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Cholera and pertussis toxins amplify prostacyclin synthesis in aortic smooth muscle cells

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Pretreatment of bovine aortic smooth muscle cells in culture with pertussis toxin (PT) or cholera toxin (CT) potentiated the synthesis of prostacyclin (PGI_2) induced by 5-hydroxytryptamine (5-HT) and phorbol-12-myristate, 13-acetate (PMA). The production of PGI_2 by explants from the bovine aortic media was also synergistically stimulated by 5-HT and CT, whereas PT was inactive. These data are consistent with the hypothesis that guanosine 5'-triphosphate binding proteins are directly involved in the control of phospholipases which release free arachidonic acid for prostaglandin synthesis.

Introduction There is growing evidence that the activity of phospholipases which release free arachidonic acid for prostaglandin synthesis can be controlled directly by guanosine 5'-triphosphate (GTP)-binding proteins (Burgoyne *et al.*, 1987). Among other arguments, this hypothesis is supported by experiments with cholera toxin (CT) and pertussis toxin (PT), which modulate the activity of some G proteins by catalyzing their ADP-ribosylation (Neer & Clapham, 1988). The action of these toxins on phospholipase A_2 activity and prostaglandin production is variable from one cell type to the other. The stimulation of prostaglandin E_2 (PGE_2) synthesis by α_1-adrenoceptor agonists is blocked by PT in FRTL5 rat thyroid cells (Burch *et al.*, 1986), while it is not modified by this toxin in MDCK canine kidney cells (Slivka & Insel, 1987). In the rod outer segments from the bovine retina, both PT and CT prevent the transducin-mediated activation of phospholipase A_2 by light (Jelsema, 1987). In contrast, CT and PT increase the basal production of PGE_2 in a murine macrophage line (Burch *et al.*, 1988) and potentiate the synthesis of prostacyclin (PGI_2) in bovine aortic endothelial cells stimulated by ATP or bradykinin (Pirotton *et al.*, 1987). In this study we have investigated the action of CT and PT on the production of PGI_2 by two distinct preparations of vascular smooth muscle: explants from the bovine aortic media, in which the cells keep a contractile phenotype and cultured cells obtained by outgrowth from these explants, which have modulated to the synthetic phenotype (Chamley-Campbell *et al.*, 1981).

Methods *Explants of the bovine aortic media* After removal of the intima and adventitia from the aorta, the media was cut into ± 1 mm squares (Ross, 1971); 4–5 such explants (20–50 mg wet weight) were put on 60 mm Petri dishes and cultured for 24 h at 37°C, under 5% CO_2/95% air, in a medium of the following composition: Dulbecco's modification of Eagle's medium (DMEM: 80%), Ham's F_{12} medium (20%), foetal calf serum (FCS: 20%), penicillin (100 u ml^{-1}), streptomycin (100 $\mu g\,ml^{-1}$), amphotericin B (2.5 $\mu g\,ml^{-1}$). PT and CT were added at the beginning and 2 h before the end of that 24 h period respectively. Then the medium was removed, the explants were rinsed and incubated for 60 min in DMEM containing cocaine 30 μM (Cory *et al.*, 1986), with or without 5-hydroxytryptamine (5-HT).

Bovine aortic smooth muscle cells Bovine aortic smooth muscle cells were obtained by outgrowth from media explants (Ross, 1971). The cells which had migrated out of the explants were subcultured in 35 mm Petri dishes in the complete culture medium (see above). Experiments were performed with confluent cells between passage 3 and 8. The cells were rinsed and incubated for 24 h in the complete culture medium (see above), except for the absence of FCS, in the presence of the agents to be tested. PT and CT were added 20 h and 1 h respectively before the start of the incubation with the agonists.

PGI_2 assay The production of PGI_2 was measured by radioimmunoassay of its stable degradation product, prostaglandin 6-keto-$F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) in the incubation medium (Van Coevorden & Boeynaems, 1984).

Statistical analysis of the results was performed using the paired *t* test.

Materials CT and its subunits were obtained from Sigma Chem. Co., and PT from List Biological

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Laboratories. All the other reagents were purchased as previously described (Demolle & Boeynaems, 1988).

Results 5-HT (10 μM), adenosine 5'-O-(3-thiotriphosphate) (ATPγS, 100 μM), phorbol-12-myristate, 13-acetate (PMA, 50 nM) and A23187 (0.1 μM) stimulated the production of PGI_2 by bovine aortic smooth muscle cells, as previously described (Coughlin *et al.*, 1984; Demolle & Boeynaems, 1988; Demolle *et al.*, 1988). Pretreatment with CT (5 $\mu g\,ml^{-1}$, 2 h) or PT (200 $ng\,ml^{-1}$, 20 h) amplified the response to 5-HT (Figure 1a and b). In the absence of toxins, 5-HT induced a 2.5 fold stimulation of PGI_2 release (mean of 10 experiments, range 1–6, $P = 0.003$): this stimulation was increased to 10 fold by PT (mean of 5 experiments, range: 3–18, $P = 0.05$) and to 11 fold by CT (mean of 10 experiments, range: 5–24, $P = 0.001$). In some experiments where no stimulation could be detected in response to 5-HT, an effect was unmasked by PT or CT. Pretreatment with both toxins potentiated the release of PGI_2 in a more than additive way (Figure 1a). The toxins increased the basal release of PGI_2 in a few experiments (3 out of 10): mean stimulations of 1.5 fold and 2 fold were observed in response to CT and PT respectively. The stimulatory effect of PMA was also potentiated by PT (Figure 1b) and CT (not shown), whereas the toxins had no significant effect on the response elicited by ATPγS and ionophore A23187 (Figure 1b). A synergism between 5-HT and CT was also observed in explants from the bovine aortic media (Figure 1c, 8 experiments, $P = 0.02$), while PT was completely inactive (3 experiments, not shown). Neither the A nor the B subunit of CT was able to mimic the action of the holotoxin either in the media explants (Figure 1c), or in the cultured smooth muscle cells (not shown). Forskolin (50 μM), an agent known to increase adenosine 3':5'cyclic-monophosphate (cyclic AMP) levels in vascular cells, was inactive in both experimental systems (not shown).

Discussion Our data suggest that one or more GTP-binding proteins are directly involved in the control of PGI_2 synthesis by bovine aortic smooth muscle cells. Previous studies have provided evidence for a coupling between G protein and phospholipase A_2 (PLA_2). GTPγS and noradrenaline increased the release of arachidonate from membranes of FRTL5 cells in a synergistic way (Burch *et al.*, 1986). The activation of PLA_2 induced by light in rod outer segments of the retina was abolished by transducin depletion and restored by addition of exogenous transducin (Jeselma, 1987): this effect was

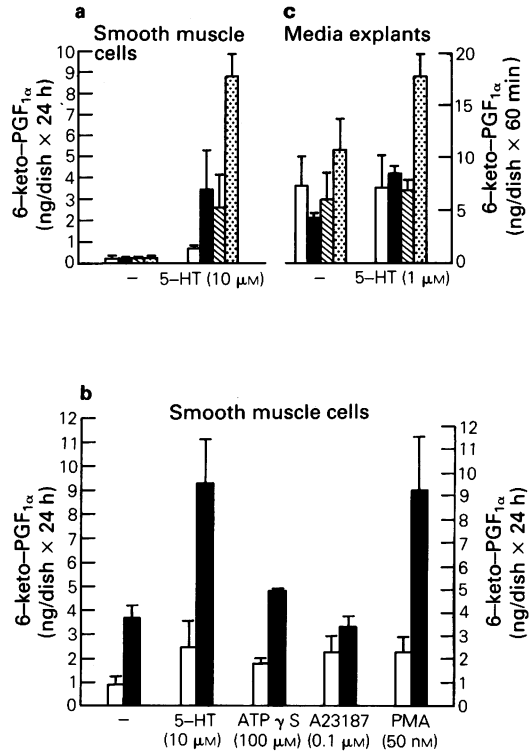

Figure 1 (a) Effect of pertussis toxin (PT) and cholera toxin (CT) on the production of prostacyclin (PGI_2) induced by 5-hydroxytryptamine (5-HT, 10 μM) in cultured bovine aortic smooth muscle cells. After a preincubation of respectively 20 h with PT (200 $ng\,ml^{-1}$) or 1 h with CT (5 $\mu g\,ml^{-1}$), the cells were incubated for 24 h in a culture medium without FCS, in presence of the various agents. Control: open columns; PT: solid columns; CT: hatched columns; PT + CT: stippled columns. (b) Selectivity of the PT action on the production of PGI_2 induced by 5-HT and PMA in cultured bovine aortic smooth muscle cells. The cells were incubated for 20 h with PT (200 $ng\,ml^{-1}$), prior to the addition of adenosine 5'-O-(3-thiotriphosphate (ATPγS, 100 μM), 5-HT (10 μM) A23187 (100 nM) or phorbol-12 myristate, 13-acetate (PMA, 50 nM): thereafter, the incubation was continued for 24 h, in the complete culture medium without FCS. Control: open columns; PT: solid columns. (c) Effect of CT on the production of PGI_2 by bovine aortic media explants stimulated by 5-HT: comparison between the holotoxin and its subunits. CT and its subunits A and B were added for 1 h to the tulture medium, each at 5 $\mu g\,ml^{-1}$. After removal of that medium and 2 washings, the explants were incubated for 1 h in presence of 5-HT (1 μM) and cocaine (30 μM). Control: open columns; CT, subunit A: solid columns; CT, subunit B: hatched columns; CT: stippled columns. For each panel, results represent the amount of 6-keto-PGF_1 accumulated in the medium (mean ± s.d. of triplicate determinations, in 1 representatative experiment out of 3).

mimicked by $\beta\gamma$-subunits and inhibited by the α-subunit (Jelsema & Axelrod, 1987). The GTP-binding proteins can be divided into four groups according to their susceptibility to ADP-ribosylation by the toxins: substrates for CT, for PT, for both toxins or for none (for review, see Neer & Clapham, 1988). PT and CT increased the basal production of PGE_2 in murine macrophages (Burch *et al.*, 1988) and potentiated the release of PGI_2 induced by ATP in bovine aortic endothelial cells (Pirotton *et al.*, 1987). Our results suggest that a similar mechanism operates in cultured bovine aortic smooth muscle cells, with some peculiarities. In smooth muscle cells, the toxins had little effect *per se*, whereas they increased the basal production of PGE_2 in murine macrophages (Burch *et al.*, 1988). In aortic endothelial cells, the toxins amplified the response to all the agonists tested (ATP, bradykinin, A23187); in smooth muscle cells, they were selective for some of them (5-HT, PMA). The action of PT and CT on PGI_2 synthesis in bovine aortic smooth muscle cells could be explained by the activation via ADP-ribosylation of a Gs-like protein, by the inhibition of a Gi-like protein (Jelsema, 1987) or by a combination of these 2 events, as suggested by Burch *et al.* (1988) to explain the synergism between PT and CT on PGE_2 synthesis in murine macrophages. Likewise, the effects of PT and CT on smooth muscle cells were more than additive.

In rat aortic myocytes, 5-HT, via 5-HT_2 receptors, induces an accumulation of inositol trisphosphate (Cory *et al.*, 1986), which results in an increase of cytosolic Ca^{2+}: this rise was inhibited by PT, which probably blocks the G protein coupling the 5-HT_2 receptor to phospholipase C (PLC) (Bruns & Marme, 1987). In FRTL5 cells, α_1-adrenoceptors are coupled to PLA_2 and PLC via different GTP binding proteins: only the G protein linked to PLA_2 was PT-sensitive (Burch *et al.*, 1986). The fact that PT potentiates the stimulatory effect of 5-HT on PGI_2 synthesis in bovine aortic smooth muscle cells is thus not incompatible with the observation that it blocks the rise of cytosolic Ca^{2+} induced by 5-HT in rat aortic smooth muscle cells.

In the explants of aortic media (contractile phenotype), only CT was able to potentiate the 5-HT effect on PGI_2 production, whereas in cultured smooth muscle cells (synthetic phenotype), PT was as effective as CT. Although, we cannot rule out the possibility that PT does not penetrate the media explants, its ineffectiveness is more likely to result from a differential sensitivity to PT of the G proteins involved in the control of PGI_2 production. For instance, PT inhibited PGE_2 synthesis induced by α_1-adrenoceptor agonists in FRTL5 rat thyroid cells (Burch *et al.*, 1986), whereas it was inactive in MDCK canine kidney cells (Slivka & Insel, 1987). The 5-HT receptor mediating the stimulation of PGI_2 synthesis in the media explants is different from the 5-HT_2 receptors involved in the cultured smooth muscle cells (Coughlin *et al.*, 1984; Demolle *et al.*, 1989). The mediators controlling PGI_2 production also seem to be specific for each system (Demolle & Boeynaems, 1988). Our observation of a differential action of the toxins constitutes an additional argument supporting the hypothesis that the modulation from a contractile to a synthetic phenotype is accompanied by a fundamental change in the mechanisms which control PGI_2 production.

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Augmentation and subsequent attenuation of Ca^{2+} current due to lipid peroxidation of the membrane caused by *t*-butyl hydroperoxide in the rabbit sinoatrial node

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Cellular electrophysiological effects of membrane lipid peroxidation by *t*-butyl hydroperoxide (TBH) were studied in the rabbit sinoatrial (SA) node. Superfusion for 1–5 min with 300 μM TBH caused an initial increase and subsequent decrease in the spontaneous firing frequency of the SA node. Voltage clamp experiments revealed that TBH initially enhanced but later blocked the Ca^{2+} current. Thus, membrane lipid peroxidation appears to accelerate and then suppress physiological automaticity by causing biphasic changes in the Ca^{2+} current.

Introduction Recent experimental evidence has linked the production of free radicals and resultant lipid peroxidation of the membrane to myocardial reperfusion injury and cardiac arrhythmias (Hess & Manson, 1984). Electrophysiological effects of free radicals on ventricular cells include reductions in the action potential amplitude and maximal rate of depolarization, loss of excitability and generation of abnormal automaticity. The action potential duration is shortened either with or without an initial prolongation, depending on the species and free radical generating systems (Nakaya *et al.*, 1987; Barrington *et al.*, 1988). However, ionic mechanisms underlying these action potential changes have not been elucidated to date. Hence, we studied the changes in the automaticity and ionic currents in the rabbit sinoatrial (SA) node caused by *t*-butyl hydroperoxide (TBH), an agent known to cause lipid peroxidation of the cardiac membrane (Nakaya *et al.*, 1987).

Methods Rabbits weighing 1.5–2.0 kg were anaesthetized by an intravenous injection of sodium pentobarbitone (30 mg kg^{-1}). The heart was quickly removed and the SA nodal tissue was dissected along the crista terminalis. A small strip of tissue, 3 mm long and 0.2 mm wide, was cut from the central portion of the node with a razor blade and ligated with fine silk sutures to obtain small nodal preparations with a final dimension of 0.2 × 0.2 × 0.1 mm. The preparations were superfused in a tissue bath at a flow rate of 5 ml min^{-1} with a modified Tyrode solution having the following composition in mM: NaCl 136.9, KCl 4.0, $CaCl_2$ 1.8, $MgCl_2$ 1.0, NaH_2PO_4 0.33 and glucose 5.5. The pH of the superfusate was adjusted to 7.4 with Na_2HPO_4 and the temperature was maintained at 37 ± 1°C. Partial oxygen pressure of the solution was maintained between 500 and 550 mmHg by 100% O_2 during the experiment. A 70% aqueous solution of TBH (Sigma) was added to the Tyrode solution at desired concentrations before individual experiments.

The membrane potential was recorded by use of conventional glass microelectrodes filled with 3 M KCl and having tip resistances of 20–50 MΩ. Voltage clamp experiments were conducted using double microelectrode techniques. The small preparation size allowed adequate spatial homogeneity. Values were expressed as mean ± s.d. Statistical analyses were made with Student's paired *t* test, and *P* values less than 0.05 were considered significant.

Results *Effects of TBH on the spontaneous action potential* Effects of lipid peroxidation by TBH (10–500 μM) on automaticity and action potential characteristics of the SA node were studied. TBH at 10 and 30 μM did not cause significant changes in the spontaneous action potential. The membrane action became evident at 100 μM, causing an initial increase with subsequent decrease in the spontaneous firing frequency. At 300 μM, these changes were exagger-

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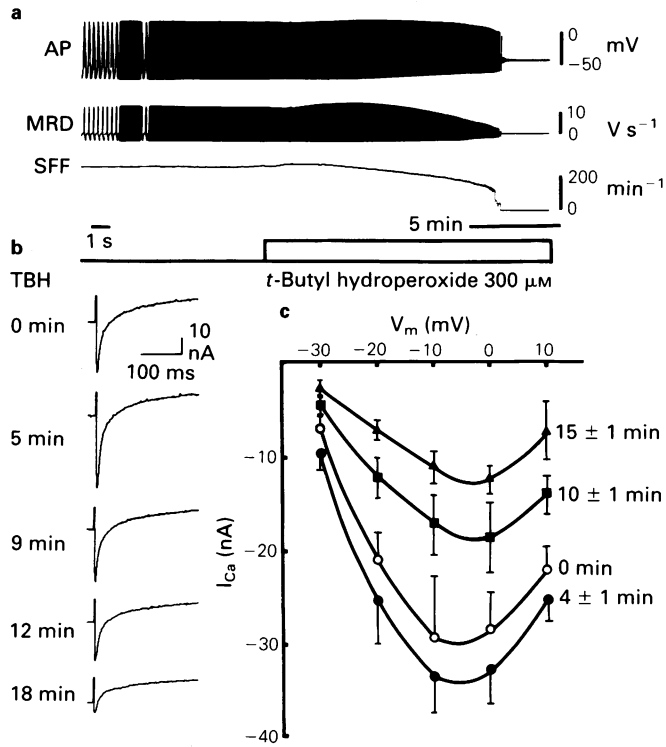

Figure 1 Effects of *t*-butyl hydroperoxide (TBH) on the sinoatrial node: (a) Positive and negative chronotropic actions of 300 μM TBH in a spontaneously beating small preparation. AP = action potential; MRD = maximal rate of depolarization; SFF = spontaneous firing frequency. In (b) is shown the effect of 300 μM TBH on the Ca^{2+} current (I_{Ca}) elicited on step depolarization from −40 to 0 mV. Note an initial increase and subsequent decrease in the current amplitude. (c) Time course of current-voltage relationships for I_{Ca} during 300 μM TBH superfusion ($n = 4$). I_{Ca} became maximal at 4 ± 1 min and then was progressively reduced. 0 min indicates the control curve. V_m = membrane potential.

ated (Figure 1a). In four such experiments, 4 ± 1 min superfusion with TBH initially increased the spontaneous firing frequency from 242 ± 25 to 261 ± 26 beats min^{-1} and the maximal rate of depolarization from 10.1 ± 2.2 to 15.9 ± 4.6 V s^{-1} ($P < 0.05$), but reduced them to 181 ± 23 beats min^{-1} and 8.1 ± 1.3 V s^{-1} ($P < 0.05$) after 8 ± 2 min superfusion. The action potential amplitude also showed a biphasic response with an initial, slight increase from 93.0 ± 0.8 to 94.5 ± 2.1 mV ($P > 0.1$) and a subsequent decrease to 85.9 ± 1.3 mV ($P < 0.05$). These inhibitory actions progressed with time. Finally, the spontaneous activity ceased after 15 ± 3 min with a resting potential of −46 ± 3 mV. At 500 μM, TBH caused similar changes although the time course was somewhat accelerated. Conversely, the action potential duration remained unchanged throughout the experiment. When the preparation was pretreated with butylated hydroxytoluene, a lipid peroxidation inhibitor, the effects of TBH on the spontaneous action potential were slowed, requiring more than 30 min to inhibit the automaticity completely. This observation would support the notion that TBH actions are mediated by membrane lipid peroxidation.

Effects of TBH on the Ca^{2+} current The action potential study suggested that the Ca^{2+} current (I_{Ca}) was initially augmented and subsequently attenuated by TBH-induced lipid peroxidation. Voltage clamp experiments were conducted to verify this assumption. Figure 1b shows representative I_{Ca} traces obtained on step depolarization to 0 mV from a holding potential of −40 mV. TBH transiently increased I_{Ca} at 5 min, and then progressively suppressed this current. The current-voltage relationships in Figure 1c summarize four such experiments. During control, the peak I_{Ca} recorded at −10 mV measured 29.3 ± 6.3 nA. After the addition of TBH, I_{Ca} was increased to 33.6 ± 6.0 nA at 4 ± 1 min

($P < 0.05$), and then decreased to 11.0 ± 1.1 nA at 15 ± 1 min ($P < 0.01$), when peak I_{Ca} was recorded at 0 mV. The delayed rectifying K^+ current was also decreased without an initial increase, whereas the hyperpolarization-activated inward current was increased throughout the experiment (not shown).

Discussion In the present study, membrane lipid peroxidation by TBH caused biphasic changes in SA node automaticity with initial acceleration and subsequent suppression. Voltage clamp experiments revealed that I_{Ca} played a major role in such biphasic changes because other pacemaker currents, the delayed rectifying K^+ current and hyperpolarization-activated inward current, were progressively decreased and increased, respectively. These results verify the contribution of I_{Ca} to pacemaker depolarization in the SA node as reported with the positive chronotropic action of adrenaline (Noma *et al.*, 1980).

The biphasic effects of TBH on I_{Ca} are consistent with recent reports that membrane lipid peroxidation initially increased and subsequently decreased (1) the developed tension of the guinea-pig papillary muscle (Nakaya *et al.*, 1987) and (2) the action potential duration in the canine ventricular muscle (Barrington *et al.*, 1988). The latter authors further noted induction of abnormal automaticity due to early and delayed afterdepolarizations when the action potential duration (and probably I_{Ca}) was increased. We also observed similar triggered activities initiated by early afterdepolarization when physiological SA node automaticity was accelerated by augmented I_{Ca}. Thus, both enhanced physiological automaticity and induction of abnormal automaticity in cardiac cells appear to be mediated by an excess transmembrane Ca^{2+} influx and/or an increased intracellular Ca^{2+} concentration.

