 50.15 ± 2.2% and 40.1 ± 4.7%, respectively. Further, when the concentration of ACh was reduced to 10^{-7} M, the low Ca^{2+} medium abolished the phasic component and inhibited the tonic component by 60%.

Discussion

Single pulse-induced contraction of the rat urinary bladder consisted of two components: an initial fast contraction which was resistant to atropine and a slow component which was sensitive. This observa-

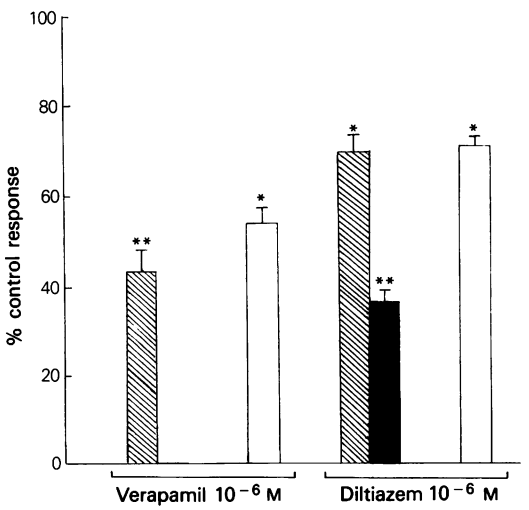

Figure 4 Effect of verapamil (10^{-6} M) and diltiazem (10^{-6} M) on ATP (10^{-4} M) and ACh (10^{-4} M)-induced contractions in rat urinary bladder: open columns, ATP; hatched columns, ACh phasic; solid column, ACh tonic.

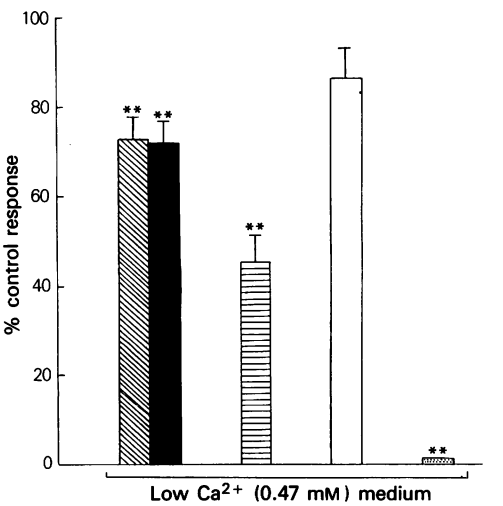

Figure 5 Effect of low Ca^{2+} medium (0.47 mM) on the contractions induced by ATP (10^{-4} M), ACh (10^{-4} M) and single pulse stimulus in rat urinary bladder: hatched column, ACh, phasic; solid column, ACh tonic; horizontally striped column, ATP; open column, single pulse fast; stippled column, single pulse, slow. (**$P < 0.01$; $n = 4$–8).

tion made in the present study confirms the earlier work of Maggi *et al.* (1985). The different time course of attaining maxima of fast (0.72 ± 0.05 s) and slow (2.80 ± 0.81 s) components indicated that at least two different neurotransmitters are involved in the neurogenic response elicited on single pulse stimulation. This is evident from the present findings that the slow component (cholinergic) was relatively more susceptible to calcium channel blockers and Ca^{2+} depletion than the non-cholinergic fast component. There are several reports implicating ACh and ATP as the putative neurotransmitters mediating cholinergic and non-cholinergic contractile responses, respectively, in mammalian urinary bladder (Brown *et al.*, 1979; Kasakov & Burnstock, 1983; Hoyle & Burnstock, 1985; Fujii, 1988). Calcium channel blockers and low Ca^{2+} medium were used in the present study to assess the extracellular Ca^{2+}-dependency of contractions elicited by endogenously applied agonists. Although both the components of the neurogenic response in rat urinary bladder were inhibited by calcium channel blockers in a dose-related pattern, at equimolar concentrations (10^{-6} M), verapamil was more potent than diltiazem in inhibiting the non-cholinergic component of the neurogenic response. Since the cholinergic component was abolished at this concentration of calcium channel blockers, it was difficult to compare their potency. The low IC_{50} values of verapamil and diltiazem with respect to inhibition of the nerve-mediated cholinergic component indicate that it is more dependent on extracellular Ca^{2+} than the non-cholinergic response. The extracellular Ca^{2+} dependency of the cholinergic contractile response is further substantiated by the observation that it was readily abolished in a low Ca^{2+} medium while the non-cholinergic contractions were little affected. If it is presumed that the cholinergic and non-cholinergic neutrotransmitters are released from different nerves, then the preferential inhibition of the cholinergic component of single pulse stimulus-induced response may be related to greater susceptibility of ACh release from the intramural nerves to the presence of Ca^{2+} channel blockers or low Ca^{2+} medium. It, therefore, appears that the neurotransmitters, released during single pulse electrical field stimulation, differentially activate the calcium channels and mobilize the extracellular Ca^{2+} to cause contraction in the rat urinary bladder.

In order to examine further the involvement of purinergic and cholinergic mechanisms in the neurogenic contractile responses of rat urinary bladder, experiments were conducted with equimolar concentrations (10^{-4} M) of ATP and ACh, which induced submaximal contractile responses. In terms of absolute tension, the contractions elicited by ATP (1.00 ± 0.17 g) and the non-cholinergic component of single pulse stimulation (1.22 ± 0.08 g) were identical. Similarities between the responses mediated by exogenously added ATP and the non-cholinergic neurotransmitter were also evident in studies with calcium channel blockers. For instance, the inhibition of the ATP-induced contractions in the rat urinary bladder by verapamil and diltiazem compares well with the inhibition of the non-cholinergic component of the neurogenic response under similar conditions. All these observations strongly support the participation of ATP as a neurotransmitter in the fast non-cholinergic component of the single pulse stimulus in rat urinary bladder. Interestingly, however, there was a difference in the susceptibility of exogenous ATP responses and non-cholinergic neurogenic responses to low Ca^{2+} medium; the former was more susceptible than the latter. Besides lending support to the role of endogenous ATP as a possible non-cholinergic neurotransmitter, these results indicate the participation of extracellular Ca^{2+} in the manifestation of contractile responses caused by exogenously added ATP. The role of extracellular Ca^{2+} is confirmed by the observation that the inhibitory effects of low Ca^{2+} were readily reversible on readmission of full Ca^{2+} to the medium.

In contrast to several similarities between ATP and the fast component of the neurogenic response, there were a number of discrepancies in the contractile responses to ACh and the slow component of the electrical field stimulation, despite the fact that the latter was abolished by atropine. The absolute tension values of the phasic component of ACh 10^{-4} M (3.06 ± 0.46 g; $n = 6$) and cholinergic component of the neurogenic response (0.69 ± 0.06 g; $n = 12$) were significantly different. Dissimilarities were also evident in altered Ca^{2+} studies. The concentration (10^{-6} M) of verapamil and diltiazem that completely abolished the slow component of the neurogenic response caused 30–56% inhibition of the response to ACh (phasic component). Similarly, while the former was absent in low Ca^{2+} medium, the latter was inhibited by about 27% only. Probably the quantum of ACh released on single pulse stimulation may be very small in comparison with the amounts added exogenously, thereby causing a response of lesser magnitude which is more susceptible to altered Ca^{2+} levels, as is evident from the greater susceptibility of the contractile responses induced by lower concentrations of ACh (10^{-6} M and 10^{-7} M) to low Ca^{2+} medium. The differential sensitivity of the responses caused by endogenously released neurotransmitter and exogenously added agonist to an altered cellular Ca^{2+} is evident in other tissues. For instance, the IC_{50} values of verapamil for inhibiting the noradrenaline-induced contractions and the adrenergic component elicited on single pulse stimulation in rat vas deferens were

$74.2 \pm 12.0\,\mu M$ and $30.6 \pm 3.4\,\mu M$, respectively (Hay & Wadsworth, 1983).

The present study thus leads to the conclusion that (1) single pulse stimulus-induced contractions are possibly mediated by ATP and ACh, (2) the cholinergic component is more susceptible to low Ca^{2+} medium than the non-cholinergic component, (3) in view of the physiological role of endogenous neurotransmitters in the regulation of bladder contractility, the differential inhibition of different components of neurogenic responses, by calcium channel blockers is of therapeutic relevance.

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Interactions of drugs acting on central dopamine receptors and cholinoceptors on yawning responses in the rat induced by apomorphine, bromocriptine or physostigmine

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1 Yawning was induced by subcutaneous (s.c.) injection of low doses of apomorphine to rats. This effect decreased with increasing doses of the drug.

2 Intraperitoneal (i.p.) pretreatment of animals with sulpiride (D_2-receptor blocker) reduced the frequency of the yawns induced by apomorphine, while SCH 23390 (D_1-receptor blocker, s.c.) pretreatment increased the small number of yawns which was induced by higher doses of apomorphine. Administration of SCH 23390 alone to rats also produced a low degree of yawning.

3 Apomorphine-induced yawning was decreased in animals treated with SK&F 38393 (D_1-agonist, i.p.), atropine (i.p.) or theophylline (i.p.).

4 Intraperitoneal injection of bromocriptine (D_2-agonist) in rats also induced dose-dependent yawning. The effect was decreased in animals pretreated with sulpiride, while SCH 23390 pretreatment did not change bromocriptine-induced yawning significantly. Pretreatment of animals with SK&F 38393, atropine or theophylline reduced the number of yawns induced by bromocriptine.

5 Physostigmine (i.p.) but not neostigmine (i.p.) also induced yawning. The effect was antagonized by atropine or theophylline but not by sulpiride. Administration of SK&F 38393 decreased yawning induced by physostigmine. This inhibitory influence of SK&F 38393 was reduced by SCH 23390 in pretreated animals. Treatment of animals with SCH 23390 or bromocriptine increased the frequency of yawns induced by physostigmine.

6 It is concluded that D_2-receptor activation elicits yawning through influence on cholinergic mechanisms, whereas D_1-receptor stimulation decreases yawning behaviour by a negative influence on the cholinergic system.

Introduction

Biochemical and pharmacological evidence indicate that two different dopamine receptors, termed D_1 and D_2 mediate the dopamine functions in brain (Garau *et al.*, 1978; Kebabian & Calne, 1979; Stoof & Kebabian, 1984; Onali *et al.*, 1985; Weiss *et al.*, 1985). These two categories of dopamine receptors are distinct molecular entities (Nielsen *et al.*, 1984; Dumbrille-Ross *et al.*, 1985) with different distributions (Altar *et al.*, 1985; Dawson *et al.*, 1985; Martres *et al.*, 1985; Scatton & Dubois, 1985).

Both D_1- and D_2-dopamine receptors, which exist in striatum, can stimulate and inhibit the striatal cyclic AMP formation respectively (Onali *et al.*, 1984; Stoof & Kebabian, 1981; 1984). Striatum contains the highest concentration of acetylcholine in the brain (Sethy *et al.*, 1973). Available evidence suggests that dopamine receptors have a regulatory role on striatal acetylcholine (Sethy & Van Woert, 1974). The opposing effects of D_1- and D_2-receptors on striatal cholinergic neurones have also been shown (Fage & Scatton, 1986).

Yawning can be induced in experimental animals by the dopamine receptor agonists apomorphine, norpropylnorapomorphine and lisuride (Mogilnicka & Klimek, 1977; Baggio & Ferrari, 1983). It has been proposed that stimulation of dopamine autoreceptors, and therefore inhibition of dopaminergic transmission in the brain, causes yawning (Yamada & Furukawa, 1980; Protais *et al.*, 1983; Baggio & Ferrari, 1983). This behaviour appears to be cen-

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trally mediated (Dourish *et al.*, 1985) and due to septal and striatal D_2-receptor activation (Yamada *et al.*, 1986), although results obtained by some workers (Morelli *et al.*, 1986) contradict the hypothesis that apomorphine produces yawning by acting on dopamine autoreceptors.

Some investigators have suggested that central cholinergic mechanisms are involved in yawning behaviour (Urbá-Holmgren *et al.*, 1977; Yamada & Furukawa, 1980; Ushijima *et al.*, 1984). Drugs acting on both cholinoceptors and dopamine receptors have been used to investigate the influence of D_1- and D_2-receptors on yawning behaviour.

Methods

Male albino rats weighing 200–250 g were used in these experiments. They were housed 10 per cage, in a room on a 12 h/12 h light-dark cycle at 20 ± 2°C. Food and water were freely available except during the time of experiments.

Behavioural observations

Rats were placed individually in a plastic cage at 20 ± 2°C during experiments and allowed to habituate for 15 min before injection of drugs. No more than two rats were observed simultaneously. Yawning was counted by direct observation after drug injection. The results were recorded and expressed as number of yawns in a 60 min period. The statistical analysis of the data was performed by ANOVA followed by Student's *t* test. Difference with $P < 0.05$ were considered statistically significant.

Drugs

The drugs used were apomorphine hydrochloride (MacFarlan Smith Ltd, England), bromocriptine (Sandoz, Switzerland), SCH 23390 (R-(+)-8-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepine-7-ol maleate; Schering, Italy), sulpiride (Delagrange, France), SK&F 38393 (1-phenyl-7,8-dihydroxy-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride; R.B.Inc. Wayland, U.S.A.), atropine sulphate (E. Merck, Germany), physostigmine salicylate (Sigma, England), neostigmine methylsulphate (amp., Waldemar-Weimer, Germany) and theophylline (Sigma, England). Bromocriptine was dissolved in saline by the use of crystalline tartaric acid and a few drops of alcohol. Other drugs were dissolved in saline. The drugs were prepared immediately before use and were injected in a volume 1 ml kg^{-1}.

Results

Effects of drugs on yawning induced by apomorphine

Figure 1 illustrates the yawning induced by different doses of apomorphine and the effects of SCH 23390 or sulpiride on this behaviour. Subcutaneous (s.c.) injection of low doses of apomorphine (0.1, 0.3 and 0.6 mg kg^{-1}) induced yawning in rats. The maximal effect was observed at 0.1 mg kg^{-1}. It decreased with higher doses. Pretreatment of animals with sulpiride (10 mg kg^{-1}, i.p., 30 min) diminished the number of yawns induced by apomorphine (0.1–0.6 mg kg^{-1}, s.c.). SCH 23390 pretreatment (0.05 mg kg^{-1}, s.c., 30 min) potentiated the frequency of the small number of yawns elicited by higher doses (0.6 mg kg^{-1}, s.c.) of apomorphine. Administration of SCH 23390 alone (0.05 mg kg^{-1}, s.c.) also caused a low degree of yawning with a mean ± s.e. of 5.9 ± 2.1 (not shown). This effect of the drug was observed in 80% of animals.

SK&F 38393 (8 mg kg^{-1}, i.p.) reduced the number of yawns produced by apomorphine injection (Table

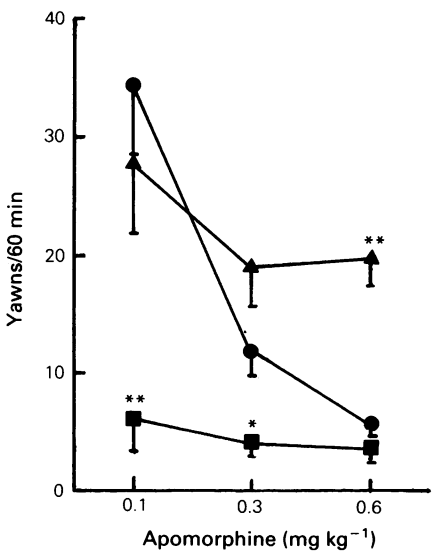

Figure 1 Yawning induced by administration of different doses of apomorphine in presence or absence of antagonists. Rats were injected subcutaneously with apomorphine alone (●) or with either SCH 23390 (▲) (0.05 mg kg^{-1}, s.c.) or sulpiride (■) (10 mg kg^{-1}, i.p.) 30 min before apomorphine injection. The number of yawns was counted immediately after administration of apomorphine for 60 min. Each point is the mean for minimum of 8 experiments; vertical bars show s.e.mean. * $P < 0.05$; ** $P < 0.001$: significantly different from apomorphine-treated group.

Table 1 Frequency of yawns induced by apomorphine (0.3 mg kg^{-1}) in rats in presence or absence of other drugs

Drug	mg kg^{-1}	Yawns/60 min Mean ± s.e.	n
Saline	1 ml	12.1 ± 2.3	8
SK&F 38393	8	2.8 ± 1.7*	6
Theophylline	25	0.2 ± 0.2**	6
Atropine	10	0.0 ± 0.0**	6

SK&F 38393 (i.p.), theophylline (i.p.) and atropine (i.p.) were injected respectively 0, 60 and 10 min before apomorphine injection (s.c.)
* $P < 0.01$; ** $P < 0.001$: significantly different from saline control group.

1). Yawning induced by apomorphine was abolished in animals pretreated with theophylline (25 mg kg^{-1}, i.p., 60 min) or atropine (10 mg kg^{-1}, i.p., 10 min).

Effects of drugs on bromocriptine-induced yawning

Dose-response curves for yawns induced by bromocriptine (2–16 mg kg^{-1}, i.p.) in the presence or absence of D_1- or D_2-receptor blockers are shown in Figure 2. Pretreatment of rats with sulpiride (10 mg kg^{-1}, i.p., 30 min) decreased the yawns induced by different doses of bromocriptine, while SCH 23390 did not change the frequency of yawns produced by the drug significantly. Administration of SK&F 38393 (4–16 mg kg^{-1}, i.p.) to rats decreased the yawning induced by bromocriptine (Figure 3). The influence of SK&F 38393 on bromocriptine-induced yawning was dose-dependent. The effect of bromocriptine was decreased by theophylline or atropine in pretreated animals (Table 2).

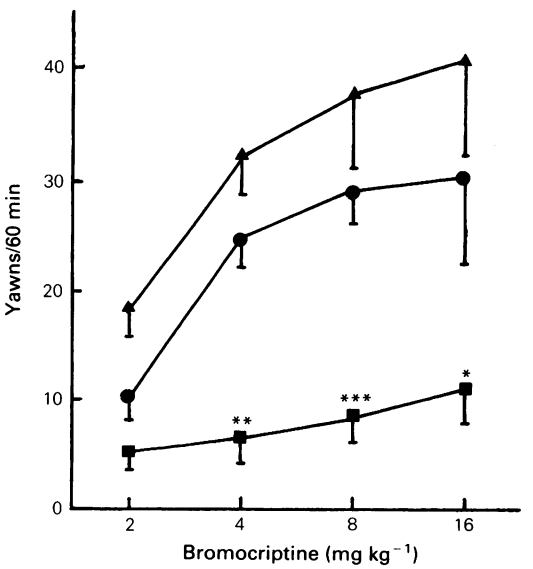

Figure 2 Yawning induced by administration of different doses of bromocriptine in presence or absence of antagonists. Rats were injected intraperitoneally with bromocriptine alone (●) or with either SCH 23390 (▲) (0.05 mg kg^{-1}, s.c.) or sulpiride (■) (10 mg kg^{-1}, i.p.) 30 min before bromocriptine injection. The number of yawns was recorded for 60 min. Each point is the mean for minimum of 8 animals; vertical bars show s.e.mean. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$: significantly different from bromocriptine-treated group.

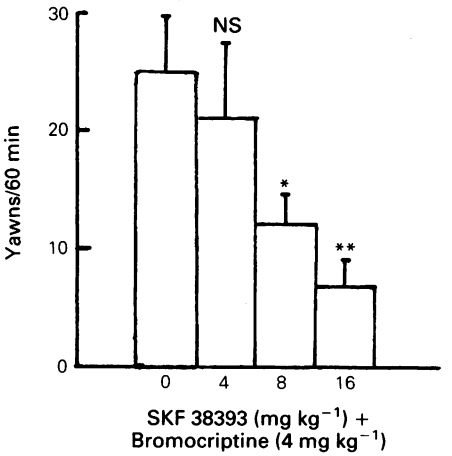

Figure 3 Yawning induced by bromocriptine (4 mg kg^{-1}, i.p.) alone or in combination with i.p. administration of different doses of SK&F 38393. Results are shown for total yawns induced during 60 min after bromocriptine injection. Each point is the mean for minimum of 8 experiments; vertical bars show s.e.mean. NS not significant; * $P < 0.05$; ** $P < 0.001$: significantly different from bromocriptine control group.

Table 2 Frequency of yawns induced by bromocriptine (8 mg kg^{-1}) in rats in presence or absence of theophylline or atropine

Drug	mg kg^{-1}	Yawns/60 min Mean ± s.e.	n
Saline	1 ml	29.1 ± 3.9	14
Theophylline	25	4.6 ± 1.6*	7
Atropine	10	0.0 ± 0.0*	6

Saline (i.p.), theophylline (i.p.) or atropine (i.p.) were injected respectively 30, 60 and 10 min before bromocriptine administration (i.p.).
* $P < 0.001$: significantly different from saline control group.

Table 3 Frequency of yawns induced by physostigmine (0.1 mg kg^{-1}) in rats in presence or absence of other drugs

Drug	mg kg^{-1}	Yawns/60 min Mean ± s.e.	n
Saline	1 ml	23.5 ± 4.4	6
Sulpiride	10	24.6 ± 11.5	6
SCH 23390	0.05	36.0 ± 6.2*	7
SK&F 38393	8	7.2 ± 2.8**	6
SCH 23390 + SK&F 38393	0.05 + 8	18.3 ± 3.0†	6
Bromocriptine	8	62.8 ± 11.0***	6
Theophylline	25	3.0 ± 1.5****	6
Atropine	10	0.0 ± 0.0****	6

Saline (i.p.), sulpiride (i.p.), SCH 23390 (s.c.), SK&F 38393 (i.p.), bromocriptine (i.p.), theophylline (i.p.) and atropine (i.p.) were administered 30, 30, 30, 0, 0, 60 and 10 min before physostigmine (i.p.) injection respectively.
* $P < 0.1$; ** $P < 0.02$; *** $P < 0.01$; **** $P < 0.001$ different from saline-treated rats.
† $P < 0.05$: different from SK&F 38393-treated animals.

Effects of drugs on physostigmine-induced yawning

As shown in Table 3, the yawns elicited by physostigmine (0.1 mg kg^{-1}, i.p.) were decreased by concomitant administration of SK&F 38393 (8 mg kg^{-1}, i.p.) and were antagonized by atropine (10 mg kg^{-1}, i.p., 10 min) or theophylline (25 mg kg^{-1}, i.p., 60 min) in pretreated animals. Neostigmine (0.1, 0.25 and 0.5 mg kg^{-1}, i.p.) did not induce yawning (data not shown). The inhibitory influence of SK&F 38393 on physostigmine-induced yawning was decreased in SCH 23390 pretreated rats; hence the number of yawns was increased. The effect of physostigmine was potentiated by concomitant administration of bromocriptine but less so by SCH 23390 pretreatment. Sulpiride had no influence on physostigmine-induced yawning.

Discussion

Apomorphine with D_1- and D_2-agonist properties (Seeman, 1980; Stoof & Kebabian, 1984) in small doses (0.1–0.6 mg kg^{-1}) induced yawning in rats. This syndrome was decreased by increasing the dose of the drug. Pretreatment of animals with sulpiride a D_2-dopamine receptor antagonist (Di Chiara *et al.*, 1976; Costall *et al.*, 1980; Kendler *et al.*, 1982; Stoof & Kebabian, 1984) reduced the ability of apomorphine to induce yawning. Our present results are in agreement with previous observations of others that apomorphine influences yawning biphasically in rats (Holmgren & Urbá-Holmgren, 1980; Yamada & Furukawa, 1980; Dubuc *et al.*, 1982; Protais *et al.*, 1983). Such a biphasic effect has been attributed to the successive involvement of D_2- and D_1-dopamine receptors; the lower doses of apomorphine stimulate D_2-receptors which decrease tonic dopaminergic transmission with a consequent induction of yawning, while the higher doses activate D_1-receptors and cause the abolition of yawning (Yamada & Furukawa, 1980; Urbá-Holmgren *et al.*, 1982). Pretreatment of animals with low doses of the specific D_1-receptor antagonist SCH 23390 (Hyttel, 1984) increased the ability of apomorphine to induce yawning. When dopamine activation of D_1-sites is impaired by SCH 23390, apomorphine activates only D_2-sites and therefore more frequent yawning can be observed. Administration of SCH 23390 alone also induced a low degree of yawning in rats, which may indicate inhibition of D_1- and unmasking of D_2-agonist properties of endogenous brain dopamine. These effects of SCH 23390 confirm the hypothesis that D_1-receptor stimulation can decrease the yawning episodes and contradict the suggestion of some investigators that autoreceptors are not involved, e.g. Morelli *et al.* (1986), who found SCH 23390 was able to antagonize yawning induced by apomorphine. For further evaluation of the opposite influences of D_1- and D_2- dopamine receptor activation on yawning, some studies were carried out with D_1- and D_2-agonists. Bromocriptine, a D_2-agonist (Di Chiara *et al.*, 1977; Gianutsos & Moore, 1980) has been reported to induce yawning through the stimulation of dopamine D_2-receptors in the rat striatum and septum (Yamada *et al.*, 1986). In the present study, bromocriptine caused yawning dose-dependently. The effect was decreased in animals pretreated with sulpiride. SCH 23390 did not alter the frequency of yawns induced by bromocriptine. These findings support the view that D_2-receptor stimulation may cause yawning.

SKF 38393 which does not induce yawning (Yamada *et al.*, 1986) is a D_1-receptor agonist devoid of D_2-receptor stimulation properties (Setler *et al.*, 1978; Tsuruta *et al.*, 1981; Stoof & Kebabian, 1982). This drug decreased yawning induced by both apomorphine and bromocriptine. These data may suggest that D_1-receptor stimulation exerts opposite influence on yawning. On the other hand, the ability of SKF 38393 to stimulate dopamine-sensitive adenylate cyclase has been shown (Setler *et al.*, 1978). It has been suggested that activation of D_1-receptors is associated with stimulation of adenylate cyclase (Kebabian & Calne, 1979) while D_2-receptor activation may cause inhibition of cyclic AMP formation in striatum (Onali *et al.*, 1984). Theophylline which increases cyclic AMP levels (Butcher & Sutherland,

1962), inhibits yawning induced by apomorphine, bromocriptine or physostigmine. Whether the opposite effects of D_1- and D_2-receptors on yawning behaviour are due to an increase or decrease of cyclic AMP levels is not clear and remains to be elucidated.

Previous investigations have pointed out the involvement of the cholinergic system in the induction of yawning syndrome (Urbá-Holmgren *et al.*, 1977; Yamada & Furukawa, 1980). The present data show that neostigmine, which is not able to enter the CNS, does not induce yawning behaviour and that atropine can antagonize episodes of yawning in rats treated with apomorphine, bromocriptine or physostigmine. This points to a possible central muscarinic component in the yawning induced by these drugs. These results are supported by the suggestion of Yamada & Furukawa (1980) who showed that apomorphine induced yawning through indirect activation of cholinergic neurones. Our results show that bromocriptine potentiates and SK&F 38393 decreases the frequency of yawns induced by physostigmine. It is therefore postulated that yawning may be induced through a cholinergic activation mechanism, while D_1- and D_2-receptor stimulation may have opposite effects on this behaviour.

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Effects of hypoxia on the pharmacological responsiveness of isolated coronary artery rings from the sheep

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1 The effects of low oxygen tension on tone and on the responsiveness to contractile and relaxant agents were examined on circumflex coronary artery rings isolated from sheep.

2 When artery rings (2–2.5 mm o.d.) were set at their optimal resting tension, introduction of hypoxia (0% O_2) caused a sustained contraction which was reversible on washing with oxygenated Krebs solution. In precontracted (40 mM KCl) arteries, hypoxia caused a similar response except that it was preceded by a transient relaxation.

3 The hypoxia-induced contraction was potentiated by the combination of phentolamine (1 μM) and propranolol (1 μM), markedly reduced by verapamil (10 μM) and either abolished or reduced by indomethacin (1 μM). Indomethacin itself caused a contraction.

4 Under hypoxic conditions, the contractile effects of U46619 (a stable thromboxane analogue) and 5-hydroxytryptamine (5-HT) and the vasodilator effects of noradrenaline, iloprost (a prostacyclin mimetic) and adenosine were markedly potentiated. In contrast, vasoconstriction to potassium or acetylcholine was depressed.

5 Changing the gases from 95% O_2 to 12% O_2 had no significant effect on the contractile effects of U46619. However, the maximum contractile effect of U46619 was significantly enhanced by changing the gases from 12% O_2 to 0% O_2.

6 Rings from a smaller branch (0.6–1.3 mm o.d.) of the circumflex coronary artery of the sheep, in the presence of hypoxia, exhibited qualitatively similar changes in the responsiveness to U46619, 5-HT and adenosine to those observed in the large artery. However, the effect of potassium was potentiated rather than depressed.

7 It is concluded that hypoxia-induced contraction may involve a modified release of cyclooxygenase products and be partly dependent upon the availability of extracellular calcium.

8 The change in the responsiveness of coronary arteries, under hypoxia, to both constrictor and dilator mediators may have clinical relevance to myocardial ischaemia and angina pectoris.

Introduction

In canine (Borda *et al.*, 1980; Rubanyi & Vanhoutte, 1985) and porcine (Rubanyi & Paul, 1985) isolated coronary arteries, hypoxia, one of the consequences of myocardial ischaemia, has been found to cause contraction. Furthermore, hypoxia has been shown to augment the contractile responses to 5-hydroxytryptamine (5-HT) and noradrenaline in isolated canine coronary arteries (Van Neuten & Vanhoutte, 1980). In addition to 5-HT (from clotting platelets) and noradrenaline (from sympathetic nerve endings), many other chemical mediators that modify coronary arterial tone are released during myocardial ischaemia. These include thromboxane A_2, prostacyclin, adenosine, potassium and acetylcholine (Yasue *et al.*, 1974; Folts *et al.*, 1976; Berne, 1980; Coker *et al.*, 1981; Kleber, 1984). However, it is not known whether the effects of these mediators on the coronary artery are modified under conditions of hypoxia.

The present experiments were designed firstly, to characterize the effect of hypoxia on the tone of isolated coronary arteries from the sheep and secondly to examine the responsiveness of these arteries, under hypoxia, to a range of substances thought to be released during myocardial ischaemia. The substances investigated were K^+, 5-HT, U46619 (the stable analogue of thromboxane A_2), noradrenaline, acetylcholine, iloprost (a prostacyclin mimetic)

and adenosine. A preliminary account of these findings has been presented to the British Pharmacological Society (Kwan *et al.*, 1988).

Methods

Experiments were performed on rings of left circumflex coronary arteries of two sizes: o.d. = 2–2.5 mm (main circumflex trunk after its first branch) and o.d. = 0.6–1.3 mm (proximal portion of second generation branch off main circumflex). The coronary arteries were dissected free from hearts of freshly slaughtered sheep and cleared of fat and adhering connective tissue before cutting into rings 4–5 mm long. Care was taken to avoid stretching and damage to the luminal surface. Lack of damage was confirmed by histological examination in a representative sample of artery rings. Rings were suspended in a water-jacketed muscle chamber (10 ml) filled with Krebs-Henseleit solution (37°C) of the following composition (mM): NaCl 119, $NaHCO_3$ 25, glucose 11, KCl 4.6, $MgCl_2$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5. The solution was aerated with gas mixtures containing 95% O_2: 5% CO_2 (oxygenated), 95% N_2: 5% CO_2 (hypoxic), or 12% O_2: 5% CO_2 in N_2 (normoxic).

The rings were suspended on a pair of stainless-steel hooks, one of which was fixed to an L-shaped rod inside the chamber and the other to a Grass isometric transducer. Arterial rings were equilibrated in Krebs-Henseleit solution gassed with 95% O_2: 5% CO_2 for an hour at their optimum resting tension of 1.5 g (o.d. = 2–2.5 mm) or 1.4 g (o.d. = 0.6–1.3 mm). The optimum resting tension of each size of artery was determined in eight preparations by comparing the tension developed by 40 mM KCl under different resting tensions. The isometric tension was calculated as force developed per cross sectional area. The cross sectional area (A) of the artery was calculated by using the equation: A = blotted weight of the artery/(h × β) where h = the distance (cm) between the two stainless steel hooks with the artery ring under optimum resting tension, and β = the density of the artery ring which was taken as 1.05 g cm^{-3}. The tissues were subjected to repeated contraction cycles with 40 mM KCl until two consecutive identical responses were observed before starting the experimental protocol.

Initially the oxygen tension of the bathing medium was measured using a blood gas analyser (Radiometer, Copenhagen). This necessitated removal by syringe of an aliquot of the bathing medium and subsequent injection into the gas analyser. In the course of these experiments, however, it was determined that this procedure resulted in contamination of the sample with atmospheric oxygen. For example, the Po_2 of the bathing medium equilibrated with 0% O_2 measured using the gas analyser and an oxygen electrode inserted into the oxygen bath was 44 ± 3 and 8 ± 2 mmHg, respectively (n = 3). Therefore all Po_2 values quoted are those measured with an oxygen electrode (Strathkelvin Instruments) immersed in the organ bath. This electrode was calibrated to zero every two weeks using sodium sulphite 100 mM in sodium tetraborate 10 mM. The pH and Pco_2 of the solution was measured using the gas analyser.

Experimental protocols

Effect of different oxygen tensions on the tone of the coronary artery: Hypoxia was introduced by changing the oxygenated Krebs-Henseleit solution (95% O_2: 5% CO_2) with one Krebs-Henseleit solution 95% N_2: 5% CO_2. The organ bath was then bubbled continuously with 95% N_2-5% CO_2 for 25 min and Po_2 values (mmHg) of 21 ± 3, 16 ± 2, 10 ± 2, 9 ± 2, 8 ± 2, were obtained at 30 s, 2, 5, 15, and 25 min respectively after the addition of hypoxic Krebs-Henseleit solution. Oxygenated conditions were re-established by washing with Krebs-Henseleit solution aerated with 95% O_2-5% CO_2. In some experiments, the effect of changing the gas from 95% O_2: 5% CO_2 to 12% O_2: 5% CO_2: 83% N_2 and from 12% O_2: 5% CO_2: 83% N_2 to 95% N_2: 5% CO_2 were examined. The Po_2 values in the organ bath after a 25 min equilibration period were 620 ± 30, 88 ± 1 and 8 ± 1 mmHg respectively for the gas mixtures of 95% O_2: 5% CO_2, 12% O_2: 5% CO_2 in N_2, and 95% N_2: 5% CO_2. For all the gas mixtures, the pH of the solution was 7.50 ± 0.06 and the Pco_2 was 34 ± 3 mmHg.

Effects of pharmacological antagonists on the hypoxic contraction: After the control hypoxic challenge, the artery was allowed to return to baseline tension before an antagonist (phentolamine and propranolol, indomethacin, verapamil or the solvent absolute ethanol) was added and allowed to equilibrate for 30 min. Resting tension was adjusted to its optimal value and the hypoxic challenge repeated in the continuing presence of the antagonist. The tension developed under hypoxia before and after the addition of each agent was compared. The experiments with antagonists were performed on the large coronary artery rings only.

Effects of hypoxia (changing from 95% O_2 to 95% N_2) on the responsiveness of the coronary artery: Cumulative concentration-response curves to each agent (under optimal resting tension: KCl, U46619, 5-HT and acetylcholine; in 40 mM KCl precontracted rings: noradrenaline, iloprost and adenosine) were constructed before and after lower-

ing the Po_2. The Po_2 was reduced for 45 min before a second curve was constructed. No significant further fall in Po_2 in the organ bath occurred after 15 min bubbling with 95% N_2: 5% CO_2. In the presence of hypoxia, each preparation was reset to its optimum resting tension before commencing the concentration-response curve to the agent. A concurrent time-matched control was set up during the noradrenaline, iloprost and adenosine experiments. The percentage change in tension, if any, observed in the response of the parallel control tissue was used to correct the response recorded in the artery rings receiving a vasodilator.

Effects of different oxygen tensions on the responsiveness of the coronary artery to U46619: Cumulative concentration-response curves to U46619 were constructed at different oxygen tensions i.e. in the presence of 95% O_2, 12% O_2 and 0% O_2. When switching from one gas mixture to another, the artery rings were allowed to equilibrate for an hour and tension was readjusted to optimal resting tension before the construction of the second concentration-response curve to U46619.

Drugs

Adenosine, 5-hydroxytryptamine creatinine sulphate complex, (±) propranolol hydrochloride, acetylcholine chloride and verapamil (all dissolved in distilled water), noradrenaline bitartrate (NA, dissolved in acidic saline), indomethacin (dissolved in absolute ethanol) (all obtained from Sigma), phentolamine mesylate (Ciba), U46619 (11α,9α-epoxymethano PGH_2, dissolved in absolute ethanol and further diluted with distilled water, Upjohn), iloprost (gift from Schering). Vehicles were tested at equal volumes.

Data analysis

Results are expressed as mean ± s.e.mean. The n value quoted is the number of hearts used. Significance tests were performed by Student's paired t test. P values of less than 0.05 were considered significant. The EC_{50} values were calculated by using a least squares curve fitting programme with concentrations of drug expressed as log concentrations. The maximum effect of K^+, U46619, 5-HT and ACh is expressed as force developed per cross sectional area while for NA, iloprost and adenosine it is expressed as % inhibition of 40 mM KCl-induced contraction.

Results

Mechanical effects of hypoxia

Under optimum resting tension, introduction of hypoxia by exchanging the oxygenated Krebs-Henseleit solution (bubbled with 95% O_2: 5% CO_2) with the pre-equilibrated hypoxic Krebs-Henseleit solution and bubbling vigorously with 95% N_2: 5% CO_2, caused an increase in the baseline tension of both large and small coronary arteries. This contraction was reversible on washing with oxygenated Krebs-Henseleit solution. Under oxygenated conditions, 40 mM KCl produced about 85% of its own maximum contraction. The hypoxic contraction was 25–33% of the tension developed by 40 mM KCl. This hypoxic contraction could be replicated at least four times in each artery. In 10 preparations, the four consecutive hypoxic-induced contractions, expressed as a % of the tension developed by 40 mM KCl, were 30 ± 4, 28 ± 4, 32 ± 2 and 31 ± 3%. An increase in tension was also observed on lowering the Po_2 from 620 to 88 or from 88 to 8 mmHg; the respective contractions being 18 ± 3% ($n = 8$) and 49 ± 2% ($n = 8$) of that induced by 40 mM KCl.

In coronary arteries precontracted with 40 mM KCl ($n = 5$, large artery; $n = 2$, small artery) hypoxia (introduced by switching from 95% O_2: 5% CO_2 to 95% N_2: 5% CO_2, without changing the Krebs-Henseleit solution) caused a transient relaxation followed by a further contraction to reach a new steady-state tension.

Effects of phentolamine plus propranolol, indomethacin and verapamil on the hypoxic contraction

Figure 1 illustrates the effects of phentolamine (1 μM) plus propranolol (1 μM), indomethacin (1 μM) and verapamil (10 μM) on baseline tension and on tension development during hypoxia. The hypoxic contraction was potentiated by the combination of phentolamine and propranolol, and markedly inhibited by verapamil. Indomethacin under oxygenated conditions caused an increase in baseline tension. However, under hypoxic conditions, two different phenomena were obtained: in early experiments indomethacin abolished the hypoxic-induced contraction and a relaxation below baseline was obtained (Figure 1). Recently, in the presence of indomethacin, the hypoxic contraction was significantly reduced (from 18.6 ± 3.4 to 11.2 ± 1.7 g cm^{-2}; $n = 12$) but not abolished. The baseline tension before the introduction of hypoxia was unaffected by phentolamine plus propranolol or by verapamil. Ethanol (2 μl 10 ml^{-1}), the solvent for indomethacin, had no effect on the baseline tension or the hypoxic contraction.

Effects of hypoxia on the pharmacological responsiveness of the sheep coronary artery

In control experiments, two replica concentration-response curves for each agonist were constructed

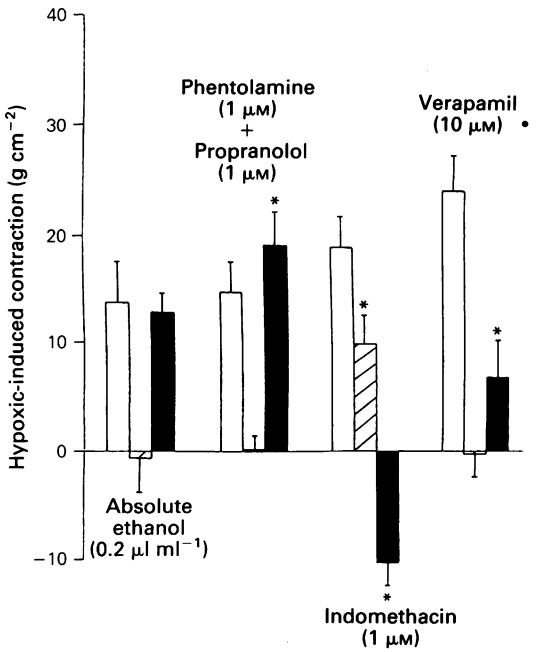

Figure 1 The hypoxia-induced contraction before (open columns, control) and during (solid columns) the action of various antagonists in left circumflex coronary artery rings isolated from sheep. The effects of the antagonists alone on baseline tension are also shown (hatched column). Vertical lines show s.e.mean. * Denotes significant difference from control at $P < 0.05$.

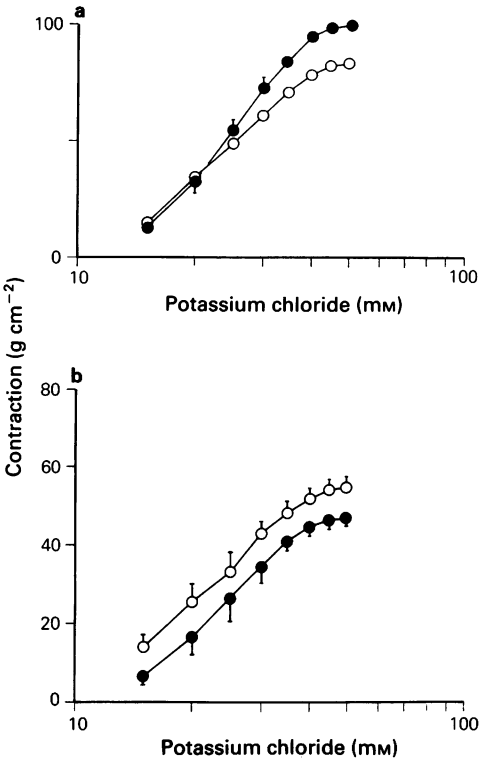

Figure 2 Concentration-response curves for potassium chloride on left circumflex coronary artery rings isolated from sheep and studied under oxygenated (●) and hypoxic (○) conditions. (a) and (b) Show the results obtained on large and small coronary arteries, respectively. Each point respresents the mean and vertical lines show s.e.mean. ($n = 10$).

under oxygenated conditions in a minimum of four preparations. In each case the second concentration-response curve was not significantly different from the first. The only exception to this was observed with 5-hydroxytryptamine in the small coronary artery. In this case, a severe desensitization to the drug was observed and no consistent second concentration-response curve could be obtained ($n = 4$). Thus, the effects of hypoxia on the responsiveness to 5-hydroxytryptamine of the small coronary artery could not be tested.

Table 1 summarizes the EC_{50} values and the maximum contraction or relaxation observed with the drugs under oxygenated and hypoxic conditions in the large coronary arteries. In both sizes of artery, hypoxia did not modify the EC_{50} of potassium. Hypoxia decreased the maximum tension developed in the large but increased the maximum tension developed by K^+ in the small arteries (Figure 2).

The thromboxane A_2 stable analogue, U46619, caused a concentration-dependent vasoconstriction under oxygenated and hypoxic conditions. In the large arteries, there was a marked increase in the maximum active tension development and a decrease in the EC_{50} of this agonist during hypoxia (Figure 3). A similar effect was observed in the small arteries; the EC_{50} (μM) being reduced from 0.26 ± 0.04 to 0.08 ± 0.02 and the maximum effect increased from 50.6 ± 19.5 to $102.3 \pm 11.6\,g\,cm^{-2}$. Changing the gas from 95% O_2: 5% CO_2 to 12% O_2: 5% CO_2 in N_2 did not alter the contractile effect of U46619. However, a change from 12% O_2: 5% CO_2 in N_2 to 95% N_2: 5% CO_2 significantly enhanced the maximum tension development by U46619 (63.08 ± 6.44 cf $23.04 \pm 5.05\,g\,cm^{-2}$) and decreased the EC_{50} value (0.08 ± 0.01 cf 0.15 ± 0.02 μM).

Both 5-hydroxytryptamine and acetylcholine, under oxygenated conditions, caused a biphasic contraction, comprising a phasic component (peaking at 20–25 s) followed by a smaller tonic component (the sizes of the phasic and tonic components are given in Table 1). In the presence of hypoxia, the biphasic

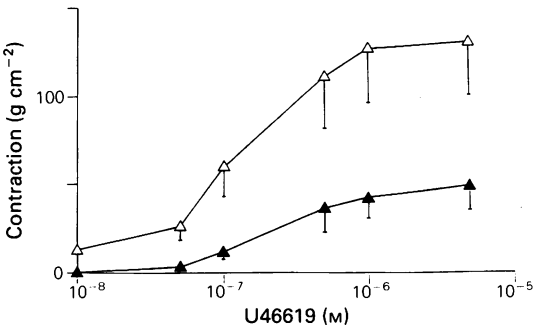

Figure 3 Concentration-response curves for U46619 on left circumflex coronary artery rings (large) isolated from sheep and studied under oxygenated (▲) and hypoxic (△) conditions. Each point represents the mean and vertical lines show s.e.mean. (n = 8).

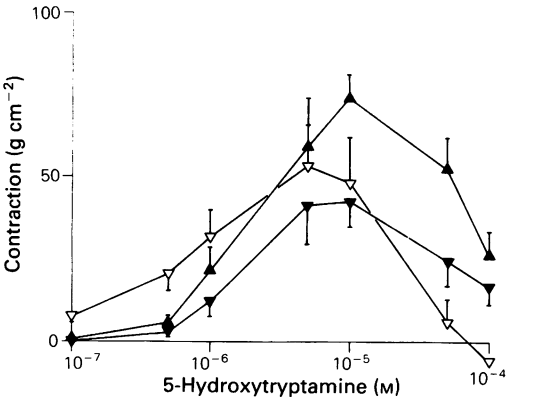

Figure 4 Concentration-response curves for 5-hydroxytryptamine on left circumflex coronary artery rings (large) isolated from sheep and studied under oxygenated (phasic contraction (▲), tonic contraction (▼)) and hypoxic (▽) conditions. Each point represents the mean and vertical lines show s.e.mean. (n = 8).

contraction to acetylcholine remained whereas 5-hydroxytryptamine elicited only a monophasic tonic contraction. By comparison with the tonic contraction observed under oxygenated conditions, there was a shift to the left and an increase in the maximum tension developed by 5-hydroxytryptamine during hypoxia (Figure 4). In contrast to the potentiation by hypoxia of the contractile effects of U46619 and 5-hydroxytryptamine, the maximum active tension development of acetylcholine was decreased and there was no change in its EC_{50} during hypoxia (Table 1).

In preliminary experiments it was shown that noradrenaline (0.01–10 μM) had no contractile effect on the large coronary arteries both under resting tension (n = 4) and precontracted (n = 2). However, noradrenaline did produce a concentration-dependent relaxation of the large coronary artery precontracted with 40 mM KCl under both oxygenated and hypoxic conditions. The vasorelaxant effect of noradrenaline was augmented significantly by hypoxia giving a decrease in the EC_{50} and an increase in the % inhibition of contraction (Figure 5).

Similar to noradrenaline, iloprost (0.1–10 μM) and adenosine (1 μM–2 mM) caused a concentration-dependent relaxation of the precontracted (by 40 mM KCl) coronary artery. Hypoxia augmented the maximum vasorelaxant effect to both drugs but increased the sensitivity only to iloprost (Table 1). Similarly, in the small arteries, the EC_{50} (mM) for adenosine was unchanged by hypoxia (0.65 ± 0.09 cf

Table 1 Effects of hypoxia on the responsiveness of large circumflex coronary artery rings obtained from sheep

	EC_{50}		*Maximum effect* (g cm^{-2})	
	Oxygenated	*Hypoxic challenge*	*Oxygenated*	*Hypoxic challenge*
K^+ (mM)	23.90 ± 1.10	23.40 ± 0.90	81.30 ± 5.80	66.40 ± 2.10*
U46619 (μM)	0.30 ± 0.03	0.19 ± 0.05*	48.60 ± 14.00	129.80 ± 29.9*
5-HT (μM)				
(P)	2.60 ± 0.66	—	74.09 ± 14.52	—
(T)	2.95 ± 0.86	0.78 ± 0.14*	42.60 ± 7.80	48.30 ± 13.80*
ACh (μM)				
(P)	0.78 ± 0.22	0.73 ± 0.26	76.04 ± 22.62	43.08 ± 17.37*
(T)	1.00 ± 0.30	0.61 ± 0.26	58.36 ± 20.33	24.69 ± 12.28*
NA (μM)	2.13 ± 0.31	0.65 ± 0.09*	48.48 ± 3.68	88.19 ± 4.78*
Ilo (μM)	1.05 ± 0.20	0.44 ± 0.07*	28.09 ± 1.81	86.15 ± 6.06*
Adeno (mM)	0.70 ± 0.10	0.60 ± 0.06	87.50 ± 8.90	154.90 ± 11.60*

Results are expressed as means ± s.e.mean from 6–10 preparations.
* Indicates a significant difference from the value obtained under oxygenated conditions. (P) and (T) relate to the phasic and tonic contraction respectively. Ilo: iloprost; Adeno: adenosine.

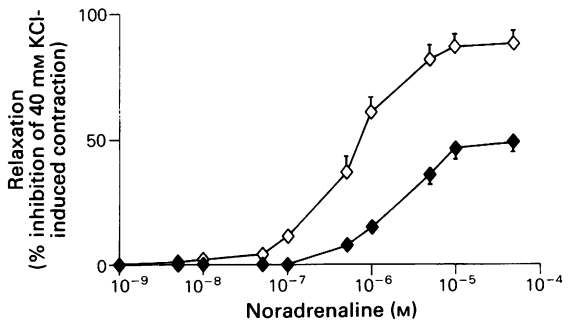

Figure 5 Concentration-response curves for noradrenaline on precontracted (by 40 mM KCl) left circumflex coronary artery rings (large) isolated from sheep and studied under oxygenated (◆) and hypoxic (◇) conditions. Each point represents the mean and vertical lines show s.e.mean. ($n = 6$).

0.58 ± 0.09) but the maximum relaxation increased from 97.5 ± 10 to 203.1 ± 7.5.

Discussion

Lowering the Po_2 of the bathing fluid from approximately 620 mmHg to 8 mmHg, cuased a sustained contraction of coronary artery rings isolated from sheep, when the rings were under resting tension. In precontracted rings, a transient relaxation followed by sustained contraction was observed during hypoxia. This phenomenon is not merely a consequence of a change from the hyperoxia employed in oxygenated Krebs solution, since lowering the Po_2 from a more physiological 88 mmHg also resulted in contraction. A similar effect of hypoxia to contract coronary arterial rings *in vitro* has been obtained for other species such as the dog (Borda *et al.*, 1980) and the pig (Rubanyi & Paul, 1985), whereas in cattle (Kalsner, 1976; Roberts *et al.*, 1981) a relaxation is observed. The mechanism(s) underlying this hypoxia-induced contraction have not been elucidated. In the present experiments indomethacin either reduced or abolished contractions caused by hypoxia. This implies that in the coronary artery of the sheep the hypoxic contraction is, in part, mediated either by the release of a vasoconstrictor prostanoid or by a reduced synthesis of a vasodilator prostanoid. The fact that indomethacin, under oxygenated conditions, produced a contraction suggests there is likely to be a basal release of a vasodilator prostanoid from the coronary artery which might be inhibited by hypoxia. In other species conflicting results with indomethacin have been described. In the dog (Rubanyi & Vanhoutte, 1985) indomethacin had no effect on the hypoxic contraction, whereas in porcine isolated arteries (Rubanyi & Paul, 1985) and in Langendorff perfused hearts of the rat (Karmazyn *et al.*, 1979) it abolished vasoconstriction during hypoxia. Therefore, it seems probable that the mediators released from the coronary artery by hypoxia are species-dependent.

Verapamil substantially reduced the hypoxic contraction indicating that part of it is dependent upon the influx of Ca^{2+} from the extracellular fluid, as has been suggested previously by Van Neuten *et al.* (1983) and Karmazyn *et al.* (1984). This dependence on extracellular Ca^{2+} supports the idea that hypoxia may release a vasoconstrictor mediator rather than solely reducing the release of a vasodilator. In canine coronary arteries hypoxia evokes release of noradrenaline which promotes contraction via β-adrenoceptor activation (Borda *et al.*, 1980). However, our data show that the combination of phentolamine plus propranolol significantly augmented the hypoxic contraction indicating that although hypoxia may release noradrenaline it has a vasodilator action in sheep coronary arteries.

Under oxygenated conditions, coronary artery rings from sheep were contracted by K^+, 5-hydroxytryptamine, U46619 (the thromboxane A_2 analogue) and acetylcholine but relaxed by noradrenaline, iloprost (a prostacyclin mimetic) and adenosine in a concentration-dependent manner. These results confirm previous findings (Schror *et al.*, 1980; Berne, 1980; Lewy *et al.*, 1981; Perez *et al.*, 1983), mostly in species other than the sheep, that K^+, U46619 and 5-hydroxytryptamine are vasoconstrictors whereas iloprost and adenosine are vasorelaxant in the coronary bed. Acetylcholine has been shown to cause an endothelium-dependent relaxation in a variety of isolated arteries (Vanhoutte & Rimele, 1983) but in sheep (Feletou *et al.*, 1986) as well as in the human coronary artery (Kalsner, 1985) it causes contraction. This probably reflects the fact that in these species, acetylcholine has a greater direct effect on the smooth muscle cells than on the endothelium to release endothelium-derived relaxant factor (EDRF). The vasorelaxation observed with noradrenaline is consistent with the observations by Brine *et al.* (1979) that sheep coronary arteries contain few if any α-adrenoceptors but that β-adrenoceptors mediate relaxation.

In the large coronary arteries, hypoxia augmented the contractile effects of U46619 and 5-hydroxytryptamine but depressed those of acetylcholine and K^+. The experiments carried out with U46619 under different oxygen tensions indicate that the potentiating effect, at least for this agent, mainly results from a lowering of the Po_2 from a physiological to a hypoxic one. The reason for the differential effect of hypoxia on the responsiveness to the various agents studied is not known. One possible explanation is that hypoxia causes a specific change in certain receptors or the intracellular signalling

systems linked to them. On the the other hand, it may be that acetylcholine, like K^+, mediates contraction via an influx of Ca^{2+} from the extracellular space whereas U46619 and 5-hydroxytryptamine release Ca^{2+} from intracellular stores. Since hypoxia appears to increase Ca^{2+} influx across the smooth muscle cell membrane it may thereby potentiate the contraction caused by U46619 and 5-hydroxytryptamine but depress the maximum contraction obtained with K^+ and acetylcholine.

Hypoxia also augmented the maximum relaxations observed with adenosine, noradrenaline or iloprost but it reduced the EC_{50} values of the latter two drugs only. The interaction between hypoxia and the vasorelaxant effect of adenosine is in agreement with Gellai *et al.* (1973) who demonstrated that the vasodilator effect of adenosine was potentiated under low oxygen tension. The mechanism(s) underlying this potentiation by hypoxia of the vasorelaxant agents has not been studied. A direct effect of hypoxia to inhibit contraction of the smooth muscle can be excluded since under these experimental conditions, it causes contraction. Hypoxia has been shown to block extraneuronal noradrenaline uptake in rat isolated heart (Inoue *et al.*, 1987) which could account for a shift to the left of the noradrenaline concentration-response curve. However, such a hypoxia-induced increase in the local concentration of noradrenaline, or of the other two substances tested, could not explain the increase in the maxium relaxation observed.

In conclusion, in isolated coronary arteries of the sheep, hypoxia caused a contraction and modified the responsiveness to both vasoconstrictor and vasodilator substances that may be released during myocardial ischaemia. These results may have clinical relevance both to the aetiology of coronary vasospasm, the underlying mechanisms of which are unknown, and to the effects of various mediators or drugs on coronary vascular tone during myocardial ischaemia.

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Hypoxia- and endothelium-mediated changes in the pharmacological responsiveness of circumflex coronary artery rings from the sheep

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1 The role(s) of the endothelium in modulating the responsiveness of isolated circumflex coronary artery rings (o.d. = 2.0–2.5 mm and o.d. = 0.6–1.3 mm) from sheep was investigated under oxygenated and hypoxic conditions.

2 Removal of the endothelium abolished the contraction produced by lowering the Po_2 from 620 to 8 mmHg (either under optimal resting tension or precontracted by 40 mM KCl). In denuded artery rings sudden hypoxia caused relaxation.

3 Under oxygenated conditions, removal of the endothelium augmented the vasoconstrictor effects of U46619, 5-hydroxytryptamine (5-HT) and K^+. In the denuded artery rings, hypoxia abolished the contractile effects of U46619 and reduced the contractile effects of 5-HT and K^+.

4 Under oxygenated conditions, the vasorelaxant effect of adenosine was depressed by removal of the endothelium. In endothelium-denuded preparations, the small relaxant effect of adenosine remaining was greatly potentiated.

5 Haemolysate (1 μl ml^{-1}) caused an endothelium-dependent contraction under oxygenated conditions. The hypoxic contraction observed in the artery ring under resting tension was significantly potentiated by haemolysate (1 μl ml^{-1}). Haemolysate 1 μl ml^{-1} had no effect on the denuded artery rings under hypoxic conditions.

6 Haemolysate (1 μl ml^{-1}) potentiated the vasoconstrictor effects of U46619 (0.5 μM), 5-HT (1 μM) and K^+ (24 mM) under oxygenated conditions.

7 These results indicate that endothelium profoundly modifies the effect of hypoxia on the responsiveness of sheep isolated left circumflex coronary artery rings.

Introduction

Since Furchgott & Zawadski (1980) demonstrated that an intact endothelial layer is required for acetylcholine to exert its vasodilator effect, abundant evidence has been obtained to show that many substances release vasoactive mediators from the endothelium (De Mey *et al.*, 1982; Cherry *et al.*, 1982; White & Angus, 1987). It has been postulated that anoxia (Morrison *et al.*, 1977) and platelets and platelet-released materials (Asada *et al.*, 1988) cause endothelial injury leading to vasospasm. Hypoxia, one of the consequences of a decrease in perfusion causes marked changes in the pharmacological responsiveness of sheep coronary artery rings (Kwan *et al.*, 1989). Although there have been many studies on the effect of removal of the endothelium from artery on its responses to different vasoactive substances under normal oxygenation, nothing is known of the interaction between hypoxia and the endothelium on the responsiveness of coronary artery rings. The current study was performed to evaluate the role(s) of the endothelium on the pharmacological responsiveness of left circumflex coronary artery rings isolated from the sheep and studied under oxygenated and hypoxic conditions. Substances that may be released during platelet aggreation and myocardial ischaemia were examined: K^+, 5-hydroxytryptamine (5-HT), the thromboxane mimetic U46619 and adenosine. A preliminary account of these findings has been presented to the British Pharmacological Society (Kwan *et al.*, 1988).

Methods

Isometric tension was recorded in rings of left circumflex coronary arteries (large, o.d. 2–2.5 mm; small, o.d. 0.6–1.3 mm) isolated from the sheep, as described in detail in the preceding paper (Kwan *et al.*, 1989). In some experiments, the endothelium was removed by deliberately rubbing the internal surface with a wooden stick. The removal of endothelium was confirmed by histological examination.

Preparation of haemolysate solution

Cat arterial blood was heparinized (16 u ml^{-1}), centrifuged (1000 *g* for 20 min at 4°C), and the plasma and buffy coat were removed by aspiration. The erythrocytes were washed twice and resuspended in phosphate-buffered isotonic saline to restore the original volume of blood. Haemolysis was effected by pipetting 1 ml of the washed erythrocyte suspension into 19 ml of hypotonic phosphate buffer (20 m osmol l^{-1}, pH 7.4), mixing and centrifuging at 20,000 *g* for 30–40 min at 4°C. The supernatant from this procedure was dialysed overnight at 4°C with stirring against distilled water to remove low molecular weight components. The haemolysate was used at a concentration of 1 μl (equivalent to 0.05 μl of whole blood) per ml.

Experimental protocol

Effect of the removal of endothelium and of haemolysate on the hypoxia-induced contraction: Hypoxia and reoxygenation were introduced as described in the preceding paper (Kwan *et al.*, 1989). After the first hypoxic challenge, haemolysate was added and allowed to equilibrate for 30 min. Resting tension was adjusted to the optimal resting tension and the hypoxic challenge repeated in the continuing presence of the haemolysate. The tension developed under hypoxia before and after the addition of haemolysate was compared in artery rings with and without endothelium.

Effect of hypoxia on the responsiveness of the denuded coronary artery rings: Cumulative concentration-response curves to each agent (under optimal resting tension: U46619, 5-HT and K^+; in KCl 40 mM precontracted rings: adenosine) were constructed before and after lowering the Po_2. The Po_2 was reduced for 45 min and in the presence of hypoxia, each preparation was reset to its optimum resting tension before starting the concentration-response curve to the agent. A concurrent time-matched control was set up during the adenosine experiments. The percentage change, if any, observed in the K^+ response of the parallel control tissue, was used to correct the response recorded in the artery rings receiving adenosine.

Effect of haemolysate on the responsiveness of the coronary artery rings to the vasoconstrictors under standard conditions: A single dose of each vasoconstrictor (U46619, 5-HT or K^+) was added to the organ bath for 5 min and washed out. After a 45 min resting period, haemolysate (1 μl ml^{-1}) was added and allowed to equilibrate for 30 min. Resting tension was readjusted to the optimum resting tension and the challenge of each vasoconstrictor was repeated in the continuous presence of 1 μl ml^{-1} haemolysate. The tension developed before and after the addition of haemolysate was compared. Using the same protocol the effects of haemolysate on the relaxation caused by substance P (1 μM) and sodium nitroprusside (0.5 μM) were also measured.

Drugs

Adenosine, 5-hydroxytryptamine creatinine sulphate complex, and papaverine hydrochloride (all dissolved in distilled water) (all obtained from Sigma), U46619 (11α,9α-epoxymethano PGH_2 dissolved in absolute ethanol and further dilution in distilled water, Upjohn). Vehicles were tested at equal volumes.

Data analysis

Results are expressed as mean ± s.e.mean. Significance tests were Student's paired or unpaired *t* test as appropriate. *P* values of less than 0.05 were considered significant.

Results

Effect of endothelium removal on the mechanical effect of hypoxia

In endothelium-containing rings of sheep circumflex coronary artery, hypoxia (reducing the Po_2 from 620 to 8 mmHg) caused a contraction (artery under resting tension) or a relaxation followed by a contraction (artery precontracted) (Figure 1). Removal of the endothelium eliminated the contractile effect and hypoxia produced a relaxation. The hypoxia-induced decrease in tension in the precontracted (by 40 mM KCl) artery rings was about 15–20% (Figure 1a). In those under resting tension, hypoxia caused a relaxation below baseline tension (Figure 1b). These experiments were performed on the large artery rings only.

The relaxation described in the denuded preparations under hypoxic conditions was not due to

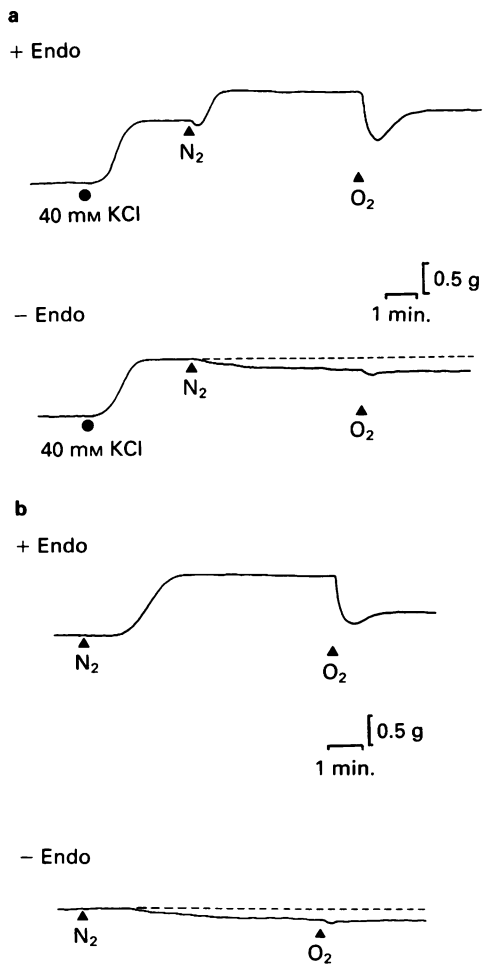

Figure 1 The mechanical effects of hypoxia on circumflex coronary artery rings (large) with endothelium intact (+ Endo) or denuded (− Endo) isolated from sheep. (a) and (b) show effects on the artery precontracted and under resting tension, respectively.

the trauma caused by the rubbing of the internal layer since rings rubbed on their outside surface produced the normal hypoxic contraction ($n = 4$). Furthermore, papaverine (100 μM) relaxed denuded artery rings under resting tension ($n = 3$) and precontracted ($n = 4$). The hypoxic relaxation was 72 ± 2% of that induced by papaverine.

Effects of endothelium removal on the responsiveness of coronary artery rings under standard and hypoxic conditions

Table 1 summarizes the effects of removal of the endothelium on the responsiveness of the large coronary artery rings to K^+, 5-HT, U46619 and adenosine under oxygenated and hypoxic conditions. In these coronary artery rings, under oxygenated conditions, removal of the endothelium enhanced the maximum active tension development by K^+ and 5-HT but had no effect on that developed by U46619. However, denudation increased the sensitivity as measured by the EC_{50}, to 5-HT and U46619 but not to K^+. The effect of denudation is illustrated for 5-HT and for U46619 in Figures 2 and 3, respectively. The vasorelaxant effect of adenosine on the precontracted (by 40 mM KCl) rings, under oxygenated conditions, was attenuated by removal of the endothelium (Table 1).

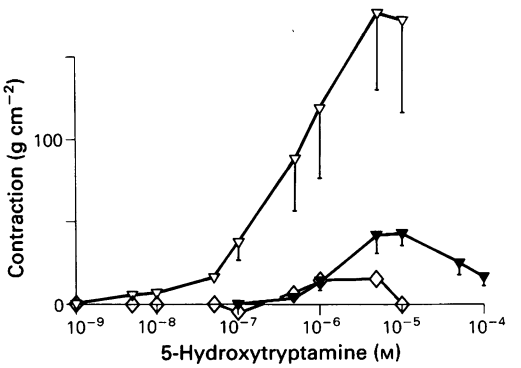

Figure 2 Concentration-response curves for 5-hydroxytryptamine (5-HT) on intact (▼) and endothelium denuded (▽) sheep coronary artery rings (large) under oxygenated conditions. The concentration response curve for 5-HT on endothelium-denuded rings during hypoxia (◇) is also shown. Each point represents the mean and vertical lines show s.e.mean.

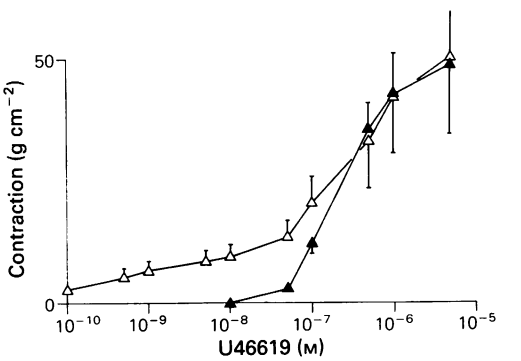

Figure 3 Concentration-response curves for U46619 on intact (▲) and endothelium denuded (△) sheep coronary artery rings (large) under oxygenated conditions. Each point represents the mean and vertical lines show s.e.mean.

Table 1 The effect of endothelium removal on the responsiveness of large coronary artery rings to potassium, 5-hydroxytryptamine (5-HT), U46619 and adenosine under oxygenated and hypoxic conditions

	Oxygenated		*Hypoxic*
	Endo +	*Endo −*	*Endo −*
Potassium			
EC_{50} (mM)	23.9 ± 1.1	23.1 ± 0.8	25.2 ± 0.7
Max. effect	81.3 ± 5.9	151.6 ± 17.8*	90.7 ± 7.5†
5-HT			
EC_{50} (μM)	2.95 ± 0.8	0.51 ± 0.08*	1.17 ± 0.35†
Max. effect	42.6 ± 7.8	175.8 ± 47.1*	10.0 ± 5.3†
U46619			
EC_{50} (μM)	0.3 ± 0.03	0.14 ± 0.04*	0
Max. effect	48.6 ± 14.0	72.9 ± 20.6*	0
Adenosine			
EC_{50} (mM)	0.70 ± 0.1	0.15 ± 0.01*	0.06 ± 0.01†
Max. effect	87.5 ± 8.9	17.3 ± 4.6*	86.4 ± 7.8†

The maximum effect of potassium, 5-HT and U46619 is expressed as force developed per cross sectional area ($g\,cm^{-2}$) and that of adenosine as % inhibition of the 40 mM KCl-induced contraction. $n = 6$–8. * $P < 0.05$, significantly different from results obtained in endothelium intact (Endo +) preparations. † $P < 0.05$ significantly different from results obtained in endothelium denuded (Endo −) preparations under oxygenated conditions.

In the large arteries, stripped of endothelium, hypoxia depressed the contractile effect of K^+ and 5-HT and abolished that of U46619 (Table 1). Hypoxia had no effect on the EC_{50} of K^+ but increased that of 5-HT. Figure 2 shows this effect of hypoxia on the contraction produced by 5-HT in denuded artery rings. Hypoxia enhanced the vasorelaxant effect of adenosine and decreased its EC_{50}.

For the most part qualitatively similar results following denudation and exposure to hypoxia were observed in the small and in the large coronary arteries. As shown in Table 2, in the small arteries under oxygenated conditions, denudation increased the maximum contractile effect of K^+, had no effect on that of U46619 but decreased the maximum relaxation observed with adenosine. A concentration-response curve to 5-HT could not be obtained in the small artery rings with endothelium intact due to severe desensitization. However, with the endothelium removed, 5-HT did cause a

Table 2 The effect of endothelium removal on the responsiveness of small coronary artery rings to potassium, 5-hydroxytryptamine (5-HT), U46619 and adenosine under oxygenated and hypoxic conditions

	Oxygenated		*Hypoxic*
	Endo +	*Endo −*	*Endo −*
Potassium			
EC_{50} (mM)	23.7 ± 1.3	23.6 ± 0.8	27.1 ± 1.0
Max. effect	54.4 ± 8.2	129.7 ± 16.6*	30.0 ± 9.8†
5-HT			
EC_{50} (μM)	—	0.35 ± 0.1	0
Max. effect	—	97.1 ± 44.0	0
U46619			
EC_{50} (μM)	0.3 ± 0.04	0.07 ± 0.02*	0.06 ± 0.005
Max. effect	50.6 ± 19.5	36.1 ± 8.6	5.9 ± 2.6†
Adenosine			
EC_{50} (mM)	0.75 ± 0.1	0.68 ± 0.08	0.66 ± 0.07
Max. effect	97.5 ± 10.0	65.3 ± 8.1*	180.4 ± 8.8†

The maximum effect of potassium, 5-HT and U46619 is expressed as force developed per cross sectional area ($g\,cm^{-2}$) and that of adenosine as % inhibition of the 40 mM KCl-induced contraction. $n = 6$–8. * $P < 0.05$, significantly different from results obtained in endothelium intact (Endo +) preparations. † $P < 0.05$ significantly different from results obtained in endothelium denuded (Endo −) preparations under oxygenated conditions.

concentration-dependent contraction which was abolished by hypoxia (Table 2). Hypoxia, as in the large arteries, markedly reduced the maximum tension developed by K^+ and U46619 and enhanced the relaxation caused by adenosine. In contrast to the results obtained in the large artery, denudation under oxygenated or hypoxic conditions did not modify the EC_{50} of adenosine (compare Tables 1 and 2).

Effect of haemolysate on the hypoxic contraction and on the responsiveness of the large coronary artery rings

Haemolysate ($1\,\mu l\,ml^{-1}$), under oxygenated conditions in endothelium intact rings, caused a sustained contraction ($9.5 \pm 1.4\,g\,cm^{-2}$). The hypoxia-induced contraction was significantly potentiated by $1\,\mu l\,ml^{-1}$ of haemolysate (16.1 ± 1.6 cf $32.9 \pm 4.8\,g\,cm^{-2}$, $n = 8$). In endothelium denuded preparations, haemolysate ($1\,\mu l\,ml^{-1}$) had no effect on baseline tension nor on the relaxation produced by hypoxia ($n = 6$).

Table 3 shows that haemolysate in a concentration ($1\,\mu l\,ml^{-1}$) that abolished or markedly reduced the relaxant effect of substance P or sodium nitroprusside, augmented the contractile effect of K^+ (24 mM), 5-HT (1 μM) and U46619 (0.5 μM).

Discussion

In isolated coronary artery rings from sheep, abrupt hypoxia causes a large, sustained contraction when the endothelium is present and a small relaxation if the endothelium has been removed. In the dog coronary artery also, the hypoxic contraction is abolished by removal of the endothelium (Rubanyi & Vanhoutte, 1985a). These results strongly suggest that the hypoxic contraction results from changes in release, caused by hypoxia, of mediators from the endothelium; there could be an increased release of a contractile mediator and/or reduced release of a vasodilator mediator. Other evidence from our laboratory suggests that the hypoxic contraction is caused by the vasoconstrictors acetylcholine and 5-HT combined with reduced release of the vasodilator, prostacyclin (Amatya *et al.*, 1988; Kwan *et al.*, 1989). From the present results we conclude that these mediators are either produced by the endothelium or released in response to another factor that is produced by the endothelium. The relaxation observed in endothelium-denuded coronary artery rings may be due to an inhibitory effect of low Po_2 on the contractile process or to release of a vasodilator from the vessel wall.

Table 3 The effect of haemolysate ($1\,\mu l\,ml^{-1}$) on the contraction caused by potassium, 5-hydroxytryptamine (5-HT) or U46619 and the relaxation produced by substance P or nitroprusside on large circumflex coronary artery rings (intact endothelium) under oxygenated conditions

	Control ($g\,cm^{-2}$)	*With haemolysate* ($g\,cm^{-2}$)
Potassium (24 mM)	18.0 ± 6.0	24.4 ± 6.6*
5-HT (1 μM)		
(P)	35.7 ± 17.6	53.4 ± 23.6*
(T)	26.8 ± 13.9	40.7 ± 17.5*
U46619 (0.5 μM)	13.5 ± 3.1	19.0 ± 3.6*
Substance P (1 μM)	8.4 ± 1.6	no relaxation
Nitroprusside (0.5 μM)	103.3 ± 2.4	11.2 ± 4.7*

P and T represent the phasic and tonic phases of contraction respectively (see Kwan *et al.*, 1989). $n = 6$. * $P < 0.05$ significantly different from control value.

Haemoglobin caused a contraction and augmented the contractile effects of KCl, 5-HT, U46619 and hypoxia. Haemoglobin is known to antagonize the effect of the vasodilator, endothelium-derived relaxant factor (EDRF, Martin *et al.*, 1985) and in these experiments was shown to reduce markedly the relaxation of substances acting via cyclic GMP. Our results suggest that there is a continuous basal output of EDRF from the sheep coronary artery rings, thus limiting basal tone and depressing the activity of vasoconstrictors. This would explain why haemoglobin, by antagonism of the effects of EDRF, causes a contraction itself and augments the effects of all contractile substances tested, a diverse group of agonists acting through different mechanisms. This would also explain why removal of the endothelium enhanced the contractile effects of K^+, 5-HT and U46619. Slight differences between the agonists (e.g. endothelial removal increases the maximal contraction to K^+, but increases sensitivity of U46619 with no change in maximal response) may indicate that the agonists themselves modify release of mediators from the vessel wall. 5-HT, for example, has been shown to cause release of EDRF from the endothelium of the dog coronary artery (Lamping *et al.*, 1985).