The increase in the resting tension caused by TBH suggests an intracellular Ca^{2+} overloading (Nakaya *et al.*, 1987), probably resulting from a depression of Ca^{2+} uptake by the sarcoplasmic reticulum and mitochondria (Hess *et al.*, 1981) and an inhibition of Na,K-ATPase activity (Kramer *et al.*, 1984). Thus, a relatively small rise in intracellular Ca^{2+} concentration may enhance Ca^{2+} entry by promoting Ca^{2+} channel phosphorylation through Ca^{2+}-activated protein kinases (positive feedback regulation of I_{Ca}) (Marban & Tsien, 1982). During the course of lipid peroxidation, intracellular Ca^{2+} concentration may further increase through additional mechanisms such as the opening of Ca^{2+} channels (Lebedev *et al.*, 1982) and destruction of Ca^{2+} channel structure as evidenced by increased membrane fluidity and permeability (Meerson *et al.*, 1982). This, in turn, would attenuate I_{Ca} by (1) a Ca^{2+}-dependent I_{Ca} inactivation mechanism (negative feedback regulation of I_{Ca}) (Eckert *et al.*, 1981), (2) a reduced driving force for I_{Ca}, and (3) a decreased phosphorylation of Ca^{2+} channel due to the lack of adenosine 5'-triphosphate. Hence, lipid peroxidation by TBH appears to regulate I_{Ca} by increased intracellular Ca^{2+} concentrations due to biochemical impairment of Na^+-K^+ and Ca^{2+} pumps, and damaged lipid microenvironment of the Ca^{2+} channel and biomembranes.

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Role of selective cyclic GMP phosphodiesterase inhibition in the myorelaxant actions of M&B 22,948, MY-5445, vinpocetine and 1-methyl-3-isobutyl-8-(methylamino)xanthine

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1 The mechanism by which M&B 22,948, MY-5445, vinpocetine and 1-methyl-3-isobutyl-8-(methylamino)xanthine (MIMAX), which have been described as selective cyclic GMP phosphodiesterase (PDE) inhibitors, relax rat aorta was investigated.

2 Three cyclic nucleotide PDEs were identified in the soluble fraction of rat aorta; a Ca^{2+}-insensitive form exhibiting substrate selectivity for cyclic GMP (cGMP PDE), a Ca^{2+}/calmodulin-stimulated form which also preferentially hydrolyzed cyclic GMP (Ca^{2+} PDE), and a form demonstrating substrate selectivity for cyclic AMP (cAMP PDE).

3 M&B 22,948 and MIMAX inhibited cGMP PDE ($K_i = 0.16\,\mu M$ and $0.43\,\mu M$, respectively) and Ca^{2+} PDE ($K_i = 9.9\,\mu M$ and $0.55\,\mu M$, respectively), but exhibited weak activity against cAMP PDE ($K_i = 249\,\mu M$ and $42\,\mu M$, respectively). MY-5445 selectivity inhibited cGMP PDE ($K_i = 1.3\,\mu M$) and vinpocetine selectively inhibited Ca^{2+} PDE ($K_i = 14\,\mu M$).

4 M&B 22,948 and MIMAX induced dose-dependent increases in the accumulation of cyclic GMP, but not cyclic AMP, in rat aorta pieces. These effects were greatly reduced by endothelial denudation and by methylene blue ($5\,\mu M$) which blocks the actions of endothelium-derived relaxant factor. MY-5445 and vinpocetine had no effect on rat aorta cyclic GMP or cyclic AMP accumulation.

5 All four compounds caused dose-related relaxation of 5-hydroxytryptamine ($10\,\mu M$) contracted, endothelium-intact rat aorta, the effects of M&B 22,948 and MIMAX being greatly reduced by methylene blue ($5\,\mu M$). Methylene blue also caused 10 fold and 100 fold rightward shifts in the dose-response curves of MY-5445 and vinpocetine, respectively.

6 The results are consistent with the smooth muscle relaxant actions of M&B 22,948 and MIMAX, but not vinpocetine and MY-5445, being mediated through a mechanism involving inhibition of cyclic GMP hydrolysis.

Introduction

Multiple forms of cyclic nucleotide phosphodiesterase (PDE) exist in mammalian tissues (Weishaar *et al.*, 1985). In vascular smooth muscle, several PDE forms have been identified: a Ca^{2+}/calmodulin-insensitive isoform exhibiting substrate selectivity for guanosine 3′:5′-cyclic monophosphate (cyclic GMP) (cGMP PDE), a Ca^{2+}/calmodulin-stimulated activity which also preferentially hydrolyzes cyclic GMP (Ca^{2+} PDE), and an isoform demonstrating substrate selectivity and high affinity for cyclic AMP (cAMP PDE) (Lugnier *et al.*, 1986). Recently, Reeves *et al.* (1987) have indicated that a second cAMP PDE isozyme corresponding to the type IV activity in cardiac muscle is present in smooth muscle from pig aorta and pulmonary artery. It is possible that these PDE isoforms are compartmentalized and each associated with discrete intracellular pools of cyclic AMP or cyclic GMP. The relevance of the different PDE isozymes to the physiological control of cyclic nucleotides remains to be established.

The development of selective inhibitors may provide useful investigative tools for delineating the roles of the PDE isoforms in regulating tissue cyclic AMP and cyclic GMP levels. Several studies (Lorenz & Wells, 1983; Hagiwara *et al.*, 1984b; Schoeffter *et al.*, 1987) have provided evidence that 'second-

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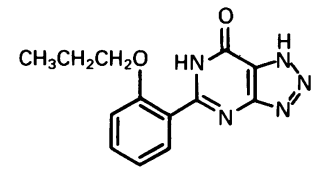
generation' PDE inhibitors exert selective effects on cyclic nucleotide metabolism. One such compound is M&B 22,948 which has proved useful in studying the role of cyclic GMP in regulating smooth muscle contractility. This azapurinone inhibits both cGMP PDE and Ca^{2+} PDE activities while exhibiting weak activity against cAMP PDE (Lugnier *et al.*, 1986). Levels of cyclic GMP, but not cyclic AMP, are elevated in vascular smooth muscle incubated with M&B 22,948 (Lugnier *et al.*, 1986; Martin *et al.*, 1986). Furthermore, M&B 22,948 potentiates the relaxant activities of agents whose actions are mediated by cyclic GMP but not those working via cyclic AMP (Martin *et al.*, 1986).

Relatively little information is available on other selective inhibitors of cyclic GMP hydrolysis. Lorenz & Wells (1983) identified a number of alkylated xanthines which increase bovine coronary smooth muscle cyclic GMP, but not cyclic AMP, and potentiate the relaxant action of sodium nitroprusside (SNP), which is mediated by cyclic GMP, but not that of isoprenaline, which is mediated by cyclic AMP. These xanthines inhibited an activity which probably comprised unresolved cGMP PDE and Ca^{2+} PDE, but only weakly inhibited cAMP PDE. The smooth muscle relaxant actions of vinpocetine have been ascribed to selective inhibition of Ca^{2+} PDE (Hagiwara *et al.*, 1984a). Smooth muscle relaxant activity has also been demonstrated for MY-5445 which is a selective inhibitor of cGMP PDE activity (Hidaka *et al.*, 1984). The structures of these compounds are presented in Figure 1. Theoretically at least, because of their selective inhibitory actions, MY-5445 and vinpocetine could prove useful in elucidating the respective roles of cGMP PDE and Ca^{2+} PDE in regulating cyclic GMP levels in smooth muscle.

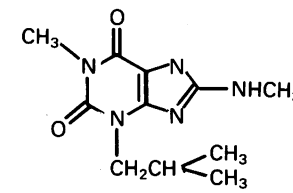
In the present studies, we have compared the effects of M&B 22,948, MY-5445, vinpocetine and 1 - methyl - 3 - isobutyl - 8 - (methylamino)xanthine (MIMAX), one of the more potent and selective inhibitors of cyclic GMP hydrolysis reported by Wells *et al.* (1981), on rat aorta relaxation and cyclic nucleotide levels. Since selective inhibitors of cyclic GMP hydrolysis should potentiate the actions of endothelium-derived relaxing factor (EDRF), which is believed to relax smooth muscle by stimulating guanylate cyclase (Martin *et al.*, 1986), we have investigated whether any endothelium-dependent component is associated with the relaxant effects elicited by the compounds.

Methods

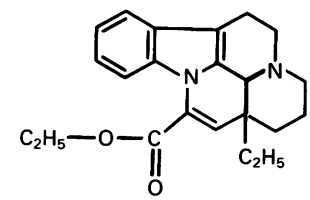
Experiments were conducted on aortae from male Wistar rats (250–400 g) purchased from Charles River U.K. Ltd. (Margate, Kent).



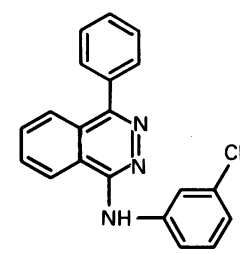
M&B22,948



MIMAX



Vinpocetine



MY-5445

Figure 1 Chemical structures of the phosphodiesterase inhibitors.

Partial purification of rat aorta phosphodiesterase activities

PDE activities were separated from rat aorta smooth muscle essentially according to the method of Lugnier *et al.* (1986). Briefly, aortae were excised from rats and extraneous tissue removed. The blood vessels were then cut open longitudinally and the intimal surface rubbed with a cotton swab to remove the endothelium. The tissue was then minced with

scissors before being homogenized in 6 vol. 20 mM Tris-HCl (pH 7.5), 2 mM magnesium acetate, 1 mM dithiothreitol, 5 mM EDTA, 2000 U ml^{-1} aprotinin, first with an Ultra Turrax (3 × 30 s bursts) and then with a scintered glass pestle homogenizer. The homogenate was then centrifuged at 105,000 *g* for 60 min and the supernatant (20 ml) applied to a DEAE-trisacryl column (IBF, Villeneuve La Garenne, France) pre-equilibrated with column buffer (Tris-HCl 20 mM, magnesium acetate 2 mM, dithiothreitol 1 mM, TLCK (N^{α}-tosyl-L-lysylchloromethane hydrochloride) 20 μM, pH 7.5). The column was washed with 50 ml of column buffer and PDE activities were eluted with 2 successive linear gradients of NaCl (0–150 mM, 210 ml and 150–400 mM, 140 ml) in column buffer; 2 ml fractions were collected and assayed and for long-term storage at −20°C, ethylene glycol was added to a final concentration of 30% (v/v). Activity was stable for several weeks under these conditions.

Measurement of phosphodiesterase activity

PDE activity was determined by the two step, radioisotope method of Thompson *et al.* (1979). The reaction mixture contained 20 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 4 mM 2-mercaptoethanol and 0.05 mg ml^{-1} bovine serum albumin. Unless otherwise stated, the concentration of substrate ([^{3}H]-cyclic AMP or [^{3}H]-cyclic GMP) was 1 μM. [8-^{3}H]-cyclic GMP (13.8 Ci $mmol^{-1}$) and [2, 8-^{3}H] cyclic AMP (41 Ci $mmol^{-1}$) were purchased from Amersham International (Amersham, Bucks).

For the determination of V_{max} and K_m values the concentration of cyclic AMP or cyclic GMP was varied while the amount of ^{3}H-labelled cyclic nucleotide remained constant. The data were analysed by non-linear least squares regression analysis to obtain values of K_m and V_{max}.

Determination of inhibition constants was carried out by selecting a grid of substrate and inhibitor concentrations which spanned the K_m and estimated K_i values of the enzyme. The data were again fitted by non-linear least-squares regression analysis to obtain the K_m and V_{max} values from which the K_i values were calculated.

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin used as the standard.

Measurement of cyclic GMP and cyclic AMP

Rat aorta cyclic nucleotides were determined by a modification of the method described previously by Bowman & Drummond (1984). Briefly, thoracic aortae were removed from rats, cleaned of extraneous tissue, cut transversely into 4 segments and incubated in 2 ml of Krebs-Henseleit bicarbonate solution (in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.6, $NaHCO_3$ 24.9, KH_2PO_4 1.2, glucose 5.6, pH 7.4) gassed with 95% O_2:5% CO_2.

Incubations were performed with intact rings of arota where the endothelium had been preserved or with aortic strips without endothelium which, prior to segmenting, had been cut open longitudinally and rubbed gently with a cotton swab. Before addition of test reagents, tissue pieces were pre-incubated for 120 min during which time the medium was changed once after 60 min. To terminate incubations, pieces of aorta were quickly removed from the medium and plunged into liquid N_2 before being thawed and homogenized with a glass pestle homogenizer in 1 ml of 5% trichloroacetic acid (TCA).

The homogenate was centrifuged at 2000 *g* for 15 min and the supernatant removed to a clean tube. TCA was removed with 3 washes of H_2O saturated ether (5 vols). The last traces of ether were removed by gassing with N_2, and sodium acetate (pH 6.2) was added to a final concentration of 50 mM. Samples were acetylated and cyclic AMP and cyclic GMP quantified by radioimmunoassay (RIA, NEN Chemicals GmbH). Protein was extracted from the TCA pellets by adding 2 ml 0.1 M NaOH containing 0.1% Triton X-100 (Rohn & Haas) and heating to 100°C in a boiling water bath and was measured in cooled samples by the method of Lowry *et al.* (1951).

Smooth muscle relaxant studies

Male Wistar rats (350–400 g) were killed by stunning and exsanguination. The aortae were carefully removed, cleaned of fat and extraneous tissue and cut transversely (5 mm) and longitudinally. The strips were mounted under 2 g of resting tension in 20 ml muscle chambers and bathed in Krebs Henseleit physiological salt solution (pH 7.4) at 37°C and gassed with 95% O_2:5% CO_2. Tension was measured isometrically using FT03 Grass force-displacement transducers and displayed on a Lectromed recorder. Tissues were equilibrated for 60 min before contraction was induced by 5-hydroxytryptamine (5-HT) (10 μM). Test compounds were added to the organ bath in increasing concentrations, each successive addition being made after maximum relaxation to the previous concentration had been achieved. Relaxation was expressed as percentage of relaxation of 5-HT-induced tone. The concentration of agent giving 50% relaxation (EC_{50}) was estimated by interpolation on the curve drawn through the mean data points for relaxation as a function of log concentration. The presence of an intact endothelium was verified by the ability of acetylcholine (1 μM) to induce relaxation.

Table 1 Kinetic properties of rat aorta smooth muscle phosphodiesterase (PDE) activities

Enzyme	K_m for cyclic GMP (μM)	K_m for cyclic AMP (μM)	Ratio V_{max} for cyclic GMP / V_{max} for cyclic AMP
cGMP PDE	0.24 ± 0.04	10.89 ± 1.38	1.09 ± 0.23
Ca^{2+} PDE − CaM	2.61 ± 0.91	2.81 ± 1.02	1.25 ± 0.33
Ca^{2+} PDE + CaM	2.47 ± 0.40	1.82 ± 0.06	2.19 ± 0.69
cAMP PDE	ND	0.27 ± 0.04	ND

Cyclic nucleotide PDE activities were separated from the cytosolic fraction of rat aorta as shown in Figure 2. The kinetic constants were determined as described in the methods section. Ca^{2+} PDE was assayed in the absence (−) and presence (+) of Ca^{2+} (2 μM) and calmodulin (2.5 u) (CaM). The results represent the means ± s.e.mean of kinetic parameters calculated from data obtained in 4 different experiments with different batches of enzymes. ND = not determined.

Drugs

M&B 22,948 (2-*o*-propoxyphenyl-8-azapurine-6-one), 1-methyl-3-isobutyl-8-(methylamino)xanthine (MIMAX) and MY-5445 [1-(3-chloroanilino)-4-phenylphthalazine] were synthesized by the Department of Pharmaceutical Chemistry, Rhône-Poulenc Ltd. Vinpocetine [ethyl(3α, 16α) eburnamen-14-carboxylate ethyl apovincamine-22-oate] was a gift from Ayerst Laboratories Research, Inc. (Princeton, NJ, U.S.A.). SK&F 94120 {5-(4-acetamido-phenyl) pyrazine-2[1H]-one} was a gift from Smith Kline and French Research Ltd., Welwyn, Herts. and Rolipram (ZK 62711; 4-(3-cyclopentyl-oxy-4-methoxy phenyl)-2-pyrrolidone) was generously provided by Schering A.G. (Hounslow, Middx.). 5-Hydroxytryptamine, methylene blue and acetylcholine were purchased from Sigma Chemical Co. (Poole, Dorset).

Statistical analysis

Data are presented as means ± standard error of the mean (s.e.mean) and analysed by Student's *t* test. Values were considered to be statistically significant at $P < 0.05$.

Results

Resolution of phosphodiesterase activities

In agreement with previous studies (Hagiwara *et al.*, 1984a; Lugnier *et al.*, 1986) three PDE activities could be distinguished in the cytosol from rat aorta following ion-exchange chromatography on DEAE-trisacryl and assaying with 1 μM substrate (Figure 2). For the purposes of this paper, the peaks of activity, in eluting order, have been designated cGMP PDE, Ca^{2+} PDE and cAMP PDE. Interestingly, the elution order of the cGMP PDE and Ca^{2+} PDE was the reverse of that found by Lugnier *et al.* (1986), although the same elution conditions were employed. The reason for this discrepancy is not clear. The total recovery of PDE activity from the column, measured with 1 μM substrate was greater than 90% for cAMP PDE and for cGMP PDE.

The kinetic parameters (K_m, V_{max} for cyclic GMP/V_{max} for cyclic AMP) of the three PDE isoforms are presented in Table 1. cGMP PDE exhibited high affinity and selectivity for cyclic GMP as substrate and was uninfluenced by addition of Ca^{2+}/calmodulin. Ca^{2+} PDE exhibited similar K_ms for the two substrates but the V_{max} for cyclic GMP was over 2 fold greater than against cyclic AMP in the presence of Ca^{2+}/calmodulin. Addition of Ca^{2+}/calmodulin to this isoform increased V_{max} (2 fold) without altering the K_m.

In agreement with previous studies (Lorenz & Wells, 1983; Hagiwara *et al.*, 1984a; Lugnier *et al.*, 1986), only one cytosolic cAMP PDE activity could be distinguished in rat aorta. This contrasts with pig aorta in which two cAMP PDE isoforms have been demonstrated with the same chromatrographic conditions (B.K. Diocee & J.E. Souness, unpublished data). On the basis of their kinetic properties and susceptibility to inhibition by rolipram, SK&F 94120 and cyclic GMP, these pig aorta PDE activities appeared to correspond to the Type III and Type IV PDEs identified in cardiac muscle (Reeves *et al.*, 1987). SK&F 94120 inhibited the cytosolic cAMP PDE in rat aorta but its potency (IC_{50} = 140 μM) was much weaker than against the heart type III enzyme (IC_{50} = 3.7 μM) (Reeves *et al.*, 1987). Rat aorta cAMP PDE was also inhibited by cyclic GMP (IC_{50} = 0.2 μM using 0.25 μM substrate) but activity was only slightly reduced by rolipram (30% inhibition at 1 mM). These data indicate the presence of a PDE activity in rat aorta which resembles the Type III isoform in cardiac muscle. Whether any Type IV enzyme is present as a minor contaminant of the cAMP PDE peak awaits further investigation.

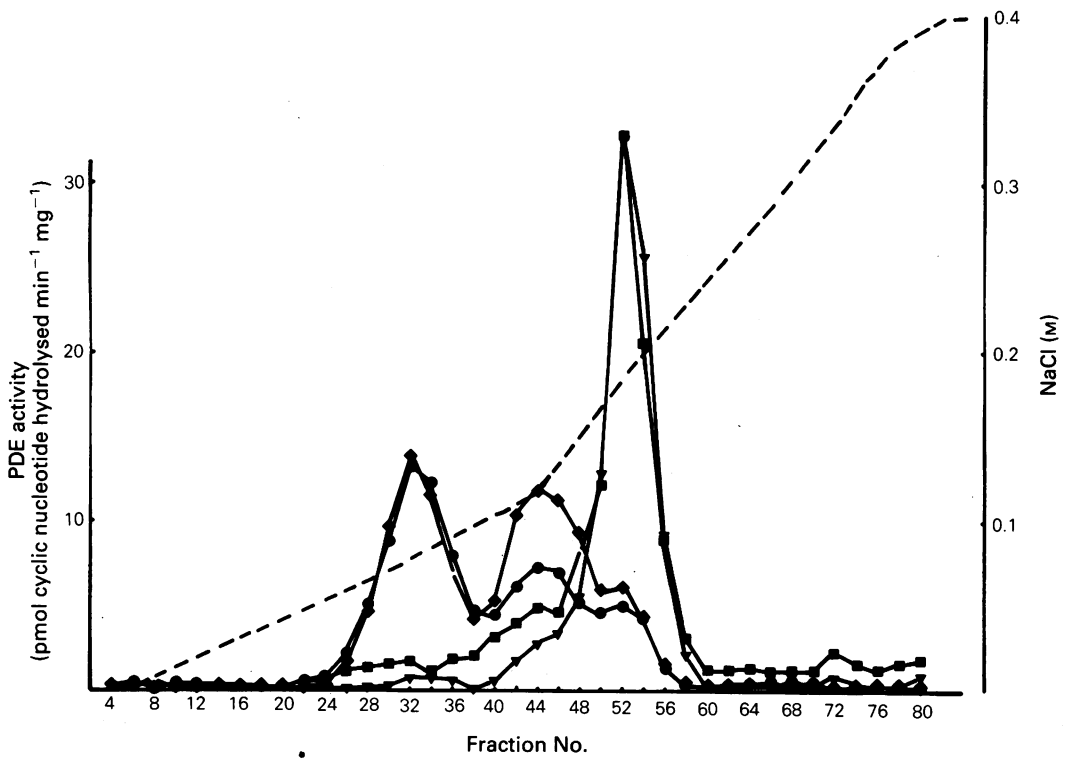


Figure 2 DEAE-trisacryl chromatography of phosphodiesterase (PDE) activity of the 105,000 *g* supernatant fraction from rat aortae. A 100,000 *g* cytosolic fraction from rat aortae was applied to a column of DEAE-trisacryl (0.9 × 7 cm). The column was washed with Tris-HCl 20 mM, magnesium acetate 2 mM, dithiothreitol 1 mM, and TLCK 20 μM (pH 7.5) and the PDE activities were eluted with successive linear gradients of NaCl (0–150 mM; 150–400 mM). PDE activity was determined using 1 μM cyclic AMP (■▼) or 1 μM cyclic GMP (●◆) in the presence of EGTA (100 μM) with (◆■) or without 2 mM $CaCl_2$ + 2.5 u calmodulin (●▼). For further studies, fractions 32–34 were pooled for cGMP PDE activity, fractions 42–46 for Ca^{2+} PDE activity and fractions 51–54 for cAMP PDE activity.