In endothelium-denuded coronary artery rings from sheep, hypoxia inhibited the contractile effects of K^+, 5-HT and U46619. This effect was particularly dramatic with the latter two agents and, under hypoxic conditions, no contraction at all was produced by U46619 in the large coronary artery or by 5-HT in the small artery. 5-HT and U46619, in most arteries, produce a contraction that depends partly on release of intracellular stored calcium, while the K^+ contraction is caused by influx of extracellular

calcium (Bolton, 1979). Our results may indicate that hypoxia reduces the ability of arterial smooth muscle to mobilize calcium, especially intracellular calcium, for contraction. We have previously shown that in endothelium-containing sheep coronary artery rings, hypoxia increases the maximal response and reduces the EC_{50} to 5-HT and U46619 (Kwan *et al.*, 1989). Thus, these effects of hypoxia in the absence of endothelium are the opposite of those observed in the presence of endothelium. One possible explanation is that a factor is released from the endothelium by hypoxia, this factor causing a contraction and enabling 5-HT and U46619 to mobilize calcium perhaps from a different source such as extracellular calcium. An alternative explanation for these results is that hypoxia causes the release of two factors: a vasoconstrictor from the endothelium and a vasodilator from the media or adventitia, the vasoconstrictor effect dominating in the intact artery.

The vasodilator action of adenosine was markedly reduced by removal of the endothelium, showing that at least part of the action of adenosine is due to release of a vasodilator from the endothelium. The reduced dilatation that remains in the endothelium-denuded ring is markedly enhanced by hypoxia. Hypoxia also enhances the relaxation produced by adenosine in endothelium containing rings (Kwan *et al.*, 1989). A unifying theme that links the effects of hypoxia on the vasodilator adenosine and on the vasoconstrictors, 5-HT and U46619 is that hypoxia in our experiments diminishes the ability of arterial smooth muscle to contract and promotes vasodilatation. In dog coronary artery rings it has been shown that the actions of adenosine are modified by removal of the endothelium (Rubany & Vanhoutte, 1985b) while other studies show no effect of removal of the endothelium (White *et al.*, 1987).

In the present study we compared a large (2–2.5 mm) and a small (0.6–1.3 mm) artery. Most results are qualitatively similar, although differences were found in 5-HT desensitization and in the effect of hypoxia on K^+ contractions. In endothelium-containing small, but not large, artery rings, 5-HT produced desensitization. No desensitization was seen after removal of the endothelium. These results may suggest that a factor released from the endothelium of the small, but not of the large, coronary artery ring interacts with 5-HT to enhance desensitization, presumably at the 5-HT receptors. In endothelium-containing rings, the K^+ contraction was augmented by hypoxia in the small rings but inhibited by hypoxia in the large rings. It is possible that the small artery rings are endowed with different nerve or other excitable cells from the large artery rings. High K^+, by depolarizing these cells, would release additional neurotransmitters or mediators in the small artery. The action of these released substances may be similar to those of 5-HT and U46619, which are augmented by hypoxia in both large and small artery rings.

In conclusion, the vasoconstrictor effect of K^+, 5-HT and U46619 and the vasorelaxant effect of adenosine on coronary artery rings isolated from sheep studied under oxygenated conditions are in part endothelium-dependent. The endothelium also profoundly modifies the response of the arterial rings to these agonists during hypoxia.

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8-OH-DPAT, flesinoxan and guanfacine: systemic and regional haemodynamic effects of centrally acting antihypertensive agents in anaesthetized rabbits.

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1 8-Hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) and flesinoxan, agents which show high affinity and selectivity for 5-HT_{1A} receptors, were administered intravenously in doses of 0.003 to 0.1 and 0.01 to 0.3 mg kg^{-1} respectively to 5 rabbits each. Their effects were compared with those of the centrally acting agent and α_2-adrenoceptor agonist, guanfacine, 0.01–0.3 mg kg^{-1}, administered to a group of 5 rabbits. Five further rabbits were used as controls and treated with the vehicle of the active agents.

2 Both flesinoxan and 8-OH-DPAT induced similar systemic and regional haemodynamic changes. Both lowered mean arterial pressure and heart rate. The principal blood pressure lowering mechanism was vasodilatation; cardiac output changed minimally despite the falls in heart rate and myocardial contractile force.

3 With guanfacine the maximal fall of blood pressure was comparable to that obtained with the 5-HT_{1A} receptor ligands; however, in contrast to the latter, the dose-response curve was U-shaped, the highest dose eliciting a pressor effect with reversal of the vasodilatation.

4 Widespread peripheral vasodilatation was found with all the agents in the splanchnic circulation and also in the brain and skeletal muscle. A weak tendency towards vasodilatation was found in the kidneys where the dose-response curve was bell-shaped for guanfacine.

5 This spectrum of activity is different from that of peripheral vasodilators, such as calcium antagonists, potassium channel activating agents or hydralazine; it is, however, consistent with the putative mechanism of action of these compounds to reduce peripheral sympathetic tone by a central mechanism of action.

Introduction

Investigation of the significance of 5-hydroxytryptamine (5-HT) in physiological and pathological function has been greatly facilitated in recent years by the availability of new pharmacological tools selective for 5-HT receptor subtypes (Bradley *et al.*, 1986; Fozard, 1987). One such agent is 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) which shows high affinity and selectivity for the central 5-HT_{1A} recognition site (Middlemiss & Fozard, 1983) and has been used extensively to define the functional correlates of this site (Middlemiss, 1986; Dourish *et al.*, 1987; Fozard, 1987; Mir *et al.*, 1988).

8-OH-DPAT has prominent cardiovascular effects in a number of species. Falls in blood pressure, generally accompanied by bradycardia, have been demonstrated in conscious spontaneously hypertensive (SH) rats, anaesthetized normotensive rats and cats and conscious renal hypertensive dogs (Gradin *et al.*, 1985; Martin & Lis, 1985; Dabire *et al.*, 1987; Fozard *et al.*, 1987; Ramage & Fozard, 1987; Doods *et al.*, 1988; Di Francesco *et al.*, 1988). In the rat and cat, compelling evidence exists that the cardiovascular effects of 8-OH-DPAT are largely, if not entirely centrally mediated, that both sympathoinhibition and an increase in vagal tone contribute to the response and that stimulation of postsynaptic 5-HT_{1A} receptors is the key receptor mechanism involved (Dabire *et al.*, 1987; Fozard *et al.*, 1987; Ramage & Fozard, 1987; Doods *et al.*, 1988; Wouters *et al.*, 1988; for review, see Mir & Fozard, 1987).

As yet, there has been no detailed analysis of the haemodynamic effects of 8-OH-DPAT in any species. The present paper describes the results from experiments carried out in anaesthetized rabbits in

which standard haemodynamic measurements have been supplemented by recordings of regional blood flow distribution using radioactive microspheres. Comparisons have been made with flesinoxan, which has high affinity and selectivity for 5-HT_{1A} receptors (Wouters *et al.*, 1988) and is undergoing clinical evaluation as an antihypertensive agent and guanfacine, a centrally acting antihypertensive agent with selectivity for α_2-adrenoceptors (Scholtysik, 1986) and negligible affinity for 5-HT_{1A} receptors (pK_D 5.2; Hoyer, personal communication).

Methods

Full details of the anaesthesia and preparation of the rabbits are given in Hof (1985). In brief, large rabbits (3–4 kg) were anaesthetized by intravenous injection of pentobarbitone followed by phenobarbitone. The animals were tracheotomized and ventilated (Loosco MK2 infant ventilator) with room air. End-expiratory CO_2 was kept between 4.0 and 4.5% at a positive end-expiratory pressure of 2 mmHg. Catheters were placed in the lower abdominal aorta, the inferior vena cava, the jugular vein and, through a thoracotomy in the left 3rd intercostal space, in the left atrium for microsphere injection. An electromagnetic flow probe (Narco RT 500) was fitted on the aortic root. It was calibrated *in vivo* by the reference flow method at the time of the last microsphere injection (Hof & Hof, 1981). A second thoracotomy in the 5th right intercostal space was used to sew a Walton-Brodie strain-gauge onto the right ventricle in parallel to the superficial muscle fibres.

Microsphere technique

The use of the microsphere method in our laboratory has been described in detail previously (Hof *et al.*, 1980; 1981; Hof & Hof, 1981). In brief, for each set of measurements we injected about 150,000 microspheres labelled with one of the following tracers: ^{125}I, ^{141}Ce, ^{51}Cr, ^{85}Sr or ^{46}Sc. The sequence of labels was changed for each experiment so that each label was used once for each measuring period. This procedure minimizes systematic errors due to the small differences in the physical properties (e.g. size, density, leaching tendency) of different batches of microspheres. At the end of the experiment the animals were killed with an overdose of pentobarbitone and the tissues dissected. The radioactivity of the tissue samples was determined in a Packard gamma counter (Model 5921) and the spectra processed on an OKI if-800 Model 30 microcomputer according to the method of Rudolph & Heymann (1967) with the modifications of the calculations described by Schosser *et al.* (1979).

Experimental protocol

After obtaining baseline measurements, 0.003 mg kg^{-1} of 8-OH-DPAT or 0.01 mg kg^{-1} of flesinoxan was infused during 10 min then measurements were taken and the 2nd to the 4th doses of the two compounds were infused using the same schedule. In preliminary dose-finding studies, all compounds maintained their activity as judged from the systemic haemodynamic variables during an observation period of 30 min after the infusion. The duration of action was thus long compared with the duration of the infusion periods and, for this reason, cumulative doses at the end of each infusion period (0.003, 0.01, 0.03 and 0.1 mg kg^{-1} for 8-OH-DPAT, 0.01, 0.03, 0.1 and 0.3 mg kg^{-1} for flesinoxan and 0.01, 0.03, 0.1 and 0.3 mg kg^{-1} for guanfacine) are given in the figures.

The substances were dissolved using minimal amounts of tartaric acid and 0.02 ml ethanol per mg of active substance and then 5% glucose was added to give a final concentration of 0.1 and 0.3 mg ml^{-1}, respectively; thus in each experiment a total vehicle volume of 1 ml kg^{-1} was administered.

Statistics

The overall effects of the test compounds were analysed by the Kruskal-Wallis test followed by the Dunn-Bonferroni test to assess which of the experimental groups was different from placebo. All statistical calculations were performed using the absolute values, which were then converted to percentage changes for the visual presentation in the figures.

Drugs

8-Hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) was purchased from Research Biochemicals, Wayland, Maryland, U.S.A. Flesinoxan was generously suppled by Duphar, Weesp, The Netherlands. Guanfacine was synthesized and supplied by Sandoz, Basel, Switzerland.

Results

Systemic haemodynamics

The mean baseline values for each parameter and for each group of rabbits are presented in Table 1. The Kruskal-Wallis test indicated that there were significant differences between the groups before drug administration for heart rate and regional conductance of adrenals and pancreas. Treatment effects were evaluated in terms of drug-induced changes

Table 1 Baseline values for all groups of rabbits

	Placebo	*8-OH-DPAT*	*Flesinoxan*	*Guanfacine*
Systemic variables				
MAP	69 ± 4	71 ± 4	69 ± 2	75 ± 3
HR*	291 ± 12	305 ± 6	317 ± 7	280 ± 6
CF	26.8 ± 3.0	27.6 ± 3.0	24.9 ± 1.0	31.4 ± 1.4
CO kg^{-1}	103.1 ± 6.3	102.6 ± 6.4	90.5 ± 10.2	108.2 ± 3.1
SVC kg^{-1}	1.50 ± 0.12	1.45 ± 0.08	1.30 ± 0.13	1.45 ± 0.1
Regional conductance (ml min^{-1} $mmHg^{-1}$ per 100 g of tissue)				
Heart	2.48 ± 0.13	2.57 ± 0.24	2.50 ± 0.26	2.85 ± 0.47
Brain	0.52 ± 0.02	0.57 ± 0.06	0.51 ± 0.07	0.59 ± 0.09
Muscle	0.08 ± 0.01	0.08 ± 0.02	0.05 ± 0.01	0.06 ± 0.01
Kidneys	4.01 ± 0.56	3.44 ± 0.37	2.57 ± 0.27	3.56 ± 0.43
Skin	0.04 ± 0.01	0.04 ± 0.00	0.03 ± 0.00	0.04 ± 0.01
Lungs	4.39 ± 0.51	4.33 ± 0.83	3.91 ± 1.02	5.06 ± 0.85
Adrenals*	2.91 ± 0.26	2.29 ± 0.28	1.54 ± 0.17	2.30 ± 0.29
Liver	0.15 ± 0.07	0.26 ± 0.11	0.13 ± 0.06	0.10 ± 0.02
Spleen	1.86 ± 0.23	1.48 ± 0.51	0.97 ± 0.19	1.03 ± 0.21
Pancreas*	1.05 ± 0.19	0.61 ± 0.05	0.64 ± 0.10	0.86 ± 0.02
Stomach	0.73 ± 0.20	0.42 ± 0.08	0.53 ± 0.11	0.67 ± 0.12
Small intestine	0.69 ± 0.08	0.70 ± 0.04	0.61 ± 0.05	0.63 ± 0.03
Colon	0.41 ± 0.03	0.41 ± 0.04	0.41 ± 0.05	0.39 ± 0.01
Caecum	0.52 ± 0.01	0.54 ± 0.04	0.48 ± 0.06	0.47 ± 0.03

Values represent means ± standard error of the mean ($n = 5$).
Abbreviations and units: MAP, mean arterial pressure (mmHg); HR, heart rate (beats min^{-1}); CF, contractile force (g); CO kg^{-1}, cardiac output per kg of body weight (ml min^{-1} kg^{-1}); SVC, systemic vascular conductance (ml min^{-1} $mmHg^{-1}$ kg^{-1}). *indicates that significant differences exist between the mean values of the four groups ($P < 0.05$; Kruskal-Wallis test).

from these pretreatment values; for the figures these were converted into percentage changes.

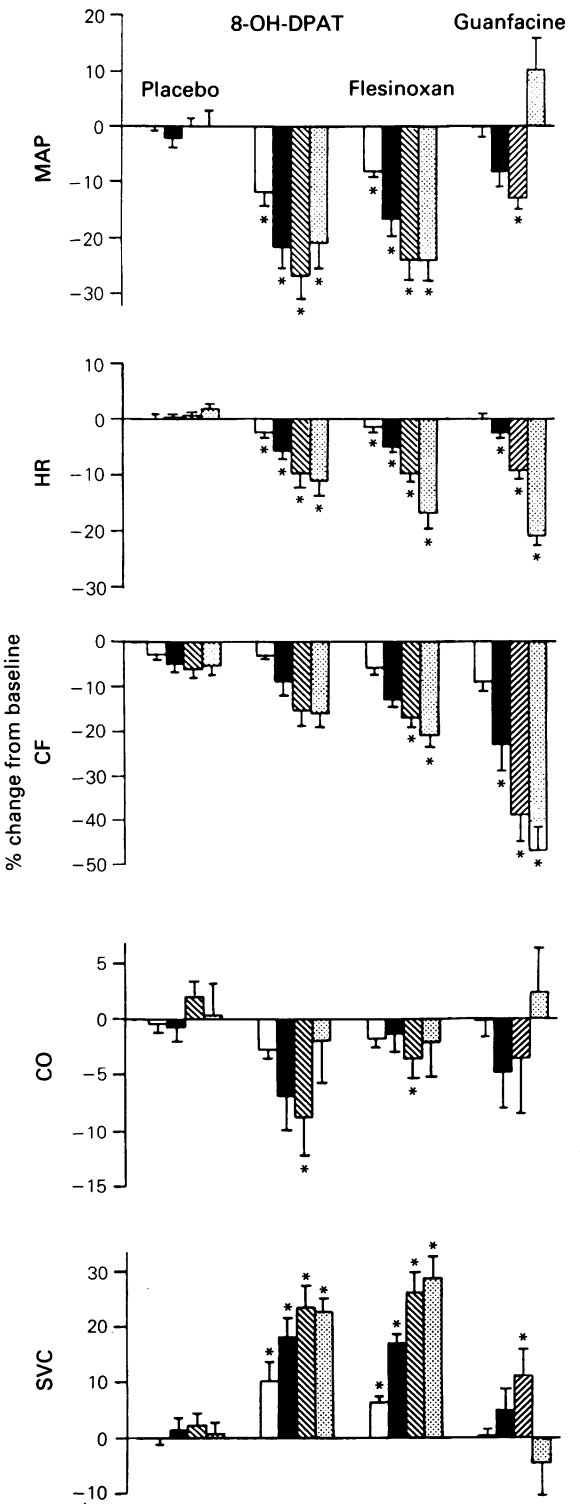
The systemic haemodynamic drug effects are shown in Figure 1. All compounds caused dose-related decreases in blood pressure which peaked at the third dose. The 8-OH-DPAT dose-effect curve was slightly bell-shaped. The blood pressure effects of guanfacine were clearly reversed at the highest dose where the peripheral pressor effects of this agent became dominant.

All compounds decreased heart rate. The effect was only modest with 8-OH-DPAT and levelled off at the third dose. Overall there were no significant differences between the effects of the three drugs. However, it should be noted that the highest dose of guanfacine was associated with a pressor effect, which by deactivating the baroreflex, could have contributed to the bradycardia.

The effects on myocardial contractile force were similar to those on heart rate. The changes tended in the same direction with all agents; however, only those of flesinoxan and, in particular, guanfacine were significant. 8-OH-DPAT and to a minimal extent flesinoxan, lowered cardiac output whereas guanfacine did not. Systemic vascular conductance increased with all agents. However, for guanfacine the dose-response curve was once again bell-shaped and at the highest dose a tendency to vasoconstriction was seen. No such tendency was found with the 5-HT_{1A} receptor ligands (Figure 1).

Regional conductance

The patterns of regional vasodilatation (Figure 2), showed several minor differences but, more importantly, many striking similarities. Flesinoxan and 8-OH-DPAT but not guanfacine, caused slight coronary and more pronounced skeletal muscle vasodilatation. All three agents dilated the vessels of the brain. The effects of 8-OH-DPAT were almost maximal at the lowest dose and no clear dose-dependence was observed. However, no significant differences from the effects of the other agents could be demonstrated by the Kruskal-Wallis test. Renal vasodilatation was weak and not dose-dependent for the 5-HT_{1A} receptor ligands, whereas it reached significance with the second dose of guanfacine. Microspheres retained in the lungs mostly reach this organ by crossing from the arterial to the venous side through the arteriovenous shunts. Neither 8-OH-DPAT nor flesinoxan altered conductance of these shunt vessels, whereas guanfacine strikingly constricted them (see 'lung' in Figure 2).

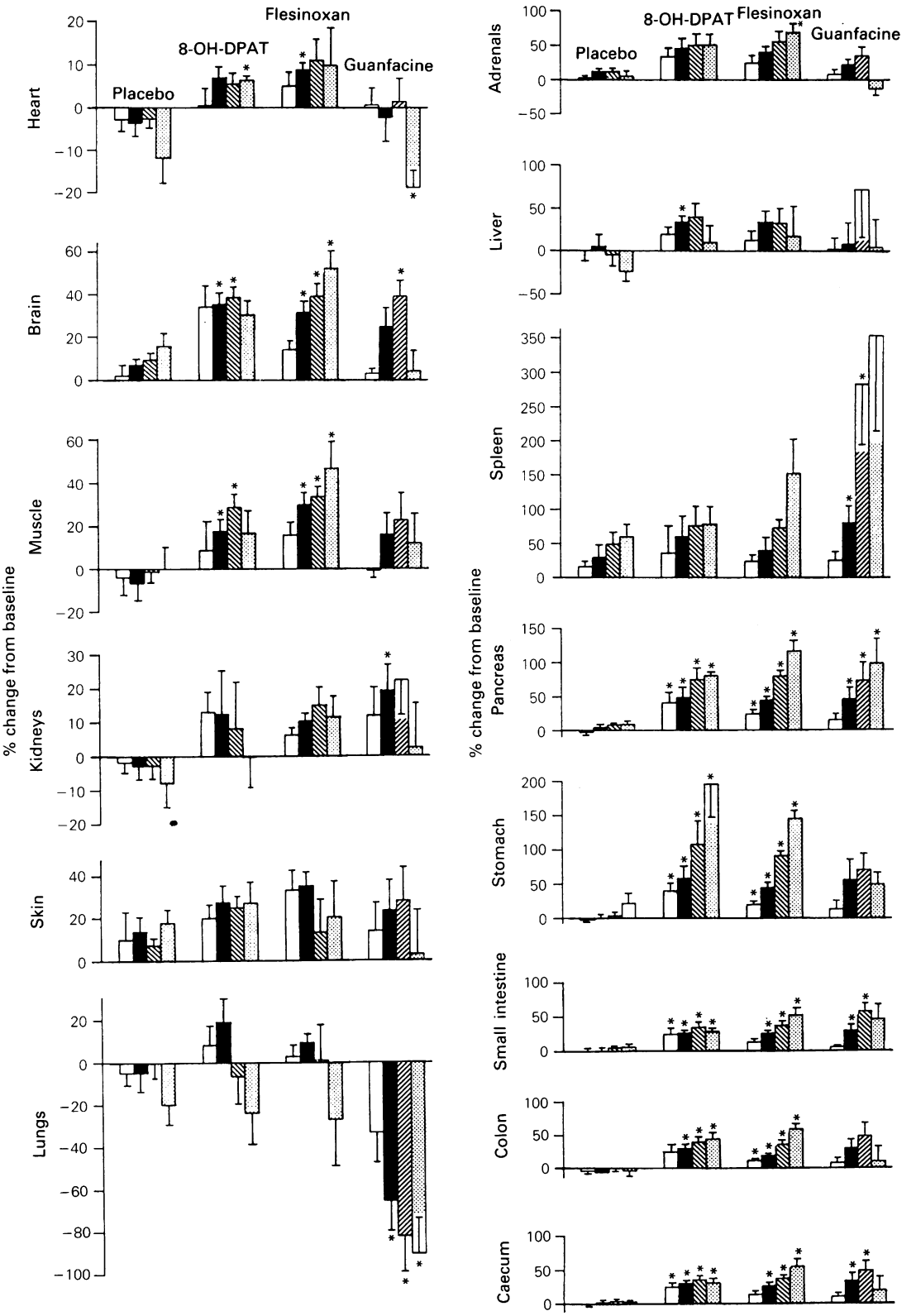


8-OH-DPAT tended to, and flesinoxan and guanfacine unequivocally did, dilate the adrenal blood vessels (Figure 2). With all three agents, the trend was to vasodilatation in the hepatic arterial circulation although in each case the changes were poorly dose-related ('liver' in Figure 2). Guanfacine strikingly dilated the vessels of the spleen; the highest dose had the largest effect but also by far the largest variability. Both 8-OH-DPAT and flesinoxan produced qualitatively similar effects although these were less pronounced than those of guanfacine. All three agents elicited dilatation in the pancreas and the gastro-intestinal tract. The effects of guanfacine tended to be more variable and its dose-response curves were generally bell-shaped in these organs (Figure 2).

Discussion

There are marked differences in the mechanisms of action of 8-OH-DPAT and flesinoxan on the one hand and guanfacine on the other; the former are selective and potent ligands at 5-HT_{1A} receptors (Middlemiss & Fozard, 1983; Wouters *et al.*, 1988) whereas guanfacine is a selective α_2-adrenoceptor agonist with minimal effects on 5-HT_{1A} receptors (Scholtysik, 1986; Hoyer, personal communication). Despite this, the present data obtained in the instrumented open-chest rabbit reveal remarkable similarities between the three compounds in the large number of haemodynamic variables measured. Thus, at low doses the two 5-HT_{1A} receptor ligands decreased blood pressure by widespread systemic vasodilatation and without inducing reflex activation of the cardiovascular system; similar effects were seen with guanfacine albeit over a narrower dose-range (second and third doses only). This spectrum of action is quite dissimilar to that seen following peripheral vasodilator agents such as hydralazine (Hof & Hof, 1984), calcium antagonists (Bolt & Saxena, 1984; Hof, 1984) or potassium channel openers (Cook & Hof, 1988) and suggests an action on the central nervous system to inhibit sympathetic

Figure 1 Systemic haemodynamic effects of the infusion of four doses of 8-OH-DPAT, flesinoxan, guanfacine or equivalent volumes of vehicle (placebo). All effects are shown as percentage change from the pretreatment baseline values (see Table 1). Bars show standard errors of the mean values ($n = 5$). Asterisks show significant differences (Kruskal-Wallis, Dunn-Bonferroni; $P < 0.05$) from changes occurring at the same stage of the experiment in vehicle-treated animals. Abbreviations: MAP, mean arterial pressure; HR, heart rate; CF, myocardial contractile force; CO, cardiac output; SVC, systemic vascular conductance.


Placebo
8-OH-DPAT
Flesinoxan
Guanfacine
% change from baseline
Heart
Brain
Muscle
Kidneys
Skin
Lungs
Adrenals
Liver
Spleen
Pancreas
Stomach
Small intestine
Colon
Caecum

outflow and/or increase vagal tone. This interpretation would accord with evidence from a number of other species that the cardiovascular effects of 8-OH-DPAT (Fozard *et al.*, 1987; Ramage & Fozard, 1987; Doods *et al.*, 1988), flesinoxan (Wouters *et al.*, 1988; Ramage *et al.*, 1988) and guanfacine (Scholtysik, 1986) arise primarily from a central mechanism of action.

Open chest animals have high sympathetic tone; this can be clearly demonstrated by the administration of a β-adrenoceptor antagonist such as propranolol which substantially decreases both the rate and force of cardiac contraction and cardiac output under these conditions, while blood pressure remains almost unchanged (Hof, unpublished observations). All three agents induced or tended to induce (8-OH-DPAT) a negative inotropic effect, as would be expected for agents inhibiting sympathetic tone. However, cardiac output changed little. Vasodilatation facilitates the ejection of blood from the left ventricle and this effect apparently compensates the decrease of myocardial contractile force.

Despite clear similarities, there are several notable differences between the 5-HT_{1A} receptor ligands and guanfacine. For instance, the highest dose of guanfacine induces a rise in blood pressure due to vasoconstriction in some vascular beds and widespread reversal of the dilator effects seen at lower doses. This is a characteristic feature of α_2-adrenoceptor agonists (Zaimis, 1970; Reid, 1986) and reflects activation of postjunctional α_2- and/or α_1-adrenoceptors in the peripheral vasculature (Timmermanns & Van Zwieten, 1981; Reid, 1986). Neither 8-OH-DPAT nor flesinoxan has a similar action, a reflection both of their selectivity for 5-HT_{1A} receptors and the fact that such sites seem not to be functionally important in the control of peripheral cardiovascular function (Mir *et al.*, 1987).

Guanfacine, in contrast to 8-OH-DPAT and flesinoxan, markedly decreased the microsphere content of the lungs. α_2-Adrenoceptors are present on arterio-venous shunt vessels which induce their constriction when activated (Saxena, 1984) and such a mechanism would readily explain the effects of guanfacine. In contrast, the 5-HT receptor(s) present on shunt vessels seem not to be of the 5-HT_{1A} subtype at least in the pig (Verdouw *et al.*, 1985; Bom *et al.*, 1988) and cat (Feniuk *et al.*, 1987) and effects from compounds with selectivity for these sites would not be expected.

The highest dose of both 5-HT_{1A} receptor ligands appeared to have an analeptic action, not shown by guanfacine. The animals exhibited spontaneous movements, despite deep anaesthesia, associated with cardiovascular instability. Although no attempt was made to quantify such observations, the effects seemed to be more marked with 8-OH-DPAT. Central nervous system stimulation spreading to the autonomic nervous system may well explain the flattening of the dose-response curve with 8-OH-DPAT with respect to heart rate and mean arterial pressure. Similar observations have been made in the conscious dog where the appearance of central excitation following 8-OH-DPAT coincided with a loss of hypotensive activity (Di Francesco *et al.*, 1988).

In conclusion, our experiments provide the first detailed haemodynamic data on 8-OH-DPAT and flesinoxan, compounds which induce cardiovascular effects by activation of central 5-HT_{1A} receptors. Differences from guanfacine, a centrally acting antihypertensive agent with selectivity for α_2-adrenoceptors, include an absence of vasoconstriction at higher doses and no effects on arterio-venous shunts. Blood pressure lowering activity arising from peripheral vasodilatation with no major changes in heart rate, myocardial contractility or cardiac output would represent an interesting profile for a putative antihypertensive agent. Thus, cardiac side effects should be minimal and regression of the cardiovascular hypertrophy that accompanies hypertension should be facilitated. However, before agonism at central 5-HT_{1A} receptors can be taken seriously as a novel antihypertensive principle, the significance of the central stimulant effects, detectable even in anaesthetized, open-chest rabbits, will have to be evaluated.

We thank Dr D. Hoyer for measuring the affinity of guanfacine for 5-HT_{1A} receptors.

Figure 2 Changes of regional conductance induced by 8-OH-DPAT, flesinoxan or guanfacine. The data were obtained from the same experiments shown in Figure 1 and are shown as percentage changes from pretreatment baseline values (see Table 1). Bars show standard errors of the mean values ($n = 5$). Asterisks show significant differences (Kruskal-Wallis, Dunn-Bonferroni; $P < 0.05$) from changes occurring at the same stage of the experiment in vehicle-treated animals. 'Liver' indicates conductance of the hepatic artery only, since portal blood is free of microspheres. 'Lungs' shows microspheres trapped in this organ. They reach the lungs mostly (>80%) through arterio-venous shunts and can therefore be taken to represent principally arterio-venous shunt conductance. Abbreviations as in Figure 1.

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Castor oil increases intestinal formation of platelet-activating factor and acid phosphatase release in the rat

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1 When castor oil was administered by gavage to rats, the duodenum and jejunum but not ileum and colon produced large amounts (5–6 fold greater than control) of platelet activating factor (Paf).

2 Intraluminal release of acid phosphatase (AP) was also markedly increased (5–6 fold greater than control) in the duodenum and jejunum of castor oil-treated rats and there was a correlation between the elevated release of AP and intestinal hyperaemia.

3 These findings support a role for Paf as a mediator of intestinal damage induced by castor oil.

Introduction

It has been recently demonstrated that platelet-activating factor (Paf), an endogenous phospholipid, produces extensive gastrointestinal hyperaemia and haemorrhage (Wallace & Whittle, 1986a). The most severely affected tissues are the stomach and jejunum while the colon is unaffected. Administration of endotoxin produces hypotension (Doebber *et al.*, 1985), diarrhoea (Tsurumi & Fujimura, 1983) and gastrointenstinal damage that is associated with the increased formation of Paf throughout the digestive tract (Whittle *et al.*, 1987). CV-3988, a Paf receptor antagonist, prevents gastric and intestinal damage which follows endotoxin administration (Wallace & Whittle, 1986b), thereby supporting the role of Paf in gastrointestinal damage.

Castor oil, used as a laxative, induces intestinal damage, termed 'chemical gastroenteritis' by Reynell & Spray (1958). Inflammatory swelling of villi with sloughing of erosion at the villus tips and desquamation of surface cells have been reported in rats and rabbits following administration either of castor oil or its active principle, ricinoleic acid (for references see Gaginella & Bass, 1978). However, the mechanism involved in this damage is not yet known.

In the present study we have determined whether oral administration of castor oil leads to changes in the levels of Paf formed by intestinal tissue and if these changes are related to intestinal damage, assessed both macroscopically and by using intraluminal acid phosphatase as a marker of cellular damage (Wallace & Whittle, 1986a).

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Methods

Male Wistar-Nossan rats (130–140 g) were deprived of food overnight but allowed water *ad libitum*. Castor oil, 2 ml per rat, was administered orally and 3 h later the animals were killed by cervical dislocation. Macroscopically visible intestinal damage was scored on a scale (0, normal, to 3, severe hyperaemia) by an observer unaware of the treatment. Intraluminal release of acid phosphatase was measured by the method of Wallace & Whittle (1986a), and enzymatic activity was measured as described by Ammendola *et al.* (1975).

Isolation and identification of Paf was carried out by a modification of the method previously described by Calignano *et al.* (1988). In brief, segments of intestinal tissues (500 mg wet weight) were suspended whole in 5 ml of 0.25% bovine serum albumin (BSA) in 154 mM NaCl (0°C). After vortexing for 20 s, the mixture was added to cold acetone (10 ml, −20°C), and after centrifugation (2000 *g* for 5 min), the acetone-water phase was extracted by vortex mixing for 10 s with chloroform (10 ml). The upper aqueous phase was discarded after separation of the mixture by centrifugation for 10 min at 2000 *g*. The organic phase containing the extracted Paf was evaporated to dryness, redissolved

in chloroform 100 μl, applied to thin layer chromatography (t.l.c.) plates and developed in a solvent system chloroform/methanol/water (65:35:6) together with authentic standard Paf (Sigma). Visualized by exposure to u.v., zones from the plate co-migrating with authentic Paf were re-extracted, the dried organic phase was resuspended in Tris buffer (25 mM, pH 8.0) containing 0.25% BSA, and the Paf activity bioassayed on the aggregation of rabbit washed platelets (Whittle *et al.*, 1987). The pro-aggregatory activity of the samples was confirmed as Paf since it was completely inhibited by pre-incubating the platelet suspension for 1 min with CV 3988 ((**RS**)-2-methoxy-3-(octadecylcarbamoxyloxy)propyl - 2 - (3 - thiazolio)ethyl - phosphate 10 μM, Takeda Chemical), a selective Paf inhibitor. In five experiments, olive oil 2 ml per rat was administered orally and 3 h later rats were killed and intestinal tissue analysed as above.

Results

Formation of Paf by segments of rat intestine was substantially increased 3 h after castor oil administration (Table 1). Paf formation was greater in the small intestine than in the colon and was greater in the duodenum (6.6 × control, $P < 0.001$) than in the jejunum (5.0 × control, $P < 0.01$). There was a similar tendency in the ileum and colon but this was not significant ($P < 0.2$–0.1).

On macroscopic examination, the whole intestinal mucosa had extensive regions of hyperaemia. The duodenum (damage score = 1.0 ± 0.3 $n = 6$, $P < 0.05$ compared to controls) and jejunum (0.8 ± 0.2, $n = 6$, $P < 0.05$) were the most severely affected regions, while the ileum (0.5 ± 0.2, $n = 6$, $P < 0.1$) and colon (0.4 ± 0.2, $n = 6$, $P < 0.1$) showed only moderate damage which was not significantly different from controls. Intestinal segments of rats treated with olive oil did not produce any increase of Paf formation (duodenum 158.4 ± 30.8, jejunum 168.3 ± 50.4, ileum 201.5 ± 50.1, colon 128.5 ± 35.8 pg g^{-1} wet tissue). In addition, the intestinal mucosa of olive oil-treated rats was completely intact, as in control animals.

Castor oil also increased the intraluminal release of acid phosphatase in the duodenum and jejunum ($P < 0.01$), with a similar trend in the ileum and colon ($P < 0.2$–0.1). However, no significant increase of acid phosphatase was observed in olive oil-treated rats.

Discussion

It has been reported that castor oil causes diarrhoea 1–2 h after administration in rats (Niemegeers *et al.*, 1972; Vischer & Casals-Stenzel, 1983). We have obtained a satisfactory and highly reproducible response 3 h after castor oil administration. In addition we have studied a 3 h time-period after castor oil because at this time extensive hyperaemia was more evident throughout the intestinal mucosa.

The ability of non-stimulated rat intestine to produce large amounts of Paf (Whittle *et al.*, 1987) has been confirmed in the present study. This substantial non-stimulated production of Paf, characterized by its chromatographic mobility and by antagonism of the biological activity with CV 3988, may be due to the manipulation of the tissue. Nevertheless, castor oil, but not olive oil, at a dose that produced diarrhoea in all the animals treated, increased the amount of intestinal Paf. This stimulated formation was markedly greater in the duodenum than in the jejunum, and less in the ileum and

Table 1 Formation of platelet-activating factor (Paf) and the intraluminal release of acid phosphatase (AP) by intestinal tissue of rats after oral administration of castor oil

	Paf (a)		*AP* (b)	
Tissue	*Control*	*Castor oil*	*Control*	*Castor oil*
Duodenum	164.3 ± 33.7	1076.8 ± 131.3 $P < 0.001$	943.8 ± 149.7	3464.7 ± 318.3 $P < 0.01$
Jejunum	182.8 ± 49.2	914.5 ± 100.1 $P < 0.01$	825.8 ± 66.3	2801.0 ± 174.0 $P < 0.01$
Ileum	193.7 ± 48.9	413.9 ± 150.0 $P < 0.2$	486.3 ± 126.3	790.7 ± 150.0 $P < 0.1$
Colon	128.5 ± 39.3	179.2 ± 63.6 $P < 0.1$	347.6 ± 123.2	776.9 ± 179.0 $P < 0.2$

Results are expressed as mean ± s.e. of 10 experiments and analysed by Student's *t* test for paired data.
In (a) results are expressed as pg g^{-1} wet tissue, and in (b) as μg of substrate transformed g^{-1} dry tissue in 30 min at 37°C.

colon. The elevated intestinal formation of Paf following castor oil was accompanied by hyperaemia, which was most severe in the duodenum but less evident in the ileum and colon. Similar results were obtained with the intraluminal release of acid phosphatase, a marker of cellular damage.

The mechanism underlying the intestine damage induced by castor oil is not yet established, but the present evidence suggests that Paf is involved. Ricinoleic acid, a C-18 aliphatic monohydroxy fatty acid, is the active principle in castor oil. It is liberated in the small bowel by lipolysis, but on account of its 12-hydroxyl group it is poorly absorbed (Ammon & Phillips, 1974). This could explain the localization of the damage induced by this agent (Ammon & Phillips, 1974; Gaginella & Bass, 1978) which consists of erosions, desquamation of surface cells and infiltration of several cell types, and changes in the microcirculation. The cause(s) for the increased formation of Paf following castor oil administration are not known. Perhaps there is a contribution from infiltrated platelets, polymorphonuclear neutrophils, monocytes, macrophages, and/or from the activation of other cell types (e.g. endothelial cells, smooth muscle). Paf is formed in cells simultaneously with 5-HETE (5-hydroxyeicosatetraenoic acid), leukotriene B_4 and prostaglandins (O'Flaherty & Wykle, 1987), and some studies indicate that eicosanoids may contribute to the cathartic effect of castor oil and other laxatives (Capasso *et al.*, 1986; 1987). It remains to be determined whether stimulation of Paf formation by the intestinal tissue contributes to the laxative actions induced by castor oil.

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EP 171: a high affinity thromboxane A_2-mimetic, the actions of which are slowly reversed by receptor blockade

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1 Replacement of the four-carbon ω-terminus in 9,11-endoxy-10a-homo prostaglandin H_2 with a *p*-fluorophenoxy group produces a compound (EP 171) with very high agonist potency at TP-receptors.

2 On six isolated smooth muscle preparations EP 171 was 33–167 times more potent as a TP-receptor agonist than U-46619 (11,9-epoxymethano PGH_2); EC_{50} values ranged from 45 to 138 pM. The actions of EP 171 were difficult to study because of their slow onset and offset. For example, on the guinea-pig trachea the time required for 50% reversal of EP 171-induced contractions during washout was about 3 h.

3 On the pig pulmonary artery, a more rapidly responding preparation, it was possible to show that the TP-receptor antagonist EP 092 blocked the contractile actions of EP 171 and U-46619 to similar extents: $pA_2 = 8.09$ and 8.15 respectively.

4 EP 171 was also a very potent activator of human blood platelets, being about 90 times more potent than U-46619. Both shape change (0.1 nM) and aggregation (1 nM) were slow in onset, a profile not previously observed for a thromboxane A_2-mimetic.

5 When potencies at TP-, EP_1-(guinea-pig fundus) and FP-(dog iris sphincter) receptors were compared, EP 171 showed a higher specificity as a TP-receptor agonist than either STA_2 or U-46619. These studies also showed that contrary to earlier reports, the guinea-pig fundus does contain TP-receptors mediating muscle contraction. However, the maximal response due to activation of TP-receptors was only about 35% of the PGE_2 maximum.

6 Established responses to EP 171 were slowly reversed following addition of a high concentration of a TP-receptor antagonist (EP 092, GR 32191 or BM 13177). Faster reversals of three less potent 16-*p*-halophenoxy prostanoids and U-46619 were obtained. Half-times for offset (and onset) of agonist action appeared to correlate with potency rather than with lipophilicity.

7 Competition between the agonists and a radio iodinated PTA_2 derivative ([^{125}I]-PTA-OH) for binding to TP-receptors on intact human platelets was studied. IC_{50} values correlated well with aggregating potency, EP 171 having the lowest IC_{50} of 2.9 nM. The true K_i for EP 171 may be about 1 nM if both its racemic nature and reduction of initial free ligand concentration due to TP-receptor binding are taken into account.

8 It is concluded from a comparison of agonist potency rankings that subclassification of the TP-receptor is not warranted at this time. The factors that may be responsible for the slow kinetics of EP 171 action are discussed.

Introduction

Manipulation of the chemical structure of a transmitter substance or hormone can result in substantial loss or, less frequently, enhancement of potency. While clearly the maximum degree of loss of potency is absolute, the maximum degree of enhancement has yet to be defined. Our early studies of TP-receptor agonists (thromboxane A_2 mimetics; see Kennedy *et al.*, 1982; 1983, for prostanoid receptor nomenclature) showed that alteration of the ω-chain structure of compounds with weak agonist activity could dramatically increase potency. For example, it was observed that the replacement of the terminal four-carbon unit of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) with a *p*-fluorophenoxy group produced an analogue

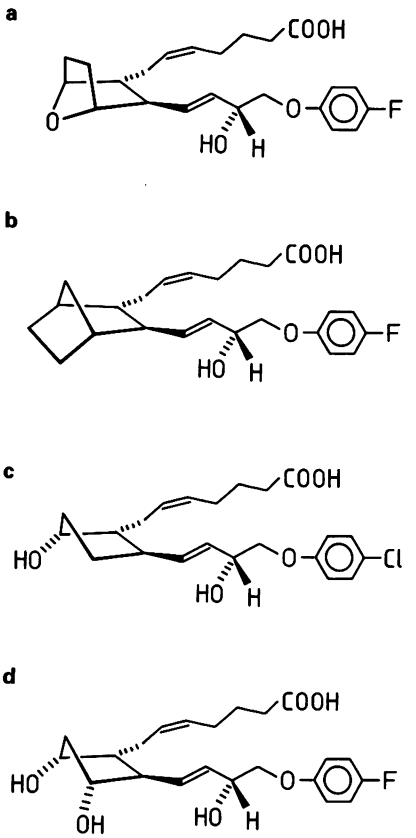

Figure 1 Structures of the 16-*p*-halophenoxy-ω-tetranor prostanoids examined in this study: (a) EP 171 (*rac*), (b) EP 031 (*rac*), (c) 16-*p*-chlorophenoxy-ω-tetranor-11-deoxy $PGF_{2\alpha}$ (*rac*) and (d) 16-*p*-fluorophenoxy-ω-tetranor $PGF_{2\alpha}$ (*nat*).

(structure d in Figure 1) with 50 fold greater contractile potency on the isolated aorta of rabbit and saphenous vein of dog (EC_{50} = 30 nM) (Jones & Marr, 1977). When a similar substitution was made on all-carbon ring analogues of the prostaglandin endoperoxide PGH_2, partial agonist activity was converted into potent full agonism. For example, EP 031 (Figure 1) has an EC_{50} value for contraction of rabbit aorta, dog saphenous vein and guinea-pig trachea of about 1 nM (Jones *et al.*, 1982). We were curious to know therefore whether the 16-*p*-fluorophenoxy-ω-tetranor modification would greatly enhance the potency of a prostanoid which was already a potent full agonist of TP-receptors (EC_{50} < 10 nM).

Our choice of molecule was influenced by the remarkably slow onset and offset of EP 031 action on isolated smooth muscle preparations. A contact time of 2–3 h is often required to achieve a stable submaximal response and the return to resting tension even with continuous wash-out of the organ bath takes 4–5 h (Jones *et al.*, 1982). We assumed that the slow onset is mainly due to removal of the highly lipophilic analogue from the extracellular space into adjacent lipid domains. Thus diffusion of the agonist to membrane receptors in the centre of the preparation is retarded and equilibrium occupation of the receptor pool is only slowly attained. Offset of action is correspondingly slow because the loss of agonist from the lipid reservoir maintains the extracellular space concentration. EP 031 is also a potent activator of human platelets (Wilson *et al.*, 1982), but its rate of action (shape change or aggregation) is only marginally slower than other TP-receptor agonists. This is not surprising if we suppose that agonist molecules, irrespective of their lipophilicity, have ready access to surface membrane receptors of discrete cell fragments in a well-stirred system. We felt therefore that a polar bicyclic ring system would favour a rapid onset/rapid offset action on smooth muscle preparations by increasing the overall water solubility of the molecule. By this reasoning, CTA_2(9,11-carba-11a-carba TXA_2), the all-carbon ring analogue of TXA_2 (Lefer *et al.*, 1980), was deemed to be an unsuitable candidate for the parent molecule. 9,11-Endoxy-10a-homo PGH_2 was much more attractive since our chemical work on precursor molecules showed that the 7-oxabicyclo[2.2.1]heptane ring endowed considerable water solubility and a preliminary report in the literature (Sprague *et al.*, 1983) indicated high agonist potency on guinea-pig trachea and human platelets. The 16-*p*-fluorophenoxy-ω-tetranor derivative of 9,11-endoxy-10a-homo PGH_2 (Figure 1) is coded EP 171 and this paper describes our attempts to determine its potency, specificity and kinetics on isolated smooth muscle preparations and human platelets.

Methods

Isolated smooth muscle preparations

Thoracic aortae were removed from male rats (250–300 g) killed by stunning and exsanguination and from male rabbits (2–3 kg) killed by exsanguination under pentobarbitone anaesthesia. Segments of saphenous vein were obtained from dogs under pentobarbitone anaesthesia, and eyes were removed from the same animals killed by air embolism. Lobar pulmonary arteries were dissected from lungs of pigs (30–35 kg) killed by exsanguination under pentobar-

bitone anaesthesia. Trachea and stomach were removed from guinea-pigs (400–700 g) of either sex killed by stunning and exsanguination. Bullock eyes were obtained from the abbatoir.

Rings, 3 mm wide, of trachea and blood vessels were suspended between stainless steel hooks in 10 ml organ baths and tension changes were recorded with Grass FT03 force displacement transducers linked to a Grass Polygraph. Tension changes in the sphincter pupillae of the iris and strips of fundic stomach (ventral surface) were measured with the same recording system, connections being made with fine silk thread. The Krebs bathing solution contained (mmol l^{-1}): NaCl 118, KCl 5.4, $MgSO_4$ 1.0, $CaCl_2$ 2.5, NaH_2PO_4 1.1, $NaHCO_3$ 25 and dextrose 10, and was gassed with 95% O_2 and 5% CO_2 and maintained at 37°C. In addition, for the pig pulmonary artery and the dog and bullock iris preparations, indomethacin (1 μM) was present and for the guinea-pig trachea and fundus indomethacin (1 μM) and atropine (20 nM).

As a general procedure, a 1 h equilibration procedure was followed by the cumulative addition of doses of the standard agonist. After washout of the organ bath by upward displacement the standard agonist cumulative dose sequence was repeated. With fast onset/fast offset test compounds a cumulative dose sequence was performed and then a final sequence of standard agonist doses was applied. With EP 171 and EP 031 the organ bath system was perfused with a suitable concentration of the agonist and when a stable level of contraction had been reached the flow was stopped. A high concentration of 11,9-epoxymethano PGH_2 (U-46619, 1 μM) was then added to establish the tissue maximum response. This procedure is termed the 'single + maximum dose method'.

Platelet activation

The preparation of human platelets for shape change/aggregation measurements has been described previously by us (Armstrong *et al.*, 1985). In this single wash procedure the platelet pellet from the centrifugation of platelet-rich plasma was suspended in Ca^{2+}-free Krebs solution (composition as above) and maintained at 37°C. The plasma protein concentration was about 1 mg ml^{-1} (from u.v. absorbance at 280 nm). Additional washing steps designed to remove the remaining plasma protein tend to reduce the sensitivity of the platelets to aggregating agents.

Shape change and aggregation were measured with a modified Cary 118C spectrophotometer (incident light wavelength = 600 nm). Each cuvette, containing 1.0 ml platelet suspension, 1.0 ml Krebs solution and 0.4 ml 0.9% NaCl solution, was held in a heated jacket at 37°C and stirring was achieved with a stainless steel rod revolving at 1000 r.p.m. Aggregating agents were added in 50 or 100 μl of 0.9% NaCl solution.

Ligand binding

Inhibition of the binding of radio iodinated 13-aza-13,14-dihydro-16-(p-hydroxy-m-iodophenyl)-ω-tetranor PTA_2 ([^{125}I]-PTA-OH) to washed human platelets was measured essentially according to the method of Narumiya *et al.* (1986). The platelet pellet obtained as described above was suspended in assay buffer (100 mM NaCl, 5 mM dextrose, 1 μM indomethacin and 50 mM Tris-HCl, pH 7.4) and PGI_2 addition, centrifugation and resuspension of the pellet was repeated. The final incubation mixture (200 μl in 1.5 ml Eppendorf tubes) contained about 5×10^7 platelets, 0.1 nM [^{125}I]-PTA-OH (75 TBq $mmol^{-1}$, Amersham), 4 nM cicaprost (Stürzebecher *et al.*, 1986) and a variable concentration of the displacing agent. After incubation for 30 min at 37°C, the tubes were centrifuged at 16,000 *g* for 1 min and then transferred onto ice. The supernatant was rapidly removed by tapping onto absorbant paper and the pellet was washed with 1 ml of ice-cold assay buffer. Radioactivity in each pellet was measured with a LKB Universal Gamma Counter.

Each treatment was run in triplicate and non-specific binding was determined with 1 μM ONO 11120 (13-aza-13,14-dihydro-16-phenyl-ω-tetranor PTA_2), a close analogue of the radioligand (Katsura *et al.*, 1983). Inhibition curves for each prostanoid were obtained on platelets from four separate donors. IC_{50} values correspond to K_i values since the radioligand concentration (0.1 nM) is much less than the K_d of the radioligand (20 nM) (Narumiya *et al.*, 1986).

Partition coefficients and h.p.l.c. retention volumes

Compounds were partitioned between 5 ml of 0.1 M $NaHPO_4/K_2HPO_4$ buffer pH 7.4 and 5 ml of chloroform. Brief centrifugation was performed to reduce the contamination of each phase through emulsification. The concentration in each phase was determined by u.v. spectroscopy or gas chromatography after suitable derivatisation. In the case of EP 031 and EP 092 the concentration remaining in the aqueous phase was insufficient for accurate measurement. The partition was therefore conducted between 0.1 M $NaHCO_3/Na_2CO_3$ buffer pH 9.0 and chloroform and the partition coefficient corrected to

pH 7.4, by use of the Henderson-Hasselbach equation and assuming a pKa of 5.0 for each compound.

As a corroborative measure, retention volumes for the chromophore-containing compounds was determined on a reversed-phase h.p.l.c. system. The octadecylsilane-bonded column (Partisil PXS 10/25 ODS, Whatman) was equilibrated with methanol/water/acetic acid 60:40:0.1 (by vol.) at a flow rate of 1 ml min^{-1}. Retention volumes (expressed as % of column volume) were BM 13177 (4-[2'-benzenesulphonamido ethyl)-phenoxy acetic acid) 97, 16-*p*-fluorophenoxy-ω-tetranor $PGF_{2\alpha}$ 128, 16-*p*-chlorophenoxy-ω-tetranor-11-deoxy $PGF_{2\alpha}$ 188, EP 171 189, EP 092 488 and EP 031 517.

Compounds

The following compounds were prepared in our laboratory: (*rac*)9α,11α-epoxy-10a-homo-15S-hydroxy-prosta-5Z,13E-dienoic acid (9,11-endoxy-10a-homo PGH_2) and its 16-*p*-fluorophenoxy-ω-tetranor derivative (EP 171), (*rac*)9α,11α-ethano-15S-hydroxy-16-*p*-fluorophenoxy-ω-tetranor-prosta-5Z,13E-dienoic acid (EP 031), (*rac*)9α,11α-ethano-1-methyl-13(N-phenylthio-carbamoyl)hydrazono-ω-heptanor-prosta-5Z-enoic acid (EP 092) and (*nat*)16-*p*-fluorophenoxy ω-tetranor $PGF_{2\alpha}$.

STA_2 (11a-carba-9,11-thia TXA_2) and ONO 11120 were gifts from the ONO Company, Japan. BM 13177 was a gift from Boehringer – Mannheim, W. Germany, misoprostol a gift from Searle Ltd., U.S.A. and GR 32191 (1α-(6'-carboxyhex-3'Z-enyl)-2β-(N-piperidino)-3α-hydroxy-5α-(4''-biphenylyl methoxy)-cyclopentane) a gift from Glaxo, U.K. 11,9-Epoxymethano PGH_2 (U-46619) was purchased from Upjohn Diagnostics, U.S.A.

SC 19220 (10-(acetyl hydrazino carbonyl)-8-chloro-10,11-dihydrodibenz (b,f)(1,4) oxazepine) and SC 25191 (10-(n-butyryl hydrazino carbonyl)-8-chloro-10,11-dihydrodibenz (b,f)(1,4) oxazepine) were gifts from Searle, U.S.A. They were added to the organ bath dissolved in ethanol such that the final ethanol concentration in the organ bath was 18 mM when the concentrations of the blockers were 30 and 10 μM respectively.

Results

Actions at TP-receptors in smooth muscle

The potency of EP 171 was compared with that of U-46619, the most commonly used standard agonist for TP-receptor studies, on six isolated smooth muscle preparations: rabbit aorta, rat aorta, dog saphenous vein, pig pulmonary artery, guinea-pig trachea (all ring preparations) and the bullock iris sphincter (two strips obtained from the one eye). The mean EC_{50} value for U-46619 lay between 4 and 12 nM on each preparation. EP 171 was highly potent and produced contractile effects at concentrations of 0.1 nM and less. To our surprise its rates of onset and offset of action were comparable to or even slower than those of EP 031. Indeed it was only on the most rapidly responding preparation, the pig pulmonary artery, that concentration-response relationships could be obtained by cumulative addition of EP 171 doses. The results, shown in Figure 2, indicate that EP 171 is about 100 times more potent than U-46619, about 50 times more potent than its parent 9,11-endoxy-10a-homo PGH_2 and about 20 times more potent than STA_2, a close structural analogue of TXA_2 (Katsura *et al.*, 1983).

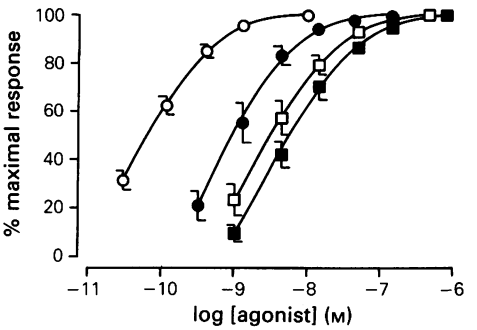

Figure 2 Log concentration-response curves for contractile action of EP 171 (○), STA_2 (●), 9,11-endoxy-10a-homo PGH_2 (□) and U-46619 (■) on the pig isolated pulmonary artery. Mean responses are derived from experiments on 4 preparations each from a different pig; vertical bars show s.e.mean.

On the other five smooth muscle preparations the onset of action of EP 171 was so slow that it was necessary to employ the single + maximum dose technique (Jones *et al.*, 1982) for the construction of concentration-response relationships. In brief, preparations which responded reproducibly to U-46619 were exposed to a single concentration of EP 171 for 100–300 min before a maximum dose of U-46619 was added. Each preparation yields a single data point and 15–20 data points are required to give an accurate log concentration-response curve for EP 171. EP 171 was a full agonist on each of the five preparations; EC_{50} values are given in Table 1. An equipotent molar ratio (e.p.m.r., U-46619 = 1.0) for EP 171 was obtained from each preparation where the EP 171 response fell between 20 and 80% of the

Table 1 Agonist potencies of EP 171, STA_2 9,11-endoxy-10a-homo PGH_2 and U-46619 at TP-receptors in smooth muscle and human platelets

Preparation	*EC_{50} for EP 171* (pM)	*Equipotent molar ratio* (± s.e.mean, U-46619 = 1.0) *EP 171*	*STA_2*	*9,11-Endoxy-10a-homo PGH_2*
Rabbit aorta	138	0.0136 ± 0.0025 (8)*	0.23 ± 0.06 (4)	0.62 ± 0.08 (4)
Rat aorta	45	0.0094 ± 0.0006 (7)*	0.15 ± 0.02 (4)	0.63 ± 0.07 (4)
Pig pulmonary artery	70	0.0093 ± 0.0008 (4)	0.21 ± 0.03 (4)	0.50 ± 0.06 (4)
Dog saphenous vein	120	0.0302 ± 0.0034 (11)*	0.30 ± 0.05 (4)	0.69 ± 0.05 (4)
Guinea-pig trachea	57	0.0096 ± 0.0012 (9)*	0.35 ± 0.04 (4)	0.44 ± 0.04 (4)
Bullock iris sphincter	72	0.0060 ± 0.0005 (5)*	0.080 ± 0.002 (4)	0.38 ± 0.02 (4)
Human platelets shape change	51	0.0110 ± 0.0020 (6)	—	—
aggregation	—	—	0.30 ± 0.05 (4)†	0.96 ± 0.05 (4)†

Values in parentheses refer to the number of preparations used.
* Single + maximum dose method was employed.
† Data from Jones *et al.*, 1987.

maximum response and a comparison could therefore be made with the corresponding U-46619 log concentration-response curve (Table 1). EP 171 is 33–167 times more potent than U-46619, 23–67 times more potent than 9,11-endoxy-10a-homo PGH_2 and 10–40 times more potent than STA_2.

Submaximal (<80%) responses to EP 171 on all six preparations were completely inhibited by the TP-receptor antagonists EP 092 (1 μM) (Armstrong *et al.*, 1985) and BM 13177 (30 μM) (Patscheke & Stegmeier, 1984). It is obviously difficult when using EP 171 as agonist to obtain accurate pA_2 values for TP-receptor antagonists by the Schild procedure (Arunlakshana & Schild, 1959). However, in the case of the pig pulmonary artery an 80% maximum response to EP 171 will return to less than 5% of maximum after 4 h continuous displacement of the bathing fluid and this allows a second cumulative series of doses to be applied. It was therefore possible to obtain a dose-ratio for antagonism of EP 171 action on one preparation and a corresponding measure of the change in sensitivity to EP 171 with time on a second (control) preparation. Using preparations from four separate pigs, the mean dose ratio for 0.25 μM EP 092 versus EP 171 was 32.0 ± 4.6 (s.e.mean) and the corresponding control mean dose-ratio 1.12 ± 0.08. The dose-ratio for 0.25 μM EP 092 versus U-46619 determined on parallel preparations from the four pigs was 36.2 ± 6.1 and the control value 1.00 ± 0.07. Using the Schild equation, a pA_2 value of 8.09 was obtained for the EP 092/EP 171 interaction and 8.15 for the EP 092/U-46619 interaction. These values (although obtained with a single antagonist concentration) are close to previous values obtained for the EP 092/U-46619 interactions at TP receptors on dog saphenous vein (7.94) and guinea-pig trachea (7.96) (Armstrong *et al.*, 1985).

Actions at FP- and EP_1-receptors in smooth muscle

The dog iris sphincter contracts to low concentrations of $PGF_{2\alpha}$ (mean EC_{50} in these experiments = 3 nM) and appears to contain only FP-receptors (Dong & Jones, 1982; Kennedy *et al.*, 1983). Responses to $PGF_{2\alpha}$ were rapid in onset and offset but tended to fade if greater than 75% of the maximum and the contact time was prolonged. EP 171, STA_2 and U-46619 behaved as full agonists. The e.p.m.rs ($PGF_{2\alpha}$ = 1.0) are given in Table 2. The action of EP 171 was slightly slower in onset and offset than $PGF_{2\alpha}$ but similar to one of the most potent FP-receptor agonists (*rac*)16-*m*-trifluoromethylphenoxy-ω-tetranor $PGF_{2\alpha}$ (ICI 81008) (Table 2). EP 092 (3 μM) had little blocking action on either $PGF_{2\alpha}$ or EP 171 (dose ratio = 1.3–1.6, $n = 3$ in each case).

Although the guinea-pig trachea and the bullock iris sphincter contain EP_1-receptors mediating muscle contraction, estimation of the EP_1-receptor agonist potency of EP 171 on these preparations would require a very effective (and possibly irreversible) blockade of TP-receptors. We therefore decided to examine the activity of EP 171 on the guinea-pig stomach fundus, a EP_1-receptor preparation thought to be devoid of TP-receptors (Kennedy *et al.*, 1983).

Table 2 Potency and specificity of prostanoid agonists

Agonist	*Equipotent molar ratio* (± s.e.mean) *Dog iris* ($PGF_{2\alpha} = 1.0$)	*Guinea-pig fundus*† ($PGE_2 = 1.0$)	*Ratio of EC_{50} values** *Dog iris/rat aorta*	*Guinea-pig fundus*†*/rat aorta*
EP 171	44 ± 4 (6)	268 ± 32 (4)	3100	14000
STA_2	151 ± 30 (4)	243 ± 53 (4)	630	1700
U-46619	196 ± 12 (4)	3230 ± 520 (4)	130	1500
ICI 81008	0.15 ± 0.05 (4)	>1000 (4)	0.00015	—
16,16-dimethyl PGE_2	>100 (4)	0.115 ± 0.015 (4)	—	0.0035

Values in parentheses are numbers of observations.
* A high ratio indicates specificity for TP-receptors over FP-receptors (dog iris) or EP_1-receptors (guinea-pig fundus).
† 3 μM EP 092 present.

The fundus was highly sensitive to the standard agonist PGE_2. On 12 preparations the shape of the PGE_2 log concentration-response curve corresponded to a single sigmoid (EC_{50} = 1.2–5.2 nM). EP 171 however showed a biphasic log concentration-response curve (Figure 3a). The more potent contractile component covered the range 0.1–10 nM and had a maximum of about 35% of the PGE_2 maximum (all % maximum values relate to the PGE_2 maximum). The EP 171 responses were slow in onset and offset and to ensure that a build up of metabolites in the organ bath did not augment the responses, the EP 171 solution was continuously replaced using a roller pump. Above 10 nM, EP 171 added as discrete doses elicited contractile responses which quickly reached a stable level (~5 min): on wash-out of the organ bath these responses decayed rapidly, but only to the 30–40% maximum response level. It seemed likely that EP 171 was specifically activating TP-receptors at concentrations of 10 nM and below, whilst at higher concentrations both TP- and EP_1-receptor activation contributed to the contractile response. The following observations support this hypothesis.

The EP_1-receptor antagonist SC 25191 (10 μM) (Sanner *et al.*, 1973) did not affect the more potent component of EP 171 action but reduced the less potent component (Figure 3a). The rightward shift of the EP 171 curve at the 50% maximal response level is similar to the shift of the PGE_2 curve due to the presence of 10 μM SC 25191: PGE_2 dose-ratio = 13.6 ± 1.4 (n = 5). In contrast, EP 092 (3 μM) did not affect responses to PGE_2 (dose ratio = 1.0–1.5, n = 4), but abolished responses to concentrations of EP 171 up to 10 nM (Figure 3a, b). EP 092 also abolished responses to STA_2 and U-46619 provided these were less than 30% of the PGE_2 maximum (Figure 3a, b). Within the 5–25% maximum range, EP 171 was about 30 and 70 times more potent than STA_2 and U-46619 respectively; these values are similar to relative potencies found on the other preparations containing TP-receptors (Table 1). In the experiments of Kennedy *et al.* (1983) a 300 μM concentration of SC 19220, a close analogue of SC25191, abolished responses to U-46619, leading to the suggestion that the agonist action was entirely due to activation of EP_1-receptors. We feel that SC 19220 may not be specific at this high concentration; in our hands 30 μM SC 19220 behaved similarly to 10 μM SC 25191, blocking PGE_2 responses but not responses to EP 171 or U-46619 when these were less than 30% of the PGE_2 maximum.

Equipotent molar ratios (Table 2) were calculated from log concentration-response curves obtained in the presence of 3 μM EP 092 (Figure 3b). We thought it possible however that the higher concentrations of the three potent TP-receptor agonists might overcome the EP 092 block. As a corroborative measure, therefore, we also obtained relative potencies on EP_1-receptors during continuous near-maximal activation of the thromboxane-sensitive system. For this purpose, the preparations were continuously bathed with 10 nM EP 171 and concentration-response relationships to PGE_2 and the three thromboxane A_2-mimetics determined. Figure 4 shows a typical experiment and Figure 3c the combined data. E.p.m.rs (PGE_2 = 1.0) were calculated at the 70% maximal response level: EP 171 170 ± 22, STA_2 131 ± 8, U-46619 2350 ± 380 (n = 4); the EP 092 blockade would appear to have been adequate.

Activation of human platelets

EP 171 is the most active thromboxane A_2 mimetic with respect to the aggregation of human platelets *in*

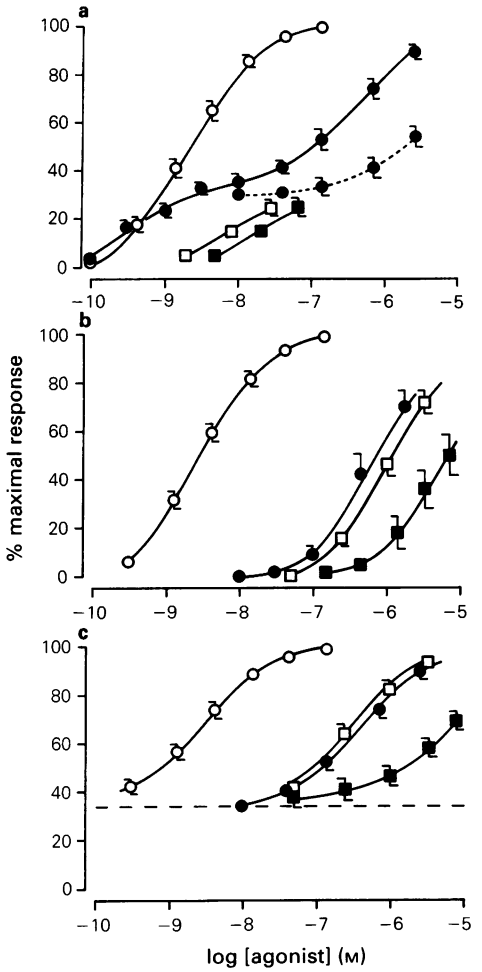

Figure 3 Log concentration-response relationships on the guinea-pig stomach fundus. Mean responses for PGE_2 (○) ($n = 8$–12), EP 171 (●), STA_2 (□) and U-46619 (■) ($n = 4$ for each) are shown; vertical bars show s.e.mean. In the group of experiments depicted in (a) the agonists act alone except for EP 171 in the presence of 10 μM SC 25191 (- - - -). In (b) the TP-receptor antagonist EP 092 (3 μM) was present and in (c) 10 nM EP 171 was present throughout, producing near-maximal activation of the thromboxane-sensitive system.

vitro that we have encountered so far. However, responses to EP 171 were quite unlike those of any other thromboxane A_2 mimetic in that primary reversible aggregation waves were never produced. With low concentrations (0.5–1 nM) the onset of aggregation was delayed such that the full extent of the shape change response was always seen (Figure 5a). Slowly developing aggregation induced by EP 171 showed little tendency to reverse. Increasing the EP 171 concentration led to increasingly faster onset of agrregation such that at 50 nM the EP 171 profile was very similar to that of a maximal irreversible response to U-46619. It was not possible to make accurate potency comparisons when the time courses of the responses to the two agonists were so different.

Shape change responses to EP 171 were also slow in onset compared to those of U-46619 (Figure 5b). However, EP 171 did produce graded stable submaximal responses and this allowed comparison with the standard agonist. EP 171 had an EC_{50} value of about 50 pM and an e.p.m.r. of 0.011 (U-46619 = 1.0) (Table 1). The cyclo-oxygenase inhibitor indomethacin (1 μM) did not influence the profile of activity of EP 171.

Reversal of agonist action by TP-receptor blockade

The observation that the smooth muscle contractile action of EP 171 mediated by TP-receptors was slowly reversed by washing whereas its contractile actions through FP- and EP_1-receptors were quickly reversed suggested to us that the interaction with the TP-receptor, rather than sequestration into lipid, could be the dominant factor in the rate of action of EP 171. We supposed that an agonist with activity at concentrations below 100 pM must have a reasonably low equilibrium dissociation constant (K_d) and, by conventional wisdom, the avid binding would be reflected in a low dissociation rate constant (k_2). It was therefore of interest to determine how quickly a high concentration of a TP-receptor antagonist would reverse an established submaximal response to EP 171 in comparison with less potent TP-receptor agonists.

Measurements of reversal rates on smooth muscle were made on the rabbit aorta and guinea-pig trachea. Each preparation was exposed to a single concentration of agonist sufficient to produce a 50–70% maximum response and the time (t_{on}) for the response to reach 50% of its final level was measured. The preparation was then treated in one of three ways: (a) washed by continuous upward displacement of the bathing fluid, (b) exposed to a TP-receptor antagonist (EP 092 or BM 13177) and (c) exposed to a physiological antagonist (the PGE analogue misoprostol on the guinea-pig trachea and atrial natriuretic peptide, ANP, on the rabbit aorta). The time (t_{off}) for the response to decay to 50% of its original magnitude was measured in each case.

In addition to EP 171, U-46619 and three other 16-*p*-halophenoxy prostanoid agonists, 16-*p*-fluorophenoxy-ω-tetranor $PGF_{2\alpha}$, 16-*p*-chloro-

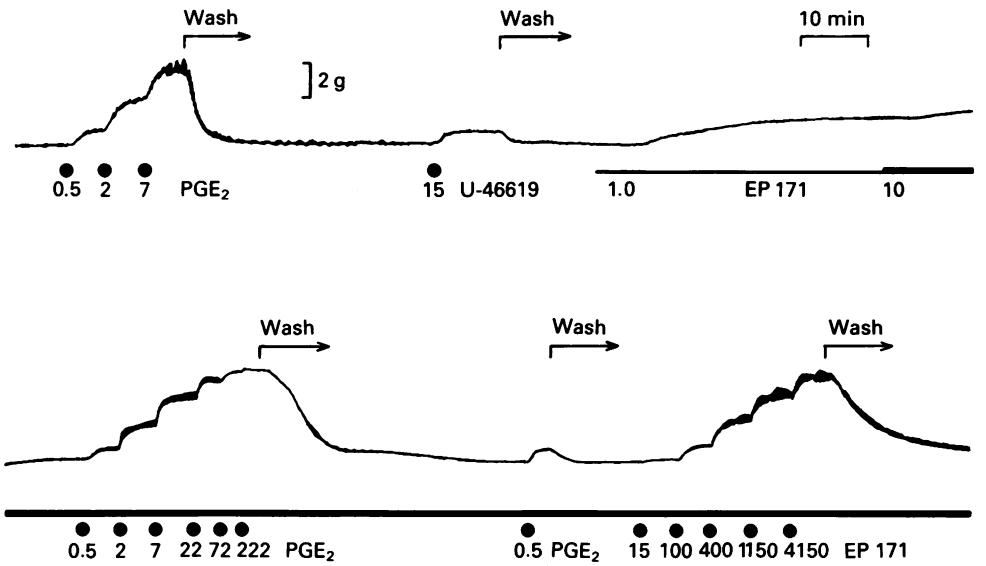

Figure 4 Estimation of the agonist potency of EP 171 at EP_1-receptors in the guinea-pig fundus strip: 1 μM indomethacin and 20 nM atropine were present throughout. EP 171 was present in the bathing fluid as indicated by the solid bars. Cumulative concentrations (nM) of PGE_2 and EP 171 are shown. The single dose of PGE_2 was added to ensure that the preparation had not been desensitized by the preceding high doses of PGE_2.

phenoxy-ω-tetranor-11-deoxy $PGF_{2\alpha}$ and EP 031 (Figure 1) were investigated. On the guinea-pig trachea contractile responses to the two PGF analogues are due to activation of both TP- and EP_1-receptors (Jones *et al.*, 1982) and consequently half-times for these two agonists were measured in the presence of 30 μM SC 19220 (see Dong *et al.*, 1986); this treatment did not affect the potencies or rate profiles of EP 031 and EP 171.

The results are shown in Figure 6a, b. The five agonists have been arranged from left to right in order of increasing potency. For example, the concentrations used to produce matching submaximal responses on the rabbit aorta were typically 15 nM for 16-*p*-fluorophenoxy-ω-tetranor $PGF_{2\alpha}$, 7.5 nM for U-46619, 2.5 nM for 16-p-chlorophenoxy-ω-tetranor-11-deoxy $PGF_{2\alpha}$, 0.6 nM for EP 031 and 0.1 nM for EP 171. There is a reasonably good correlation between agonist potency and both t_{on} and wash-t_{off}. The correlation between lipophilicity and t_{on} and wash-t_{off} is poorer; partition coefficients (PC) between chloroform and pH 7.4 water are given in Table 3. Measurements were also made on a diastereoisomer of EP 171, in which the ω-chain is *cis* to the α-chain and the 15-hydroxyl is β-orientated. This compound is slightly more lipophilic than EP 171 but about 500 times less potent (e.p.m.r. = 4.8 $\pm$ 1.3, $n = 4$; U-46619 = 1.0, guinea-pig trachea). It showed rapid onset/offset characteristics similar to 16-*p*-fluorophenoxy-ω-tetranor $PGF_{2\alpha}$.

The slowest decay of contractile action following addition of the TP-receptor antagonists is seen with EP 171 on both preparations. The similar half-times for the three least potent agonists may reflect the rate at which the antagonist penetrates the tissue and binds to TP-receptors and/or the rate at which the contractile process is reversed once the stimulatory input has been removed. It should be noted that the two antagonists differ in affinity and particularly in lipid solubility. EP 092 has a reasonably high affinity ($pA_2 = 7.26$ and 7.96 for rabbit aorta and guinea-pig trachea respectively) and is highly lipophilic (PC = 1900), whereas BM 13177 is a weaker blocker ($pA_2 = 6.24$ and 6.30 respectively, our results using U-46619 as agonist) and has low lipid solubility (PC = 0.013). Contractions to each of the five agonists were rapidly reversed by the physiological antagonists.

Similar kinetic studies were performed on washed human platelets using shape change (70–80% of maximum) as the response parameter (Figure 6c). However, wash-t_{off} could not be determined since there is no satisfactory method for rapidly removing the agonist from the solution bathing the platelets whilst simultaneously recording light transmission.

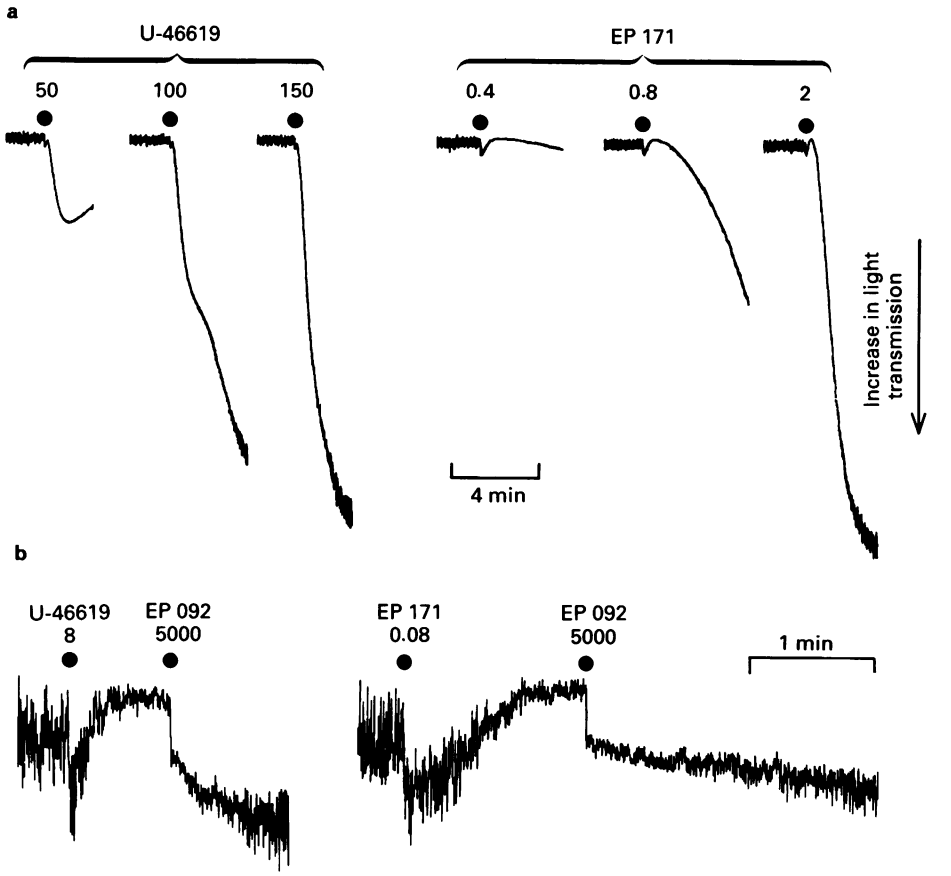

Figure 5 Comparison of the stimulant action of EP 171 and U-46619 on washed human platelets. Light transmission tracings from two experiments. Cuvette concentrations (nM) are indicated. (a) The rapid change in signal strength on addition of drug is due to dilution of the cuvette contents, the upward deflection with loss of oscillations to the platelet shape change and the downward deflection to aggregation. (b) Shape-change responses recorded at higher gain and faster chart speed than in (a). The TP-receptor antagonist EP 092 rapidly reverses the shape change induced by U-46619, but only slowly reverses the EP 171 response.