Inhibition of rat aorta phosphodiesterase activities by M&B 22,948, MIMAX, MY-5445 and vinpocetine

The inhibitory activities (K_i values) of M&B 22,948, MIMAX, MY-5445 and vinpocetine against the three resolvable PDE activities in the cytosol of rat aorta are presented in Table 2. M&B 22,948 was a potent competitive inhibitor of cGMP PDE. Ten fold weaker activity was exhibited against the Ca^{2+} PDE and only weak inhibition against cAMP PDE was observed. MIMAX exhibited similar inhibitory potency against cGMP PDE and Ca^{2+} PDE while only weakly inhibiting cAMP PDE. As with M&B 22,948, MIMAX was shown to be a competitive inhibitor of both cGMP PDE and Ca^{2+} PDE. MY-5445 selectively and competitively inhibited cGMP PDE, and vinpocetine selectively and non-competitively inhibited Ca^{2+} PDE.

Actions of phosphodiesterase inhibitors on cyclic nucleotide levels in rat aorta with and without endothelium

The effects of PDE inhibitors on rat aorta cyclic nucleotide levels were studied in rat aortic rings with endothelium intact and endothelium-denuded (rubbed) strips of rat aorta. The effective removal of endothelium by the rubbing technique was confirmed by the abolition of endothelium-dependent stimulation of cyclic GMP levels by acetylcholine without any effect on the direct action of SNP. In intact rings, acetylcholine (0.1 μM) elevated cyclic GMP levels from 1.1 ± 0.5 to 27.2 ± 2.5 pmol mg^{-1} protein (n = 3). Rubbing aortic strips lowered cyclic GMP levels to 0.1 ± 0.1 and acetylcholine only increased levels to 0.4 ± 0.2 pmol mg^{-1} protein (n = 3). SNP (1 μM), on the other hand, elevated

Table 2 Effects of inhibitors on separate phosphodiesterase activities

Compound	*cGMP PDE*	*Ca^{2+} PDE (+)CaM*	*cAMP PDE*
M&B 22,948	0.16 ± 0.04 (C)	9.9 ± 1.4 (C)	249 ± 22 (C)
MIMAX	0.43 ± 0.05 (C)	0.55 ± 0.08 (C)	42 ± 5 (C)
MY-5445	1.3 ± 0.4 (C)	>1000	915 ± 211 (C)
Vinpocetine	>1000	14 ± 2 (NC)	>1000

K_i values (μM) were determined as described in the Methods section. The Ca^{2+} PDE was assayed in the presence of Ca^{2+} (2 mM) + calmodulin (2.5 u) (CaM). The results represent the mean ± s.e.mean of K_i values calculated from the K_m or V_{max} values obtained from 4 experiments using 4 different inhibitor concentrations. The letters in parentheses denote competitive (C) or non-competitive (NC) inhibition.

cyclic GMP to 56.9 ± 7.0 pmol mg^{-1} protein ($n = 3$) in intact tissue and to 52.8 ± 6.4 pmol mg^{-1} protein ($n = 3$) in rubbed tissue.

Both M&B 22,948 (1–100 μM) and MIMAX (1–100 μM) dose-dependently increased cyclic GMP levels, the effect being greatly reduced in endothelium-denuded strips of tissue. Neither vinpocetine nor MY-5445 increased cyclic GMP levels at concentrations up to 100 μM. These data are summarised in Figure 3. None of the four PDE inhibitors influenced aorta cyclic AMP levels over the concentration range tested (basal levels, 11.65 ± 1.41 pmol mg^{-1} protein, $n = 4$, intact; 6.57 ± 1.35 pmol mg^{-1} protein, $n = 4$, rubbed). The effect, or lack of effect, of the 4 PDE inhibitors on both cyclic nucleotides was unaffected by 10 μM 5-HT (Figure 4), 60 mM K^+ or 1 μM noradrenaline (data not shown).

Methylene blue (5 μM) which abolishes EDRF stimulation of soluble guanylate cyclase (Martin *et al.*, 1985) completely blocked the endothelium-dependent elevation of cyclic GMP levels elicited by M&B 22,948 (100 μM) and MIMAX (100 μM) (Figure 4).

Smooth muscle relaxant actions of phosphodiesterase inhibitors

The dose-response relationships for the smooth muscle relaxant actions of the four PDE inhibitors in the presence and absence of methylene blue (5 μM) are presented in Figure 5. All four PDE inhibitors dose-dependently relaxed intact (endothelium-preserved) rat aortic strips contracted with 5-HT (10 μM). In agreement with previous studies (Martin *et al.*, 1986), the relaxant effects of M&B 22,948 ($EC_{50} = 15$ μM; 100% relaxation at 100 μM, $n = 4$) were almost totally abolished by prior addition of methylene blue (5 μM), suggesting that the compound acts by enhancing the relaxant actions of tonically released EDRF.

Similarly, MIMAX induced a relaxation of intact rat aorta ($EC_{50} = 2$ μM; 100% relaxation at 10 μM, $n = 4$) which at concentrations less than 10 μM was abolished by methylene blue; however, at higher concentrations, methylene blue was only partially effective in reducing the action of the xanthine.

MY-5445 also dose-dependently relaxed intact strips of rat aorta ($EC_{50} = 4$ μM; maximum relaxation, 95% at 60 μM, $n = 4$). However, unlike M&B 22,948 and MIMAX, the relaxant effect of MY-5445 was largely maintained in the presence of methylene blue, the dose-response curve only being shifted 10 fold to the right.

Vinpocetine potently inhibited the 5-HT-induced contractions in endothelium-intact rat aorta ($EC_{50} = 1$ μM; maximum relaxation, 100% at 100 μM, $n = 4$). Methylene blue inhibited the relaxant actions of low concentrations (<10 μM) of vinpocetine but was less effective in blocking the relaxant effects of higher concentrations of the drug.

Discussion

The evidence supporting a smooth muscle relaxant role for cyclic GMP is overwhelming (for review, see Murad, 1986); however, much has still to be learnt of the processes regulating the metabolism of this important second messenger. The presence of more than one enzyme capable of selectively hydrolyzing cyclic GMP in vascular smooth muscle (Figure 2) may suggest a complex degradatory interplay between the enzymes. Perhaps Ca^{2+} PDE takes on a dominant role when smooth muscle is exposed to an excitatory stimulus leading to influx of Ca^{2+}. Certainly, increased activity of this enzyme has been reported following exposure of vascular smooth muscle to depolarizing K^+ (Miller & Wells, 1987). Alternatively, Ca^{2+} PDE and cGMP PDE may regulate separate intracellular pools of cyclic GMP. Evidence for compartmentalised cyclic AMP coupled to distinct pools of cAMP-dependent protein kinase in heart has recently emerged (Gristwood *et al.*, 1986; England & Shahid, 1987; Murray *et al.*, 1987). The finding that an inhibitor of Type III cAMP PDE (SK&F 94120) increases contractile force and elevates cyclic AMP content of

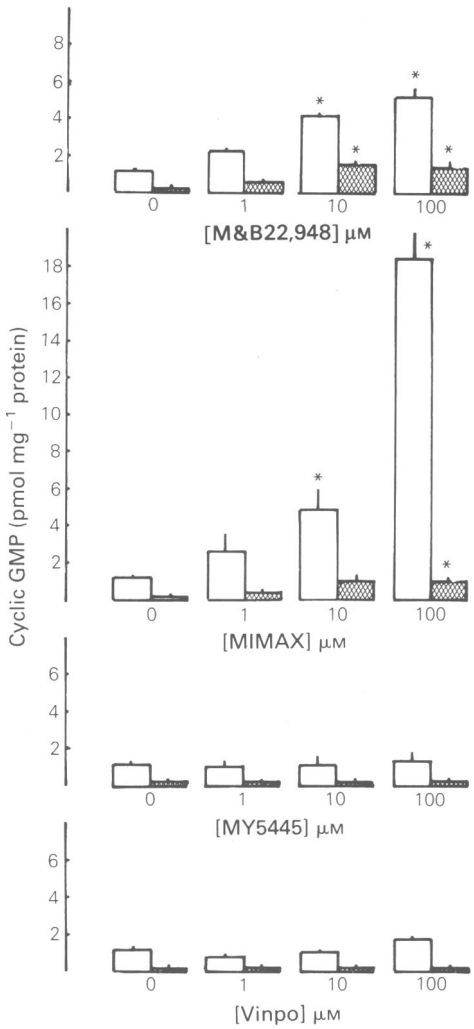


Figure 3 Effects of phosphodiesterase (PDE) inhibitors on the cyclic GMP content of endothelium-intact and endothelium-denuded pieces of rat aorta. Rubbed (endothelium removed) strips (cross hatched columns) and intact (endothelium-preserved) rings (open columns) of rat aorta were incubated with PDE inhibitors for 10 min. Cyclic GMP was extracted and quantified as described in the methods section. The results represent the means with s.e.mean (vertical bars) of 4 separate determinations. * Represents a significant difference from control values (absence of PDE inhibitors) $P < 0.05$.

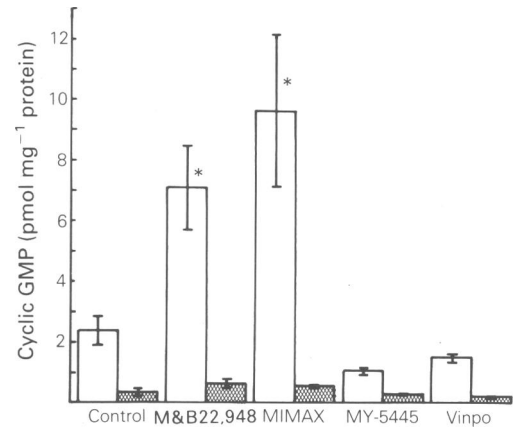


Figure 4 The effect of phosphodiesterase (PDE) inhibitors on cyclic GMP levels in endothelium-intact pieces of rat aorta in the presence and absence of methylene blue. Intact rings of rat aorta were pre-incubated with (cross hatched columns) or without (open columns) methylene blue (5 μM) for 10 min before being contracted by exposure to 5-hydroxytryptamine (5-HT, 10 μM) for 1 min. The tissue was then exposed to PDE inhibitors (100 μM) for a further 10 min in the continued presence of 5-HT. Cyclic GMP was extracted and quantified as described in the methods section. The results represent the means of 3 incubations with s.e.mean shown by vertical bars. * Represents a significant difference from control values (absence of PDE inhibitors) $P < 0.05$.

ventricular muscle strips while a Type IV cAMP PDE inhibitor (Rolipram) raised ventricular cAMP levels without inducing a positive inotropic effect (Gristwood *et al.*, 1986) suggests that different cAMP PDE isozymes are linked to distinct pools of cyclic AMP. Whether discrete pools of cyclic GMP associated with different cGMP PDEs exist in vascular smooth muscle in an analogous manner is a question which, to our knowledge, has not been addressed.

The advent of compounds which selectively inhibit individual PDEs has proved useful for the characterization of the different isozymes present in mammalian tissues. Furthermore, these inhibitors may prove to be useful in understanding the respective roles of the multiple PDEs in regulating cyclic nucleotide metabolism. M&B 22,948 is a selective inhibitor of cyclic GMP hydrolysis and has been an important tool in establishing a causal role for cyclic GMP in a number of biological events. This azapurinone inhibits both cGMP PDE and Ca^{2+} PDE (see Table 2, Lugnier *et al.*, 1986), exhibiting approximately 60 fold greater potency against the former activity. In agreement with previous studies (Martin *et al.*, 1986), exposure of rat aorta to M&B 22,948 resulted in an increase in cyclic GMP, but not cyclic AMP, (Figures 3, 4), and reduction in tone by enhancing the actions of EDRF (Figure 5). MIMAX exhibited similar potency against Ca^{2+} PDE and cGMP PDE, while only weak activity against cAMP PDE was observed (Table 2). It behaved in a similar manner to M&B 22,948 in inducing an elevation of cyclic GMP

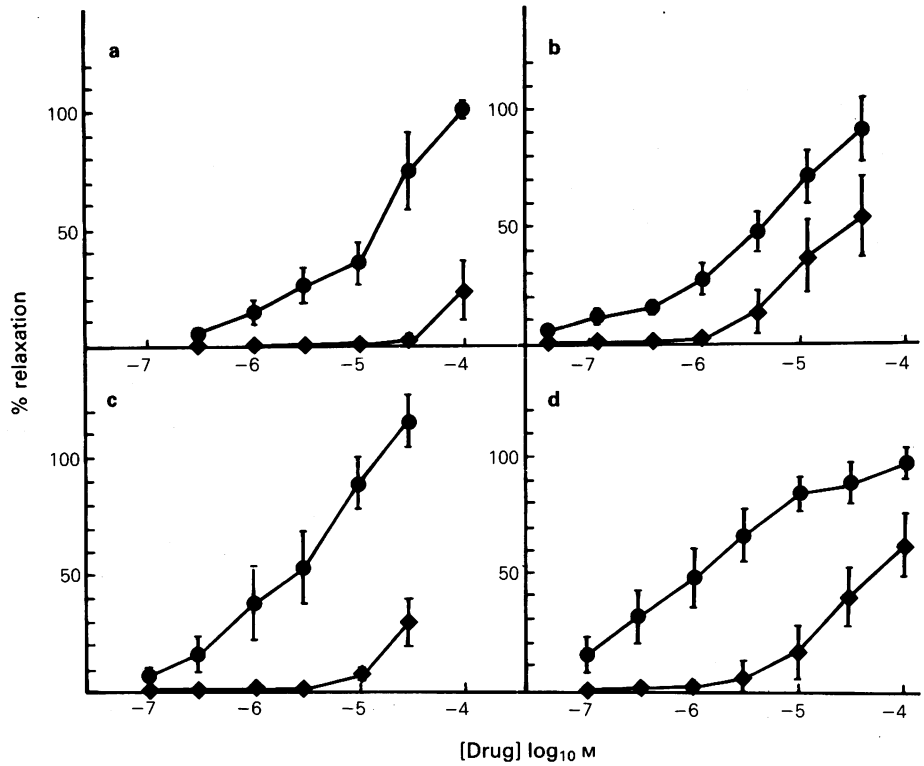


Figure 5 Dose-response curves of the smooth muscle relaxant effects of M&B 22,948, MIMAX, vinpocetine and MY-5445 in endothelium-intact strips of rat aorta in the presence and absence of methylene blue. Aortic strips were contracted with 5-hydroxytryptamine (10 μM). Plotted points show relaxation elicited by M&B 22,948 (a), MY-5445 (b), MIMAX (c) and vinpocetine (d) in aortic strips in the presence (◆) and absence (●) of methylene blue (5 μM). Each point is the mean and vertical bars the s.e.mean of 4 separate experiments.

and a relaxant response which was greatly reduced by abolishing EDRF (Figures 3, 4, 5); however, methylene blue only partially inhibited relaxation induced by 30 μM MIMAX (Figure 5), although the increase in cyclic GMP caused by an even higher concentration (100 μM) of the xanthine was completely abolished (Figure 4). Although this cyclic GMP-dependent component of the myorelaxant actions of MIMAX was observed at a concentration approaching its K_i value against the cAMP PDE ($K_i = 42 \pm 5\,\mu$M, $n = 4$), cyclic AMP involvement would appear to be ruled out as a mediatory mechanism since no increase in the level in rat aorta could be detected at MIMAX concentrations as high as 100 μM (data not shown).

Although M&B 22,948 and MIMAX are of use in studying cyclic GMP-mediated processes, they are of little value in delineating the respective roles of cGMP PDE and Ca^{2+} PDE in regulating cyclic GMP breakdown because of their inability to discriminate sufficiently between these enzymes. The potency of M&B 22,948 required to elevate cyclic GMP and reduce vascular tone ($EC_{50} = 15\,\mu$M) more closely correlated with its inhibitory activity against Ca^{2+} PDE ($K_i = 9.9 \pm 1.4\,\mu$M) than that against cGMP PDE ($K_i = 0.16 \pm 0.04\,\mu$M). From this it has been inferred that the Ca^{2+} PDE is of predominant importance in regulating smooth muscle cyclic GMP hydrolysis (Ahn *et al.*, 1987). However, a number of factors may contribute to discrepancies between the activity of a compound against the isolated enzyme and potency in influencing intracellular cyclic GMP concentrations and the biological response linked to this second messenger. Therefore, ideally, chemical structures which selectively inhibit the activity of one or other of these cyclic GMP hydrolyzing enzymes are required. Very few compounds with such activity have been reported in the literature. The anti-platelet action of MY-5445 has been ascribed to elevation of cyclic GMP levels via a selective inhibition of cGMP PDE (Hagiwara *et al.*, 1984b). This compound has also

been reported to induce relaxation in helical strips of rabbit aorta (Hidaka *et al.*, 1984). It was of interest, therefore, to determine whether this smooth muscle relaxant effect is mediated via a mechanism involving cyclic GMP. The results presented herein (Table 2, Figures 3, 4, 5) indicate, surprisingly, that although MY-5445 selectively inhibited rat aorta cGMP PDE its relaxant action is independent of cyclic GMP-mediated events.

The smooth muscle relaxant action of vinpocetine has also been attributed to blocking cyclic GMP hydrolysis but via inhibition of Ca^{2+} PDE (Hagiwara *et al.*, 1984a). We also demonstrated vinpocetine to be a selective, though relatively weak ($K_i = 14 \pm 2\,\mu M$) inhibitor of Ca^{2+} PDE (Table 2); however, although an endothelium-dependent component of the relaxant action of vinpocetine, especially at low concentrations, was suggested by the inhibition of this effect by methylene blue (Figure 5) our inability to observe an elevation of cyclic GMP (Figures 3, 4) appears to eliminate an involvement of this second messenger in the actions of the drug, at least in rat aorta. Whether methylene blue interferes with the myorelaxant actions of vinpocetine and the other PDE inhibitors through a mechanism independent of EDRF generation and guanylate cyclase activation warrants further investigation. Our results differ from those reported by others (Hagiwara *et al.*, 1984a; Ahn *et al.*, 1987) in which smooth muscle cyclic GMP levels increased following exposure to vinpocetine. Their studies were performed on rabbit aorta, suggesting, perhaps, differences between the contractile processes in vascular smooth muscle from the two species. Possibly the contribution of Ca^{2+} PDE to the regulation of intracellular cyclic GMP may be of less significance in rat aorta. It is possible, as previously mentioned, that the contribution of Ca^{2+} PDE and therefore the effectiveness of vinpocetine in influencing intracellular hydrolysis of cyclic GMP would only become apparent following elevation of cytosolic Ca^{2+} after exposure of the smooth muscle to an excitatory agonist; however, we have been unable to detect any stimulatory effect of vinpocetine on rat aortic cyclic GMP following exposure of the tissue to either 5-HT (Figure 4), depolarizing K^+ (60 mM) or noradrenaline (1 μM).

In conclusion, our results suggest that MIMAX, like M&B 22,948, relaxes rat aorta in an endothelium-dependent manner which is likely to result from the inhibition of the breakdown of smooth muscle cyclic GMP whose accumulation is stimulated by tonically released EDRF. Based on data presented in the present paper, it seems unlikely that such a mechanism can account for the smooth muscle relaxant actions of vinpocetine and MY-5445. The elucidation of the roles of the cGMP PDE and Ca^{2+} in regulating vascular smooth muscle cyclic GMP levels awaits the development of more potent and selective inhibitors. We are at present seeking to identify such compounds to understand better the cyclic GMP degradatory processes which exist in vascular smooth muscle.

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Effects of benzodiazepines and non-benzodiazepine compounds on the GABA-induced response in frog isolated sensory neurones

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1 The effects of benzodiazepines and non-benzodiazepine compounds on the γ-aminobutyric acid (GABA)-induced chloride current (I_{Cl}) were studied in frog isolated sensory neurones by use of a concentration-jump (termed 'concentration-clamp') technique, under single-electrode voltage-clamp conditions. The drugs used were classified into four categories as follows: full benzodiazepine receptor agonists (diazepam, clonazepam, nitrazepam, midazolam, clotiazepam and etizolam), partial agonists (CL 218,872, Ro 16-6028, Ro 17-1812 and Ro 23-0364), inverse agonists (Ro 15-3505, FG 7142 and β-CCE) and a benzodiazepine receptor antagonist, Ro 15-1788 (flumazenil).

2 All full agonists at concentrations of 3×10^{-6} M or less increased dose-dependently the peak amplitude of I_{Cl} elicited by 3×10^{-6} M GABA to twice to three times larger than the control. However, no further augmentation of the GABA response was observed at concentrations of 1×10^{-5} M or higher. Partial agonists also showed a dose-dependent augmentation of the GABA response at concentrations ranging from 3×10^{-8} M to 3×10^{-5} M, but their efficacies of augmentation of the GABA response were only about half or less of those of full agonists. Of the inverse agonists, β-CCE had a unique dose-dependent effect on the GABA response. β-CCE reduced dose-dependently the GABA response at concentrations of less than 3×10^{-6} M, but augmented it at concentrations of 3×10^{-5} M and 6×10^{-5} M. The inverse agonists reduced dose-dependently the GABA response. The benzodiazepine antagonist, flumazenil, slightly augmented the GABA response at concentrations between 3×10^{-7} M and 3×10^{-5} M.

3 These results show clear differences in the effects on the GABA response between these four categories of compounds known to affect the benzodiazepine recognition site of the GABA/benzodiazepine receptor-chloride channel complex. Our experimental system of frog isolated sensory neurones and a 'concentration-clamp' technique appears to be useful for evaluating efficacy of compounds on responses mediated by the GABA/benzodiazepine receptor-chloride channel complex.

Introduction

Anxiety is a ubiquitous symptom associated with medical as well as psychiatric disorders, some of which can be diagnosed and effectively treated. Currently, the most useful drugs seem to be the benzodiazepine derivatives which facilitated γ-aminobutyric acid (GABA) responses via the benzodiazepine receptor at the GABA/benzodiazepine receptor-ionophore complex (Costa *et al.*, 1975; Haefely *et al.*, 1975; Möhler & Okada, 1977). However, as the dose of benzodiazepines is increased, the anxiolytic effect progresses to sedation, sedation to sleep and/or anticonvulsive activity (Harvey, 1985; Petersen *et al.*, 1986). In the treatment of anxiety, other pharmacological actions of the drugs become undesirable side effects. Therefore, compounds that possess an anxioselective anxiolytic effect (pure anxiolytic action) are desirable. Recently, non-benzodiazepine compounds have been studied, for example cyclopyrolones, triazolopyridazines, pyrazoloquinolines and quinoline derivatives (Blachard *et al.*, 1979; Lippa & Crichett, 1979; Williams, 1983; Blanchard & Jolon, 1983). Many of them lack sedative, hypnotic and/or anticonvulsive activity. However, the compounds which affect the benzodiazepine receptor at the GABA/benzodiazepine receptor-ionophore complex can be

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Table 1 Compositions of internal and external test solutions (in mM)

Internal solution	CsCl 95	Cs-aspartate 10	TEA-Cl 25	HEPES 10	EGTA-Ca^{2+} 1×10^{-8} M	buffer Ca^{2+}
External solution	Tris-Cl 89	CsCl 2	$MgCl_2$ 5	TEA-Cl 25	Glucose 5	HEPES 10

The pH of internal and external solutions was adjusted to 7.2 and 7.4, respectively, with appropriate Tris-base.

classified into at least three categories: benzodiazepine receptor agonists (full and partial), inverse agonists and antagonists (Richards & Möhler, 1984). Such a classification has been based mainly on their pharmacological profile after their affinities to the benzodiazepine receptor had been established biochemically (Polc *et al.*, 1982; Möhler & Richards, 1983; Richards & Möhler, 1984); there have been relatively few systematic comparisons made with electrophysiological techniques (Chan & Farb, 1985; Sigel & Baur, 1988).

For the last five years we have carried out physiological and pharmacological studies on the $GABA_A$ response with single neurones isolated from frog dorsal root ganglia and a 'concentration-clamp' technique. Evidence has accumulated which suggests that this experimental system is useful for evaluating activity at the GABA receptor-ionophore complex (Hattori *et al.*, 1984; 1986; Akaike *et al.*, 1985a,b; 1986; 1987a,b,c; Inoue *et al.*, 1986; Akaike, 1989). In the present study, we have compared the effects of several benzodiazepines and non-benzodiazepine compounds on the $GABA_A$ receptor response to see if our experimental system adequately distinguishes the different compounds originally classified in biochemical studies. Part of this study has been presented in a preliminary form in Japanese (Akaike *et al.*, 1987d).

Methods

The experimental methods used were as described previously (Hattori *et al.*, 1984; Ishizuka *et al.*, 1984; Akaike *et al.*, 1986). In brief, dorsal root ganglia isolated from bull-frog (*Rana catesbiana*) were digested in normal Ringer solution containing 0.3% collagenase and 0.05% trypsin at pH 7.4 for 15 to 20 min at 37°C. During the enzyme treatment, the preparation was gently agitated by bubbling 95% O_2 and 5% CO_2. Thereafter, single neurones were mechanically isolated from the digested ganglia with fine pins under a binocular microscope. Isolated neurones were maintained in a solution which contained equal parts of Eagle's minimum essential medium (Nissui, Tokyo, Japan) and normal Ringer solution at a room temperature of 22 to 25°C. Isolated neuronal cell bodies were perfused internally and externally with respective test solutions (Table 1) for recording the chloride current (I_{Cl}), by a suction pipette technique (Ishizuka *et al.*, 1984). Neurones were voltage-clamped at a holding membrane potential of −50 mV with a single-electrode voltage-clamp system (Ishizuka *et al.*, 1984). Drugs were applied by use of a 'concentration-clamp' technique (see Figure 1 of Akaike *et al.*, 1986).

Clonazepam, nitrazepam, midazolam, flumazenil, Ro 15-3505 (ethyl-7-chloro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate), Ro 16-6028 (tert-butyl(s)-8-bromo-11,12,13,13a-tetra-hydro-9-oxo-9H-imidazo[1,5-a]-pyrrolo-[2,1-c][1,4]benzodiazepine-1-carboxylate), Ro 17-1812 (cyclopropylmethyl(s)-8-chloro-12,12a-dihydro-9-oxo-9H,11H-aceto-[2,1-c]-imidazo-[1,5-a]-[1,4]benzodiazepine-1-carboxylate) and Ro 23-0364

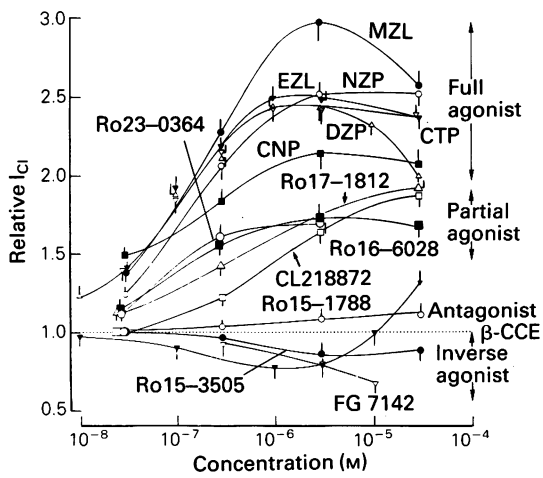

Figure 1 Concentration-response relationships for the facilitatory and inhibitory actions of the benzodiazepines and non-benzodiazepine compounds on the response elicited by 3×10^{-6} M γ-aminobutyric acid (GABA). Each point shows the mean value of four to six experiments; vertical lines show s.e.mean. The dotted line indicates the current amplitude induced by 3×10^{-6} M GABA alone (the control level). All values are normalized to the control level. MZL (midazolam), NZP (nitrazepam), CNP (clonazepam), CTP (clotiazepam), DZP (diazepam) and EZL (etizolam). Holding potential was −50 mV.

(6-(2-chlorophenyl)-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxamide) were provided by Dr Scherschlicht (F. Hoffman – La Roche & Co., Ltd., Basel, Switzerland). Etizolam, clotiazem, CL 218872 (3-methyl-6-(3-trifluoromethyl-phenyl)-1,2,4-triazolo[4,3-b]pyridazine), FG 7142 (N-methyl-β-carboline-3-carboxamide) and β-CCE (ethyl-β-carboline-3-carboxylate) were synthesized and provided by Yoshitomi Pharmaceutical Industries (Yoshitomi, Japan). Diazepam was purchased from Sigma (St. Louis, U.S.A.). All test drugs were initially dissolved in dimethyl sulphoxide (DMSO, Sigma) and diluted with the external test solution just before use. DMSO at final concentrations (0.2% or less) did not affect the GABA response. Only GABA, of the drugs tested, directly elicited the I_{Cl} at the concentrations used in this study. The different agents (the benzodiazepines and non-benzodiazepine compounds) were simultaneously applied to the neurones with 3×10^{-6} M GABA; at this concentration GABA produces very slow desensitization (Akaike *et al.*, 1985a; Hattori *et al.*, 1986). All values of the current amplitude were normalized, relative to the current amplitude produced by 3×10^{-6} GABA. Control current amplitude of the GABA response was 0.437 ± 0.009 nA (mean ± s.e., $n = 68$).

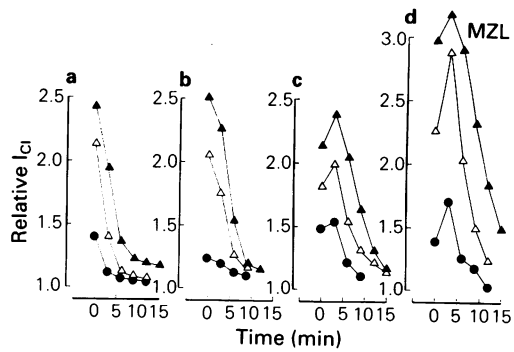

Figure 2 Time course of the recovery from the drug-induced augmentation of the response to γ-aminobutyric acid (GABA) (3×10^{-6} M) after washout of (a) diazepam, (b) nitrazepam, (c) clonazepan and (d) midazolam. The compounds were used at 3 different concentrations: (●) 3×10^{-8} M, (△) 3×10^{-7} M and (▲) 3×10^{-6} M. Abscissae indicate the time after washout of the agent. Zero point shows the GABA response just before washout of the agent. Ordinate scales: the current amplitude of the GABA response relative to that elicted by 3×10^{-6} M GABA alone. The results are typical of observations obtained from four experiments.

Results

As shown in Figure 1, the simultaneous application of the full benzodiazepine receptor agonists (diazepam, clonazepam, nitrazepam, midazolam, etizolam and clotiazepam) at concentrations between 3×10^{-7} M and 3×10^{-6} M potentiated the GABA response to two to three times the control level induced by GABA alone. Increasing the concentration of these agents beyond 3×10^{-6} M did not produce further augmentation of the GABA response. The dose-response relationships for both midazolam and diazepam were bell-shaped. Recovery from the augmentation, induced by the concentrations used in Figure 1, was complete within 30 min after washing out the agent. However, the time course of recovery from the augmentation of the GABA response induced by midazolam or clonazepam was different from those of the other full agonists. After wash out of midazolam or clonazepam, a further increase of the GABA response was observed ('after-wash' augmentation), while with diazepam and nitrazepam the response returned smoothly toward the control level after wash out (Figure 2). The partial agonists (CL 218,872, Ro 16-6028, Ro 17-1812 and Ro 23-0364) produced dose-dependent increases in the GABA response at a greater range of concentrations (3×10^{-8} M to 3×10^{-5} M), but their effects on the GABA response were only about half or less than those induced by the full agonists. Recovery was also complete within 30 min of washout. The benzodiazepine bases are generally lipophilic and might well penetrate into the membrane bilayer. Trapping in the membrane could be the reason for a slow recovery and/or 'after-wash' augmentation. Removal of an overoptimal concentration of a benzodiazepine in the extracellular medium by the first wash could well liberate enough trapped benzodiazepine to yield a somewhat higher response to GABA.

Data from ligand binding experiments suggest that Ro 15-1788 is a benzodiazepine antagonist (for a review, Richards & Möhler, 1984), but in our experiment it slightly increased the GABA response, indicating that flumazenil is a very weak partial agonist rather than a 'pure' benzodiazepine antagonist. Recovery was complete within 30 min after washout of this agent. Two inverse agonists (Ro 15-3505 and FG 7142) dose-dependently reduced the GABA-induced current amplitude at concentrations of 3×10^{-7} M or more. However β-CCE produced a unique dose-dependent effect on the GABA response (Figure 1). At concentrations of 1×10^{-8} M to 1×10^{-6} M it dose-dependently reduced the GABA response (Figure 3a). However, the inhibitory action of β-CCE on the GABA response was reduced at concentrations between 3×10^{-6} M and 1×10^{-5} M and it potentiated the GABA response at concentrations of 3×10^{-5} M and 6×10^{-5} M (Figure 3b,c).

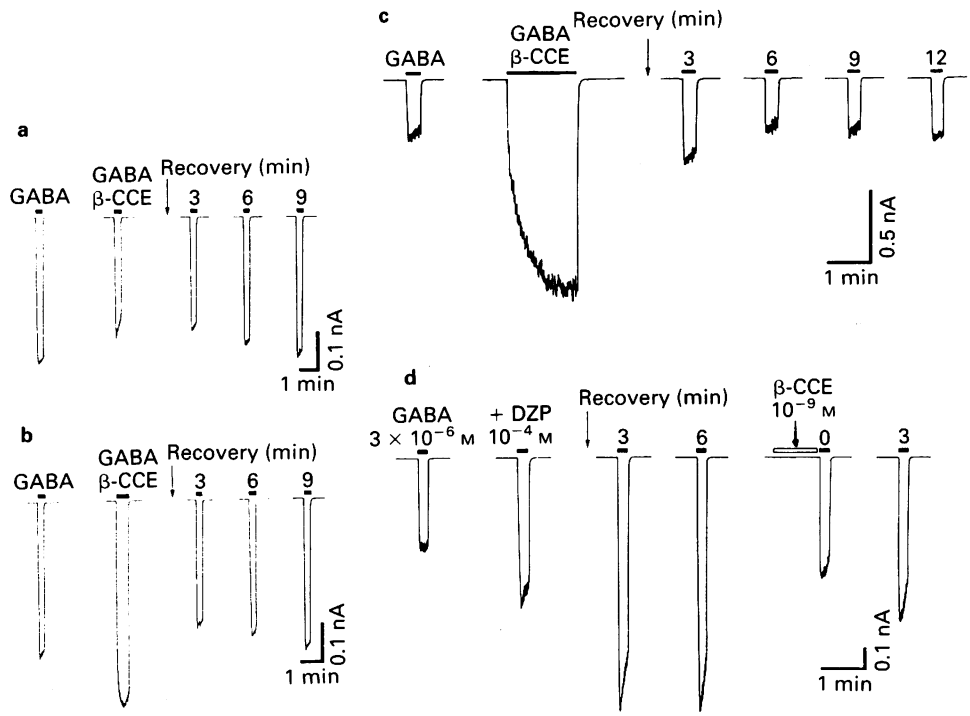

Figure 3 Inhibitory and facilitatory actions of β-CCE on the response elicited by 3×10^{-6} M γ-aminobutyric acid (GABA). Concentrations of β-CCE were 1×10^{-6} M (a), 3×10^{-5} M (b), 6×10^{-5} M (c) and 1×10^{-9} M (d). β-CCE was simultaneously applied to the neurone with 3×10^{-6} M GABA in each case. In (d), 1×10^{-4} M diazepam (DZP) was simultaneously applied with 3×10^{-6} M GABA. To see the antagonistic action of β-CCE on the GABA response modulated by diazepam the neurone was treated with 1×10^{-9} M β-CCE for 1 min before applying 3×10^{-6} M GABA alone. Holding potential was −50 mV.

Recovery from the effect of this inverse agonist was complete within 20 min of washout. However, the time course of recovery from the effect of β-CCE varied with different concentrations. As shown in Figure 3a, the current amplitude of the GABA response time-dependently recovered toward the control level at a concentration of 1×10^{-6} M. A similar change in the current amplitude after washout was observed at a β-CCE concentration of 3×10^{-5} M, although at this concentration β-CCE increased with GABA response (Figure 3b). With 6×10^{-5} M β-CCE, the GABA response was still larger than the control shortly after washing, it then recovered with a similar time course to that seen with 1×10^{-6} M and 3×10^{-5} M β-CCE, i.e. the current amplitude of the GABA response became smaller than the control and then returned to the control level. Another interesting effect of β-CCE was observed in the experiment shown in Figure 3d. Diazepam at 1×10^{-4} M slightly augmented the GABA response, as expected from its 'bell-shaped' dose-response curve. When a high concentration of diazepam was used, it augmented the GABA response after washout, as seen with midazolam and clonazepam. Such potentiation by 1×10^{-4} M diazepam persisted for about 20 min after washing. This effect was greatly reduced by a low concentration (1×10^{-9} M) of β-CCE, which did not affect the GABA response directly.

Discussion

As illustrated in Figure 1, our experimental system using a 'concentration-clamp' technique and frog isolated single neurones could clearly distinguish effects of the different categories of drugs, known to act on the benzodiazepine receptor and defined by pharmacological and biochemical studies (Polc *et al.*, 1982; for a review, Richards & Möhler, 1984). Of the full agonists examined, midazolam and diazepam produced a 'bell-shaped' dose-dependent effect on the

GABA response at concentrations ranging from 1×10^{-8} M to 3×10^{-5} M (Figure 1). The reason for the 'bell-shaped' dose-response curve is unknown, but high concentrations of these benzodiazepines may directly block GABA-gated chloride channels or may non-specificially perturb membrane functions. Although they have similar dose-dependent effects, there are interesting differences between them. With midazolam, the 'after-wash' augmentation of the GABA response was observed at all the concentrations used (3×10^{-8} M or more), whereas this was only observed with diazepam at a high concentration (1×10^{-4} M) (Figure 2). Yet both agents equally augmented the GABA response in the concentration range 3×10^{-8} M to 3×10^{-5} M. Therefore, it is possible that some full agonists have two actions on the GABA/benzodiazepine receptor-ionophore complex with a different dose-dependence: one is to increase the affinity of the GABA/benzodiazepine receptor to GABA and the other may be to hinder directly some functions of the GABA receptor-ionophore complex or the GABA-GABA receptor interaction, not yet specified. As the dose of the benzodiazepines is increased, the anxiolytic effect progresses to sedative, sedative to hypnosis and/or anticonvulsive effects. Therefore, partial agonists may be ideal anxioselective anxiolytics, since their potencies in augmenting the GABA response are relatively weak and dose-dependent changes in their actions on the GABA response are moderate (Figure 1). As shown in Figure 1, the degree of potentiation of the GABA response induced by partial agonists (CL 218,872, Ro 16-6028, Ro 17-1812 and Ro 23-0364) at the maximum concentration (3×10^{-5} M) seems to be obtained at concentrations around 1×10^{-7} M when full agonists are used. Clonazepam (CNP) has been regarded for many years as a full agonist and has been used as an antiepileptic. Recently, it has been shown, in a preliminary study, that CNP in a certain dose range antagonizes the effect of diazepam in a behavioural test (Bonetti *et al.*, 1987). As shown in Figure 1, the maximum efficacy of CNP is located between those of full agonists and partial agonists. Therefore CNP may be a partial agonist with a higher intrinsic efficacy than most other partial agonists.

Flumazenil has been shown to bind with high affinity to the benzodiazepine receptor site and to antagonize a variety of the actions induced by the benzodiazepines (Hunkeler *et al.*, 1981). However, two opposite, excitatory and inhibitory, actions on the GABA response have been obtained in electrophysiological studies (Chan & Farb, 1985; Sigel & Baur, 1988). Thus, at the concentration range 3×10^{-8} M to 1×10^{-6} M, flumazenil augmented the GABA response of cultured neurones isolated from embryonic chick spinal cord (Chan & Farb, 1985), while it reduced the response mediated by the chick forebrain GABA receptor expressed in *Xenopus* oocytes (Sigel & Baur, 1988). At present there is no explanation for this difference. In our case flumazenil slightly, but dose-dependently, increased the GABA response at concentrations similar to those previously used (Chan & Farb, 1985; Sigel & Baur, 1988). In spite of the wide use of the term 'benzodiazepine antagonist', flumazenil seems to possess a small intrinsic action and it may thus be termed a partial antagonist in our case. Of the inverse agonists examined here, Ro 15-3505 and FG 7142 reduced the GABA response as expected. However, β-CCE produced complicated actions on the GABA response, as observed previously (Akaike *et al.*, 1987d; Sigel & Baur, 1988). These results suggest that β-CCE may be an inverse agonist possessing 'intrinsic activity' on the benzodiazepine receptor. However, the inverse agonistic activity of the agent could be observed at a concentration of 1×10^{-9} M when the GABA receptor-ionophore complex was previously modulated by a full agonist (Figure 3d). Therefore, the specificity of β-CCE as an inverse agonist is thought to be high.

In conclusion, our experimental system of frog isolated sensory neurones and a 'concentration-clamp' technique appears to be a useful one for evaluating the efficacy of benzodiazepines and non-benzodiazepine compounds on the responses mediated by the GABA receptor-ionophore complex.

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Pharmacological characterization of postjunctional α-adrenoceptors in cerebral arteries from the sheep

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1 The responsiveness to noradrenaline was characterized in cerebral arteries from the sheep, since this species was large enough to permit a comparison of arteries from different parts of the cerebral vasculature.

2 Noradrenaline caused contraction of the basilar artery, middle cerebral artery and small pial arteries by stimulation of α_1-adrenoceptors.

3 The maximum contraction to noradrenaline was small in the basilar artery (28% of the 5-hydroxytryptamine (5-HT) maximum) but larger in the middle cerebral artery (78% of the 5-HT maximum) and pial artery (92% of the 5-HT maximum) of the sheep.

4 Cocaine (10 μM) potentiated noradrenaline-induced contractions in the sheep middle cerebral artery but not in the sheep basilar artery.

5 The noradrenaline contraction, relative to the 5-HT contraction, was not affected by removal of the endothelium in either the sheep basilar or middle cerebral artery.

6 The results showed a variation within the sheep cerebral vasculature in the response to noradrenaline which cannot be explained by regional differences in α-adrenoceptor subtypes, noradrenaline uptake mechanisms or endothelial function.

Introduction

The cerebral arteries of many species have been shown to have a dense adrenergic innervation (Iwayama *et al.*, 1970; Dahl, 1973). However, in various species it has been found that the cerebral arteries, in contrast to those of the periphery, are contracted weakly by noradrenaline (NA) in comparison to other agonists such as 5-hydroxytryptamine (5-HT), histamine and KCl (Nielsen & Owman, 1971; Toda & Fujita, 1973; Duckles & Bevan, 1976; Harder *et al.*, 1981; Skarby *et al.*, 1983; Usui *et al.*, 1985; Tsukahara *et al.*, 1986).

Postjunctional α_2-adrenoceptors appear to be the predominant adrenoceptor subtype present in the cerebral arteries of several species including cats (Skarby *et al.*, 1983), dogs (Sakakibara *et al.*, 1982), rats (Skarby *et al.*, 1984), pigs (Busija & Leffler, 1987) and cattle (Tsukahara *et al.*, 1983). It has been suggested that the small contractile response to NA may be limited by the receptor response mechanisms of these α_2-adrenoceptors (Sakakibara *et al.*, 1982) or by the number of receptors present (Laher & Bevan 1985b). Binding studies have shown both α_1- and α_2-adrenoceptors to be present in monkey and human cerebral arteries (Usui *et al.*, 1985). Functional studies in human cerebral arteries have shown the NA-induced contraction to be mediated only by α_1-adrenoceptors (Toda, 1986). Similarly, in the guinea-pig basilar artery the NA-induced contraction appears to be mediated by stimulation of α_1-adrenoceptors (Chang *et al.*, 1988). The rabbit basilar artery appears to contain functional α_1-receptors (Laher & Bevan, 1985a) though there is some contradictory evidence from binding studies which have failed to show their presence (Ferran *et al.*, 1984).

The first objective of the present study was to characterize and compare the α-adrenoceptors in several different arteries from the cerebral circulation since previous studies have usually used only one artery, generally the basilar. For this reason sheep cerebral arteries were used as the various arteries were large enough for comparative studies to be carried out. Differences in responsiveness to NA could be caused by regional variation in receptor

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subtypes, NA uptake processes or in endothelial function as endothelium-derived relaxing factor (EDRF) has been shown to be released by agonists such as NA and 5-HT in some arteries (Cocks & Angus, 1983). The possibility of an endothelium-derived contracting factor (EDCF) which is also released by NA in cerebral arteries has also been suggested (Usui *et al.*, 1987) and investigated in this study. Preliminary results from these experiments have been reported previously (Gaw *et al.*, 1987).