Reversal by a third TP-receptor antagonist, GR 32191 (Lumley *et al.*, 1987), was also studied. At a concentration of 30 μM, the BM 13177 blockade is surmountable and the U-46619 dose ratio is about 50 ($pA_2 = 6.2$). With 5 μM EP 092 ($pA_2 = 7.9$) and 5 μM GR 32191 ($pA_2 = 8.8$) the U-46619 dose ratio is greater than 300. The five agonists have the same potency ranking on the platelet system as on the smooth muscle preparations: EC_{50} values are given in Table 3. Responses to the three weakest agonists were rapid in onset and also in offset with all three receptor antagonists and with the physiological antagonist PGE_1 (50 nM) (Figure 6c). Responses to EP 031 were slightly slower in onset and offset (except with PGE_1). EP 171 showed the slowest onset and the slowest offset due to receptor blockade (Figure 5b); its rate of reversal with PGE_1 did not differ from that of the other four agonists.

Inhibition of [^{125}I]-PTA-OH binding to washed human platelets

The binding constants (K_i) for the interaction of U-46619 and the four *p*-halophenoxy prostanoids with the human platelet TP-receptor were estimated from concentration-inhibition curves using [^{125}I]-PTA-OH as radioligand (Figure 7 and Table 3). The ranking of K_i values correlates well with the potency of the analogues as inducers of the platelet shape change.

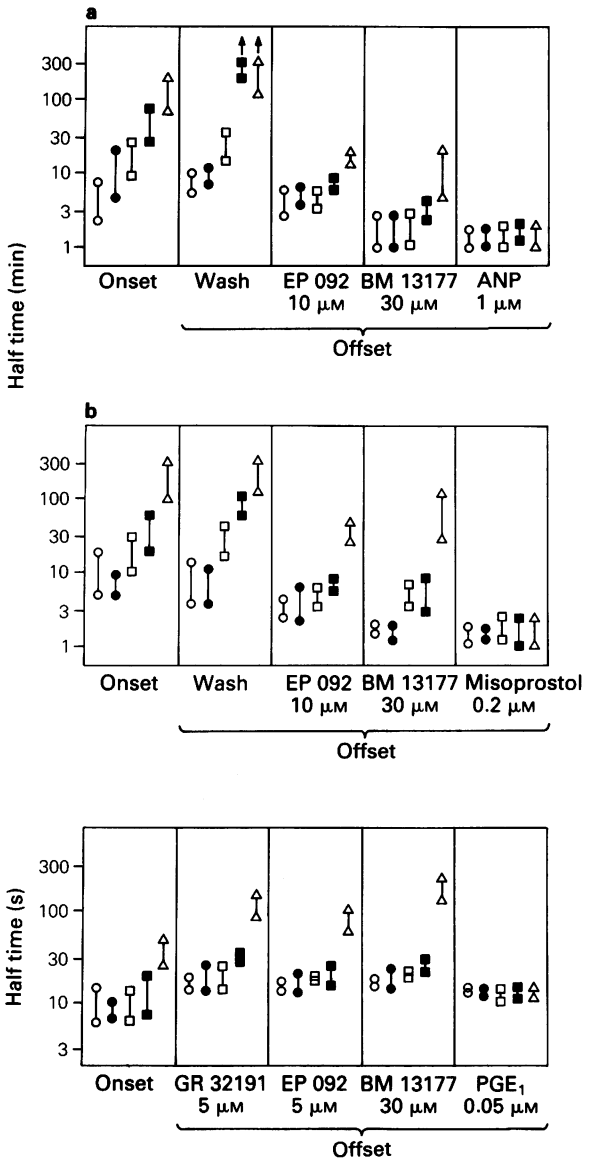

Figure 6 Half times for onset and offset of action at TP-receptors in (a) rabbit aorta, (b) guinea-pig trachea and (c) human platelets (shape change). The agonists are 16-*p*-fluorophenoxy-ω-tetranor $PGF_{2\alpha}$ (○), U-46619 (●), 16-*p*-chlorophenoxy-ω-tetranor-11-deoxy $PGF_{2\alpha}$ (□), EP 031 (■) and EP 171 (△). The ranges for 12–16 separate observations for onset and 4 observations for each offset regime are shown. The arrows in the EP 031 and EP 171 columns for offset due to washing on the rabbit aorta indicate that 2 of the 4 preparations had not relaxed to 50% of the original response level after 300 min of continuous washing. Note the different ordinate scale for the platelet observations.

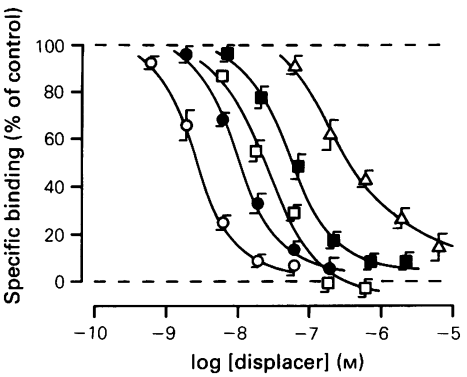

Figure 7 Inhibition of [^{125}I]-PTA-OH binding to intact human platelets by EP 171 (○), EP 031 (●), 16-*p*-chlorophenoxy-ω-tetranor-11-deoxy $PGF_{2\alpha}$ (□), U-46619 (■) and 16-*p*-fluorophenoxy-ω-tetranor $PGF_{2\alpha}$ (△). Means ($n = 4$) are shown; vertical bars show s.e.mean.

Discussion

Comparison of EP 171 with its natural ω-chain parent convincingly demonstrates the ability of the 16-*p*-fluorophenoxy substituent to enhance agonist potency at TP-receptors, even when the parent compound itself is a potent full agonist. Indeed EP 171 is the most potent thromboxane A_2 mimetic reported so far and its EC_{50} values of 45–138 pM compare favourably with those of the most potent agonists on other receptor systems.

When agonist activity at TP-receptors is compared with that at FP- or EP_1-receptors, EP 171 has a higher specificity as a TP-receptor agonist than either U-46619 or STA_2. This is shown in Table 2, where the ratio of EC_{50} values on a TP-receptor preparation (rat aorta) and either a FP-receptor (dog iris) or a EP_1-receptor (guinea-pig fundus) preparation has been calculated for each agonist. The larger the ratio the greater is the specificity as a TP-receptor agonist. For the purpose of distinguishing between TP- and FP-receptors the agonist combination of EP 171 and ICI 81008 could be very useful. In the case of TP-receptors and EP_1-receptors EP 171 in combination with 16,16-dimethyl PGE_2 would suffice. Although we suspect that the development of TP-receptor agonists with greater potency than EP 171 will be difficult, increasing the specificity of action may be possible by a reduction in potency at other prostanoid receptors. EP 171 does after all activate FP- and EP_1-receptors at concentrations of 50–150 nM and could hardly be classed as a low potency agonist.

The relative potencies (and to a large extent the absolute potencies) of EP 171, STA_2, 9,11-endoxy-

Table 3 Comparison of U-46619 and *p*-halophenoxy prostanoids in terms of platelet activation, inhibition of [^{125}I]-PTA-OH binding and partition coefficient

	Human platelets		
Agonist	*EC_{50} for shape change* (nM)	*Inhibition of [^{125}I]-PTA-OH binding: K_i* (nM)	*Partition coefficient: $CHCl_3/H_2O$, pH 7.4*
EP 171	0.065 ± 0.011	2.9 ± 0.4	10
EP 031	0.55 ± 0.08	11.0 ± 1.0	1150
16-*p*-Chlorophenoxy-ω-tetranor-11-deoxy $PGF_{2\alpha}$	2.7 ± 0.6	23 ± 4	3.0
U-46619	5.4 ± 0.9	69 ± 14	15
16-*p*-Fluorophenoxy ω-tetranor $PGF_{2\alpha}$	27 ± 8	440 ± 32	0.029

Values on human platelets are means ± s.e.mean of 4 determinations.

10a-homo PGH_2 and U-46619 as TP-receptor agonists on the eight preparations studied here are quite similar (guinea-pig fundus is included). When these results are combined with our earlier structure-activity data (Jones *et al.*, 1982; Armstrong *et al.*, 1985) there appears to be no obvious division of the TP-receptor into subtypes on the basis of different agonist rankings.

The slow onset and offset of EP 171 action on TP-receptor preparations rules out any possibility of this compound replacing U-46619 as a standard agonist. Indeed, of the thromboxane A_2 mimetics we have examined, U-46619 has the most favourable combination of potency, specificity and rapidity of action. Nevertheless the mechanisms underlying the slow kinetics of EP 171 action are of considerable interest. The ligand binding experiments indicate a K_i for EP 171 of 2.9 nM. The true K_i may be somewhat lower for two reasons. First, EP 171 is racemic and it is possible that only one enantiomer competes effectively with the radioligand in the binding assay. In the case of the parent compound, the isomer formally related to PGH_2/TXA_2 is about 100 times more potent than its mirror image as an activator of human platelets (Sprague *et al.*, 1985). Secondly, some reduction in the initial free EP 171 concentration may occur due to TP-receptor binding. Assuming that each platelet possesses 1700 TP-receptors (Armstrong *et al.*, 1983) the concentration of TP-receptors will be about 0.7 nM. The IC_{50} value of 1.45 nM for the active species of EP 171 would therefore be reduced to about 1 nM (the initial free concentration of the less potent competing ligands is likely to be reduced by less than 10%). Could an equilibrium dissociation constant of about 1 nM account for the slow kinetics of EP 171 action? Let us deal with the organisationally simpler platelet system first. The antagonist-t_{off} value for EP 171 of about 110 s (Figure 6c) will reduce to 70 s when EP 171 concentration is substituted for response magnitude (a reduction in shape change response from 80% to 40% of maximum corresponds to a 3.0 fold reduction in EP 171 concentration). A t_{off} of 70 s corresponds to a dissociation rate constant (k_2) for the EP 171/TP-receptor interation of 0.01 s^{-1}, and an association rate constant (k_1) of 1×10^7 $M^{-1}s^{-1}$ derives from a K_d of 1 nM. The rate constant for receptor occupation (assuming no reduction in initial free ligand concentration) is given by $k_1[A] + k_2$ (Paton & Rang, 1965). At low concentrations of EP 171 (0.1 nM or less) the term $k_1[A]$ becomes negligible and the rate of receptor occupation is dependent only on k_2. The half-times for onset and offset of EP 171 action should therefore be very similar. However, t_{on} for EP 171 is clearly less than t_{off} (Figure 6c). The discrepancy may lie in an overestimation of t_{off}, since we have observed that the rate of reversal of shape change produced by a fast acting agonist (e.g. U-46619) declines if the agonist is allowed more than 3 min contact before the TP-receptor antagonist is added. In addition, the shape change becomes more difficult to reverse if the platelets are used more than 2 h after PGI_2 decay. It is possible that the intracellular events associated with TP-receptor agonist-induced shape change tend to lose some of their ability to reverse during prolonged agonist contact.

The suggested magnitude of k_2 for the EP 171/TP-receptor interaction is small for an agonist/receptor interaction (see Ginneken, 1977) but not for an antagonist-receptor interaction. For example, the best non-ligand binding estimate of the rate constants for the atropine/muscarinic receptor interaction is probably that of Bolton (1977) using iontophoretic application of drugs to guinea-pig taenia coli: $k_1 = 1 \times 10^7 M^{-1}s^{-1}$, $k_2 = 0.011 s^{-1}$ and $K_d = 1.1$ nM. Ligand binding experiments with radiolabelled EP 171 could provide information on the magnitudes of k_1 and k_2. However, the radioligand would require to have a high specific activity since the free ligand concentration would be in the 1–10 nM range. The simple expedient of partially

replacing the 15β-proton with tritium (Armstrong *et al.*, 1983) may not be sufficient for this purpose.

The t_{on} and t_{off} values obtained on the rabbit aorta and guinea-pig trachea are considerably greater than those found for the human platelet system, although similar trends are seen. To obtain half-times related to agonist concentration the values shown in Figure 6a, b must be divided by 1.55 for both rabbit aorta and guinea-pig trachea. It seems unlikely that half-times in excess of 60 min simply reflect the rate constant for occupation of TP-receptors by EP 171 and one must look for mechanisms whereby the access of agonist to cells in the centre of the tissue is restricted. Factors potentially controlling the rate of drug action in densely packed tissues such as smooth muscle or nerves fibres have been considered by several workers (Furchgott, 1964; Rang, 1966; Colquhoun & Ritchie, 1972; Colquhoun *et al.*, 1972). In the exact diffusion equation approach of Colquhoun *et al.* (1972) it is suggested that diffusion of the drug in and out of the tissue is rate-limiting, with equilibration at receptors being relatively rapid. Diffusion of drug through the extracellular fluid (e.c.f.) is slowed by one or more cell membrane-based processes which abstract drug molecules from the e.c.f. These processes could include saturable binding to cell surface receptors, active uptake into the cell and passive transfer into lipophilic areas of the cell. In the case of saturable binding the diffusion coefficient is reduced by a factor $1 + M/K_d V$, when the drug concentration is much smaller than K_d (M is the binding capacity and V the volume of the extracellular space). For tetrodotoxin (TTX) binding ($K_d = 3$ nM) to the desheathed rabbit vagus nerve it was suggested that the above mechanism could slow the rate of equilibration of TTX by more than a thousand fold. If the K_d of EP 171 binding to TP-receptors in smooth muscle is similar to that determined for human platelets, then this mechanism rather than sequestration into lipid control also account for the slow kinetics of EP 171 action; the parameter we are lacking to complete the comparison with the TTX data is the TP-receptor binding capacity of the smooth muscle systems. It is of interest that in a quite distinct system, steroid inotropic activity on guinea-pig papillary muscle, the rate of onset of action decreased as potency of the analogue increased (Ebner, 1987). It was also shown that the rate of onset decreased as aqueous diffusion distance (muscle diameter) increased, and that this was most marked with highly potent steroids (e.g. digitoxin). The author favoured reduced diffusion through the e.c.f. due to binding to steroid receptors rather than lipophilic uptake into the myocytes to account for these findings.

On the rabbit aorta the half-time for EP 171 offset due to TP-receptor blockade was much smaller than the half-time for offset due to washing (Figure 6a). However, on the guinea-pig trachea the difference was less striking (Figure 6b) and the EP 171 contraction decayed slowly ($t_{off} \sim 40$ min) even with a high concentration (10 μM) of EP 092 (predicted dose-ratio = 1000). More limited observations with two other potent TP-receptor antagonists, GR 32191 (10 μM) and ONO 11120 (5 μM), gave similar profiles. A somewhat different situation from that found with EP 171 on the guinea-pig trachea is seen with salmeterol, a salbutamol analogue in which a 11-phenyl-6-oxaundecyl group replaces the *t*-butyl group (Bradshaw *et al.*, 1987). Salmeterol activates β_2-adrenoceptors to produce tracheal relaxation which is very slowly reversed by washing. However, addition of the β-blocker propranolol rapidly reverses the inhibition (Ball *et al.*, 1987). Further investigations are obviously required to ascertain whether the slow offset of EP 171 action due to receptor blockade can be mainly attributed to its high affinity for the TP-receptor.

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Arterial catecholamine levels in morphine-treated rats subjected to sympathetic nerve stimulation

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1 The effect of acute or chronic morphine treatment on the changes in arterial noradrenaline and adrenaline levels in response to sympathetic nerve stimulation was studied in rats.

2 Rats which had been chronically treated with morphine in their drinking fluid for 21 days were shown to be morphine-tolerant, as revealed by the tail-immersion test for analgesia.

3 It was found that animals given either acute or chronic morphine treatment had similar basal concentrations of arterial catecholamines to their controls.

4 Sympathetic nerve stimulation produced significant increases in arterial noradrenaline and adrenaline levels in both the control and morphine-treated animals. However, the degree of arterial noradrenaline elevation was significantly less in morphine-tolerant animals.

5 This phenomenon was not observed in acutely morphine-treated rats or at 2 weeks following opiate withdrawal in animals which had been treated previously with morphine for 3 weeks.

6 The findings suggest that chronic morphine treatment in rats not only leads to opiate tolerance but also reduces catecholamine release in response to sympathetic nerve stimulation.

Introduction

Although it has been postulated that morphine and many other opioids may influence the release of neurotransmitters in opiate-sensitive neuronal tissues, their mechanism of action is not clear. In most studies, their effects on the central or peripheral nervous systems were deduced from isolated tissue preparations or cell cultures (Snyder, 1978; Terenius, 1978; Beaumont & Hughes, 1979). There are relatively few studies on peripheral sympathetic function in whole animals which have been acutely or chronically treated with morphine (Jurna & Rummel, 1984), or on the profile of catecholamine release following peripheral sympathetic nerve stimulation in morphine-treated animals (Wada *et al.*, 1938; Radosevich *et al.*, 1984).

Prolonged morphine administration is known to produce physical dependence and tolerance. It has been reported that, depending on the species and opioid-exposure time, experimental animals also exhibit other physiological alterations after chronic opiate treatment which are different from those observed after acute treatment (Schulz & Goldstein, 1973; Kromer & Steigemann, 1982; Rae & De Moraes, 1983; Dai *et al.*, 1985; Martin & Takemori, 1986). Recently, it has been observed that chronically morphine-treated rats exhibit a significantly lower incidence and delayed onset of early ventricular arrhythmias, and a marked decline in ventricular noradrenaline concentration, during acute myocardial ischaemia (Chan *et al.*, 1987; Ko *et al.*, 1988). A previous study has shown that the cardiovascular responses to sympathetic nerve stimulation are significantly reduced following prolonged morphine administration (Leung *et al.*, 1986b). Thus, it is possible that chronic opiate treatment may influence the alterations in arterial catecholamine levels elicited by sympathetic nerve stimulation.

The present study examines the effects of sympathetic nerve stimulation on arterial noradrenaline (NA) and adrenaline (Ad) levels in acutely or chronically morphine-treated rats, and in a group of animals from which morphine had been withdrawn for 2 weeks after receiving morphine for 3 weeks.

Methods

Animals

Female Sprague–Dawley rats, weighing 140–160 g, were fed a standard rat pellet diet (Ralston Purina

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Co., U.S.A.) and given tap water to drink *ad libitum*. They were housed in a temperature (23 ± 1°C)- and humidity (65 ± 5%)-controlled room, and were exposed to a 12 h light/12 h dark cycle.

Morphine treatment

In the studies on the effects of acute morphine treatment, animals were injected i.p. with an analgesic dose of morphine sulphate (Macfarlan Smith, Middlesex) 16 mg kg^{-1}, expressed as the salt, 15 min before sympathetic nerve stimulation. Control rats were given a solution of 0·9% w/v NaCl (saline) 1 ml kg^{-1} by the same route and at the same time.

In the case of chronic morphine treatment, the opiate was administered in the drinking fluid for 21 days. A solution of 5% w/v sucrose in ordinary tap water was used as drinking fluid in order to mask the bitter taste of morphine (Dai *et al.*, 1984). Morphine sulphate, weight expressed as the salt, was given in increasing concentrations (48 h apart) of 0·1, 0·2, 0·3 and finally 0·4 mg ml^{-1}. The rats continued to receive the final concentration until the end of the 3 week experimental period (Dai *et al.*, 1984). Control animals were given a 5% sucrose solution to drink. All drinking solutions were provided *ad libitum*. Daily intakes of fluid and morphine were determined as previously described (Dai *et al.*, 1984; Leung *et al.*, 1986a,c). The average daily intake of morphine sulphate 0·4 mg ml^{-1} solution (from day 7 onwards) was 60 ml per rat; the estimated daily intake of morphine sulphate during this period was, therefore, about 120 mg kg^{-1}. The average daily consumption of 5% sucrose solution by the control group during this period was about 62 ml per rat.

For observing recovery from the effects of chronic opiate treatment, a group of rats was first treated chronically with morphine solution for 21 days as described above. The opiate was withdrawn from the drinking water of these animals from day 22; they were given only a sucrose solution to drink for a further 2 weeks. At the end of this 2 week withdrawal period, the degree of opiate tolerance and the arterial level of catecholamines in response to sympathetic nerve excitation were examined. Control animals received sucrose solution for the whole 5 week experimental period.

Sympathetic nerve stimulation

Rats, anaesthetized with diethyl ether (BDH), were pithed through the right orbit (Gillespie *et al.*, 1970; Leung *et al.*, 1986b) and then artificially ventilated (82 srokes min^{-1}, 1 ml 100 g^{-1}) by a respirator (Palmer, U.K.). The pithing rod comprised two parts: an external movable 6 cm long trocar and an internal 13 cm stainless steel electrode. The whole electrode, except for the terminal 4.5 cm, was enclosed in an insulating teflon sleeve. The exposed part of the electrode was used to stimulate the autonomic preganglionic fibres between T_1–T_{12} in the spinal canal. The indifferent needle electrode was inserted into the subcutaneous tissues on the back of the animal, approximately opposite and parallel to the terminal end of the stimulating electrode. An abdominal laparotomy was subsequently performed and the descending aorta cannulated proximally with a heparinised polyethylene tube (internal diameter 1 mm) for blood sampling. The left jugular vein was also cannulated with a polyethylene tube (internal diameter 0·5 mm) for drug injection. After the cannulation procedures were completed, the rats were left undisturbed for 15 min before sympathetic nerve stimulation was started. Electrical impulses (70 V, 10 Hz, 0·5 ms pulse width), provided by a square-wave stimulator (Palmer, C.V.P. model), were given for 5 s. Immediately after stimulation, 6–7 ml of blood was collected from the aorta into a heparinised test tube containing 4.5 mg sodium metabisulphite (M&B) which acted as an antioxidant (Taylor and Simpkins, 1984); the collection time did not exceed 1.5 min. Contraction of skeletal muscle during electrical stimulation was reduced by i.v. treatment with gallamine triethiodide (Sigma) 5 mg kg^{-1}, expressed as the salt; the effects of possible stimulation of the parasympathetic nerves were minimised by i.p. injection of atropine sulphate (E. Merck) 1 mg kg^{-1}, expressed as the salt. Both cholinoceptor blocking drugs were given 15 min before sympathetic nerve stimulation was started. The exact position of the stimulating electrode in each rat was confirmed by *post-mortem* examination. A separate group of rats was subjected to the same pithing procedure but no electrical stimulation was delivered through the pithing electrode (pithed control). All animals were killed after collection of the aortic blood samples.

Measurement of arterial catecholamines

Arterial plasma NA and Ad were extracted and purified with aluminium oxide (BDH), and measured by high performance liquid chromatography (h.p.l.c.) together with electrochemical detection (Causon *et al.*, 1981; Eriksson & Persson, 1982; Weicker *et al.*, 1984). The mobile phase was prepared as described by Eriksson & Persson (1982). The h.p.l.c. system consisted of a pump (Altex 100A) and a stainless-steel column (Waters, Bondapak C-18) which were connected to a LC-4B electrochemical detector (BAS) and a paper recorder (Kipp and Zonen BD41). Standards used were adrenaline (Codex), (−)-noradrenaline (Sigma) and dihydroxybenzylamine (DHBA) (Sigma). Calibration was achieved during each measurement by comparing the ratios of the

catecholamine/DHBA peak heights (Causon *et al.*, 1981; Eriksson & Persson, 1982; Weicker *et al.*, 1984).

Morphine tolerance

Development or disappearance of tolerance to the analgesic action of morphine was examined in separate groups of chronically morphine-treated rats or of post opiate-withdrawal animals by employing the tail-immersion test for analgesia, as previously described (Dai *et al.*, 1984). Twenty min after i.p. injection of morphine sulphate 2, 4, 8 or 16 mg kg^{-1}, or an equivalent volume of saline, the terminal 3 cm of the rat tails were immersed in hot water (55°C). The reaction time was taken as the period elapsing between immersion and flicking of the tails to avoid the painful simulus. This test was performed on day 21 of the 3 week morphine treatment period or at 2 weeks following cessation of the 3 week opiate treatment.

Statistical analysis

Data are expressed as means ± s.e.mean and analysed by Student's unpaired *t* test.

Results

Chromatographic patterns of arterial catecholamines

The sequence of elution in the h.p.l.c. was NA at 5 min 30 s, Ad at 6 min 40 s and DHBA at 7 min 15 s (Figure 1). The basic chromatographic patterns of the arterial plasma samples from the acutely or chronically morphine-treated rats were similar to those of their controls.

Arterial catecholamine levels

Table 1 shows the effects of acutely administered morphine on arterial catecholamine concentrations.

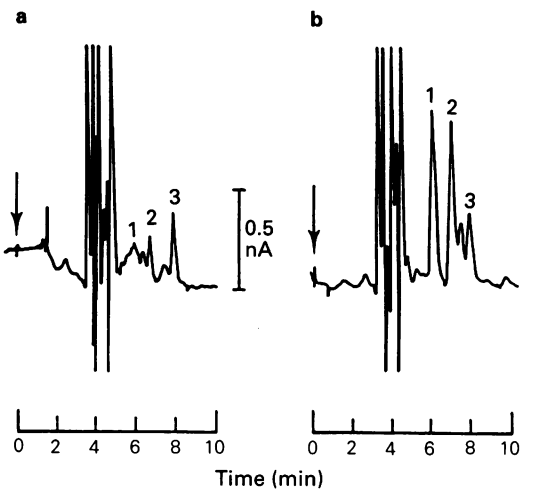

Figure 1 Separation of catecholamines extracted from 2 ml of aortic plasma obtained from rats subjected to pithing procedure without (a) or with sympathetic nerve stimulation (b). ↓ = injection into port; 1 = noradrenaline; 2 = adrenaline; 3 = dihydroxybenzylamine.

In the pithed controls, treatment with morphine 16 mg kg^{-1} did not significantly alter the basal levels of either arterial NA or Ad. Arterial concentrations of both catecholamines were significantly higher in rats subjected to sympathetic nerve stimulation. Acute morphine treatment did not influence these responses; the arterial NA or Ad concentrations were not statistically different between the saline- and morphine-treated groups.

The effects of chronic morphine treatment on arterial catecholamine levels are shown in Table 2. In the pithed controls, the values of arterial NA or Ad concentrations in sucrose- or morphine-treated rats were not significantly different. Sympathetic nerve stimulation significantly increased arterial NA and Ad in both treatment groups; however, the

Table 1 Effects of acute morphine treatment on arterial catecholamine responses to sympathetic nerve stimulation

Experimental condition	*Treatment* (i.p.)	*Number of rats*	*Arterial catecholamine concentrations* (ng ml^{-1}) *Noradrenaline*	*Adrenaline*
Pithed control				
Saline	1 ml kg^{-1}	10	0.63 ± 0.09	0.43 ± 0.08
Morphine	16 mg kg^{-1}	10	0.51 ± 0.16	0.35 ± 0.10
Sympathetic nerve stimulation				
Saline	1 ml kg^{-1}	10	3.08 ± 0.18*	3.06 ± 0.32*
Morphine	16 mg kg^{-1}	10	2.95 ± 0.15*	2.96 ± 0.29*

The values are the means ± s.e.mean.
* $P < 0.001$ when compared with the corresponding values of the pithed controls receiving the same treatment.

Table 2 Effects of chronic morphine treatment on arterial catecholamine responses to sympathetic nerve stimulation

Experimental condition	*Drinking solution*	*Number of rats*	*Arterial catecholamine concentrations* ($ng\,ml^{-1}$) *Noradrenaline*	*Adrenaline*
Pithed control				
	5% sucrose solution	10	0.71 ± 0.08	0.45 ± 0.14
	Morphine in 5% sucrose solution	9	0.74 ± 0.18	0.52 ± 0.08
Sympathetic nerve stimulation				
	5% sucrose solution	12	3.11 ± 0.21*	2.96 ± 0.30*
	Morphine in 5% sucrose solution	9	2.25 ± 0.25*†	2.45 ± 0.23*

The values are the means ± s.e.mean.
* $P < 0.001$ when compared with the corresponding values of the pithed cotrols of the same chronic treatment group.
† $P < 0.05$ when compared with the corresponding values of the chronic sucrose-treated controls subjected to sympathetic nerve stimulation.

arterial NA concentration in morphine-treated animals was lower ($P < 0.05$) than that in their sucrose-treated controls. The arterial Ad level in the morphine-treated group also appeared to be less than that in sucrose-treated animals, but the difference was not statistically significant.

The arterial catecholamine concentrations of rats which were formerly morphine-treated and of their sucrose-drinking controls, subjected to sympathetic nerve stimulation or similar pithing procedure but without stimulation at the end of the 2 week morphine abstinence, are shown in Table 3. All pithed controls exhibited similar basal levels of arterial NA or Ad. Sympathetic nerve stimulation increased the arterial NA and Ad concentrations in both the previously morphine-treated rats and their sucrose-drinking controls ($P < 0.001$ for all). There were no significant differences between the arterial catecholamine levels of these two groups.

The development and disappearance of opiate tolerance

Figure 2 shows the effects of acutely administered morphine on the reaction time to pain, as determined by the tail-immersion test, at the end of the 3 week morphine-drinking period. Intraperitoneal injections of various doses of morphine sulphate (2, 4, 8 or 16 $mg\,kg^{-1}$) significantly prolonged ($P < 0.05$ for all doses) the reaction time in the sucrose-drinking controls, but not in the chronically morphine-treated rats. The reaction times of the

Table 3 Arterial catecholamine levels in previously morphine-treated rats subjected to sympathetic nerve stimulation at 2 weeks after morphine withdrawal

Experimental condition	*Previously chronic treatment (drinking fluid)*	*Number of rats*	*Arterial catecholamie concentrations* ($ng\,ml^{-1}$) *Noradrenaline*	*Adrenaline*
Pithed control				
	5% sucrose solution	8	0.68 ± 0.20	0.52 ± 0.13
	Morphine in 5% sucrose solution	9	0.51 ± 0.15	0.41 ± 0.11
Sympathetic nerve stimulation				
	5% sucrose solution	8	3.15 ± 0.23*	2.97 ± 0.26*
	Morphine in 5% sucrose solution	8	3.21 ± 0.19*	2.85 ± 0.25*

The values are the means ± s.e.mean.
* $P < 0.001$ when compared with the corresponding values of the pitched controls of the same previously morphine-treated group.

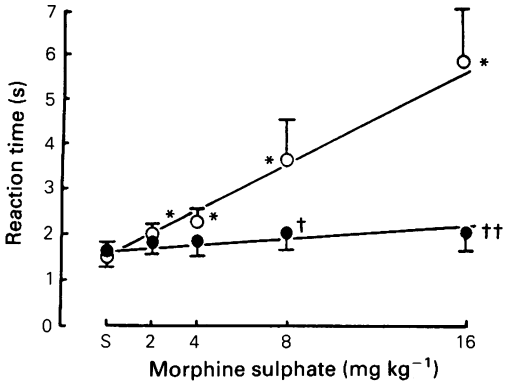

Figure 2 Effects of acutely administered morphine on the reaction time to pain in rats chronically treated with morphine in 5% sucrose (●, $n = 9$ for each dose) or with 5% sucrose (○, $n = 10$ for each dose) drinking solution. The values plotted are the means with s.e.mean shown by vertical bars. S = saline. $*P < 0.05$ when compared with the saline-treated control of the same group. $\dagger P < 0.05$, $\dagger\dagger P < 0.001$ when compared with the corresponding values of the sucrose-drinking controls.

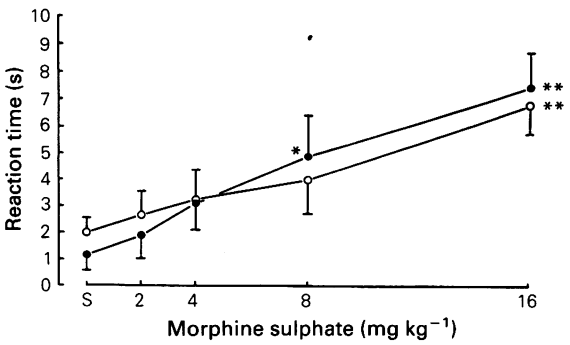

Figure 3 Effects of acutely administered morphine on the reaction time to pain at 2 weeks after morphine withdrawal in 5% sucrose-drinking controls (○, $n = 8$ for each dose) or in previously 3-week morphine-treated rats (●, $n = 8$ for each dose). The values plotted are the means with s.e.mean shown by vertical bars. S = saline. $*P < 0.05$, $**P < 0.001$ when compared with the saline-injected controls of the same group. No significant differences were found between the two groups.

chronically opiate-treated animals given acute doses of morphine 8 or 16 mg kg^{-1} were also significantly less ($P < 0.05$ and $P < 0.001$, respectively) than the corresponding values of the sucrose-drinking controls.

After 2 weeks of morphine withdrawal, i.p. injection of morphine sulphate into the previously morphine-treated rats significantly prolonged the reaction time to pain in the tail-immersion test ($P < 0.05$ and $P < 0.001$ for 8 and 16 mg kg^{-1}, respectively). The values of their reaction time were not significantly different from those of the sucrose-drinking controls (Figure 3). The patterns of their dose-response curves were also similar.

Discussion

It has been reported that the acute administration of opioids suppresses the electrical activity of sympathetic nerves in animals (Laubie *et al.*, 1977; Willette *et al.*, 1982; Jurna & Rummel, 1984). However, it was found in the present investigation that acute i.p. administration of morphine sulphate 16 mg kg^{-1} did not significantly influence the arterial concentrations of NA or Ad in pithed controls and in rats subjected to sympathetic nerve stimulation. Assuming that the arterial levels of catecholamines reflect sympathetic activities, these findings, therefore, suggest that acute administration of this dose of morphine does not suppress electrical activity of the sympathetic nerves in pithed rats. It is unlikely that the negative findings are due to a pharmacologically ineffective amount of injected morphine because this dose of the opiate, 16 mg kg^{-1}, produces significant analgesia in rats (Figure 2).

Chronic treatment with morphine has been reported not only to lead to the development of opiate tolerance and dependence in experimental animals but also to produce other functional changes which may not be seen after acute morphine administration (Schulz & Goldstein, 1973; Llorens *et al.*, 1978; Collier, 1980; Kromer & Steigemann, 1982; Rae & De Moraes, 1983). The present study demonstrates that the increases in arterial NA due to sympathetic nerve stimulation are significantly depressed by chronic morphine treatment. These findings support a past observation that the cardiovascular responses to sympathetic nerve stimulation are impaired in chronically morphine-treated rats (Leung *et al.*, 1986b). The present method of administering morphine in drinking fluid has been shown to be effective in inducing opiate dependence (Leung *et al.*, 1986a). As the rats had developed tolerance to the analgesic effects of acute doses, 8 or 16 mg kg^{-1}, of the opiate, after they had been drinking a morphine solution for 21 days, the current results support the idea that the occurrence of morphine tolerance is accompanied by decreased responses to sympathetic stimulation which are due to a functional change in the sympathetic system. It is conceivable that the observed phenomenon may be due to an enhanced neuronal reuptake mechanism or increased activities of monoamine oxidase or catechol-*O*-methyltransferase by chronic opiate administration; this would facilitate the clearance of catecholamine from

the circulation and subsequently lead to decreased cardiovascular changes and arterial catecholamine elevation in response to sympathetic nerve stimulation. This possibility is, however, unlikely because past findings indicate that the cardiovascular responses of chronically morphine-treated rats to acutely administered NA are not significantly different from those of naive animals (Leung *et al.*, 1986b). Direct measurement of catecholamine metabolites was carried out in a preliminary study. However, this was found to be technically difficult because the level of the major catecholamine metabolities, such as 3-methoxy-4-hydroxyphenylethylene glycol and 3-methoxy-4-hydroxymandelic acid, in the aortic plasma samples following electrical stimulation were extremely small and close to the zero baseline of the electrochemical detector recording. This finding is not at variance with the conclusions of Esler *et al.* (1984) that plasma samples from arterial blood contain extremely low amounts of catecholamine metabolites.

The present study also reveals that rats previously treated with morphine for 3 weeks had indeed recovered from opiate tolerance after 2 weeks of morphine abstinence. Sympathetic nerve stimulation in these animals elicited similar changes in arterial catecholamine levels to those in the corresponding controls, indicating that the sympathetic responses to excitation had returned to normal levels.

Since the changes in sympathetic function commence and disappear in parallel with the phenomenon of opiate tolerance, it is possible that these physiological changes are associated with the occurrence of opiate tolerance. However, the time-course of their causal relationship is still not clear and requires clarification.

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Agonist-induced glycogenolysis in rabbit retinal slices and cultures

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1 The effects of different putative retinal transmitters and/or modulators on glycogenolysis in rabbit retinal slices and in retinal Müller cell cultures were examined.

2 Incubation of rabbit retinal slices or primary retinal cultures (either 3–5 day-old or 25-30 day-old) in a buffer solution containing [^{3}H]-glucose resulted in the accumulation of newly synthesized [^{3}H]-glycogen.

3 Noradrenaline (NA), isoprenaline, vasoactive intestinal peptide (VIP), 5-hydroxytryptamine (5-HT) and 8-hydroxy-dipropylaminetetralin (8-OH-DPAT) stimulated the hydrolysis of this newly formed ^{3}H-polymer. The potency order of maximal stimulations was: VIP > NA > isoprenaline > 5-HT > 8-OH-DPAT.

4 The putative retinal transmitters, dopamine, γ-aminobutyric acid (GABA), glycine and taurine and the muscarinic agonist carbachol (CCh) had no effect on [^{3}H]-glycogen content.

5 The glycogenolytic effects of NA/isoprenaline and 5-HT/8-OH-DPAT appear to be mediated by β-adrenoceptors and 5-HT_1 receptors (possibly 5-HT_{1A}), respectively while the VIP-induced response involved another receptor subtype.

6 Agonists which mediated [^{3}H]-glycogen hydrolysis also stimulated an increase in adenosine 3′:5′-cyclic monophosphate (cyclic AMP) formation. Both responses are blocked to a similar extent by the same antagonists and so are probably mediated via the same receptor subtypes. Moreover, dibutyryl cyclic AMP (db cyclic AMP) promoted tritiated glycogen breakdown in the three retinal preparations.

7 Not all receptors linked to cyclic AMP production however promote glycogenolysis. Dopamine and apomorphine stimulated cyclic AMP formation via D_1-receptors without influencing glycogenolysis. These receptors are exclusively associated with neurones.

Introduction

Glycogen is the major energy reserve in the avascular rabbit retina (Kuwabara & Cogan, 1961; Mizuno & Sata, 1975). Histochemical studies on the rabbit retina showed that glycogen stores are predominantly associated with Müller cells (Kuwabara & Cogan, 1961; Mizuno & Sata, 1975). Furthermore, these are major sites for glycogen synthesis (Kuwabara & Cogan, 1961). It is known that a number of neurotransmitters including neuropeptides induce glycogen breakdown in mouse cerebral cortex (Magistretti *et al.*, 1981; 1984; 1986; Quach *et al.*, 1978; 1980; 1982), astrocytic cultures (Magistretti *et al.*, 1983), leech segmental ganglia (Seal & Pentreath, 1985) and in cultured glial cells of the chick retina (Koh *et al.*, 1984). Recent evidence has indicated that transmitter-like substances induce their glycogenolytic effects in the CNS by stimulating adenosine 3′:5′-cyclic monophosphate (cyclic AMP) production (Magistretti *et al.*, 1983; 1986).

The purpose of the present study was to investigate the effects of different putative retinal transmitters and/or modulators on glycogenolysis in rabbit retinal slices and to correlate these actions with the ability of the individual substances to stimulate cyclic AMP production. Experiments were also carried out on retinal cultures containing primarily

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Müller cells (Osborne *et al.*, 1988; Ghazi & Osborne, 1988a) to determine the cellular site of glycogenolysis.

Methods

Adult albino rabbits (2–3 kg) were obtained from the University Breeding Farm. These rabbits had free access to food and water while being maintained at a temperature of 18–20°C.

Intact retina

Agonist-induced stimulation of glycogenolysis Rabbits were anaesthetised with an intramuscular injection of Hypnorm (0.5 ml kg^{-1}) and killed by an overdose injection of sodium pentabarbitone. Eyes were immediately enucleated and the retinas quickly dissected and washed in oxygenated modified Krebs-Ringer bicarbonate medium (composition, mM: NaCl 120, KCl 5, $CaCl_2$ 2.6, $MgSO_4$ 0.67, KH_2PO_4 1.2, glucose 5.5 and $NaHCO_3$ 27.5) at pH 7.4. Retinal slices (0.25 × 0.25 mm thick) were obtained with a McIlwain tissue chopper, suspended in Krebs-Ringer solution and preincubated for 15 min at 37°C under a constant stream of 95%O_2:5%CO_2. The slices were then washed with fresh buffer and resuspended in oxygenated buffer (2.5 ml per retina). Aliquots of the tissue slices suspension (0.29 ml) were pipetted into individual tubes to which were added 0.01 ml of a buffer solution containing [^{3}H]-glucose (final conc. 50 nM). The tubes were then incubated for 30 min at 37°C in a shaking water bath. Buffer (10 μl) with or without antagonist was added to each tube and the incubation continued for 5 min. Following this period 0.01 ml of drug or buffer was added to individual samples and the incubation continued for 30 min at 37°C under gentle agitation. The reactions were stopped by rapid centrifugation (1000 *g* for 5 min). The supernatants were discarded, replaced with fresh buffer and the slices immediately sonicated for about 9 s. An aliquot (approx. 0.25 ml) of each of the resulting homogenates was quickly transferred to another tube and placed in boiling water for 10 min, followed by a short centrifugation. The supernatants of these samples were assayed for [^{3}H]-glycogen content. Another sample of each of the homogenates was taken for determination of protein content by the method of Bradford (1976).

An ethanol filter paper technique (Solling & Esmann, 1975) was used to isolate and assay for [^{3}H]-glycogen. Briefly, 0.15 ml aliquots of the deproteinized supernatant were spotted on filter paper discs, air dried and then immersed in approximately 10 ml of ice-cold 60% ethanol/10% trichloroacetic acid for 10 min. This solution was replaced with 66% ethanol at room temperature for 10 min. This procedure was repeated 6 times. The filter discs were then placed in acetone for 5 min and dried for 20 min. Each disc was subsequently placed in a scintillation counting vial and the [^{3}H]-glycogen extracted by the addition of 2 ml of boiling water. After cooling, 10 ml of 'Insta-gel scintillation fluid were added and the radioactivity determined in a liquid scintillation counter (Wallac 1211 Rackbeta Liquid Scintillation Counter, LKB, Finland).

Control experiments showed that radioactivity of [^{3}H]-glucose spotted onto filter paper discs was not carried through to provide a source of error. Moreover, treating samples of the supernatant before the glycogen extraction procedure, with or without 4 units ml^{-1} of amylo-1-6-glucosidase demonstrated that as little as 3.0% of the ^{3}H-isolated product was not tritiated glycogen. Since it is known that amylo-1-6-glucosidase hydrolyzes glycogen to form glucose (Nahorski & Rogers, 1972; Passonneau & Lauderdale, 1974) the contamination may be glucose-containing gangliosides and/or cerebrosides (Nahorski & Rogers, 1972).

Agonist-induced stimulation of cyclic AMP production With minor modifications, the method described by Kebabian *et al.* (1972) was used to stimulate cyclic AMP production by the various agents in homogenates of rabbit retinas. The retinas were dissected, weighed and quickly homogenized in an ice-cold solution of a Tris I buffer (0.02 ml per mg of wet weight). Tris I buffer consists of: Tris 2 mM, maleate 2 mM and EGTA 2 mM; pH 7.4. Aliquots of 0.13 ml of Tris II buffer solution [Tris II buffer consists of: Tris 80 mM, $MgSO_4$ 2 mM, theophylline 10 mM or isobutylmethylxanthine (IBMX) 1 mM, EGTA 0.2 mM; pH 7.4] and 0.01 ml of the agonist (made up in Tris II buffer) were added to assay tubes in triplicate. Aliquots of 0.02 ml of retinal homogenates were then added to each of the tubes. When antagonists were used, 0.01 ml of the antagonist solution replaced 0.01 ml of Tris II buffer. Basal levels of cyclic AMP in retinal homogenates were determined in assay tubes containing only the homogenate and 0.14 ml of Tris II buffer. Stimulation of cyclic AMP production was initiated by adding 0.05 ml of ATP (made up in Tris II buffer) in each assay tube. The tubes were incubated for 5 min at 37°C in a gently shaking water bath. The reaction was stopped by placing the tubes in boiling water for 3 min, followed by centrifugation at 11,500 *g* for 5 min. Aliquots (0.05 ml) of the supernatant were removed and kept frozen before being assayed for cyclic AMP content. The method of Brown *et al.* (1972), which is based on a competition between [^{3}H]-cyclic AMP and unlabelled cyclic AMP for specific binding sites to a

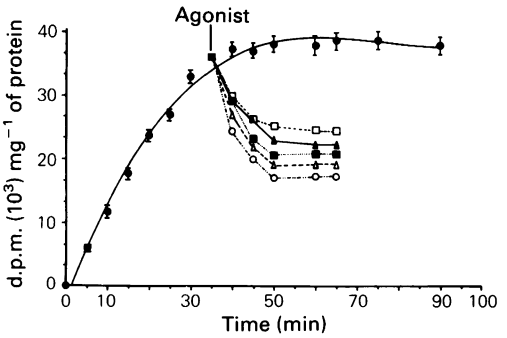

Figure 1 Time course of the synthesis of [^{3}H]-glycogen in rabbit retinal slices and of its hydrolysis by various agents. Slices were incubated in a modified Krebs-bicarbonate solution (see Methods section) containing [^{3}H]-glucose and the content of [^{3}H]-glycogen determined at the appropriate times as described in the Methods section. After 35 min of incubation, either drugs or buffer solutions were added (arrow) and the reaction was stopped at increasing intervals and the amount of [^{3}H]-glycogen determined. Points are means ± s.e. (error bars) from 4 experiments. Assay conditions and presentation of results are as described in Table 2. Noradrenaline (△); isoprenaline (■); 5-hydroxytryptamine (▲); vasoactive intestinal peptide (○) and 8-hydroxy-dipropylaminetetralin (□).

binding protein was used to quantify cyclic AMP levels.

Preparation of retinal cultures

Retinas from 1 to 5 day postnatal rabbit pups were dissociated by a modified trypsinisation procedure as previously described (Beale *et al.*, 1982; Osborne & Beaton, 1986). The dissociated cells were suspended in culture medium consisting of 85% Eagles Minimal Essential medium with Earle's salt (Gibco). The cells in 0.5 ml of culture medium were placed on a 13 mm diameter glass coverslip coated with poly-L-lysine. The coverslips were in Multidish (Falcon 3008) wells and the density of the cells was between 4000–5000 per mm^2. The cultures were grown at 35.5°C in a humidified atmosphere containing 5% CO_2. The culture medium was changed after 3 days and thereafter at 2- to 3-day intervals.

Agonist-induced stimulation of [^{3}H]-glycogen hydrolysis Retinal cultures corresponding to between 3–5 and 25–30 days following seeding, were washed twice with previously oxygenated Krebs-Ringer bicarbonate buffer (pH 7.4): 0.8 ml of this buffer solution containing [^{3}H]-glucose (final conc. 50 nM) was added to each well and the cultures were incubated for 70 min (3–5 day) or 85 min (25–30 day) in a humidified 95%O_2:5%CO_2 atmosphere at 35.5°C. Either antagonist or buffer (0.1 ml of either) was added to each culture well and the incubation continued for 5 min. These incubation times were used since time course experiments indicated that the rate of [^{3}H]-glucose incorporation into glycogen decreased significantly at 75 min (3–5 day) and 90 min (25–30 day), to eventually reach a plateau. Each well then received another 0.1 ml of drug or buffer and the incubation was continued for 30 min at 35.5°C in the same humidified atmosphere. The reactions were terminated by thrice washing the cultures with buffer, removing the coverslips and placing them in a plastic vial containing 0.5 ml of fresh buffer. The coverslips were then crushed, vortexed, sonicated for 20 s and placed in boiling water for 10 min, followed by a short centrifugation (1000 *g* for 5 min). The supernatant of each sample was analysed for its content of [^{3}H]-glycogen in the same manner as for retinal slices, and the protein content was determined by the method of Bradford (1976).

Agonist-induced stimulation of cyclic AMP production The cultures were washed twice with culture medium and incubated for 5 min in Eagles Minimal Essential medium containing Tris 2 mM, maleate 2 mM, EGTA 2 mM; pH 7.4; (Tris I/MEM). Two coverslips containing the cultures were then transferred to individual plastic tubes already containing 0.14 ml of Tris II/MEM (MEM, Tris 80 mM, EGTA 0.2 mM, theophylline 10 mM or IBMX 1 mM, $MgSO_4$ 2 mM; pH 7.4) and 0.01 ml of antagonist (made up in Tris II/MEM) where appropriate. Following an incubation for 5 min, 0.01 ml of the agonist (made up in Tris II/MEM) was added to each tube followed by 0.05 ml of ATP (made up in Tris II/MEM) to initiate the reaction. The tubes were then incubated for 5 min in a shaking water bath at 37°C. Control tubes contained no agonist or antagonist and all experiments were carried out in triplicate. Following centrifugation, cyclic AMP was determined in 0.05 ml aliquots of the supernatants.

Materials

D-[6-^{3}H]-glucose (30.2 Ci mmol^{-1}) and [8-^{3}H]-cyclic AMP (41.7 Ci mmol^{-1}) were purchased from Amersham International plc, UK. Prazosin was obtained from Pfizer, UK; ketanserin and Hypnorm (a central depressant) from Janssen Pharmaceuticals, Germany; methysergide, spiroxatrine and mianserin from Sandoz, Switzerland; vasoactive intestinal peptide (VIP) from Bachem U.K. Ltd. MDL-72222 (1αH,3α,5αH-tropan-3-yl-3,5-dichlorobenzoate) was a gift from Dr Fozard (Merrell Dow) and sodium pentobarbitone was from Willows Francis Veterinary, West Sussex, England. RU24696 (5-methoxy-

3(1,2,3,6-tetrahydropyridin-4-yl)1H indole) was obtained from Le Centre de Recherche Roussel UCLAF. All other chemicals and drugs were purchased from Sigma, U.K.

Statistical analysis

Statistical significance was determined by the Mann-Whitney test. A P value of <0.05 was considered significant.

Results

Retinal slices

Rabbit retinal slices incubated in the presence of [^{3}H]-glucose (50 nM) for different time periods, followed by the determination of [^{3}H]-glycogen content demonstrated that the accumulation of newly synthesized [^{3}H]-glycogen was linear for the first 30 min. Thereafter a plateau was reached and maintained for at least 60 min (Figure 1).

Retinal slices which had been preincubated for 35 min with [^{3}H]-glucose and then exposed to 500 μM noradrenaline (NA), isoprenaline, 5-HT, 8-OH-DPAT or 5 μM VIP resulted in maximal increases in the hydrolysis of [^{3}H]-glycogen (see Table 1). All the agents produced a rapid rate of decrease in [^{3}H]-glycogen content, though NA, isoprenaline and VIP induced a much more pronounced decrease than 5-HT or 8-OH-DPAT. Dibutyryl cyclic AMP at 250 μM also induced a substantial reduction in [^{3}H]-glycogen content (Table 1). Figures 2 and 3 demonstrate that the glycogenolytic effects of the agonists were dose-dependent resulting in EC_{50} values of 22.7 nM (NA), 27.9 nM (isoprenaline), 0.422 nM (VIP), 306 nM (5-HT) and 194 nM (8-OH-DPAT). Dopamine, CCh, GABA, glycine and taurine did not induce glycogenolysis in retinal slices (Table 1).

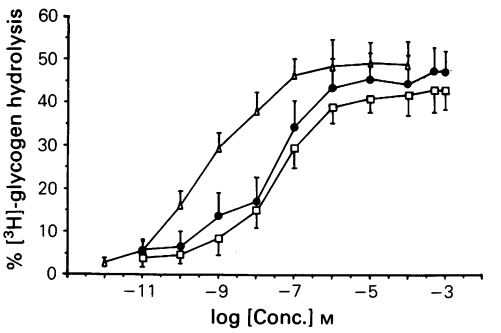

Figure 2 Dose-response curves of [^{3}H]-glycogen hydrolysis elicited by noradrenaline (●), isoprenaline (□) and vasoactive intestinal peptide (△) in rabbit retinal slices. Conditions of the experiments are as described in Table 2 except that antagonists were not used. Points represent means ± s.e. (error bars) from 4 separate experiments.

Prazosin (10 μM), yohimbine (10 μM) or phentolamine (10 μM), three specific α-adrenoceptor antagonists, and spiroxatrine (10 μM), a 5-HT_{1A} receptor antagonist (Nelson & Taylor, 1986; Göthert & Schli-

Table 1 Effect of various agents on % of [^{3}H]-glycogen hydrolysis (glycogenolysis) in rabbit retinal slices or % stimulation of cyclic AMP production over basal levels in retinal homogenates

Agent	*Conc* (μM)	% [^{3}H]-*glycogen hydrolysis*	% *cyclic AMP production*
Buffer	—	0	0
NA	500	46.33 ± 1.62	269.63 ± 13.35
Isoprenaline	500	42.01 ± 0.90	114.44 ± 6.34
VIP	5	49.98 ± 3.89	357.88 ± 43.01
5-HT	500	37.37 ± 1.95	139.02 ± 7.39
8-OH-DPAT	500	31.81 ± 2.30	128.86 ± 10.07
GABA	500	5.65 ± 2.58 NS	9.10 ± 2.61 NS
Glycine	500	7.42 ± 1.33 NS	7.83 ± 2.14 NS
Taurine	500	5.22 ± 2.32 NS	5.08 ± 3.87 NS
Dopamine	500	11.43 ± 1.36 NS	199.82 ± 8.56
Apomorphine	500	4.94 ± 0.99 NS	184.57 ± 13.81
Carbachol	500	7.40 ± 6.49 NS	−24.78 ± 8.66
db cyclic AMP	250	62.13 ± 1.28	

Values are means ± s.e.mean where n is from 6–14 separate experiments. NA = noradrenaline, VIP = vasoactive intestinal peptide, 8-OH-DPAT = 8-hydroxy-dipropylaminetetralin, GABA = γ-aminobutyric acid, and db cyclic AMP = dibutyryl cyclic AMP. NS = insignificant compared with controls.

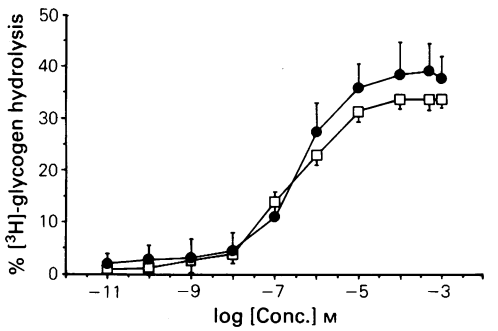

Figure 3 Dose-response curves of [^{3}H]-glycogen hydrolysis elicted by 5-hydroxytryptamine (●) and 8-hydroxy-dipropylaminetetralin (□) in rabbit retinal slices. Conditions of the experiments are as described in Table 2 except that antagonists were not added. Points represent means ± s.e. (error bars) from 4 separate experiments.

ker, 1987), had no effect on either the NA- (Table 2) or isoprenaline-induced glycogenolytic effect. In contrast, propranolol (1 μM), a β-adrenoceptor blocker, which had no significant action of [^{3}H]-glycogen content strongly inhibited both the NA- and isoprenaline-induced responses (Table 2). The effects of the dopamine receptor antagonists, haloperidol, spiroperidol and (+) and (−)-butaclamol (10 μM), on the responses induced by the two agonists were tested. It was found that these antagonists had no effect on the isoprenaline-induced response, however the NA-induced response was slightly inhibited by haloperidol but not by either (+) and (−)-butaclamol or spiroperidol. This is interesting since neither dopamine nor apomorphine stimulated glycogenolysis in retinal cells, but strongly stimulated the production of cyclic AMP (Table 1). It is therefore concluded that NA and isoprenaline influenced β-adrenoceptors to promote glycogenolysis in the

Table 2 Effect of various antagonists on noradrenaline/isoprenaline-induced [^{3}H]-glycogen hydrolysis or cyclic AMP production

Agent	*Antagonist added*	*% [^{3}H]-glycogen hydrolysis*	*% cyclic AMP production*
Buffer		0	0
NA (500 μM)		44.87 ± 3.05	266.81 ± 13.02
NA	Propranolol (1 μM)	*10.46 ± 1.55	*150.22 ± 10.41
NA	Propranolol (10 μM)	*7.48 ± 1.14	*107.26 ± 5.54
NA	Haloperidol (1 μM)	*25.71 ± 4.15	*87.14 ± 3.29
NA	Spiroperidol (1 μM)	NS 44.08 ± 2.09	*155.12 ± 2.63
NA	(+)-Butaclamol (1 μM)	NS 40.85 ± 1.85	*151.63 ± 12.54
NA	(−)-Butaclamol (1 μM)	NS 44.83 ± 2.96	NS 255.82 ± 5.31
NA	Prazosin (10 μM)	NS 43.43 ± 1.03	NS 263.96 ± 10.25
NA	Yohimbine (10 μM)	NS 43.50 ± 2.99	NS 270.03 ± 15.10
NA	Phentolamine (10 μM)	NS 44.90 ± 1.16	NS 259.97 ± 11.71
NA	Spiroxatrine (10 μM)	NS 43.71 ± 0.85	NS 272.06 ± 9.91
Isoprenaline (500 μM)		40.63 ± 5.26	114.40 ± 6.32
Isoprenaline	Propranolol (1 μM)	*5.22 ± 1.38	*14.62 ± 6.48
Isoprenaline	Propranolol (10 μM)	*1.92 ± 1.03	*5.18 ± 2.33
Isoprenaline	Haloperidol (1 μM)	NS 41.65 ± 3.80	NS 109.93 ± 4.11
Isoprenaline	Spiroperidol (1 μM)	NS 39.81 ± 2.97	NS 112.19 ± 4.72
Isoprenaline	Phentolamine (10 μM)	NS 41.21 ± 0.78	NS 120.16 ± 8.65
Isoprenaline	Spiroxatrine (10 μM)	NS 40.35 ± 0.90	NS 117.76 ± 9.22
Propranolol (1 μM)		1.61 ± 0.63	2.16 ± 0.34
Prazosin (10 μM)		0.98 ± 0.37	1.73 ± 0.25
Yohimbine (10 μM)		2.35 ± 1.01	3.55 ± 0.92
Phentolamine (10 μM)		3.26 ± 1.64	1.39 ± 0.51
Spiroxatrine (10 μM)		4.18 ± 2.83	2.85 ± 1.12
Haloperidol (1 μM)		0.68 ± 0.29	1.76 ± 0.91
Spiroperidol (1 μM)		1.22 ± 1.94	1.06 ± 0.61
(+)-Butaclamol (1 μM)		1.42 ± 0.86	1.33 ± 1.65
(−)-Butaclamol (1 μM)		0.78 ± 0.45	0.06 ± 1.14

None of the antagonists used stimulated glycogenolysis or cyclic AMP production. Results are expressed in terms of the % of [^{3}H]-glycogen hydrolysis relative to control and % stimulation of cyclic AMP production above basal levels, and are given as means ± s.e.mean where n is from 8–12 separate experiments. NA = noradrenaline. * and NS denotes significantly and insignificantly different from agonist-induced response alone.

Table 3 Effects of various antagonists on 5-hydroxytryptamine (5-HT)/8-OH-DPAT induced [^{3}H]-glycogen hydrolysis or cyclic AMP production

Agent	*Antagonist added*	*% [^{3}H]-glycogen hydrolysis*	*% cyclic AMP production*
Buffer		0	0
5-HT (500 μM)		38.90 ± 1.46	139.02 ± 7.36
5-HT	Spiroxatrine (1 μM)	*6.70 ± 0.77	*25.42 ± 9.58
5-HT	Spiroxatrine (10 μM)	*3.28 ± 0.42	*24.24 ± 8.08
5-HT	Ketanserin (10 μM)	NS 36.59 ± 1.45	NS 130.01 ± 9.14
5-HT	Mianserin (10 μM)	NS 39.44 ± 1.80	NS 136.85 ± 10.05
5-HT	Methysergide (10 μM)	NS 37.18 ± 0.66	NS 132.04 ± 13.64
5-HT	Propranolol (10 μM)	NS 37.27 ± 0.94	NS 132.58 ± 3.37
5-HT	MDL-72222 (10 μM)	NS 38.99 ± 1.87	NS 141.35 ± 12.90
5-HT	Haloperidol (10 μM)	NS 39.27 ± 0.53	NS 134.97 ± 5.82
8-OH-DPAT (500 μM)		33.06 ± 1.82	128.86 ± 10.07
8-OH-DPAT	Spiroxatrine (1 μM)	*5.08 ± 1.19	*16.88 ± 6.20
8-OH-DPAT	Spiroxatrine (10 μM)	*2.18 ± 0.73	*9.02 ± 2.12
8-OH-DPAT	Methysergide (10 μM)	NS 32.23 ± 3.02	NS 115.04 ± 7.30
8-OH-DPAT	MDL-72222 (10 μM)	NS 33.70 ± 4.60	NS 130.31 ± 12.84
8-OH-DPAT	Propranolol (10 μM)	NS 32.45 ± 3.48	NS 124.93 ± 11.73
8-OH-DPAT	Haloperidol (10 μM)	NS 34.01 ± 2.75	NS 126.33 ± 8.17
RU24696 (500 μM)		31.93 ± 1.61	104.85 ± 7.98
RU24696	Spiroxatrine (1 μM)	*4.18 ± 0.21	*10.74 ± 0.96
RU24696	Spiroxatrine (10 μM)	*1.83 ± 0.96	*4.60 ± 0.48
RU24696	Methysergide (10 μM)	NS 31.59 ± 2.23	NS 107.28 ± 8.62
RU24696	MDL-72222 (10 μM)	NS 32.26 ± 1.30	NS 106.79 ± 6.93
RU24696	Haloperidol (10 μM)	NS 31.51 ± 0.91	NS 101.98 ± 2.19
Spiroxatrine (1 μM)		2.12 ± 1.36	−1.21 ± 3.67
Ketanserin (10 μM)		0.43 ± 0.65	1.42 ± 0.77
Mianserin (10 μM)		1.87 ± 1.45	4.19 ± 2.08
Methysergide (10 μM)		2.70 ± 1.55	0.74 ± 1.72
MDL-72222 (10 μM)		0.79 ± 0.37	2.63 ± 1.05
Haloperidol (10 μM)		1.90 ± 1.75	−0.19 ± 1.66

None of the antagonists used stimulated glycogen hydrolysis or cyclic AMP production. Results are expressed in terms of the % of [^{3}H]-glycogen hydrolysis relative to control and % stimulation of cyclic AMP production above basal levels, and are given as means ± s.e.mean. *n* is from 10–14 separate experiments, except for RU24696 results where $n = 3$. 8-OH-DPAT = 8-hydroxy-dipropylaminetetralin. * and NS denote significantly and insignificantly different from agonist-induced response alone.

retina while dopamine receptors were not significantly involved in glycogenolysis.

Ketanserin (10 μM), mianserin (10 μM) and methysergide (10 μM), all 5-HT$_2$ antagonists, did not influence [^{3}H]-glycogen hydrolysis induced by either 5-HT or 8-OH-DPAT (Table 3). Furthermore, MDL-72222 (10 μM), a peripheral 5-HT$_3$ receptor antagonist (Fozard, 1984), propranolol and haloperidol (10 μM) were without effect (Table 3). In contrast, spiroxatrine (1 μM), a 5-HT$_{1A}$ antagonist was a potent blocker of the 5-HT- and 8-OH-DPAT-induced responses (Table 3). To characterize further the 5-HT-induced response, RU24696 (a 5-HT$_{1A/B}$ agonist) was examined. This agonist stimulated [^{3}H]-glycogen hydrolysis to an extent similar to 8-OH-DPAT and the response was inhibited by spiroxatrine while other receptor antagonists had no effect (Table 3). Since 8-OH-DPAT is a 5-HT$_{1A}$ receptor agonist (Middlemiss & Fozard, 1983; Hamon *et al.*, 1984; Hoyer *et al.*, 1986), it is concluded that 5-HT and 8-OH-DPAT promote glycogenolysis in the retina most probably via 5-HT$_{1A}$ receptor sites.

The effect of VIP on the hydrolysis of [^{3}H]-glycogen in rabbit retinal slices also appears to be mediated through specific receptors. Propranolol (10 μM), yohimbine (10 μM), spiroxatrine (10 μM), haloperidol (10 μM) and methysergide (10 μM) were all without effect upon the VIP-induced stimulation of glycogenolysis. Thus the VIP-induced response appeared not to be mediated by adrenoceptors, 5-HT or dopamine receptors.

The involvement of cyclic AMP in mediating glycogenolysis is demonstrated in Tables 1, 2 and 3. Except for dopamine, only substances which stimulated cyclic AMP production in retinal homogenates promoted glycogenolysis in rabbit retinal slices (Table 1). Additionally, db cyclic AMP (250 μM), a synthetic analogue of cyclic AMP which crosses the cell membrane (Posternak *et al.*, 1962), induced a more than 62% increase in glycogen breakdown in retinal slices, and neuronal and Müller cell cultures. Interestingly CCh induced a slight decrease in cyclic AMP production but had no effect on [^{3}H]-glycogen levels. Tables 2 and 3 show the influence of various antagonists upon the stimulation of cyclic AMP production but had no effect on [^{3}H]-glycogen levels. Tables 2 and 3 show the influence of various antagonists upon the stimulation of cyclic AMP production caused by different agonists. These results suggest that in the rabbit retina, NA/adrenaline, 5-HT and VIP increase cyclic AMP production and induce an enhanced rate of glycogen breakdown through the same receptor types.

Retinal cultures

Rabbit retinal cultures at 3–5 and 25–30 days old were used in an effort to determine the cellular site of the agonist-induced [^{3}H]-glycogen hydrolysis. This is possible since 3–5 day old cultures contain both neurones and Müller cells, whereas only non-neuronal retinal (Müller) cells are found in the 25–30 days cultures (Osborne, 1987; Osborne *et al.*, 1988; Ghazi & Osborne, 1988b). In both young and old cultures, NA, isoprenaline, VIP, 5-HT and 8-OH-DPAT stimulated the hydrolysis of [^{3}H]-glycogen to an extent similar to that found in intact retinal slices (Tables 4 and 5). Furthermore, pharmacological characterization of this response in both culture types proved to be identical to that found in retinal slices. Tables 4 and 5 demonstrate to what extent the varous agents stimulated the production of cyclic AMP in 3–5 and 25–30 day old cultures. It is evident that these cyclic AMP responses are fairly similar to those shown in Table 1 for retinal homogenates, with two exceptions: (1) isoprenaline (500 μM) induced a significantly higher stimulation of cyclic AMP production in both culture types than in retinal homogenates and (2) dopamine (500 μM) and apomorphine (500 μM) were only able to elicit a cyclic AMP response in young 3–5 day old retinal cultures. It appears that the adenylate cyclase-linked dopamine receptors are only located on neurones, as no response was found in the Müller cell cultures (25–30 days). The results obtained in the 25–30 day old cultures suggest the presence of functional muscarinic receptor, β-adrenoceptor, 5-HT$_1$(5-HT$_{1A}$) and VIP receptors, linked to cyclic AMP production, on Müller cells.

Discussion

NA, isoprenaline, VIP, 5-HT and 8-OH-DPAT induced [^{3}H]-glycogen hydrolysis in rabbit retinal slices and retinal cultures of different ages. The NA/

Table 4 Percentage stimulation of [^{3}H]-glycogen hydrolysis (glycogenolysis) or cyclic AMP production over basal levels in 3–5 day old rabbit retinal cultures by various agents

Agent	*Conc* (μM)	% [^{3}H]-*glycogen hydrolysis*	% *cyclic AMP production*
Buffer	—	0	0
NA	500	52.41 ± 1.85	164.23 ± 4.74
Isoprenaline	500	50.32 ± 5.42	235.59 ± 14.40
VIP	5	54.72 ± 2.14	393.23 ± 29.89
5-HT	500	37.77 ± 1.91	107.14 ± 5.57
8-OH-DPAT	500	33.05 ± 2.20	90.17 ± 4.04
GABA	500	NS 4.03 ± 0.58	NS 5.88 ± 1.23
Glycine	500	NS 3.73 ± 0.73	NS 4.45 ± 3.22
Taurine	500	NS 1.78 ± 0.98	NS 1.82 ± 1.52
Dopamine	500	NS 8.28 ± 1.53	143.77 ± 6.16
Apomorphine	500	NS 2.45 ± 0.79	165.92 ± 3.20
Carbachol	500	NS 8.59 ± 2.96	−35.40 ± 3.80
db cyclic AMP	250	68.79 ± 1.74	

Results are given as means ± s.e.mean where n is from 6–14 separate experiments. NA = noradrenaline, VIP = vasoactive intestinal peptide, 8-OH-DPAT = 8-hydroxy-dipropylaminetetralin, GABA = γ-aminobutyric acid and db cyclic AMP = dibutyryl cyclic AMP. NS denotes insignificant compared with controls.

Table 5 Percentage stimulation of [^{3}H]-glycogen hydrolysis (glycogenolysis) or cyclic AMP production over basal levels in 25–30 days old rabbit retinal cultures (primarily Müller cells) by various agents

Agent	*Conc* (μM)	% [^{3}H]-*glycogen hydrolysis*	% *cyclic AMP production*
Buffer	—	0	0
NA	500	50.38 ± 1.61	186.72 ± 3.89
Isoprenaline	500	49.47 ± 1.68	223.09 ± 26.33
VIP	5	49.18 ± 2.19	392.02 ± 39.38
5-HT	500	35.68 ± 1.26	88.00 ± 5.58
8-OH-DPAT	500	32.96 ± 1.86	66.99 ± 6.01
GABA	500	NS 6.00 ± 0.99	NS 4.62 ± 1.19
Glycine	500	NS 6.60 ± 0.96	NS 4.15 ± 1.36
Taurine	500	NS 3.34 ± 1.42	NS 2.90 ± 0.86
Dopamine	500	NS 3.90 ± 1.33	NS 6.74 ± 3.65
Apomorphine	500	NS 4.25 ± 1.24	NS 3.33 ± 0.88
Carbachol	500	NS 4.28 ± 0.58	−30.38 ± 6.75
db cyclic AMP	250	69.28 ± 1.76	

Values are means ± s.e.mean where n is from 6–14 separate experiments. NA = noradrenaline, VIP = vasoactive intestinal peptide, 8-OH-DPAT = 8-hydroxy-dipropylaminetetralin, GABA = γ-aminobutyric acid, and db cyclic AMP = dibutyryl cyclic AMP. NS denotes insignificant compared with controls.

isoprenaline and 5-HT/8-OH-DPAT responses appear to be mediated via β-adrenoceptors and 5-HT$_1$ receptors (possibly 5-HT$_{1A}$), respectively, while the VIP-induced response is mediated through another receptor subtype. The results obtained in the non-neuronal (Müller) cell cultures establish the presence of functional receptors on these cells. With the exception of dopamine and apomorphine, agonists which stimulated the formation of cyclic AMP also promoted glycogenolysis. Agonist-induced stimulation of glycogenolysis and cyclic AMP formation were blocked to a similar extent by the same antagonist and both responses are probably mediated through the same receptor subtypes. Furthermore, db cyclic AMP strongly induced glycogenolysis in both retinal slices and cultured retinal cells. It seems probable that agonist-induced [^{3}H]-glycogen hydrolysis correlates with stimulated production of cyclic AMP.

The effect of noradrenaline on glycogenolysis in the retina resembles observations made elsewhere. NA is known to decrease newly synthesized intracellular glycogen in mouse cortical slices (Quach *et al.*, 1978; Magistretti *et al.*, 1981) and in cultured astroglia (Magistretti *et al.*, 1983) by at least 60%. In astrocytoma cells, cultured and transformed astrocytes, NA and isoprenaline significantly increased the breakdown of glycogen (Passonneau & Crites, 1976; Cummins *et al.*, 1983a,b). Isoprenaline was also found to induce glycogenolysis in rat striatal slices (Wilkening & Makman, 1976; 1977) and in chick cerebral hemispheres (Edwards *et al.*, 1974). NA- and isoprenaline-mediated effects were through β-adrenoceptors. Interestingly, haloperidol partially blocked the NA-induced stimulation of cyclic AMP production and glycogen breakdown in intact retina and 3–5 day retinal cultures but not in the older Müller cell cultures. Whilst haloperidol is a classical dopamine antagonist it would appear that this effect is elicited via adrenoceptors rather than dopamine receptors. Other dopamine receptor antagonists, viz spiroperidol and butaclamol, did not nullify the NA-induced responses. Furthermore neither dopamine nor apomorphine stimulated glycogenolysis. Since both dopamine and apomorphine strongly stimulated cyclic AMP production without influencing glycogen breakdown, it is clear that not all receptors linked to cyclic AMP production promote glycogenolysis. Interestingly, an inactive form of cyclic AMP-dependent protein kinase containing bound cyclic AMP has been purified and characterized (Cobb *et al.*, 1987). This may help explain the inability of either dopamine or apomorphine to stimulate glycogen breakdown.

As is the case for mouse cortical slices (Quach *et al.*, 1978), leech segmental ganglia (Seal & Pentreath, 1985) and cultured brain astroglia (Magistretti *et al.*, 1983), 5-HT induced a significant hydolysis of [^{3}H]-glycogen in the various retinal preparations. 8-OH-DPAT and RU24696, known as 5-HT$_{1A}$ receptor agonists (Middlemiss & Fozard, 1983; Hamon *et al.*, 1984; Sills *et al.*, 1984), mimicked the effect produced by 5-HT and were likewise strongly inhibited by the 5-HT$_{1A}$ receptor antagonist, spiroxatrine. Furthermore the dose-response curves and EC$_{50}$ values for both 5-HT- and 8-OH-DPAT-induced effects on

[^{3}H]-glycogen hydrolysis were very similar. Thus it appears that 5-HT induces glycogenolysis in the retina via 5-HT$_{1A}$ receptors.

VIP was the most potent of all the agents tested in inducing glycogenolysis in the different retinal preparations. VIP has a similar potent effect on glycogenolysis in mouse cortical slices, cultured rat astroglia (Magistretti *et al.*, 1981; 1983), hepatocytes (Wood & Blum, 1982), cultured chick retinal cells (Koh *et al.*, 1984) and a human colon cell line (Rousset *et al.*, 1981) and vertebrate retina (Schorderet *et al.*, 1984). The glycogenolytic effect of VIP in retinal tissue and cell culture was not inhibited by any of the antagonists tested, including those that blocked the response induced by either NA or 5-HT. These results, particularly from the culture studies, show that the VIP response is not mediated indirectly by the release of either NA or 5-HT from other cells but rather point to an involvement of a specific VIP receptor type in mediating [^{3}H]-glycogen breakdown.