Methods

Experiments were carried out on sheep basilar arteries, middle cerebral arteries (MCA), circle of Willis, anterior cerebral arteries and first generation pial arterial branches of the middle cerebral artery (pial). The sheep heads were obtained daily from an abattoir. The brain was rapidly excised and transferred to a beaker of oxygenated physiological salt solution (composition, mM: NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0 and glucose 11.1). The basilar and MCA were dissected off the base of the brain from the circle of Willis. The diameters of the unstressed arteries were: basilar 0.7–0.9 mm, MCA 0.6–0.8 mm, circle of Willis 0.8–1.0 mm, anterior cerebral 0.4–0.6 mm, pial 0.3–0.5 mm. The responsiveness of the MCA to NA and 5-HT was found to be representative of most of the cerebral arteries investigated and was therefore used for comparitive studies with the basilar artery.

Each artery was cut into two sections one of which was then rubbed intraluminally with a double twisted fine wire (100 μm diameter). Two ring preparations, 3–4 mm long, were prepared from each section. Each ring was suspended on two intraluminal parallel wires of 100 μm diameter in a manner similar to that described by Nielsen & Owman (1971). They were then placed under 0.2 g of tension for 1 h before application of the optimal preloads for each artery, (1.0 g basilar, 1.0 g MCA, 1.0 g circle of Willis, 0.5 g anterior cerebral and 0.5 g pial). Optimal preload was determined in preliminary experiments ($n = 4$ for each ring) by repeated contraction with KCl over a range of set preloads. The tissues were then left for a 30 min equilibration period, during which they attained a steady resting tension. The arteries were then exposed to a maximal concentration of 5-HT (10 μM), washed at 0, 2, 4, 6, 8, and 10 min and left for a further 30 min.

Endothelium and noradrenaline uptake studies

NA was added cumulatively to the arteries (100 nM–100 μM) to obtain concentration-response curves. The arteries were then washed once more and cocaine (10 μM) was added to one rubbed and one unrubbed artery, the other two arteries serving as controls. The cocaine was left in contact with the tissues for 30 min before repetition of the NA concentration-response curve. The tissues were washed, as above, and left for a 30 min period. The arteries were then precontracted with 5-HT (3 μM), which gave a maximal contraction, before exposure to acetylcholine (1 μM). If acetylcholine did not cause a relaxation it was assumed that the endothelium had been removed. Responses were expressed as a percentage of the maximum response to NA obtained in the absence of cocaine and the curves for each artery were then plotted to determine whether cocaine or endothelium removal had any effect. The NA results were also expressed as a percentage of the maximum response to 3 μM 5-HT to determine whether a difference existed in the responsiveness of NA relative to 5-HT between different arteries.

Receptor characterization

Cocaine (10 μM) was added to four ring segments from unrubbed basilar, MCA, pial and circle of Willis arteries and remained in contact with the tissues for the duration of the experiment. Following a 30 min equilibration period in the presence of cocaine, NA was added cumulatively and a concentration-response curve was obtained in each preparation. The rings were washed at 0, 2, 4, 6, 8 and 10 min before the addition of prazosin (1 nM, 3 nM or 10 nM) or the drug vehicle. The preparations were then left for 30 min to allow equilibration before a second cumulative addition of NA was carried out. The rings were again washed at 0, 2, 4, 6, 8 and 10 min and left for a further 30 min to equilibrate. The α_2-adrenoceptor agonist UK14304 was then added to a final bath concentration of 100 μM to see if it had any contractile effect.

The responses to NA in each preparation were expressed as a percentage of the maximum NA response in the absence of prazosin in the same preparation and curves for each artery were plotted. Concentration-ratios were determined from the EC_{50} values (the concentration of agonist necessary to produce 50% of the maximum response). The NA concentration ratios for the prazosin-treated rings were divided by the concentration-ratios of the vehicle control rings to provide a corrected concentration-ratio (there was no significant difference between the first and second curves in these control experiments). The results were then subjected to Schild analysis. In some experiments where only one concentration of prazosin was investigated the concentration-ratios were obtained and then used to calculate apparent pA_2 values.

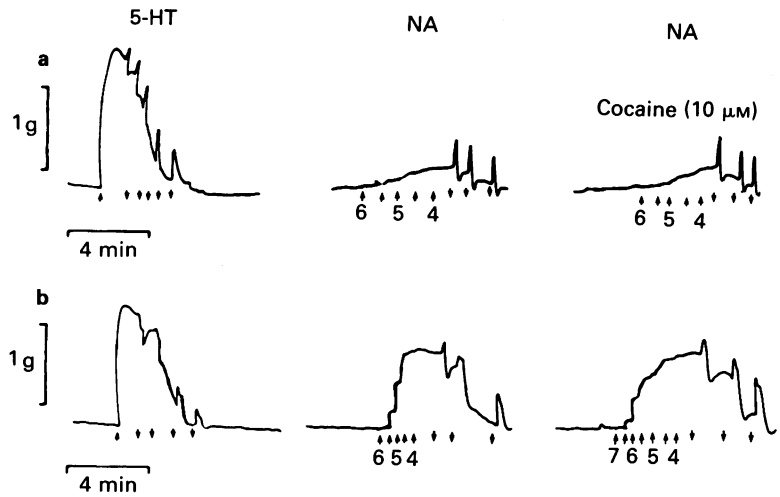

Figure 1 Typical responses obtained to a single application of 5-hydroxytryptamine (5-HT) (10 μM) and to cumulative addition of noradrenaline (NA) in both the absence and presence of cocaine (10 μM). The traces are representative of the basilar artery (a) and middle cerebral artery (b). Addition and washout of drugs are represented by upward and downward pointing arrows respectively. Drug concentrations are given as the negative logarithm of the final bath concentration (M).

Drugs

The following drugs were used: (−)-noradrenaline hydrochloride (Sigma); cocaine hydrochloride; 5-hydroxytryptamine creatinine sulphate (Sigma); prazosin hydrochloride (Pfizer Ltd. Sandwich.); UK14304 (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline bitartrate) (Pfizer Ltd. Sandwich.). The drugs were dissolved in distilled water to form a stock solution of 10 mM with subsequent dilutions made in 0.9% saline solution. Exceptions to this were noradrenaline hydrochloride which was dissolved in 0.9% saline containing 1 mM ascorbic acid and prazosin which was made up at a 100 μM stock concentration in 1% lactic acid.

Statistical analysis

The data were expressed as means ± s.e.mean: n refers to the number of heads used. Statistical comparisons between groups were made by Student's t test. P values <0.05 were considered to be statistically significant.

Results

Results from preliminary studies on the basilar artery, circle of Willis, MCA, anterior cerebral artery and pial branches showed 5-HT to be a very potent

Table 1 Noradrenaline EC_{50} values and maximal contractions (expressed as a percentage of the maximum response to 5-hydroxytryptamine ($5\text{-}HT_{max}$)

Artery		pEC_{50} *No cocaine*	pEC_{50} *Cocaine* (10 μM)	*5-HT max Force* (g)	*% NA max 5-HT max*
Basilar	(+E)	5.47 ± 0.08	5.51 ± 0.07	1.71 ± 0.18	27.6 ± 3.8%[c]
	(−E)	5.32 ± 0.11	5.27 ± 0.13	1.25 ± 0.13[a]	22.7 ± 3.2%[c]
MCA	(+E)	5.55 ± 0.07	6.37 ± 0.08[b]	2.08 ± 0.33	78.1 ± 3.6%[c]
	(−E)	5.33 ± 0.13	6.12 ± 0.15[b]	1.18 ± 0.31[a]	77.1 ± 4.3%[c]

Values are mean ± s.e.mean, $n = 6$.
+E = endothelium present; −E = endothelium removed by rubbing.
MCA = middle cerebral artery.
[a] Significant difference caused by endothelium removal.
[b] Significant difference caused by cocaine addition.
[c] Significant difference between arteries.

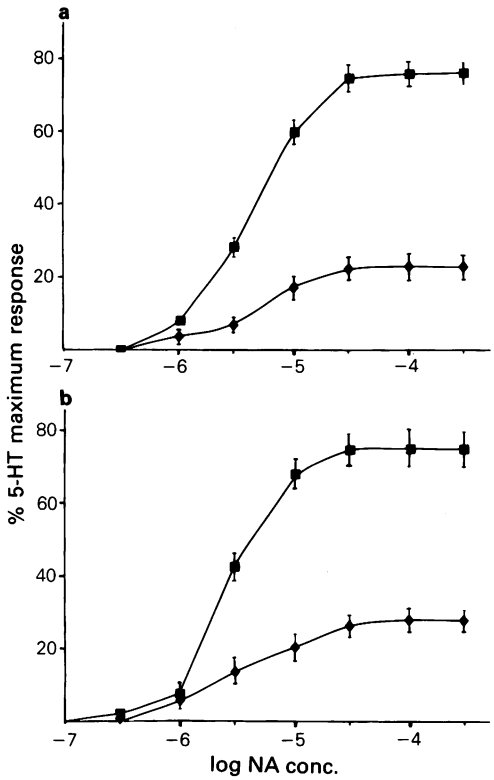

Figure 2 A comparison of the responsiveness of basilar and middle cerebral arteries (MCA), both rubbed (a) and unrubbed (b), to noradrenaline (NA). The results are expressed as a percentage of the maximum contraction induced by 5-hydroxytryptamine (5-HT) and are shown as the mean of 6 experiments; s.e.mean indicated by vertical bars except when obscured by the symbols (◆ = basilar, ■ = MCA).

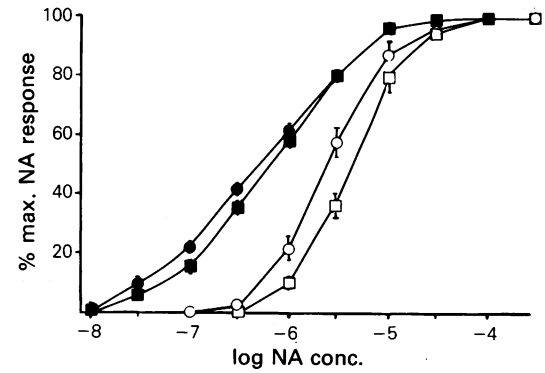

Figure 3 Modification by cocaine (10 μM) of the contractile response to noradrenaline (NA) in unrubbed and rubbed middle cerebral arteries (○, ● = unrubbed; □, ■ = rubbed). The maximum contraction elicited by NA, before the addition of cocaine, is expressed as 100%. The results shown are the mean of 6 experiments. Open symbols represent results obtained before cocaine and solid symbols represent the results obtained in the presence of cocaine (10 μM). The s.e.mean are represented by vertical bars, when not obscured by the symbols used.

and effective contractile agent on all the arteries. NA did not produce as great a contractile response as 5-HT though most of the arteries responded in a similar way (the NA-induced maximum contraction, expressed as a percentage of the 5-HT (3 μM) induced maximum contraction, was $74.7 \pm 4.4\%$, $78.1 \pm 3.6\%$, $91.6 \pm 8.3\%$ and $85.4 \pm 1.5\%$ in the circle of Willis, MCA, pial and anterior cerebral arteries respectively, $n > 4$). The response to NA, relative to 5-HT, was exceptionally low in the basilar artery compared to the other arteries investigated (approximately 20–30% of the 5-HT maximum). The MCA was taken to be representative of the other arteries and was used for comparison with the basilar artery in all the subsequent studies carried out. A further investigation of the pial branches was also carried out to determine whether there was any difference between the adrenoceptors of the MCA, a major conducting vessel, and these smaller arteries.

Endothelium and noradrenaline uptake studies

The magnitude of the NA-induced contraction, relative to the 5-HT-induced contraction, was much smaller in the basilar artery than in the MCA (Figure 1). Removal of the endothelium caused a significant reduction in the maximum contraction of the sheep cerebral arteries to NA, however the response to 5-HT was also significantly reduced with the endothelium removed (Table 1). The responses to NA and 5-HT were reduced by similar proportions (Figure 2) which suggests that this reduced ability to contract may have been caused by mechanical damage to the blood vessel wall. There was also a tendency for the rubbed arteries to be less sensitive (higher pD_2) to NA than the unrubbed arteries though this was not found to be significant (Table 1).

The addition of cocaine caused a significant potentiation in the responsiveness of the MCA to NA in both the rubbed and unrubbed arteries ($P < 0.01$, Figure 3) without increasing the maximum response. In the basilar artery cocaine did not significantly affect the potency of NA in either the rubbed or unrubbed artery (Table 1).

Receptor characterization

The α_1-adrenoceptor antagonist prazosin exerted a concentration-dependent displacement of the NA

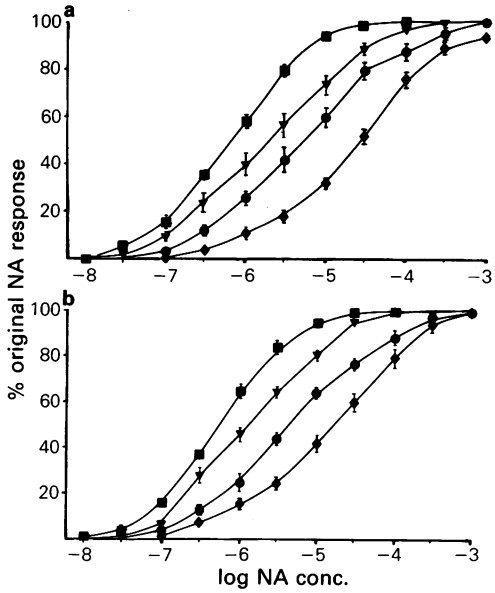

Figure 4 Contractile responses of sheep middle cerebral (a) and pial arteries (b) to noradrenaline (NA) and their modification by prazosin (▼ = 1 nM, ● = 3 nM, ◆ = 10 nM). Cocaine (10 μM) was present throughout. Results are expressed as the mean of 6 experiments. The s.e.mean are represented as vertical bars, except when obscured by symbols.

concentration-response curve to the right in both the MCA and pial artery (Figure 4). Its potency in both the MCA ($pA_2 = 9.76 \pm 0.13$, slope $= 0.96 \pm 0.13$, $n = 6$) and pial arteries ($pA_2 = 9.59 \pm 0.04$, slope $= 1.00 \pm 0.04$, $n = 6$) is indicative of prazosin having a selective and competitive blocking action at α_1-adrenoceptors. Preliminary results with prazosin (10 nM) in the circle of Willis and anterior cerebral arteries gave pA_2 values of 9.8 ± 0.3 ($n = 4$) and 9.3 ± 0.3 ($n = 4$) respectively. In the basilar artery prazosin (1 nM) caused a marked antagonism of the NA response which was also suggestive of an action at α_1-adrenoceptors. The action of prazosin was so pronounced in the basilar artery that there was difficulty in obtaining a measurable contraction to the highest concentrations of NA which prevented calculation of the prazosin pA_2 value for this artery.

The α_2-adrenoceptor agonist UK14304 did not produce any contractile response in the basilar, MCA or pial arteries in concentrations up to 100 μM. Neither the addition of cocaine nor removal of the endothelium affected this result.

Discussion

The results of this study demonstrated a marked difference in the action of NA on various arteries in the sheep cerebrovascular bed. In the basilar artery the maximal contraction to NA was less than in the MCA or pial branches but the pD_2 for NA was similar in all the branches tested. However, the potency of NA in the MCA and pial arteries was increased following inhibition of neuronal uptake with cocaine. This potentiation did not occur in the basilar artery which suggests that the low reactivity of NA in this artery is not because of a high neuronal uptake. The removal of the endothelium did not potentiate the NA response which suggests that NA did not cause a significant release of EDRF in the sheep cerebral vasculature. The possibility that NA, but not 5-HT, causes release of an endothelium-dependent contracting factor (Usui *et al.*, 1987) seems unlikely as there was a significant reduction in the size of the 5-HT-induced contraction as well. The removal of the endothelium did not significantly affect the potency of NA in the arteries. It therefore seems more likely that, in sheep cerebral arteries, the lowered contractile effect of NA is due to mechanical damage either to the medial layer or to smooth muscle cells during the rubbing process.

The lack of effect of the α_2-adrenoceptor agonist UK14304 suggests that there is no significant population of α_2-adrenoceptors in any of the sheep cerebral arteries investigated. This conclusion is supported by the finding that the NA-induced response was entirely mediated by α_1-adrenoceptors in the MCA and pial artery as judged by the pA_2 and slope for prazosin. Although it was not possible to determine a pA_2 value for prazosin in the basilar artery, 'the α-adrenoceptor is likely to be of the α_1-subtype since UK14303 was unable to elicit a response and prazosin completely eliminated the NA-induced contraction.

It has been suggested that the poor contractile response to NA in the cerebral arteries of many species is due to the properties of the α_2-adrenoceptor. However, our results show that this explanation is not valid for the sheep. The prazosin experiments showed that α_1-receptors predominate in the sheep MCA, basilar and pial arteries. The sheep basilar artery had a markedly smaller response to NA than the other cerebral arteries, but this was not because of neuronal uptake of noradrenaline or the release of vasodilator mediators from the endothelium. The possibility that endothelial removal could increase the maximum response to certain agonists cannot be discounted because the mechanical damage that the rubbing of these small arteries causes reduces their maximum contractile force. The most probable explanations for the poor responsiveness of the sheep basilar artery are either a lower adrenoceptor number (Laher & Bevan, 1985a) or an inferior coupling of the adrenoceptors to the calcium signalling process.

This study has shown that a variation exists in the response to NA in different regions of the sheep cerebrovascular bed. Inhibition of neuronal uptake potentiated the response to NA in the MCA and pial artery but had no effect on the sheep basilar artery. Noradrenaline caused contraction by activation of α_1-adrenoceptors in the MCA and pial artery and probably in the basilar artery as well. The potency of NA did not appear to be affected by the release of endothelium-derived mediators in any of the cerebral arteries studied.

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A patch-clamp study of effects of dihydroavermectin on *Ascaris* muscle

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1 Effects of bath-application of the anti-parasitic agent 22,23 dihydroavermectin B_{1a} (DHAVM 20 fM to 0.2 μM) on outside-out and inside-out patches from collagenase-treated muscle membranes of *Ascaris suum* were examined.

2 DHAVM was tested as a γ-aminobutyric acid (GABA) agonist by application to outside-out patches. DHAVM failed to open characteristic GABA channels even when high concentrations (20 nM to 0.2 μM) were used. These high concentrations of DHAVM also failed to potentiate opening of channels activated by application of 3–4 μM GABA; instead they acted to depress GABA-activated channel currents by reducing mean conductances and P_{open}.

3 In outside-out patches, low concentrations of DHAVM (1 pM to 100 pM) produced opening of 0–13 channels, the number depending on the patch. The progressive opening of channels gave rise to a 'Staircase' effect. The conductance of these channels was 9–15 pS and open times were long (greater than 100 mS). Ion substitution experiments showed these channels to be permeable to Cl. The channels were not blocked by 20 μM picrotoxin. There was a long delay (greater than 15 s) between DHAVM application and channel opening; this delay and lipophilic nature of DHAVM suggested a site of action in the lipid phase of the membrane.

4 The effect of bath-application of low concentrations of DHAVM on inside-out patches was investigated under low-Ca conditions (to avoid Ca-activated Cl channels). DHAVM (20 fM to 1 nM) did not produce opening of Cl channels but produced opening after a long delay (mean 2.5 minutes) of noisy cation-selective channels which had conductances of 5–30 pS.

5 The actions of DHAVM are discussed.

Introduction

Avermectins, a family of highly lipid soluble macrocyclic lactones, derived from *Streptomyces avermitilis* (Burg *et al.*, 1979), have a biological activity against parasitic nematodes and insects but not against tape worms, flukes or their hosts (Egerton *et al.*, 1979; Campbell & Benz, 1984). The selective action and potency of avermectins means that these drugs can be used effectively in animals for treating a wide range of nematode infestations including intestinal nematodes like *Ascaris suum* (Campbell & Benz, 1984). In man an avermectin is used to control the nematode *Onchocerca volvulus* which causes river blindness.

The naturally occurring avermectins are separated into four major groups (A_{1a}; A_{2a}; B_{1a}; B_{2a}) and four minor groups (A_{1b}; A_{2b}; B_{1b}; B_{2b}). The B groupings are more active biologically. A synthetic B derivative (22-23 dihydroavermectin, known as ivermectin) has been developed for animal use.

One suggested mode of action for avermectins is that they are γ-aminobutyric acid (GABA) agonists or potentiators (Wang & Pong, 1982; Wright, 1986). This hypothesis has the advantage of explaining the immobilizing effect on nematode parasites and the reported lack of effect on parasites which do not possess GABA receptors; it can also explain the increase in membrane Cl conductance which has been associated with the action of avermectins (Fritz *et al.*, 1979; Duce & Scott, 1985; Lees & Beadle, 1986). However, this interpretation does not explain why avermectins can increase Cl conductances in preparations not sensitive to GABA (Duce & Scott, 1985; Lees & Beedle, 1986).

The effects of 22,23 dihydroavermectin B_{1a} (DHAVM) have been investigated on isolated patches of the bag region of muscle membrane from the nematode parasite of the pig, *Ascaris suum*. This part of the muscle is not innervated but has been

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shown to possess extrasynaptic GABA-activated Cl channels (Martin, 1980; 1985). It is shown that DHAVM is not a GABA agonist or potentiator but produces opening of channels permeable to Cl when applied to outside-out patches; DHAVM does not open Cl channels but produces non-selective cation channels when applied to inside-out patches under low-Ca conditions. Preliminary accounts of some of this work have appeared (Martin & Pennington 1988; 1989).

Methods

The methods have been described in detail previously (Martin, 1985) but are outlined below.

The preparation

Specimens of *Ascaris suum* were collected from the local abattoir and maintained in Locke solution (replaced daily) at 32°C in a water bath. The *Ascaris* were used for experiments within four days. The 2 cm flap preparations were pinned onto 'Sylgard' in an experimental chamber maintained at 22°C. Clean membranes were prepared by use of collagenase (Type 1A Sigma; $1\,mg\,ml^{-1}$) for 10 to 60 min, depending on the preparation.

Solutions

The preparations were maintained in a Ringer solution containing (mM): NaCl 140, KCl 2.0, $CaCl_2$ 5, $MgCl_2$ 2.0, glucose 3.0 and HEPES 10, pH 7.2 with NaOH. Electrodes for outside-out patches were filled with (mM): CsCl 140, $CaCl_2$ 1, $MgCl_2$ 2, EGTA 11, HEPES 10, pH 7.2 with CsOH. Electrodes for inside-out patches were filled with (mM): CsCl 150, $MgCl_2$ 5, HEPES 10, pH 7.2 with CsOH for low-Ca conditions. Once an isolated patch had been obtained the bath solution was changed as indicated in the results. Tris (5 mM) with maleic acid was used instead of HEPES for pH control of isolated patches in some experiments with no apparent effect on the observations.

Recording

Standard patch-clamp techniques were used to obtain outside-out or inside-out patches. Patch-electrodes were made from micro-haematocrit capillary tubes (Garner Glass 7052) and had a resistance of 1–3 mΩ: 'Sylgard' was used for coating the electrodes to improve frequency responses (typically greater than 2 kHz). Currents were recorded using a List EPC-7 and a modified Sony digital audio processor with a Sony Betamax video system for making records on tape. An agar-plug bath electrode was used to minimize changes in junction potentials as a result of solution changes. Despite this, small changes (3 mV) in junction potential still occurred as a result of changing Cl concentrations in the bath. Corrections were made for this effect when estimating reversal potentials.

Data processing

Records were analysed by playing back records on tape into a CED 1401 interface connected to DCS 286 PC computer and using the PAT software (Dr J. Dempster, Strathclyde University). Amplitude histograms produced by this software express the time spent at particular current levels as a percentage of the total signal time analysed. It does not equate simply with the relative frequency at which the various conductance states occur but does allow estimates of the proportion of time the channel passes particular levels of current. Attempts to fit these current amplitude histograms by multiple Gaussian functions to identify the probability of observing distinctive subconductance states were not generally successful because the subconductance states were not sufficiently separated. Cumulative distributions of open channel conductances were therefore constructed to illustrate the probability of observing conductance levels. Slope conductances from channel I/V plots were obtained by linear regression. Relative permeabilities of ions were calculated from reversal potentials (Hille, 1984).

Drugs

DHAVM was obtained as gifts from Pfizer Central Research and Merck, Sharp and Dhome. DHAVM was usually dissolved in dimethylsulphoxide (DMSO) to make a 1 μM stock which was maintained at −12 C°; it was diluted in Ringer, with 0.1% DMSO present as the carrier, to the concentrations described in the results. In some experiments ethanol was used as a carrier. The rest of the chemicals were purchased from Sigma.

Effect of the solvent

Most studies with avermectins have used DMSO (1% to 2%) as a carrier. Kass *et al.* (1984) reported that 1% DMSO had no effect on electrophysiological recordings. Duce & Scott (1985) found that in the leg muscle of locust DMSO (0.5 to 4%) increases input conductance and hyperpolarizes the membrane by increasing K but not Cl conductance. They also reported that at 2% DMSO, there was a reduction of GABA-induced Cl conductance changes. In this

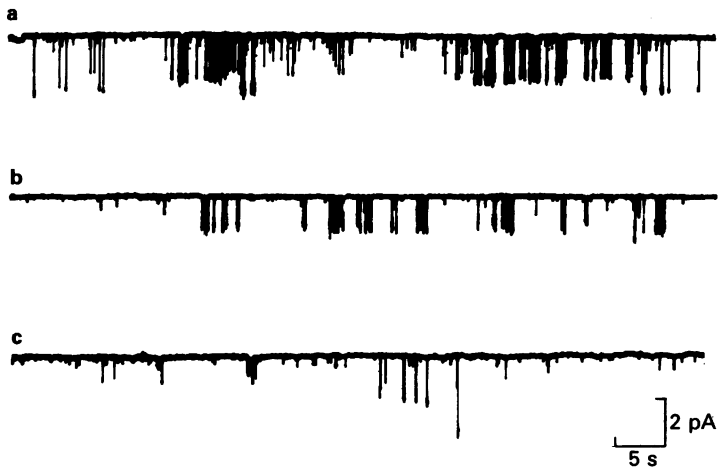

Figure 1 Effect of DHAVM (20 nM and 200 nM) on GABA-activated channels. (a) Control effect of 4 μM GABA; (b) in the presence of 4 μM GABA and 20 nM DHAVM; (c) in the presence of 4 μM GABA and 200 nM DHAVM. Outside-out patch, membrane potential: (a) and (b) -50 mV; (c) -75 mV. Pipette solution (mM):CsCl 140, $MgCl_2$ 2, $CaCl_2$ 1, EGTA 11, Tris 5, pH 7.2. Bath solution (mM):NaCl 140, KCl 2, $MgCl_2$ 2, Tris 5, glucose 3, $CaCl_2$ 5, pH 7.2.

study we used 0.1% DMSO; control experiments showed that it was without effect. As a further control 0.1% ethanol was also used as a carrier; no difference in the results was observed. Control applications of solutions containing carrier but no drug did not open channels in either outside-out or inside-out patches.

Results

The following experiments were carried out on 104 patch preparations from muscle cell bag regions. Effects of DHAVM (20 fM to 0.2 μM) were examined using 24 outside-out and 80 inside-out patches.

DHAVM is not a GABA agonist but acts as an antagonist

Wang & Pong (1982) have suggested that avermectins may act as GABA agonists or potentiators. It was possible to test this in *Ascaris* using the extra-synaptic GABA receptors present on the bag region of the somatic muscle. GABA agonists combine with extra-synaptic receptors on the outside membrane and open Cl channels which have conductances of 15 to 40 pS and mean open times of less than 35 ms; bursts and clusters of openings are also expected (Martin, 1985). DHAVM was therefore tested as a GABA agonist by applying it in the bath to outside-out patches. DHAVM in concentrations as high as 0.2 μM failed in all cases to open characteristic GABA channels.

DHAVM was also tested as a GABA potentiator. Outside-out patches were used and GABA channels activated initially by bath-application of 3 to 4 μM agonist; DHAVM (20 nM to 0.2 μM) was then added as well. The results of a typical experiment are shown at a low time resolution in Figure 1. The top trace shows GABA channels at -50 mV in the presence of agonist alone; the second trace shows GABA channels at -50 mV in the presence of 20 nM DHAVM; the bottom trace shows the GABA channels at -75 mV in the presence of 0.2 μM DHAVM. It can be seen that there was a dose-dependent reduction in the frequency of channel opening and that the amplitude of the channel currents was depressed in the presence of DHAVM; there was no potentiation.

The depressive effect of DHAVM on GABA channel currents at a higher time resolution is illustrated in Figure 2 for the same experiment as above. Figure 2a shows a typical GABA channel burst and current amplitude histogram in the presence of GABA alone (control): the current amplitude histogram had a peak at 1.8 pA:P_{open} was 0.2 with the channel conductance peak at 36 pS. Figure 2b shows the same in the presence of 20 nM DHAVM: P_{open} was 0.13 with the channel conductance peak at 28 pS. Figure 2c shows the same in the presence of 0.2 μM DHAVM:P_{open} was 0.11 with the channel conductance peaking near 20 pS.

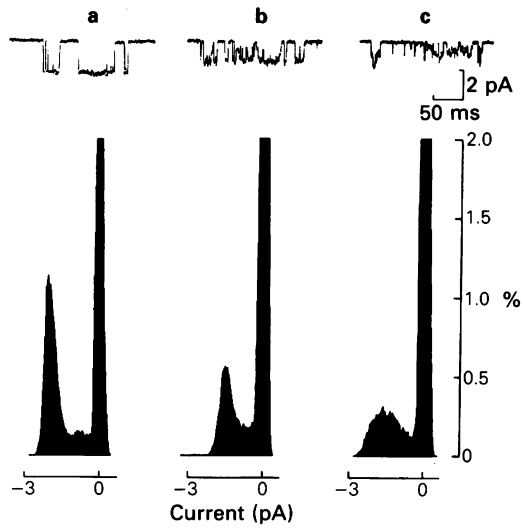

Figure 2 GABA-activated Cl channels and current amplitude histograms. (a) 4 μM GABA; −50 mV. (b) 4 μM GABA and 20 nM DHAVM; −50 mV. (c) 4 μM GABA and 200 nM DHAVM; −75 mV. From the same patch recording as Figure 1.

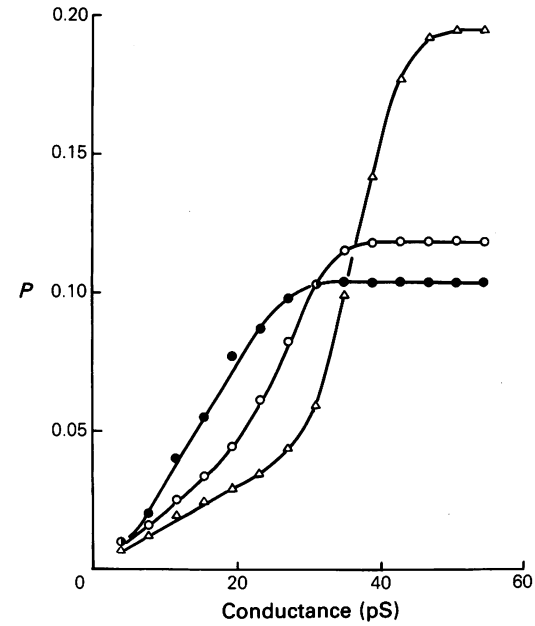

Figure 3 Effect of DHAVM on GABA-activated channel cumulative-conductance distributions; (△) 4 μM GABA, −50 mV; (○) 4 μM GABA and 20 nM DHAVM; −50 mV; (●) 4 μM GABA and 200 nM DHAVM, −75 mV. Distributions from the data shown in Figure 2. The ordinate scale is the probability of the channel being open (greater than 5 pS) and having a conductance less than or equal to that shown on the abscissa scale.

GABA channel currents are characterized by the presence of multiple conductance states (Bormann *et al.*, 1987); subconductance levels are also seen in *Ascaris* (Martin, 1985). It was possible that DHAVM may affect the relative proportion of time spent at different conductance levels. The effect of DHAVM on GABA channel conductance was examined by looking at cumulative distributions of channel conductances greater than 5 pS (i.e. open). Figure 3 illustrates these distributions which were obtained from the same experiments as those shown in Figures 1 and 2. These observations consistently showed that one effect of DHAVM is to depress the amplitude of the channel conductance and to reduce the proportion of time spent at higher conductance levels. The depressive effect of DHAVM on GABA channel currents shows that DHAVM acts as a blocker/antagonist on the *Ascaris* muscle GABA receptor.

Activation of channels in outside-out patches by DVAHM in the absence of GABA

In the experiments described in the previous section where DHAVM was added by itself to outside-out patches to test for a GABA agonist action, it was noticed that DHAVM produced open times and long burst durations with mean open times greater than 100 mS; some open times lasted for several seconds. The channels were readily distinguished from GABA-activated channels which had much shorter open times and showed shorter bursts and clusters of bursts.

Low concentrations (2 pM to 0.1 nM) of DHAVM were also tested and found to open 0–13 of these channels depending on the patch. The most dramatic response seen in four patches was the progressive opening of channels by DHAVM which gave rise to a 'Staircase' effect (Figure 4a and b). There was always a long delay between the addition of DHAVM to the bath and the onset of channel opening; this delay was much longer than required for GABA to open channels. Figure 4a shows the application of 2 pM DHAVM (vertical arrow) to an outside-out patch at −75 mV: 1.1 pA channels (conductance: 14.6 pS) were produced after a delay of 66 s (star); a second channel was activated after 80 s; a third channel was activated after 134 s. The mean open channel lifetime was measured as 164 ms. Figure 4b shows the opening of 13 channels after the addition of 20 nM DHAVM; the opening of channels starts after 32 s.

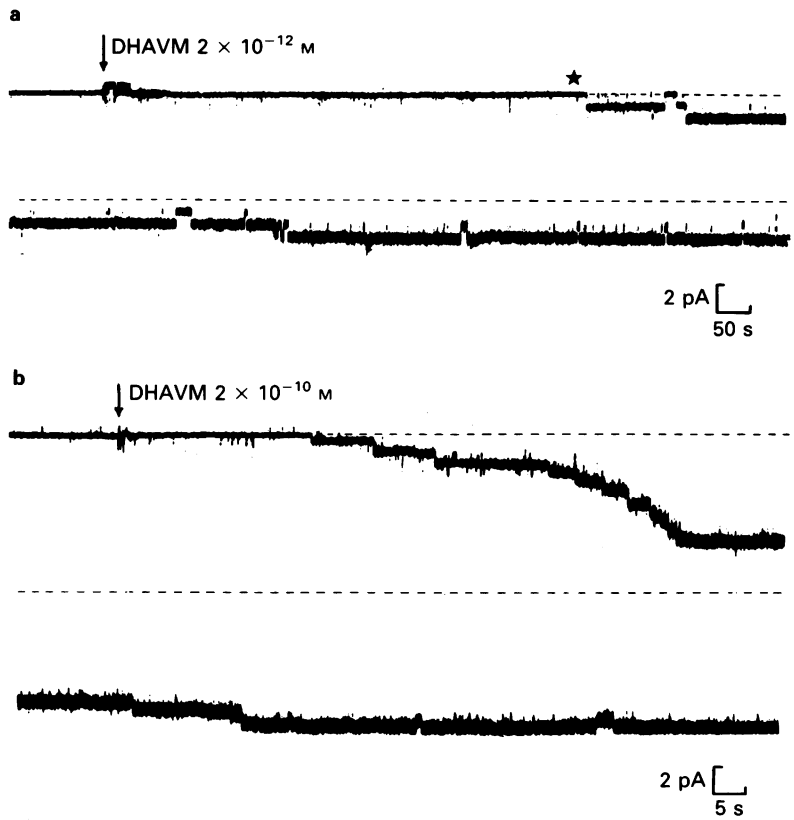

Figure 4 Staircase production of channels in outside-out patches by DHAVM. The dashed lines show the closed states. Pipette and bath solutions as for Figure 1. (a) −75 mV; 2 pM DHAVM after a delay of 66(*), 80 and 134 s produces opening of channels. The mean open time of 164 ms was measured during the period when only one channel was open. (b) −50 mV; 200 pM DHAVM produces 13 channels (from 32 to 86 s).

The permeability of the channels involved was investigated in three outside-out patches by examining the effect of Cl ion substitution. Figure 5 shows the I/V plot of DHAVM-activated channels obtained from the experiment shown in Figure 4b, first with 156 mM Cl and then with 51 mM Cl on the outside of the membrane. The channel conductance was reduced from 11.6 ± 0.3 pS (mean ± s.e.) to 9.1 ± 0.4 pS while the reversal potential shifted from near 0 mV to +23 mV. These potentials are close to the predicted values (of −1.7 and +26.7 mV respectively) of the Nernst equation for Cl and are consistent with the channel being permeable to Cl. Prolonged washing in DHAVM free Ringer containing 0.1% DMSO did not reverse channel opening; this was not unexpected since DHAVM is lipophilic. Picrotoxin (20 μM) applied after activation of the channels did not block the channel currents.

In order to estimate further the proportion of patches that contained Cl channels activated by DHAVM, a series of experiments on 14 isolated inside-out patches with 100 pM DHAVM present in the patch pipette was carried out. Low-Ca conditions were used with asymmetric Cl concentrations (reversal potential = −33.5 mV) to allow identification of Cl channels. Cl channels with conductances and open times similar to those described above were seen in 4 (29%) of the patches.

DHAVM activated channels when applied to inside-out patches

The effect of DHAVM (20 fM to 1 nM) on inside-out patches was tested in low-Ca conditions to avoid Ca-activated Cl channels (Thorn & Martin, 1987). Inside-out patches were formed with pipettes filled with 150 mM CsCl as the main constituent; Ca was not added. The patches were perfused with a bath Ringer which contained EGTA to buffer Ca to less than 10^{-8} M. Under these conditions it was found

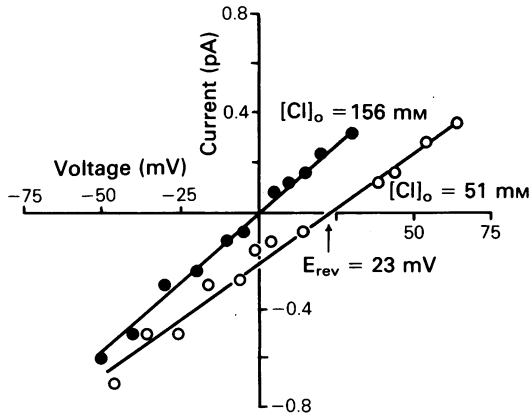

Figure 5 DHAVM-activated channel I/V plots from an outside-out patch in different Cl concentrations. Pipette solution (mM):CsCl 140, $MgCl_2$ 2, $CaCl_2$ 1, EGTA 11, Tris 5; pH 7.2 156 mM-Cl bath solution (mM):NaCl 140, KCl 2, $MgCl_2$ 2, Tris 5, glucose 3, $CaCl_2$ 5, pH 7.2. 51 mM-Cl bath solution (mM):NaCl 35, Na acetate 105, KCl 2, $MgCl_2$ 2, Tris 5, glucose 3, $CaCl_2$ 5, pH 7.2. The slope conductance in 156 mM. $[Cl]_0$ was 11.6 ± 0.3 pS (mean ± s.e.) with a reversal potential near 0-mV. In 51 mM-$[Cl]_0$ the slope conductance was reduced to 9.1 ± 0.4 pS (mean ± s.e.) and the reversal potential was +23 mV. Ringer-agar plug bath electrodes were used and corrections (3 mV) made for changes in junction potentials on solution change.

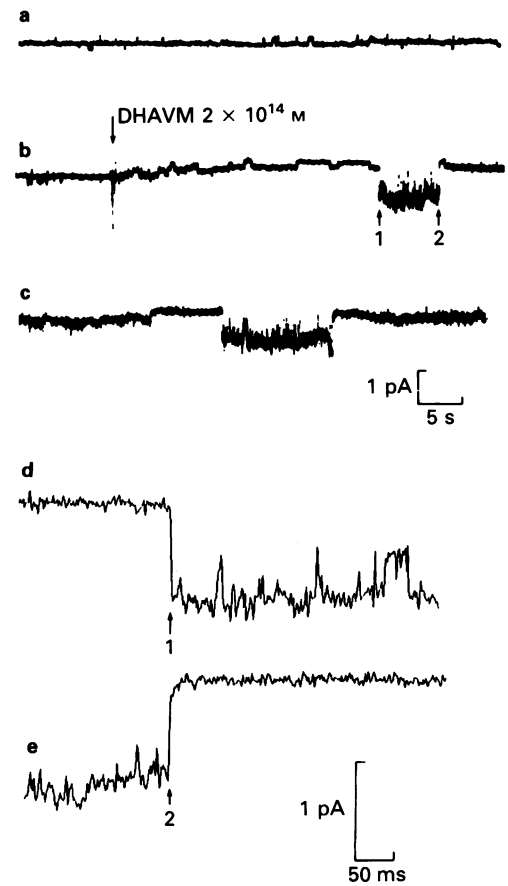

Figure 6 Production of channels in an inside-out patch by DHAVM, −50 mV. Pipette solution (mM):CsCl 150, HEPES 10, $MgCl_2$ 5, pH 7.2. Bath solution (mM):CsCl 150, HEPES 10, EGTA 11, $CaCl_2$ 1, 0.1% DMSO, pH 7.2. (a) Control recording, note the presence of spontaneous channels which have a low conductance; (b) 20 fM DHAVM added rapidly to the bath produces a long channel burst (6.7 s, vertical arrows 1 and 2) after a delay of 28 s, similar bursts follow; (c, d and e) show the burst of (b) at a higher time resolution.

that DHAVM (20 fM–1 nM) produced opening of 'noisy' channels in 52 of 66 patches, usually after a long delay (mean 2.5 min). The results of a typical experiment are shown in Figure 6: after the addition of 20 fM DVAVM there is a 28 s delay which is followed by a long 6.7 s channel burst. Subsequently similar noisy channel bursts followed. Figure 6d and e shows the channel burst of Figure 6b at a higher time resolution and illustrates further the noisy nature of the open channel.

Figure 7 shows current amplitude histograms of three DHAVM bursts obtained from the same experiment as Figure 6. The histograms consistently showed several peaks indicating the presence of multiple subconductance states. The range of subconductance states here was between 8 and 28 pS. The most common state had a conductance of 20 pS. The presence of multiple conductance states appeared to be the origin of the channel noise.

DHAVM-activated channels in inside-out patches are cation selective

The permeability of channels produced by DHAVM in inside-out patches was routinely examined by increasing the concentration of CsCl in the bath from 150 mM to 300 mM. Under these conditions it is predicted from the Nernst potential that a channel selective for Cs will have a reversal potential of −17.6 mV; a channel selective for Cl will have a reversal potential of +17.6 mV. The I/V plots in Figure 8 show that the effect on reversal potential was to produce a shift of −16.5 mV and to increase the conductance of the channel from 19.7 to 22.3 pS. The potential shift was close to that predicted by the Nernst equation for Cs. Similar results were obtained from all inside-out patches. It was con-

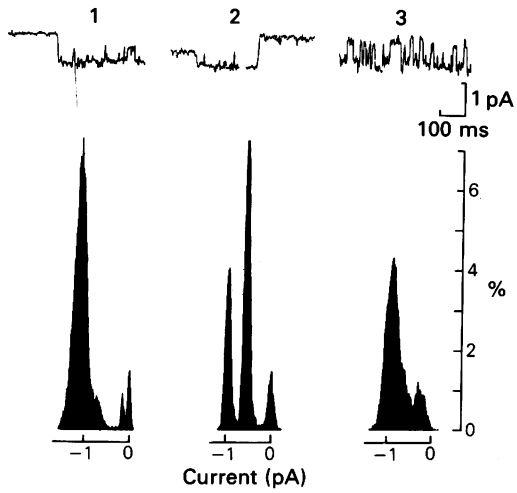

Figure 7 Three representative DHAVM channel bursts at high time resolution and current amplitude histograms from the same experiment as in Figure 6. Histograms are for a 7 s length of recording and show multiple subconductance states. The closed state is marked as zero current.

cluded that these channels were selective for Cs. The Cl/Cs permeability ratio was then calculated from the equation modified from Hille (1984):

$$E = RT/F \ln \frac{Cs_o + \alpha\, Cl_i}{Cs_i + \alpha\, Cl_o}$$

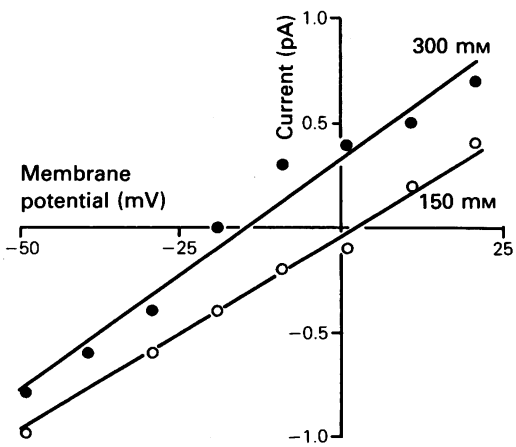

Figure 8 Effect of bath CsCl concentration on channel I/V plots. Results from the same experiment as Figure 6: (○) with 150 CsCl; (●) with 300 mM CsCl. Slope conductances 19.7 pS (○) and 22.3 pS (●). Shift in reversal potential −16.5 mV. Permeability ratio: Cl/Cs = 0.03.

where E = −16.5 mV; Cs_o is 150 mM, the concentration of Cs in the pipette; Cs_i is 300 mM, the concentration of Cs in the bathing Ringer; Cl_o is 150 mM, the concentration of Cl in the pipette; Cl_i is 300 mM, the concentration of Cl in the bathing Ringer; α is the Cl/Cs permeability ratio; R, T and F have their usual meaning. α is then estimated to be 0.03. Results obtained from other experiments were similar with α being less than 0.1: no evidence for activation of channels selectively permeable to Cl was ever seen under low-Ca conditions in inside-out patches. No 'Staircase' effect was seen in any of these inside-out patches.