The results described in this study suggest that agonist-induced glycogen hydrolysis involves the generation of cyclic AMP. However, other factors may contribute to the glycogenolytic effects of the different agonists. For example, in the CNS, as in other tissues, phosphorylase *b* kinase is activated by agonists which mediated a Ca^{2+} influx (this issue is discussed below) (Cohen, 1982; Ververken *et al.*, 1982). Additionally, Opler & Makman (1972) demonstrated that, in contrast to NA, histamine induces glycogenolysis in rat astrocytoma cells without activating adenylate cyclase activity. These authors also concluded from their studies that the histamine effect may be mediated by an elevation of cyclic AMP levels through an action on the phosphodiesterase activity (Opler & Makman, 1972). More recently, Pearce *et al.* (1985) have produced data to suggest that α-adrenoceptors, through a cyclic AMP-independent mechanism, stimulate glycogen turnover. In the present study no evidence was found for the involvement of α-adrenoceptors in either stimulating cyclic AMP production or promoting glycogenolysis. Moreover, CCh does not stimulate glycogen breakdown or cyclic AMP production. Furthermore, NA in the presence of propranolol can promote inositol phosphates (IPs) accumulation but has no effect on cyclic AMP formation or glycogen hydrolysis These results argue against the involvement of the IPs/DG 2nd messenger system in mediating glycogenolytic events in retinal cells.

In the present study, only agents which stimulated cyclic AMP formation also promoted glycogen breakdown. Moreover, the EC_{50} values for NA, 5-HT and VIP are similar for both stimulation of cyclic AMP formation (unpublished results) and glycogenolysis, thus arguing in favour of the idea that the same receptors are involved in the two responses and that similar receptor occupancies are necessary to elicit each of the effects. This is not the case in mouse cortical slices where a lower receptor occupancy is required for the stimulation of [^{3}H]-glycogen hydrolysis than for promoting cyclic AMP production (Quach *et al.*, 1978). The possibility that external Ca^{2+} might be involved in the agonist-induced stimulation of glycogenolysis in retinal tissues is unlikely, because similar results were obtained in Ca^{2+}-free medium for each of the agonists tested (unpublished results). Another possible mechanism is that agonists induce an activation of glycogen hydrolysis by mobilizing intracellular stores of Ca^{2+}. This is very unlikely since agents such as VIP, isoprenaline and 8-OH-DPAT do not induce a rise in free intracellular Ca^{2+} (Ghazi & Osborne, 1988b) but significantly stimulate glycogenolysis in retinal cells. Moreover, NA and 5-HT induce a rise in free intracellular Ca^{2+} levels that are respectively blocked by prazosin and ketanserin and these two antagonists have no effect on glycogenolysis (Ghazi & Osborne, 1988b).

Previous investigations in rat striatal slices and chick cerebral hemispheres also showed the involvement of cyclic AMP in the glycogenolytic responses induced by isoprenaline (Edwards *et al.*, 1974; Wilkening & Makman, 1976; 1977). In the present study the synthetic cyclic AMP analogue, db cyclic AMP, displayed a very potent glycogenolytic effect in rabbit retinal slices and retinal cultures as described before for rat astrocytoma cells (Opler & Makman, 1972). This suggests that the cyclic AMP-dependent phosphorylation cascade is one of the operative mechanisms in hydrolysing glycogen in retinal cells. This is supported by the finding that NA stimulated the conversion of phosphorylase *b* into its more active form phosphorylase *a* which hydrolyses the glycogen chain through increasing cyclic AMP (Edwards *et al.*, 1974). Furthermore an increase in the amount of phosphorylase *a* in rat astrocytoma and neuroblastoma cells has been reported following treatment with a phosphodiesterase inhibitor (Passonneau & Crites, 1976).

The primary site for glycogen storage and synthesis in the rabbit retina is the inner portion of the Müller cells (Kuwabara & Cogan, 1961; Magalhaes & Coimbra, 1970; 1972). Nearly all glucose-6-phosphatase activity, which mainly hydrolyses glucose-6-phosphate that emanates from glycogen breakdown, has been localised in the inner portion of the Müller cells (Magalhaes & Coimbra, 1972). These observations suggest that Müller cells are the main storage area of glycogen and glucose formation; retinal neurones and photoreceptors possess negligible glycogen stores. Since systemic glucose and oxygen are not directly supplied to the inner layers of the rabbit

retina, the glucose formed and released from the Müller cells could be utilized in anaerobic glycolysis in the deprived parts of the retina. Additionally, Koh *et al.* (1984) have demonstrated that VIP and glucagon stimulate glycogen breakdown in cultured Müller cells from the avian retina. Thus the present results could be interpreted as suggesting that substances like NA, VIP and 5-HT are released directly by retinal neurones following a physiological event or alternatively transported as hormones to activate glycogenolysis in the Müller cells. This results in the formation of a supply of glucose for ultimate utilization by surrounding neurones.

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Differential antagonism of the negative inotropic effect of gentamicin by calcium ions, Bay K 8644 and isoprenaline in canine ventricular muscle: comparison with cobalt ions

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1 Gentamicin (10^{-4}–10^{-2} M) and Co^{2+} (10^{-4} – 10^{-2} M) produced a decrease in developed tension of canine isolated ventricular muscles leading to near abolition at 10^{-2} M. Their negative inotropic effects developed rapidly and wore off shortly after wash-out.

2 The concentration-negative inotropic effect curves for gentamicin were shifted to the right in a parallel manner by increasing external Ca^{2+}, or by the presence of Bay K 8644 (10^{-7}–10^{-5} M) or isoprenaline (10^{-7}–10^{-5} M). IC_{50} values for gentamicin increased about 3-fold with about a 6 fold increase in external Ca^{2+}. The Schild plot yielded a pA_2 of 2.29 for Ca^{2+} and its slope was -1.17 ($r = -0.79$).

3 The concentration-negative inotropic effect curves for Co^{2+} were shifted to the right in a parallel manner by increasing external Ca^{2+}, or by the presence of isoprenaline (10^{-7}–10^{-5} M). IC_{50} values for Co^{2+} increased about 5 fold with about a 6 fold increase in external Ca^{2+}. The Schild plot gave a pA_2 value of 2.60 for Ca^{2+} and its slope was -1.11 ($r = -0.86$).

4 The concentration-positive inotropic effect curves for Ca^{2+}, which were computer-fitted to a logistic equation, gave 2.88×10^{-3} M for EC_{50}. This value was very close to the K_{Ca} calculated from pA_2 values for Ca^{2+} based on antagonism between gentamicin or Co^{2+} and Ca^{2+} (5.13×10^{-3} and 2.51×10^{-3} M).

5 It is concluded that like Co^{2+}, gentamicin molecules compete with Ca^{2+} for the same binding sites presumably located at the outer orifice of Ca-channels in the cardiac sarcolemma.

Introduction

Aminoglycoside antibiotics like streptomycin and neomycin have long been known to produce respiratory depression (by impairing neuromuscular transmission), hypotension (by relaxation of vascular smooth muscle) and myocardial depression. Since these effects were readily reversed by raising extracellular concentrations of Ca^{2+}, interference of aminoglycosides with the function of Ca^{2+} was suggested as the underlying mechanism (Swain *et al.*, 1956; Vital Brazil & Corrado, 1957; Corrado, 1963). The hypothesis has later been refined in such that at least at the prejunctional membrane of the motor endplate, aminoglycoside molecules compete with Ca^{2+} for binding sites presumably located near the outer orifice of Ca-channels to impede the Ca^{2+}-influx (Vital Brazil & Prado-Franceschi, 1969; Pittinger & Adamson, 1972). In cardiac muscle the negative inotropic effect of gentamicin, an aminoglycoside antibiotic, has also been found to be antagonized by Ca^{2+} apparently in a competitive manner (Adams, 1975). Thus, the mechanism of action of gentamicin may be to affect adversely either the transport system responsible for Ca^{2+} movement through Ca-channels, the availability of Ca^{2+} for transportation to these sites, or both (Adams & Durrett, 1978).

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The present experiments were designed to gain evidence for the hypothesis that aminoglycosides produce a negative inotropic effect by competing with Ca^{2+} for the same binding sites at the outer orifice of Ca-channels in the cardiac sarcolemma, as postulated at the motor endplate. For this purpose, gentamicin was chosen, because it has clearly been demonstrated to block the inward Ca^{2+}-current in guinea-pig papillary muscles (Hino *et al.*, 1982). The interaction of gentamicin with procedures that increase trans-sarcolemmal Ca^{2+} influx through different mechanisms (increase in extracellular Ca^{2+} concentrations, Bay K 8644 and isoprenaline) was investigated in canine isolated ventricular muscle. The positive inotropic effect of Bay K 8644 has been ascribed to the increased inward Ca^{2+}-current resulting from the prolonged open state of Ca-channels (Ochi *et al.*, 1984; Hess *et al.*, 1984) and that of isoprenaline to their increased opening probability (Reuter, 1983). For comparison, the antagonism of the negative inotropic effect of Co^{2+} by Ca^{2+} and isoprenaline was also investigated, because Co^{2+} is known to compete with Ca^{2+} for the same binding sites at the outer orifice of Ca-channels (Hagiwara & Byerly, 1981).

Methods

Mongrel dogs of either sex weighing 5 to 15 kg were anaesthetized with pentobarbitone sodium (30 mg kg^{-1}, i.v.) and hearts were excised. Right ventricular trabeculae were dissected from the hearts and mounted in 20 ml organ baths containing Krebs-Henseleit solution or HEPES buffered solution. Krebs-Henseleit solution was gassed with 95% O_2 and 5% CO_2 and HEPES buffered solution with 100% O_2 at a temperature of 37°C. The composition (mM) of the solutions was as follows: (1) Krebs-Henseleit solution: NaCl 118, KCl 4.7, $CaCl_2$ 2.55, $MgSO_4$ 1.18, KH_2PO_4 1.18, $NaHCO_3$ 24.9 and glucose 11.1; (2) HEPES buffered solution: HEPES 3, NaCl 140, KCl 4.7, $CaCl_2$ 2.55, $MgCl_2$ 2.5 and glucose 11.1 (pH 7.4). HEPES buffered solution was used to avoid the precipitation of insoluble salts in the experiments with Co^{2+} or to obtain the maximum developed tension by increasing Ca^{2+} concentrations.

Muscle preparations were stretched to a resting tension of about 500 mg and stimulated by square-wave pulses of a voltage of about 20% above threshold and 5 ms duration at a frequency of 0.5 Hz. Muscles were allowed to equilibrate for 1–1.5 h in Krebs-Henseleit solution and washed once or twice during the equilibration period. The developed tension was recorded on a thermal pen writing oscillograph (NEC San-ei Instrument, 8K-23) by the use of force displacement transducers (Shinkoh, UL-10). Four to eight muscles were isolated from each heart and run in parallel. One of them always served as control; a cumulative concentration-negative inotropic effect curve for gentamicin was determined at 2.55×10^{-3} M external Ca^{2+} in the absence of Bay K 8644 or isoprenaline. In the remaining muscles, similar concentration-effect curves were determined at various concentrations of external Ca^{2+} or in the presence of Bay K 8644 or isoprenaline at 2.55×10^{-3} M external Ca^{2+}. In these muscles gentamicin was administered when the developed tension had reached a plateau after changing external Ca^{2+} concentrations or adding Bay K 8644 or isoprenaline. The time required for the developed tension to reach a plateau was about 20, 60 or 10 min after changing external Ca^{2+} concentrations or adding Bay K 8644 or isoprenaline, respectively. Concentrations of gentamicin were increased at 5 min intervals, because each concentration of gentamicin produced its full effect within 5 min after administration. Experiments with Bay K 8644 were done under a sodium lamp.

Essentially similar experiments were done with Co^{2+} in the place of gentamicin in HEPES buffered solution except that in the equilibration period muscles were bathed in Krebs-Henseleit solution. In experiments in which the concentration of Ca^{2+} that produced a half maximum positive inotropic effect (EC_{50}) was determined, after the equilibration period Krebs-Henseleit solution was replaced by HEPES buffered solution containing 6.4×10^{-4} M Ca^{2+}. Concentrations of Ca^{2+} were increased cumulatively up to 2.0×10^{-2} M. The maximum developed tension was obtained at $1.0–1.25 \times 10^{-2}$ M Ca^{2+}. In all experiments preparations showing unstable resting tension or less than 100 mg of the developed tension were not used.

The drugs used were gentamicin sulphate (Shionogi), cobalt chloride (Wako), methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate (Bay K 8644, Bayer) and (−)-isoprenaline hydrochloride (Sigma). Bay K 8644 was dissolved in 99.5% ethanol at a concentration of 2×10^{-3} M and this stock solution was diluted with distilled water to the desired concentrations. Other drugs were dissolved in distilled water in the desired concentrations. These drug solutions were added to the organ baths in a volume of 50 or 100 μl.

Experimental values are given in terms of mean ± s.e.mean or geometric mean with 95% confidence limits in parentheses. For cumulative concentration-negative inotropic effect curves for gentamicin or Co^{2+}, effects were expressed as per-

centage suppression of the developed tension just before administration of this agent, whether positive inotropic intervention was absent (control) or present. The concentrations of gentamicin or Co^{2+} that produced a half maximum negative inotropic effect (IC_{50}) were determined in the following way. Each concentration-negative inotropic effect curve was computer-fitted to a logistic equation:

$$E = 100 \times A^p/(K^p + A^p) \quad (1)$$

where E is an effect of gentamicin or Co^{2+} expressed as above at a concentration A, K is the IC_{50} value of gentamicin or Co^{2+} and p is a slope parameter (Parker & Waud, 1971). Gentamicin failed to produce a 100% suppression of the developed tension at 10^{-2} M which was the maximum concentration obtainable because of its limited solubility, and instead the tension remained about 10%. However, it was assumed that gentamicin would produce a 100% suppression if further higher concentrations were available, because the concentration-negative inotropic curves for gentamicin did not seem saturated at 10^{-2} M. The concentration-ratios (r) of the IC_{50} of gentamicin or Co^{2+} were determined based on the IC_{50} values at 1.25×10^{-3} M external Ca^{2+}. pA_2 values were calculated from the Schild equation (Arunlakshana & Schild, 1959):

$$\log (r - 1) = pA_2 - a \times \log B \quad (2)$$

where a is the slope of linear regression and B is the concentration of Ca^{2+}.

The EC_{50} of Ca^{2+} and the slope parameter p of the concentration-positive inotropic effect curves for Ca^{2+} were determined by computer-fitting the curves expressed as percentages of the maximum developed tension to the logistic equation (1), where E is an effect of Ca^{2+} at a concentration A, K is the EC_{50} of Ca^{2+} and p is a slope parameter (Parker & Waud, 1971).

The developed tension increased by 10^{-5} M isoprenaline gradually decayed with time (decreased to 92% of its maximum 30 min after administration). Therefore, the negative inotropic effect of each concentration of gentamicin or Co^{2+} in the presence of 10^{-5} M isoprenaline was expressed as percentage decrease from the averaged developed tension obtained from the muscles ($n = 6$) in the presence of only 10^{-5} M isoprenaline at the corresponding time. Each preparation was subjected to only one concentration of external Ca^{2+}, Bay K 8644 or isoprenaline.

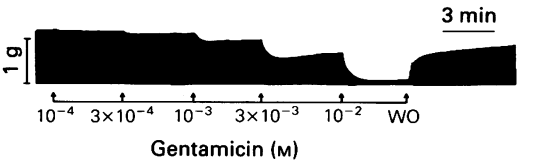

Figure 1 Negative inotropic effect of gentamicin in canine ventricular muscle. Gentamicin was administered cumulatively (10^{-4}–10^{-2} M). The negative inotropic effect of gentamicin developed rapidly and wore off shortly after wash-out.

Statistical significance between mean values was estimated by Student's *t* test. Analyses of parallelism of concentration-effect curves were carried out on the slope parameters described above. A *P* value less than 0.05 was considered significant.

Results

Effect of gentamicin on developed tension

Gentamicin (10^{-4}–10^{-2} M) produced a concentration-dependent suppression of the developed tension of ventricular muscles amounting to about 90% at 10^{-2} M (Figure 1). Although the effect of gentamicin in further higher concentrations of gentamicin was not obtainable because of its limited solubility, it seemed that gentamicin would produce a 100% suppression of the developed tension if its further higher concentrations were available. The negative inotropic effect of gentamicin emerged rapidly to reach a peak within 5 min after administration, and wore off rapidly with wash-out.

Negative inotropic effect of gentamicin in the presence of various concentrations of external Ca^{2+}

A reduction of external Ca^{2+} from 2.55×10^{-3} to 1.25×10^{-3} M resulted in a decrease in developed tension to nearly half the basal value, whereas elevations of external Ca^{2+} (to 5.05×10^{-3} and 7.55×10^{-3} M) increased developed tension; the increase was about 135% of the basal value at 7.55×10^{-3} M Ca^{2+}. The concentration-negative inotropic effect curves for gentamicin were shifted to the right in a concentration-dependent and parallel manner by increases in external Ca^{2+} (Figure 2). About a 6 fold increase in external Ca^{2+} resulted in about a 3 fold increase in the IC_{50} of gentamicin (Table 1). The Schild plot yielded the regression line within a slope of -1.17 ($r = -0.79$) and pA_2 value of 2.29 for Ca^{2+} (Figure 2, inset).

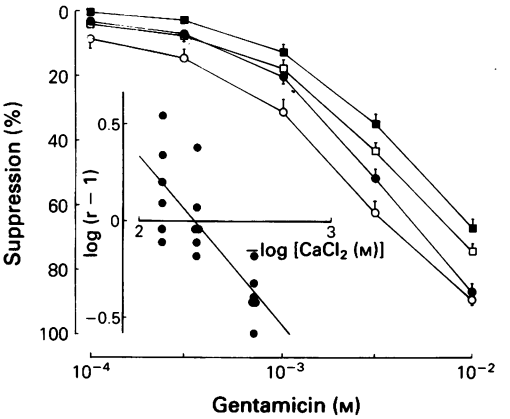

Figure 2 Concentration-negative inotropic effect curves for gentamicin in the Krebs-Henseleit solution containing 1.25 (○) ($n = 6$), 2.55 (●) ($n = 6$), 5.05 (□) ($n = 6$) and 7.55 (■) ($n = 6$) $\times 10^{-3}$ M Ca^{2+}. Effects are expressed as percentage suppression of the developed tension just before administration of gentamicin. Inset: Schild plot showing the competitive antagonism of the negative inotropic effect of gentamicin by Ca^{2+}. The pA_2 value was 2.29 and the slope of the regression line was −1.17. The correlation coefficient was −0.79.

Negative inotropic effect of gentamicin in the presence of Bay K 8644

As reported previously (Ishii *et al.*, 1985), Bay K 8644 (10^{-7}–10^{-5} M) produced a concentration-dependent increase in developed tension which was about 100% of the basal value at 10^{-5} M. The concentration-negative inotropic effect curves for gentamicin in the presence of Bay K 8644 are shown in Figure 3. The curves for gentamicin were shifted by these concentrations of Bay K 8644 to the right in a concentration-dependent manner to a comparable extent with changing external Ca^{2+} concentrations. These curves were parallel except for that in the presence of 10^{-5} M Bay K 8644. As shown in Table 1, the IC_{50} values of gentamicin were increased about 3 fold by 10^{-5} M Bay K 8644.

Negative inotropic effect of gentamicin in the presence of isoprenaline

Isoprenaline (10^{-7}–10^{-5} M) produced an increase in developed tension and at 10^{-5} M the developed tension nearly doubled. In the presence of isoprenaline the concentration-effect curves for gentamicin

Table 1 IC_{50} values of gentamicin and Co^{2+} in the absence (control) and in the presence of various concentrations of external Ca^{2+}, Bay K 8644 or isoprenaline

		n	*Developed tension* *Basal* (mg)	*Increase* (%)	*Mean* IC_{50} (10^{-3} M) (*Confidence limits*)
Gentamicin					
Ca^{2+} (10^{-3} M)	1.25	6	888 ± 223	−53.0 ± 3.7	1.86 (1.49–2.33)
	2.55	6	809 ± 161		2.88** (2.41–3.45)
	5.05	6	482 ± 159	53.5 ± 13.5	3.80** (3.17–4.55)
	7.55	6	419 ± 54	134.8 ± 40.8	5.50** (4.39–6.89)
Control		6	844 ± 143		2.51 (2.00–3.15)
Bay K 8644 (M)	10^{-7}	7	627 ± 168	33.5 ± 7.5	3.55 (2.59–4.87)
	10^{-6}	6	164 ± 9	91.1 ± 25.0	5.89** (3.92–8.84)
	10^{-5}	6	346 ± 67	95.1 ± 30.1	8.13* (3.77–17.51)
Control		5	1008 ± 283		2.63 (2.10–3.30)
Isoprenaline (M)	10^{-7}	6	1030 ± 175	53.6 ± 11.3	4.27 (3.25–5.59)
	10^{-6}	5	1552 ± 199	65.3 ± 33.3	5.25* (4.19–6.58)
	10^{-5}	6	748 ± 180	92.1 ± 19.3	6.46* (4.71–8.86)
Co^{2+}					
Ca^{2+} (10^{-3} M)	1.25	7	495 ± 95	−54.9 ± 5.3	0.63 (0.53–0.76)
	2.55	5	371 ± 83		1.29** (0.94–1.77)
	5.05	5	485 ± 137	47.0 ± 10.9	2.19** (1.67–2.87)
	7.55	5	238 ± 38	81.6 ± 12.0	3.24** (2.83–3.71)
Control		5	964 ± 393		1.32 (1.26–1.38)
Isoprenaline (M)	10^{-7}	5	522 ± 129	48.5 ± 14.7	1.20 (1.00–1.44)
	10^{-6}	5	1463 ± 355	66.2 ± 9.6	2.24* (1.49–3.36)
	10^{-5}	5	590 ± 144	124.0 ± 17.1	2.00** (1.83–2.18)

*$P < 0.05$; **$P < 0.01$ against IC_{50} values in the presence of 1.25×10^{-3} M Ca^{2+} or in the control conditions.

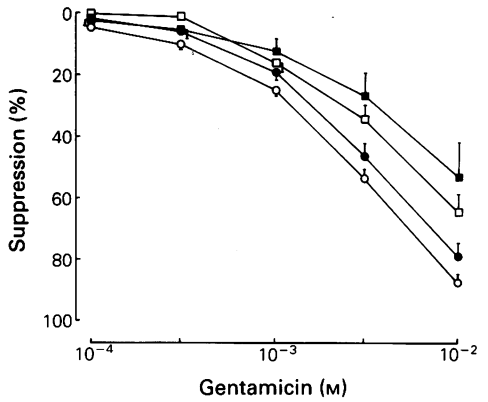

Figure 3 Concentration-negative inotropic effect curves for gentamicin in the absence (○) ($n = 6$) and presence of Bay K 8644 at 10^{-7} M (●) ($n = 7$), 10^{-6} M (□) ($n = 6$) or 10^{-5} M (■) ($n = 6$).

were shifted to the right in a concentration-dependent and parallel manner to a comparable extent with increasing external Ca^{2+} concentrations or Bay K 8644 (Figure 4). The IC_{50} values of gentamicin were increased about 2.5 fold by 10^{-5} M isoprenaline (Table 1).

Negative inotropic effect of Co^{2+} in the presence of different concentrations of external Ca^{2+}

In the presence of the normal concentration of external Ca^{2+} (2.55×10^{-3} M) Co^{2+} (10^{-4}–10^{-2} M) produced a concentration-dependent negative inotropic effect and at 10^{-2} M the developed tension was nearly abolished. The onset and wearing off with wash-out of the negative inotropic effect of Co^{2+} were rapid as were those of gentamicin. With a reduction of external Ca^{2+} from 2.55×10^{-3} to 1.25×10^{-3} M the concentration-negative inotropic effect curve for Co^{2+} was shifted to the left, and with elevations of external Ca^{2+} to 7.55×10^{-3} M the concentration-effect curves for Co^{2+} were shifted to the right in a concentration-dependent and parallel manner (Figure 5). The IC_{50} values of Co^{2+} increased about 5 fold with an approximately 6 fold increase in external Ca^{2+} (Table 1). The Schild plot yielded the regression line with a slope of -1.11 ($r = -0.86$) and gave a pA_2 value of 2.60 for Ca^{2+} (Figure 5, inset).

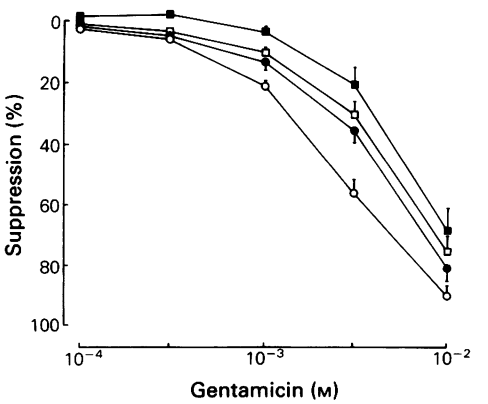

Figure 4 Concentration-negative inotropic effect curves for gentamicin in the absence (○) ($n = 5$) and presence of isoprenaline at 10^{-7} M (●) ($n = 6$), 10^{-6} M (□) ($n = 5$) or 10^{-5} M (■) ($n = 6$).

Negative inotropic effect of Co^{2+} in the presence of isoprenaline

In the presence of isoprenaline (10^{-7}–10^{-5} M) the concentration-effect curves for Co^{2+} were shifted to the right in a concentration-dependent and parallel manner to a similar extent to the shift of the concentration-effect curves for gentamicin. The IC_{50} values of Co^{2+} were increased about 1.5 fold by 10^{-5} M isoprenaline (Table 1).

Concentration of Ca^{2+} that produced a half maximum developed tension

The concentration-positive inotropic effect curves for external Ca^{2+} were obtained in HEPES buffered solution by increasing concentrations of Ca^{2+} from 6.4×10^{-4} to 1.25×10^{-2} M. The maximum developed tension obtained was $3{,}276 \pm 439$ mg ($n = 8$). Computer-fitting of each concentration-effect curve to the logistic equation (1) yielded 1.89 ± 0.10 as the slope parameter and gave 2.88 (2.51–3.31) $\times 10^{-3}$ M for the EC_{50}.

Discussion

In the present experiments gentamicin produced a negative inotropic effect with rapid onset and wearing off upon wash-out in canine ventricular muscle, as has been observed in rat atria (Adams, 1975). Co^{2+} produced a similar negative inotropic effect. In the presence of increasing external Ca^{2+}, the concentration-negative inotropic effect curves for gentamicin were shifted to the right in a parallel

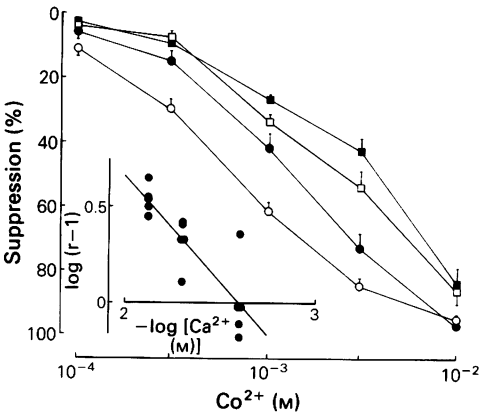

Figure 5 Concentration-negative inotropic effect curves for Co^{2+} in the HEPES buffered solution containing 1.25 (○) ($n = 7$), 2.55 (●) ($n = 5$), 5.05 (□) ($n = 5$) and 7.55 (■) ($n = 5$) $\times 10^{-3}$ M Ca^{2+}. Inset: Schild plot showing the competitive antagonism of negative inotropic effect of Co^{2+} by Ca^{2+}. The pA_2 value was 2.60 and the slope of the regression line was −1.11. The correlation coefficient was −0.86.

manner. The Schild plot of antagonism of the negative inotropic effect of gentamicin by Ca^{2+} yielded unity as the absolute value for the slope of the regression line. This antagonism was very similar to that seen with Co^{2+} and Ca^{2+}; the concentration-negative inotropic effect curves for Co^{2+} were shifted to the right in a parallel manner by increasing external Ca^{2+} and the Schild plot gave unity as the absolute value of the slope of the regression line. Co^{2+} has been known to compete with Ca^{2+} for the same binding sites at the outer orifice of Ca-channels in excitable membrane (Hagiwara & Byerly, 1981). Thus, like Co^{2+}, gentamicin molecules as polyvalent cations may compete with Ca^{2+} for the same binding sites at the outer orifice of Ca-channels in the cardiac sarcolemma. This supposition is in line with that proposed at the prejunctional membrane in the motor endplate (Vital Brazil & Prado-Franceschi, 1969; Pittinger & Adamson, 1972). However, the present experiments give no direct evidence that gentamicin and Co^{2+} are competing for the same site with Ca^{2+} and some other form of interaction remains a possibility.

The Schild plot of the antagonism of the negative inotropic effect of gentamicin by Ca^{2+} gave a pA_2 value of 2.29 for Ca^{2+}. The Schild plot of the antagonism of the negative inotropic effect of Co^{2+} also yielded a pA_2 value of 2.60 for Ca^{2+}. The close proximity of two pA_2 values for Ca^{2+} determined from antagonism against the different agonists (difference between two pA_2 values was less than 0.5) suggests that Ca^{2+}, gentamicin and Co^{2+} bind to the same type of binding sites (Furchgott, 1972). The pA_2 values for Ca^{2+} yielded 5.13×10^{-3} and 2.51×10^{-3} M for dissociation constants, K_{Ca}, between Ca^{2+} and its binding sites. The EC_{50} value for Ca^{2+} was 2.88×10^{-3} M. The close proximity of K_{Ca} and the EC_{50} value for Ca^{2+} suggests that when a half population of Ca^{2+} binding sites presumably located at the outer orifice of Ca-channels is occupied by Ca^{2+}, the Ca^{2+}-influx through Ca-channels occurring as a result would be enough to produce a half maximum positive inotropic effect.

The negative inotropic effect of gentamicin or Co^{2+} was far less efficiently antagonized by Bay K 8644 or isoprenaline than by Ca^{2+}, a 100 fold increase in concentration of Bay K 8644 or isoprenaline being required for about a 2 fold increase in the IC_{50} of gentamicin or Co^{2+}. This is understandable because it is unlikely that competition of gentamicin or Co^{2+} with Ca^{2+} for the same binding sites at the outer orifice of Ca-channels is influenced by whether the open state of Ca-channels is prolonged by Bay K 8644 (Ochi *et al.*, 1984; Hess *et al.*, 1984) or the opening probability of Ca-channels is increased by isoprenaline (Reuter, 1983).

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Effects of hydroxyethyl rutosides upon the permeability of single capillaries in the frog mesentery

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1 We have investigated the effects of a standardised mixture of hydroxyethyl rutosides (HR) upon the permeability of the walls of single capillaries and venules of the frog mesentery.

2 In each experiment a single vessel was perfused via a micropipette with frog Ringer solutions containing bovine serum albumin ($10\,mg\,ml^{-1}$) and Ficoll 70 ($40\,mg\,ml^{-1}$) first in the absence of HR and then with HR added to the perfusate. The permeability of the vessel walls was assessed during each perfusion by using a development of the Landis micro-occlusion technique to estimate their hydraulic permeability (L_p) and the effective osmotic pressure ($\sigma\Delta\pi$) exerted across them by the perfusate macromolecules.

3 Measurements were made both in vessels which appeared to be healthy and in vessels showing signs of stasis or inflammation before perfusion.

4 HR at concentrations of 1.0, 0.1 and $0.01\,mg\,ml^{-1}$ reduced hydraulic permeability to approximately half of its value in the absence of HR. It increased $\sigma\Delta\pi$ to macromolecules at concentrations of 10, 1.0, 0.1, 0.01 and $0.001\,mg\,ml^{-1}$. The effects of HR upon permeability were not reversed within 10 min of perfusion with an HR-free solution.

5 Ultrastructural examination of a number of vessels in which initial high values of permeability were reduced to values within the normal range of permeabilities by HR, showed clear signs of damage to the endothelium, with large gaps between adjacent endothelial cells.

6 These observations suggest that HR does reduce microvascular permeability both in healthy vessels and vessels showing signs of inflammation. The reduction in permeability of inflamed vessels does not appear to be the result of closure of the gaps between adjacent endothelial cells.

Introduction

It has been shown that flavonoids, such as the derivatives of rutin, can reduce the oedema of the lower limbs of patients suffering from venous hypertension and that they do so by promoting a reduction in capillary filtration (Roztocil *et al.*, 1977). Flavonoids have been shown to diminish histamine and bradykinin induced leakage of albumin in rat skin (Gerdin & Svensjo, 1983) and to reduce the permeability of the microvasculature of the dog paw to dextrans following thermal injury (Hilton, 1982). Their mode of action, however, remains uncertain.

To investigate more fully the effects of flavonoids on permeability, we have examined their effects upon the permeability of single perfused capillaries and venules in the frog mesentery. We have measured the hydraulic permeability (L_p) of the walls of these vessels and the effective osmotic pressure ($\sigma\Delta\pi$) exerted across them by perfusate macromolecules using a development of the Landis micro-occlusion technique (Michel *et al.*, 1974; Michel, 1980). Measurements have been made on the same vessel during perfusion with a control solution and during perfusion with a solution containing a standardised mixture of β-hydroxyethyl rutosides (HR). By working on single vessels, changes in permeability can be measured independently of changes in microvascular surface area, microvascular flow and microvascular pressure, all of which may complicate the investigation of changes in permeability in whole vascular beds.

We have examined the effects of HR upon vessels which appeared healthy and upon vessels which showed signs of inflammation. At the completion of six experiments, the tissues of the mesentery were

fixed, prepared for electronmicroscopy and the ultrastructure of the vessel, in which a permeability change had been measured, was then examined.

The results have been presented in part to The Physiological Society (Blumberg *et al.*, 1988) and to the British Microcirculation Society (Blumberg *et al.*, 1987).

Methods

Experiments were performed on single capillaries and venules of the mesenteries of *Rana temporaria* and *Rana pipiens* whose brain and upper spinal cord were destroyed by pithing. The mesentery was exposed through a lateral incision in the abdominal wall and draped over a small Perspex pillar raised in the stage of a Wild M8 stereomicroscope. The microcirculation was viewed using transmitted light from a cold light source and the tissue prevented from drying by continuous superfusion with a cooled, oxygenated frog Ringer solution.

Solutions

All solutions were made up on the day of experimentation and stored in the refrigerator before use. A standardised mixture of β-hydroxyethyl rutosides (by courtesy of Zyma U.K.) was used in all experiments. The composition of this mixture (HR) is given in Table 1.

The Ringer superfusate contained (mmol l^{-1}): NaCl 111.1, KCl 2.4, $MgSO_4 7H_2O$ 1.0, glucose 5.5, $CaCl_2$ 1.1, adjusted to pH 7.2–7.4 with $NaHCO_3$ (10 mmol l^{-1}). The perfusate solutions were Ringer solutions to which had been added bovine serum albumin at a concentration of 10 mg ml^{-1} and the neutral macromolecule, Ficoll 70 at a concentration of 40 mg ml^{-1}, which together had a mean *in vitro* oncotic pressure of 24 cmH_2O. Hydroxyethyl rutosides (HR) were added to the perfusate solution at concentrations of between 0.0001 mg ml^{-1} and 10 mg ml^{-1}. The perfusate solutions contained no bicarbonate and their pH was adjusted before use by addition of 0.23 M NaOH. The *in vitro* osmotic pressures of the HR-containing perfusates ranged from 23 cmH_2O to 27 cmH_2O. A few washed human red cells were added to all the perfusate solutions to act as markers of fluid flow.

Table 1 Composition of standard mixture of hydroxyethyl rutosides

Component	*Molecular weight*	*Concentration in mixture* (g 100 g^{-1})
Rutin	610.53	0.1–1.0
Monohydroxyethylrutosides	654.58	5–10
Dihydroxyethylrutosides	698.63	30–38
Trihydroxyethylrutosides	742.69	45–55
Tetrahydroxyethylrutosides	786.74	3–12

Measurement of permeability

Long straight unbranched capillaries and venules were selected and each vessel cannulated with a sharpened glass micropipette (tip diameter 10 to 15 μm) and perfused via the pipette at a pressure of 30 cmH_2O. Five to ten min after the start of perfusion, the vessel was briefly occluded some distance downstream from the site of cannulation and the movement of red cells trapped within the occluded segment recorded on videotape for later analysis.

Fluid flux across unit area of capillary wall (J_v/A) was calculated from the movements of the red cells within the occluded segment of vessel at two perfusion pressures (P_c) of approximately 30 and 15 cmH_2O, using the methods described by Michel (1980). Several occlusions were made at each pressure during every perfusion. The relation between J_v/A and P_c has been shown to be linear over a wide range of values of P_c (Michel *et al.*, 1974; Mason *et al.*, 1977; Curry *et al.*, 1976)). The slope of the relation is the hydraulic permeability, L_p, and its intercept with the P_c axis is the effective osmotic pressure exerted across the capillary walls, $\sigma\Delta\pi$ (see Figure 1).

Experimental protocol

In the first series of experiments, designed to investigate the effects of HRs on the permeability of single capillaries and venules, permeability was initially assessed whilst the vessel was perfused with the control perfusate (see solutions). The vessel was then recannulated and reperfused with a second solution to which had been added hydroxyethyl rutoside at a known concentration. The permeability coefficients of the vessel were re-estimated after 5 to 10 min perfusion with the HR-containing solutions.

In order to investigate the reversibility of the effects of HR upon permeability, a second series of experiments were performed in which three measurements of permeability were made. The first two were as above. The vessels were then cannulated for a third time and reperfused with the control solution, and permeability in the absence of HR was reassessed 5 to 10 min after the start of reperfusion.

Nine further experiments were performed in which the vessels were cannulated and perfused twice, but where both the first and second perfusates were the control solution containing no HR. In this way the effects of repeated cannulation and perfusion upon

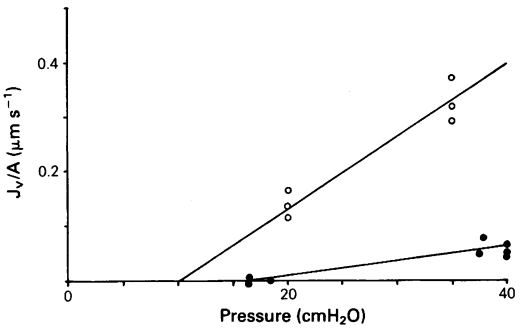

Figure 1 Effects of hydroxyethyl rutosides (HR) upon the permeability of a single vessel perfused initially with Ringer solution containing 40 mg ml^{-1} Ficoll 70 and 10 mg ml^{-1} BSA (○) and then with a similar solution containing HR (0.1 mg ml^{-1}) (●). J_v/A = fluid flux across unit area of capillary wall. The slope of the relation gives the hydraulic permeability (L_p) and the intercept with the pressure axis the effective osmotic pressure ($\sigma\Delta\pi$) exerted by the colloids across the capillary wall.

the permeability coefficients of a single vessel could be investigated.

Preparation for electron microscopy

In six experiments in which permeability had been assessed before and during perfusion with HR-containing solutions, the ultrastructural appearance of the vessels was examined using transmission electron microscopy. The tissues were fixed by flooding the mesentery with 2.5% glutaraldehyde, 2.5% formaldehyde in 0.1 M cacodylate buffer whilst perfusion of the vessel with HR-containing solution was maintained at a pressure of 30 cmH_2O. Following post fixation in 0.2% osmium tetroxide, the tissue was embedded and ultrathin transverse sections of the perfused section of vessel examined in a Jeol 100CX transmission electron microscope. Particular attention was paid to the appearance of the endothelium and the endothelial cell clefts of the walls of the perfused vessels.

Statistics

L_p was estimated from the slope of the regression line relating values of J_v/A to P_c during perfusion with a given solution. The intercept of this line on the pressure axis was used to estimate $\sigma\Delta\pi$.

Paired t tests were used to estimate the effects of HR on L_p and $\sigma\Delta\pi$, as shown in Table 2.

Results

The effects of HR upon hydraulic permeability (L_p) were investigated in capillaries and venules perfused with solutions containing HR at concentrations ranging from 0.001 to 10 mg ml^{-1}. Figure 1 shows the results of one experiment where a single vessel was perfused first with Ringer solution containing 40 mg ml^{-1} Ficoll 70 and bovine serum albumin (BSA, 10 mg ml^{-1}) and then with a similar solution to which HR was added at a concentration of 0.1 mg ml^{-1}.

Table 2 Effects of different perfusate concentrations of hydroxyethyl rutosides (HR) on the permeability of single frog mesenteric capillaries

	Concentration of HR in perfusate (mg ml^{-1})						
	0	*0.0001*	*0.001*	*0.01*	*0.1*	*1.0*	*10*
L_{po} (before HR)	40.18	22.94	22.60	20.53	20.61	18.12	11.88
cm s^{-1} cmH_2O^{-1} × 10^{-7}	10.57	6.57	4.69	3.32	4.40	4.92	2.52
± s.e.mean							
L_{pHR} (after HR)	39.62	31.31	18.99	5.87*	11.15**	6.26*	10.87
cm s^{-1} cmH_2O^{-1} × 10^{-7}	10.88	7.77	5.18	1.05	2.25	1.11	2.72
± s.e.mean							
$\sigma\Delta\pi$ (before HR)	9.33	10.58	11.83	11.84	8.55	11.17	17.70
cmH_2O	1.50	1.31	3.01	3.53	1.63	1.12	0.78
± s.e.mean							
$\sigma\Delta\pi_{HR}$ (after HR)	10.11	863	20.90**	16.77**	13.88***	13.99	21.77*
cmH_2O	1.34	297	3.63	0.96	1.59	1.46	1.57
± s.e.mean							
n	9	6	6	7	16	10	8

* $P \leq 0.05$; ** $P \leq 0.02$; *** $P < 0.001$.

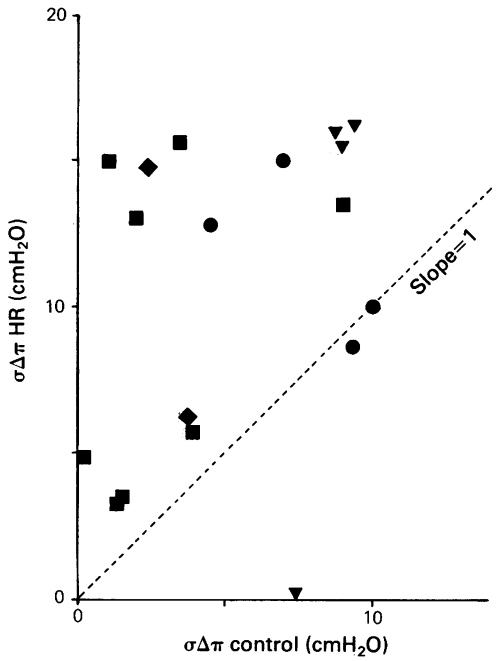

Figure 2 Effect of hydroxyethyl rutosides (HR) upon the effective osmotic pressure ($\sigma\Delta\pi$) of leaky vessels ($\sigma\Delta\pi$, control < 10 cmH_2O) perfused with solutions containing 40 $mg\,ml^{-1}$ Ficoll 70 and 10 $mg\,ml^{-1}$ BSA. Different symbols indicate different concentrations of HR: (◆) 0.001 $mg\,ml^{-1}$, (▼) 0.01 $mg\,ml^{-1}$, (■) 0.1 $mg\,ml^{-1}$, (●) 1.0 $mg\,ml^{-1}$.

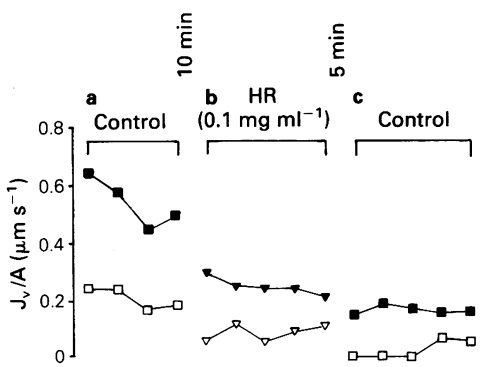

Figure 3 Measurements of fluid flux (J_v/A) across the walls of a single vessel perfused (a) first with a solution containing no hydroxyethyl rutosides (HR), then (b) with the HR solution and (c) for a third time with a solution containing no HR. Solid symbols represent J_v/A when capillary pressure was 30 cmH_2O; open symbols, J_v/A when pressure was 15 cmH_2O.

The data have been plotted as a relation between fluid filtration per unit area of vessel wall (J_v/A) and capillary pressure (P_c) (Figure 1). It is seen that HR reduces the slope of the relation, L_p, from a value of 11.8×10^{-7} $cm\,s^{-1}\,cmH_2O^{-1}$ to one of 2.8×10^{-7} $cm\,s^{-1}$ cmH_2O^{-1}. The effective osmotic pressure opposing filtration, $\sigma\Delta\pi$, increased from 10.3 cmH_2O (control) to 16.2 cmH_2O in the presence of HR. When the osmotic pressures of the same solutions were measured *in vitro* in a membrane osmometer, they had a value of 25 cmH_2O. The increase in $\sigma\Delta\pi$ represents a reduction in the permeability of the vessel wall to the perfusate macromolecules. Thus in this vessel HR reduced both L_p and permeability to macromolecules.

Between six and sixteen experiments were carried out at each perfusate concentration of HR. In nine additional experiments which acted as controls, the second perfusate contained no HR. All these data are summarized in Table 2.

When present in concentrations ($mg\,ml^{-1}$) of 1.0, 0.1 and 0.01, HR reduced L_p and increased $\sigma\Delta\pi$. At concentrations of 10 $mg\,ml^{-1}$ and 0.001 $mg\,ml^{-1}$ it increased $\sigma\Delta\pi$ without significantly reducing L_p. No effect on either L_p or $\sigma\Delta\pi$ was observed when the HR concentration was 0.0001 $mg\,ml^{-1}$. In nine experiments where the second perfusate contained no HR, the values of L_p and $\sigma\Delta\pi$ did not differ significantly between successive perfusions. Thus the procedure of recannulation and perfusion of the vessels did not change permeability.

Since HR gave rise to similar percentage changes in L_p in normal and leaky vessels, data from both types of vessel have been used to calculate the mean values in Table 2. The effect of HR upon $\sigma\Delta\pi$ of leaky vessels, however, is shown in Figure 2 and deserves further comment. For these purposes leaky vessels are defined as those where $\sigma\Delta\pi$ was less than 10 cmH_2O in the absence of HR. In 10 out of 18 vessels of this kind, varying concentrations of HR raised $\sigma\Delta\pi$ into the range of 12.5 cmH_2O to 16.5 cmH_2O. In 7 of the remaining 8 vessels, HR had relatively small effects on $\sigma\Delta\pi$ and in one vessel it appeared to reduce it.

Figure 2 also reveals that there was no correlation between the increase in $\sigma\Delta\pi$ and the concentration of HR in the perfusate. The lack of correlation between perfusate concentration of HR and the effect of HR on permeability is also evident in Table 2.

Non-reversibility of effects of HR in the short term

The extent to which the effects of 0.01 $mg\,HR\,ml^{-1}$ could be reversed was examined in six vessels. Each vessel was perfused first with a solution containing no HR, then with the HR solution and finally with a solution containing no HR. Several estimates of J_v/A

were made at both 30 cmH_2O and 15 cmH_2O during each perfusion. The third (HR free) perfusion was always continued for 10 min and usually for longer. Data from such an experiment are shown in Figure 3 where it is seen that HR reduced J_v/A at both 15 cmH_2O and 30 cmH_2O and these reductions of J_v/A were not reversed by subsequently perfusing with the control solution, even though the latter perfusion continued for 14 min. This finding was characteristic of the observations made on all six vessels where HR had reduced fluid filtration. In no vessel could fluid filtration be increased by perfusing with the control solution: in some vessels J_v/A continued to fall during reperfusion with the control solution as a result of a continuing fall of L_p.

Ultrastructural studies

The ultrastructural appearance of the walls of six vessels perfused with HR-containing solutions was examined using transmission electron microscopy. In all these vessels the initially high values of L_p had been considerably reduced by perfusion with HR. All six vessels showed signs of endothelial damage (Figure 4) and attenuation.

In some vessels, gaps as large as 1.0 μm to 5.0 μm were seen between adjacent endothelial cells. Intercellular gaps and other obvious signs of damage were not seen in two other vessels where L_p was initially low, nor are such appearances normally seen in vessels free from signs of inflammation *in vivo* (Mason *et al.*, 1979).

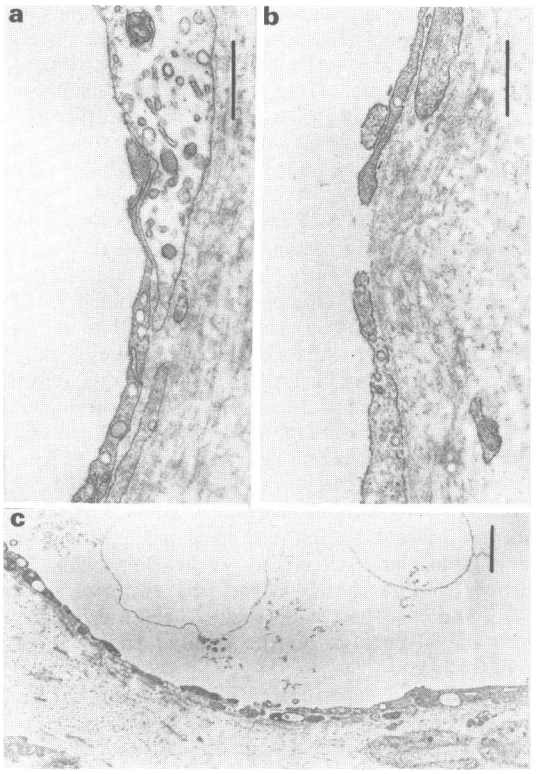

Figure 4 Electron micrographs of vessels perfused with hydroxyethyl rutoside (HR)-containing solutions before fixation. In each the initially high values of hydraulic permeability (L_p) had been considerably reduced by perfusion with HR. Note the attenuated endothelium and the large gaps between adjacent cells. Bar = 0.5 μm (a and b), 10 μm (c).

Discussion

Our observations show that the permeabilities of frog mesenteric capillaries to fluid and macromolecules are reduced in the presence of HR. This reduction appears to be independent of the concentration of HR in the perfusate over the range of 0.001 $mg\,ml^{-1}$ to 10 $mg\,ml^{-1}$ and it persists for 15 min or more after HR has been removed from the perfusate.

Although some experiments were carried out on vessels where initially the permeability was normal, the majority of experiments were carried out on leaky vessels. This is appreciated when the values for L_p in Table 2 are compared with mean values for the 'normal L_p' of frog mesenteric capillaries. In a number of studies in our laboratory on the L_p of the frog mesenteric microvessels, mean values of between 2×10^{-7} $cm\,s^{-1}\,cmH_2O^{-1}$ and 6×10^{-7} $cm\,s^{-1}$ cmH_2O^{-1} have been found for mid-capillaries and mean values of 1×10^{-6} to 1.5×10^{-6} $cm\,s^{-1}\,cmH_2O^{-1}$ for venous capillaries (Michel *et al.*, 1974; Curry *et al.*, 1976; Michel, 1980; Michel & Phillips, 1985; 1987). Mean values of $\sigma\Delta\pi$ to perfusates containing the same concentrations of Ficoll 70 and BSA have been found to be between 16 cmH_2O and 19 cmH_2O in normal frog mesenteric vessels (Michel & Phillips, 1985; 1987). It should be noted that $\sigma\Delta\pi$ is less than the perfusate oncotic pressure in normal vessels not only because, σ, the osmotic reflection coefficient to perfusate macromolecules, is less than unity but also because the presence of perfusate macromolecules in the pericapillary spaces reduces the difference in osmotic pressure ($\Delta\pi$) across the vessel walls below the perfusate oncotic pressure, π_p. Under conditions of high steady filtration, $\sigma\Delta\pi$ approximates to its maximal value which is $\sigma^2\pi_p$ (Michel & Phillips, 1987). Thus the rise in $\sigma\Delta\pi$ associated with perfusion with HR indicates a rise in σ to Ficoll 70.

Whereas L_p is determined by the dimensions and frequency of the channels conducting fluid through the microvascular walls, σ is determined by channel dimensions alone. Thus a fall in L_p indicates a narrowing of the channels. Where the endothelium is continuous, the fluid conducting channels are believed to be located in the intracellular clefts (Karnovsky, 1967; Wissig & Williams, 1978; Bundgaard, 1984) and there is good evidence that this is true in frog mesenteric capillaries (Clough & Michel, 1988b). Many believe that the limiting dimensions of the channels are determined by the widths of the clefts, i.e. the distance within the cleft between the outer leaflets of the membranes of adjacent cells (e.g. Crone & Levitt, 1984). On this hypothesis, the molecular filtration of macromolecules occurs at the narrow regions of the cleft (Ward *et al.*, 1988) or at pores which penetrate the lines of fusion in the junctional regions (Bundgaard, 1984). An alternative hypothesis considers that a lattice of fibrous molecules reinforced by plasma protein acts as a molecular filter (Curry & Michel, 1980). This lattice or fibre matrix covers the entrance to the clefts and fills their wide regions, being continuous with the glycocalyx on the luminal surface of the endothelium.

When vascular permeability is increased in inflammation or after the application of a wide variety of mediators, the clefts between the endothelial cells of the post-capillary venules are opened into large ($\geq 0.1\,\mu m$) gaps (Majno & Palade, 1961). Until recently these gaps have usually been regarded as holes, offering unrestricted passage to fluid and macromolecules between the vessel lumen and the interstitial space. Occasionally this view has been questioned (Renkin *et al.*, 1974; Diana *et al.*, 1972), on the grounds that such large holes in microvascular walls should increase permeability more than has been measured. Recent studies from our own laboratory have led to the suggestion that the gaps are not empty holes but contain material which acts as a barrier to the movement of fluid and macromolecules (Clough *et al.*, 1988; Clough & Michel, 1988a).

The observations presented in this paper, that large gaps may persist between the endothelial cells after permeability has been reduced by HR is further evidence for a barrier to fluid and macromolecules within the gaps. Whereas HR could reduce L_p by reducing the frequency of the gaps, a simple explanation of the increase in $\sigma\Delta\pi$ would be that HR increases σ in those gaps that persist. We can only speculate on how such an increase in σ might be achieved. HR could bind directly to existing structures within the gaps, cross-linking plasma proteins and fibrous molecules of the glycocalyx and basement membrane into a more selective filter. Alternatively it could act on the endothelial cells, stimulating them to secrete more fibre matrix or to manipulate the material that is already present (e.g. by secretion of enzymes). The failure to show a clear concentration-dependence of the effects of HR on permeability and the persistence of its effects after washing with HR-free solutions would favour a cellular mechanism for the action of HR. They do not, however, exclude the possibility of an extracellular mechanism.

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Kinins act on B_1 or B_2 receptors to release conjointly endothelium-derived relaxing factor and prostacyclin from bovine aortic endothelial cells

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1 Bradykinin (Bk) induced the coupled release of endothelium-derived relaxing factor (EDRF) and prostacyclin (PGI_2) from bovine aortic endothelial cells grown in culture. The B_2 kinin receptor antagonist, [D-Arg^0,Hyp^3,Thi^{5,8},D-Phe^7]-Bk, abolished this release by Bk.

2 Des-Arg^9-Bk, a B_1 kinin receptor agonist, also induced the release of EDRF and PGI_2, but much higher concentrations were required to obtain a similar release to that induced by Bk.

3 [Leu^8],des-Arg^9-Bk, a B_1 receptor antagonist, significantly reduced the response to des-Arg^9-Bk without affecting the release induced by Bk.

4 The release of EDRF and PGI_2 induced by arachidonic acid or ADP was not significantly affected by the B_2 or the B_1 antagonist.

5 We conclude, therefore, that Bk and des-Arg^9-Bk were acting respectively on B_2 and B_1 bradykinin receptors.

6 The possible role of kinin receptors in the release of EDRF and PGI_2 from endothelial cells is discussed.

Introduction

Bradykinin (Bk), among other vasoactive agents, is a potent stimulant for the release of endothelium-derived relaxing factor (EDRF) from bovine (Cocks *et al.*, 1985; de Nucci *et al.*, 1988a) and porcine (Gryglewski *et al.*, 1986) aortic endothelial cells (EC). Bk and its analogues induce endothelium-dependent relaxation of canine arterial rings via B_2 receptors for kinins (Regoli *et al.*, 1986) and of rabbit mesenteric artery strips via B_1 receptors (Churchill & Ward, 1986; Deblois & Marceau, 1987). Kinins stimulate release of prostacyclin (PGI_2) from bovine pulmonary artery endothelial cells, Bk being 100 times more potent than des-Arg^9-Bk (Crutchley *et al.*, 1983). Bk also induces a coupled release of EDRF and PGI_2 from bovine aortic EC through the activation of B_2 receptors (de Nucci *et al.*, 1988a). We describe here a further characterization of the functional B_2 receptors found in the EC by the use of a selective B_2 antagonist: [D-Arg^0,Hyp^3,Thi^{5,8},D-Phe^7]- Bk (Vavrek & Stewart, 1985). In addition, we have studied the release of EDRF and PGI_2 by the N-terminal octapeptide of Bk through the activation of B_1 receptors. Hence, Bk and des-Arg^9-Bk induce a coupled release from EC through distinct kinin receptor populations.

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Methods

Preparation of bovine aortic endothelial cells

Endothelial cells were isolated by treatment of bovine aortae with 0.02% w/v collagenase. The cells were grown to confluence in plastic vessels and then removed by treatment with 0.05% w/v trypsin and seeded onto cytodex 3 microcarrier beads. The beads were stirred for 3–7 days until the cells became confluent (de Nucci *et al.*, 1988a). Then, 2–3 ml of beads covered with 10–20 $\times$ 10^6 cells were packed into a jacketed column, and perfused (5 ml min^{-1} at 37°C) with gassed (95% O_2/5% CO_2) Krebs solution.

Bioassay

New Zealand white rabbits (2.5–3.0 kg) were anaesthetized with a solution of pentobarbitone sodium

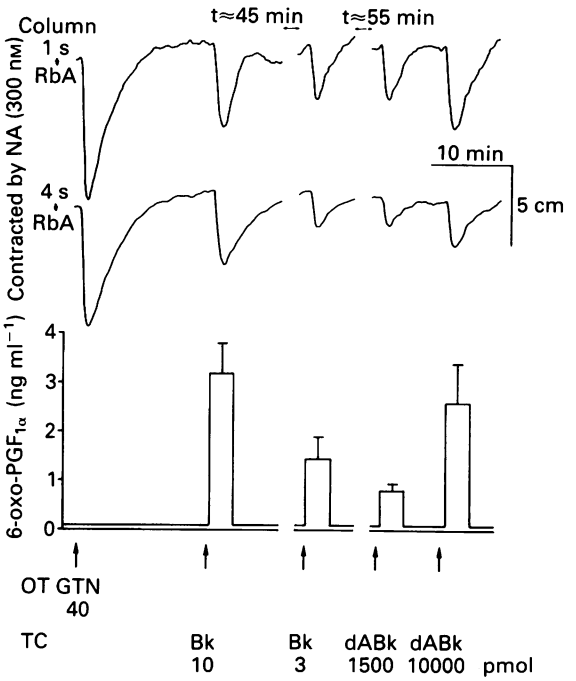

Figure 1 Release of endothelium-derived relaxing factor (EDRF) and prostacyclin (PGI_2) by bolus injections of bradykinin (Bk) and des-Arg^9-Bk (dABk) from a column of bovine aortic endothelial cells. The effluent from the column superfused a cascade of four rabbit aortic strips (RbAs); the first two are shown. The tissues were precontracted with noradrenaline (NA; 300 nM). Drugs were injected through the column (TC) or directly over the tissues (OT). Bk (3 and 10 pmol) or dABk (1,500 and 10,000 pmol) injected TC released EDRF and PGI_2. Superoxide dismutase (10 u ml^{-1}) was infused TC throughout the experiment. PGI_2 was measured as its breakdown product, 6-oxo-$PGF_{1\alpha}$. Glyceryl trinitrate (GTN; 40 pmol) was used as control.

(30–35 mg kg^{-1}) by injection through an ear vein. The animals were then exsanguinated. The aortae were freed *in situ* of adipose tissue, and strips were spirally cut from the vessels. The endothelial layer was removed from the strips by delicate rubbing of the intimal surface with filter paper. The strips were then suspended and perfused in cascade (Vane, 1964) with Krebs solution gassed with 95% O_2/5% CO_2 at 37°C containing indomethacin (5 μM) and sometimes [Leu^8],des-Arg^9-Bk (2 μM) to eliminate the contractile effect of des-Arg^9-Bk (Regoli *et al.*, 1977). The assay tissues were contracted by U46619 (30 nM) or noradrenaline (NA, 300 nM). When a stable plateau was obtained, the tissues were calibrated by the relaxant effects of glyceryl trinitrate (GTN) and the sensitivity of the recording of the 4 tissues was adjusted electronically to be roughly equal (Gryglewski *et al.*, 1986).

Preparation and superfusion of the column and assay of EDRF

The effluent from the column of EC superfused the four aortic strips at a flow rate of 5 ml min^{-1}, reaching the consecutive rabbit aortae (RbAs) after 1, 4, 7 and 10 s. Drugs were injected over the aortic strips (OT) as a control or through the column (TC) containing the EC. Superoxide dismutase (SOD; 10 u ml^{-1}) was infused TC (Gryglewski *et al.*, 1986; de Nucci *et al.*, 1988a). Antagonists were given as infusions (0.1 ml min^{-1}) over the tissues or through the column of EC.

Radioimmunoassay of prostacyclin

Prostacyclin released from EC was assayed in the column effluent as 6-oxo-$PGF_{1\alpha}$ determined by a specific radioimmunoassay (RIA) after suitable dilution (1:2–1:10) in RIA buffer, without prior extraction or purification. The specificity of the antiserum used in these RIA has been established previously (Salmon, 1978).

Drugs

Bradykinin (Bk), des-Arg^9-Bk, glyceryl trinitrate (GTN), superoxide dismutase (SOD) and adenosine diphosphate (ADP) were all obtained from Sigma (Poole, Dorset).

Indomethacin (Sigma, Poole, Dorset) was dissolved in 5% (w/v) $NaHCO_3$ solution and diluted in Krebs solution before use. Arachidonic acid (AA; Sigma) was stored in an hexane solution (10 mg ml^{-1}) at −20°C and diluted with Tris (50 mM, pH 7.4) before use. U46619 (9,11-dideoxy-9α,11α-methanoepoxy-prostaglandin $F_{2\alpha}$) was a generous gift from Dr J. Pike, Upjohn, Kalamazoo.

The 6-oxo-$PGF_{1\alpha}$ antiserum was kindly provided by Dr J. Salmon, Wellcome Research Laboratories U.K. The antagonists of B_2 and B_1 receptors namely [D-Arg^0,Hyp^3,Thi^{5,8},D-Phe^7]-Bk and [Leu^8],des-Arg^9-Bk were generously provided by Dr D. Regoli, University of Sherbrooke, P.Q., Canada.

Sodium pentobarbitone (Sagatal) was obtained from M&B Pharmaceuticals, Poole, Dorset.

Statistics

Results are shown as mean values ± s.e.mean for n experiments. Student's paired t test was used to determine the significance of differences between means and P values of <0.05 were considered as significant.

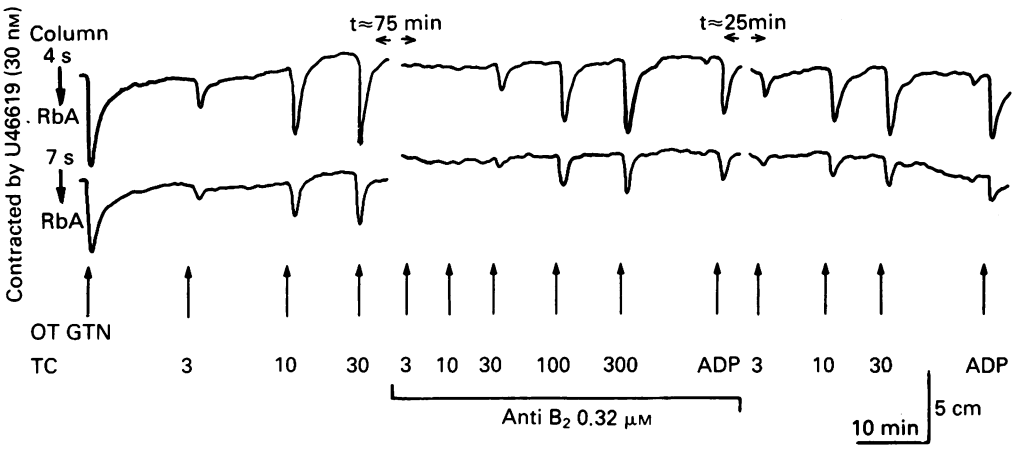

Figure 2 Release of endothelium-derived relaxing factor (EDRF) from a column of bovine aortic endothelial cells by bradykinin (Bk, 3–300 pmol) and ADP (2 nmol). Drugs were injected through the column (TC) or over the tissues (OT). The tissues were contracted with U46619 (30 nM). Experimental details as in Figure 1. The second and third aortae are represented. An infusion of the B_2 kinin receptor antagonist, [D-Arg^0,Hyp^3,Thi^{5,8},D-Phe^7]-Bk (0.32 μM) reduced the release of EDRF induced by Bk but not that induced by ADP. Superoxide dismutase (10 u ml^{-1}) was infused TC throughout the experiment. Glyceryl trinitrate (GTN; 40 pmol) was used as control.

Results

Release of EDRF and PGI_2 by bradykinin and des-Arg^9-Bk

Bolus injections of Bk (3 or 10 pmol) induced the release of both EDRF and PGI_2 from the EC which was concentration-dependent and rapidly reversible. Des-Arg^9-Bk (1.5–10 nmol) also induced a concentration-dependent release of EDRF and PGI_2 from the EC (Figure 1).

Kinin-induced release of EDRF by B_2 receptor stimulation

The infusion OT of [D-Arg^0,Hyp^3,Thi^{5,8},D-Phe^7]-Bk did not alter the effects on the Bk-induced release of EDRF or GTN (not shown), neither did the antagonist have a releasing effect by itself when infused TC (Figure 2). Infusions of the B_2 antagonist TC (0.13 or 0.32 μM) abolished the release of EDRF induced by the lower doses of Bk, and significantly reduced the release induced by the higher concentrations (30–300 pmol) of Bk. The same concentrations of the B_2 antagonist did not affect the responses to ADP or AA (results not shown).

The antagonism was reversible, for the release of EDRF from the EC by Bk returned 25 min after the cessation of the infusion of the B_2 antagonist (Figure 2).

Kinin-induced release of EDRF by B_1 receptor stimulation

The release of EDRF by intermediate doses of Bk, AA and des-Arg^9-Bk is illustrated in Figure 3. Any contractile effect of the B_1 receptor agonist on the RbAs, was prevented by an antagonist of B_1 receptors [Leu^8],des-Arg^9-Bk (2 μM) infused continously OT. This procedure did not alter the sensitivity of the assay tissues to EDRF or GTN (Figure 3). Without showing any agonist effect per *se*, the infusion of the B_1 antagonist through the column of EC significantly reduced the release of EDRF induced by des-Arg^9-Bk without affecting the release of EDRF induced by Bk, AA or ADP (not shown). Partial return of the release of EDRF induced by des-Arg^9-Bk was seen 15 min after the infusion of the B_1 antagonist was stopped and a complete return of the release was seen 30 min after the cessation of the infusion.

In another set of experiments the selectivity of the B_2 antagonist on EDRF release induced by Bk and des-Arg^9-Bk was assessed. The infusion of the B_2 antagonist did not affect the release of EDRF induced by des-Arg^9-Bk (2 nmol) whereas the release of EDRF induced by Bk was abolished ($n = 4$; Figure 4).

Effects of B_1 and B_2 antagonists on the release of prostacyclin

As shown in Table 1, [D-Arg^0,Hyp^3,Thi^{5,8},D-Phe^7]-Bk at 1 μM abolished the release of PGI_2 induced by Bk without significantly affecting the release induced by des-Arg^9-Bk, ADP or AA.

The release from the EC of PGI_2 induced by des-Arg^9-Bk was significantly reduced by [Leu^8],des-Arg^9-Bk. Release of PGI_2 by Bk, ADP and AA was not significantly affected by the B_1 antagonist (Table

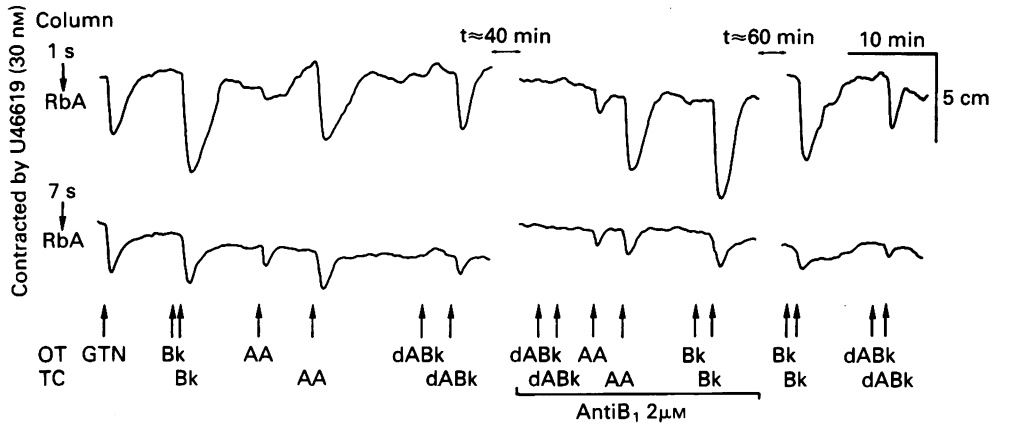

Figure 3 Release of endothelium-derived relaxing factor (EDRF) from a column of bovine aortic endothelial cells by bradykinin (Bk; 10 pmol), des-Arg^9-Bk (dABk; 750 pmol) and arachidonic acid (AA, 75 nmol). Drugs were injected through the column (TC) or over the tissues (OT). The tissues were contracted with U46619 (30 nM). Experimental details as in Figure 1. The first and third aortae are represented. An infusion of B_1 antagonist, [Leu^8]-des-Arg^9-Bk (2 μM), blocked the release of EDRF induced by dABk but not that induced by Bk or AA. Superoxide dismutase (10 u ml^{-1}) was infused throughout the experiment. Glyceryl trinitrate (GTN, 40 pmol) was used as control.

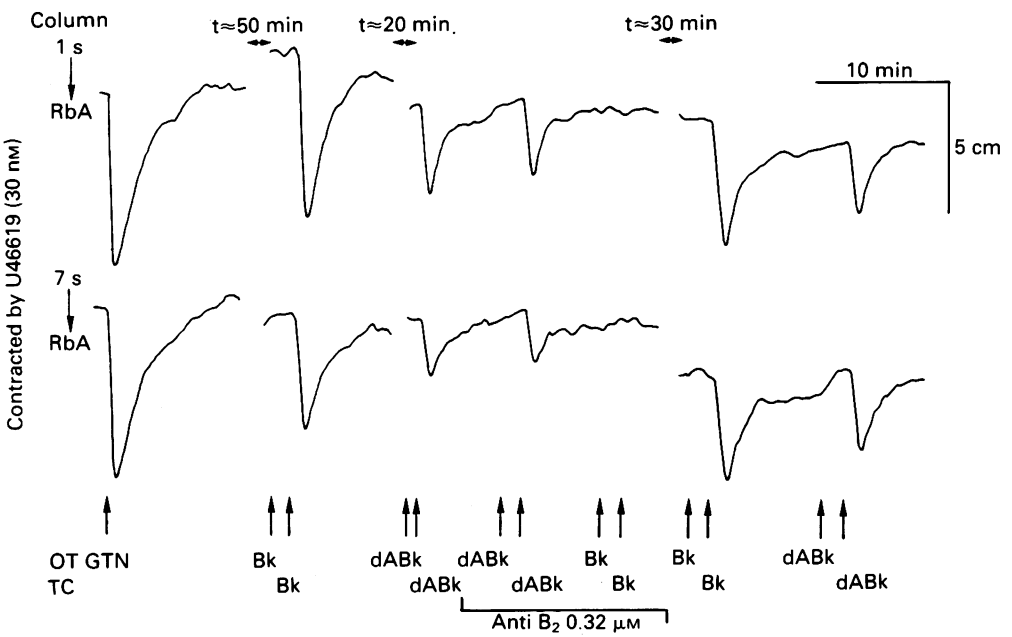

Figure 4 Release of endothelium-derived relaxing factor (EDRF) from a column of bovine aortic endothelial cells by bradykinin (Bk; 10 pmol) and des-Arg^9-Bk (dABk, 2 nmol). Drugs were injected over the tissues (OT) or through the column (TC). The tissues were contracted with U46619 (30 nM). Experimental details as in Figure 1. The first and third aortae are represented. The infusion of B_2 antagonist, [D-Arg^0,Hyp^3,Thi^{5,8},D-Phe^7]-Bk (32 nM) did not affect the release of EDRF induced by dABk but abolished that induced by Bk. Superoxide dismutase (10 u ml^{-1}) was infused throughout the experiment. Glyceryl trinitrate (GTN, 40 pmol) was used as control.

Table 1 Effect of B_2 (aB_2) and B_1 (aB_1) antagonists on the release of prostacyclin (PGI_2) induced by bradykinin (Bk), des-Arg^9-Bk, adenosine diphosphate (ADP) and arachidonic acid (AA).

Agonist	*Dose*	*aB_2-OT*	*aB_2-TC*	n	*aB_1-OT*	*aB_1-TC*	n
Bk	10 pmol	2.6 ± 0.3	0.7 ± 0.1*	(12)	2.6 ± 1.6	2.7 ± 1.6	(6)
des-Arg^9-Bk	7.5 nmol	2.1 ± 0.8	1.4 ± 0.4	(7)	1.7 ± 0.4	0.7 ± 0.3*	(10)
ADP	2 nmol	1.7 ± 0.5	1.6 ± 0.3	(7)	1.4 ± 0.6	1.5 ± 0.3	(4)
AA	3 nmol	10.7 ± 4.0	6.6 ± 2.3	(7)	17.0 ± 7.4	18.7 ± 7.5	(3)

Anti-B_2: [D-Arg^0,Hyp^3,Thi^{5,8},D-Phe^7]-Bk infused at a concentration of 1 μM.
Anti-B_1: [Leu^8],des-Arg^9-Bk infused at a concentration of 5 μM.
Release of PGI_2 expressed as 6-oxo-$PGF_{1\alpha}$ (ng ml^{-1}).
* $P < 0.01$; n = number of experiments.

1). Neither the B_1 nor the B_2 antagonist increased the basal release of PGI_2 by the cells.

Discussion

Bradykinin induces endothelium-dependent relaxations of many arteries and veins from various species *in vitro* (Altura & Chand, 1981; Cherry *et al.*, 1982; Forstermann *et al.*, 1984). The present results confirm the observation made by de Nucci *et al.* (1988a) that Bk induces a coupled release of EDRF and PGI_2 via B_2 receptor stimulation in EC. Although the mechanism of action of this coupled release from EC is not yet well defined, there is evidence for the involvement of phospholipase C (de Nucci *et al.*, 1988a). Bk stimulates a calcium-dependent release of arachidonate from porcine EC (Van de Velde *et al.*, 1986). Furthermore, Morgan-Boyd *et al.* (1987) have recently demonstrated using [D-Arg^0,Hyp^3,Thi^{5,8},D-Phe^7]-Bk, that kinins mobilize intracellular Ca^{2+} via B_2 receptors in bovine pulmonary artery EC. Thus, the stimulation of B_2 receptors may activate a calcium-dependent phospholipase C in both types of bovine endothelial cells.

The inhibition by [Leu^8],des-Arg^9-Bk of the release of EDRF and PGI_2 induced by des-Arg^9-Bk shows that the EC also possess receptors of the B_1 type. In our experiments, the infusion of the B_1 antagonist over the precontracted vascular tissues allowed the assessment of the complete dose-response curve to des-Arg^9-Bk, the effect of the concentration-dependent release of EDRF being otherwise masked by a contractile effect of des-Arg^9-Bk on the aortic strips. The B_1 receptors are of comparatively low affinity relative to the B_2 receptors present in the EC. This agrees with the observations made by Sung *et al.* (1988) who have demonstrated the presence of high affinity B_2 receptor sites and low affinity B_1 sites in the bovine pulmonary EC.