Permeability sequence of the cation channel

In order to predict possible effects of the cation channel we looked at the permeability of the channel to different cations (in four preparations). The permeability sequence was found to be: Cs > Rb > K > Na > Li > Ca (Table 1) which is an Eisenman Type 1 series (Hille, 1984). This is compatible with a channel whose permeability is determined by dehydration energies of the permeant ions and which has a 'weak-field-strength' binding site controlling permeability; Cs is most easily dehydrated and therefore the most permeant. This channel is permeable to K, Na and Ca and can therefore be described as a 'non-selective cation channel'. The conductance sequence of the channel was not evaluated because of the presence and complication of subconductance states.

Discussion

We have shown that DHAVM depresses the opening of GABA channels and can produce the progressive opening of channels permeable to Cl with a conductance of 9–15 pS when applied to outside-out patches. DHAVM did not open Cl channels when applied to the inside of inside-out patches under low-Ca conditions. There was always a long delay between the onset of effect and application of the drug.

Depressive effects of DHAVM on GABA channel conductances

It has been reported previously that GABA channel current may exist in a number of subconductance states (Bormann *et al.*, 1987). GABA channel currents in *Ascaris* also show subconductance states (Martin, 1985). The separation of the subconductance states in this study was not usually sufficient to allow fitting of multiple Gaussian curves to

Table 1 Shifts in reversal potentials and relative permeability to cations shown by DHAVM-activated channels recorded in an inside-out patch in low-Ca conditions

	Cs^+	Rb^+	K^+	Na^+	Li^+	Ca^{2+}
Reversal potential (mV)	−14.3	−10.7	−5.6	+9.5	+10.7	−4.3
Relative permeability (P_x/P_{cs})	1.0	0.87	0.71	0.39	0.37	0.32

Pipette solution (mM):CsCl 150, HEPES 10, pH 7.2. Bath solution initially (mM):CsCl 150, HEPES 10, EGTA 11, $CaCl_2$1, 0.1% DMSO; pH 7.2. The 150 mM CsCl in the bath solution was then replaced by 300 mM CsCl and the shift in reversal measured as −14.3 mV. The other cations (X) using a 300 mM concentration of the Cl salt were then tested. The permeability relative to Cs was calculated from the equation:

$$E_{rev,Cs} - E_{rev,X} = RT/F \cdot \ln \frac{P_x[X]}{P_{Cs}[Cs]}$$

where E_{rev} was the observed reversal potential with the 300 mM concentration of the Cl salt present. The relative permeability of Ca was estimated from a modified form of this equation appropriate for divalent cations (Lewis, 1979). The main channel conductance varied between 22 pS and 10 pS in the different cation solutions but the conductance sequence was not evaluated because of the presence and complication of subconductance states.

the open channel current amplitude histograms. However the histograms showed that the depressive effect of DHAVM was associated with a reduction in the proportion of time the channels spend at the higher conductance levels. One explanation for this is that DHAVM decreased the relative frequency of higher conductance states. Alternatively DHAVM could produce states of very rapid opening and closing beyond the time resolution of the recording system; this could also appear to reduce the mean channel current.

Long delay before effect

The delay between effect and application observed here is interesting. It seems likely that DHAVM works in the lipid phase of the membrane because of its hydrophobic properties; the long delay may occur as DHAVM passes from the bathing solution into the membrane. The relationship between DHAVM concentration and the latency of channel opening was not obvious. Despite this we observed differences between outside-out and inside-out patches suggesting that DHAVM may not easily traverse the bilayer. It is unlikely that DHAVM acts in the aqueous phase. Similar long delays have been described for DHAVM in *Ascaris* (Kass *et al*; 1984) where effects on the nerve cord were examined and took 40 min to reach a maximum. In the locust preparation (Duce & Scott, 1985) DHAVM acted slowly on the non-GABA muscle bundle 33 with Cl conductances increasing over a period of at least 60 min.

Dose-dependent and reversible effects of DHAVM

We were not able to demonstrate clear-cut dose-response relationships for DHAVM. This was because of the practical problems of the finite life time of patch recording, the long delay and non-stationary nature of the response; DHAVM responses usually continued to increase with time in responsive patches. Reversibility was not seen after prolonged washing in DHAVM free Ringer containing 0.1% DMSO. However we did find in two patches that prolonged washing of the preparation with 4% DMSO in DHAVM-free Ringer produced a reduction in the number of channels open. We interpreted this observation as showing that the response may be reversed.

Effects of DHAVM on electrical properties of Ascaris suum

Effects of bath-application of avermectin on the electrical properties of *Ascaris* muscle flap preparations using intracellular recording electrodes have been reported by Holden-Dye *et al.* (1988). Avermectin was found to inhibit GABA-evoked increases in conductance and the associated membrane hyperpolarization with an apparent affinity constant in the mid-micromolar range. The depressant effect on GABA channel currents described here is consistent with these observations but was observed at concentrations 10 to 20 times lower. The difference in concentration may be related to the removal of connective tissue with collagenase for the preparation of 'clean' membranes for patch formation. It is unlikely that the mode of action of DHAVM as an antiparasitic agent involves only a GABA antagonistic effect on the muscle receptor as the action of DHAVM on the whole parasite would then be to produce muscle contraction rather than the observed paralysis (Wright 1986).

Another reported effect of 1 μM avermectin (Holden-Dye *et al.* 1988) was the block of sponta-

neous spiking activity after a delay of 25 min; this block appeared to be Cl-dependent since it did not occur in Cl-free Ringer. However no change in membrane potential or conductance was detected. The activation of low conductance Cl channels as reported here may underly this effect. The density of these Cl channels in this study appeared to be low since they were not present in large numbers in most patches; avermectin induced conductance changes may go undetected in the bag region of the muscle when using intracellular techniques. The very low concentrations of DHAVM required to activate these channels suggests these channels as a therapeutic site of action; this effect may explain the paralysing action of avermectins on nematodes. It is possible that when DHAVM acts on the whole worm that it also has an effect on the nervous system (Kass *et al.*, 1984) which could contain the same Cl channel as described here in the muscle.

Cation channel in inside-out patches

The therapeutic significance of the cation channels produced in inside-out patches is difficult to evaluate at this stage since it is not known if or how DHAVM moves across lipid bilayers. DHAVM may act via the inner membrane and bind with and open non-selective cation channels in the membrane. Alternatively DHAVM may act via the inner membrane as a pore-former to produce the channel itself.

Activation of the cation channel in intact cell preparations could produce depolarization and spiking since the relative permeability of Na/K is equal to 0.49. Although no depolarizing effect has been reported in *Ascaris*, Lees & Beadle (1985) have reported that application of DHAVM produces an excitatory effect in addition to an increase in Cl conductance in some cultured insect neurones.

Movement of Ca through the DHAVM cation channel could produce secondary changes as a result of an increase in cytoplasmic Ca. The secondary effects could include activation of Ca-activated Cl channels in *Ascaris* (Thorn & Martin, 1987) or the release of transmitter. Ca-dependent effects of DHAVM reported in other preparations include: a Ca-dependent potentiation of the frog endplate potential and miniature endplate potential frequency (Fritz *et al.*, 1979); release of GABA, dopamine, noradrenaline and acetylcholine from rat brain synaptosomes (Wang & Pong, 1982); and a potentiation of GABA-mediated transmission in rats (Williams & Yarborough, 1979). Some of these results might be explained by DHAVM producing non-selective cation channels. The relationship between the effect of DHAVM on non-selective cation channels and paralysis of the nematode parasites is uncertain.

Conclusion

This study has shown in a nematode parasite preparation that DHAVM is not a GABA agonist or potentiator and that its mode of action may involve the opening of non-GABA-activated Cl channels and non-selective cation channels. The speed of onset of effects is slow.

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S-nitrosothiols as vasodilators: implications regarding tolerance to nitric oxide-containing vasodilators

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1 The formation of an S-nitrosothiol compound, S-nitroso-N-acetylcysteine (SNAC) has recently been proposed to mediate the augmentation of the anti-aggregatory and haemodynamic effects of glyceryl trinitrate observed in the presence of N-acetylcysteine. This study investigated the effects on an isolated coronary artery preparation of acute and prolonged exposure to S-nitrosothiol compounds and nitric oxide (NO).

2 Single doses of NO and of the S-nitrosothiol compounds, SNAC and S-nitroso-N-acetylpenicillamine (SNAP), induced rapid, but transient, relaxations in U46619-contracted bovine isolated coronary artery rings. Peak relaxation responses to SNAP and NO were attenuated in the presence of N-acetylcysteine, cysteine, ascorbic acid and methylene blue. The duration of the relaxation responses to SNAC was two to three times longer than those to SNAP and NO. In the presence of N-acetylcysteine (but not cysteine, ascorbic acid or methylene blue) the duration of the relaxation responses to SNAP and NO (but not to SNAC) was markedly increased. H.p.l.c. assay confirmed that, in the presence of N-acetylcysteine, SNAP and, to a lesser degree, NO were converted to the relatively more stable and longer acting vasodilator, SNAC.

3 When compared to control rings, coronary artery rings superfused with glyceryl trinitrate were subsequently markedly less responsive to the vasodilator actions of glyceryl trinitrate, whereas responsiveness to SNAC or NO was only marginally reduced. On the other hand, coronary artery rings superfused with SNAC or NO were subsequently less responsive to glyceryl trinitrate, SNAC and NO. Thus prolonged vascular exposure to SNAC or NO induced a form of tolerance different from that induced with glyceryl trinitrate and which is possibly associated with impaired guanylate cyclase activity.

4 Coronary artery rings superfused with NO were markedly less responsive to glyceryl trinitrate and NO, whereas responses to the endothelium-dependent vasodilator A23187 and to theophylline were not significantly attenuated.

5 It is concluded that formation of the more stable vasodilator SNAC occurs on incubation of N-acetylcysteine with SNAP or NO. While coronary artery responsiveness to SNAC and NO is virtually unchanged in the presence of glyceryl trinitrate-induced tolerance, after prolonged exposure to SNAC or NO tolerance may develop to these vasodilators with cross-tolerance to glyceryl trinitrate but not A23187. Thus, formation or therapeutic utilization of SNAC may acutely circumvent the problem of glyceryl trinitrate-induced tolerance but, during prolonged vascular exposure to SNAC, attenuation of vascular responsiveness may occur to a wide range of vasodilators.

Introduction

Tolerance to glyceryl trinitrate and related organic nitrate vasodilators poses a significant clinical problem which may limit the long-term efficacy of these agents in the management of patients with angina pectoris or congestive heart failure (Abrams, 1986; Horowitz & Henry, 1987; Packer *et al.*, 1987). Previous studies have indicated that glyceryl trinitrate, as well as other nitric oxide (NO)-containing vasodilators, activate soluble guanylate cyclase via an extensive cascade of biochemical events (Ignarro *et al.*, 1981; 1984) and that the resultant increase in intracellular cyclic guanosine 3',5'-monophosphate

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(cyclic GMP) concentrations induces vasodilatation. Tolerance to glyceryl trinitrate is associated with reduced metabolic bioconversion, possibly reflecting reduced conversion to dinitrates and nitrite, the initial metabolic step (Brien *et al.*, 1986).

As a number of other agents have been shown to activate guanylate cyclase without undergoing this initial bioconversion (Ignarro *et al.*, 1984), it might be anticipated that the development of pharmacological tolerance to these agents might be limited or avoided. However, little information is available in this regard, with virtually no clinical studies currently published.

Several investigations have suggested that glyceryl trinitrate may combine with tissue sulphydryls, inducing the formation of S-nitrosothiols (Ignarro *et al.*, 1981), which combine vasodilator (Kowaluk *et al.*, 1987) and anti-platelet effects (Loscalzo, 1985). In a number of clinical investigations, the short-term efficacy of intravenously infused glyceryl trinitrate has been increased and glyceryl trinitrate-induced tolerance reversed in the presence of the sulphydryl donors N-acetylcysteine and methionine (Horowitz *et al.*, 1983; Packer *et al.*, 1987; Levy *et al.*, 1988). This change has been attributed to formation of S-nitroso-N-acetylcysteine (SNAC; Loscalzo, 1985; Fung *et al.*, 1988). The question arises as to whether SNAC or other S-nitrosothiols might be useful therapeutically and, in particular, whether or not the phenomenon of haemodynamic tolerance to organic nitrate vasodilators might be ameliorated by their use.

In the current experiments, we describe some aspects of the vasodilator activity of two S-nitrosothiols, SNAC and S-nitroso-N-acetylpenicillamine (SNAP). In particular, we examined the acute vasodilator effects of SNAP, SNAC and NO and their modulation by agents including N-acetylcysteine, cysteine, ascorbic acid and methylene blue. In addition the effects of continuous vascular exposure to SNAC or NO on subsequent vascular responses to NO-containing and endothelium-dependent vasodilators were compared to the effects of tolerance induced by prolonged exposure to glyceryl trinitrate. The results of these investigations, while supporting a potential therapeutic role for S-nitrosothiols, reveal the existence of a further site of tolerance in the organic nitrate bioconversion cascade, potentially inhibiting the vasodilator actions of these, and related, agents.

Methods

Bovine isolated coronary artery rings

Hearts from freshly slaughtered cattle were placed in cold Krebs-bicarbonate solution and transferred to the laboratory (within 30 min). The left anterior descending coronary artery was removed from surrounding myocardium and cut into 3 mm long segments. Bovine isolated coronary artery rings were suspended under 4 g resting tension in a water-jacketed organ bath containing 10 ml Krebs-bicarbonate solution (pH 7.4) bubbled with 95% O_2 plus 5% CO_2. The composition of the Krebs-bicarbonate solution was (in mM): NaCl 117, KCl 5.36, $MgSO_4$ 0.57, KH_2PO_4 1.03, $NaHCO_3$ 25.0, $CaCl_2$ 2.5 and glucose 11.1. Changes in coronary artery ring tension were measured by Grass FTO3-C force-displacement transducers connected to a Grass Model 7D Polygraph. Rings were equilibrated for at least 45 min before any drug addition.

Unless otherwise stated, effects of vasodilators were determined in coronary artery rings contracted with the thromboxane analogue U46619 (EC_{70}–EC_{80} concentration, range 0.05–0.2 μM). Within 15 min, U46619-induced contractions reached a plateau level of elevated tone that was maintained for several hours in the absence of vasodilator drugs.

Single dose experiments

Addition of a single dose of either SNAP, SNAC or NO induced a rapid relaxation response which, after attaining a peak response, returned spontaneously to the previous level of elevated tone. The peak response was expressed as percentage reversal of the U46619-induced contraction. The period between the attainment of, and recovery to, 50% of the peak response was used as the measure of the 'duration of relaxation' induced by the vasodilator. We examined the effect of several agents (N-acetylcysteine, cysteine, ascorbic acid and methylene blue) on the peak response and the 'duration of relaxation' induced by the vasodilators. In these experiments, coronary artery rings were exposed to a single dose of vasodilator at a concentration which induced a peak response of between 30 and 70%. On recovery to the previous level of elevated tone, rings were exposed to N-acetylcysteine (100 μM), cysteine (100 μM), ascorbic acid (30 μM) or methylene blue (3 μM) and 2 min later re-exposed to the same single dose of vasodilator. The peak response was variably reduced in the presence of each of these agents. To overcome this inhibition (such that the peak response induced by the vasodilator in the presence of inhibitor was comparable to peak response observed in its absence) rings were re-exposed to a second, higher concentration of vasodilator. A comparison of this relaxation response with the initial relaxation response (i.e. when no inhibitor was present) was used to determine the effect of these agents on the 'duration of relaxation' induced by the vasodilators. A control

preparation was repeatedly exposed to a single dose of vasodilator to monitor any spontaneous changes in coronary artery responsiveness.

Superfusion experiments

We examined the effects of continuous coronary artery exposure to SNAC, NO and glyceryl trinitrate on subsequent vascular responses to a series of vasodilators. To minimize the potential loss of activity of these agents that may occur during prolonged incubation with vascular tissue bathed in oxygenated Krebs-bicarbonate solution, coronary artery rings were superfused for 60 min with Krebs-bicarbonate solution (4 ml min^{-1}, 37°C, carbogenated) into which SNAC, NO or glyceryl trinitrate was infused (20 μl min^{-1}) immediately before contact with the rings. A paired control ring was concomitantly superfused with Krebs-bicarbonate solution that did not contain SNAC, NO or glyceryl trinitrate. The concentrations of glyceryl trinitrate (10 μM), SNAC (3 μM) and NO (5 μl NO stock solution ml^{-1} in Krebs-bicarbonate solution) in the superfusate were chosen on the basis of data obtained from preliminary experiments. In these preliminary experiments, the EC_{50} values (95% confidence limits, number of preparations) of glyceryl trinitrate, SNAC and NO for relaxation of coronary artery rings superfused with a Krebs-bicarbonate solution containing 30 mM KCl were respectively: 0.13 μM (0.086–0.19, $n = 12$), 0.042 μM (0.015–0.12, $n = 6$) and 0.049 μl (0.034–0.072, $n = 6$) NO stock solution ml^{-1} in Krebs-bicarbonate solution. Thus, the concentrations of glyceryl trinitrate, SNAC and NO chosen for tolerance induction in the superfusion experiments were approximately 100 times greater than their respective EC_{50} values obtained for relaxation of control coronary artery rings. Following the 60 min superfusion, coronary artery rings were placed in an organ bath containing Krebs-bicarbonate solution and repeatedly washed for 15 min. Rings were then exposed to U46619 and upon attainment of a plateau level of contraction (15 min) a single cumulative concentration-effect curve to glyceryl trinitrate, NO, SNAC, A23187 or theophylline was completed.

Formation of SNAC; h.p.l.c. studies

Solutions of N-acetylcysteine (0, 10, 100 μM) and ascorbic acid (100 μM) prepared in Krebs-bicarbonate solution were bubbled with 95% O_2 plus 5% CO_2 and maintained at 37°C. To these solutions, SNAP (22.7 μM) was added, and aliquots were taken at 0.5 and 2 min for the determination of SNAP, SNAC and N-acetylpenicillamine concentrations. These aliquots (40 μl) were analysed by h.p.l.c. by use of a C_{18}-μBondapak column (Waters Associates) and a Model 450 variable wavelength detector (Waters Associates) set to detect absorbance at 214 nm. The mobile phase consisted of 30% methanol and 70% ammonium phosphate buffer (10 mM, pH 2.5) with a flow rate of 0.8 ml min^{-1}. The retention times for SNAC, N-acetylpenicillamine and SNAP were respectively 2.5, 3.5 and 8 min. Standard curves were derived for SNAC, N-acetylpenicillamine and SNAP based on peak heights. For h.p.l.c. determination of SNAC formation from reaction mixtures of N-acetylcysteine (100 μM) and NO at 37°C in Krebs-bicarbonate solution, a mobile phase consisting of 12% methanol and 88% ammonium phosphate buffer (10 mM, pH 2.5) was used. The retention time for SNAC under these conditions was 6 min. All other conditions for h.p.l.c. assay of SNAC were as described above.

Preparation of solutions of SNAC and NO

SNAC was prepared by reacting NO with a solution of N-acetylcysteine under acidic, O_2-free conditions. Gas-tight vials containing 10 mM N-acetylcysteine in HCl (pH 2.0) were evacuated and then bubbled with N_2 for 30 min at room temperature (approximately 20°C). The N-acetylcysteine solution was cooled to 4°C and bubbled with 50 ml NO gas over a 3 min period, during which time the solution turned from colourless to rose-pink. The vial was placed on ice for 20 min and then bubbled with N_2 for 20 min to remove NO. Dilutions of the SNAC solution were made in gas-tight vials containing HCl (pH 2.0) that had been evacuated and bubbled with N_2 for 30 min at room temperature. Saturated solutions of NO were prepared in gas-tight vials under O_2-free conditions. Vials containing 10 mM K_2HPO_4 (pH 6.0) were bubbled with N_2 for 30 min at room temperature and then cooled to 4°C. Solutions were then bubbled for 4 min with NO gas (50 ml).

Drugs

SNAP was a gift from Professor L.J. Ignarro (UCLA). Solutions of SNAP were prepared in ethanol (bubbled with N_2) and stored in gas-tight vials on ice. Glyceryl trinitrate (Merck, Darmstadt) was stored as a 10 mM solution in ethanol, and dilutions were made into saline. Solutions of A23187 (Sigma) were stored and diluted in ethanol. Theophylline, methylene blue, cysteine, N-acetylcysteine, N-acetylpenicillamine were purchased from Sigma and dilutions were made into water, unless otherwise stated. U46619 ((15S)-hydroxy-11α,9α-(epoxymethano)prosta-5Z,13-dienoic acid) was purchased from Caymen Chemical Company (Denver);

1 mg ml^{-1} stock solutions of U46619 in ethanol were stored at −20°C and dilutions to 1 μM were made in 2.4 mM $NaHCO_3$.