The low affinity of the B_1 receptors located in the bovine EC suggests a relatively unimportant role for this receptor type in these cells. However, one cannot rule out a functional role for B_1 receptor stimulation in certain vascular beds, as des-Arg^9-Bk is a potent agonist in the mesenteric vascular bed of the rabbit (Regoli & Barabé, 1980; Deblois & Marceau, 1987) and of the rat (Mastrangelo *et al.*, 1987). Furthermore, Cahill *et al.* (1988) demonstrated that kinins induce release of PGI_2 by activating high affinity B_2 and B_1 receptor sites in calf pulmonary EC.

The finding that both the B_1 and B_2 antagonists reduce the release of PGI_2 and EDRF induced by kinins suggests that the release of PGI_2 and EDRF by Bk and des-Arg^9-Bk are receptor-mediated in the EC. These results do not support the view that PGI_2 generation is caused by the breakdown of Bk by angiotensin converting enzyme, as proposed by Sawada *et al.* (1986). They are in agreement with de Nucci *et al.* (1988b) who demonstrated that captopril does not inhibit the coupled release of EDRF and PGI_2 induced by Bk from the EC.

The contribution of B_2 receptor activation *in vivo* to the action of Bk is better documented. [D-Arg^0, Hyp^3,Thi^{5,8},D-Phe^7]-Bk markedly reduced the hypotensive response to Bk in normotensive and hypertensive rats (Waeber *et al.*, 1987; Aubert *et al.*, 1988). Carbonell *et al.* (1988a) found that the B_2 antagonist *in vivo* promoted a biphasic change in the rat mean arterial blood pressure; the hypotensive effect of the antagonist being mediated by a release of prostanoids. In our EC model, the B_2 antagonist did not modify the basal release of prostanoids from the EC, thus suggesting that the antagonist might induce a release of AA metabolites from sources other than the endothelial cells of vascular beds *in vivo*.

What is the function of bradykinin receptors in the endothelium? Clearly we do not understand the presence of acetylcholine (ACh) receptors in EC which release EDRF and prostacyclin, for it is most unlikely that they will ever encounter ACh carried in the bloodstream. Concentrations of Bk in the circulation are increased in states of shock such as haem-

orrhagic hypotension (Berry *et al.*, 1970) and anaphylactic shock but normal concentrations of bradykinin are vanishingly small (Scicli *et al.*, 1982). However, the findings that inhibitors of angiotensin converting enzyme decreased blood pressure in hypertensive subjects with normal and low renin (Gavras *et al.*, 1978), whereas saralasin, a competitive antagonist of angiotensin II did not (Case *et al.*, 1976), suggest a role for bradykinin in the control of vascular tone. This is further supported by the experiments demonstrating that antibodies to kinins blocked part of the acute response to captopril in two kidney, one clip hypertensive rats (Carretero *et al.*, 1981) and those which showed that kinin antagonists can also reverse the acute antihypertensive effect of angiotensin converting enzyme inhibitors in this model (Benetos *et al.*, 1986; Carbonell *et al.*, 1988b). Thus, a better understanding of the functional kinin receptors *in vivo* is desirable to allow the development of bradykinin analogues which could release both EDRF and prostacyclin without having an algesic action.

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Mechanisms of positive inotropic effects and delayed relaxation produced by DPI 201-106 in mammalian working myocardium: effects on intracellular calcium handling

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1 We used the bioluminescent protein aequorin, which emits light when it combines with Ca^{2+}, to test the hypothesis that the inotropic and lusitropic actions of DPI 201-106 are due to changes in intracellular Ca^{2+} handling in papillary muscles from ferrets and guinea-pigs.

2 DPI 201-106 increased peak isometric tension (T) in a dose-dependent manner, with an 83% increase in T as the concentration of DPI 201-106 was increased to 1×10^{-5} M; however, peak $[Ca^{2+}]_i$ did not increase significantly until the concentration of DPI 201-106 reached 3×10^{-6} M, suggesting a sensitization of the contractile apparatus to Ca^{2+}.

3 Tetrodotoxin (1×10^{-6} M), which did not reduce the tension response significantly before DPI 201-106, decreased both $[Ca^{2+}]_i$ and T in the presence of 1×10^{-5} M DPI 201-106, suggesting involvement of a sodium channel activation mechanism; however, tetrodotoxin did not completely reverse the calcium sensitization.

4 The shift of the $[Ca^{2+}]_i$ versus T relationship was not observed in the presence of another sodium channel agonist, veratridine (3×10^{-7}–1×10^{-6} M).

5 In the guinea-pig, DPI 201-106 markedly prolonged relaxation of tension (increase of 60% in the time from peak to 50% tension regression), which was accompanied by the appearance of a second component in the aequorin light signal; effects on relaxation were less prominent in the ferret.

6 Tension prolongation and the second component of the $[Ca^{2+}]_i$ transient in the guinea-pig were exacerbated by increased $[Ca^{2+}]_o$ and decreased by tetrodotoxin. Ryanodine (3×10^{-7} M) markedly diminished the calcium transient in controls and the initial component of the calcium transient in the presence of DPI 201-106, but had only a modest effect on the second component.

7 We conclude that although sodium agonism plays a role, sensitization of the contractile apparatus to Ca^{2+} is an important mechanism in the positive inotropic action of DPI 201-106.

8 The negative lusitropic action of DPI 201-106 varies between ferret and guinea-pig, possibly reflecting differences between these two species in subcellular Ca^{2+} handling.

Introduction

Beginning in 1978 with the introduction of amrinone (Bennotti *et al.*, 1978; Alousi *et al.*, 1979), a growing number of new non-glycoside inotropic agents have been developed, reflecting an increased interest in drugs alternative to digitalis in the treatment of congestive heart failure. For most of these compounds however, inhibition of phosphodiesterase activity and the consequent increase in intracellular adenosine 3′:5′ cyclic monophosphate (cyclic AMP) levels appears to be the main mechanism of action (Scholz & Meyer, 1986; Endoh *et al.*, 1986). The clinical use of phosphodiesterase inhibitors as positive inotropic agents might therefore be seriously limited by cyclic AMP-induced positive chronotropic and arrhythmogenic effects which often accompany their inotropic actions.

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Recently, DPI 201-106 (DPI), a novel piperazinyl-indole has been reported to possess a unique pharmacological profile which shows α- and β-adrenoceptor and cyclic AMP-independent cardiotonicity with negative chronotropic and antiarrhythmic actions (From *et al.*, 1984; Scholtysik *et al.*, 1985); Salzmann *et al.*, 1986). Initial studies indicated that the agent has a site of action in the sarcolemma, exerting a sodium channel agonist effect with prolongation of action potential duration (From *et al.*, 1984; 1985; Scholtysik *et al.*, 1985; Buggisch *et al.*, 1985; Salzmann *et al.*, 1986). Initial studies indi-Studies of skinned porcine ventricular trabeculae showed a direct effect of DPI on the myofilaments causing an increase in the sensitivity to Ca^{2+} (Herzig & Quast, 1984; Scholtysik *et al.*, 1985). Inhibition of the slow Ca^{2+} channels in the guinea-pig myocardium (Buggisch *et al.*, 1985; Holck & Osterrieder, 1988) or in the rabbit aortic ring (Hof & Hof, 1985) has also been reported. The overall effects of DPI on the myocardium therefore appear to reflect a complex combination of actions on different subcellular components. Although synergistic combination of these cellular mechanisms might produce a favourable effect in myocardium with depressed contractility, a delayed relaxation (negative lusitropic effect) has been described in myocardium isolated from the kitten (Scholtysik *et al.*, 1985) and guinea-pig (Scholtysik *et al.*, 1985; Buggisch *et al.*, 1985). This has raised questions regarding DPI's potential clinical utility, since impaired relaxation is a common abnormality in many patients with heart failure (Grossman & Barry, 1980). The mechanism of this delayed relaxation caused by DPI and its relation to the inotropic action is unknown.

Because most inotropic interventions in the heart either alter intracellular Ca^{2+} handling, change the responsiveness of the contractile apparatus to Ca^{2+}, or exert both types of effects (Morgan & Blinks, 1982), the most direct way of determining which action an inotropic intervention might have is to monitor the intracellular Ca^{2+} transients and correlate them with mechanical performance. This correlation provides the basis for a functional classification scheme (Morgan & Morgan, 1984a) that can be used to investigate drugs with unknown mechanisms of action. The purpose of the present study, therefore, was to delineate the effects of the new inotropic agent, DPI, on intracellular Ca^{2+} handling in mammalian working myocardium by use of aequorin, a bioluminescent protein that emits light when it combines with Ca^{2+} (Blinks *et al.*, 1982a). We found that sensitization of the myofilaments to Ca^{2+} is a mechanism of the inotropic action of DPI, and that delayed relaxation (negative lusitropy) is caused mainly by the increased duration of the slow Ca^{2+} current which directly activates the myofilaments. Since, under some conditions, the inotropic mechanism appears to be essentially independent of the lusitropic action, we also suggest the possibility of separating the lusitropic from the inotropic actions of this drug.

Methods

Papillary muscles of 0.8 mm or less in diameter were excised from the right ventricles of hearts removed from adult male ferrets (650–700 g) or from adult male guinea-pigs (450–550 g), while the animals were anaesthetized with chloroform. The methods for preparation and the instrumentation used in these studies have been described previously (Morgan *et al.*, 1984; Gwathmey & Morgan, 1985). The excised muscles were bathed in a bicarbonate-buffered physiological salt solution that was bubbled with a gaseous mixture of 95% O_2 and 5% CO_2 to pH 7.4 and was maintained at 30°C. The preparations were paced at a basal rate of 0.33 Hz with pulses of 5 ms duration and voltages $\leqslant 10\%$ above threshold, applied through a punctate platinum electrode located at the base of the papillary muscle. An initial 2-h equilibration period was employed routinely, during the first hour of which the muscles were subjected to repeated stretches until the length was attained yielding maximal force development.

In one group of muscles (ferret $n = 19$, guinea-pig $n = 10$), cumulative concentration-response, and frequency-response relationships were determined. Peak developed tension, time to peak tension, and time from peak to 50% and 80% tension regression were measured on chart strip paper which recorded the tension response and the stimulus artifact simultaneously at $100\,mm\,s^{-1}$. All measures were made under steady state conditions. In three ferret preparations, the action potential was recorded by standard methods simultaneously with the tension response from superficially located myocardial cells. In order to avoid precipitation of Ca^{2+} during the cumulative calcium concentration-response study, a phosphate-free solution was used and calcium was added in incremental doses up to 16 mM (Gwathmey & Morgan, 1985). For DPI concentration-response curve determinations, the perfusate contained a 1.0 mM concentration of calcium.

In a second group of muscles (ferret $n = 21$, guinea-pig $n = 16$), aequorin was loaded chemically as described elsewhere (Morgan *et al.*, 1984; Morgan & Morgan, 1984b). Muscles were discarded that showed a decrease in peak tension (less than 80% of the control value) or a significant change in time course after the loading procedure. Light signals were recorded with a photomultiplier (EMI 9635qA) using a light collecting apparatus of a design

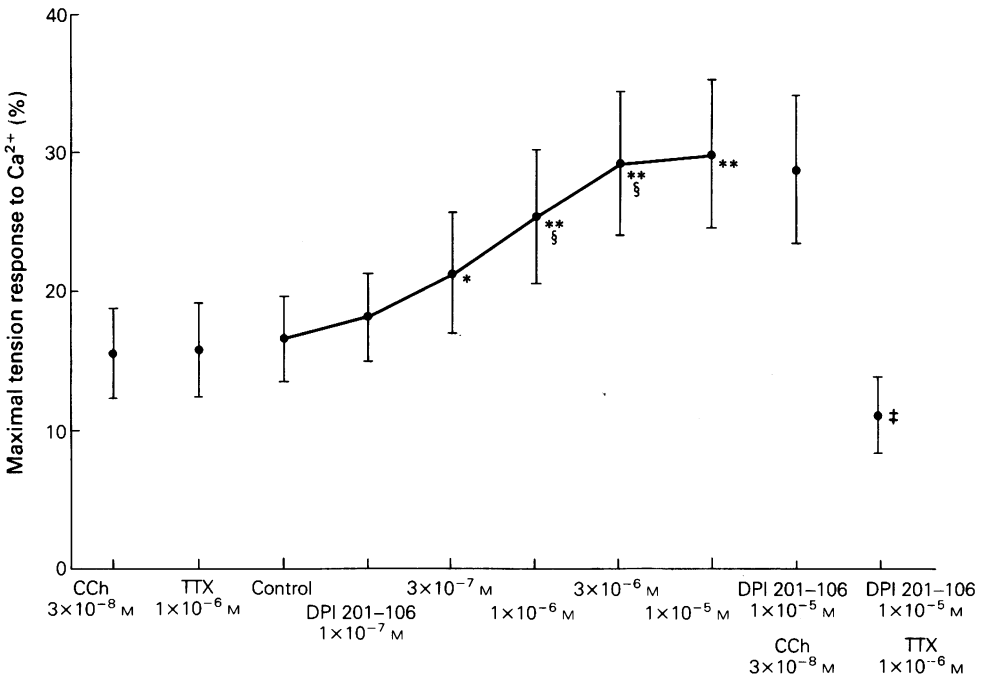


Figure 1 Cumulative concentration-response relationship of DPI 201-106 in the ferret papillary muscle, stimulated to contract at 0.33 Hz; 30°C. The ordinate scale shows % of maximal response to $[Ca^{2+}]_o$. Carbachol (CCh) or tetrodotoxin (TTX) were randomly added in the control and at the top of cumulative concentration-response to DPI 201-106. In the control, TTX or CCh was washed out by changing the solution in the bath before testing DPI 201-106. The same control value was obtained before and after washing the effect of these drugs. $n = 8$; all points are means with s.e.mean shown by vertical bars. * $P < 0.05$; ** $P < 0.01$ versus the control value before DPI 201-106; § $P < 0.05$ versus the effect of previous concentration of DPI 201-106; ‡ $P < 0.01$ versus the effect of 1×10^{-5} M DPI 201-106.

described by Blinks (1982). To obtain a satisfactory signal-to-noise ratio, it was usually necessary to average successive signals (from 16 to several hundred depending on the light intensity). Signal averaging was performed only after responses had reached a steady state. The light and tension responses and the stimulus artifact were recorded simultaneously both on magnetic tape and on chart strip recording paper. The light signal was measured in nanoamperes of anode current.

It is possible for drugs to interact directly with aequorin and modify the luminescent reaction or the sensitivity of aequorin to Ca^{2+} (Blinks *et al.*, 1982b). Therefore, each of the drugs used in these experiments was tested *in vitro* using the basic method and calibration device described by Blinks *et al.* (1978). Briefly, aequorin was added to deionized, chelated water (Ca^{2+} contamination was less than 1×10^{-7} M); under these conditions a low level of luminescence persists for several minutes until the aequorin is gradually consumed. After initiating the luminescent reaction, drugs in concentrations equal to or greater than the concentrations used in the experiments were added to the reaction cuvette. In particular, blank dimethylsulphoxide (DMSO) and DPI in concentrations as high as 3×10^{-5} M were tested. It was difficult to test above this concentration because of the insolubility of DPI and the occurrence of precipitation. In no case did the drugs tested affect the intensity or time course of luminescence, indicating that they do not interact directly with aequorin in a way that would alter the experimental results.

Drugs

The aequorin used in these experiments was purchased from the laboratory of Dr J.R. Blinks of Rochester, Minnesota, U.S.A.; veratridine and tetrodotoxin from Sigma Chemical Co. DPI 201-106

Table 1 Maximum effects of DPI 201-106 on amplitude and time course of tension responses in ferret and guinea-pig papillary muscles

	Peak tension (mN mm^{-2})		*TPT* (ms)		*RT50%* (ms)		*RT80%* (ms)	
	Control	*DPI*	*Control*	*DPI*	*Control*	*DPI*	*Control*	*DPI*
Ferret	4.0 ± 1.7	7.1 ± 2.0**	169 ± 19	178 ± 13	88 ± 15	94 ± 15*	138 ± 20	147 ± 19*
$n = 6$		183 ± 18%		106 ± 4%		108 ± 4%		106 ± 4%
Guinea-pig	1.2 ± 0.6	2.2 ± 1.0**	276 ± 31	285 ± 32	159 ± 20	261 ± 69**	252 ± 35	381 ± 73**
$n = 7$		186 ± 36%		101 ± 4%		160 ± 37%§		149 ± 23%§

Muscles were stimulated to contract at 0.33 Hz, at 30°C. Maximum effects during cumulative concentration-responses are presented as data in DPI 201-106. Normalized values (% control) were used for comparisons between species. All values are means ± s.d.
Abbreviations: DPI = DPI 201-106; TPT = time to peak tension; RT50% = time from the peak to 50% regression of tension; RT80% = time from the peak to 80% regression of tension; * $P < 0.05$ versus control; ** $P < 0.01$ versus control; § $P < 0.01$ versus the corresponding change in ferret muscles.

was generously supplied by Sandoz Ltd., Basle, Switzerland, and ryanodine by Merck, Sharpe and Dohme.

Statistics

In cumulative concentration-response and frequency-response studies, the Neuman-Keuls multiple comparison test with analysis of variance was used for statistical analysis (Zar, 1974). In others, Student's *t* test was used for paired data from a muscle or for unpaired data from different species. *P* values <0.05 were considered significant.

Results

Effects of DPI on isometric tension development

Figure 1 shows the concentration-dependent effects of DPI on peak isometric tension development in ferret papillary muscle stimulated at 0.33 Hz. The tension was normalized for the maximally developed tension in high calcium solution ($[Ca^{2+}]_o$: 8–16 mM) in each muscle. Before DPI, the effects of tetrodotoxin (TTX) and carbachol were tested. At a concentration of 1×10^{-6} M, TTX did not attenuate the control tension response significantly (96 ± 5%, NS); however, a consistent reduction of peak tension was observed at a TTX concentration of 3×10^{-6} M (85 ± 7%, $P < 0.05$ in the control preparations, $n = 8$). Carbachol (3×10^{-8} M) did not affect tension development (97 ± 4%, NS, $n = 8$) under the same control conditions. During cumulative DPI concentration-effect determinations, the stable maximal change was reached after 30–45 min of exposure to each concentration of the drug. A significant and concentration-dependent increase in peak tension was observed in the range of concentrations from 3×10^{-7} to 1×10^{-5} M. Since the compound showed poor solubility in the perfusate, the upper range of the curve ($>3 \times 10^{-5}$ M) was difficult to examine. In contrast to the effect on the control preparations, TTX (1×10^{-6} M) markedly attenuated the tension at the top of the DPI cumulative concentration-response curve (65 ± 7% of control $P < 0.05$ versus the control value; $P < 0.01$ versus the effect of 1×10^{-5} M DPI). Carbachol did not have any effect before or after DPI.

Table 1 shows that DPI significantly increased the time to 50% and time to 80% decline from peak

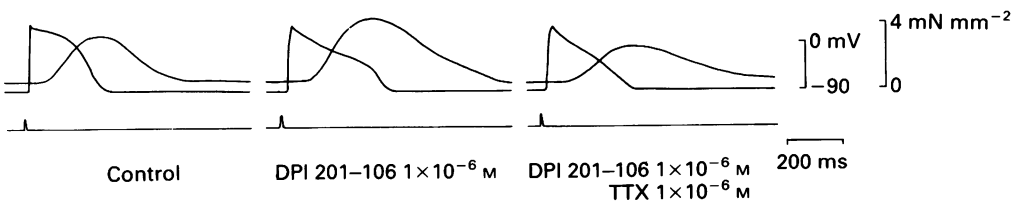


Figure 2 Effects of DPI 201-106 and tetrodotoxin (TTX) on tension response and action potential in a ferret papillary muscle. The muscle was stimulated to contract at 0.33 Hz; 30°C; $[Ca^{2+}]_o = 1.0$ mM. In the presence of 1×10^{-6} M DPI, 1×10^{-6} M TTX reduced the peak tension and reversed the prolonged action potential. Note that, after TTX, while the peak tension was attenuated below the control level, the tension prolongation was not reversed completely.

tension in the ferret. There was also a tendency to increase the time to peak tension, but this was not statistically significant. More interestingly, while 1×10^{-6} M TTX at the top of the DPI concentration-response curve decreased the peak tension to below the control level, it did not reverse the prolonged tension time course completely (Figure 2). These changes in tension time course were relatively modest in the ferret preparations; however, in the papillary muscles from the guinea-pig, increases in the bath concentration of DPI were accompanied by marked increases in the overall twitch duration and by prolongation of the relaxation time (Table 1, Figure 8). While the inotropic responses in both species were of the same magnitude, the difference between the ferret and the guinea-pig with regard to the drug-related changes in the tension time course was satistically significant. Although the prolongation of relaxation seemed to parallel the increase in peak tension in the guinea-pig, the difference between the species suggests that the inotropic effect of DPI is not necessarily accompanied by a lusitropic action.

To elucidate the mechanism of action of DPI, we examined the frequency-dependence of its inotropic action. The inotropic effect of DPI at 30°C was assessed in muscles from the ferret driven at frequencies ranging from 0.03 to 1.0 Hz. Figure 3 shows the normalized steady-state frequency-force relationships under drug-free conditions and in the presence of 1×10^{-6} M DPI. Twitch amplitude was measured at each frequency after steady-state was reached, and was normalized in each muscle by the maximum of control twitch amplitudes observed in the response, and then averaged. Under drug-free conditions, increased twitch tension was observed as the frequency was increased from 0.03 to 1.0 Hz. In the presence of DPI, the curve was shifted above the control. The inotropic response to DPI was more marked at frequencies above 0.2 Hz and diminished at frequencies lower than 0.2 Hz. These data suggest that the inotropic mechanism of DPI is at least partially dependent on the stimulation frequency.

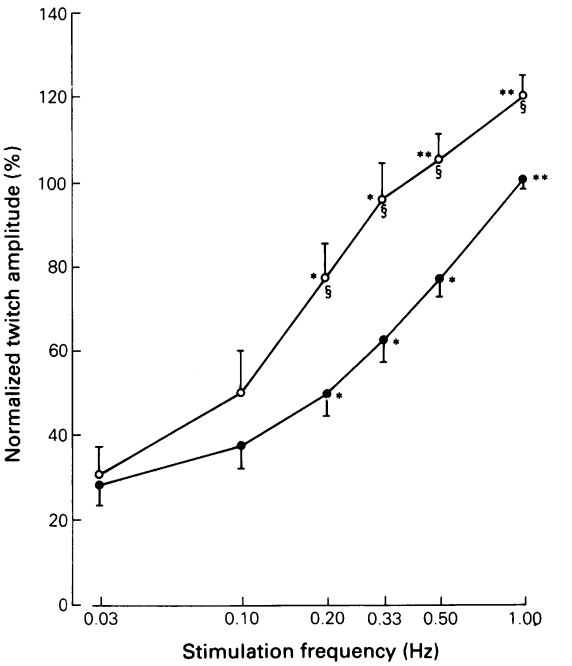


Figure 3 Effects of DPI 201-106 on frequency-response curve in ferret papillary muscles: (●) amplitudes of tension in the control; (○) after DPI 201-106, 1×10^{-6} M. Values plotted on ordinate scale were normalized by the maximal amplitude of tension obtained in the control in each muscle. $n = 8$; all points are mean values with s.e.mean shown by vertical bars. * $P < 0.05$; ** $P < 0.01$ versus the value at 0.03 Hz in each curve; § $P < 0.05$ versus the corresponding value of the control curve.

Mechanisms of inotropic action

Figure 4 shows a representative record of the aequorin light signal and tension response of a ferret papillary muscle before and after exposure to DPI. There are two distinct findings; first, during cumulative Ca^{2+} concentration-response determinations, both the light signal and tension responses increased in a concentration-dependent manner (Figure 4a); however, in the presence of 3×10^{-7}–1×10^{-6} M DPI, the peak of light signal did not increase significantly ($107 \pm 15\%$ at 1×10^{-6} M DPI) while peak tension increased by $44 \pm 9\%$ ($P < 0.05$ of control; Figure 4b). In concentrations greater than 3×10^{-6} M, DPI increased both peak light and peak tension significantly ($123 \pm 12\%$, $P < 0.05$; $170 \pm 15\%$, $P < 0.01$, at 3×10^{-6} M DPI, respectively). This concentration of DPI produced a similar increase in tension as 4 mM $[Ca^{2+}]_o$ in the absence of DPI; peak tensions were $170 \pm 15\%$ and $175 \pm 16\%$ of the control, respectively (NS). However, when the light signals were compared, the increase in peak light with DPI was significantly less than that in response to increased Ca^{2+} concentrations ($123 \pm 12\%$ and $210 \pm 25\%$, respectively $P < 0.01$, $n = 8$). Second, records superimposed by normalizing the peak amplitudes reveal that, after DPI, the time course of the light signal was abbreviated despite prolongation of the tension response (Figure 4c). In guinea-pig preparations ($n = 7$), the same disproportionate increase in peak light versus peak tension was observed (between 4.0 mM Ca^{2+} and 3×10^{-6} M DPI: $P < 0.05$, in % increases in light

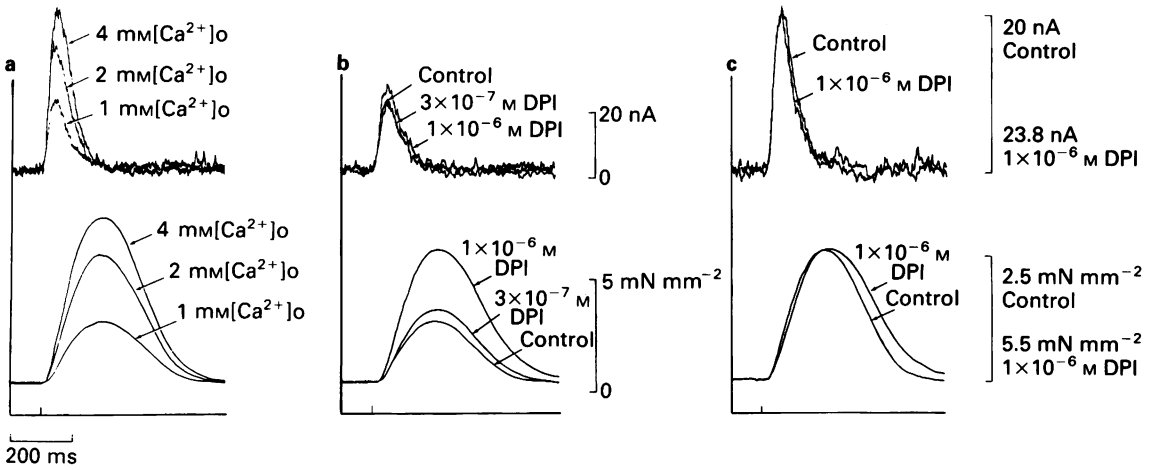


Figure 4 Cumulative concentration-response relationships of $[Ca^{2+}]_o$ (a) and DPI 201-106 (b) in an aequorin-loaded ferret papillary muscle. In (c), the vertical gains of signals have been electronically adjusted to the same amplitudes to elucidate the effect of DPI 201-106 on the time courses of aequorin signals and isometric contractions. Each trace represents the average of 48 steady state responses at 3 s intervals of stimulation; 30°C.

signal; NS, in % increases in peak tension). To investigate the relationship between intracellular Ca^{2+} and developed tension, the peak amplitude of the aequorin signal and tension response were plotted during the determination of concentration-effect curves for $[Ca^{2+}]_o$ under control conditions and in the presence of a single concentration of DPI in the same preparation. As shown in Figure 5, the simple repetition of the Ca^{2+} cumulative-concentration response curve was reproducible and did not shift the relationship ($n = 2$). However, in the presence of DPI, the relationship consistently shifted upwards ($n = 9$ in the ferret, $n = 7$ in the guinea-pig). After the Ca^{2+} concentration in the bath was increased to 4 or 8 mM in the presence of DPI, TTX (1×10^{-6} M) reduced both the peak light and peak tension values significantly ($P < 0.01$ for decrease in light and in tension, both in the ferret and in the guinea-pig); however, the light versus tension relationship in TTX remained positioned on the shifted curve.

We tested further the hypothesis that the sodium channel agonist effect of DPI and the subsequent increase in the amount of Ca^{2+} available to the contractile proteins is not the only mechanism of its inotropic action by comparing in the same muscles the peak light versus peak tension relationships after DPI and after the administration of veratridine, a ceveratrum alkaloid. Veratridine is known to interact with the sodium channels of mammalian myocardium and to potentiate their opening probability and, consequently, their sodium permeability (Figure 6). Because an increase in frequency of stimulation

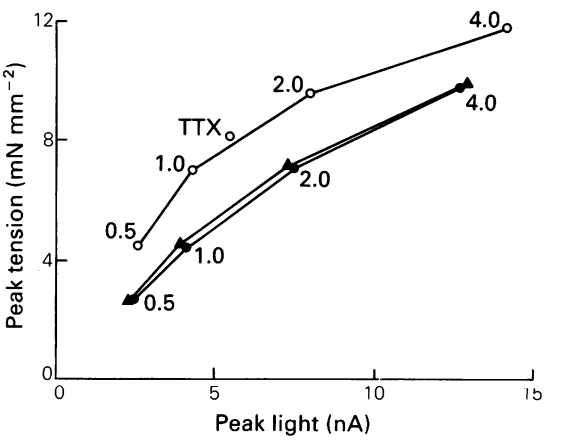


Figure 5 Comparison of the relationship between peak tension and peak light measured in the same muscle during the determination of concentration-effect curves for $[Ca^{2+}]_o$ before (●, ▲) and after (○) DPI 201-106 1×10^{-6} M. The numbers beside the points indicate $[Ca^{2+}]_o$ (mM). Calcium concentration-effect responses were repeated twice in this muscle in order to show their reproducibility (●: the first run; ▲: the second run). After the increase in $[Ca^{2+}]_o$ to 4 mM in the presence of DPI 201-106, tetrodotoxin (TTX) 1×10^{-6} M was added to the bath. The relation shifted to the point indicated by TTX. Ferret papillary muscle, temperature 30°C, stimulus interval 3 s; each point represents the average of 16 steady-state responses.

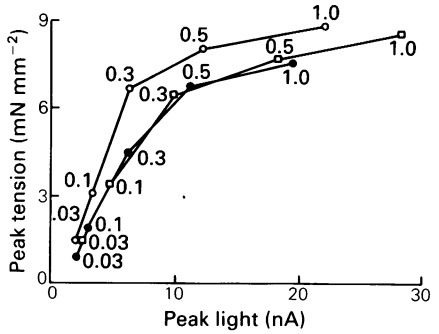
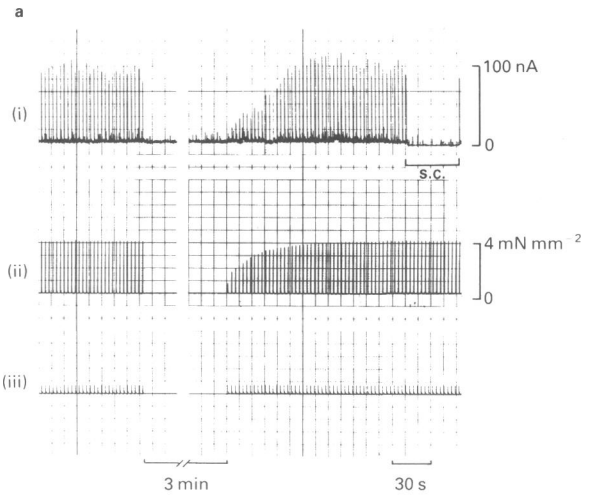


Figure 6 Comparison of the relationship between peak tension and peak light measured in the same muscle during the determination of frequency-effect curves in the control, in the presence of veratridine 3×10^{-7} M, or DPI 201-106, 1×10^{-6} M. The curves were determined in the following order: veratridine, 3×10^{-7} M (□); control after washing veratridine (●); DPI 201-106, 1×10^{-6} M (○). The numbers beside the points indicate the frequency of stimulation (Hz). Ferret papillary muscle, temperature 30°C; $[Ca^{2+}]_o$ 1.0 mM. Stimulus rates varied from 0.03 to 1.0 Hz. Each point represents the average of 32 steady-state responses.



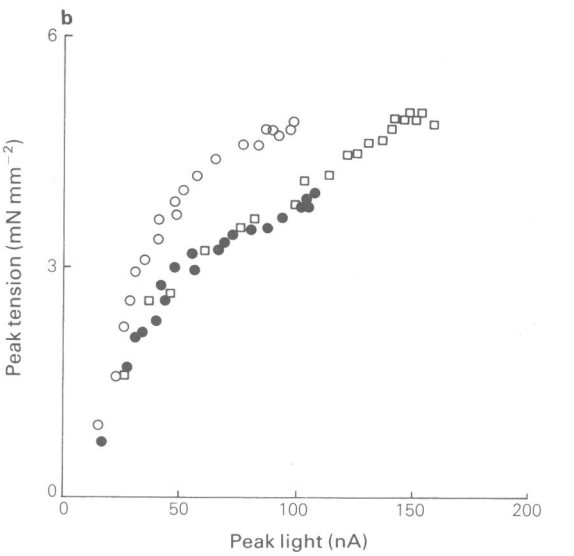


Figure 7 Effects of veratridine and DPI 201-106 on the relationship between peak tension and peak light during the post-rest recovery. In (a) the original tracing of light (i), tension (ii), and stimulus artefact (iii) before and after the 3-min pause in the control is presented. The base-line of the light signals is shown at the end of the tracing by closing a shutter of the light-collecting system (S.C.). In (b) the light vs tension relationships were determined in the following order: veratridine, 3×10^{-7} M (□); control (after washing veratridine) (●): DPI 201-106, 1×10^{-6} M (○). Ferret papillary muscle, temperature 30°C, $[Ca^{2+}]_o$ 1.0 mM. In each restitution, 20 beats after re-substitution of the stimulus (0.33 Hz) were plotted.

would be expected to potentiate the inotropic effects of sodium channel agonists by means of increased sodium and calcium influx per unit time, we determined the force-frequency relationship before and after the addition to the perfusate of DPI or veratridine ($n = 5$). The results showed that the light-tension relationship after veratridine was superimposable on that of the control. In contrast, the frequency-response relationship after DPI consistently shifted upwards. In two preparations in which the individual light signal for each contraction could be recorded without the need for signal averaging, peak light versus peak tension relationships were obtained by measuring every single twitch after 3-min rest, under control conditions, in the presence of veratridine, and DPI (Figure 7). The result was consistent with that obtained in the steady-state force-frequency relationship; namely, DPI, but not veratridine, shifts the relationship between the $[Ca^{2+}]_i$ and tension response.

Mechanism of lusitropic action

To elucidate the mechanism of the negative lusitropic action of DPI, we examined further the time courses of the aequorin light signal and tension response. In contrast to the findings in the ferret preparation mentioned earlier, in the guinea-pig, the descending phase of the Ca^{2+} transient in the

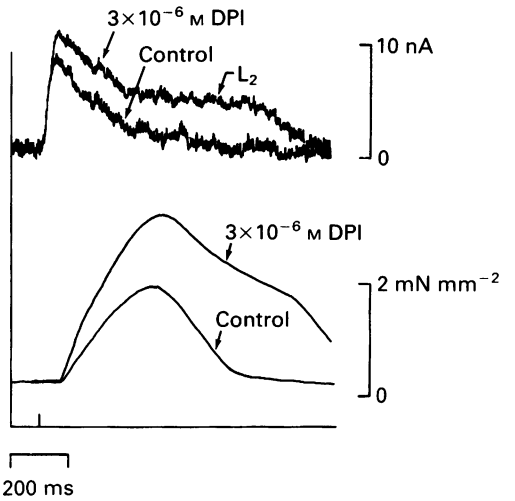


Figure 8 Effects of DPI 201-106 on light and tension in an aequorin-loaded guinea-pig papillary muscle. The muscle was stimulated to contract at 0.33 Hz; 30°C, $[Ca^{2+}]_o$ = 2.5 mM, and signals represent the average of 96 steady-state responses. Note the marked prolongation of the tension time course in the presence of DPI 201-106, 3×10^{-6} M which is associated with appearance of an abnormal component of light signal, indicated by L_2.

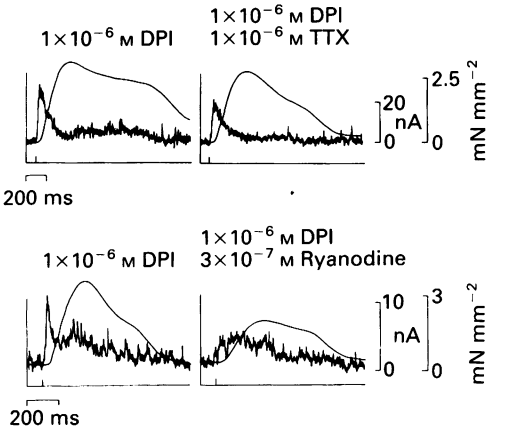


Figure 9 Effects of tetrodotoxin (TTX) and ryanodine on light and tension in aequorin-loaded guinea-pig papillary muscles in the presence of DPI 201-106. The signals represent the average of 72 steady-state responses in the upper panel, and 144 responses in the lower panel; stimulus interval 3 s, temperature 30°C. 1×10^{-6} M DPI 201-106 and 8 mM $[Ca^{2+}]_o$ were present throughout.

presence of DPI was markedly prolonged, and was associated with a remarkable delay in tension relaxation. A representative recording is shown in Figure 8. The comparison of tension-time courses between the control and DPI-treated muscles suggested that the change might consist of an addition of a second component to the twitch which followed the normal contraction. This second component of the twitch was associated with the appearance of a new component of the Ca^{2+} transient (L_2) which emerged during the descending phase of the initial component (L_1) and produced a sustained plateau in the signal for 400–800 ms. This L_2 component was seldom observed in any ferret preparations we examined.

To determine whether these two components of the light signal which appeared in the guinea-pig preparation in the presence of DPI were derived from different sources of Ca^{2+} or were controlled by different subcellular mechanisms, we studied the differential effects of TTX and ryanodine on these components. Figure 9 shows effects of these drugs on the light signal and tension responses after exposure to 1×10^{-6} M DPI and high Ca^{2+} (8 mM). Both TTX and ryanodine reduced the amplitude of L_1 and L_2 and decreased the peak tension responses; however, the degrees of inhibition of the two light components were markedly different. Ryanodine (3×10^{-7} M) preferentially reduced the amplitude of L_1 by $70 \pm 26\%$ compared with L_2 by $8 \pm 10\%$ ($n = 6$, $P < 0.05$). The peak of tension response shifted to the later portion of twitch. In contrast, TTX (1×10^{-6} M) predominantly inhibited the amplitude of L_2 (by $74 \pm 20\%$) compared with L_1 (by $34 \pm 28\%$, $n = 7$, $P < 0.05$) and the prolonged relaxation of the twitch was abbreviated. Moreover, the preserved L_2 and tension response after ryanodine were suppressed by the addition of TTX. These data are consistent with the hypothesis that the two components of the intracellular Ca^{2+} transient are mediated by different subcellular mechanisms; L_1 (the normal component) is predominantly controlled by the intracellular calcium storage system, presumably, the sarcoplasmic reticulum, whereas, L_2 (the component appearing in the presence of DPI) is derived from trans-sarcolemmal Ca^{2+} influx.

Discussion

Mechanism of inotropic action of DPI

Although calcium sensitization of the myofibrils by DPI has been suggested from results in skinned fibre preparations (Herzig & Quast, 1984; Scholtysik *et al.*, 1985), we have confirmed this effect for the first time in intact and actively contracting mammalian

myocardium. As shown in Figure 4, changes in the pattern of the aequorin light signal and tension response were complex and apparently paradoxical; the increase in the amplitude of the Ca^{2+} transient was disproportionate to that of the tension responses. At relatively low concentrations of DPI, the peak tension increased without a significant increase in the corresponding peak Ca^{2+} transient. Closer inspection revealed that there was a slight abbreviation of the light signal despite a prolonged tension time course. The most likely explanation for the dissociation between the effects of DPI on the light and tension responses is an increase in the affinity of troponin C for Ca^{2+}, since this protein is quantitatively one of the most important Ca^{2+} sinks in myocardial cells, as well as being the Ca^{2+} receptor site for the myofilaments that controls actin and myosin interactions. The pattern of change we observed is consistent with previous findings (Allen & Kurihara, 1982; Allen & Orchard, 1983; Blinks & Endoh, 1986). They observed a similar complex of responses during increases in muscle length (Allen & Kurihara, 1982; Blinks & Endoh, 1986), during alkalinization of the solution (Allen & Orchard, 1983), or after phenylephrine (Blinks & Endoh, 1986) in similar experimental settings to the present study; most of these interventions have been shown to increase the Ca^{2+} sensitivity of skinned cardiac muscle preparations.

Relating the peak light signal to the corresponding peak tension response provides an approach for testing whether an intervention modifies the Ca^{2+} sensitivity of the contractile apparatus in aequorin-loaded working myocardium (Morgan, 1985; Blinks & Endoh, 1986). The DPI-induced upward shift of this relationship (Figures 5–7) is consistent with DPI-induced sensitization of the myofilaments for Ca^{2+}. Since the amplitude of the light signal is not only dependent on the amount of $[Ca^{2+}]_i$ released from subcellular Ca^{2+} storage sites but also is affected by the rates of Ca^{2+} release and re-uptake, it must be remembered that the use of the peak amplitude of the aequorin signal essentially ignores changes in the time course of $[Ca^{2+}]_i$ transients (Blinks & Endoh, 1986). In the present study, however, DPI produced a minimal change in the time course of the Ca^{2+} transient, suggesting that these effects might be small. Another possible criticism of our results is that the upward shift could represent the consequence of aequorin consumption, because the lipophilic properties of DPI makes it difficult to wash the drug out of preparations (Scholtysik *et al.*, 1985; Buggisch *et al.*, 1985; Kohlhardt *et al.*, 1986) in order to repeat the control protocol to check data reproducibility. To test this possibility, L_{max} was obtained as the integrated light signal during the experiment and during exposure to 2% Triton X-100 at the end of the experiment (Wier *et al.*, 1983). The consumption rate before DPI was 2.7% of L_{max} per hour in 2.5 mM Ca^{2+} using muscles stimulated to contract at 0.33 Hz; 30°C ($n = 2$), whereas, the rate was 2.3% per h during the period from just before exposure to 1×10^{-6} M DPI and throughout the subsequent 1 h ($n = 2$), suggesting that there was no significant aequorin consumption in the presence of DPI. Our preliminary studies (see Methods) ruled out direct inactivation of aequorin by DPI. Taken together, these data do not support the possibility of 'pseudo-' shifts of the relationships.

The effects of DPI on the frequency-force relationship are shown in Figure 3, which is consistent with the observation by Buggisch *et al.* (1985) that DPI may be a sodium channel agonist. The positive staircase of the steady-state force-frequency relationship in mammalian working myocardium may be produced, at least in part, by the net increase in sodium influx per unit time that occurs at higher frequencies. This, in turn, increases the net Ca^{2+} influx via Na^+/Ca^{2+} exchange. (Reiter & Stickel, 1968; Lado *et al.*, 1982; Craig & Fozzard, 1983; Brill & Wasserstrom, 1968). It is therefore reasonable to propose that a sodium channel agonist like DPI or veratridine which increases the opening probability of the channels might shift the relationship upward at any given frequency (Honerjager & Reiter, 1975). In addition, the magnitude of inotropic effect of such a compound might also be expected to show a frequency-dependence, because longer diastole would allow more time for elimination of much more of the primary increase in sodium and calcium in the cell. Such effects were reported in a study by Honerjager & Reiter (1975) using veratridine (see Figure 13 of that study) and by Beress *et al.* (1982) who tested the effect of *Anemonia sulcata* toxin (ATX II) on the frequency-response relationship (see Figure 2 of that study). Therefore, the results presented in Figure 3 are consistent with a sodium channel agonist action of DPI.

However, there are important differences in the actions of DPI compared to other sodium channel agonists. When the relationship of peak light versus peak tension was plotted against the $[Ca^{2+}]_i$-tension relationship, we found that the relationship for veratridine was the same as for Ca^{2+}, whereas the DPI curve was shifted upwards. These data concerning veratridine are consistent with previous observations using an intracellular microprobe, which demonstrated that an increase in intracellular sodium concentration and the subsequent increase in $[Ca^{2+}]_i$ are responsible for the inotropic effect of this agent (Brill & Wasserstrom, 1986). In contrast, the relationship for DPI clearly demonstrated that the increase in $[Ca^{2+}]_i$ is not essential to its inotropic effect.

In two preparations from the ferret, which were sufficiently bright to record individual $[Ca^{2+}]_i$ transients during every twitch, we determined the peak light versus peak tension relationship while the tension showed non-steady-state recovery from a 3 min period of rest (Figure 7). This relationship showed that the post-rest recovery of tension is essentially a $[Ca^{2+}]$-dependent process. Moreover, the $[Ca^{2+}]_i$-tension relationship was consistently modified in the presence of DPI, but not by veratridine. Therefore, the comparison of frequency-response curves in terms of the $[Ca^{2+}]_i$-tension relationship between these two compounds emphasizes the differences between their mechanisms of action.

In previous studies, it has been suggested that DPI can increase Ca^{2+} influx by means of maintaining the open state of sodium channels and subsequently increasing Na^+/Ca^{2+} exchange (From *et al.*, 1984; Buggisch *et al.*, 1985; From *et al.*, 1985; Scholtysik *et al.*, 1985; Scholtysik, 1986; Kohldhardt *et al.*, 1986). However, we observed that the increase in $[Ca^{2+}]_i$ is not essential to the positive inotropic effect of DPI. Especially in the range of relatively low DPI concentrations, the Ca^{2+} transient did not show a substantial increase, which seems inconsistent with its action as a sodium channel agonist. DPI may exert Ca^{2+} channel antagonistic effects in the same concentration range we examined (Buggisch *et al.*, 1985; Holck & Osterrieder, 1988), or may stimulate Ca^{2+} activated efflux mechanisms which counteract and regulate excessive Ca^{2+} influx and prevent a subsequent additional increase in $[Ca^{2+}]_i$ (Bers, 1985). Prolonged diastolic periods at lower frequencies may lead the compensatory mechanism to override the primary increase in the Ca^{2+} influx, as shown in a study of the *Anemonia sulcata* toxin, ATX II (Beress *et al.*, 1982).

Mechanisms of the negative lusitropic action of DPI

Our results show that the tension prolongation observed in the guinea-pig in the presence of DPI is accompanied by a characteristic calcium transient (L_2) (Figure 8). We and others have shown that TTX abbreviates the DPI-induced prolongation of action potential duration (APD) (Figure 2), presumably by antagonizing sodium channel activation (Buggisch *et al.*, 1985; Scholtysik, 1986; Kohlhardt *et al.*, 1986; Slawsky & Morgan, 1987). Therefore, the calcium transient induced by prolonged depolarization or by an increase in sodium loading would be expected to be diminished in the presence of TTX (Narahashi, 1974). In contrast, since ryanodine has been shown to block the release of Ca^{2+} from the sarcoplasmic reticulum (SR) (Sutko & Kenyon, 1983), the calcium transient derived from the SR should be abolished by this agent. Thus, L_2 appeared to originate from the trans-sarcolemmal Ca^{2+} influx and not from the SR, the latter of which is generally accepted as a major source of activator calcium in mammalian cardiac muscle (Chapman, 1983).

We observed that the second component of the Ca^{2+} transient (L_2) appears to arise from the sarcolemma (Figure 9); however, we could not specify the mechanism which mediates the increased trans-sarcolemmal Ca^{2+} influx. One possibility is via the voltage-dependent slow Ca^{2+} channels. The TTX-sensitive nature of this Ca^{2+} current (Figure 9) could be explained by the reduction of the prolonged APD and the subsequent inactivation of the voltage-dependent currents. Recently, however, it has been shown that outward creep currents (implying Ca^{2+} influx across the sarcolemma) in sodium-loaded voltage-clamped atrial cells are mediated by a Na^+/Ca^{2+} exchange carrier (Bielefeld *et al.*, 1986). According to this concept, the TTX-sensitive nature of L_2 can be explained more directly, because the carrier activity is controlled by a combination of transmembrane gradients for Na^+ and Ca^{2+} and the membrane potential (Mullins, 1979).

It is known that certain conditions can induce biphasic contractions that have two distinct components (Endoh *et al.*, 1982; King & Bose, 1983; Malecot *et al.*, 1986). These include rested state contractions (Allen *et al.*, 1976; Beresewicz & Reuter, 1979), or constant frequency contractions at low temperature under the influence of noradrenaline (Bogdanov *et al.*, 1979), with the combination of isoprenaline and theophylline (Endoh *et al.*, 1982) and after replacement of Ca^{2+} with Sr^{2+} (King & Bose, 1983). Biphasic contractions are also known to be induced when the plateau of the action potential is artificially lengthened (Coraboeuf, 1974). The mechanisms underlying biphasic contractions are not known with certainty; however, it has been proposed that each component of the contraction is due to a specific type of cation release; namely, the early component of contraction is related to Ca^{2+} release from the SR, whereas, the late component is due to trans-sarcolemmal Ca^{2+} influx (Bogdanov *et al.*, 1979). Our results from guinea-pig preparations in the presence of DPI provide direct evidence that two distinct components in the Ca^{2+} transient mediate the two components of the contraction.

It is of interest that veratridine has also been reported to cause biphasic contractions (Honerjager & Reiter, 1975). Veratridine prolongs the APD in mammalian myocardium (Honerjager & Reiter, 1975; Brill & Wasserstrom, 1986), and, in our studies in the guinea-pig, veratridine induced a second component in the Ca^{2+} transient accompanied by a markedly prolonged tension time course (data not shown). This implies that the negative lusitropic

properties of DPI are not specific to this agent but are characteristic of sodium channel agonists and other agents that might promote Ca^{2+} influx via the sarcolemma.

At present, we cannot explain the differential lusitropic effects of DPI on guinea-pig versus ferret myocardium. It appears unlikely that changes in the APD in the presence of DPI are responsible for the differences, because ferret preparations also showed a prolongation of APD which was abbreviated by TTX (Figure 2) in the same manner as reported for the guinea-pig. Different activation levels of the voltage-dependent slow inward current or Na^+/Ca^{2+} exchange mechanisms between the two animal species may be the cause. However, it is attractive to relate the species difference of the drug action to potential differences in their intracellular Ca^{2+} handling (Page & Surdyk-Droske, 1979; Sutko & Willerson, 1980; Bers, 1985). The ryanodine-sensitive nature of the ferret preparation indicates that most of the activator Ca^{2+} in this animal is derived from the SR, while the lower sensitivity of the guinea-pig implies that other sources of activator Ca^{2+} may be relatively more important than in the ferret (Sutko & Willerson, 1980; Bers, 1985). Of importance, several manoeuvres that are known to induce biphasic contractions act by reducing the amount of Ca^{2+} released from the SR (Endoh *et al.*, 1982; Malecot *et al.*, 1986). Taken together, these observations suggest that ferret myocardium may contain SR with sufficient capacity to handle excessive Ca^{2+} influx even in the presence of sodium channel activation. Although, at present we do not have data to directly support this hypothesis, these species differences should be considered when selecting an animal model for investigating the inotropic and lusitropic effects of newly developed agents. Preliminary studies from our laboratory suggest that the balance of effects of DPI on normal and failing human myocardium may be different from that in the ferret or guinea-pig (Briggs *et al.*, 1987). On the other hand, comparisons of these responses between animals could provide further understanding about the subcellular effects of these newly developed agents as well as differences in the subcellular Ca^{2+} handling among various species.

In conclusion, this study indicates that although the sodium agonist properties of DPI might play a role, the predominant mechanism of its inotropic action is related to an increased responsiveness of the contractile apparatus to Ca^{2+}, at least in working mammalian myocardium from the species we tested. Second, prolongation of tension relaxation (negative lusitropic property of DPI) was related to abnormal intracellular Ca^{2+} movement, which does not originate from intracellular Ca^{2+} stores but arises by trans-sarcolemmal Ca^{2+} entry during mid-to-late systole. The Ca^{2+} sensitization of the myofilaments exaggerates the tension prolongation; however, its role may be small. Finally, the abnormal intracellular Ca^{2+} movement and the subsequent tension prolongation show significant species differences.

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Caesium ions: a glycine-activated channel agonist in rat spinal cord neurones grown in cell culture

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1 The chloride (Cl^-) currents activated by caesium ions (Cs^+), glycine and γ-aminobutyric acid (GABA) were compared following their application to rat neurones that had been grown in cell culture. Recordings were made using the whole-cell patch clamp technique under voltage clamp conditions.

2 In spinal cord neurones, bicuculline methiodide antagonized GABA-activated currents more effectively than Cs^+- or glycine-activated currents. However, strychnine was more effective at blocking the currents activated by Cs^+ or glycine than those activated by GABA.

3 Of the 3 agonists, only GABA activated currents in cells from the intermediate lobe of the rat pituitary.

4 In spinal neurones the size of the currents activated by 70 mM Cs^+ was correlated to the size of the currents activated by 15 μM glycine ($P < 0.005$; $n = 10$, Spearman's rank correlation), but there was no significant correlation between the size of the currents activated by these agents and 10 μM GABA.

5 The joint application of glycine and Cs^+ activated currents that were approximately twice as big as the sums of the currents activated by separate applications of the same doses. This synergism was consistent with Cs^+ acting at the same receptor as glycine (7 μM glycine being equivalent to 31 ± 7 mM Cs^+).

6 It was concluded that Cs^+ activates the same Cl^- channel as the inhibitory neurotransmitter glycine.

Introduction

Curiously, the monovalent cation caesium (Cs^+) has recently been shown to activate a chloride (Cl^-) channel in rat spinal neurones (McBurney *et al.*, 1985; Hughes *et al.*, 1987). The physiologically important alkali metal ions, sodium and potassium, were dismissed as candidates for the role of the endogenous ligand of this channel as they did not mimic the effect of Cs^+. However, the observation that the Cs^+-activated channel exhibited the same conductance states (Hughes *et al.*, 1987) as the Cl^- channels activated by glycine and γ-aminobutyryic acid (GABA) (Smith *et al.*, 1988), prompted us to compare some other properties of these agonists. We present the results of these studies here and conclude that Cs^+ is a glycine channel agonist. Some of these results have been presented previously in abstract form (Smith, 1987a; Smith & McBurney, 1987).

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Methods

Tissue culture

Cultures of spinal cord neurones were prepared as described previously (Smith, 1987b). Briefly, spinal cords were dissected from 13–15 day old embryonic rats, dissociated and plated onto a near-confluent bed of cortical astrocytes. Culture medium consisted of a mixture of Dulbecco's minimum essential medium (MEM), Ham's F12 and α-MEM in the ratio 3:6:1, that was augmented with rat serum (4%) and chick embryo extract (1%). Glucose (28 mM), L-glutamine (2 mM), sodium bicarbonate (10.5 mM) and 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid

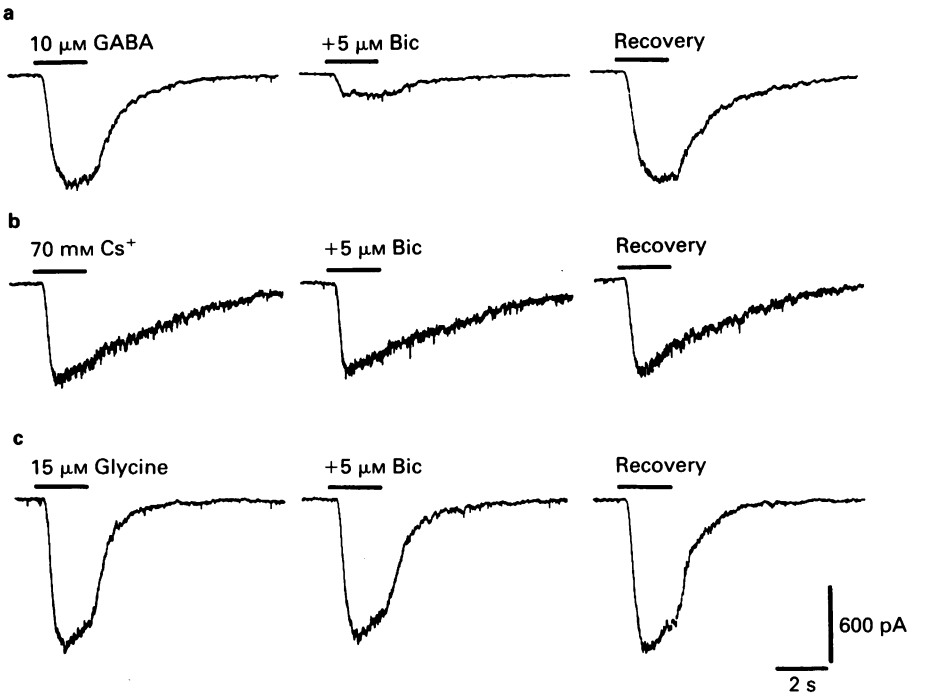

Figure 1 The action of 5 μM bicuculline methiodide (Bic) on the currents activated by 3 Cl^- channel agonists. (a) GABA (10 μM), (b) Cs^+ (70 mM), (c) glycine (15 μM). All applications were made with a U-tube tool for 2 s, with at least 70 s between applications. Cs^+ test solutions were produced by replacing 70 mM of the Na^+ in the bathing medium and the patch electrode contained ES4 ($V_H = -70$ mV, $E_{Cl} = -5$ mV).

(HEPES, 4.2 mM) were also added to the culture medium. All components except the rat serum were obtained from GIBCO. The neurones were incubated at 37°C in 5% CO_2 and 95% air for 11–35 days before use. Pars intermedia cells from the rat pituitary were prepared as described by Kehl *et al.* (1987).

Recording technqiues

Coverslips supporting the cells were placed in a recording chamber mounted on the stage of an inverted phase contrast microscope (Invertoscope D. Zeiss) and viewed at $\times 400$ magnification. Recording electrodes were prepared with outer diameters of about 1–2 μm and heat polished. Whole-cell recordings (Hamill *et al.*, 1981) were made from spinal neurones with cell bodies of 10–25 μm in diameter.

All recordings were made at room temperature (19–22°C) with an EPC-7 amplifier (List Electronics) that was modified to permit the offsetting of junction potentials of up to ± 100 mV. The current and voltage signals were stored following pulse code modulation (modified Sony 701ES: Lamb, 1985) on a video recorder (NV-830, Panasonic).

Solutions

During recordings, the cells were continuously superfused with a medium containing (in mM): NaCl 140, KCl 3.5, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10 and glucose 5. The electrode filling solutions were either ES3 (Na isethionate 114, NaCl 6, $CaCl_2$ 1, $MgCl_2$ 2, EGTA 11 and HEPES 10) or ES4 (CsCl 125, $CaCl_2$ 1, $MgCl_2$ 2, EGTA 11 and HEPES 10). All solutions were titrated to pH 7.2 with NaOH, except for ES3 where TEAOH was used. The Cl^- activities of all solutions were estimated by interpolation from standard tables (Robinson & Stokes, 1959) and used to calculate the Cl^- equilibrium potential (E_{Cl}). Test solutions were made up in the bathing medium and applied to whole cells from a modified U-tube tool (Hughes *et al.*, 1987). Cs^+-containing solutions were made by replacing Na^+ on a stoichiometric basis. Membrane potentials were corrected to account for

the liquid junction potentials that resulted from the different ionic composition of electrode and bathing solutions (Kaneko & Tachibana, 1986).

Data analysis

The majority of recordings were replayed from tape, filtered at 1 or 3 kHz (−3 dB) and captured on the screen of an oscilloscope (Gould 4020). Measurements were then made directly from chart records, subsequent to the traces being dumped at low speed onto a chart recorder (2400S, Gould). Measurements are described in the format aritmetic mean ± s.d.

Results

The actions of bicuculline and strychnine on GABA-, Cs^+- and glycine-activated currents

The receptors for glycine and GABA have been distinguished by their different sensitivity to the antagonists strychnine and bicuculline (Curtis *et al.*, 1971a,b). In an attempt to determine the site of action for Cs^+ we therefore compared the effects of these antagonists on the currents activated by GABA, Cs^+ and glycine.

Figure 1 illustrates the effect of bicuculline methiodide on similar sized currents activated by GABA, Cs^+ and glycine in a spinal neurone (holding voltage (V_H) = 70 mV). Solutions containing antagonists also contained the control level of agonist. The GABA (10 μM)-activated current was reversibly reduced by 81% of its control value by 5 μM bicuculline methiodide (Figure 1a), whilst the same concentration of bicuculline methiodide reduced the Cs^+- and glycine (70 mM and 15 μM respectively; Figure 1b,c)-activated currents by 11 and 8%, respectively. In 6 similar recordings 5 μM bicuculline methiodide reduced the GABA-activated currents by 82 ± 5% but had no significant effect on the Cs^+ (50 or 70 mM) – or glycine (10 or 15 μM)-activated currents (reductions of 3 ± 15 and 5 ± 7%, respectively). However, in 2 of these neurones, 10 μM bicuculline methiodide reduced the Cs^+ (70 mM)-activated currents by 20% and the glycine (15 μM)-activated currents by 19 and 22%.

In recordings from 6 other spinal neurones, using the same recording protocol, strychnine (5 μM) reduced the peak currents activated by GABA (10 μM), Cs^+ (70 or 100 mM) and glycine (15 μM) by 48 ± 9, 80 ± 11 and 94 ± 6%. One such recording is illustrated in Figure 2. Unlike the GABA-activated currents, the responses to Cs^+ and glycine were truncated in the presence of strychnine; peaking sooner and falling at greater rates than in the control applications (see Figure 2). In 3 of these cells the strychnine concentration was increased to 10 μM, and

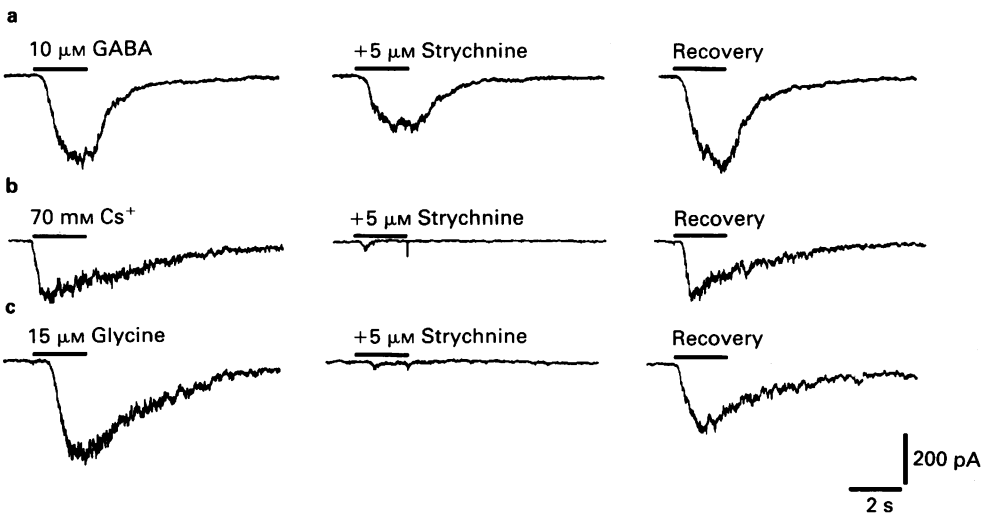

Figure 2 The action of 5 μM strychnine on the currents activated by 3 Cl^- channel agonists. (a) GABA (10 μM), (b) Cs^+ (70 mM), (c) glycine (15 μM). Solutions were as described in Figure 1 and were applied from a U-tube tool (V_H = −70 mV).

this inhibited the GABA-activated currents by $73 \pm 4\%$.

The different sensitivity of the responses to bicuculline methiodide and strychnine, was inconsistent with Cs^+ and GABA activating the same receptor. However, the degree of inhibition of the peak currents by 5 μM strychnine was also different for Cs^+ and glycine ($P < 0.05$, paired t test). This difference may have reflected different mechanisms of action for Cs^+ and glycine, or problems arising from making measurements under non-equilibrium conditions. In an attempt to differentiate between these 2 possibilities the effect of a lower concentration of strychnine (2 μM) was examined in greater detail.

As before, control solutions containing GABA (10 μM), Cs^+ (70 mM) and glycine (15 μM) were applied with a U-tube tool to spinal neurones ($V_H = -70$ mV). Test solutions contained in addition 2 μM strychnine, and reduced the peak responses to Cs^+ and glycine by 62 ± 11 and $81 \pm 9\%$ on average, in the 5 neurones tested, GABA (10 μM) was applied to 4 of these neurones, and the resulting currents were reduced by $21 \pm 6\%$ by 2 μM strychnine.

The effects of 2 μM strychnine on glycine- and Cs^+- activated currents are compared in Figure 3 for a typical cell. Even when test and control currents were both measured at the time at which the control currents peaked (denoted by double arrow heads in Figure 3a), the glycine-activated current was antagonized by a greater amount than the Cs^+-activated current (93 ± 6 and $78 \pm 9\%$, respectively; $P < 0.05$, paired t test). However, this difference was at least partly explained by the observation that the current activated by glycine took longer to peak than the Cs^+- activated current. The glycine-activated currents peaked, on average, 960 ± 210 ms after the switching of the U-tube valve, compared with 650 ± 90 ms for the Cs^+-activated currents ($P < 0.05$, paired t test, $n = 5$).

The currents activated by both Cs^+ (70 mM) and glycine (15 μM) usually desensitized during the course of an application (see Figures 1, 2 and 3). This factor complicated our attempts to obtain a meaningful measure of the level of antagonism produced by strychnine at equilibrium. However, by measuring the test current: control current ratio (r_0) at 100 ms intervals throughout the application period (Figure 3b) the maximum level of antagonism that occurred during an application was estimated. This r_0 value will reflect the antagonism by strychnine if the rates of desensitization are unchanged in the presence and absence of the antagonists. In this cell the r_0 values fell during the application period to 0.02 and 0.08 for glycine and Cs^+ respectively. On average the levels of antagonism increased during the applications to give r_0 values of 0.01 ± 0.01 and 0.09 ± 0.08 for glycine- and Cs^+-activated currents respectively

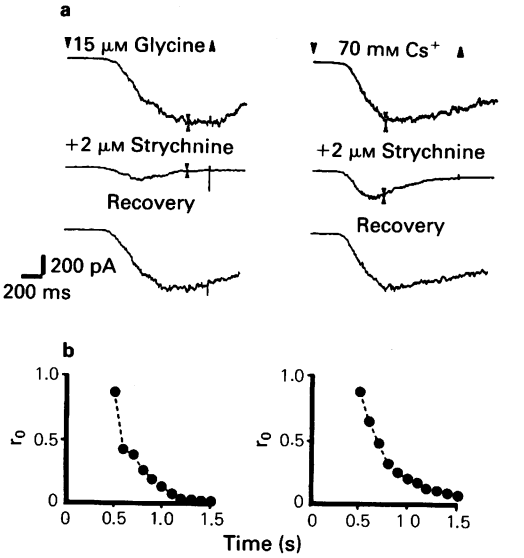

Figure 3 Antagonism of glycine- and Cs^+-activated currents by 2 μM strychnine. (a) Responses aligned relative to the beginning of the 1.5 s application period, which is denoted by the arrowheads above the highest traces. The 2 smaller arrowheads on either side of the current traces indicate the time at which the control responses to glycine and Cs^+ peaked. (b) Plots of r_0 against time for glycine- and Cs^+-activated currents, where r_0 is the ratio of the test current: control current. Bath and electrode solutions were as described in Figure 1.

($P = 0.08$, paired t test). It is pertinent to note that whilst the degree of antagonism of the responses to glycine achieved an apparently steady level by the end of the application period, this was not the case for the responses to Cs^+.

The actions of GABA, glycine and Cs^+ on cells of the rat pituitary

From the comparisons of the actions of bicuculline methiodide and strychnine on GABA-, Cs^+- and glycine-activated currents it seemed more probable that Cs^+ was an agonist of glycine-activated channels than of GABA-activated channels. To determine whether Cs^+ had any action on GABA-activated channels, we compared the effects of GABA, glycine and Cs^+ on cells from the pars intermedia of the rat pituitary, as these cells contain GABA-activated Cl^- channels (Kehl *et al.*, 1987) but are insensitive to glycine (data not shown).

The cells were voltage clamped at 0 mV ($E_{Cl} = -64$ mV) and the test solutions applied with a

U-tube tool for 500 ms at intervals of 80–120 s. GABA (100 μM) activated outward currents of 294–1314 pA (mean = 699 pA) but glycine (100 μM) and Cs^+ (140 mM) produced no detectable effect in the same 4 cells.

A comparison of the sizes of the currents activated by GABA, glycine and Cs^+

Unlike the pars intermedia cells, spinal neurones were always found to respond to GABA, glycine and Cs^+. However, using fixed concentrations of agonists the ratios of the sizes of the currents were not always constant. In 10 spinal neurones GABA, glycine and Cs^+ were applied at concentrations of 10 μM, 15 μM and 70 mM to give mean currents of 421 ± 345, 509 ± 379 and 358 ± 240 pA, respectively. The ratios of the currents elicited for Cs^+:GABA, Cs^+:glycine and glycine:GABA were 1.38 ± 1.93, 0.78 ± 0.19 and 1.77 ± 2.4. It can be seen that the standard deviation of the Cs^+:glycine ratio is over an order of magnitude smaller than the values for the other 2 ratios.

The variation in the relative magnitude of the currents can be seen more clearly in Figure 4. Here the sizes of the current activated by each agonist are plotted against the size of the currents activated by the other 2 agents. There is a significant correlation between the sizes of the currents activated by glycine and Cs^+ ($P < 0.005$, Spearman's rank correlation test) but none between GABA- and Cs^+- or GABA- and glycine-activated currents at the $P = 0.05$ level.

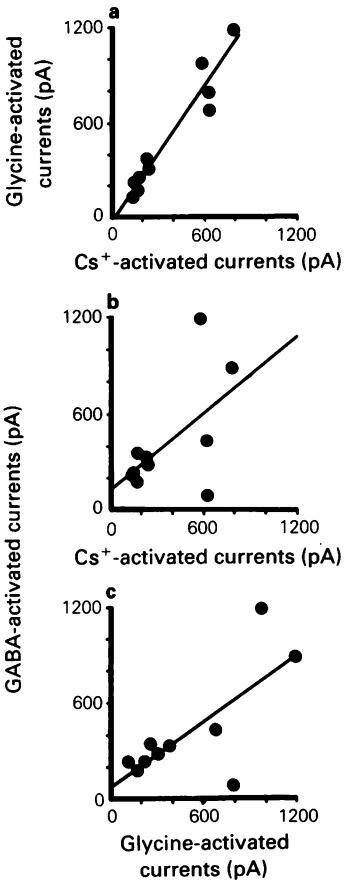

Figure 4 Correlations between the size of the currents activated by GABA (10 μM), glycine (15 μM) and Cs^+ (70 μM) in 10 spinal neurones. All cells were voltage clamped at −70 mV and superfused with BM1 (Cs^+ test solutions produced by replacing an equivalent concentration of Na^+). The electrode solution was ES4 ($E_{Cl} = -5$ mV). (a) Glycine- against Cs^+-activated currents. Spearman's correlation coefficient (r_s) = 0.94. (b) GABA- against Cs^+-activated currents; $r_s = 0.47$. (c) GABA- against glycine-activated currents; $r_s = 0.60$. Lines represent linear regression to the data points.

Does Cs^+ activate the same channel as glycine?

The above results are consistent with Cs^+ activating the same channel as glycine but not GABA. However, they do not exclude the possibility that Cs^+ and glycine activate similar but distinct types of channel. We considered 3 different receptor-channel configurations to explain the activation of Cl^- channels by Cs^+, all of which were based on the assumption that Cs^+ acts through a single type of receptor. It was proposed that Cs^+ and glycine act at distinct receptors that are both coupled to similar, but independent channels, or that they act at distinct receptors that are both coupled to the same Cl^- channel, or that both agonists act at the same receptor. To differentiate between these hypotheses we compared the effects of applying Cs^+ and glycine separately and jointly to the same neurone; for if either of the first two hypotheses are correct the sums of currents activated by the separate application of both agonists, should be equal to or greater than the currents elicited by the simultaneous application of glycine and Cs^+. However, it is known that double logarithmic transformations of the Cs^+ and glycine dose-response plots are fitted by lines with mean slopes of 1.9 and 2.0, respectively (Smith, 1987b). Therefore, if Cs^+ and glycine compete for the same receptor, their joint application should activate larger currents than the sum of the currents elicited by separate applications.

In Figure 5a a spinal neurone was voltage clamped at −70 mV ($E_{Cl} = -5$ mV) and test solutions applied from a U-tube tool. The sums of the

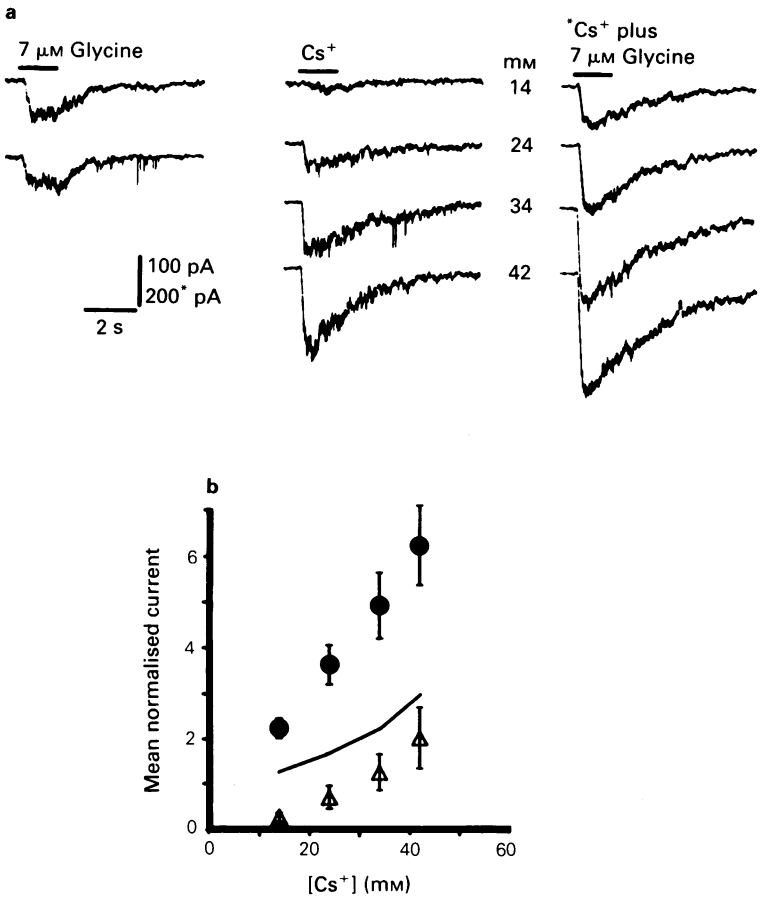

Figure 5 Glycine and Cs^+ act synergistically on spinal neurones. (a) Currents activated by glycine, Cs^+ or both agonists together. Only 2 of the 5 applications of 7 μM glycine are illustrated. Agonist concentrations are given next to each trace. (b) The plot of the mean normalized currents against Cs^+ concentration with (●) and without (△) 7 μM glycine for 3 spinal neurones. Currents were normalized by being divided by the mean response to 7 μM glycine for each neurone. Straight lines were drawn between the points representing the sums of the responses to separate applications of glycine and Cs^+. Solutions were as described in Figure 4.

currents activated by glycine (7 μM) and Cs^+ (14, 24, 34 and 42 mM) were less than the peak currents elicited by the simultaneous application of glycine and Cs^+ (Figure 5a, note the reduced gain in the right-hand panel). The mean data from 3 such experiments are illustrated in (Figure 5b). Each current was normalized by being divided by the mean current ($n = 5$) activated by 7 μM glycine in that particular cell. The means ($n = 3$ cells) of these normalised currents were plotted against the concentration of Cs^+ (Figure 5b). Straight lines were drawn between the points representing the sums of the responses to separate applications of glycine and Cs^+. These lines clearly lie below the currents measured during the simultaneous applications of both agents (●), and so the first and second hypotheses are untenable.

In an attempt to account for the apparent synergism between Cs^+ and glycine quantitatively the data from the 3 cells were replotted (Figure 6). Double logarithmic plots were constructed of the Cs^+-activated currents against Cs^+ concentration (△), and the points fitted to a line by linear regression. The 3 lines have slopes of 1.9 (a), 1.7 (b) and 1.9 (c). The concentration of Cs^+ required to produce a current equal to that activated by 7 μM glycine ($[Cs^+]_{eqv}$) was interpolated from each line (26, 27 and 39 mM in a, b and c respectively). Using these values the data derived from the simultaneous appli-

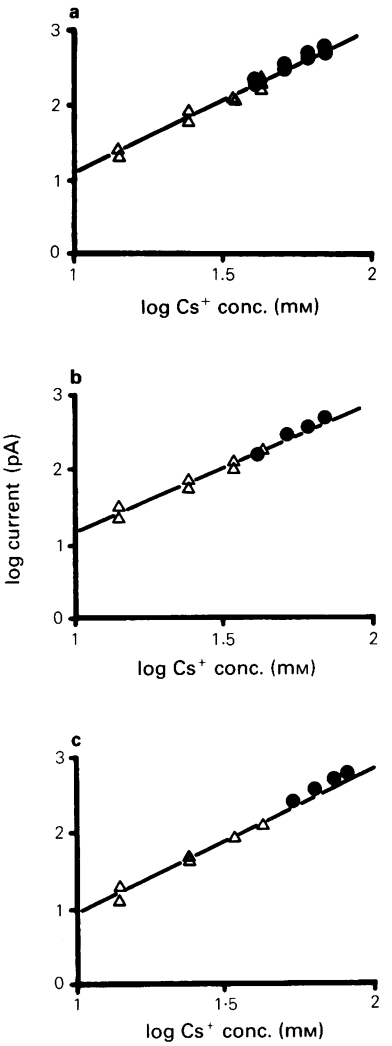

Figure 6 The synergism between Cs^+ and glycine can be explained by assuming they act at the same receptor. The Cs^+-activated currents (△) from the 3 cells described in Figure 5 were replotted on double logarithmic axes and fitted to a straight line by linear regression. Values for $[Cs^+]_{eqv}$ were obtained by interpolation. (a) Slope = 1.9; $[Cs^+]_{eqv}$ = 26 mM. (b) Slope = 1.7; $[Cs^+]_{eqv}$ = 27 mM. (c) Slope = 1.9; $[Cs^+]_{eqv.}$ = 39 mM. The responses to joint applications of Cs^+ and glycine were replotted using the actual $[Cs^+]$ plus the $[Cs^+]_{eqv}$ (●) as the values on the abscissa scale.

cation of both agents were replotted (●) so that the $[Cs^+]$ equalled the actual value plus $[Cs^+]_{eqv}$. These additional points lie close to the lines derived from the responses to Cs^+ alone, which would be expected if both agonists bound to the same receptor and their concentration-effect relationships had the same form over the range of concentrations used.

Discussion

Cs^+ is not a GABA channel agonist

Cs^+ has been shown to activate Cl^- channels with conductance properties similar to those activated by glycine and GABA, in spinal neurones (Hughes *et al.*, 1987). The inability of bicuculline methiodide (5 μM) to reduce Cs^+-activated currents, while antagonizing similar-sized responses to GABA, argues against Cs^+ acting at the same receptor as GABA. The different effects of strychnine on Cs^+- and GABA-activated currents is also inconsistent with them binding to a common receptor. If 2 agonists activate the same channel, then under conditions of voltage clamp it is expected that the ratio of the elicited currents will be constant for fixed concentrations of agonist. The poor correlation between the size of currents activated by fixed concentrations of Cs^+ and GABA, indicated that these 2 agents activated different populations of Cl^- channels. However, such data did not exclude the possibility that Cs^+ acted in some part, by causing the opening of channels sensitive to GABA. Pars intermedia cells possess GABA-activated Cl^- channels which are modulated by benzodiazepines and antagonized by bicuculline (Kehl *et al.*, 1987), but they do not respond to Cs^+ (140 mM). If the same level of Cs^+ insensitivity is exhibited by the GABA-activated channel in cells from the pars intermedia and spinal neurones, then the observed effects of Cs^+ on spinal neurones would not have involved the activation of the GABA sensitive channel at all. Therefore, the major effect of Cs^+ in spinal neurones probably involves activation of a GABA-insensitive Cl^- channel.

Cs^+ is a glycine channel agonist

Glycine- and Cs^+-activated currents were similarly sensitive to bicuculline methiodide. A comparison of the effects of strychnine on the similarly sized currents activated by Cs^+ and glycine, showed that the latter were apparently more sensitive to strychnine. Maximum blockade by strychnine occurred more slowly than the maximum activation of channels by either glycine or Cs^+ and during control applications, Cs^+-activated currents peaked earlier than those activated by glycine. These factors may explain

why the level of antagonism by strychnine was greater for glycine- than for Cs^+-activated currents, when it was assessed by measuring test: control peak current ratios (Figure 2).

From the r_0 values for the duration of application, the amount of antagonism was observed to increase with time as the relative effects of strychnine and agonist approached 'equilibrium' (Figure 3). Strychnine was also slightly more effective against glycine than Cs^+ when judged by this method. However, this small, insignificant difference ($P = 0.08$) may be accounted for by the following explanations. The level of antagonism of the Cs^+-activated current was still increasing at the end of the application (Figure 3b), whereas the effect of strychnine on glycine had apparently achieved a steady state. This could be responsible for some of the observed difference and presumably stems from the tendency for responses to Cs^+ to persist longer than the comparable currents activated by glycine (see Figures 1, 2 and 5). One other possibility is that the currents activated by Cs^+ (70 mM) may have involved the occupation of a greater proportion of the total number of receptors than when glycine (15 μM) was applied. This may seem unlikely because the ratio of the sizes of currents for Cs^+:glycine was 0.78. However, it was evident that Cs^+-activated currents showed more desensitization than the glycine-activated currents, and so the relative size of the currents activated by the 2 agonists is an imperfect estimate of the relative degrees of receptor occupation. Taking into consideration these explanations, the effects of strychnine are consistent with Cs^+ and glycine activating the same type of receptor.

The absence of a response to Cs^+ or glycine in pars intermedia cells, and the close correlation between the sizes of current activated by fixed concentrations of Cs^+ and glycine are 2 factors consistent with Cs^+ activating the same channel as glycine. Comparisons of the frequency of occurrence of the different conductance states of the relevant Cl^- channels (see Hughes *et al.*, 1987; Smith, 1987b), are also consistent with the proposal that Cs^+ activates the same channel as glycine but not GABA.

Of the three models (see Results section) relating the receptor sites for glycine and Cs^+ the first two can be dismissed on account of the synergism observed when the agonists were applied jointly. Moreover, the data were consistent qualitatively with Cs^+ and glycine acting at the same receptor.

By using the peak currents as approximations of the currents at equilibrium, it was possible to estimate $[Cs^+]_{eqv}$ (Figure 6). The assumption was then made that 7 μM glycine would have the same effect as $[Cs^+]_{eqv}$. The validity of this assumption depends on the kinetic scheme for the interaction between the agonists and its receptor. For instance with even a relatively simple scheme such as that used to describe the activation of other channels with 2 binding sites (Colquhoun *et al.*, 1987), and supposing $k_1 = k_2$ and $k_{-1} = k_{-2}$:

$$A + R \underset{k_{-1}}{\overset{2k_1}{\rightleftharpoons}} AR + A \underset{2k_{-2}}{\overset{k_2}{\rightleftharpoons}} A_1R \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} A_2R^*$$

the assumption only holds if the ratio β/α is the same for Cs^+ and glycine, or if the open probability for the channels is suitably low (e.g. for β/α ratios of 3 and 30 the error < 10% if the open probability < 0.025). However, although these conditions are quite plausible a satisfactory kinetic scheme has not yet been derived for glycine (see Smith, 1987b) and so the original assumption may be invalid. In favour of the assumption, it has been shown that the doube logarithmic dose-response relationships for Cs^+ and glycine are both described by straight lines with similar slopes for the range of concentrations used in Figure 6 (Smith, 1987b), which is consistent with the assumption that 7 μM glycine will behave like $[Cs^+]_{eqv}$. The currents elicited with the joint applications of Cs^+ and glycine were therefore replotted using the actual $[Cs^+]$ plus $[Cs^+]_{eqv}$ and it is evident that these data lie close to the line extrapolated from the Cs^+ dose-response plot (Figure 6). This result is consistent with the third model, which predicts that both agents compete for the same receptor. This approach is not as rigorous as would be preferred; however, it does show that the synergism can be accounted for in terms of only one type of receptor. Hence, the data do not necessitate the postulation of an additional modulatory site, equivalent to those activated by barbiturates and steroids and involved in the enhancement of GABA-activated channels (Barker & Ransom, 1978; Cottrell *et al.*, 1987).

On the sites of action of Cs^+

In conclusion there is good evidence that Cs^+ activates the same channel as glycine, probably via the same receptor, in rat spinal cord neurones. Nevertheless it is difficult to envisage how a simple monovalent alkali metal cation can interact with a receptor that is normally activated by glycine; an amino acid which exists almost completely as a zwitterion at pH 7.2 (White *et al.*, 1973).

The large number of shells of electrons around the nucleus of Cs^+ makes it difficult to estimate the shape of the hydrated ion. Thus we have been unable to compare the hydrated structures of Cs^+ and glycine.

Interestingly, other similarities between the actions of Cs^+ and glycine have been described recently.

Biscoe *et al.* (1987), using mouse spinal neurones, showed that glycine blocks a potassium channel that is also blocked by Cs^+. So whilst there is no obvious structural similarity between Cs^+ and glycine they have similar effects on at least 2 types of channel. However, Cs^+ does not enhance N-methyl-D-aspartate-activated currents (Smith & McBurney, unpublished observation) in the way described for glycine (Johnson & Ascher, 1987).

In addition, to its well documented action of blocking K^+ channels (Hille, 1984), and its ability to activate Cl^- channels, Cs^+ also blocks the activation of acetylcholine-activated channels in chromaffin and sympathetic ganglion cells (Hirano *et al.*, 1987; A. Mathie, personal communication). Surprisingly, Cs^+ antagonizes acetylcholine-activated currents without causing a change in the noise spectrum, and it is therefore possible that Cs^+ is acting as a competitive antagonist at this receptor.

It is clear that Cs^+ can no longer be considered solely as a blocker of K^+ channels, and in future, its other actions should be considered whenever it is used.

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Role of endothelium in hypoxic contraction of canine basilar artery

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1 Reversible contraction of canine basilar artery, produced by hypoxia, persisted after mechanical and chemical removal of the endothelium. The removal of endothelium was confirmed by scanning electron microscopy as well as by the abolition or reversal of the relaxant response to acetylcholine or arginine[8]-vasopressin.

2 Hydroquinone, believed to block selectively endothelium-mediated relaxation, also preferentially attenuated hypoxic contractions even in the absence of endothelium but did not reduce responses to 5-hydroxytryptamine (5-HT) or high external potassium.

3 Contractions induced by red blood cell haemolysate, which occur independently of the endothelium, were also selectively attenuated by hydroquinone.

4 Contractions caused by hypoxia were inhibited by pretreatment with adenosine or by its application after contraction had developed.

5 Hypoxic contraction in canine basilar artery may result partly from a direct effect on smooth muscle as well as through the endothelium.

6 Hydroquinone may have an additional locus of action in smooth muscle cells besides its well known effect on the endothelium.

Introduction

Canine isolated basilar artery rings exhibit an increase in active tension when made hypoxic. These contractions are not antagonized by either β- or α-adrenoceptor blockers, 5-hydroxytryptamine (5-HT) receptor blockers, muscarinic receptor blockers, or by inhibitors of cyclo-oxygenase (Katusic & Vanhoutte, 1986, Mallick *et al.*, 1987). It was previously suggested that hypoxic contractions were due to release of endothelium-derived constricting factors (Katusic & Vanhoutte, 1986). The release of a vasoconstrictor substance from bovine cultured coronary endothelial cells (Hickey *et al.*, 1985), and canine coronary endothelium (Rubanyi & Vanhoutte, 1986) has been described previously.

In this paper evidence will be presented that hypoxic contraction is only partially dependent on the endothelium because significant hypoxic contraction persisted in the absence of endothelium. Nevertheless, hydroquinone, an agent known to block endothelium-mediated relaxation (Furchgott, 1984), was also able to block hypoxic contraction as well as that due to haemoglobin (also believed to act directly on vascular smooth muscle) (Fujiwara & Kuriyama, 1984; Conner & Feniuk, 1987). Finally this work also shows that hypoxic contraction is reversible and that it is markedly reduced by adenosine.

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Methods

Basilar arteries were obtained from mongrel dogs (5–11 kg) of either sex, anaesthetized with sodium

pentobarbitone (30 mg kg^{-1} i.v.). Five mm long segments were suspended by two L-shaped stainless steel wires (30 AWG) in 20 ml organ baths containing Krebs-Henseleit solution bubbled with 95% O_2: 5% CO_2 and maintained at 37°C. The composition of the solution (in mM) was: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.4, $NaHCO_3$ 25 and dextrose 11. Isometric tension was measured at a preload of 2 g. Hypoxia was initiated by changing the gas mixture bubbling through the bath to 95% N_2: 5% CO_2.

Experimental protocols

To test for the presence of a functional endothelium, functional and histological criteria were adopted.

Functional testing Contraction of the vessel ring was first produced by the application of 5-hydroxytryptamine (5-HT, 0.1 μM). Subsequent addition of acetylcholine (ACh, 50 μM) or arginine^8-vasopressin (AVP, 0.01 μM) to the bath caused relaxation of vascular tone. This is indicative of the presence of endothelium and the release of endothelium-dependent relaxing factor(s) (Furchgott *et al.*, 1981). Once the vessels relaxed to these agents the preparation was washed and the response to a 10 min period of hypoxia was recorded. The endothelium of the vessels was then removed and the responses to the various drugs were observed and compared to their own control. The response of the vessels to high extracellular K^+ (100 mM) was recorded before and after removal of endothelium to check for the effect of the procedure for endothelium removal on vascular smooth muscle function.

Scanning electron microscopy In each of 3 experiments 2 vascular preparations were first mounted in the bath and tested for relaxant response to acetylcholine and arginine^8-vasopressin, as indicated above. One of the vessels was fixed for histological examination at this point. The lumen and outer surface of the remaining vessel were treated with 0.1% Triton X-100 for 1 min. After washout of the detergent, abolition of the relaxant response to acetylcholine and arginine^8-vasopressin was always observed. At this point the vessel was removed and fixed for electron microscopy. The vessel was transversely cut into 1 mm lengths and fixed in a solution of 2.5% paraformaldehyde, 0.02 mM $CaCl_2$ and 2% glutaraldehyde in 0.12 M phosphate buffer at 4°C for 24 h. This was followed by three rinses overnight in a solution containing 8% dextrose, 0.02 mM $CaCl_2$ in 0.12 M phosphate buffer. After washing in twice distilled water for 2 h the preparations were dehydrated in increasing concentrations of ethanol and dried to critical point with CO_2. The specimens were mounted with conductive silver paint onto spuds and examined with a JEOL JSM 35C scanning electron microscope at a magnification of 2000 ×.

Hydroquinone treatment and preparation of haemoglobin suspension

Responses to several contractile agents were evaluated in the absence and presence of hydroquinone. Contractions were elicited by addition of 5-HT (0.1 μM), KCl (100 mM), hypoxia and red blood cell haemolysate (haemoglobin; 0.2 μM), to the bath before, during, and after exposure of the muscle to hydroquinone (HQ, 40 μM). Ten ml blood was obtained from dogs at the same time as the vessel, via a major artery and collected in a heparinized tube. It was then centrifuged at 5000 *g* for 10 min and the buffy coat aspirated to leave the red blood cells (RBC), which were washed 2 times with 10 ml isotonic saline and centrifuged at 15,000 *g* for 20 min; this left about a 5 ml suspension of RBC which was diluted to 30 ml in distilled water. Ten μl aliquots of this suspension (0.2 μM haemoglobin) were added to the bath.

Adenosine treatment

The effect of adenosine on hypoxic contraction was determined by treatment of the vessel with varying concentrations of adenosine either 5 min before induction of hypoxia, or at the peak of hypoxic contraction. The inhibitory effect of pretreatment with adenosine on the hypoxic contraction was assessed by measuring the area under the contraction curve over a 10 min period and this was compared to a comparable response in the absence of adenosine. The area under the contraction curve was measured with a Houston Instrument Hipad Digitizer (Model DT114) connected to a microcomputer which integrated the digitized data.

Removal of endothelium

After the control responses were obtained the endothelium was removed by either intimal rubbing with a 23 gauge stainless steel needle or intraluminal exposure to 0.1% Triton X-100 for 1 min. Triton treatment has been shown to remove effectively the endothelial layer from small cerebral vessels (Conner & Feniuk, 1987). In some experiments the artery was gently everted before mounting. This facilitated the subsequent removal of the endothe-

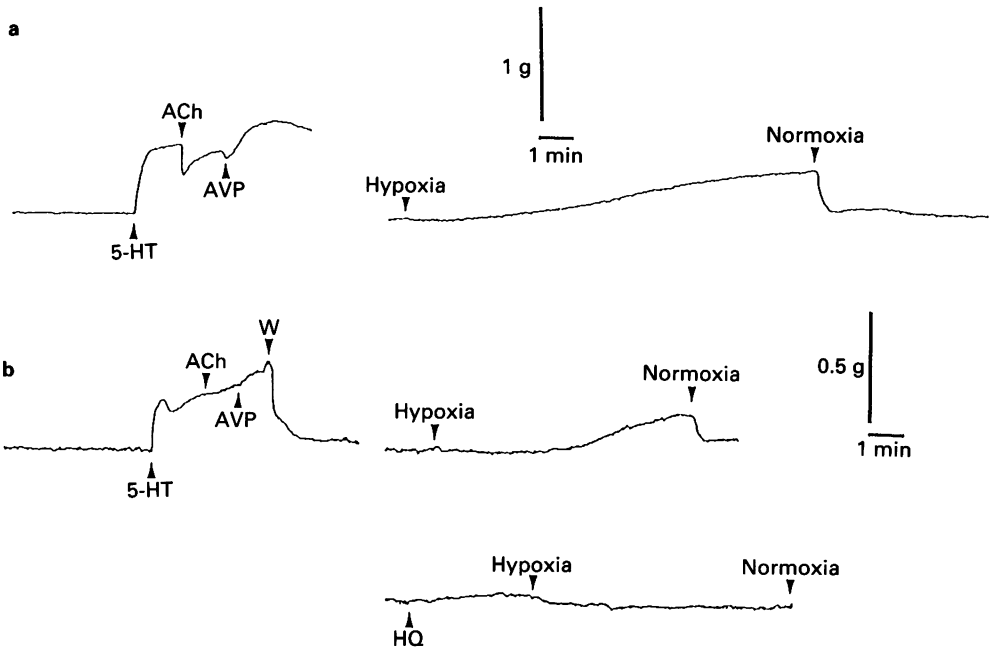

Figure 1 Increase in isometric tension tracing from a canine basilar artery ring showing hypoxic contraction in the presence and absence of endothelium. (a) Contraction due to 100 nM 5-hydroxytryptamine (5-HT). Subsequent relaxation by 50 μM acetylcholine (ACh) and 0.01 μM arginine^8-vasopressin (AVP) indicates presence of functional endothelium. Ten min of hypoxia caused contraction which was reversed by normoxia. (b) Same tissue as in (a), after intraluminal exposure to 0.1% Triton X-100 for 1 min. Acetylcholine (ACh) and arginine-vasopressin (AVP) relaxations were converted to contraction, indicating impairment of endothelial influence. Hypoxic contraction is present but is blocked by 40 μM hydroquinone (HQ).

lium by gentle rubbing of the outer surface of the vessel. In some cases though, eversion of the vessel alone abolished the relaxation induced by ACh and arginine^8-vasopressin, indicating damage to the endothelium. Therefore these experiments, where there were no internal controls even though they contracted in response to hypoxia, were removed from the series.

Drugs

The following chemicals were used: 5-hydroxytryptamine, arginine^8-vasopressin, acetylcholine chloride (all from Sigma Chemical Co.), Triton X-100 (Fisher Scientific Co.), hydroquinone (J.T. Baker Chemical Co.).

Statistical evaluation

Data are shown as means $\pm$ s.e.mean and were analysed by one way analysis of variance and Duncan's multiple range test with $P \leq 0.05$ indicating statistical significance.

Results

Effect of endothelium removal on contractions and relaxations elicited by various agonists

Canine isolated basilar artery rings contracted when exposed to 5-HT (0.1 μM) and subsequently relaxed on exposure to ACh (50 μM) or AVP (0.01 μM) (Figure 1). This was indicative of a functional endothelial layer, capable of releasing endothelium-derived relaxing factors. A scanning electron micrograph representative of the luminal face of normal basilar artery specimens from 3 separate animals is shown in Figure 2a. The exposed surface is largely invested with endothelium. When a normal

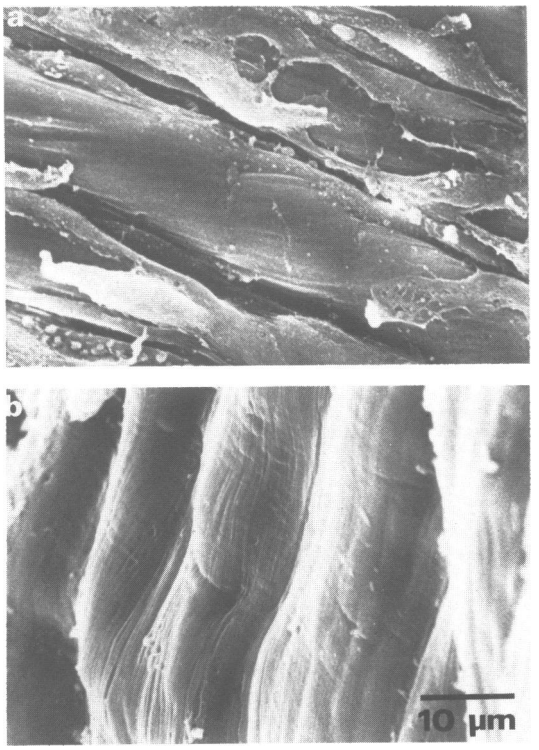

Figure 2 Scanning electron micrographs of luminal surface of (a) normal basilar artery showing largely preserved endothelial covering and (b) in Triton X-100-treated basilar artery showing removal of endothelial coating. The Triton X-100-treated vessel failed to relax in response to acetylcholine and vasopressin compared to the normal vessel.

vessel was made hypoxic ($Po_2 < 30$ mmHg) a contraction was produced which was relaxed upon restoration of normoxia ($Po_2 > 600$ mmHg). Removal of the endothelium by rubbing or with the use of the detergent, Triton X-100, partially reduced contractions due to hypoxia, KCl and 5-HT but this reached statistical significance in the case of hypoxia and 5-HT (Table 1). Impairment of contraction of all stimuli may represent a generalized deleterious effect of endothelium removal. If one takes this into account then the true dependence of hypoxic contraction on the endothelium appears to be less marked than the data indicate. Scanning electron micrographs obtained from Triton X-100-treated basilar arteries ($n = 3$) showed complete removal of the endothelium (Figure 2b). More importantly, endothelium-dependent relaxations produced by ACh and AVP, were either abolished or converted to small contractions (Table 2). The latter test is more representative of the state of the entire blood vessel preparation than is the sampling done of the histological specimens.

Effect of hydroquinone on basilar artery contractions

Pretreatment of basilar artery rings with hydroquinone (40 µM) significantly attenuated contraction due to hypoxia or red blood cell haemolysate without affecting those due to KCl (Table 3). Hydroquinone potentiated the contractile effects of 5-HT (Table 3), but this finding was not further investigated in this study. The fact that hydroquinone attenuated endothelium-independent haemoglobin-induced contractions as effectively as those produced by hypoxia, suggests that hydroquinone may have a direct smooth muscle locus of action.

Hypoxic contraction and the effect of adenosine

Canine basilar artery rings contracted by hypoxia were completely relaxed by exposure to adenosine (5 µM; Figure 3). Pretreatment with adenosine also attenuated hypoxic contraction in a dose-dependent manner, with a 92% decrease in contraction size with 5 µM adenosine ($n = 6$; Figure 4).

Table 1 Effect of presence or absence of endothelium on tension increase due to hypoxia, KCl or 5-hydroxytryptamine (5-HT)

	Developed isometric tension (g wt.)					
	Hypoxia	n	*KCl* (100 mM)	n	*5-HT* (0.1 µM)	n
+ Endothelium	0.73 ± 0.11	8	0.95 ± 0.36	3	0.76 ± 0.10	6
− Endothelium	0.30 ± 0.06*	8	0.66 ± 0.14	3	0.54 ± 0.13*	6

Data shown are mean ± s.e.mean.
* $P \leqslant 0.05$.

Table 2 Effect of presence or absence of endothelium on acetylcholine (ACh) or arginine vasopression (AVP)-induced relaxation

	% change in active tension with ACh (50 μM)	n	AVP (0.01 μM)	n
+ Endothelium	−31.34 ± 2.81	10	−67.65 ± 9.18	6
− Endothelium	1.03 ± 1.63*	10	9.46 ± 4.63*	5

Data shown are means ± s.e.mean.
* $P \leqslant 0.05$.

Discussion

The major finding of our study is that hypoxia-induced contraction of canine isolated basilar artery is not wholly dependent on the endothelium. This is in agreement with previous work done in our laboratory (Mallick *et al.*, 1987) and only partially with those experiments carried out by Katusic & Vanhoutte (1986). In contrast to the earlier study showing the abolition of hypoxic contraction in unstimulated basilar artery rings devoid of endothelium (Katusic & Vanhoutte, 1986), we saw appreciable hypoxic contraction even after mechanical and/or chemical removal of the endothelium. When the generalized depression of contraction due to KCl and 5-HT, agents which act directly on the smooth muscle, is taken into account then the decrease in hypoxic contraction appears to be less than the data indicate. Successful endothelium removal in our experiments was confirmed by reversal of the vasodilator effects of vasopressin (Katusic *et al.*, 1984) or acetylcholine. Although previous work indicated only a weak (15%) relaxation with ACh (0.1 μM) of a contraction produced by prostaglandin $F_{2\alpha}$ (3 μM) in the canine basilar artery (Kanamura *et al.*, 1987), we consistently obtained 30% relaxation of a 5-HT (0.1 μM)-induced contraction with ACh (50 μM). Our results are similar to that seen in the rabbit basilar artery (Fujiwara *et al.*, 1986) and slightly less than the relaxation produced by ACh of a 5-HT-induced contraction in the rabbit aorta (Griffith *et al.*, 1987). Our data indicate that the hypoxic contraction, at least in part, is not dependent on the endothelium.

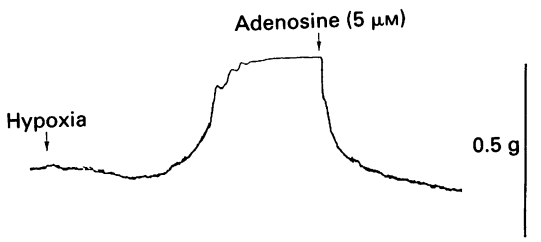

Figure 3 The effect of exogenously applied adenosine on hypoxic contraction in canine basilar artery. A typical experiment is shown in which adenosine (5 μM) added at the peak of an hypoxic contraction completely relaxed the vessel past its baseline tension.

Hydroquinone, despite being a very effective reducing agent, is generally used as a tool to block endothelium-mediated relaxations. Its mechanism is believed to be either a disruption of endothelial integrity (Furchgott, 1984), or production of free radicals leading to the breakdown of an endothelial factor (Moncada *et al.*, 1986). Superoxide dismutase or hydroquinone modify endothelium-mediated responses (Furchgott *et al.*, 1981; Rubanyi & Vanhoutte, 1985, Moncada *et al.*, 1986). The effect of hydroquinone becomes irreversible upon longer exposure (Furchgott *et al.*, 1981). In our experiments, hydroquinone was able to block reversibly hypoxic contraction in normal as well as endothelium-

Table 3 Effect of hydroquinone (HQ, 50 μM) on contraction due to hypoxia, KCl, 5-HT or haemoglobin

	Developed isometric tension (g wt.) *Hypoxia*	n	*KCl* (100 mM)	n	*5-HT* (0.1 μM)	n	Haemolysate (0.2 μM haemoglobin)	n
Before HQ	0.69 ± 0.04	7	1.23 ± 0.16	7	0.51 ± 0.06	4	0.39 ± 0.05	6
During HQ	0.01 ± 0.01*	7	1.30 ± 0.27	4	1.12 ± 0.21*	3	0.01 ± 0.01*	7
After wash	0.64 ± 0.15	5	1.08 ± 0.21	6	0.78 ± 0.14	4	0.35 ± 0.08	4

Data shown are means ± s.e.mean.
* $P \leqslant 0.05$.

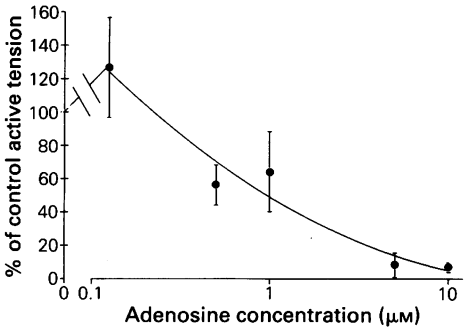

Figure 4 The inhibitory effect of adenosine pretreatment on hypoxic contraction. The concentration of adenosine added before hypoxia has been plotted against the % active tension in the vessel measured as the area under the hypoxic contraction curve. Adenosine concentrations of 5 and 10 μM decreased the area to 8.14 $\pm$ 7.36% (n = 6) and 6.92 $\pm$ 2.96% (n = 5) of the pre-adenosine contraction, respectively. Mean values (with vertical lines indicating s.e.mean) after various doses from 3–6 experiments are shown. The experimental points were fitted by 4th order polynomial regression.

denuded preparations of the canine basilar artery. This finding is a novel one considering that this is the first indication that hydroquinone is able to alter contractile properties of vascular smooth muscle. The ability to block the response of the artery to hypoxia without attenuating the response to membrane depolarization (high K^+) or to a vasoactive agent (5-HT) suggests a specific action against the effect(s) of hypoxia or haemoglobin. The fact that hydroquinone potentiated the response to 5-HT somewhat is further evidence that this agent is not acting by non-specific depression of smooth muscle contraction.

In cerebral vessels, contraction to haemoglobin (or red blood cell haemolysate) is known to be independent of the endothelium (Fujiwara & Kuriyama, 1984; Conner & Feniuk, 1987). In our study we have also shown that haemolysate produces a contraction of the canine basilar artery and this effect is completely abolished by pretreatment with hydroquinone. These results suggest that, in addition to its better known effect on the endothelium, hydroquinone may also act directly on the smooth muscle in a selective manner against hypoxia and haemoglobin-induced contractions. Therefore caution is recommended in ascribing its actions solely to the endothelium.

It is well known that *in vivo* the cerebral vasculature dilates in response to hypoxic conditions (Haggendal & Johansson, 1965; Jones *et al.*, 1978; 1981; Busija & Heistad, 1981) and this is thought to be as a result of the release of adenosine (Wahl & Kuschinsky, 1976; Winn *et al.*, 1981; Phillis *et al.*, 1987; Sollevi *et al.*, 1987) from the brain parenchyma. It was therefore surprising to observe a contraction with hypoxia in the isolated basilar artery. With this in mind and with the knowledge that the larger cerebral vessels in the cat (Kontos *et al.*, 1978) and the rat (Harper *et al.*, 1984) are at least as important as the microvasculature in adjusting cerebral blood flow to changes in oxygen tension, we decided to determine the effects of exogenously applied adenosine on hypoxic contraction in the canine isolated basilar artery. Adenosine (5 μM) added to the bath at the peak of hypoxic contraction caused a complete relaxation of the vessel. Pretreatment with adenosine before hypoxia attenuated hypoxic contraction in a dose-dependent manner. Adenosine concentrations of 5–10 μM, which were similar to the *in vivo* concentrations of adenosine achieved during hypoxia (Winn *et al.*, 1981; Phillis *et al.*, 1987; Park *et al.*, 1987), almost completely abolished hypoxic contraction. This lends further evidence to the theory that adenosine could be a major factor in the regulation of cerebral blood flow during hypoxia.

It is known that hypoxic contraction, which is blocked by EGTA (2.5 mM) and the calcium channel blocker methoxyverapamil (D600, 10 μM) (Mallick *et al.*, 1987), is dependent on extracellular calcium influx. Preliminary data from our laboratory indicates that the calcium channel agonist Bay K 8644, poteniates the effects of hypoxia on this vessel (Elliott, unpublished observations), which suggests that the voltage-gated calcium channel may play a role in hypoxic contraction in the canine basilar artery.

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Involvement of a pertussis toxin-sensitive G-protein in the pharmacological properties of septo-hippocampal neurones

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1 The physiological and pharmacological properties of identified septo-hippocampal neurones (SHNs) have been studied in rats pretreated with the bacterial toxin, pertussis toxin (PTX).

2 In rats anaesthetized with urethane and pretreated with PTX, the axonal conduction velocity was unchanged while the mean spontaneous activity was significantly increased.

3 PTX pretreatment had no effect on responses of SHNs to the iontophoretic application of γ-aminobutyric acid (GABA) and cholinoceptor agonists (acetylcholine or carbachol).

4 Baclofen and 5-hydroxytryptamine (5-HT), almost exclusively inhibitory in control rats, had little effect or an excitatory effect in PTX pretreated rats.

5 These results suggest the involvement of a pertussis toxin-sensitive G-protein in responses mediated by 5-HT and $GABA_B$-receptors but not in responses mediated by cholinoceptors and $GABA_A$-receptors in medial septum neurones projecting into the hippocampus.

Introduction

The septo-hippocampal projection is one of the major central cholinergic pathways and may be involved in memory processes (for references see Hepler *et al.*, 1985). The pharmacological properties of the septal neurones which project into the hippocampus in the rat have been described using microiontophoresis application *in vivo* (Lamour *et al.*, 1984; Dutar *et al.*, 1986). However, the nature of transduction mechanisms possibly involved in the pharmacological properties of these neurones is still unknown. Recently, a number of guanine nucleotide binding proteins (G-proteins) have been identified as coupling some neurotransmitter receptors to a variety of effectors in different tissues (for review see Stryer & Bourne, 1986). Some of these G-proteins (Gi or Go) are inactivated by pertussis toxin (PTX) which ADP- ribosylates the alpha subunit of the protein (Murayama & Ui, 1983). This inactivation blocks in turn the effect mediated by the protein (Kurose *et al.*, 1983). PTX-sensitive G-proteins are present in the central nervous system and have recently been shown to be involved in the pharmacological effects of various neurotransmitters in the hippocampus (Andrade *et al.*, 1986; Clarke *et al.*, 1987), locus coeruleus (Aghajanian & Wang, 1987), substantia nigra and raphe nucleus (Innis & Aghajanian, 1987a,b). The purpose of this experiment was to study the changes occurring in the pharmacological properties of septo-hippocampal neurones (SHNs) in rats pretreated with PTX.

Methods

Experiments were performed in Male Sprague Dawley rats (200–300 g, $n = 22$) anaesthetized with urethane (1.5 g kg^{-1}, i.p.). Among these rats, 10 were used as control rats and did not receive any pretreatment. The others ($n = 12$) were treated with pertussis toxin (PTX, List Biological Laboratories) 3 days before the experiment. PTX was injected bilaterally in the cerebral ventricles using a stereotaxic approach (2 μg diluted in sterile water, pH 7, at coordinates A 7.5, L 1, H 1.5 (König & Klippel, 1963)) in rats anaesthetized with chloral hydrate (400 mg kg^{-1}, i.p.). We have shown previously that the i.c.v. injection of NaCl does not modify the properties of the

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SHNs (Jobert *et al.*, 1989). As a consequence, we used in this experiment non-injected rats as controls instead of sham operated saline-injected rats.

Extracellular recordings were obtained from single neurones located in the medial septum and in the nucleus of the diagonal band of Broca (MS-nDBB). These neurones were identified as projecting to the hippocampus by their antidromic response to the electrical stimulation of the fimbria-fornix (square pulses of 0.2 ms, 40–800 μA). The stimulating pulses were applied between two monopolar electrodes positioned in the fimbria on each side of the midline at the coordinates A 6060, H 1.5 of the atlas of König & Klippel (1963). The recording electrode was filled with 1 M NaCl and 2% pontamine sky blue in order to localize subsequently the position of the recorded neurones. This electrode was attached to multibarrelled micropipettes filled with solution for testing by iontophoresis: acetylcholine chloride (0.2 M, pH 4), carbamylcholine chloride (CCh, 0.2 M, pH 4), γ-aminobutyric acid (GABA, 0.2 M, pH 4), 5-hydroxytryptamine creatinine sulphate complex (5-HT, 50 mM, pH 4) all purchased from Sigma, and baclofen (40 mM, pH 4, Ciba Geigy). Automatic current balancing was routinely used. The mean spontaneous activity of each neurone was recorded over a period of 1 min before any drug application. The frequency of the neuronal discharge and the changes induced by drug applications were displayed on a rectilinear strip chart recorder. Iontophoretic applications (baclofen 80 to 110 nA, GABA 5 to 50 nA, 5-HT 80 to 110 nA, CCh 40 to 60 nA) were used in control and PTX-treated animals to allow a comparative study between the two groups. Only standard iontophoretic applications (baclofen 80 nA for 20 s, GABA 20 nA for 10 s, 5-HT 80 nA for 20 s, CCh 40 nA for 15 s) were used for the statistical analysis. The mean percentage of excitation or inhibition of the spontaneous activity was measured as the ratio between the discharge frequency of the neurone during the drug application (increase or decrease in the discharge frequency measured at the peak of the response) and before the drug application. At the end of the experiment, animals were perfused through the heart with a solution of formaldehyde. Sections of the brain were cut and stained with cresyl violet to check the location of the recorded neurones and of the PTX injection site. Additional technical details have been published previously (Lamour *et al.*, 1984; Dutar *et al.*, 1986).

Results

Physiological properties of SHNs

In PTX pretreated rats, the mean latency ($\pm$ s.e.mean) of the antidromic response of the SHNs was not significantly different from that in control rats: 1.4 ± 0.1 ms, ($n = 121$) vs 1.5 ± 0.1 ms, ($n = 95$) respectively (Student's t test, $t = 0.95$, d.f. = 214). However, the spontaneous activity of the SHNs ($\pm$ s.e.mean) was significantly higher in PTX pretreated rats than in control rats: 21.6 ± 1.6 spikes s^{-1} ($n = 117$) vs 13.2 ± 1.2 spikes s^{-1} ($n = 89$) respectively. (Student's t test, $t = 3.91$, d.f. = 204, $P < 0.001$).

Table 1 Effect of drugs on the discharge rate of septo-hippocampal neurones in 10 control (A) and 12 pertussis toxin (PTX) pretreated (B) rats

(A) *Control rats*

	Neurones tested	*Neurones excited*	*No effect*	*Neurones inhibited*
Carbachol	20	14	1	5
5-HT	19	0	1	18
GABA	43	0	1	42
Baclofen	61	0	2	59

(B) *PTX pretreated rats*

	Neurones tested	*Neurones excited*	*No effect*	*Neurones inhibited*
Carbachol	51	49	1	1
5-HT	28	14	10	4
GABA	54	0	1	53
Baclofen	83	12	49	22

Statistical analysis, chi-squared tests with the Yates correction are given in the text.

Pharmacological properties

Responses of SHNs to CCh, GABA, baclofen and 5-HT were compared between PTX pretreated and control rats. The results are summarized in Table 1. In both control and PTX-treated animals, carbachol excited the majority of SHNs (14 out of 20 in controls vs 49 out of 51 in PTX treated rats) (see Table 1) (the proportion of the various types of responses to carbachol was not statistically different between control and PTX-pretreated rats, chi-squared test with the Yates correction). Carbachol (standard application: 40 nA for 15 s) induced a similar increase in firing rate in both groups ($121 \pm 23\%$ in controls vs $107 \pm 9\%$ in PTX pretreated animals) (mean $\pm$ s.e.mean) (Figures 1 and 2). The difference between these two percentages was not statistically significant (Student's t test, $t = 0.7$, d.f. = 56).

GABA inhibited 53 out of 54 SHNs in PTX pretreated rats and 42 out of 43 SHNs in control rats (see Figure 1 and Table 1). The mean percentage inhibition of the spontaneous activity of SHNs was similar in the two populations: $95.4 \pm 1.3\%$ ($n = 51$)

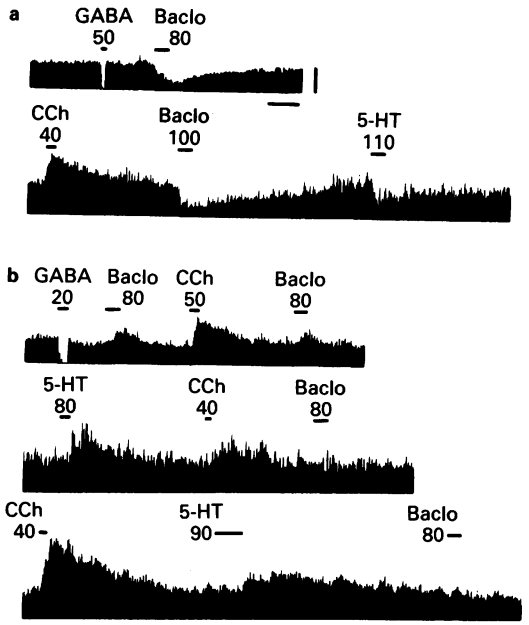

Figure 1 (a) Examples of responses to the iontophoretic application of γ-aminobutyric acid (GABA), 5-hydroxytryptamine (5-HT), baclofen (Baclo) and carbachol (CCh) in 2 septo-hippocampal neurones (SHNs) recorded from control rats. Notice the strong and long-lasting inhibitory effect of baclofen and 5-HT. (b) Examples of responses of 3 different SHNs recorded from pertussis toxin (PTX) pretreated rats. Notice the excitatory effect of baclofen (i) or its lack of effect (ii and iii), the excitatory effect of 5-HT often of long duration (ii and iii). In contrast, the depressant effect of GABA and the excitatory effect of carbachol are similar in normal (a) and PTX pretreated rats (b). Drugs were applied during the time periods indicated by the horizontal bars. Numbers indicate the current applied in nA. Vertical scale: 30 spikes s^{-1} (a,b (ii) and b (iii)). 15 spikes s^{-1} (b(i)). Horizontal scale: 1 min.

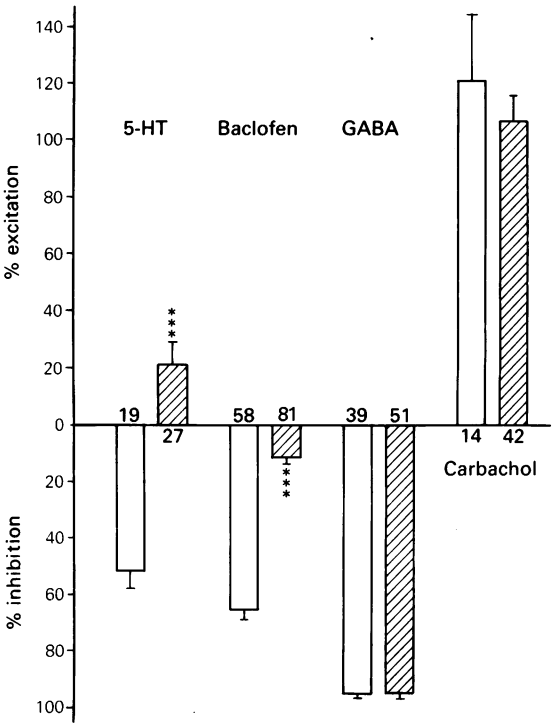

Figure 2 Mean percentage change in spontaneous firing rate of septo-hippocampal neurones (SHNs) during 5-hydroxytryptamine (5-HT), baclofen, γ-aminobutyric acid (GABA) or carbachol application in normal (open columns) and pertussis toxin (PTX) pretreated rats (hatched columns). Number of SHNs tested are indicated at each column base. *** Statistically significant changes (Student's t test, $P < 0.001$).

in PTX pretreated rats and 95.5 ± 1.3% ($n = 39$) in control rats (see Figure 2) (Student's t test, $t = 0.05$, d.f. = 88). The proportion of neurones inhibited or unaffected by GABA was not significantly different in control as compared to PTX pretreated rats (chi-squared test with the Yates correction, $\chi^2 = 0.3$).

In contrast, (Figures 1 and 2, Table 1), the responses to standard applications of baclofen, differed significantly between PTX pretreated and control rats. Baclofen, which strongly inhibited the activity of the SHNs in control rats (97% of neurones inhibited, $n = 61$) (Figure 1a) had either no effect in PTX pretreated rats (59% of the neurones studied, $n = 83$) or an excitatory effect (14% of the neurones) (Figure 1b) (the proportion of neurones excited or inhibited was significantly different between control and PTX pretreated rats, chi-squared test with the Yates correction, $\chi^2 = 20.7$, $P < 0.001$). In these animals, only 26% of the SHNs were inhibited by baclofen. The mean percentage inhibition of spontaneous activity was 65.4 ± 3.2% in control rats ($n = 58$) and 12.3 ± 2.4% in PTX pretreated rats ($n = 81$). The difference was statistically significant (Student's t test, $t = 13.4$, d.f. = 137, $P < 0.001$) (see Figure 2).

The effect of 5-HT was also markedly affected by PTX pretreatment. In control rats, 5-HT had an inhibitory effect in 95% of the SHNs ($n = 19$) (see Table 1 and Figure 1a). In contrast, in PTX pretreated rats, 5-HT inhibited only 14% of the SHNs, had no effect in 36% and excited 50% of the neurones ($n = 28$) (Figures 1b and 2) (chi-squared test with the Yates correction, $\chi^2 = 19.6$, $P < 0.001$). The 5-HT-induced excitations often occurred after a long latency and were long lasting. In some cases a clear afterdischarge of long duration was present (see

Figure 1b, bottom trace). The mean percentage inhibition of the spontaneous activity was $52.5 \pm 5.7\%$ ($n = 19$) in control rats; in contrast, in PTX pretreated rats, 5-HT was excitatory on the average: $20.5 \pm 8.4\%$ ($n = 27$) (see Figure 2). The difference between these percentages was statistically significant (Student's t test, $t = 6.7$, d.f. $= 44$, $P < 0.001$).

Discussion

Spontaneous activity of SHNs was increased in PTX pretreated rats, possibly reflecting a modification of the inputs to these neurones. A monoaminergic innervation (see Lindvall & Stenevi, 1978; Köhler *et al.*, 1982; Gall & Moore, 1984) and a GABAergic innervation of the septal area (Panula *et al.*, 1984) have been described. The monoamines have a prominent inhibitory effect on SHNs (Lamour *et al.*, unpublished results) and their mechanism of action is linked to a PTX-sensitive G-protein in different structures (see Innis & Aghajanian, 1987a,b) including the medial septum (this study). We can hypothesize that after PTX treatment, the inactivation of 5-HT receptor or $GABA_B$-receptor-mediated effects could lead to a disinhibition of the SHNs. On the other hand, the mean latency of antidromic activation of the SHNs was unchanged by PTX. The inactivation of Gi and Go seems to have had no effect on the conduction velocity of the axons of these neurones.

Our results show that some neurotransmitter systems do not seem to be physiologically linked to a PTX-sensitive G-protein: responses to CCh and GABA were unaffected by PTX. The fact that a PTX-sensitive G-protein is not involved in the mediation of the cholinergic responses in the medial septum contrasts with results obtained in the heart where a G-protein couples directly muscarinic receptors to potassium-channels (Breitwieser & Szabo, 1985; Pfaffinger *et al.*, 1985). However, our results are in agreement with those obtained in the hippocampus where the effects mediated by muscarinic agonists persist in PTX pretreated rats (Dutar & Nicoll, 1988a). This suggests differences in the coupling mechanisms between muscarinic receptors and effector systems depending on the structure studied.

The major point of this study concerns the involvement of a PTX-sensitive G-protein in the responses to baclofen and 5-HT. The hyperpolarizing response induced by baclofen (acting on $GABA_B$-receptors) or 5-HT in hippocampus are associated with an increase in potassium conductance (Newberry & Nicoll, 1985; Inoue *et al.*, 1985; Andrade *et al.*, 1986). Furthermore, in hippocampal CA_1 neurones (Andrade *et al.*, 1986) and CA_3 neurones (Thalmann, 1987), the opening of these potassium channels involves a PTX-sensitive G-protein. The present results are in good agreement with these previous studies and suggest that a similar coupling mechanism exists in the medial septum. By inactivating Gi, PTX can block effects mediated by receptors negatively coupled to adenylate cyclase (see Gilman, 1984). Some effects of 5-HT or baclofen have been linked to adenylate cyclase inhibition (Wojcik & Neff, 1983; Clarke *et al.*, 1987) in the brain while other effects have not (Andrade *et al.*, 1986; Thalmann, 1987). In the medial septum, the possible link between the G-protein and adenylate cyclase or other biochemical effectors requires additional *in vitro* studies.

The unusual excitatory effect of baclofen observed in 14% of the SHNs recorded in PTX pretreated rats is surprising. One possible hypothesis is that the remaining response is due to the activation of presynaptic receptors. Indeed, a previous study in the hippocampus showed that presynaptic $GABA_B$-receptors in contrast with postsynaptic ones are not sensitive to PTX (Dutar & Nicoll, 1988b). The excitatory effect of 5-HT observed in 50% of the neurones in PTX pretreated rats has also been observed in the hippocampus (Andrade & Nicoll, 1987). In fact, in this structure, 5-HT has a dual effect: a hyperpolarizing effect followed by a long lasting depolarizing effect often masked by the hyperpolarization. In the hippocampus, only the hyperpolarization is dependent on the G-protein and is blocked by PTX, allowing the depolarizing effect of 5-HT to appear. A similar mechanism in SHNs could explain the high proportion of neurones excited by 5-HT in PTX pretreated rats.

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Effects of 5-hydroxytryptamine on human isolated placental chorionic arteries and veins

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1 Effects of 5-hydroxytrypamine (5-HT) on cylindrical segments of human chorionic arteries and veins were investigated. Concentrations of 5-HT (up to 3×10^{-6} M) produced concentration-dependent contractions; higher concentrations induced a reduction of the maximal response. These responses were antagonized by methysergide and ketanserin in a non-competitive manner. The contractions elicited by low 5-HT concentrations were more affected by methysergide (10^{-7} M) than by ketanserin (10^{-7} M). Ketanserin apparently increased the responses to high 5-HT concentrations in veins. Arteries appeared to be more sensitive to both drugs than veins. Single concentrations of 5-HT elicited transient contractions in both kinds of vessel.

2 Marked tachyphylaxis was seen in segments exposed to high concentrations of 5-HT or in which a concentration-response curve was determined.

3 Contractions induced by 5-HT were reduced in a Ca^{2+}-free medium. Veins were more affected by the Ca^{2+} antagonists, nifedipine (10^{-7} M), nicardipine (10^{-5} M) and diltiazem (10^{-5} M) than arteries.

4 5-HT (10^{-6} M) enhanced $^{45}Ca^{2+}$ uptake in those vessels in which a concentration-response curve had not been previously determined. In veins, this increase was reduced by the three Ca^{2+} antagonists.

5 The results indicate that 5-HT responses in these vessels were greatly dependent on extracellular Ca^{2+}. A type of 5-HT_1-receptor may mediate responses to low 5-HT concentrations, while higher concentrations may activate 5-HT_2-receptors. 5-HT may desensitize the latter by interconversion between a high affinity and low affinity state, as suggested by others, a change prevented in part by ketanserin.

Introduction

It has been demonstrated that 5-hydroxytryptamine (5-HT) induces a substantial increase in the tone of the human placental vascular bed (Aström & Samelius, 1957) and of isolated umbilical vessels (Altura *et al.*, 1972; Mak *et al.*, 1984). In addition, the concentration of 5-HT in the placental circulation, maternal blood and placental tissue increases from late pregnancy until spontaneous vaginal delivery (Koren *et al.*, 1965; O'Reilly & Loncin, 1967; Jones & Rowsell, 1973). It is suggested that 5-HT may be involved in the closure of the umbilical blood vessels at birth (Mak *et al.*, 1984), and in the pathogenesis of pre-eclampsia (Montenegro *et al.*, 1985).

The umbilical and placental vessels, which lack autonomic innervation (Walker & McLean, 1971; Reilly & Russel, 1977), are appropriate for studying the direct effect of 5-HT on the vascular smooth muscle. In other blood vessels, an indirect adrenergic component contributes to the total action of this amine (Marín & Sánchez, 1980; Marín *et al.*, 1981). The action of 5-HT has been more widely studied on umbilical cord than on placental chorionic vessels. The latter contribute more to placental vascular

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resistance than the larger umbilical cord vessels (Bhargava & Raja, 1970).

The aim of the present study was to analyse the effects of 5-HT in human chorionic arteries and veins, in terms of: (1) the subtype of 5-HT receptor involved, (2) the degree of dependence of 5-HT-induced contractions on extracellular Ca^{2+}, and (3) the effects of different Ca^{2+} antagonists on 5-HT-evoked contraction and $^{45}Ca^{2+}$ influx.

Methods

Vascular preparations

The human placental vessels used in the present study were arteries and veins (1.8–2.3 mm o.d.) of chorionic plate near to the point of insertion of the umbilical cord. The placentas were obtained from full-term normal, vaginal deliveries from apparently healthy women. Immediately following delivery, the vessels were carefully isolated and immersed in Krebs-Henseleit solution (KHS) at 4°C and transported to the laboratory. Then, they were divided into cylindrical segments 5 mm in length, which were cleaned of traces of blood and adherent tissues.

Analysis of drug effects on vascular tone

For isometric tension recording, each vascular cylinder was set up in an organ bath according to the method described elsewhere (Marín *et al.*, 1981). The organ bath contained 6 ml of KHS at 37°C continuously bubbled with 95% O_2: 5% CO_2, which gave a pH of 7.4. Two stainless steel pins, 250 μm in diameter, were passed through the lumen of the vascular segment. One pin was fixed to the organ bath wall while the other was connected to a strain gauge for isometric tension recording. The latter pin was parallel to the former and was movable, thus permitting the application of resting tension in a plane perpendicular to the long axis of the vascular cylinder. The isometric contraction was recorded through a force-displacement transducer (Grass FTO3C) connected to a Grass Model 7D polygraph. The segments were submitted to a tension of 1.5 g (optimal resting tension), which was readjusted every 15 min during a 120 min equilibration period before addition of drugs.

The vessels were exposed, at the beginning of the experiment, to 75 mM K^+ to check their functional integrity. Subsequently, the bath medium was changed several times until the resting tone recovered and then cumulative concentration-response curves (CRCs) to 5-HT were determined.

Contractile responses induced by 5-HT were expressed in mg or as a percentage of the response induced by previous administration of 75 mM K^+. 5-HT concentrations producing 50% of maximum contractile responses (EC_{50}) were calculated according to Fleming *et al.* (1972).

When Ca^{2+} antagonists (nifedipine, diltiazem or nicardipine) were used, they were added to the bath 15 min before and during the administration of 5-HT (10^{-6} M).

To study the effect of extracellular Ca^{2+} on 5-HT contractions, segments were exposed for 10 min to solutions containing different concentrations of Ca^{2+} (2.5, 1, 0.5, 0.25, 0 mM and 0 plus 10^{-3} M EGTA). Furthermore, the effect of Ca^{2+} removal and subsequent Ca^{2+} addition on the 5-HT contraction was investigated; thus, segments were exposed for 10 min to Ca^{2+}-free solution and then 10^{-6} M 5-HT was administered. Once the amine produced its effects, a CRC to $CaCl_2$ was determined.

$^{45}Ca^{2+}$ uptake

$^{45}Ca^{2+}$ uptake in 5 mm segments of chorionic artery and vein was determined by the La^{3+}-method (Godfraind, 1976). Briefly, segments were tied at one end with silk thread to a glass rod, the lumen remaining open. Thereafter, they were immersed in 4 ml of oxygenated KHS at 37°C for 60 min (stabilization period) and placed for different time intervals (30 s to 60 min) in KHS at 37°C containing $^{45}Ca^{2+}$ (0.6 μCi ml^{-1}).

Segments were rinsed with KHS at 4°C for 10–15 s (to remove surface $^{45}Ca^{2+}$) and then incubated with 200 ml of a Ca^{2+}-free solution containing La^{3+} (50 mM) for different periods of time (2 to 60 min). La^{3+} displaces the extracellular Ca^{2+} and blocks Ca^{2+} fluxes (Van Breemen *et al.*, 1972; Godfraind, 1976), therefore, the method gives an estimation of intracellular $^{45}Ca^{2+}$ uptake. Different exposure times to La^{3+} solution were employed to determine the optimum uptake. Finally, the segments were blotted, weighed and digested in vials containing 1 ml of H_2O_2 (30%) at 100°C for 5 h. Two ml of Ready-Solv HP (Beckman) was added and the radioactivity present in the vials measured in a scintillation counter (Beckman LS 2800).

The $^{45}Ca^{2+}$ uptake was calculated from the formula (Turlapaty *et al.*, 1979):

$$^{45}Ca^{2+}\ (\text{mmol kg}^{-1}\ \text{wet wt}) = \frac{\text{c.p.m. in muscle}}{\text{wet wt (kg)}} \times \frac{\text{mmol Ca}^{2+}\ \text{l}^{-1}\ \text{medium}}{\text{c.p.m. l}^{-1}\ \text{medium}}$$

In order to determine the effect of 5-HT (10^{-6} M) on $^{45}Ca^{2+}$ uptake, this amine was added to the bath during incubation of the vessels with $^{45}Ca^{2+}$. Ca^{2+}

antagonists were administered 15 min before, and during, the incubation period (5 min). Influx predominates over efflux or redistribution during this time interval. Once the CRC to 5-HT had been determined a marked desensitization was observed, i.e. the vessels did not respond to 5-HT. Some experiments were designed to investigate if this effect was parallel to a loss of the ability of 5-HT to induce Ca^{2+} influx. After the curve to 5-HT had been constructed, the segments were rinsed several times with KHS until control basal tone was restored (around 20 min) and were then exposed to a solution containing $^{45}Ca^{2+}$ plus 5-HT (10^{-6} M).

Solutions, drugs and statistical evaluation

The composition of KHS was (mM): NaCl 115, KCl 4.6, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 2.5, $MgSO_4 \cdot 7H_2O$ 1.2, glucose 11.1, Na_2EDTA 0.03. The Ca^{2+} concentration was changed in modified KHS with no compensation. Ca^{2+}-free KHS was prepared by omitting $CaCl_2$ and in some cases, 1 mM ethyleneglycol-*bis*(beta-aminoethyl-ether) N,N'-tetra-acetic acid (EGTA) was added. La^{3+} (50 mM) solution contained (mM) (Turlapaty *et al.*, 1979): NaCl 118, KCl 5.9, $MgSO_4 \cdot 7H_2O$ 1.2, glucose 10, tris hydroxymethyl-aminomethane 5, $LaCl_3 \cdot 7H_2O$ 50. The final pH was adjusted to 7 with HCl (0.1 M), due to the basicity of $LaCl_3$ solution.

Stock solutions (10^{-3} M) of 5-HT, methysergide and ketanserin were prepared in physiological saline solution (0.9% NaCl) containing 0.01% (w/v) ascorbic acid. Those of nifedipine and nicardipine were prepared in ethanol 99.8% and protected from the light, and that of diltiazem in distilled water. Both dihydropyridines were used, at the appropriate concentrations in KHS, under sodium vapour light. All the stock solutions were kept at −20°C. The effect of Ca^{2+} antagonists could not be reversed after several washing periods. For this reason, the segments were used once, and the same was done with vessels in which a CRC to 5-HT was determined, due to desensitization.

Drugs used were: 5-hydroxytryptamine creatinine sulphate (Sigma), ketanserin tartrate (a gift from Janssen), methysergide bimaleate (a gift from Janssen); ^{45}calcium chloride (specific activity 36.5 mCi mg^{-1}, New England Nuclear); lanthanum chloride (Sigma), nifedipine hydrochloride (a gift from Bayer), nicardipine hydrochloride (a gift from Zambeletti) and diltiazem hydrochloride (a gift from Esteve).

Results are given as means ± s.e.mean. Statistical significance was evaluated by Student's t test for paired or unpaired values and P values of 0.05 or less were considered significant.

Results

Reactivity experiments

5-HT (up to 3×10^{-6} M) induced concentration-dependent contractions in chorionic arteries and veins; higher concentrations produced a reduction of the maximal contraction (Figures 1 and 2). EC_{50} values are shown in Table 1. 5-HT (10^{-6} M) did not induce contractile responses in vessels in which a CRC had been determined in the preceding 60 min or which had been exposed to high concentrations of 5-HT (10^{-4} M). After 2 h, the responses elicited by 5-HT were usually similar to those obtained in the control situation. In contrast, 75 mM K^+ produced contractions in these desensitized vessels, which were similar to those obtained at the beginning of the experiment (Figure 1). Single concentrations of this amine (up to 10^{-6} M) did not usually produce desensitization. Single concentrations of 5-HT elicited transient contractile responses. The maximal contraction was obtained during the first 4–5 min and then the vascular tone began to diminish (Figure 1).

The CRC to 5-HT was determined in the presence (15 min preincubations) of ketanserin (10^{-7} and 10^{-6} M) or methysergide (10^{-7} M), antagonists of 5-HT_2- or both 5-HT_1- and 5-HT_2-receptors, respectively (Figures 2 and 3). These blockers reduced contractions caused by 5-HT; arteries appeared to be more sensitive than veins. Ketanserin apparently increased the contractions to high 5-HT concentra-

Table 1 Effect of methysergide and ketanserin (15 min preincubations) on the concentrations of 5-hydroxytryptamine (5-HT) producing 50% of maximum contractile responses (EC_{50})

	EC_{50} (M)	
	Arteries	*Veins*
Control	2.96 (1.09–7.94) × 10^{-7}	2.55 (0.95–6.46) × 10^{-7}
Methysergide (10^{-7} M)	1.96 (0.7–10) × 10^{-6}*	1.1 (0.26–4.58) × 10^{-6}
Ketanserin (10^{-7} M)	1.97 (0.66–5.68) × 10^{-7}	6.99 (3.36–14.22) × 10^{-7}
Ketanserin (10^{-6} M)	3.05 (1.22–6.72) × 10^{-5}*	5.04 (2.9–8.6) × 10^{-5}*

95% confidence limits are shown in parentheses. $n \geq 6$, in each case. * $P < 0.05$ with respect to control.

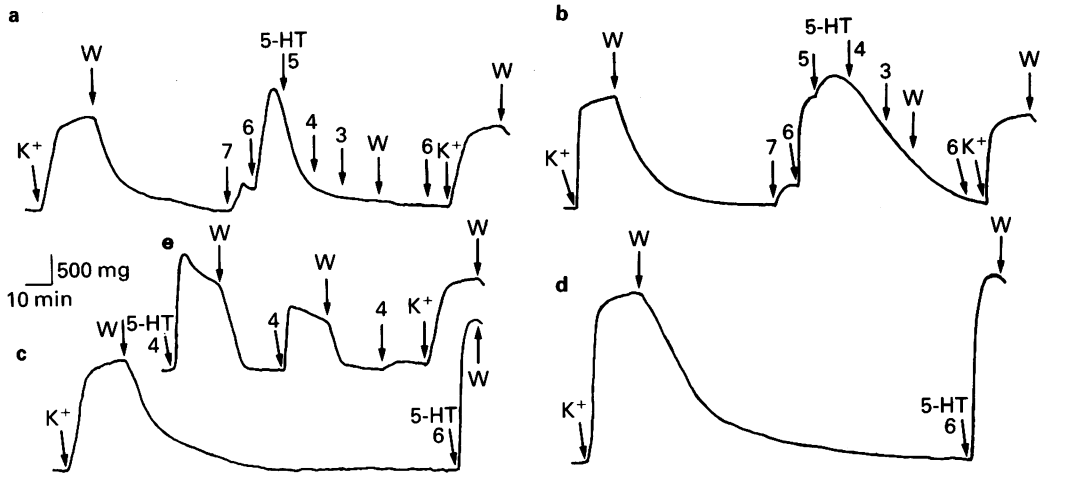

Figure 1 Typical recordings showing the biphasic responses induced by the cumulative administration of 5-hydroxytryptamine (5-HT) in cylindrical segments of human chorionic arteries (a) and veins (b), and the loss of the ability of this amine, but not of K^+ (75 mM), to contract these segments. In segments of artery (c) and vein (d) not exposed to this amine, this desensitization was not observed. The addition of high concentrations of these drugs (10^{-4} M) to arteries also produced a marked desensitization (e). In veins, this process was similar. Single concentrations of 5-HT elicited transient contractions. The concentrations of 5-HT administered are indicated as $-\log_{10}$ M. W = wash.

tions in veins (Figure 3). Neither ketanserin (10^{-8} M) nor methysergide (3×10^{-8} M) modified the contractions caused by 5-HT in either kind of vessel. The presence of 10^{-6} M ketanserin enhanced the EC_{50} values of 5-HT in both types of vessel (Table 1). Increasing the preincubation time with ketanserin from 15 min to 30 or 60 min did not modify the inhibitory effect of this antagonist (results not shown).

The effect of different extracellular Ca^{2+} concentrations on the CRC to 5-HT was analysed. Figure 4 shows that contractions are largely dependent on extracellular Ca^{2+}, but possess a small component independent of this source of Ca^{2+}. In addition, responses induced by 10^{-6} M 5-HT were greatly reduced by Ca^{2+} removal. Subsequent cumulative Ca^{2+} addition produced concentration-dependent contractions (Figure 5).

Contractions induced by 5-HT (10^{-6} M) in arteries and veins were reduced by nifedipine (10^{-7} M),

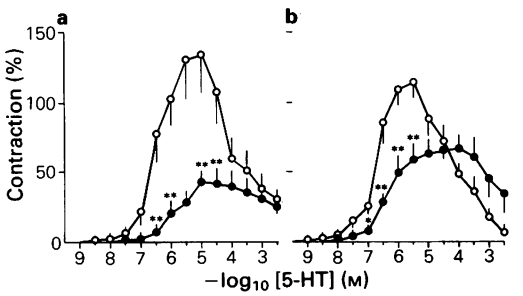

Figure 2 Effect of methysergide (●, 10^{-7} M, $n = 6$) on the concentration-response curve to 5-hydroxytryptamine (5-HT, 0, $n = 8$) in cylindrical segments of human chorionic artery (a) and vein (b). Responses to 5-HT were expressed as percentages of the previous contraction induced by 75 mM K^+ (1340 ± 240 mg in arteries, 1394 ± 218 mg in veins). Values are means and vertical lines show s.e.mean. $*P < 0.05$, $**P < 0.01$.

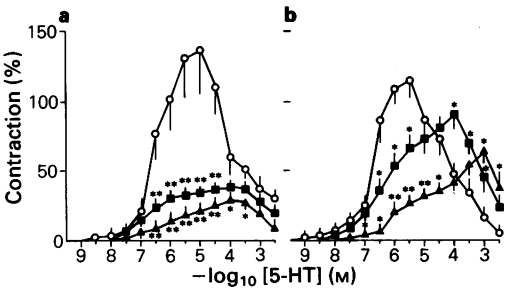

Figure 3 Effect of ketanserin (■, 10^{-7}, and ▲, 10^{-6} M, $n = 6$) on the concentration-response curve to 5-hydroxytryptamine (5-HT, 0, $n = 8$) in cylindrical segments of human chorionic artery (a) and vein (b). Responses to 5-HT were expressed as percentages of the previous contraction induced by 75 mM K^+ (1326 ± 221 in arteries, 1245 ± 166 mg in veins). Values are means and vertical lines show s.e.mean. $*P < 0.05$, $**P < 0.01$.

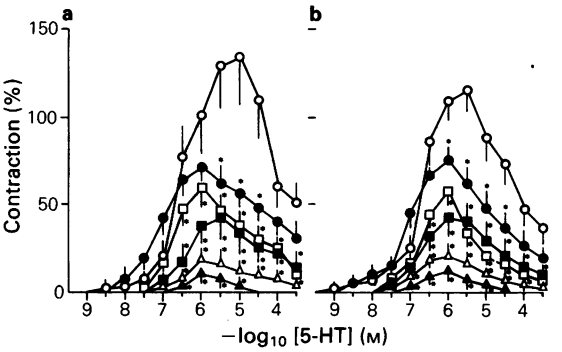

Figure 4 Effect of different Ca^{2+} concentrations (mM) (○, 2.5; ●, 1; □, 0.5; ■, 0.25; △, 0 and ▲, 0 + 1 EGTA; $n = 5$–8) on the concentration-response curve to 5-hydroxytryptamine (5-HT) in segments of human chorionic artery (a) and vein (b). Responses to 5-HT were expressed as percentages of the previous contraction induced by 75 mM K^+ (1250 ± 230 mg in arteries, 1200 ± 210 mg in veins). Values are means and vertical lines show s.e.mean. * $P < 0.05$.

nicardipine (10^{-5} M) and diltiazem (10^{-5} M). Arteries were less sensitive to Ca^{2+} antagonists than veins, the reduction caused by diltiazem in arteries did not reach significance (Figure 6). The concentrations of Ca^{2+} antagonists used were chosen because they produced maximal relaxant responses in segments contracted with 75 mM K^+.

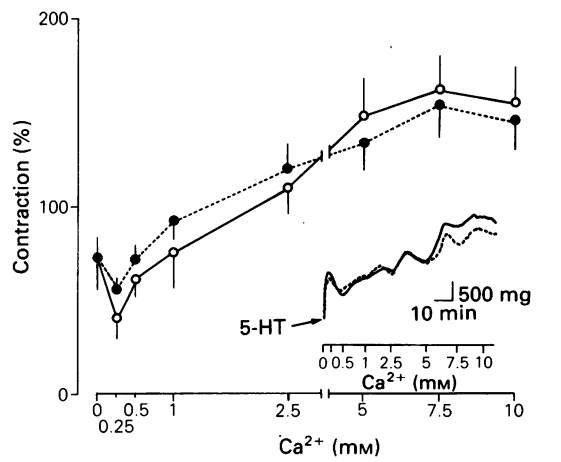

Figure 5 Effect of Ca^{2+} removal and subsequent Ca^{2+} addition on contractions induced by 5-hydroxytryptamine (5-HT, 10^{-6} M) in segments of human chorionic artery (○) and vein (●). Responses to 5-HT were expressed as percentages of the previous contraction induced by 75 mM K^+ (1775 ± 375 mg in arteries, 1550 ± 306 mg in veins). The inset shows typical recording of these effects.

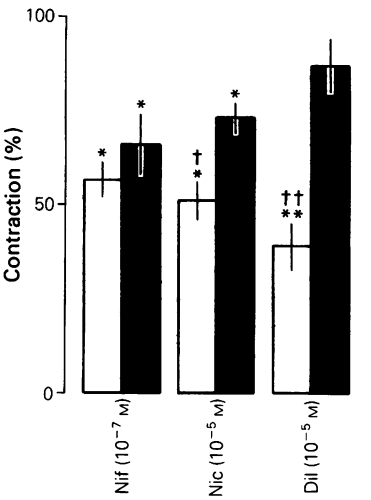

Figure 6 Inhibitory effect of nifedipine (Nif), nicardipine (Nic) and diltiazem (Dil) (15 min preincubations) on the contractions induced by 5-hydroxytryptamine (5-HT, 10 M) in segments of human chorionic artery (solid columns) and vein (open columns). Responses to 5-HT were expressed as percentages of the previous contraction induced by 5-HT (10^{-6} M) (1650 ± 285 mg in arteries, 1870 ± 426 mg in veins). * $P < 0.05$, ** $P < 0.01$, with respect to controls; † $P < 0.05$; †† $P < 0.01$, with respect to the remaining contractions obtained in arteries.

$^{45}Ca^{2+}$ uptake

To measure intracellular $^{45}Ca^{2+}$ uptake it is necessary to remove the large amount stored in the extracellular space and adhering to the plasma membrane. In these experiments, extracellular Ca^{2+} was removed by the addition of La^{3+} (Van Breemen *et al.*, 1972) at a concentration of 50 mM (Godfraind, 1976). For this purpose, the vessels were incubated for 15 min in KHS containing $^{45}Ca^{2+}$ and then rinsed with La^{3+} solution, which produced a rapid $^{45}Ca^{2+}$ loss in the first 10 min. At this time, steady state Ca^{2+} efflux was reached, and after 20 min washing with La^{3+} medium the Ca^{2+} loss was stopped (Figure 7); this incubation time was used in the following experiments determining $^{45}Ca^{2+}$ uptake.

The time course of $^{45}Ca^{2+}$ uptake, with or without 5-HT (10^{-6} M), is illustrated in Figure 7. In the control situation, the $^{45}Ca^{2+}$ content was augmented with time of incubation until 15 min, when the steady-state was reached. 5-HT significantly increased $^{45}Ca^{2+}$ uptake at 5 and 10 min incubations in both kinds of vessel.

The interference by nifedipine (10^{-7} M), nicardipine (10^{-5} M) and diltiazem (10^{-5} M) with the $^{45}Ca^{2+}$

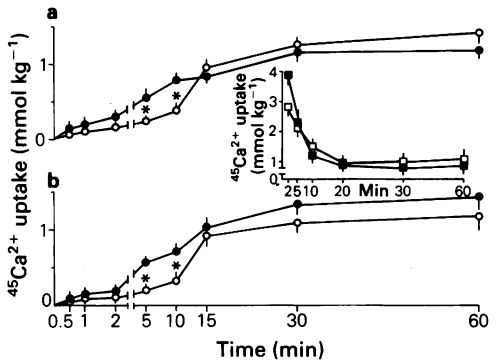

Figure 7 Time course of intracellular $^{45}Ca^{2+}$ uptake in the presence (●) and absence (○) of 5-hydroxytryptamine (5-HT, 10^{-6} M) in segments of human chorionic artery (a) and vein (b), incubated for different times in 4 ml KHS containing $^{45}Ca^{2+}$ and subsequently rinsed with La^{3+} (50 mM) solution for 20 min. The inset represents the time course of Ca^{2+} loss after different periods of washing with this La^{3+} solution in arteries (□) and veins (■). Each point represents the mean and vertical lines show s.e.mean of 6 experiments. * $P < 0.05$.

accumulation induced by 5-HT (10^{-6} M, 5 min incubation) is shown in Table 2. These Ca^{2+} antagonists significantly reduced Ca^{2+} content only in veins; basal $^{45}Ca^{2+}$ uptake was not modified. In addition, we investigated whether there was a reduction in the $^{45}Ca^{2+}$ uptake in desensitized vessels. If 5-HT receptors gate Ca^{2+} entry, then a reduction in Ca^{2+} influx would be predicted in desensitized vessels. Vessels were exposed to cumulative concentrations of 5-HT, and after rinsing with KHS and a period of stabilization in which the tension returned to baseline (around 20 min), $^{45}Ca^{2+}$ and 5-HT (10^{-6} M) were added to the bath. Control vessels were submitted to similar conditions. Uptake was significantly reduced in desensitized arteries (control: 0.67 ± 0.19; desensitized: 0.40 ± 0.06 mmol kg^{-1}; $P < 0.04$, $n = 5$) and veins (control: 0.55 ± 0.08; desensitized: 0.33 ± 0.02 mmol kg^{-1}; $P < 0.03$, $n = 6$).

Table 2 Effect of nifedipine, nicardipine and diltiazem (15 min preincubations) on intracellular $^{45}Ca^{2+}$ uptake (mmol kg^{-1}) induced by 5-hydroxytryptamine (5-HT, 10^{-6} M) in segments of human chorionic artery and vein

	Arteries	*Veins*
Control	0.58 ± 0.08	0.57 ± 0.04
Nifedipine (10^{-7} M)	0.61 ± 0.09	0.45 ± 0.03*
Nicardipine (10^{-5} M)	0.56 ± 0.09	0.47 ± 0.02*
Diltiazem (10^{-5} M)	0.53 ± 0.08	0.43 ± 0.04*

The vessels were incubated for 5 min in 4 ml KHS containing $^{45}Ca^{2+}$, with or without 5-HT, and subsequently rinsed with La^{3+} (50 mM) solution for 20 min.
Values are means ± s.e.mean. $n = 6$ in each situation. * $P < 0.05$.

Discussion

In this study it was shown that 5-HT induces strong, transient contractile responses in chorionic arteries and veins. The cumulative addition of this amine produced concentration-dependent contractions up to 3×10^{-6} M in veins and 10^{-5} M in arteries. The ability of 5-HT to elicit potent and transient increases in tension has been observed in bovine coronary arteries (Ratz & Flaim, 1984) and rabbit thoracic aorta (Purdy *et al.*, 1987). This amine also produces powerful contractions, greater than other agents, in human and animal umbilical or placental vessels (Dyer, 1970; Altura *et al.*, 1972; Nair & Dyer, 1974; Tulenko, 1979; Mak *et al.*, 1984; Maigaard *et al.*, 1986). In addition, it has been demonstrated that the concentration of 5-HT in the maternal blood and blood present in umbilical vessels at term (vaginal delivery) is around 10^{-7} M (in both cases measured in the whole blood) (O'Reilly & Loncin, 1967; Jones & Rowsell, 1973), i.e., similar to our EC_{50} values for 5-HT on chorionic vessels. It has been suggested that 5-HT may contribute to spasm and closure of the umbilical vessels after birth (Tulenko, 1979; Mak *et al.*, 1984). It is interesting to note that the concentration of placental 5-HT increases from the start of pregnancy until delivery. In contrast, monoamine oxidase (MAO) activity is decreased. This might trigger 5-HT release from uterine and placental stores and induce delivery (Koren *et al.*, 1965), and contraction of placental vessels. The increase in placental 5-HT concentration is probably due to an augmentation in the production of 5-HT in the developing foetus, since the placenta appears to lack the ability to synthesize 5-HT (Jones & Rowsell, 1973).

The 5-HT receptors involved in the contractions were investigated, using ketanserin and methysergide, antagonists of 5-HT_2 and both 5-HT_1 and 5-HT_2 receptors, respectively (Van Nueten *et al.*, 1981; Houston & Vanhoutte, 1986). Methysergide diminished responses induced by 5-HT, as did ketanserin. However, methysergide (10^{-7} M) reduced the effects of low 5-HT concentrations while ketanserin (10^{-7} M) did not. This suggests that the high sensitivity component of the effect of 5-HT is mediated through a 5-HT_1-like receptor, while high 5-HT concentrations act through 5-HT_2 receptors. The vessels may, thus, possess two types of 5-HT receptor (5-

HT_1 and 5-HT_2), which mediate the actions of 5-HT. A similar conclusion has been obtained by other authors in human umbilical arteries (Diemer *et al.*, 1985). The antagonism between 5-HT and each of these blockers was non-competitive, since the CRCs to 5-HT were not displaced in a parallel manner to the right, and the maximal response was reduced. Both antagonists have other properties which might account for these results, e.g. ketanserin may block α-adrenoceptors (Brazenor & Angus, 1982; Nishimura *et al.*, 1987). Similar results and conclusions have been obtained in human hand veins (Arneklo-Nobin *et al.*, 1985). Alternatively, 5-HT might stimulate α-adrenoceptors in these vessels (see Purdy *et al.*, 1987). If 5-HT acts by these mechanisms in chorionic vessels, this might explain the non-competitive antagonism previously mentioned. In other vessels, ketanserin and/or methysergide are competitive antagonists (Brazenor & Angus, 1982; Frenken & Kaumann, 1985; Arneklo-Nobin *et al.*, 1985), non-competitive antagonists (Brazenor & Angus, 1982; Arneklo-Nobin *et al.*, 1985) or show no effect (Bradley *et al.*, 1986), supporting the contention that different 5-HT receptor subtypes exist on vascular smooth muscles.

It is interesting to note that the antihypertensive effect of ketanserin (Hedner *et al.*, 1983) occurs at a peak plasma concentration of around 10^{-7} M (Williams *et al.*, 1986). This concentration caused a significant reduction of 5-HT contractions in chorionic vessels. Further, chorionic arteries were more sensitive to both ketanserin and methysergide than veins. Thus, the placental circulation may be markedly affected by the administration of these drugs, especially at the end of delivery when concentrations of 5-HT are high.

The responses induced by 5-HT were decreased with a reduction of Ca^{2+} in the medium. When this ion was removed (with or without EGTA), the responses were greatly diminished. 5-HT (10^{-6} M)-induced contractions were also markedly reduced in a Ca^{2+}-free medium and recovered on subsequent Ca^{2+} addition. These results show that the responses to 5-HT are largely dependent on extracellular Ca^{2+}. The dependence of 5-HT-induced contractions on intracellular and extracellular Ca^{2+} has been observed in rabbit ear and bovine coronary arteries (Maggi *et al.*, 1983; Ratz & Flaim, 1984), whereas others, such as cerebral arteries, are markedly dependent on extracellular Ca^{2+} (Rusch *et al.*, 1985; Marin, 1988).

The ability of 5-HT to induce Ca^{2+} influx was demonstrated by tracer experiments (La^{3+}-method) and by the use of Ca^{2+} antagonists. La^{3+} displaces extracellular Ca^{2+} and blocks Ca^{2+} fluxes (Van Breemen *et al.*, 1972; Godfraind, 1976). Washing chorionic vessels preincubated with $^{45}Ca^{2+}$ with La^{3+} solution displaced most Ca^{2+} in the first 10 min, and Ca^{2+} fluxes were stopped by 20 min. Godfraind (1976) observed in rat aorta, using the same La^{3+} concentration, that there was a rapid loss of $^{45}Ca^{2+}$ in the first 5 min but, subsequently, loss was markedly diminished although not abolished. This discrepancy might be due to vessel differences. Control and stimulated $^{45}Ca^{2+}$ uptake were fast in these vessels, until 15 min of incubation when a steady state was reached. A similar pattern of $^{45}Ca^{2+}$ uptake has been observed in human umbilical vessels (Ozaki *et al.*, 1981).

The effect of Ca^{2+} antagonists on 5-HT-induced $^{45}Ca^{2+}$ influx was studied using a short period of incubation (5 min) when inward flux predominates (Meisheri *et al.*, 1981). 5-HT-stimulated uptake was significantly reduced by nifedipine, nicardipine and diltiazem only in veins. Unstimulated $^{45}Ca^{2+}$ uptake was not affected by these Ca^{2+} antagonists, which agrees with results obtained with verapamil in umbilical vasculature (Ozaki *et al.*, 1981) and with other Ca^{2+} antagonists in different vessels (Cauvin *et al.*, 1983). The ability of these drugs to reduce 5-HT-induced $^{45}Ca^{2+}$ influx in veins was correlated with their greater capacity to inhibit 5-HT contractions in veins compared to arteries (Figure 6). In contrast, ketanserin and methysergide were more effective in blocking 5-HT responses in arteries. These data indicate that although the contraction caused by 5-HT is dependent on extracellular Ca^{2+} in both chorionic arteries and veins, 5-HT might produce a greater depolarization in vein smooth muscle cells and thus be more susceptible to Ca antagonists. The ability of 5-HT to produce depolarization has been observed previously (Harder & Waters, 1983).

In chorionic vessels, 5-HT produced transient contractions, a CRC which was not sigmoid, but biphasic, and pronounced tachyphylaxis. However, the response induced by K^+ in desensitized segments was unaffected. These results suggest that exposure to high 5-HT concentrations produces a rapid and long-lasting desensitization of the receptors. This is supported by the finding that $^{45}Ca^{2+}$ uptake was reduced in this situation. This desensitization appeared to be agonist specific, because responses to histamine or noradrenaline were not diminished (results not shown). It is interesting to note that ketanserin appeared to enhance the contractions to high 5-HT concentrations in veins. These results possibly indicate that desensitization of 5-HT receptors by 5-HT might be due to interconversion of the receptors from a high affinity state to another of low affinity, an interconversion prevented in part by ketanserin. Interconvertible states and this kind of action of ketanserin on 5-HT_2 receptors in their normal active state has been postulated recently (Kaumann & Frenken, 1985; Frenken & Kaumann,

1988). The tachyphylaxis observed in chorionic vessels could have a physiological role in preventing a prolonged reduction of placental blood flow.

In conclusion, 5-HT produced strong transient contractions in human chorionic arteries and veins, largely dependent on extracellular Ca^{2+}. Responses in arteries were more affected by methysergide and ketanserin than those in veins, while the opposite occurred with the Ca^{2+} antagonists, nifedipine, nicardipine and diltiazem. These contractions appear to be mediated by 5-HT_2- and by 5-HT_1-like receptors.

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Electrophysiological effects of E-3753, a new antiarrhythmic drug, in guinea-pig ventricular muscle

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1 The electrophysiological effects of E-3753, a new antiarrhythmic drug, were studied in guinea-pig papillary muscles.

2 E-3753 (10^{-7}–10^{-4} M) produced a concentration-dependent decrease in the action potential amplitude and V_{max} of the upstroke, shortened the action potential duration (APD) and shifted the resting membrane potential to less negative values. E-3753 also shortened the effective refractory period (ERP), lengthening the ERP relative to APD.

3 E-3753 (10^{-5} M) shifted the membrane responsiveness curve along the voltage axis to more negative potentials.

4 In the presence of E-3753 (10^{-5} M) trains of stimuli at rates between 0.5 and 3 Hz led to an exponential decline in V_{max} (onset rate at 3 Hz, 0.05 ± 0.009 per action potential), to a new steady-state level. This use-dependent V_{max} block was augmented at higher rates of stimulation. The time constant for the recovery of V_{max} from the use-dependent block was 41.1 ± 4.8 s.

5 E-3753 (10^{-5}–10^{-4} M) had no effect on the characteristics of the slow action potentials elicited by isoprenaline in ventricular fibres depolarized by 27 mM KCl.

6 The slow onset of use-dependent block during repetitive activity and the slow offset kinetics of use-dependent V_{max} block suggest that E-3753 exhibits class Ic antiarrhythmic actions in ventricular muscle fibres but does not exhibit class IV (Ca antagonist) antiarrhythmic actions.

Introduction

E - 3753, 1H - imidazol - 2 - methanol,α - (3 - trifluoro - methylphenyl) - α - methyl - 1 - methyl - 0 - (3 - terbutyl - aminopropane)-2ol (Figure 1), is a new antiarrhythmic drug which exhibits local anaesthetic properties (Colombo *et al.*, 1987). E-3753 has been shown to be efficacious in suppressing cardiac arrhythmias in a variety of experimental models. Thus, E-3753 was as potent as lignocaine in reversing ventricular arrhythmias induced by ouabain in the anaesthetized guinea-pig and 2–3 times more potent than quinidine, lignocaine and lorcainide in suppressing the experimental arrhythmias induced by mechanical lesions in rat isolated right ventricles (Farré *et al.*, 1988). In contrast, E-3753 had no effect on the positive inotropic and chronotropic responses induced by isoprenaline in guinea-pig isolated atria or on the maximum upstroke velocity of the slow action potentials induced in K-depolarized guinea-pig papillary muscles (Delpón *et al.*, 1988). These results suggested that E-3753 had no class II (antisympathetic) or class IV (Ca antagonist) antiarrhythmic actions. However, preliminary microelectrode studies in guinea-pig atrial and ventricular muscle fibres indicated that E-3753 decreased action potential upstroke velocity (V_{max}), suggesting a class I mechanism of action, i.e., fast sodium channel block (Delpón *et al.*, 1988).

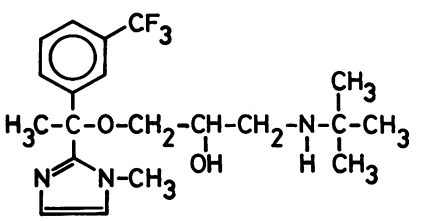

Figure 1 Chemical structure of E-3753.

Thus the present experiments were undertaken to: (1) characterize the cellular electrophysiological effects of E-3753 in guinea-pig ventricular muscle fibres and (2) determine the effects of this drug on the onset and recovery kinetics of the sodium channel as reflected by its effects on V_{max}. Moreover, since the

prevailing classifications of antiarrhythmic drugs (Harrison, 1983; Vaughan Williams, 1984) are based on changes in action potential duration, refractoriness and use-dependent block of fast sodium channels, the present experiments were also carried out in order to (3) place E-3753 in the appropriate class I subgroup (i.e, class Ia, Ib, Ic).

Methods

General procedure

Guinea-pigs of either sex weighing 250–350 g were killed by a blow on the head. The hearts were rapidly excised and papillary muscles 2 to 3 mm in length and less than 1 mm in diameter were isolated from both ventricles. The muscles were pinned to the bottom of a Lucite chamber and superfused continuously at a constant rate of 7 ml min^{-1} with Tyrode solution of the following composition (mM): NaCl 137, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.05, $NaHCO_3$ 11, NaH_2PO_4 0.42, glucose 5.5. The solutions were bubbled with 95%O_2 and 5%CO_2 and maintained at 34 ± 0.5°C. The preparations were initially driven at 1 Hz and a period of 1 h was allowed for equilibration during which a stable impalement was obtained. Driving stimuli were rectangular pulses (2 ms duration, twice threshold strength) delivered to the preparation from a multipurpose programmable stimulator (Cibertec CS-220). Electrical stimulation was applied to the surface of the preparation through Teflon-coated bipolar electrodes of silver wire.

Transmembrane action potentials (AP) were conventionally recorded through glass microelectrodes filled with 3 M KCl (tip resistance of 10–20 MΩ). The microelectrode was connected via Ag-AgCl wire to high impedance, capacity neutralizing amplifiers (WPI model 701). The maximal rate of depolarization (V_{max}) of the action potential was obtained by electronic differentiation (Tamargo & Delgado, 1985). The differentiator used had an upper limit of linearity of 1000 V s^{-1} and possessed variable input filters (3 Hz–260 kHz). The suitable frequency filter for minimizing noise without reducing the V_{max} was elected for each individual expeirment. Another distortion of V_{max} can arise from the formation of the foot of the rising phase of the electrical stimulus. This phenomenon is accompanied by a reduced interval between stimulus artifact and the upstroke of the action potential, particularly when strong electrical stimuli are applied. In order to diminish this artefact, stimulus intensity and duration were adjusted throughout each experiment to maintain a constant latency (1–2 ms) between stimulus and upstroke of the action potential to minimize latency-induced alterations in V_{max} (Valenzuela *et al.*, 1988). Both action potential and V_{max} were displayed on a storage oscilloscope (Tektronix 5104N) and the oscilloscope traces were photographed with a kymographic camera (Grass C4). The effective refractory period (ERP) was measured by introducing premature test-stimuli of twice threshold strength at different intervals from the preceding basic action potential. Interpolation and shift along the voltage axis were carried after every eighth basic drive stimulus. All experimental results were obtained from a single continuous impalement through the whole experiment.

To study the rate-dependent effects of E-3753 on V_{max}, preparations were driven at a basal rate of 0.05 Hz. Following the equilibration period, the preparations were driven by trains of stimuli at varying rates ranging from 0.5 to 3 Hz for 40 s. Rest periods of 5 min, which were sufficient to ensure full recovery from use-dependent decrease in V_{max}, were interpolated between the trains of stimuli (Valenzuela *et al.*, 1988). A similar experimental protocol was followed after exposure to 10^{-5} M E-3753. This experimental protocol indicated that E-3753 at any given rate produced two types of V_{max} inhibition, a tonic and a phasic blockade. Tonic blockade is the decrease of V_{max} of the first action potential preceded by the rest periods, whereas phasic blockade is the decrease of V_{max} during a train from the value of the first action potential to a new steady-state.

Recovery of V_{max} from rate-dependent block was studied by applying a single test stimulus at various coupling intervals after a stimulation train for 5 s at 4 Hz. The intensity and duration of the test and conditioning stimuli were adjusted to obtain a constant latency from the stimulus artefact to the initiation of the upstroke of the action potential. The effect of E-3753 on the relationship between membrane resting potential and V_{max}, i.e. membrane responsiveness, was studied in papillary muscles driven at 1 Hz and the membrane potential was lowered by adding to the perfusate KCl in steps from 2.7 mM to a final concentration of 15 mM (Valenzuela *et al.*, 1987).

Drugs

E-3753 (Esteve, S.A.) as a powder was prepared by dissolving 10 mg in 0.4 ml of 0.1 N HCl and adding 9.6 ml of H_2O to yield a stock solution of 1 mg ml^{-1}. Further dilutions were carried out in Tyrode solution to obtain final concentrations between 10^{-7} M and 10^{-4} M (0.039–39.9 μg ml^{-1}). After 1 h equilibration, control measurements were performed. The preparations were then superfused with Tyrode solutions containing the drug at various concentrations for 30 min.

Throughout the paper, data are given as

mean ± s.e.mean and Student's t test was used to estimate the significance of differences from control values. For statistical comparison of more than two groups, a one-way analysis of variance and Scheffe's test for critical difference was used (Wallenstein et al., 1980). A P value of less than 0.05 was considered to be significant.

Results

Effects of E-3753 on transmembrane action potentials

The effects of E-3753 in a range of concentrations between 10^{-7} and 10^{-4} M were studied in ventricular muscle fibres. The effects of E-3753 were usually evident within 10 min of beginning the perfusion and reached steady-state values within 30 min. Results obtained under control conditions and 30 min after each increment in drug concentration are shown in Table 1. In ventricular muscle fibres E-3753 at concentrations higher than 10^{-7} M produced a significant decrease ($P < 0.05$) in V_{max}, which at concentrations $\geqslant 10^{-5}$ M was accompanied by a significant decrease in action potential amplitude ($P < 0.05$) and a progressive shift of the resting membrane potential to less negative values. At concentrations between 10^{-7} and 10^{-5} M E-5753 had no effect on ventricular repolarization, whereas at higher concentrations it increased the slope of phases 2 and 3 which led to a significant shortening of both APD_{50} and APD_{90} values ($P < 0.05$).

In 11 papillary muscles the ventricular effective refractory period (ERP) averaged 179.8 ± 9.1 ms. At 5×10^{-5} M and 10^{-4} M, E-3753 shortened the ERP to 157.5 ± 14.3 ms ($P < 0.05$) and 143.5 ± 10.8 ms ($P < 0.05$), respectively. This shortening was accompanied by a greater shortening in the APD_{90} values, and therefore, at both concentrations E-3753 also produced a significant decrease in the ERP/APD ratio from 1.01 ± 0.03 to 1.11 ± 0.03 ($P < 0.05$) and 1.18 ± 0.06 ($P < 0.05$).

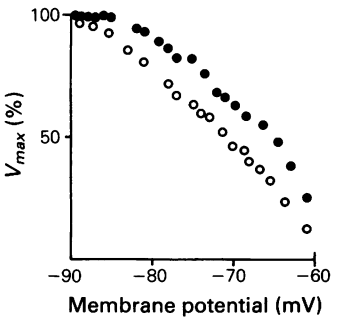

Figure 2 Effect of E3753 on the relationship between V_{max} and the resting membrane potential at which the action potential is generated, i.e. membrane responsiveness. Ordinate scale: normalized V_{max} values. Abscissa scale: resting membrane potential (mV). (●) Controls. (○) E-3753 10^{-5} M.

Effects of membrane responsiveness

The relationship between V_{max} and the membrane resting potential from which the action potential originates, i.e. membrane responsiveness, was examined in four papillary muscles driven at basal rate of 1 Hz. The membrane resting potential was depolarized in steps from its original resting level to approximately −60 mV by increasing the K^+ concentration in the Tyrode solution from 2.7 to 15 mM. The results of a typical experiment are shown in Figure 2. In the absence of the drug, the membrane potential at which V_{max} was reduced to half of its maximum value (Vh) was 61.0 ± 1.2 mV ($n = 6$). Addition of E-3753 shifted the membrane responsiveness curve along the voltage axis to more negative potentials. The average shift in Vh determined in 6 preparations exposed to 10^{-5} M E-3753 was −6.2 ± 1.2 mV. These results

Table 1 Electrophysiological effects of E-3753 on transmembrane potentials in guinea-pig papillary muscles

Concentration (M)	*Resting potential* (mV)	V_{max} ($V s^{-1}$)	*APA* (mV)	APD_{50} (ms)	APD_{90} (ms)
0	−86.5 ± 0.6	194.9 ± 7.3	118.8 ± 0.7	151.6 ± 11.5	176.8 ± 11.0
10^{-7}	−85.7 ± 0.6	185.0 ± 8.4	117.7 ± 0.9	143.4 ± 15.7	167.9 ± 16.3
10^{-6}	−85.0 ± 0.6	176.0 ± 7.8*	117.4 ± 0.8	148.2 ± 15.1	174.1 ± 15.5
10^{-5}	−82.7 ± 0.6***	133.2 ± 7.7***	116.4 ± 0.6*	143.2 ± 14.8	169.1 ± 15.4
5×10^{-5}	−80.7 ± 0.8***	80.0 ± 4.1***	111.2 ± 0.6***	116.7 ± 12.7*	142.2 ± 14.6*
10^{-4}	−78.8 ± 0.8***	50.6 ± 3.7***	101.7 ± 2.2***	97.0 ± 9.0**	126.6 ± 10.4**

Mean values ± s.e.mean, $n = 11$.
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.
APA = action potential amplitude. APD = action potential duration measured at 50% (APD_{50}) and 90% (APD_{90}) or repolarization.

indicated that the effect of E-3753 was more pronounced at low (less negative) than at normal resting membrane potentials.

Use-dependent effects of E-3753

The effectiveness of many class I antiarrhythmic drugs at inhibiting I_{Na} is strongly dependent on the frequency of stimulation. Thus, the influence of stimulation frequency on the depressant effect of E-3753 on V_{max} was studied in papillary muscles by applying trains of pulses of 40 s in duration at different rates (0.5–3 Hz) which were separated by a 5 min period of rest. In untreated muscles the value of V_{max} remained almost unchanged during the stimulation trains at rates ranging from 0.5 to 3 Hz (Figure 3). Following the perfusion with 10^{-5} M E-3753 the V_{max} of the first action potential in each train was reduced from 224 $V s^{-1}$ to 207 $V s^{-1}$, i.e. there was a 6.4% tonic (resting) V_{max} block. During the trains of pulses there was a gradual decrease of V_{max} from beat to beat until a new steady-state occurred (use-dependent or phasic block), which depended on the stimulation frequency. In the absence of the drug, the percentage decrease of V_{max} from the first action potential of the train to a new steady-state level increased progressively when the frequency of stimulation increased from 0.5 (2.8 ± 0.7%) to 3 Hz (10.8 ± 1.0%). In the presence of 10^{-5} M E-3753 the use-dependent V_{max} block significantly increased at all stimulation rates tested ($P < 0.001$), but this increase was more marked at 2 (51.1 ± 2.6%) and 3 Hz (54.2 ± 4.6%) than at 0.5 (27.8 ± 2.0%) and 1 Hz (37.7 ± 2.5%), i.e. was more marked at fast than at slow frequencies of stimulation.

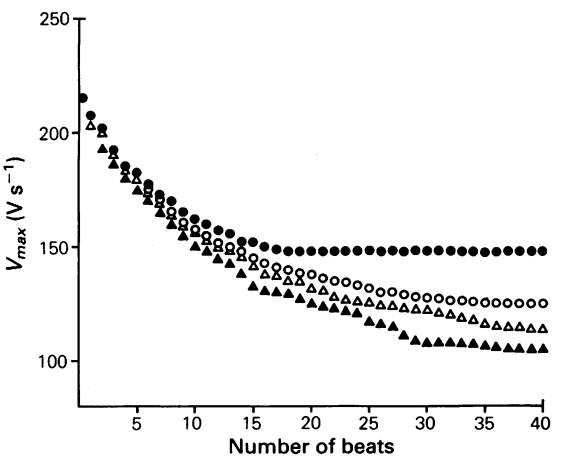

Figure 3 Onset of use-dependent depression of V_{max} induced by 10^{-5} M E-3753 in guinea-pig papillary muscles driven by trains of stimuli at various rates (0.5–3 Hz). Stimulation caused a use-dependent block which disappears, whereas the tonic V_{max} block persisted. The tonic block (%) results from [1 − V_{max} (first beat, drug)/V_{max} (rested, drug free)] × 100% and the use-dependent block (%) from [1 − V_{max} (ss, drug)/V_{max} (first beat, drug)]. V_{max} (ss) is the steady-state value attained during continuous stimulation and V_{max} (first beat) is the value of the first beat of each train of stimuli (●) 0.5 Hz, (○) 1 Hz, (△) 2 Hz, (▲) 3 Hz.

Table 2 Effect of 10^{-5} M E-3753 on the onset kinetics of use-dependent V_{max} block

Frequency of stimulation (Hz)	τ_{on} (s)	*Onset rate per action potential* (AP^{-1})
0.5	10.7 ± 0.7	0.18 ± 0.01
1.0	10.3 ± 0.6	0.08 ± 0.007***
2.0	9.5 ± 0.3	0.05 ± 0.004***
3.0	5.6 ± 0.5***	0.05 ± 0.009***

Mean values ± s.e.mean, $n = 6$.
*** $P < 0.001$.

Onset kinetics of phasic V_{max} blockade can be defined either in terms of a time- or an event-dependent process. In both cases, the decline of V_{max} during stimulation trains represents a first order reaction and can be well fitted by a single exponential. Thus, the time-constant (τ_{on}) and the onset rate per action potential (AP^{-1}) at which V_{max} fell to the new steady-state level could be calculated from the regression lines in semilogarithmic plots. Table 2 shows that between 0.5 and 2 Hz τ_{on} values were almost similar (10.7 ± 0.7 s, 10.3 ± 0.6 s and 9.5 ± 0.3 s, respectively. $P > 0.05$), whereas at 3 Hz the τ_{on} significantly shortened to a value of 5.6 ± 0.7 s ($P < 0.05$). On the other hand, at frequencies of stimulation between 1 and 3 Hz the rate-constants (K) showed similar values, but K was significantly increased ($P < 0.05$) at the lower stimulation frequency.

Recovery kinetics of phasic V_{max} *block*

The effect of E-3753, 10^{-5} M, on the kinetics of the offset (recovery) of phasic V_{max} block was studied in 6 papillary muscles driven at a basal frequency of 0.05 Hz. Muscles were driven by a train of stimuli at a frequency of 4 Hz for 5 s and a test-stimulus was applied at variable coupling intervals from 2 to 36 s. Recovery from use-dependent V_{max} block occurs as a monoexponential process for which a single time constant (τ_{re}) can be estimated by regression analysis of the data. Under control conditions, the τ_{re} was 26.5 ± 1.3 ms whereas in the presence of E-3753 the τ_{re} was prolonged to 41.1 ± 4.8 ms ($P < 0.001$). This value indicates that E-3753 must be considered as belonging to the kinetically slow group of class I antiarrhythmic drugs (Campbell, 1983a).

Effect on the slow action potentials

The effects of E-3753 were also studied on slow action potentials in muscles driven at a basal rate of 0.12 Hz. When the K concentration in the Tyrode solution was increased from 5.4 (control) to 27 mM, the membrane potential was depolarized to -43.0 ± 1.0 mV ($n = 6$) and the preparations became inexcitable due to voltage-dependent inactivation of the Na channels. Excitability, i.e. slow action potentials, were induced by adding 10^{-6} M isoprenaline to the perfusate. E-3753 at any of the concentrations tested (10^{-5}, 5×10^{-5} and 10^{-4} M) did not modify the resting membrane potential, amplitude, V_{max} and duration of the slow action potentials. These results suggest that it did not exhibit, in addition, class IV antiarrhythmic actions.

Discussion

In this paper we studied the effects of E-3753, a new antiarrhythmic class I drug, on the electrophysiological properties of guinea-pig ventricular muscles fibres. Also, the onset and recovery kinetics of use-dependent V_{max} block were analysed in order to determine the subclassification of E-3753.

The present results indicated that in ventricular muscle fibres E-3753 produced a concentration-dependent decrease of V_{max} values; this effect occurred even at concentrations which had no effect on other AP characteristics or resting membrane potential. These findings suggested that the depressive effect on V_{max} is probably due to an inhibition of the fast inward sodium current, and the drug can be classified as a class I, i.e. membrane stabilizing antiarrhythmic agent (Vaughan Williams, 1984). In this regard, E-3753 has about the same degree of local anaesthetic activity as lignocaine (unpublished data). At higher concentrations, E-3753 decreased the action potential amplitude and shifted the resting membrane potential to less negative values, thus producing a further decrease in phase 0 characteristics. Moreover, E-3753 produced a progressive shortening of the APD and ERP, although the shortening of the APD_{90} was greater than that of the ERP and therefore the ERP/APD_{90} ratio increased.

There is evidence that during the fast upstroke of the cardiac action potential, the majority of ionic current crossing the cell membrane at the time of V_{max} is a sodium current (Hondeghem, 1978; Walton & Fozzard, 1979). Thus, although the use of changes in V_{max} to measure peak sodium current and sodium conductance has been questioned (Cohen *et al.*, 1984), there is little doubt that V_{max} is mainly generated by the fast inward sodium current. Whether or not possible non-linearities between V_{max} and sodium conductance (G_{Na}) affect the present results remains to be determined in reliable voltage-clamp experiments, under the same conditions of temperature and external sodium concentration as used in this study.

The modulated-receptor hypothesis (Hondeghem & Katzung, 1977; 1984) is a useful model for investigating the use-dependent effects of class I antiarrhythmic drugs. According to this hypothesis, reduction of sodium inward current is due to an accumulation of drug-associated non-conducting channels, the affinity of the drug being modulated by the state (resting, activated, inactivated) of the channel. At therapeutic concentrations most class I antiarrhythmic drugs exhibit little or no tonic V_{max} block (Campbell 1983a,b; Hondeghem & Katzung, 1984; Tamargo *et al.*, 1988) and E-3753 is no exception. The fact that little or no resting block was found at normally polarized ventricular fibres suggests that E-3753 has little affinity for the resting state of the sodium channel. As compared to periods of quiescence, during the periods of stimulation channels spend proportionately more time in the open state during the upstroke and in the inactivated state during the plateau. Thus, if like other antiarrhythmic drugs E-3753 has higher affinity for the activated and inactivated sates of the channel (Hondeghem & Katzung, 1984), an accumulation of blocked channels during the stimulation period leading to a use-dependent V_{max} block would be expected.

The decrease of V_{max} increased progressively when the preparations were stimulated at progressively increasing driving rates and the onset rate of use-dependent block became slower as the stimulation rate was increased. The slope of the curves relating the intensity of the use-dependent block to the frequency of stimulation was not as steep as those described with lignocaine (Chen & Gettes, 1976; Hondeghem & Katzung, 1980), amiodarone (Varro *et al.*, 1985) and mexiletine (Campbell, 1983a), but flat like those of flecainide, lorcainide (Campbell & Vaughan Williams, 1983), propafenone (Kohlhardt & Seifert, 1983) and 5-hydroxypropafenone (Valenzuela *et al.*, 1988). Moreover, at driving rates (2 and 3 Hz) that reduced V_{max} by about 50%, the onset rate of the use-dependent V_{max} block per action potential was 0.05 ± 0.009 AP^{-1}. These values are similar to those for procainamide which has been proposed as an intermediate kinetics class I drug by Campbell (1983a,b).

The recovery from use-dependent V_{max} block (reactivation) by E-3753 was far slower than those of most antiarrhythmic drugs (Campbell, 1983a,b; Courtney, 1980a,b; 1987), probably reflecting the unbinding of the drug from inactivated sodium channels. In fact, the values found in this study are

intermediate between those described with CCl 22277 (Campbell, 1982) and encainide (Campbell, 1983a). E-3753 also shifted the membrane responsiveness curve along the voltage axis to more negative membrane potentials, indicating a greater inhibition of V_{max} at less negative potentials. This effect suggests that this drug possesses a somewhat enhanced affinity for the inactivated state of the sodium channel. However, voltage-clamp studies are required to determine whether E-3753 has a higher affinity for the open and/or inactivated state of the channel. Thus, it can be predicted that E-3753 might cause greater inhibition of excitability and conduction velocity in depolarized cardiac tissues due to ischaemia or other pathological conditions than in normally polarized cardiac tissues. Therefore, since E-3753 exhibits slow onset and offset kinetics of use-dependent block, it can be classified as a kinetically slow class Ic agent according to the classification of Vaughan Williams (1984). The kinetics of the onset and particularly of the offset seem to correlate reasonably well with the physicochemical properties of the drug (Courtney, 1980a,b; Sada & Ban, 1981; Campbell, 1983a). It has been demonstrated that binding and unbinding of class I antiarrhythmic drugs to the receptor site are favoured by low molecular weight and high lipophilicity and inhibited by ionization (Courtney, 1980a,b; 1987). The present data seem consistent with this hypothesis, since E-3753 exists mostly in an ionized form at physiological pH and has a larger molecular weight (399) compared to class I antiarrhythmic drugs with intermediate and fast kinetics (Campbell, 1983a; Courtney, 1987).

Class I antiarrhythmic drugs characterized by a slow onset of and recovery from V_{max} block are often very effective in suppressing both early and late premature beats since they will find a significant fraction of non-conducting sodium channels (Hondeghem & Katzung, 1980; 1984). Moreover, in the presence of tachyarrhythmias sodium channels are in activated and/or inactivated states for a longer period and there is less time for recovery from block between beats. Thus, it would be possible to select a dose of an antiarrhythmic drug with long τ_{re} (i.e., E-3753) which suppressed conduction in the presence of tachycardia but produced minimal changes at normal sinus rates (Hondeghem & Katzung, 1984; Tamargo *et al.*, 1988).

The present data also reveal that E-3753 shortens the APD. This shortening might result from: (1) a decrease in the slow inward Ca current, (2) an inhibition of the slow inward Na 'window current' flowing during the plateau (Carmeliet & Saikawa, 1982) and/or (3) an increase in the time-dependent outward K current (Carmeliet & Vereecke, 1979). The first possibility was investigated using slow action potentials. E-3753 was without effect on the slow action potentials elicited by isoprenaline in K-depolarized fibres, thus suggesting that it did not exhibit class IV activity in guinea-pig papillary muscles. A more likely explanation for the shortening in APD would be a reduction in the Na current flowing during the plateau. This current is thought to utilize the rapid Na channel forming the basis for a relationship between V_{max} inhibition and APD shortening (Carmeliet & Saikawa, 1982), which can thus be considered to be a manifestation of the class I action exhibited by E-3753. However, an increase of outward K current may also be responsible for the shortening of the APD, but this possibility was not investigated in this study.

In conclusion, on the basis of the present results obtained in guinea-pig atrial and ventricular muscle fibres, E-3753 can be classified as class I antiarrhythmic agent with effects similar to those previously described for class Ic drugs.

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A comparison of the effects of a series of anti-anginal agents in a novel canine model of transient myocardial ischaemia

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1 An anaesthetized canine model of transient myocardial ischemia (TMI) has been developed in which reproducible and reversible electrocardiographic (ECG) and haemodynamic responses are exacerbated by electrical pacing.

2 The model could separate the ECG and haemodynamic effects of compounds with anti-ischaemic properties.

3 Compounds known to possess peripheral or coronary vasodilator properties did not necessarily alleviate the ECG consequences of TMI since glyceryl trinitrate was active whereas dipyridamole was not. The effects of verapamil were complicated by its adverse conduction effects while lidoflazine inhibited the ECG changes only during the ischaemic phase and the 'metabolic modulator' oxfenicine worsened the ECG response.

4 In a model considered to lack coronary reserve, improvements observed in the ischaemic ECG and global ventricular function were considered to result from a direct myocardial effect of the drugs examined rather than by a vascular influence. This was provided to the greatest degree by the Ca^{2+}-entry blockers nifedipine and nicardipine, with flunarizine adopting an intermediate position.

Introduction

The assessment of changes in S-T segment deflections obtained by epicardial electrocardiographic (ECG) mapping has been used experimentally as a means of dynamically representing the myocardial O_2 supply/demand ratio. Szekeres *et al.* (1976) described a canine model of electrical stress-pacing induced 'angina' employing a critical stenosis to reduce coronary blood flow (CBF) with epicardial recording of ECGs providing a gross electrophysiological index of oxygen debt. In addition it has been indicated that the ischaemic changes in this type of model were accompanied by increases in myocardial lactic acid and carbon dioxide production (Szekeres *et al.*, 1976; Szekeres & Udvary, 1983; Allely & Alps, 1987). Such models of transient myocardial ischaemia (TMI) have provided the basis of the version employed in the present study where temporary complete occlusion of the left anterior descending coronary artery (LAD) mirrors the ischaemic component of the disease state and superimposed electrical pacing provides a component of work-induced stress.

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The present study was carried out in order to: (a) evaluate the reproducibility and reversibility of the model and (b) assess a series of novel and clinically-employed 'anti-anginal' agents in terms of their electrophysiological and gross haemodynamic effects.

Methods

Experimental preparation

Adult beagles of either sex (10 to 20 kg) were deprived of food overnight and premedicated with acepromazine (0.2 mg kg^{-1} i.m.). General anaesthesia was induced with sodium pentobarbitone (Sagatal, 25 mg kg^{-1} i.v.) and maintained with this agent as required. The trachea was intubated and the dogs ventilated (respirator, Harvard) with room air at a rate of 10–12 min^{-1}, tidal volume 200–300 ml according to body weight. Body temperature was maintained at 38 ± 1°C by means of a rectal probe thermometer attached to a homeothermic blanket control unit (CFP 8185).

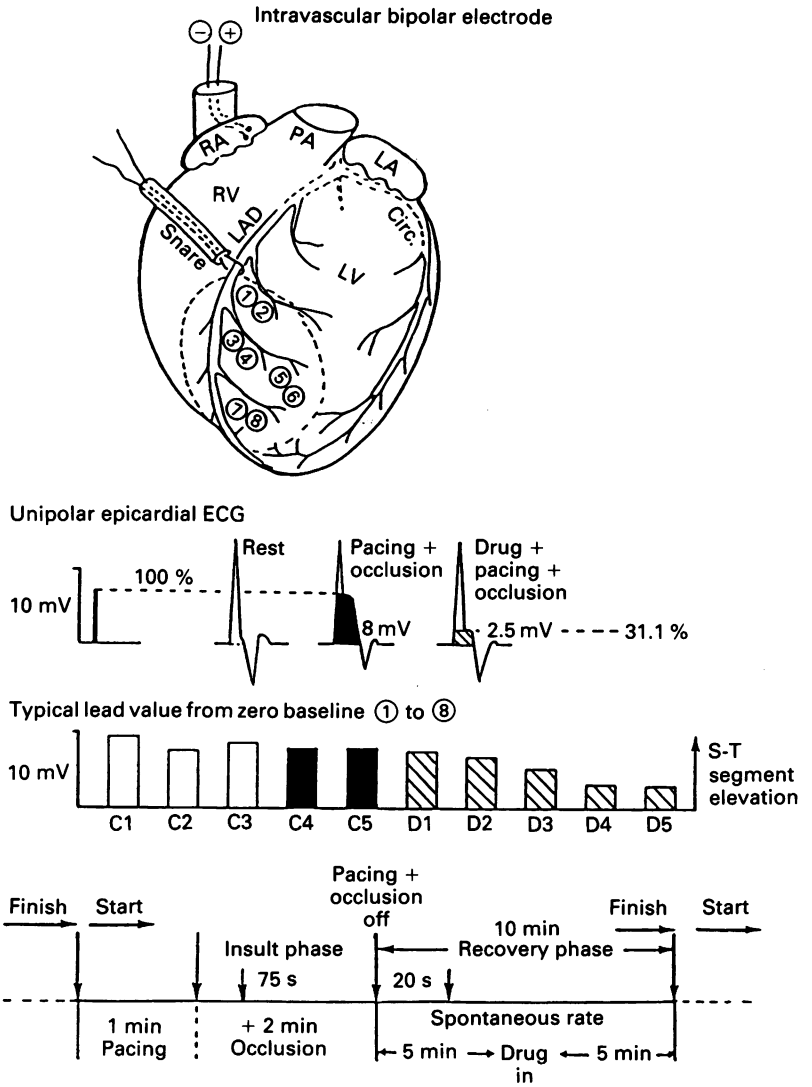

Figure 1 Arrangement of bipolar epicardial recording electrodes and experimental protocol to determine the electrocardiographic changes produced by transient myocardial ischaemia in the pentobarbitone-anaesthetized dog.

Haemodynamics

The left femoral vein was catheterized for drug administration. Ringer-lactate solution (250 ml) was infused before surgery as fluid replacement therapy. Phasic aortic blood pressure was recorded by means of a catheter inserted into the left femoral artery attached to a pressure transducer (Siemens, 746). Left ventricular systolic (LVSP) and end diastolic (LVEDP) pressures were measured by means of a catheter introduced into the left ventricular (LV) cavity via the left carotid artery and attached to a pressure transducer (Siemens, 746). The expression $dP\,dt^{-1}\,P^{-1}$ max was derived (Siemens 868 calculator) from LVSP to give a value for LV contractile force (CF). All haemodynamic data were recorded on a Mingograf (Siemens 804 inkspray oscillograph).

Coronary occlusion and S-T segment recording

Left lateral thoracotomy was performed via the 5th intercostal space. The lungs were retracted and the pericardial sac drawn up to 'cradle' the heart. A

length of the LAD was dissected free and a silk ligature loosely applied and drawn up a nylon tube for application of transient occlusion. Four close pairs of unipolar epicardial recording electrodes were sewn onto the potential ischaemic zone of the left ventricle (see Figure 1) for epicardial ECG recordings made via a Siemens 850 ECG amplifier and recorded on a Mingograf (Siemens, 804 inkspray oscillograph) made at a sensitivity of 1 mm = 1 mV. Pacing of the heart was performed by a pacing catheter inserted into the right atrium via the right jugular vein at 5 V, 1 ms duration (Grass S88 stimulator).

Experimental protocol

A schematic representation of the experimental protocol is shown in Figure 1.

The preparation was left to stabilize for 30 min. Pacing of the heart at a rate of 50–80 beat min^{-1} above resting heart rate (HR) was performed for 1 min before application of the LAD occlusion. Pacing plus occlusion was then carried out for a further 2 min. The next pacing event was after 10 min rest. Episodes of pacing and occlusion were repeated at least four times or until two consecutive challenges produced the same degree of S-T segment elevation with a return to baseline between challenges.

S-T segment elevations in response to each challenge were calculated for two distinct phases: (a) during the rising peak insult from ECG recordings made at 45, 60, 75, 90 and 105 s in the LAD occlusion period (5 time values). (b) Early during the recovery phase at 2 s intervals for the first 20 s (10 time values) following switching off electrical pacing and releasing LAD occlusion.

For each electrode the ischaemic effect was determined from a summation of the S-T segment voltages for a single heartbeat at the above time points. The two conditioning pre-drug S-T segment elevations were averaged and this value taken as 100%. Each test event thereafter was expressed as a percentage of this baseline value and a mean (±s.e.) percentage calculated. In 4 animals no drug was administered and without interrupting the timing cycle a further 8 challenges were performed to provide control responses. In treated animals a drug dose was administered 5 min into the reperfusion phase and the protocol repeated.

Drugs

Dipyridamole HCl (Boehringer Ingelheim); flunarizine HCl (Janssen Pharmaceuticals); glyceryl trinitrate (Wellcome); lidoflazine HCl (Janssen Pharmaceuticals); nicardipine HCl (Syntex); nifedipine (Bayer); oxfenicine HCl (Pfizer); verapamil HCl (Sigma).

Statistical analysis

Statistical significance of results was assessed by Student's two-tailed t test (Robson, 1973).

Results

Electrocardiographic parameters

Effects of repeat ischaemic episodes The initial 1 min pacing period produced only minimal effects on the S-T segment of the ECG. Pacing plus LAD occlusion produced a time-related rise in the S-T segment which gradually returned to its pre-insult level on reperfusion. Successive repeated ischaemic episodes subsequent to the conditioning challenges did not alter the electrocardiographic response to the challenge in the control dog group ($n = 4$) and those values are included for comparison in each figure.

Drug effects The effects of nicardipine ($n = 7$) and nifedipine ($n = 5$) on S-T segment elevations during the ischaemic and reperfusion phases are depicted in Figure 2a. Nifedipine had a greater effect than nicardipine during the ischaemic phase but both drugs produced almost identical effects during reperfusion.

Figure 2b shows the effects of dipyridamole (DPPP; $n = 5$) and glyceryl trinitrate (GTN; $n = 5$). Apart from the 5 $\mu g\,kg^{-1}$ dose during the ischaemic phase, GTN exerted a significant effect during both phases. DPPP reduced S-T segment elevation only during the ischaemic phase.

The effects of flunarizine ($n = 5$) and verapamil ($n = 5$) are shown in Figure 2c. Both drugs reduced S-T segment elevations to about the same extent at 200 $\mu g\,kg^{-1}$ during both phases, the effects being more pronounced during ischaemia. At 400 $\mu g\,kg^{-1}$ verapamil produced a 2:1 atrioventricular block in 2 dogs on electrical pacing. The S-T segment value is therefore artificially low at this point since the degree of ischaemia was not as great in these dogs and this data point has been omitted from the figure. Figure 2d depicts the effects of lidoflazine ($n = 5$) and oxfenicine ($n = 3$). Lidoflazine produced a significant effect during the ischaemic phase but on reperfusion it was ineffective at doses up to 1 $mg\,kg^{-1}$. Oxfenicine was also active in the ischaemic phase at doses of 200 $\mu g\,kg^{-1}$ and above but at low doses it actually exacerbated the S-T segment elevations. A small reduction was noted at 1 $mg\,kg^{-1}$ but this had disappeared by cumulative addition of 10 $mg\,kg^{-1}$.

General haemodynamics

Effects of repeat ischaemic episodes In the absence of drug treatment, the overall effect of the ischaemic challenge in any group was to reduce mean arterial

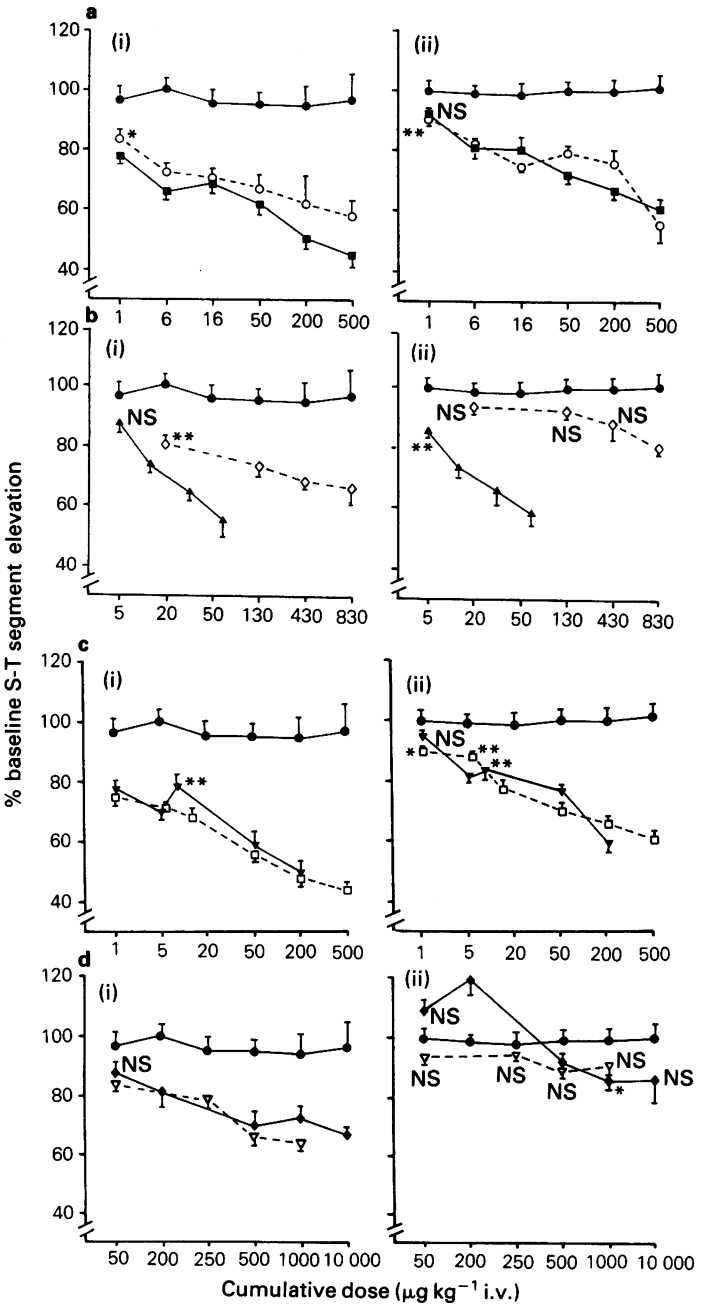

Figure 2 The effects of (a) nicardipine (○; $n = 7$) and nifedipine (■; n = 5); (b) glyceryl trinitrate (▲; $n = 5$) and dipyridamole (◇; $n = 5$); (c) flunarizine (□; $n = 5$) and verapamil (▼; $n = 5$); (d) lidoflazine (▽; $n = 5$) and oxfenicine (◆; $n = 5$), on epicardial S-T segment elevations during the ischaemic (i) and reperfusion (ii) phases of transient myocardial ischaemia in the pentobarbitone-anaesthetized dog. Values represent mean and vertical lines s.e.mean. All points are significantly lower than values in control dogs (●; $n = 4$) at $P < 0.001$ except as indicated NS, $*P < 0.05$ and $**P < 0.01$, Student's two-tailed t test.

blood pressure (MABP) and increase LVEDP while CF remained essentially unchanged. Changes in systolic and diastolic BP followed similar directional changes and the impact of ischaemia on the pump was predominantly reflected through the systolic component. For convenience, the data presented relates to MABP. MABP appeared to recover well between challenges on reperfusion but this was not always the case with LVEDP in some dogs where it remained elevated. Variability in haemodynamic responses to the challenge between animals in any given group appeared to depend upon how much of the myocardium was involved in each animal. A consideration of this variability and the possible impact of drug intervention is made later (Limitations of study section).

Drug effects Drug effects on haemodynamic parameters (excluding HR) are shown in Table 1. The values at the dose producing the optimal reduction in S-T segment elevation (or the highest dose examined in the absence of a reduction) are also given.

Nicardipine: Nicardipine ($16\,\mu g\,kg^{-1}$) had no effects on HR but it decreased resting MABP. The falls in MABP associated with ischaemia and reperfusion were not altered by nicardipine. Nicardipine reduced resting LVEDP and reduced the LVEDP elevated during the challenge. Resting CF was increased and this increase persisted during the challenge.

Nifedipine: Nifedipine ($16\,\mu g\,kg^{-1}$) produced a small fall in resting HR (about 6%). MABP was unaltered at rest or during ischaemia but the reduction associated with reperfusion was abolished. Resting LVEDP was reduced and the increase produced by the challenge was also decreased. No effects were noted on baseline CF but the depression associated with the challenge was abolished.

Table 1 The effects of nicardipine (Nic), nifedipine (Nif), glyceryl trinitrate (GTN), dipyridamole (DPPP), flunarizine (Flu), verapamil (Ver), lidoflazine (Lid) and oxfenicine (Oxf) on mean arterial blood pressure (MABP, mmHg): left ventricular end diastolic pressure (LVEDP, mmHg) and left ventricular contractility (CF, s) in a pentobarbitone-anaesthetized canine model of transient myocardial ischaemia

				Challenge			
		Baseline		*Ischaemia*		*Reperfusion*	
Drug	*Parameter*	*Pre*	*Post*	*Pre*	*Post*	*Pre*	*Post*
Nic	MABP	104 ± 4	88 ± 6	92 ± 6	85 ± 6	97 ± 4	96 ± 4
$n = 7$	LVEDP	5.1 ± 0.7	4.1 ± 1.1	7.3 ± 0.9	6.6 ± 1.4	7.2 ± 0.8	6.1 ± 1.3
(16)	CF	129 ± 7	140 ± 14	135 ± 9	149 ± 13	138 ± 10	152 ± 11
Nif	MABP	106 ± 3	102 ± 7	68 ± 9	69 ± 6	85 ± 9	92 ± 8
$n = 5$	LVEDP	4.4 ± 0.5	3.8 ± 0.9	6.7 ± 0.4	5.6 ± 0.6	7.5 ± 0.4	6.0 ± 0.5
(16)	CF	132 ± 8	132 ± 10	120 ± 9	145 ± 22	124 ± 5	132 ± 6
GTN	MABP	91 ± 8	91 ± 8	78 ± 5	76 ± 9	79 ± 6	75 ± 8
$n = 5$	LVEDP	6.4 ± 0.5	5.8 ± 1.1	9.5 ± 0.7	7.8 ± 1.5	9.8 ± 0.6	8.1 ± 0.7
(35)	CF	125 ± 12	123 ± 14	131 ± 10	158 ± 7	115 ± 12	116 ± 8
DPPP	MABP	124 ± 8	97 ± 13	87 ± 7	88 ± 11	83 ± 10	80 ± 12
$n = 5$	LVEDP	6.7 ± 0.6	7.8 ± 0.6	7.2 ± 0.6	7.5 ± 0.6	9.1 ± 1.1	10.4 ± 1.5
(830)	CF	127 ± 20	130 ± 9	136 ± 8	129 ± 40	109 ± 7	110 ± 13
Flu	MABP	111 ± 7	109 ± 7	72 ± 12	68 ± 7	101 ± 14	93 ± 3
$n = 5$	LVEDP	4.2 ± 0.3	4.4 ± 1.2	7.3 ± 0.7	8.2 ± 1.6	7.6 ± 0.5	7.2 ± 1.1
(16)	CF	142 ± 10	154 ± 27	140 ± 20	116 ± 21	143 ± 12	130 ± 23
Ver	MABP	104 ± 9	99 ± 10	96 ± 9	101 ± 13	99 ± 9	100 ± 12
$n = 5$	LVEDP	3.1 ± 0.6	3.2 ± 1.1	4.1 ± 0.7	3.8 ± 0.8	4.8 ± 0.7	4.5 ± 1.1
(50)	CF	119 ± 4	110 ± 9	137 ± 3	136 ± 6	127 ± 6	120 ± 9
Lid	MABP	109 ± 12	97 ± 11	85 ± 9	94 ± 11	92 ± 7	97 ± 11
$n = 5$	LVEDP	4.9 ± 0.8	4.1 ± 1.0	6.7 ± 0.9	6.9 ± 1.8	7.2 ± 0.2	6.5 ± 1.7
(1000)	CF	127 ± 12	138 ± 10	128 ± 4	128 ± 7	139 ± 8	140 ± 7
Oxf	MABP	79 ± 12	81 ± 3	65 ± 12	62 ± 6	77 ± 11	92 ± 4
$n = 3$	LVEDP	6.6 ± 0.8	6.7 ± 1.4	7.0 ± 0.8	6.8 ± 1.3	9.0 ± 1.6	6.2 ± 1.9
(10,000)	CF	127 ± 12	140 ± 20	122 ± 18	113 ± 33	121 ± 16	103 ± 17

Each of the 'pre-values' for baseline and peak challenge represent the mean ± s.e.mean values for the pre-drug test cycles with each animal contributing its average value of the final two conditioning events at each point. The 'post-values' indicate the new level for each parameter associated with the cumulative dose of the drug concerned producing the optimal reduction in S-T segment elevation, or the highest dose examined in the absence of a reduction. The group n values are given at the doses ($\mu g\,kg^{-1}$ i.v.) shown in parentheses

Glyceryl trinitrate: HR was essentially unaltered by GTN ($35\,\mu g\,kg^{-1}$) while MABP was only slightly reduced on reperfusion. Resting LVEDP was reduced by GTN and the increase noted during the challenge also decreased. CF was increased only during reperfusion.

Dipyridamole: DPPP at $830\,\mu g\,kg^{-1}$ (a dose which only just began to affect S-T segment elevations on reperfusion) had no effects on MABP. Resting LVEDP was increased by DPPP and the increases produced by the challenge were not lessened. CF was not altered.

Flunarizine: Flunarizine ($16\,\mu g\,kg^{-1}$) did not affect resting MABP or HR and hardly affected the depressor effects of the challenge. There was no alteration of LVEDP during the resting or reperfusion phases but it increased during ischaemia. CF was increased at rest but decreased during the challenge.

Verapamil: Verapamil exerted little effect on MABP or HR at $50\,\mu g\,kg^{-1}$. Resting LVEDP was unaltered by verapamil and the elevated LVEDP noted during the challenge was very slightly reduced. CF was reduced slightly at rest and on reperfusion.

Lidoflazine: Lidoflazine ($1\,mg\,kg^{-1}$) had no effects on resting HR or MABP, but a slight lessening of the depressor effect of the challenge was noted. Resting and reperfusion LVEDP were decreased. Resting CF was increased with no alteration during the challenge.

Oxfenicine: Oxfenicine did not affect resting or ischaemic MABP but the depressor effect of reperfusion was reversed to a pressor effect. Resting and ischaemic LVEDP values were unaltered while the rise on reperfusion was abolished. Resting CF was slightly increased but CF was decreased during the challenge.

Discussion

The two-component model (ischaemia and reperfusion) of TMI described in the present study and the method of data analysis has provided a reproducible and reversible epicardial ECG response to the ischaemic conditioning of the canine myocardium to an on/off stress-pacing protocol.

The compounds studied appeared to exert a range of effects on the ischaemic myocardium. The onset of useful haemodynamic and ECG effects of a drug did not always coincide. It was evident that not all drugs demonstrated ECG activity in both phases of the challenge, although agents which have aroused clinical interest were clearly active in both phases. This applied to nicardipine, nifedipine, verapamil, GTN and flunarizine. Where LVEDP was reduced and CF enhanced, ventricular performance was considered improved and this was a feature seen with several drugs during the ischaemic phase. In the event that this feature persisted into the reperfusion phase, as with nicardipine and nifedipine, metabolic recovery must have been facilitated, with a resultant global increase in ventricular compliance. Rousseau *et al.* (1985, 1986) have shown that nicardipine improved biochemical function of the human ischaemic myocardium with only marginal effects on coronary blood flow (CBF). The haemodynamic and ECG properties of nicardipine appear to be separated: at doses below $16\,\mu g\,kg^{-1}$ it lowered S-T segment rises in the absence of haemodynamic effects.

Where CF was not enhanced on reperfusion as with lidoflazine, GTN and verapamil, LVEDP could still be lowered. Here it was evident that the ventricle could still operate effectively at a reduced muscle fibre length.

Nifedipine is a vasodilator with little direct myocardial effect *in vivo* and has also shown good activity in a dog TMI model of critical LAD stenosis (Alps *et al.*, 1985). In the present study its effect in reducing S-T segment elevations was very similar to that of nicardipine. Our observations for GTN were in keeping with the findings of Szekeres & Udvary (1983). It would appear that the ECG and haemodynamic effects of this drug are not directly linked. Although in the non-ischaemic canine ventricle there is no transmural flow gradient (Jennings & Reimer, 1979), there is the possibility of an association of the haemodynamic and ECG effects of GTN via its reported improvement of subendocardial blood flow to the ischaemic zone, by direct coronary conductance vessel dilatation which may not be of a sufficient magnitude to affect our overt haemodynamic recordings (Cohen & Kirk, 1973; Szekeres & Udvary, 1983). The cardioinhibitory activity of verapamil has been described by Himori *et al.* (1976) who found it to be a negative inotrope in the same dose range as that producing coronary vasodilatation. It is interesting to note that the beneficial electrocardiographic effect of verapamil at $1–200\,\mu g\,kg^{-1}$ overlapped the dose range $100–200\,\mu g\,kg^{-1}$ which according to Warltier *et al.* (1981) produced coronary vasodilatation. Our earlier studies with verapamil in the critical stenosis model also showed activity for this drug at low doses (Alps *et al.*, 1985). The doses producing S-T segment reduction and those producing detrimental myocardial conduction effects ($400\,\mu g\,kg^{-1}$ in the present study with a pacing frequency-dependent atrioventricular block at $100–400\,\mu g\,kg^{-1}$ in a previous study – Allely & Alps, 1988) do not appear to be very well separated.

Lew & Ban-Hayashi (1985) demonstrated that by elevating LVEDP above the ischaemia-induced

increase, the global mechanical disadvantage imposed by the ischaemic zone was diminished. The three drugs in this study demonstrating such an effect during ischaemia with corresponding ECG improvements were flunarizine, DPPP and lidoflazine. The ECG benefit of flunarizine was sustained throughout both phases of the challenge but it failed to improve ventricular performance; CF fell whilst LVEDP rose. The other two drugs failed to confer ECG improvement on reperfusion. In their model of critical stenosis Szekeres *et al.* (1976) also found DPPP to be inactive ($60\,\mu g\,kg^{-1}$). This is an interesting observation since DPPP is a coronary dilator exerting its effects primarily on coronary resistance vessels (Becker, 1976) thus increasing subendocardial flow in the ischaemic zone and, as with GTN, this may be responsible for the onset of ECG improvement at high doses. DPPP certainly did not improve ventricular performance. Like DPPP, lidoflazine is a long-acting coronary artery dilator (Schaper *et al.* 1966; Gobel, 1980). It has been shown to be of benefit in the long-term treatment of effort angina (Bernstein & Peretz, 1972), a property attributed to its calcium entry blocking activity (Van Neuten & Wellens, 1979), yet it has negligible effects on the myocardium (Vanhoutte & Van Neuten, 1980).

Oxfenicine was included in the present study since it has been shown to increase cardiac glucose oxidation in ischaemia at the expense of utilizing free fatty acids (Higgins *et al.*, 1981), thus yielding more ATP than aerobic oxidative pathways alone (Drake-Holland & Passingham, 1983). The haemodynamic activity of oxfenicine could be interpreted cautiously as providing an overall improvement in left ventricular function and efficiency since the ventricle was able to contract to a low LVEDP. Since a fall in CF was accompanied by an increased blood pressure on reperfusion, a systemic pressor effect may be exerted during this phase which falls back to baseline at rest. However, these haemodynamic effects must also have occurred at the expense of improving segmental efficiency, since there was no decrease in S-T segment elevations. Indeed, at lower doses the increase in S-T segment elevations was exacerbated, suggesting that a global preload improvement on reperfusion accompanied a local worsening of ventricular performance.

The overall findings of the study indicate that the canine model of TMI developed here is a useful predictor of the anti-anginal efficacy of a compound. The differentiation of the onset of ECG and haemodynamic effects at different doses of a compound may indicate a separation of its mechanisms of action and these can be determined in the model. The two compounds shown to be inactive on the ECG effects during the reperfusion (lidoflazine and DPPP) are those which have aroused most clinical controversy in terms of whether or not they alleviate the symptoms of angina pectoris and the side-effects which they produce (Sbar & Schlant, 1967; Hanley & Hampton, 1983; Loeb *et al.*, 1983; Fazekas & Kiss, 1984). The compound which actually exacerbated the S-T segment elevations during the recovery phase at lower doses (oxfenicine) exerted a complex, seemingly contradictory, haemodynamic profile and a clinical application for such a metabolic modulator with no ECG benefit is uncertain. Nicardipine and nifedipine were not cardiodepressant at doses showing optimum ECG benefit during the challenge. They improved ventricular performance during the challenge while flunarizine displayed intermediate effects. In contrast, in the case of verapamil and GTN this feature was projected predominantly only during the ischaemic phase with little influence on ventricular function during reperfusion. Verapamil did not improve ventricular CF at doses associated with optimum beneficial ECG effects, and increasing the dose precluded any effective further benefit due to the A-V block incurred.

Limitations of the study

Inter-dog variability, with regard to the pattern and distribution of epicardial coronary arteries and their anastomoses, determined that the occluded vascular beds would be unequal in size and volume between hearts. This in turn dictated for any given animal whether TMI would have sufficient impact on the ventricular segment involved to embarrass mechanically the whole cardiac pump, even though the ECG evidence indicated serious local metabolic dysfunction.

We do know from other studies using our model that the reversible S-T segment changes are accompanied by reversible changes in lactic acid and CO_2 production, plasma K^+ levels and pH in local myocardial venous blood collected during ischaemia (Allely *et al.*, 1987), even though the hearts involved were not obviously compromised mechanically.

In an anaesthetized, thoracotomized animal the ischaemic heart cannot compensate by dilating to overcome the mechanical disadvantage imposed by the ischaemic segment. Rather, compensation features ballooning with an elevated LVEDP. In some animals this was obvious, and made worse by repeated ischaemic challenges, while in others no changes were evident. Thus, some hearts did not recover to their pre-ischaemic condition and featured a persistently elevated 'baseline' LVEDP. This condition did appear to be improved following the application of some drugs (e.g. nicardipine). Variation in haemodynamic responses to ischaemia made it impossible to apply statistical analysis methods to such changes and the effects of drugs on them in

small groups of animals. Nevertheless, when obvious haemodynamic responses did occur in any group the apparent effectiveness of a particular drug treatment was considered to be 'clinically' relevant to the known pharmacological profile of the agent.

Under the experimental conditions operated in the study, allowing 10 min between challenges, we were able to make only limited observations on the ability of drugs to favour the extent, as opposed to the rate which we measured, of recovery of the ischaemic myocardium. Nevertheless, as previously stated, the recovery period does allow time for reversal of local ECG and biochemical changes.

Whilst appreciating that the myocardium has been 'stunned' by repeated insults, and that not all hearts recovered haemodynamically between challenges, the time intervals followed have been evolved to standardize the acute experimental conditions. The assumption has been made that TMI is largely reversible, as in angina, otherwise the heart would infarct or fail, and thus the acute effects created in the model simulate some of the classical features of the human disease.

The technique is one used for *in vivo* 'screening' of drugs with unknown mechanism, it identifies different classes of drugs of known clinical interest and allows multiple doses to be examined in a reasonably short-duration study.

It is known from other studies in our laboratory (unpublished findings) employing the technique of radiolabelled microspheres, to measure regional and total CBF, that in this model CBF is only approximately 34% of normal during ischaemia. This residual flow must include contributions made from any pre-existing collateral circulation. Under these conditions doses of nicardipine which would cause marked coronary dilatation in normal hearts relieve S-T segment changes but do not increase ischaemic blood flow, other than to favour slightly the epicardial/endocardial gradient, and do not cause coronary steal.

Thus the model of TMI as operated here is one which has very limited coronary reserve and appears to identify pharmacological effects which are exerted predominantly on the myocardium and not via changes in blood supply. Certainly, when the critical stenosis technique is used there does appear to be coronary reserve and excessive dilatation compromises the benefit of direct myocardial action.

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ICI 204448: a κ-opioid agonist with limited access to the CNS

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1 A number of compounds were evaluated in an attempt to identify a κ-opioid receptor agonist with limited access to the central nervous system.

2 Quaternary derivatives of the κ-opioid agonists tifluadom, U-50488H and ethylketocyclazocine were essentially devoid of opioid activity in a range of isolated tissue preparations.

3 A novel compound – ICI 204448 – is described which produced a potent and naloxone-reversible inhibition of electrically-evoked contraction of the guinea-pig ileum, mouse vas deferens and rabbit vas deferens preparations. ICI 204448 was shown to displace the binding of the κ-opioid ligand [^{3}H]-bremazocine from guinea-pig cerebellum membranes.

4 *Ex vivo* binding studies in mice showed ICI 204448 to be well absorbed following subcutaneous administration. The brain levels achieved by ICI 20448 were substantially lower than those produced by κ-agonists such as U-50488H and tifluadom.

5 A good correlation was found for a range of opioids between lipophilicity and degree of CNS penetration.

Introduction

In addition to the well-characterized actions of opioids in the CNS, there is increasing evidence of a role for opioid receptors in the periphery. It has long been recognized that functional opioid receptors are present in a number of tissues such as the guinea-pig ileum (Trendelenburg, 1917) and the mouse vas deferens (Henderson *et al.*, 1972). More recently it has been suggested that opioid receptors are also present on the peripheral terminals of primary afferent neurones (Bartho & Szolcsanyi, 1981) and that activation of these sites may contribute to the antinociceptive action of opioids (Lorenzetti & Ferreira, 1982; Ferreira *et al.*, 1984).

Investigation of the actions of opioids at sites outwith the CNS has been hampered by a lack of specific tools. The classical opiates produce profound central effects, and attempts to confer peripheral selectivity by chemical modification of these agents has met with only limited success. The quaternary analogues of drugs such as morphine, nalorphine and naltrexone have been demonstrated to have restricted access to the CNS, but these agents cannot necessarily be assumed to retain the potency or selectivity of their parent compounds. A more recent development is the peripherally-selective enkephalin analogue BW-443C (Follenfant *et al.*, 1987; Lorenzetti & Ferreira, 1987). This compound appears to act predominantly at opioid μ-receptors and should prove to be of benefit in elucidating the role of these sites.

The need remains, however, for a pharmacological tool with which to investigate the role of peripheral κ-receptors. This paper describes a novel compound, ICI 204448 ((**R**,S)-N-[2-(N-methyl-3,4-dichlorophenylacetamido)-2-(3-carboxyphenyl)-ethyl]pyrrolidine hydrochloride), which exhibits high selectivity towards κ-receptors and low CNS penetration.

Methods

Isolated tissue preparations

Isolated tissue studies were performed as previously described (Miller *et al.*, 1986). Briefly, vasa deferentia were obtained from Alderley Park strain mice weighing between 23 and 28 g, and from New Zealand White rabbits weighing 2.5 to 3.0 kg. Guinea-pig ileum longitudinal muscle-myenteric plexus strips were prepared according to the method of Paton & Zar (1968). All tissues were mounted in 5 ml organ baths containing Krebs solution at 37°C. For the

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mouse vas deferens the Mg^{2+} was omitted. Contractions were elicited by stimulating through ring electrodes mounted above and below the tissues. For the guinea-pig ileum and rabbit vas deferens single, 1 ms pulses at supramaximal voltage were delivered every 10 s. Trains of 1 ms pulses (50 Hz for 100 ms, supramaximal voltage) were delivered at 10 s intervals to the mouse vas deferens preparation. Contractions were recorded isotonically under a tension of 0.2 g (0.4 g for rabbit vas deferens).

After 60 min equilibration, cumulative dose-response curves were constructed to agonists both before, and 30 min after addition of naloxone. The prototypic κ-agonist ethylketocyclazocine (EKC) was tested in all tissues for comparison.

κ-Receptor binding

[^{3}H]-bremazocine (0.2 nM) binding was performed in HEPES buffer (pH 7.4) using guinea-pig cerebellum membranes prepared according to the method of Magnan *et al.* (1982). Assays were run for 40 min at 25°C and non-specific binding was defined using 10 μM naloxone.

Determination of brain levels

Groups of 6 female Alderley Park mice weighing between 22 and 25 g were dosed subcutaneously with saline or test drug. Thirty min later the brains were rapidly dissected onto dry-ice and stored in liquid N_2. Pooled groups of saline- or drug-treated mouse brains were homogenized in cold HEPES buffer (pH 7.4) using a Polytron homogenizer (setting 5 for 30 s). Homogenates were stored on ice, and assayed at a final tissue concentration of 20 mg ml^{-1} wet weight.

Ex vivo κ-receptor binding was performed in HEPES buffer using the non-selective opioid ligand [^{3}H]-bremazocine (0.2 nM). Assays were run for 40 min at 25°C in the presence of 3 μM [D-Ala^2,D-Leu^5]enkephalin (DADLE), to suppress binding to the μ- and δ-receptors, using 200 μl of mouse brain homogenate. Non-specific binding was defined using 10 μM naloxone.

Specific binding values were obtained for both saline control and drug-treated groups of mice, and the inhibition of binding due to the test compound calculated. Homogenates from the saline control animals were also used to generate calibration curves to the test drug added *in vitro*, in the same experiment. The concentrations of test compound present in the brains of treated mice were then calculated from these calibration curves and the molar concentrations converted to pg mg^{-1} tissue wet weight. All determinations were the result of three or more separate experiments.

Determination of plasma levels

Groups of Alderley Park mice were dosed subcutaneously and 30 min later were bled under ether anaesthesia. Plasma samples were collected and stored at −20°C.

[^{3}H]-bremazocine (1 nM) binding assays were performed using guinea-pig whole brain tissue in HEPES buffer, in the presence of 3 μM DADLE, and the equivalent of 100 μl of test or control plasma. Other conditions were as described above. Calibration curves were generated to the test drugs added *in vitro*, and compared with the inhibition of binding due to the test samples. Plasma levels were then read off the calibration curves and converted to μg ml^{-1}. All values were determined in triplicate.

Determination of distribution coefficients

Samples were partitioned between octan-1-ol and pH 7.4 phosphate buffer. Octanol is the solvent of choice since it is generally believed to be the best mimic of a biological membrane (Smith *et al.*, 1975). At equilibrium, concentrations in each phase were determined by u.v. spectroscopy and the results were expressed as:

$$\log D = \log_{10} \frac{[\text{octan-1-ol}]}{[\text{aqueous layer}]}$$

Materials

[^{3}H]-bremazocine (specific activity 30 Ci mmol^{-1}) was obtained from New England Nuclear. Drugs were obtained from the following sources: naloxone HCl, DuPont; levallorphan tartrate, Roche; nalorphine HBr, Wellcome; ethylketocyclazocine methanesulphonate (EKC) and cyclazocine HCl, Sterling-Winthrop. The remaining compounds were synthesized within the Chemistry Department of ICI Pharmaceuticals. U-50488H is *trans*-3,4-dichloro-N-methyl-N-(2-(1-pyrrolidinyl)cyclohexyl)-benzeneacetamine. The structures of ICI 197067, ICI 205640 and ICI 204448 are shown in Figure 1. These compounds were prepared as the HCl salts. ICI 204448 is the subject of British Patent Application No. 8801304.

Results

Potencies of quaternary agonists

The quaternary derivatives of three prototypic κ-agonists, EKC, tifluadom and U-50488H, were tested for agonist activity in a number of opioid-sensitive isolated tissue preparations. Of the compounds tested only 4-methyl tifluadom retained the

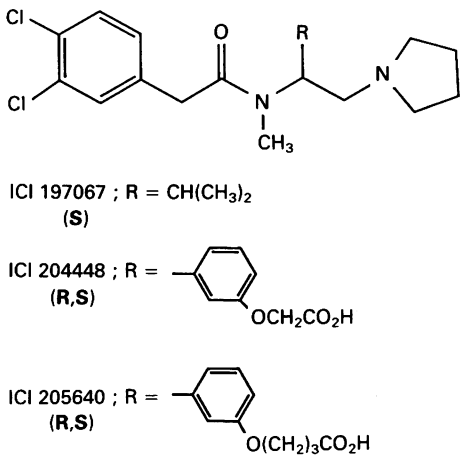

Figure 1 Chemical structures of ICI 197067, ICI 204448 and ICI 205640.

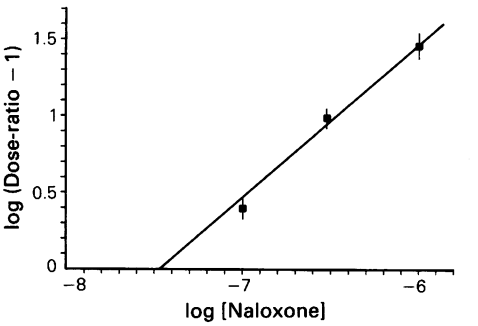

Figure 2 Schild plot for the antagonism of ICI 204448 by naloxone on the field-stimulated mouse vas deferens preparation. Each point is the mean (with vertical lines showing s.e.mean) of between 4 and 6 determinations. The slope of the regression line (1.08 ± 0.14) does not deviate significantly from unity. Consequently the Ke value (34.0 nM) was calculated after constraining the line to a slope of 1.

ability to inhibit the electrically-evoked contractions of the guinea-pig ileum, the mouse vas deferens and the rabbit vas deferens. However, the potency of this analogue was substantially less (140 to 220 fold) than tifluadom (Table 1). Since none of these compounds exhibited antagonist activity, their loss of potency appears to reflect a reduction in affinity rather than a diminished efficacy.

Receptor profile of ICI 204448

ICI 204448 produced a concentration-dependent inhibition of the electrically-evoked contractions of the field-stimulated mouse vas deferens, rabbit vas deferens and guinea-pig ileum preparations (Table 2). In all tissues the potency of ICI 204448 was found to be similar to, or slightly greater than that of the prototypic κ-agonist EKC. In each of the isolated tissue preparations the action of ICI 204448 was found to be naloxone-reversible. However, in two of the tissues, the guinea-pig ileum and the mouse vas deferens, the naloxone Ke values were somewhat greater than those obtained against EKC and U-50488H, although the slope of the Schild plot did not deviate significantly from unity (Figure 2). To investigate the possibility that these high Ke values may indicate that ICI 204448 possesses agonist activity at the δ-receptor, an attempt was made to antagonize its action on the mouse vas deferens with the selective δ-antagonist ICI 174864. No antagonism was observed at concentrations up to 1 μM (results not shown).

The possibility that ICI 204448 may possess affinity for the μ-receptor was investigated using the field-stimulated rat vas deferens preparation. At concentrations up to 5 μM ICI 204448 was devoid of agonist activity in this tissue. Furthermore, at similar concentrations the compound failed to antagonize

Table 1 Potencies of quaternary derivatives of opioid κ agonists on a range of isolated tissue preparations

	Mouse vas deferens	*Guinea-pig ileum*	*Rabbit vas deferens*
Tifluadom	2.2 ± 0.29[1]	2.8 ± 0.75[1]	2.8 ± 0.9
4-Methyl tifluadom	0.01 ± 0.005	0.016 ± 0.003	0.02 ± 0.01
Ethylketocyclazocine	1	1	1
Q. EKC	<0.001	<0.001	<0.001
U-50488H	0.11 ± 0.02[1]	0.09 ± 0.04[1]	0.04 ± 0.006
Q. U-50488H	<0.001	<0.001	<0.001

All values are molar potency ratios relative to ethylketocyclazocine (EKC) assayed in the same tissue. Results are means ± s.e.mean of at least 4 determinations. The mean IC_{50} values for EKC were 66.1 ± 5.8 nM in the mouse vas deferens, 1.63 ± 0.18 nM in the guinea-pig ileum and 31.3 ± 17.1 nM in the rabbit vas deferens.

[1] Data from Costello *et al.* (1988).

Table 2 Characterization of ICI 204448 on opioid-sensitive isolated tissue models

	Mouse vas deferens		*Guinea-pig ileum*		*Rabbit vas deferens*	
	Potency (×EKC)	*Ke naloxone* (nM)	*Potency* (×EKC)	*Ke naloxone* (nM)	*Potency* (×EKC)	*Ke naloxone* (nM)
EKC	1	15.8 ± 1.6[1]	1	15.4 ± 4.5[1]	1	18.8 ± 2.2
U-50488H	0.11 ± 0.02[1]	19.3 ± 1.7	0.09 ± 0.04[1]	10.4 ± 1.2	0.04 ± 0.006	24.5 ± 5.1
ICI 204448	1.1 ± 0.05	34.0 ± 3.5	2.1 ± 0.4	29.2 ± 7.2	2.9 ± 0.4	18.2 ± 4.2

Potencies are expressed as molar potency ratios relative to ethylketocyclazocine (EKC) assayed in the same tissues. All values are means ± s.e.mean of at least 6 determinations.
[1] Data from Costello *et al.* (1988).

the selective μ-agonist [D-Ala^2, MePhe^4, Gly-ol^5] enkephalin (results not shown).

In radioligand binding studies ICI 204448 produced a concentration-dependent displacement of [^{3}H]-bremazocine from guinea-pig cerebellum membranes (Table 3). However, in contrast to the isolated tissue preparations where ICI 204448 displayed a potency equal to or greater than EKC, in the binding assay it exhibited only 12% of the activity of EKC.

In a μ-receptor binding assay utilizing [^{3}H]-naloxone in a rat brain preparation (Carroll *et al.*, 1984), ICI 204448 was devoid of activity at concentrations up to 50 μM (results not shown).

Measurement of CNS penetration

To ensure that ICI 204448 was absorbed following subcutaneous administration, the plasma levels achieved by this compound were compared to those produced by U-50488H. Plasma samples from control mice were compared with those from drug-treated animals for their ability to displace [^{3}H]-bremazocine binding from a guinea-pig whole brain membrane preparation. It is clear from the results (Table 4) that ICI 204448 is absorbed to a similar extent to U-50488H following subcutaneous injection.

Ex vivo binding studies were conducted on a range of κ-agonists and partial agonists which had previously been shown to displace [^{3}H]-bremazocine binding from guinea-pig cerebellum membranes. The compounds were selected to represent a wide range of lipophilicity (log D) values (Table 5).

[^{3}H]-bremazocine binding was measured in brain homogenates from mice treated with each of the compounds and the reduction in binding compared to saline-treated mice was calculated (Table 5). The doses used were chosen from an initial sighting study and were designed to produce approximately 50% inhibition of binding. However, with the two most hydrophilic compounds – 4-methyl tifluadom and ICI 204448 – only 20 to 25% inhibition could be achieved at the highest doses tested.

In parallel with the *ex vivo* binding studies, displacement curves were obtained to each of the compounds added *in vitro* to brains from saline-treated mice. Using these curves it was then possible to estimate the concentration of each drug present in the brains of the treated mice. These values are shown in Table 5.

An index of CNS penetration was determined by dividing the brain level achieved by the dose of drug administered (Table 5). These results reveal that ICI 204448 has a very limited ability to enter the CNS

Table 3 Inhibition of [^{3}H]-bremazocine binding to guinea-pig cerebellum membranes

	IC_{50} (nM) (*95% confidence limits*)
Ethylketocyclazocine	3.87 (2.73–5.48)
U-50488H	15.7 (11.0–22.3)
4-Methyl tifluadom	89.4 (61.3–130)
Levallorphan	1.9 (1.4–2.6)
Nalorphine	25.1 (20.3–30.1)
ICI 197067	1.19 (0.66–2.16)
ICI 204448	33.2 (23.1–47.9)
ICI 205640	17.1 (14.3–20.3)

Values represent pooled data from at least 3 experiments, each comprising triplicate determinations at a minimum of 6 concentrations.

Table 4 Plasma concentrations of ICI 204448 and U-50488 determined by *ex vivo* binding following subcutaneous drug administration

	Dose (mg kg^{-1} s.c.)	*Binding*[1] (% Inhibition)	*Plasma concentration* (μg ml^{-1})
U-50488H	2.2	10.7 ± 3.4	0.22
	11.0	33.0 ± 2.4	1.07
ICI 204448	7.5	37.6 ± 5.7	1.42
	30.0	50.2 ± 3.0	3.99

[1] Inhibition by 100 μl plasma of [^{3}H]-bremazocine (1 nM) binding to guinea-pig whole brain membranes in HEPES buffer containing 3 μM DADLE.

Table 5 Estimation of brain concentrations of opioids by *ex vivo* binding in the mouse

	Dose ($mg\,kg^{-1}$)	*% inhibition of binding*	*Brain level* ($pg\,mg^{-1}$ tissue)	*CNS penetration index*	*Log D*
4-Methyl tifluadom	75	21.9 ± 3.8	165	2.2	0.2
ICI 204448	50	24.3 ± 4.7	435	8.7	0.3
ICI 205640	32	51.9 ± 5.7	1240	23.9	1.0
Nalorphine	3.9	39.7 ± 0.3	191	54.6	1.5
Cyclazocine	0.28	47.4 ± 4.6	36	128.6	1.3
Levallorphan	0.3	46.4 ± 1.4	94	313.3	2.4
U-50488H	3.2	34.0 ± 0.6	1050	328.1	1.7
ICI 197067	0.23	46.4 ± 5.5	119	517.4	2.5

Log D is the octanol: aqueous partition coefficient at pH 7.4.
CNS penetration index = brain level ÷ dose.

whilst its more lipophilic analogue ICI 197067 exhibited the highest level of CNS penetration. Furthermore, it is clear that an excellent correlation exists between the ability of each compound to enter the CNS and its log D (lipophilicity) value (Figure 3).

Discussion

Whilst the actions of opioids on the CNS have long been recognized, there is now an increasing awareness that opioid receptors are widely distributed in the periphery. The role of these peripheral opioid receptors is poorly understood; largely because of the lack of specific pharmacological tools. The recently described enkephalin analogue BW-443C has limited access to the CNS, and appears to be selective for the μ-receptor (Lorenzetti & Ferreira, 1987; Follenfant *et al.*, 1987). However, there are as yet no tools with which to investigate the role of peripheral κ-receptors.

The aim of the present study was to identify a hydrophilic opioid with κ-receptor selectivity which could be used to investigate the role of κ-receptors outside the CNS. A well-established means of limiting the CNS penetration of drugs is to prepare their

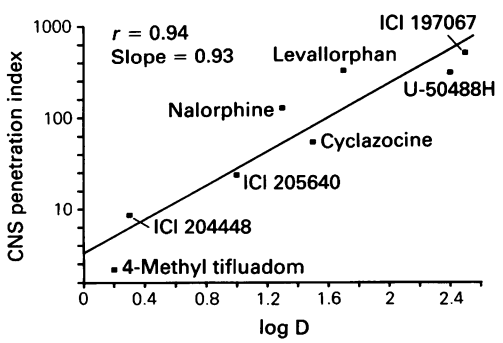

Figure 3 The correlation between CNS penetration and lipophilicity (log D) for a range of opioids administered subcutaneously to mice.

quaternary derivatives, and several quaternary opioid antagonists have been described in the literature. Whilst the quaternary antagonists all have lower affinities than their parent compounds, they have been shown in several studies to possess a high degree of peripheral selectivity. N-methyl nalorphine and both the N-allyl and N-methyl analogues of levallorphan have been shown to antagonize the inhibitory effect of morphine on gastrointestinal motility, whilst not affecting the analgesic action (Dragonetti *et al.*, 1983; Notarnicola *et al.*, 1983).

Attempts to restrict the CNS penetration of existing κ-agonists by preparing their quaternary derivatives proved unsuccessful. The compounds examined were almost completely devoid of activity. Attempts were therefore made to develop a non-quaternary hydrophilic κ-agonist. The resulting compounds – ICI 204448 and ICI 205640 – are close analogues of the potent κ-agonist ICI 199441 (Costello *et al.*, 1988), but derive their low lipophilicity from the addition of a carboxylic acid group. Of these, ICI 204448 was investigated in detail.

In isolated tissue studies ICI 204448 was found to possess agonist potency similar to, or greater than that of EKC in three preparations. The high potency of ICI 204448 in the rabbit vas deferens preparation; a tissue demonstrated to possess only the κ-type of opioid receptor (Oka *et al.*, 1980) strongly suggests that the agonist effect of this compound is κ-receptor mediated. Moreover, the Ke value for naloxone to antagonize ICI 204448 in the rabbit vas deferens was similar to that found for other κ-agonists in a variety of isolated tissue models (Hayes & Kelly, 1985; Miller *et al.*, 1986). However, the naloxone Ke values in both the mouse vas deferens and guinea-pig ileum were higher than those obtained using the κ-agonists EKC and U-50488H. This does not appear to be due to an action at the δ-receptor since in the mouse vas deferens preparation ICI 204448 was resistant to antagonism by the δ-antagonist ICI 174864. A non-

opioid action is unlikely since the dose-response curves to ICI 204448 were consistently shifted in a parallel manner by naloxone, and the Schild plot was apparently linear with a slope close to unity. These observations therefore remain unexplained.

Examination of the binding data revealed an apparent anomaly. Whereas in the isolated tissue assays ICI 204448 consistently had a potency comparable to EKC, and was considerably more potent than U-50488H, in binding studies the affinity of ICI 204448 was found to be lower than either of the standard agents. This discrepancy appears to be unrelated to the low lipophilicity of ICI 204448 since no such problem was observed with the quaternary analogue of tifluadom. Moreover, a similar discrepancy has been obtained with a considerably more lipophilic analogue – ICI 204879 (Costello *et al.*, 1988). One interpretation of this finding is that the agonist activity of ICI 204448 involves receptors either different from, or in addition to the κ-receptor. However, the possibility that ICI 204448 acts at the δ-site can be discounted since the selective δ-antagonist ICI 174864 (Cotton *et al.*, 1984) had no effect. An action at μ-receptors can also be ruled out since ICI 204448 was devoid of activity in a μ-receptor binding assay and in the field-stimulated the rat vas deferens preparation. Furthermore, the Ke of naloxone against μ-agonists is much lower (between 2 and 4 nM) than was observed with ICI 204448 (Miller *et al.*, 1986).

An alternative explanation is that results from the κ-receptor binding assay do not predict κ-agonist potency in isolated tissue models. For example, in the rabbit vas deferens U-50488H was approximately 70 times less active than EKC. This value agrees closely with the findings of other studies (Hayes & Kelly, 1985). In contrast, the affinity of U-50488H in the binding assay is only 8 times less than that of EKC. The high relative potency of U-50488H in binding assays is a consistent finding in the literature (Lahti *et al.*, 1985), with some workers demonstrating almost identical IC_{50} values for the two compounds (Frances *et al.*, 1985). It is therefore clear that the relative affinities of κ-agonists determined in standard binding assays differ markedly from potency measurements in isolated tissue preparations. Thus the problem is not unique to ICI 204448. The reason for these discrepancies is not entirely clear. However, binding assays are generally not performed under physiological conditions, and it is known that the affinities of opioids for other sites such as the μ-receptor can be markedly influenced by factors such as buffer composition (Frances *et al.*, 1985; Carroll *et al.*, 1988).

It is clear from the *ex vivo* binding studies that ICI 204448 was well absorbed following subcutaneous administration. However, in comparison to U-50488H, EKC and tifluadom, ICI 204448 displayed a substantially reduced ability to enter the CNS. This low CNS penetration appears to be a consequence of the hydrophilicity of the compound since an excellent correlation was observed between the CNS penetration and log D values of all the compounds tested. This supports the conclusion of an earlier study by Herz & Teschemacher (1971) who demonstrated a relationship between the partition coefficients of a range of μ-agonists and the ratio between their analgesic potencies following intravenous and intracerebroventricular administration.

In summary, ICI 204448 is a potent and selective agonist at the κ-opioid receptor. The compound is well absorbed following subcutaneous administration, but has a greatly reduced ability to enter the brain. ICI 204448 should therefore prove to be a valuable tool with which to investigate the function of κ-receptors outside the CNS.

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The distribution of γ-adrenoceptors and P_2 purinoceptors in mesenteric arteries and veins of the guinea-pig

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1 Membrane potential changes and contractions were recorded from mesenteric arteries and veins of the guinea-pig, during perivascular nerve stimulation or application of noradrenaline or adenosine triphosphate (ATP).

2 After α-adrenoceptor blockade, noradrenaline activated low affinity adrenoceptors (γ-adrenoceptors) causing depolarization and arterial contraction only in the presence of an inhibitor of catecholamine uptake.

3 Noradrenaline did not cause depolarization or contraction of the vein after α-adrenoceptor blockade even after catecholamine uptake was blocked.

4 Adenosine triphosphate caused depolarization and contraction of both arteries and veins. These responses were abolished by α-,β-,methylene adenosine triphosphate (Me-ATP).

5 Me-ATP abolished rapid excitatory junction potentials (e.j.ps) caused by perivascular nerve stimulation of arteries but had no effect on arterial responses mediated by γ-adrenoceptors.

6 In veins, perivascular nerve stimulation evoked slow e.j.ps which persisted in the presence of Me-ATP but were abolished after blockade of α-adrenoceptors.

7 The observations indicate that P_2 purinoceptors are present on both mesenteric artery and vein whilst γ-adrenoceptors are localized near the neuromuscular junction of the artery. However γ-adrenoceptors do not appear to be directly involved in the generation of arterial e.j.ps.

Introduction

In a number of arterial beds, a part of the response to sympathetic nerve stimulation persists in the presence of both α- and β-adrenoceptor antagonists. Examples are the neurally activated vasoconstrictions of rabbit mesenteric and saphenous arteries (Kugelgen & Starke, 1985; Burnstock & Warland, 1987), dog mesenteric arteries (Muramatsu, 1986) and the increase in peripheral resistance of rabbit hindlimbs (Hirst & Lew, 1987).

Sympathetic nerve stimulation initiates a rapid excitatory junction potential (e.j.p.) in most mammalian systemic arteries (Hirst, 1977; Holman & Surprenant, 1979; Surprenant, 1980; Kuriyama & Suzuki, 1981; Kuriyama & Suyama, 1983; Kreulen, 1986). If the e.j.p. is sufficiently large or if successive e.j.ps are summed together, the depolarization leads to the activation of voltage-dependent calcium channels and constriction follows (Hirst & Edwards, 1988). In all systemic arteries studied, e.j.ps have been found to be resistant to α-adrenoceptor blockade but are abolished or reduced in amplitude by drugs interfering with neuronal release (Holman & Surprenant, 1979; 1980; Hirst & Neild, 1980; Cheung, 1982; Fujiwara *et al.*, 1982; Hirst *et al.*, 1982; Suzuki & Kou, 1983).

Two hypotheses have been suggested to explain the non α-adrenoceptor-mediated e.j.ps in systemic arteries. One suggests that released noradrenaline activates specialized junctional adrenoceptors termed γ-adrenoceptors which are located close to sympathetic nerve terminals (Hirst & Neild, 1980; 1981; Hirst *et al.*, 1982; Luff *et al.*, 1987). The other suggests that adenosine triphosphate (ATP), or a closely related compound, co-released with noradrenaline from the nerve terminal acts on P_2 purinoceptors to produce the rapid e.j.p. (Sneddon & Burnstock, 1984; Kügelgen & Starke, 1985; Ishikawa, 1985). This hypothesis is supported by the finding that α-,β-,methylene ATP (Me-ATP), a stable analogue of ATP desensitizes P_2 purinoceptor effects

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of ATP and blocks e.j.ps (Sneddon & Burnstock 1984; Ishikawa, 1985).

In contrast to arteries, rapid e.j.ps are not initiated by sympathetic nerve stimulation in veins, perhaps with the exception of dog mesenteric and rat saphenous veins (Cheung, 1981; Suzuki, 1984). Rather, repetitive sympathetic nerve stimulation typically produces a depolarization which is slow in onset and very long lasting (Suzuki, 1981; Kreulen, 1986). These slow e.j.ps are associated with contraction. Both the slow e.j.p. and the contraction are abolished by α-adrenoceptor antagonists (Suzuki, 1981; Kreulen, 1986).

The experiments described in this paper were designed to examine the distribution of γ-adrenoceptors and purinoceptors on mesenteric arteries and veins. The nature of the response to sympathetic nerve stimulation was also determined. It was found that, whereas γ-adrenoceptors were restricted to mesenteric arteries, P_2 receptors were present on both arterial and venous tissues. γ-Adrenoceptor responses in mesenteric arteries persisted in the presence of Me-ATP; γ-adrenoceptors are therefore unlikely to be involved in the initiation of e.j.ps.

Methods

Experiments were carried out on mesenteric arteries and veins arising from the superior mesenteric artery or vein of guinea-pigs (male or female, 150–300 g). Animals were stunned and exsanguinated. Sections of small intestine with attached arcades of mesenteric arteries and veins were rapidly removed and placed in a dissecting dish containing physiological saline (composition mM: NaCl 120, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 2.0, NaH_2PO_4 0.1, $NaHCO_3$ 25 and glucose, 11; gassed with 95% O_2 and 5% CO_2).

Electrical recordings

Lengths (1 cm) of artery and adjacent vein were removed from the arcade and pinned side by side in a recording chamber (volume 0.2 ml). To obtain rapid changes in bath composition a rapid-action two-way valve (Polyplan. Glen Waverly, Vic.) was placed adjacent to the bath (see Hirst *et al.*, 1982). In some experiments a larger volume chamber (1 ml, see Hirst, 1977) was used to enable a stimulating electrode to be placed around the proximal ends of the artery and vein. Perivascular nerves were stimulated at supramaximal parameters (20–60 V, 0.2–0.5 ms pulse width). Preparations were superfused at a rate of 6 ml min^{-1} with gassed physiological saline warmed to 37°C.

Intracellular recordings were made with microelectrodes pulled from fibre-containing glass tubes (Clark electromedical, GC150F-15) with Brown-Flaming micropipette puller (model P.77, Sutter Instrument Co; San Francisco, California) and filled with 0.5 M KCl. Electrodes with resistances in the range 80–160 MΩ were used. Recordings were digitized and stored on disc for later analysis. Data acquisition and analysis were performed using programmes written under the DAOS software package (version 7.0, Laboratory Software Associates).

Mechanical recordings

Lengths of artery and vein of 5 mm were used for tension records. Vessels were attached to two perspex 'feet' by means of 50 μm tungsten wire (Goodfellow Metals, Cambridge) placed through the lumen. One foot was attached to a micromanipulator (Narashige model mm-3) whilst the other was attached to a Kistler-Morse deflection sensor (DSC-46.1003.01) for recording isometric changes in force. The voltage signal was amplified and filtered using a strain gauge conditioner (Analog devices model 2B31J) and the output was recorded on a pen recorder (Riken Denshi model SP-GC3). A resting tension of 500 mg was maintained on arteries and 200 mg on veins. Vessels were left to equilibrate for a period of 30 min before experiments were begun. Physiological saline, as used in the electrophysiological studies, was warmed to 35°C and flowed through a 5 ml bath at 7 ml min^{-1}.

Irreversible blockade of α-adrenoceptors was achieved by perfusing preparations with 10 μM benextramine (BNX) for 30 min followed by a 30 min wash period before recordings were made (Melchiorre *et al.*, 1978; Hirst & Lew, 1987).

Drugs used were: adenosine 5′ triphosphate (ATP), α-,β-,methylene adenosine 5′ triphosphate (Me-ATP), benextramine hydrochloride, noradrenaline bitartrate ((−)-arterenol) (all obtained from Sigma chemicals), bretylium toxylate (Serva), desmethylimipramine (free base) (Ciba Geigy).

Results

Responses to sympathetic nerve stimulation recorded from mesenteric arteries and veins

The resting membrane potential of mesenteric arterial smooth muscle cells was -66 ± 0.6 mV ($n = 87$). When arterial perivascular nerves were stimulated, e.j.ps were initiated. Single supramaximal stimuli evoked an e.j.p. with a peak amplitude of 3.9 ± 0.4 mV, a rise time of 47.8 ± 1.5 ms and a time constant of decay of 295 ± 35.9 ms ($n = 6$) (Figure 1a). E.j.ps were not associated with a visible contrac-

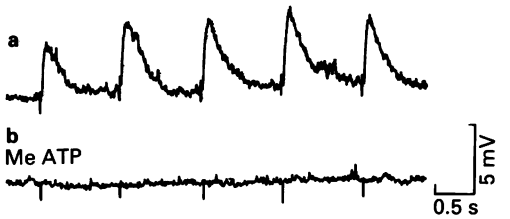

Figure 1 E.j.ps recorded from a mesenteric artery following sympathetic nerve stimulation. The record shown in (a) illustrates the response to successive supramaximal perivascular stimuli at a frequency of 1 Hz. The lower record (b) shows that the e.j.ps are abolished during prolonged superfusion with a solution containing 10 μM Me-ATP. Calibration bars apply to both records.

tion. When short trains of stimuli (20 Hz) were applied, e.j.ps summed together and triggered an action potential. This in turn initiated arterial constriction. The irreversible α_1/α_2-adrenoceptor antagonist benextramine (10 μM) did not alter the

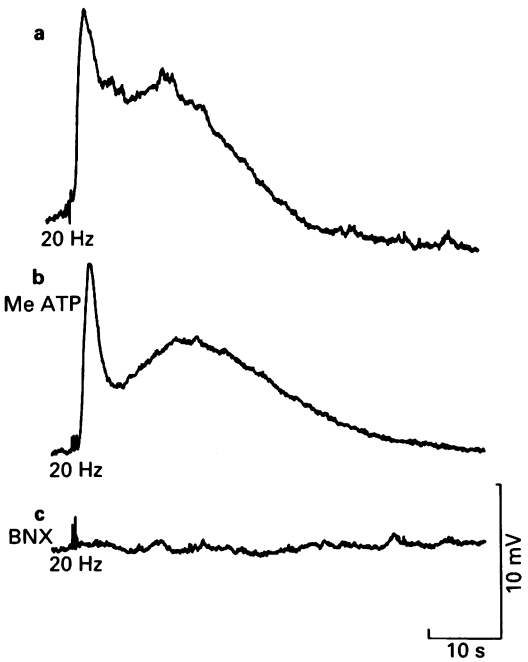

Figure 2 Depolarizations of mesenteric veins produced by stimulation of sympathetic nerves. In all three records, perivascular nerves were stimulated at a frequency of 20 Hz for 750 ms with supramaximal voltages. The record in (a) shows the effect of stimulation in control solution. The middle record (b) shows the depolarizations are little affected by prolonged incubation in 10 μM Me-ATP. The lower record (c) shows that the response to nerve stimulation is abolished after incubation in 10 μM benextramine (BNX).

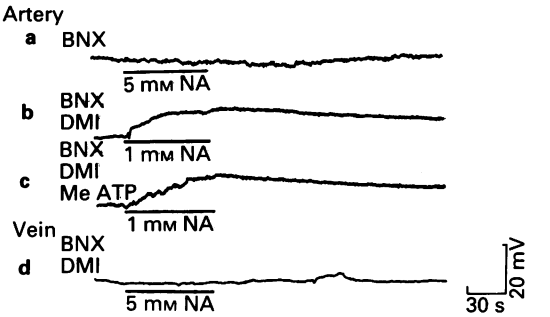

Figure 3 Noradrenaline (NA) responses in benextramine (BNX) treated mesenteric arteries and veins. Record (a) shows perfusion with NA after BNX treatment alone. The record in (b) shows that perfusion with the uptake inhibitor desmethylimipramine (DMI) reveals a depolarization in response to NA perfusion. Record (c) shows that incubation in Me-ATP has little effect on this depolarizing response to NA. Application of NA to veins in the presence of DMI (record d) produces little response. Perfusion times are shown by bars. Calibration bars apply to all records.

size of arterial e.j.ps (amplitude = 4.0 ± 0.2 mV, $n = 5$), whilst the adrenergic neurone blocker bretylium (10 μM) abolished e.j.ps after approximately 30 min.

Venous smooth muscle cells had a resting membrane potential of −70 ± 0.9 mV ($n = 23$). A single stimulus applied to the perivascular nerves of veins failed to initiate either a rapid e.j.p or any other detectable depolarization. Repetitive nerve stimulation (> 10 Hz) caused a slow e.j.p. which usually showed two components, an initial peak followed by a slower depolarization (Figure 2a). At fixed frequencies this slow e.j.p. increased in amplitude with increasing stimulus voltages until a maximum was reached. Further amplitude increases could be achieved by then increasing stimulus frequency. Stimulation at 20 Hz for 750 ms at supramaximal voltages initiated a slow e.j.p. in which the initial component was larger with an amplitude of 8.9 ± 1.9 mV ($n = 6$) (Figure 2a). The slow e.j.p. and contraction following venous nerve stimulation was abolished by 10 μM benextramine ($n = 4$) (Figure 2c).

Distribution of γ-adrenoceptors and P_2 receptors on mesenteric arteries and veins

Noradrenaline (1–10 μM), applied by superfusion, caused contraction of both arteries and veins. In both tissues these responses were prevented by incubating the tissues in benextramine. In either tissue, increasing the concentrations of noradrenaline up to 5 mM failed to produce a detectable depolarization but occasionally produced a small hyperpolarization (Figure 3a). After the addition of the neuronal amine

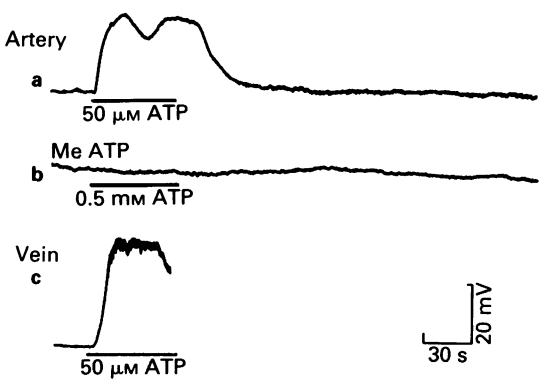

Figure 4 Effect of adenosine triphosphate (ATP) on the membrane potential of mesenteric arteries and veins. The upper record (a) shows a rapid depolarization in response to 50 μM ATP. The middle record (b) shows that the response to 0.5 mM ATP is abolished by incubation in 10 μM Me-ATP. The lower record (c) shows a rapid depolarization to 50 μM ATP in a vein. Bars indicate perfusion time. Calibration bars apply to all records.

uptake blocker desmethylimipramine (DMI) (1 μM), noradrenaline (>0.2 mM) caused arterial depolarization and contraction (Figure 3b). The amplitude of depolarization varied between preparations and electrodes were often dislodged due to contraction. The mean depolarization produced by noradrenaline (0.5 mM) was 12.6 ± 1.4 mV (n = 12).

Application of very high concentrations of noradrenaline to veins after benextramine treatment did not lead to a membrane potential change (n = 4). However, unlike arteries the addition of desmethylimipramine did not reveal an α-antagonist-resistant response (Figure 3d).

ATP (>0.1 mM) caused substantial membrane depolarization in both arteries and veins (Figure 4a,c) with 0.5 mM ATP causing 18 ± 4 mV (n = 5) depolarization in arteries and 23 ± 4 mV (n = 3) for veins. These depolarizations often caused action potentials and were associated with rapid contractions which usually dislodged the electrodes.

Mechanical responses

Contraction of arteries to exogenous noradrenaline had a threshold of 5 μM (Figure 5a). After benextramine treatment in the presence of desmethylimipramine, arteries proved unresponsive to even high concentrations (1 mM) of noradrenaline if the concentration was allowed to rise slowly. Rapid contractions were produced if the bath concentration of noradrenaline was rapidly increased to its final concentration (0.5–2 mM) by microinjection into the bath (Figure 5a) (cf. Byrne & Large, 1986). Contractions produced by 1 mM noradrenaline were 1099 ± 252 mg, (n = 6). ATP (1 mM) also produced a rapid contraction although never as large as that to noradrenaline (678 ± 135 mg, n = 5) (Figure 5a).

Veins contracted with much less force than arteries. The maximal contraction caused by increasing the potassium concentration to 70 mM was 254 ± 26 mg, n = 8 (Figure 5b). Noradrenaline (0.1 mM) caused a contraction of 127 ± 18 mg (n = 7) (Figure 5b). This contraction was abolished by incubation in 10 μM benextramine. After incubation in benextramine and in the presence of DMI, noradrenaline in concentrations of up to 2 mM added by microinjection, failed to produce a detectable contraction (Figure 5b). ATP (1 mM) caused a contraction in all venous preparations, the mean peak tension (247 ± 41 mg, n = 8) was similar to that produced during a maximal potassium contraction (Figure 5b).

Effects of Me-ATP

Perfusing the preparation with 10 μM Me-ATP caused a rapid depolarization of arteries and veins. After 30 min the vessels had repolarized to control values. Measurements of tension showed that Me-ATP 10 and 100 μM produced a rapid transient contraction which soon returned to a stable resting tension (Figure 5a). In the presence of Me-ATP, sympathetic nerve stimulation now failed to produce an e.j.p. in the artery. However, the slow e.j.p. detected in veins after sympathetic nerve stimulation was little affected (mean peak amplitude 7.6 ± 2.9 mV, n = 5) (Figure 2b).

The benextramine-resistant depolarization of arteries, (γ-adrenoceptor response), was unaffected by the presence of 10 μM Me-ATP, whilst the response to ATP (0.5 mM) was abolished (n = 4) (Figure 4b).

Tension measurements in arteries showed that the benextramine-resistant peak contraction to noradrenaline was unchanged (1393 ± 99 mg, n = 3) whilst the response to ATP was greatly attenuated 31.75 ± 31 mg (n = 3) (Figure 5).

Discussion

This study compared the membrane potential changes that occur in mesenteric arteries and veins during sympathetic transmission. The distributions of P_2 purinoceptor and γ-adrenoceptor types on these vessels were also determined. As has been shown previously, rapid e.j.ps were initiated in mesenteric arteries (Kuriyama & Suzuki, 1981; Suzuki, 1981; Ishikawa, 1985; Kreulen, 1986). Rapid e.j.ps persisted after treatment with the irreversible α-adrenoceptor antagonist, benextramine, but were abolished by bretylium, which prevents the release of

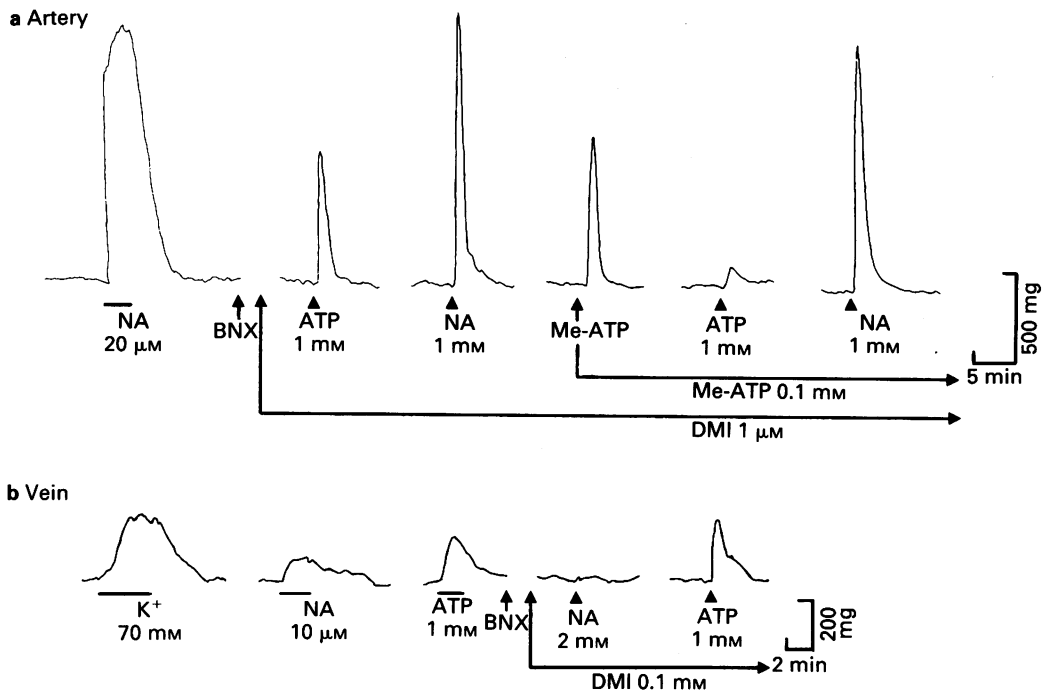

Figure 5 The effect of noradrenaline (NA) and ATP on tension in mesenteric arteries and veins. The upper trace (a) is of an artery showing that the contraction produced by NA after benextramine (BNX) treatment requires a relatively high NA concentration and is little affected by incubation in 0.1 mM Me-ATP, whilst the response to ATP is almost abolished. The preparation was continuously perfused with 1 μM desmethylimipramine (DMI) following BNX treatment. Continuous perfusion with Me-ATP caused an initial contraction which relaxed to resting levels. The lower trace (b) is of a vein showing the presence of α-adrenoceptors and P_2 purinoceptors but an absence of response to NA after BNX treatment. The preparation was continuously perfused with DMI following BNX treatment. Bars indicate perfusion times. Arrow heads (▲) indicate microinjection into the organ bath. The arrows below the trace indicate continuous perfusion of Me-ATP or DMI.

noradrenaline from sympathetic nerves (Bowman & Rand, 1980). In contrast, rapid e.j.ps were not recorded from mesenteric veins following sympathetic nerve stimulation, rather repetitive nerve stimulation initiated a slow long lasting membrane depolarization. This slow e.j.p. was associated with constriction and was prevented by benextramine. Similar observations have been made from a number of venous preparations (Suzuki, 1981; Kreulen, 1986; van Helden, 1988).

Both arteries and veins contracted when low concentrations of noradrenaline were applied. These responses were abolished by benextramine treatment. Neither arterial nor venous preparations responded to high concentrations of noradrenaline after benextramine treatment. However when the tissues were further incubated with DMI, an inhibitor of neuronal uptake, the arteries alone responded to noradrenaline. At these concentrations of noradrenaline, $Uptake_1$ should be saturated (Lightman & Iversen, 1969) and inhibition of uptake would be expected to have no effect on the concentration of noradrenaline reaching the receptor. The simplest explanation of this is that the γ-adrenoceptors activated by exogenous noradrenaline lie next to an uptake site which is not saturated. This could occur if γ-adrenoceptors are localised near the sympathetic neuromuscular junction and diffusional access to noradrenaline is limited by a narrow junctional gap. This suggestion is in accord with the observation that γ-adrenoceptors are restricted to regions of the artery near sympathetic varicosities (Hirst & Neild, 1981; Luff, 1987).

The lack of response of veins to noradrenaline after α-adrenoceptor blockade even in the presence of DMI suggests that they are devoid of γ-adrenoceptors. Tension studies on a variety of rabbit blood vessels found that non-α-adrenoceptors were restricted to systemic arteries but not veins (Laher *et al.*, 1986). The distribution of γ-adrenoceptors, present in arteries but not veins, is therefore correlated with the presence of α-antagonist resistant

neuronal responses. A similar finding has been made in rat middle cerebral arteries where γ-adrenoceptors were found in proximal innervated arterioles but not in the distal arterioles which never receive an innervation (Edwards *et al.*, 1989).

Both arteries and veins responded to ATP. Therefore the distribution of P_2 purinoceptors shows no correlation with the ability of sympathetic nerves to initiate an e.j.p. If sympathetic nerves did indeed release appreciable amounts of ATP, then non-α-adrenoceptor neuronal responses should be detected in veins unless the junctional cleft width is large or innervation is sparse. Where measured, the junctional cleft width in veins has been shown to be similar to that of arteries and often narrow (Rowan & Bevan, 1983). Mesenteric veins of the guinea-pig have been shown to be well innervated and although they appear less dense in wholemounts than arteries (Furness, 1971), the medial layer is much thinner and nerve density may be equivalent. Given a similar neuromuscular junction to the artery, either the sympathetic nerves which innervate veins must fail to release ATP, or P_2 purinoceptors may be located away from points of transmitter release. P_2 purinoceptors are relatively low affinity receptors and may only be activated in a junctional position where the local concentration of transmitter is high.

The slow e.j.p. in veins was not seen until stimulation frequency was increased. Similarly, at the low stimulus parameters required to activate arterial e.j.ps, no responses attributable to α-adrenoceptors were found. However, relatively high frequency stimulation has been reported to activate α-adrenoceptors in these arteries (Hottenstein & Kreulen, 1987). Such a dependence on high frequencies could indicate an extrajunctional position for α-adrenoceptors.

In rat basilar arteries, Me-ATP has been shown to be an antagonist to both γ-adrenoceptors and P_2 purinoceptors (Byrne & Large, 1986). This clearly is not the case for mesenteric vessels. Me-ATP abolished the responses produced by ATP in both arteries and veins but did not abolish the responses produced by γ-adrenoceptor activation in mesenteric arteries. The different sensitivity of γ-adrenoceptors in the rat basilar artery (Byrne & Large, 1986) and those of the guinea-pig mesenteric artery to Me-ATP, suggests that more than one class of γ-adrenoceptors may exist. The proposal of more than one γ-adrenoceptor has been previously suggested for the rat basilar artery where a biphasic depolarization to exogenous noradrenaline exists, neither component being due to α-adrenoceptor activation (Byrne *et al.*, 1986). The first component of this depolarization was easily desensitized and was only seen if noradrenaline was micro-injected into the bath. That is, if the bath concentration rose quickly (Byrne *et al.*, 1986). No biphasic γ-adrenoceptor response could be detected in this study even though the bath concentration would have risen quickly (see Methods).

Since Me-ATP abolished the rapid e.j.ps recorded from arteries, it seems unlikely that the activation of γ-adrenoceptors can underlie the initiation of an e.j.p. Thus arteries have a population of adrenoceptors that are located near sympathetic nerve terminals and are only activated by high concentrations of noradrenaline. It has already been pointed out that arteries generate muscle action potentials during neuromuscular transmission. Since the ability of arterioles to support inward calcium movement is related to the density of sympathetic nerves (Hill *et al.*, 1986), it could be that γ-adrenoceptors are closely linked to voltage-dependent calcium channels. This suggestion has been previously made by Benham & Tsien (1987), who found that calcium entry into single isolated arterial cells was increased in the presence of noradrenaline, the receptor involved was neither an α- nor a β-adrenoceptor.

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α_1-Adrenoceptor antagonist activity of novel pyrimidine derivatives (SHI437 and IK29) in rabbit aorta and trigone of the bladder

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1 In the rabbit isolated aorta and trigone of the bladder, noradrenaline, phenylephrine and clonidine elicited concentration-dependent contractions, which may be caused through activation of postsynaptic α_1-adrenoceptors.

2 SHI437, IK29, prazosin and yohimbine competitively antagonized the contractile responses induced by noradrenaline in the aorta and trigone. The pA_2 values of SHI437, IK29, prazosin and yohimbine were 7.35 ± 0.09, 7.47 ± 0.10, 8.55 ± 0.02 and 6.28 ± 0.05 in the aorta, and 8.07 ± 0.04, 8.30 ± 0.03, 8.22 ± 0.04 and 6.46 ± 0.04 in the trigone, respectively.

3 SHI437, IK29, prazosin and yohimbine also possessed competitive α_2-adrenoceptor blocking properties, judging from their antagonism of the clonidine-induced inhibitory effect on the twitch responses in the electrically stimulated vas deferens of the rat. The pA_2 values of SHI437, IK29, prazosin and yohimbine were determined to be 4.76 ± 0.02, 4.74 ± 0.02, 5.06 ± 0.03 and 7.86 ± 0.04, respectively.

4 SHI437, IK29 and prazosin inhibited the contractile responses elicited by transmural electrical stimulation without affecting the evoked ^{3}H-overflow from the [^{3}H]-noradrenaline-preloaded rabbit aorta. Yohimbine augmented the contractile responses and ^{3}H-overflow.

5 SHI437 and IK29 at a concentration sufficient to inhibit noradrenaline-induced contraction failed to attenuate the contractile responses of aorta to KCl, 5-hydroxytryptamine and prostaglandin $F_{2\alpha}$, and of the trigone to acetylcholine and histamine.

6 The present results suggest that SHI437 and IK29 are highly selective α_1-adrenoceptor antagonists, especially in the trigone of the bladder.

Introduction

We have demonstrated that novel pyrimidine derivatives, SHI437 (2-[2-{4-(2-methoxyphenyl)- 1-piperazinyl} ethyl] amino-5-acetyl-4,6- dimethylpyrimidine 3HCl) and IK29 (2-[4-{4-(2-methoxyphenyl)-1-piperazinyl} butyl] amino-5-ethoxy-carbonyl-4,6-dimethylpyrimidine 3HCl) (Figure 1) produce a hypotensive action, which is caused, at least in part, by the α_1-adrenoceptor antagonist activity of these compounds (Ito *et al.*, 1985). However, their precise pharmacological properties remain unknown. Thus, the present experiments were undertaken to investigate whether SHI437 and IK29 possess selective α_1-adrenoceptor antagonist activity in such smooth muscle preparations as rabbit aorta and trigone of the bladder.

α-Adrenoceptors have been differentiated into two sub-types, α_1 and α_2. According to Docherty & Starke (1981), α-adrenoceptors mediating contraction of the rabbit aorta are of the α_1 type, not of the

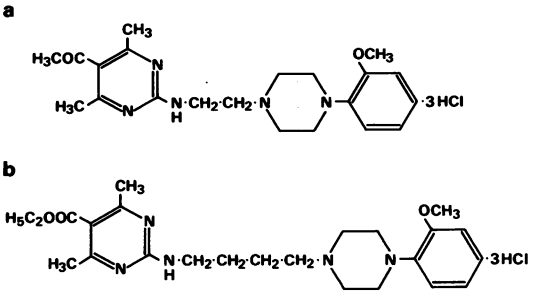

Figure 1 Chemical structures of SHI437(a) and IK29(b).

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α_2 type. In the urinary tract, postsynaptic α_1- and α_2-adrenoceptors are present in the male rabbit urinary bladder base and urethra, but α_1-adrenoceptors predominate in both tissues (Ueda *et al.*, 1984). It has also been shown that the postsynaptic α-adrenoceptors of the rabbit trigone, urethra and prostate mediating contraction belong to the α_1 subtype (Honda *et al.*, 1985). In the present studies, we first tried to characterize the α-adrenoceptors in smooth muscles of the male rabbit aorta and trigone of the bladder. The differentiation between α_1- and α_2-adrenoceptors can be made by comparing the responsiveness of the target tissues to appropriate α-agonists and antagonists. For this purpose, noradrenaline with a similar selectivity for the two adrenoceptor sites, phenylephrine which stimulates preferentially α_1-adrenoceptors, and clonidine which stimulates preferentially α_2-adrenoceptors were used as agonists. Then, antagonism of SHI437 and IK29 toward the contractile responses to noradrenaline were analysed and compared with those of prazosin and yohimbine in the aorta and trigone of the bladder.

We show here that SHI437 and IK29 are highly selective postsynaptic α_1-adrenoceptor antagonists, especially in the trigone.

Methods

Male albino rabbits weighing 2.2 to 2.5 kg were anaesthetized with sodium pentobarbitone (35 mg kg^{-1}, i.v.) and exsanguinated from the common carotid arteries. The thoracic aorta and urinary bladder were rapidly excised and placed in modified Krebs solution. After removal of fat and connective tissue, helical strips (3 × 25 mm) of the thoracic aorta, and longitudinal strips (3 × 15 mm) of the posterior wall of the trigone (urinary bladder base) were prepared. Helical strips were denuded of endothelium to avoid any complicating effects of endothelium-derived factors (Furchgott & Zawadzki, 1980; De Mey & Vanhoutte, 1982; Azuma *et al.*, 1986a). The preparations were mounted vertically in an organ bath containing 20 ml of modified Krebs solution continuously bubbled with 95% O_2 and 5% CO_2 at 37°C. One end of each strip was secured to the bottom of the organ bath and the other was attached to a force-displacement transducer (TB-611T, Nihon Kohden Kogyo Co.). Isometric changes in tension were recorded on a pen-writing oscillograph (Wi-681G, Nihon Kohden Kogyo Co.). The length of the strips was adjusted several times until a stable tension of 2 g for aorta and 1 g for trigone was attained. Before beginning the experiments, strips were allowed to equilibrate for at least 60 min in the bathing solution and during this period, the bathing solution was replaced every 20 min with fresh solution. The composition of modified Krebs solution (in mM) was as follows: NaCl 115.0, KCl 4.7, $MgSO_4 7H_2O$ 1.2, $CaCl_2 2H_2O$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 10.0. In all cases, the Krebs solution contained 10^{-6} M propranolol to block β-adrenoceptors. The concentration-contractile response curves to noradrenaline, phenylephrine and clonidine were compared in the aorta and trigone and were constructed by increasing the bath concentration of the agonists in a cumulative fashion. E_{max} (the maximum response) and ED_{50} (the concentration causing 50% of the E_{max}) values for the agonists were obtained from the log concentration-response curves. The relative potencies of the agonists were calculated as the ratio of the ED_{50} of noradrenaline to the ED_{50} of the agonists tested (Furchgott, 1972; Wikberg, 1978; 1979).

In order to assess the potency of α-adrenoceptor antagonists, cumulative concentration-response curves to noradrenaline were constructed before and after 20 min contact with SHI437, IK29, prazosin or yohimbine. Each antagonist was investigated at 3 different concentrations in the same tissue. The dose-ratio was obtained from the ratio of an ED_{50} value of noradrenaline in the presence and absence of an antagonist. The log (dose-ratio -1) was plotted against the log of the molar concentration of the antagonist (Schild plot), the regression line, pA_2 and slope of the curve were calculated (Arunlakshana & Schild, 1959).

Release of [^{3}H]-*noradrenaline*

Aortic strips were preincubated with [^{3}H]-noradrenaline (3 × 10^{-7} M) in modified Krebs solution containing ascorbic acid 5.7 × 10^{-4} M and corticosterone 10^{-5} M for 90 min at 37°C. Then the strips were mounted in a superfusion apparatus. The superfusion apparatus and the procedure were essentially the same as described by Su & Bevan (1970) and Azuma *et al.* (1986b). Briefly, the strip was mounted vertically between a stationary supporting Lucid rod and a force-displacement transducer, under an initial load of 2 g, and was constantly superfused with modified Krebs solution containing ascorbic acid 5.7 × 10^{-4} M using a peristaltic pump (Varioperpex II pump, LKB, Sweden) at a flow rate of 1 ml min^{-1}. The agents to be tested were diluted with oxygenated and warmed Krebs solution immediately before use. The strips were equilibrated for 90 min before starting the experiments. The superfusate was collected at 1 min intervals and the radioactivity determined by counting in a liquid scintillation counter (Packard 460-C). Six ml of Instagel (United Technologies Packard) was used as a scintillant. After the spontaneous ^{3}H-overflow had

Table 1 E_{max} and ED_{50} values of concentration-response curves for α-agonists in the aorta and trigone

		Aorta				Trigone		
Agonist	n[a]	E_{max} (g)[b]	ED_{50} ($\times 10^{-7}$ M)	*Ratio*[c]	n	E_{max} (g)	ED_{50} ($\times 10^{-6}$ M)	*Ratio*
Noradrenaline	14	4.58 ± 0.18	1.27 ± 0.19	1.00	9	5.03 ± 0.39	4.47 ± 0.38	1.00
Phenylephrine	10	4.14 ± 0.34	1.90 ± 0.38	0.67	8	4.97 ± 0.41	8.91 ± 1.90	0.50
Clonidine	8	3.15 ± 0.22**	10.60 ± 0.55**	0.12	8	1.07 ± 0.25**	28.20 ± 2.52**	0.16

[a] Number of experiments; [b] maximum developed tension; [c] ratio was calculated as the relative sensitivity for agonists. ** Significant difference between noradrenaline and a particular agonist at $P < 0.005$. Results are given as means ± s.e.mean.

decreased exponentially and reached a plateau at 60 to 90 min, the change in ^{3}H-overflow with transmural electrical stimulation (0.3 ms duration and supramaximum voltage at a frequency of 10 Hz for 10 s) was determined. In these experiments, although a part of [^{3}H]-noradrenaline taken up by the vascular tissue is metabolized, it has been found that the total ^{3}H-overflow correlates well with the release of [^{3}H]-noradrenaline from vascular tissue (Pinto & Trifaro, 1976; Vanhoutte *et al.*, 1981; Karaki *et al.*, 1984). We did not analyse the composition of the ^{3}H-effluent in the present experiments.

Experiments with rat vas deferens

The proximal portion of the rat vas deferens was suspended vertically under a loading tension of 1 g in an organ bath containing modified Krebs solution continuously bubbled with 95% O_2 and 5% CO_2 at 37°C. α_2-Adrenoceptor antagonist activity was evaluated by determining the pA_2 value against the inhibitory effect of clonidine on the contractile response to electrical transmural stimulation (0.3 ms duration, supramaximum voltage at a frequency of 0.1 Hz). Propranolol at a concentration of 10^{-6} M was present in the bathing solution.

Chemicals

SHI437 (2-[2-{4-(2-methoxyphenyl)-1- piperazinyl} ethyl]amino-5-acetyl-4,6-dimethylpyrimidine 3HCl) and IK29 (2-[4-{4-(2-methoxyphenyl)-1-piperazinyl}butyl]amino - 5 - ethoxycarbonyl - 4,6 - di - methylpyrimidine 3HCl) (Figure 1) were synthesized in our laboratory (Ito *et al.*, 1985). The following chemicals were used in these experiments: (±)-[17-^{3}H(N)]-noradrenaline hydrochloride (specific activity 11.8 mCi mmol^{-1}, New England Nuclear), (−)-noradrenaline bitartrate, tetrodotoxin (TTX), corticosterone acetate, histamine dihydrochloride, 5-hydroxytryptamine creatinine sulphate (5-HT), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (all from Sigma), yohimbine hydrochloride (Nakarai), phenylephrine hydrochloride (Kowa) and acetylcholine chloride (ACh, Daiichi). Prazosin hydrochloride and clonidine hydrochloride were generous gifts from Dr I. Ohtsuki, Taito Pfizer Co. and Dr H. Kohei, Japan Boehringer Ingelheim Co., respectively.

Statistical analysis

All data are expressed as the mean ± s.e.mean or the mean with 95% confidence limits. Statistical difference between the two means was determined by Student's *t* test. Regression lines were calculated by the least squares method.

Results

Contractile responses of aorta and trigone to noradrenaline, phenylephrine and clonidine

Noradrenaline, phenylephrine and clonidine produced concentration-dependent contractions in the rabbit thoracic aorta and trigone of the bladder. The E_{max} and ED_{50} values for noradrenaline in both preparations were not significantly different from those values for phenylephrine. However, ED_{50} values for noradrenaline were significantly ($P < 0.005$) smaller and E_{max} values for noradrenaline were significantly ($P < 0.005$) greater than those for clonidine in the aorta and trigone, indicating that clonidine acts as a partial agonist, whereas phenylephrine is a full agonist (Table 1).

Postsynaptic α_1-adrenoceptor antagonist activity

The effects of SHI437, IK29, prazosin and yohimbine on the concentration-response curve for noradrenaline were studied in the rabbit thoracic aorta and trigone of the bladder. In the aorta and trigone, SHI437 (3×10^{-8}, 10^{-7} and 3×10^{-7} M), IK29 (3×10^{-8}, 10^{-7} and 3×10^{-7} M), prazosin

Table 2 pA_2 values and slopes of Schild plot of SHI437, IK29 and other α-antagonists for antagonism of noradrenaline in rabbit thoracic aorta and trigone of the bladder, and of clonidine in rat vas deferens

	Postsynaptic α_1-adrenoceptors[a]					*Presynaptic α_2-adrenoceptors*[b]				
	Rabbit thoracic aorta			*Rabbit trigone*			*Rat vas deferens*		*Ratio*	
Test agent	n	pA_2	*Slope*	n	pA_2	*Slope*	n	pA_2	*Slope*	$\alpha_1(Th)/\alpha_2$[c]	$\alpha_1(Tr)/\alpha_2$[d]
SHI437	8	7.35 ±0.09	1.08 (0.85–1.31)	10	8.07** ±0.04	1.03 (0.96–1.10)	8	4.76** ±0.02	0.85 (0.71–0.99)	389	2042
IK29	8	7.47 ±0.10	1.16 (1.00–1.32)	7	8.30** ±0.03	1.05 (0.94–1.16)	7	4.74** ±0.02	0.79 (0.69–0.98)	537	3631
Prazosin	9	8.55 ±0.02	1.10 (0.96–1.24)	8	8.22** ±0.04	1.09 (0.88–1.30)	9	5.06** ±0.03	0.90 (0.81–0.99)	3162	1445
Yohimbine	8	6.28 ±0.05	0.97 (0.91–1.03)	8	6.46* ±0.04	0.73 (0.46–1.00)	8	7.86** ±0.04	0.97 (0.84–1.10)	0.026	0.040

[a] Antagonism of noradrenaline-induced contractions in the rabbit thoracic aorta and trigone of the bladder.
[b] Antagonism of the twitch inhibitory effect of clonidine in the neuronally stimulated rat vas deferens.
[c] Antilogarithm of the difference between pA_2 values in the rabbit aorta and rat vas deferens.
[d] Antilogarithm of the difference between pA_2 values in the rabbit trigone and rat vas deferens. Results are given as means ± s.e.mean or the mean with 95% confidence limits in parentheses. *n*: number of experiments * and **: Significant difference at $P < 0.01$ and $P < 0.005$ vs corresponding pA_2 values in the rabbit aorta, respectively.

(3×10^{-8}, 10^{-7} and 3×10^{-7} M) and yohimbine (10^{-6}, 3×10^{-6} and 10^{-5} M) produced parallel shifts to the right of the concentration-response curve for noradrenaline without causing a decrease in the maximum response. The pA_2 values and the slope of Schild plot for all antagonists tested in two preparations are summarized in Table 2. The slopes of the Schild plot for all antagonists were not different from unity. These data indicate that SHI437, IK29, prazosin and yohimbine competitively antagonized the contractile response induced by noradrenaline in the aorta and trigone. The postsynaptic α_1-adrenoceptor antagonist activity of SHI437, IK29 and yohimbine was significantly ($P < 0.01$ or $P < 0.005$) more potent in the trigone (pA_2 values were 8.07 ± 0.04, 8.30 ± 0.03 and 6.46 ± 0.04, respectively) than in the aorta (pA_2 values were 7.35 ± 0.09, 7.47 ± 0.10 and 6.28 ± 0.05, respectively), whereas prazosin was significantly ($P < 0.005$) more potent in the aorta (pA_2 was 8.55 ± 0.02 vs 8.22 ± 0.04 in the trigone).

Presynaptic α_2-adrenoceptor antagonist activity

The effects of SHI437, IK29, prazosin and yohimbine on presynaptic α_2-adrenoceptors were studied in the neuronally stimulated rat vas deferens using clonidine as an agonist. The concentration-response curve for clonidine was shifted to the right in a parallel and concentration-dependent manner in the presence of SHI437 (3×10^{-6}, 10^{-5} and 3×10^{-5} M), IK29 (3×10^{-6}, 10^{-5} and 3×10^{-5} M), prazosin (3×10^{-6}, 10^{-5} and 3×10^{-5} M) and yohimbine (3×10^{-8}, 10^{-7} and 3×10^{-7} M). The slopes of the Schild plots for these four antagonists were not different from unity (Table 2). None of the antagonists tested affected baseline tone of the rat vas deferens.

From the results obtained in the rabbit thoracic aorta and trigone, and the rat vas deferens, it was possible to compare the selectivity of individual antagonists for postsynaptic α_1-adrenoceptors. For this purpose, the antilogarithm of the difference between the pA_2 values obtained in the aorta or trigone (postsynaptic α_1-adrenoceptors) and the vas deferens (presynaptic α_2-adrenoceptors) was calculated. Although SHI437, IK29 and prazosin were more selective for the postsynaptic α_1-adrenoceptors than for the presynaptic α_2-adrenoceptors, the selectivity for the α_1-adrenoceptors of these three antagonists was different in the different tissues. That is, both of SHI437 and IK29 were more selective for the postsynaptic α_1-adrenoceptors in the trigone of the bladder (2042 and 3631 times, respectively) than in the aorta (389 and 537 times, respectively), whereas prazosin showed more selective antagonist activity in the aorta (3162 times vs 1445 times in the trigone). On the other hand, yohimbine was approximately 25 to 40 times more selective for presynaptic α_2-adrenoceptors (Table 2).

Effects on the transmural responses in the rabbit thoracic aorta

Transmural electrical stimulation produced a transient contraction and increase in ^{3}H-overflow in the

[^{3}H]-noradrenaline preloaded aorta. Tetrodotoxin (3.1×10^{-7} M) and guanethidine (10^{-5} M) abolished these responses, which were also attenuated by clonidine (3×10^{-8} M). This inhibitory effect of clonidine was blocked by yohimbine (10^{-7} M) (data not shown). SHI437, IK29 and prazosin at concentrations tested had no significant effects on the basal and evoked ^{3}H-overflow, but inhibited the contractile responses elicited by transmural electrical stimulation in a concentration-dependent manner. Yohimbine (3×10^{-8} and 10^{-7} M) augmented both the contractile response and ^{3}H-overflow evoked by transmural stimulation. However, 3×10^{-7} M yohimbine augmented ^{3}H-overflow but did not augment the contractile response (Figure 2). This may reflect postsynaptic α_1-adrenoceptor blockade by this high concentration of the antagonist.

Effects on the contractile response of aorta and trigone to KCl, 5-HT, $PGF_{2\alpha}$, ACh and histamine

SHI437 and IK29, at a concentration (3×10^{-7} M) sufficient to inhibit the noradrenaline-induced contraction in the aorta and trigone, failed to attenuate the contractile response of aorta to KCl, 5-HT and $PGF_{2\alpha}$, and of trigone to ACh and histamine (Table 3).

Discussion

Clonidine acts not only as an α_2-agonist at presynaptic sites with ED_{50} values between 3×10^{-9} and 3×10^{-8} M in the rat isolated vas deferens and guinea-pig ileum, but also as a partial agonist at postsynaptic sites with ED_{50} values between 10^{-6} and 10^{-5} M in the rabbit isolated aorta and rat vas deferens (Wikberg, 1978; 1979; Ruffolo *et al.*, 1981). The ED_{50} values for clonidine obtained in the rabbit aorta and trigone in the present experiments were similar to those found for postsynaptic α_1-adrenoceptor sites, but greater than those obtained for presynaptic α_2-adrenoceptor sites, suggesting that clonidine activates mainly postsynaptic α_1-adrenoceptors of the aorta and trigone. Moreover, the pA_2 values obtained for the inhibitory effect of prazosin and yohimbine against contractile responses to noradrenaline are consistent with an interaction at α_1-adrenoceptors. These results indicate that the contractile responses of these two preparations to noradrenaline, phenylephrine and clonidine can be elicited by activation of postsynaptic α_1-adrenoceptors. This speculation is supported by the demonstration that the α-adrenoceptor subtype mediating contraction of the smooth muscle of the aorta and trigone is of the α_1 type, but not α_2

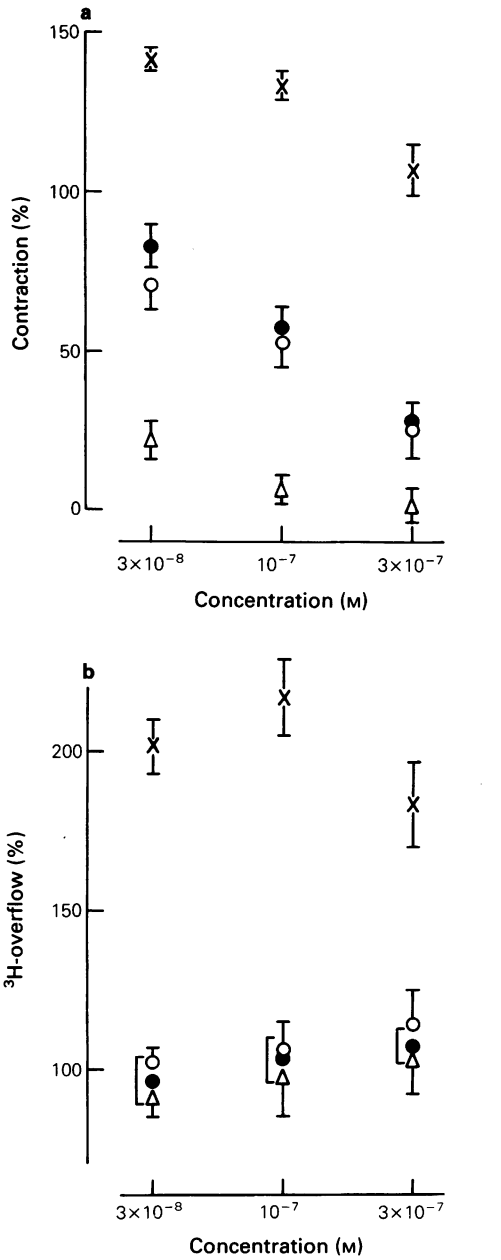

Figure 2 Effects of SHI427 (○), IK29 (●), prazosin (△) and yohimbine (×) on the contractile responses (a) and ^{3}H-overflow (b) induced by transmural electrical stimulation (10 Hz for 10 s) in the [^{3}H]-noradrenaline preloaded rabbit aorta. The contractile response and ^{3}H-overflow induced by electrical stimulation before treatment with agents were taken as 100%, respectively. Each value is the mean, and vertical lines show s.e.mean, of 4 experiments.

Table 3 Effects of SHI437 and IK29 on the contractile responses of rabbit thoracic aorta (a) and trigone (b) of the bladder to KCl, 5-hydroxytryptamine (5-HT), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), acetylcholine (ACh) and histamine

a Aorta

			KCl			*5-HT*			$PGF_{2\alpha}$	
Agent	*Concentration* (M)	n	E_{max} (g)	ED_{50} ($\times 10^{-2}$ M)	n	E_{max} (g)	ED_{50} ($\times 10^{-7}$ M)	n	E_{max} (g)	ED_{50} ($\times 10^{-6}$ M)
SHI437	—	5	4.55 ± 0.38	2.51 ± 0.32	6	3.45 ± 0.29	3.80 ± 0.15	7	3.65 ± 0.23	1.29 ± 0.22
	3×10^{-7}	5	4.62 ± 0.34	2.57 ± 0.26	6	3.39 ± 0.31	4.27 ± 0.53	7	3.63 ± 0.41	1.31 ± 0.15
IK29	—	5	4.32 ± 0.26	2.69 ± 0.31	7	3.60 ± 0.18	3.89 ± 0.42	6	3.74 ± 0.48	1.00 ± 0.18
	3×10^{-7}	5	4.34 ± 0.25	2.57 ± 0.31	7	3.43 ± 0.20	4.47 ± 0.66	6	3.77 ± 0.55	1.07 ± 0.22

b Trigone

			ACh			*Histamine*		
Agent	*Concentration* (M)	n	E_{max} (g)	ED_{50} ($\times 10^{-5}$ M)	n	E_{max} (g)	ED_{50} ($\times 10^{-5}$ M)	
SHI437	—	5	6.18 ± 0.62	3.71 ± 0.35	6	3.16 ± 0.40	2.51 ± 0.31	
	3×10^{-7}	5	6.21 ± 0.71	4.07 ± 0.65	6	3.14 ± 0.51	2.63 ± 0.25	
IK29	—	5	6.24 ± 0.65	4.26 ± 0.51	6	2.97 ± 0.63	2.00 ± 0.35	
	3×10^{-7}	5	6.22 ± 0.59	3.89 ± 0.44	6	3.11 ± 0.55	2.14 ± 0.43	

Results are given as means ± s.e.mean. *n*: number of experiments.

type in male rabbit (Docherty & Starke, 1981; Honda *et al.*, 1985).

In the rabbit isolated aorta and trigone, SHI437, IK29 and prazosin antagonized the contractile responses to noradrenaline under conditions where β-adrenoceptors were inhibited. The nature of the antagonism toward noradrenaline by these chemicals was competitive in character, since the slope of the Schild plot was close to the theoretical value of unity. SHI437, IK29 and prazosin also possessed competitive presynaptic α_2-adrenoceptor blocking properties, judging from their antagonism of the clonidine-induced inhibitory effect on the twitch responses in the electrically stimulated rat vas deferens. Although SHI437, IK29 and prazosin were competitive antagonists at both subtypes of α-adrenoceptor, these compounds displayed a much higher affinity (380 to 3600 times) for postsynaptic α_1-adrenoceptors than for presynaptic α_2-adrenoceptors. From Pa_2 values obtained in the aorta or trigone, and rat vas deferens, the α_1/α_2-adrenoceptor selectivity ratios were calculated. It was found that SHI437 and IK29 had a higher affinity for α_1-adrenoceptors in the trigone than in the aorta, whereas prazosin was more effective in the aorta. The mechanisms responsible for the different potencies of SHI437, IK29 and prazosin as α_1-adrenoceptor antagonists between the aorta and the trigone are not clear from the present experiments, but the following factors should be considered: (1) different affinities of the antagonists, (2) different conformations of the α_1-adrenoceptors and (3) different uptake capacities of the two tissues.

The contractile response and ^{3}H-overflow evoked by transmural stimulation in the [^{3}H]-noradrenaline preloaded aorta were abolished or attenuated by tetrodotoxin, guanethidine or clonidine; the effect of clonidine was blocked by pretreatment with yohimbine. In addition, at concentrations selective for α_1-adrenoceptor antagonism, prazosin strongly inhibited the stimulation-evoked contraction with almost no effect on the stimulation-evoked ^{3}H-overflow. Thus, it is suggested that rabbit thoracic aorta contains presynaptic α_2-adrenoceptors at the nerve terminals which mediate inhibition of transmitter release evoked by nerve stimulation (Starke, 1981), and α_1-adrenoceptors at postsynaptic sites which mediate contraction (Docherty & Starke, 1981). SHI437 and IK29 showed similar effects to prazosin on the stimulation-evoked contraction and ^{3}H-overflow, whereas the selective α_2-adrenoceptor antagonist, yohimbine, augmented the stimulation-evoked contraction as well as ^{3}H-overflow. On the other hand, SHI437 and IK29 at concentrations sufficient to inhibit noradrenaline-induced contraction had little or no effect on the contractile responses to KCl, 5-HT and $PGF_{2\alpha}$ in the aorta, and ACh and

histamine in the trigone. All these results suggest that the antagonistic action of SHI437 and IK29 is highly selective for postsynaptic α_1-adrenoceptors.

In conclusion, the present *in vitro* results suggest that the novel pyrimidine derivatives, SHI437 and IK29, are highly selective α_1-adrenoceptor antagonists, especially in the trigone of the bladder compared to the aorta, and indicate that SHI437 and IK29 may be useful pharmacological tools with which to investigate and characterize α-adrenoceptors.

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Papers will be considered for publication on all aspects of pharmacology, including chemotherapy.

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MEETINGS CALENDAR

3–4 April 1989

British Neuroendocrine Group Meeting, Bristol, U.K. (Dr. C.D. Ingram, Department of Anatomy, The Medical School, University Walk, Bristol BS8 1TD).

5–7 April 1989

British Pharmacological Society Spring Meeting, Bristol, U.K. (Closed meeting for members and guests)

6–7 April 1989

International Symposium on Psychiatric Neuropathology and Neurochemistry, London, U.K. (Dr. R.W. Kerwin, Department of Neuropathology, Institute of Psychiatry, De Crespigny Park, London SE5).

7 April 1989

British Pharmacological Society. Symposium on the NMDA Receptor, Bristol, U.K. (Dr. G.L. Collingridge, Department of Pharmacology, The Medical School, University of Bristol, Bristol, BS8 1TD, U.K.)

21–22 April 1989

Satellite Symposium of Int. Soc. for Neurochemistry Congress, Adenosine Receptors, Agarne, Portugal (Professor J.A. Ribeiro, Laboratory of Pharmacology, Gulbenkian Institute of Science, 2781 Oeiras, Portugal).

30 April–5 May 1989

Fifth Int. Symposium on the Adrenal Chromaffin Cell as a Neuroendocrine model; from Basic to Clinical Aspects, Jerusalem, Israel (Dr. K. Rosenheck, Dept. of Membrane Research, Weizmann Institute of Science, Rehovot 76100, Israel).

8–12 May 1989

3rd International Conference on PAF and Structurally related Alkyl ether Lipids, Tokyo, Japan. (Professor S. Nojima, Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Tsukin, Kanagawa 199, Japan).

9–12 May 1989

International group for Tryptophan Research. 6th International Meeting, Baltimore, USA. (Dr R. Schwarcz, Maryland Psychiatric Research Center, PO Box 21247, Baltimore, MD 21228, U.S.A.)

21–23 May 1989

Current and Future Trends in Anticonvulsant, Anxiety and Stroke Therapy, Princeton, USA. (Dr J. Emmett, Dialogue in Science, Packhorse Place, Kensworth, Beds LU6 3QU, U.K.)

22–26 May 1989

Second Meeting of the International Neurotoxicology Association, Barcelona, Spain. (Dr. E. Rodriquez Farre, Dept. of Pharmacology & Toxicology, CSIC, Jorge Girona Salgado, 18–26, E-08034, Spain).

25–27 May 1989

4th International Symposium on Calcium Antagonists, Florence, Italy. (Secretariat, Fondazione Giovanni Lorenzini, Via Monte Napoleone 23, 20121 Milan, Italy).

1–2 June 1989

International Workshop on Sensory Neuropeptides, Florence, Italy. (Miss A. Giannini, Istituto Farmacobiologico Malesci, S.p.A., Dept. Pharmacology, Via Porpora 22, 50144 Florence, Italy).

1–3 June 1989

International Symposium on Kidney, Proteins & Drugs, Montecatani Terme, Italy. (Dr. Claudio Bianchi, Centro Nefrologico, Clinica Medica Generale 2, University of Pisa, 56100 Pisa, Italy).

28 June–2 July 1989

Ethnicity & Cardiovascular Diseases Conference, Nairobi, Kenya. (Ms. C. Cate, Int. Society on Hypertension in Blacks, 69 Butler Street, S. E., Atlanta, GA 30303, U.S.A.).

12–14 July 1989

British Pharmacological Society Summer Meeting. Leeds, U.K. (Closed meeting for members and guests)

16–21 July 1989

Vth International Congress of Toxicology, Brighton, U.K. (IUTOX '89, Congress House, 65 West Drive, Sutton, Surrey SH2 7NB, U.K.)

23–25 July 1989

New Strategies in Pain Control, Princeton, USA. (Dr J. Emmett, Dialogue in Science, Packhorse Place, Kensworth, Beds, LU6 3QU, U.K.)

7–12 August 1989

Therapy with Amino Acids and Analogues, Ist International Congress, Vienna, Austria. (G. Lubec, University of Vienna, Dept. of Paediatrics, A-1090 Vienna, Währinger Gürtel 18, Austria).

1–3 September 1989

Second International FIP Symposium on Disposition & Delivery of Peptide Drugs, Leiden, The Netherlands. (Dr. J. Verhoef, Centre for Bio-Pharmaceutical Sciences, PO Box 9502, 2300 RA Leiden, The Netherlands).

13–15 September 1989

British Pharmacological Society Autumn Meeting. Manchester, U.K. (Closed meeting for members and guests)

17–20 September 1989

1st International $GABA_B$ Symposium, Cambridge, U.K. (Professor N.G. Bowery, Department of Pharmacology, School of Pharmacy, 29/39 Brunswick Square, London WC1N 1AX.)

19–21 September 1989

Electrochemical Detection, HPLC and *in vitro* monitoring in the Biosciences, Nottingham, U.K. (Dr I.A. MacDonald, Department of Physiology & Pharmacology, Medical School, Queen's Medical Centre, Nottingham, U.K.)

20–21 September 1989

9th Annual Meeting of the Bayliss and Starling Society. Peptides: Physiology, Pharmacology and Therapeutics, Not-

tingham, U.K. (Professor T. Bennett, Dept. of Physiology & Pharmacology, Medical School, Queen's Medical Centre, Nottingham, U.K.)

28 September 1989

Leukotrienes. London, U.K. (Society for Drug Research, c/o Institute of Biology, 20 Queensbury Place, London SW7 2DZ, U.K.)

22–25 October 1989

Molecular and Cellular Biology of IL-1, TNF and Lipocortins in Inflammation and Differentiation. Siena, Italy. (L. Parente, Sclavo Research Centre, via Florentina 1, 53100 Siena, Italy).

8–11 November 1989

Tenth International Symposium on Drugs affecting Metabolism, Houston, USA. (Meeting Manager, 4550 Post Oak Place, Suite 248, Houston, Texas 77027, U.S.A.)

27–29 November 1989

Biological Actions of Extracellular ATP, Philadelphia, USA. (Conference Dept., New York Academy of Sciences, 2 East 63rd Streed, New York, NY 10021, U.S.A.)

6–8 December 1989

Presynaptic Receptors — an Examination of Different Views, New York City, USA. (Conference Department, New York Academy of Sciences, 2 East 63rd Street, New York, NY 10021, U.S.A.)

3–5 January 1990

British Pharmacological Society Winter Meeting. London, U.K. (Closed meeting for members and guests)

1–6 April 1990

6th World Congress on Pain, Adelaide, Australia. (Int. Association for the Study of Pain, 909 NE 43rd Street, Suite 306, Seattle, WA 98105, U.S.A.)

18–20 April 1990

British Pharmacological Society Spring Meeting. Sheffield, U.K. (Closed meeting for members and guests)

29 May–1 June 1990

Fondazione Giovanni Lorenzini Conference on Prostaglandins and Related Compounds, Florence, Italy (Fondazione Giovanni Lorenzini, Via Monte Napoleone, 23, 20121 Milan, Italy).

27–30 June 1990

International Society for Heart Research XI European Section Meeting, Glasgow, UK. (Professor J.R. Parratt, Dept. of Physiology & Pharmacology, Royal College, University of Strathclyde, Glasgow G1 1XW).

1–6 July 1990

IUPHAR 11th International Congress of Pharmacology, Amsterdam, Netherlands (Contact to be advised).

The Indices to Volumes 96, 97 and 98 together with the Cumulative Contents will be published with the December 1989 issue

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