Statistics

Results are expressed as mean ± s.e.mean. For statistical evaluation of the effects of drugs or pretreatment on vasodilator concentration-effect curves, one-way analysis of variance with repeated measures was completed by means of the programme BMDP2V of the BMDP series (Dixon & Brown, 1977). For comparison of means, analysis of variance was performed followed by Student's t test for unpaired observations. The Bonferroni method was used for multiple comparisons (Wallenstein *et al.*, 1980). P values less than 0.05 were regarded as significant.

Results

Effects of N-acetylcysteine, cysteine, ascorbic acid and methylene blue on vascular responses to S-nitrosothiols and NO

In U46619-contracted bovine isolated coronary artery rings, single doses of SNAP induced reproducible, concentration-dependent relaxations that were of rapid onset and transient duration (Figure 1a and b). SNAP-induced relaxations were significantly attenuated ($P < 0.05$) in the presence of either N-acetylcysteine or ascorbic acid (Figures 1c, d and 2b) whereas neither N-acetylcysteine nor ascorbic acid had any significant effect on concentration-effect curves to glyceryl trinitrate or to the endothelium-dependent vasodilator, A23187 (Figure 2a and c). Although N-acetylcysteine reduced the peak relaxation response produced by single, submaximal concentrations of SNAP, the 'duration of relaxation' was significantly enhanced in the presence of N-acetylcysteine (Figure 3a and c). Similarly, N-acetylcysteine inhibited peak relaxation responses to NO but prolonged its 'duration of relaxation' ($P < 0.05$), although to a lesser extent than that observed to SNAP (Figure 3b). On the other hand, inhibition of peak responses to SNAP by ascorbic acid, cysteine or methylene blue was not associated with any significant change in the 'duration of relaxation' produced by SNAP (Figure 3a). As shown in Figure 3b, the 'duration of relaxation' produced by SNAC was significantly greater ($P < 0.001$) than that produced by either SNAP or NO alone and moreover, was similar to that produced by SNAP in the presence of N-acetylcysteine. In the presence of 100 μM N-acetylcysteine, peak relaxation responses

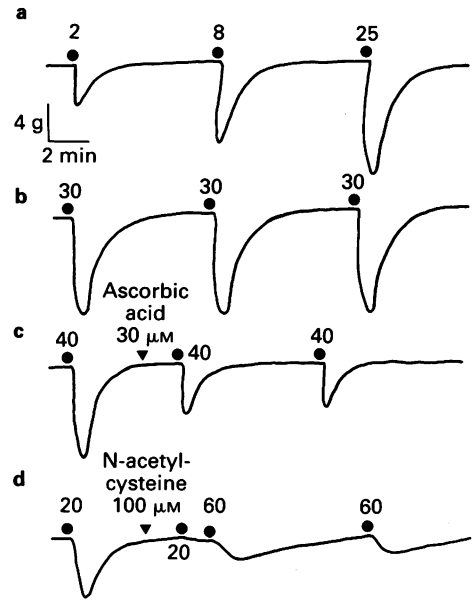

Figure 1 Isometric tension recordings of S-nitroso-N-acetylpenicillamine (SNAP)-induced relaxations in U46619-contracted bovine isolated coronary artery rings. Single doses of SNAP (●) induced rapid relaxation responses that returned to the previous level of induced tone without washout of the coronary artery preparation. SNAP-induced relaxations were concentration-dependent (a), reproducible (b) and were inhibited by ascorbic acid (c) and N-acetylcysteine (d). Numbers refer to SNAP concentrations in nM.

induced by SNAC were slightly inhibited. However, the 'duration of relaxation' was not affected (Figure 3b and c).

Formation of SNAC from N-acetylcysteine

As shown in Figure 4, N-acetylcysteine and ascorbic acid markedly enhanced the breakdown of SNAP with the concomitant formation of N-acetylpenicillamine. In the presence of N-acetylcysteine, but not ascorbic acid, there was also rapid formation of SNAC. Generation of SNAC was also observed during the co-incubation of N-acetylcysteine and NO, although the extent of SNAC generation was considerably less than that observed during incubation of SNAP with N-acetylcysteine.

Effect of prolonged vascular exposure to glyceryl trinitrate, SNAC and NO on subsequent vascular responses to selected vasodilators

Bovine isolated coronary artery rings superfused for 60 min with glyceryl trinitrate, SNAC or NO were significantly less responsive ($P < 0.05$) to subse-

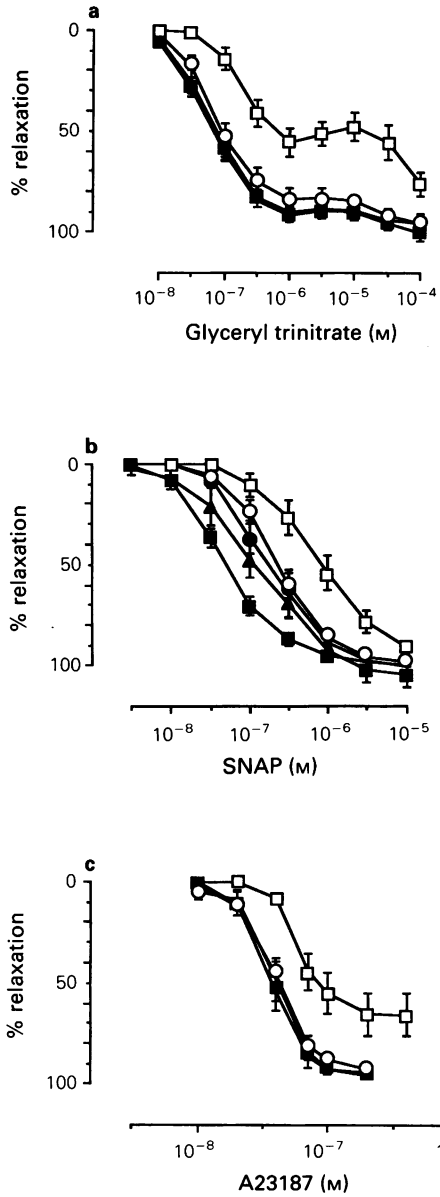

Figure 2 Mean cumulative concentration-effect curves to glyceryl trinitrate (a), S-nitroso-N-acetylpenicillamine (SNAP) (b) and A23187 (c) in the absence (■) and presence of 10 μM N-acetylcysteine (●), 100 μM N-acetylcysteine (○), 30 μM ascorbic acid (▲) and 3 μM methylene blue (□) in U46619-contracted bovine isolated artery rings. Shown are the mean responses from 6–12 coronary artery rings. Vertical lines indicate s.e.mean. In these experiments, N-acetylcysteine, ascorbic acid or methylene blue were added 5 min before the start of the vasodilator concentration-effect curve.

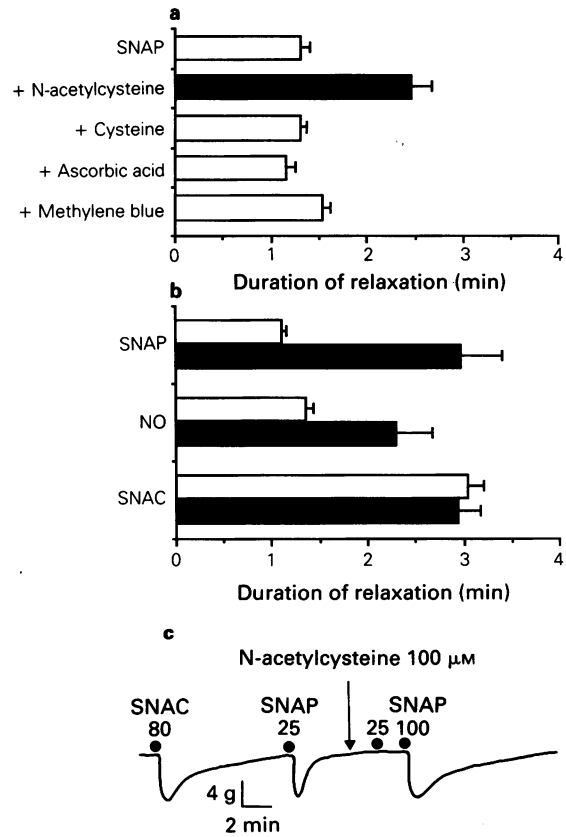

Figure 3 (a) Effect of N-acetylcysteine (100 μM), cysteine (100 μM), ascorbic acid (30 μM) and methylene blue (3 μM) on the 'duration of S-nitroso-N-acetylpenicillamine (SNAP)-induced relaxations'. The 'duration of relaxation' was estimated from SNAP-induced relaxations that produced, on average, 52% relaxation of the U46619-induced contraction. Shown are the mean of responses from 9–15 experiments. (b) 'Duration of relaxations' induced by SNAP, NO and SNAC in the absence (open columns) and presence (solid columns) of 100 μM N-acetylcysteine. Shown are the mean of responses from 6–10 experiments. (c) Isometric tension recording in a single coronary artery showing responses to S-nitroso-N-acetylcysteine (SNAC), and to SNAP in the presence and absence of 100 μM N-acetylcysteine (▼). Numbers refer to SNAP and SNAC concentrations in nM. In (a) and (b) bars show s.e.mean.

quently administered glyceryl trinitrate than were paired control rings (Figure 5). Relaxations induced by SNAC and NO were markedly attenuated ($P < 0.01$) in rings superfused with SNAC or NO, respectively, but only marginally attenuated in rings superfused with glyceryl trinitrate (Figure 6). Although rings superfused with NO were subsequently significantly less responsive to NO than were

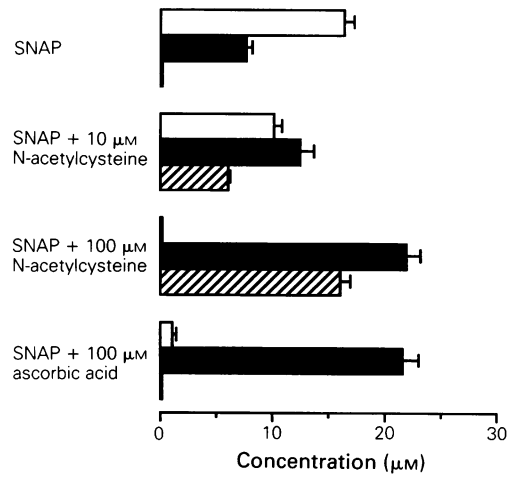

Figure 4 Concentrations of S-nitroso-N-acetylpenicillamine (SNAP) (open columns), N-acetylpenicillamine (solid columns) and SNAC (cross-hatched columns) determined from aliquots of reaction mixtures of SNAP alone (22.7 μM), and SNAP in the presence of either N-acetylcysteine (10 or 100 μM) or ascorbic acid (100 μM). Shown are the mean concentrations ($n = 4$) determined after a 2 min incubation in Krebs-bicarbonate solution bubbled with 95% O_2 plus 5% CO_2 at 37°C. Bars shows s.e.mean.

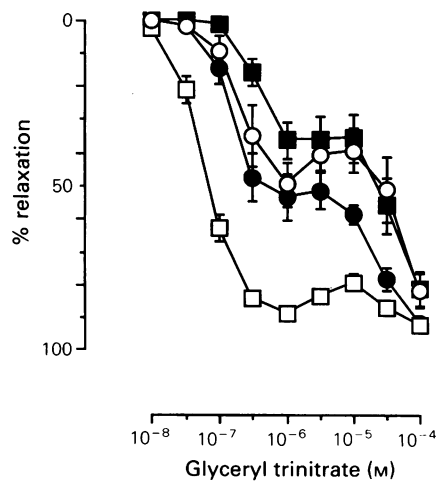

Figure 5 Mean concentration-effect curves to glyceryl trinitrate in control coronary artery rings (□) and in rings superfused for 60 min with a Krebs-bicarbonate solution containing glyceryl trinitrate (10 μM, ●), S-nitroso-N-acetylcysteine (SNAC) (3 μM, ■) or NO (5 μl NO stock ml^{-1} in Krebs-bicarbonate solution, ○). Shown are the mean responses from 8–20 experiments. Vertical lines indicate s.e.mean.

control rings ($P < 0.01$), relaxations induced by A23187 were only marginally attenuated in rings pre-exposed to either NO or glyceryl trinitrate (Figure 7). Similarly, theophylline was not significantly less potent in coronary artery rings superfused with glyceryl trinitrate, SNAC or NO than in control rings (Figure 7).

Discussion

S-nitrosothiol compounds have previously been shown to be potent activators of guanylate cyclase (Ignarro *et al.*, 1981) and have been postulated to be intermediates of the vasodilator and anti-aggregatory effects produced by NO-containing compounds (Loscalzo, 1985; Fung *et al.*, 1988; Stamler *et al.*, 1988a). Two S-nitrosothiols of particular interest are SNAP and SNAC. Ignarro *et al.* (1981) showed that SNAP has a relatively greater stability than S-nitrosothiols derived from cysteine, mercaptoethylamine and 3-mercaptopropanoic acid and is a potent vasodilator *in vitro* and *in vivo*. In addition, we have previously shown that, whereas the vasodilator activities of glyceryl trinitrate, isosorbide dinitrate, sodium nitroprusside and 3-morpholinosydnonimine are markedly reduced in coronary arteries pre-exposed to glyceryl trinitrate, the vasodilator activity of SNAP is essentially unimpaired (Henry *et al.*, 1989). More recent attention has focussed on the actions of SNAC. The extracellular formation of SNAC has been postulated to be responsible for the enhanced vasodilator and anti-aggregatory effects observed for glyceryl trinitrate in the presence of N-acetylcysteine (Loscalzo, 1985; Fung *et al.*, 1988). We were therefore interested in examining further the vasodilator effects of SNAP and SNAC, with particular emphasis on their formation and interconversion, and also on the effects of prolonged vascular exposure to S-nitrosothiol compounds.

Administration of a single dose of SNAC and, more particularly, SNAP to a constricted coronary artery ring induced a rapid, but transient, relaxation response. The brief duration of relaxation was most probably due to the decomposition of SNAP and SNAC rather than to the desensitization of the vascular tissue to their vasodilator actions because, firstly, re-exposure of the tissue to subsequent doses of S-nitrosothiol produces similar vasodilator actions, and, secondly, S-nitrosothiol compounds are generally unstable chemically and often undergo thermal decomposition at room temperature (Oae & Shinhama, 1983).

Peak relaxation responses induced by SNAP were significantly inhibited not only in the presence of the thiol compounds N-acetylcysteine and cysteine, but

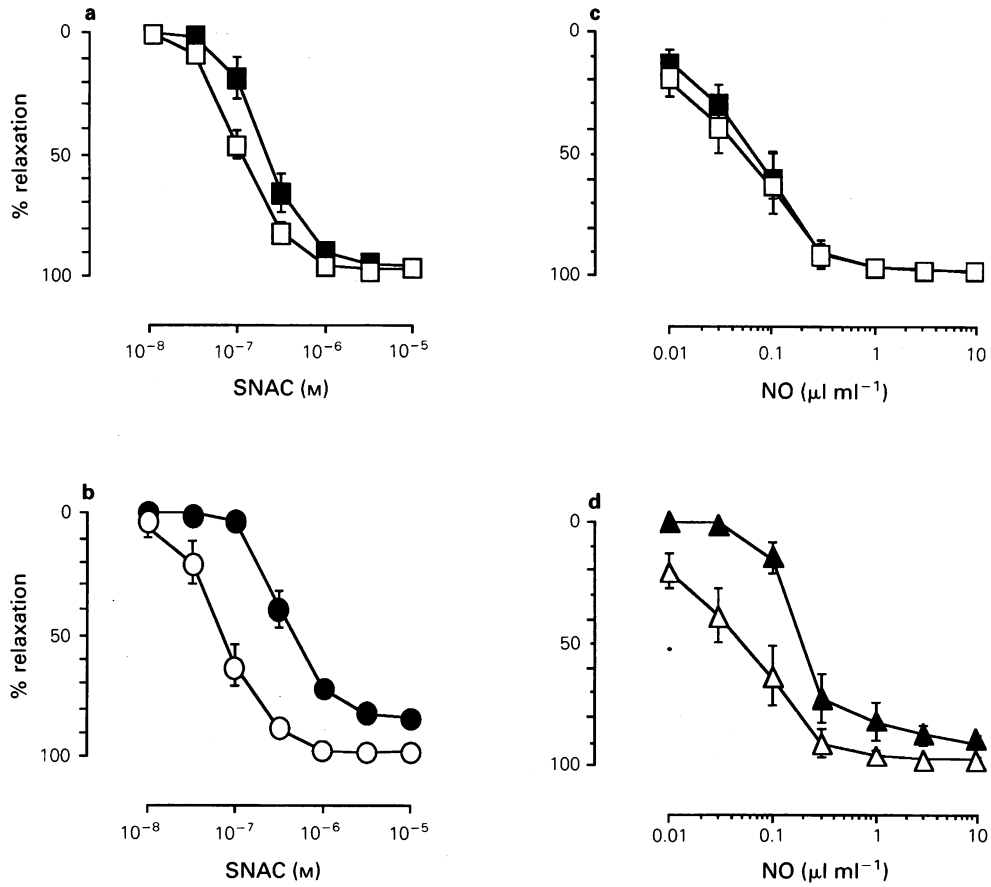

Figure 6 Mean concentration-effect curves to (a, b) S-nitroso-N-acetylcysteine (SNAC) and (c, d) NO in control rings (open symbols) and in rings superfused for 60 min with Krebs-bicarbonate solution containing (a, c) glyceryl trinitrate (10 μM, ■), (b) SNAC (3 μM, ●) or (c) NO (5 μl NO stock ml^{-1} in Krebs-bicarbonate solution, ▲). Shown are the mean responses from 6–7 experiments. Vertical lines indicate s.e.mean.

also in the presence of the non-thiol anti-oxidant ascorbic acid. Although co-incubation of SNAP with N-acetylcysteine markedly inhibited peak relaxation responses, the 'duration of relaxation' to SNAP was prolonged two to three fold. No such prolongation was observed during the co-incubation of SNAP with either the related thiol cysteine or with ascorbic acid. Similarly, methylene blue, an inhibitor of soluble guanylate cyclase, also inhibited peak relaxation responses to SNAP without affecting its 'duration of relaxation'. In view of these results, we investigated the possibility that N-acetylcysteine, cysteine and ascorbic acid inhibited the peak relaxation responses to SNAP by promoting SNAP decomposition, and that the prolongation of SNAP-induced relaxations by N-acetylcysteine was due to the formation of a significantly longer acting vasodilator, SNAC. Consistent with this proposal was the initial observation that relaxations induced by SNAC were of significantly greater duration than those induced by SNAP and, moreover, were of similar duration to those induced by SNAP in the presence of N-acetylcysteine. With the use of the h.p.l.c. assay we confirmed that co-incubation of SNAP and N-acetylcysteine increased the rate of breakdown of SNAP, with the concomitant formation of N-acetylpenicillamine and SNAC. Under the experimental conditions studied, the interconversion of SNAP to SNAC was almost complete within 30 s. At present the transfer of the NO moiety from an S-nitrosothiol to a thiol compound is not well documented and, in general, reactions between S-nitrosothiol and thiol compounds have been used as convenient methods for preparing mixed disulphides

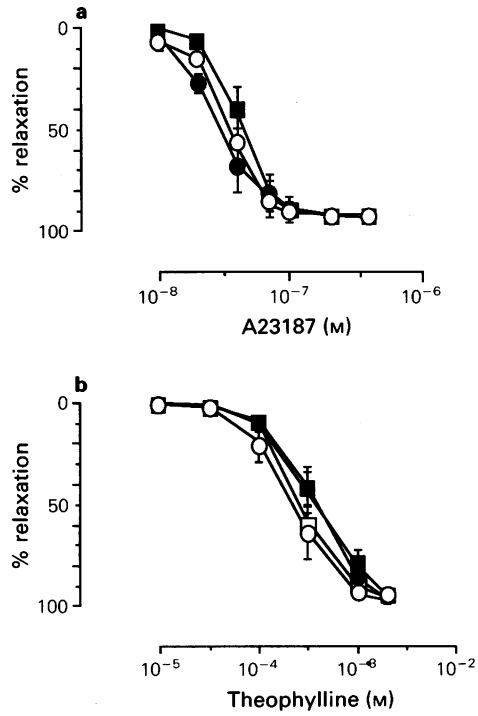

Figure 7 Mean concentration-effect curves to (a) A23187 and (b) theophylline in control coronary artery rings (●) and in rings superfused for 60 min with Krebs-bicarbonate solution containing glyceryl trinitrate (10 μM, ■), SNAC (3 μM, □) or NO (5 μl NO stock ml^{-1} in Krebs-bicarbonate solution, ○). Shown are the mean responses from 5–7 experiments. Vertical lines indicate s.e.mean.

rather than S-nitrosothiols (Oae & Shinhama, 1983). In the current study we have not examined the potential formation of SNAC from the incubation of N-acetylcysteine with S-nitrosothiols prepared from thiols of physiological significance, such as cysteine or glutathione, although such a reaction may be expected with S-nitrosocysteine given its relatively greater chemical instability than SNAP (Ignarro *et al.*, 1981).

In view of the recent findings that endothelium-derived relaxing factor (EDRF) is probably NO (Palmer *et al.*, 1987), we examined the possibility that incubation of N-acetylcysteine with NO also produced SNAC. Whereas N-acetylcysteine and NO react rapidly in an acidic, anaerobic environment to form SNAC, its formation was significantly less at neutral pH in an oxygenated aqueous buffer. However, in view of the recent findings of Fung *et al.* (1988), that SNAC is readily generated during co-incubation of glyceryl trinitrate and N-acetylcysteine in rat and human plasma but not in aqueous buffer, we cannot exclude the possibility that significant amounts of SNAC would not be produced from NO in the presence of N-acetylcysteine *in vivo*. Furthermore, such SNAC generation might contribute to the recently described augmentation of anti-aggregatory effects of EDRF in the presence of N-acetylcysteine (Stamler *et al.*, 1988b).

In addition to investigating some acute effects of S-nitrosothiol compounds and NO on an isolated coronary artery preparation, we also examined some effects of prolonged exposure to these agents on the vasculature. When compared to control coronary artery rings, those superfused with a physiological solution containing SNAC or NO were subsequently markedly less responsive to the vasodilator actions of these compounds and of glyceryl trinitrate. On the other hand, coronary artery ring responses to SNAC and NO were not impaired by prolonged superfusion with glyceryl trinitrate. These findings indicate that the tolerances induced by sustained vascular exposure to glyceryl trinitrate or to either SNAC or NO were unlikely to have occurred by similar mechanisms. It has been proposed that NO-containing vasodilators share all, or parts of, a bioconversion cascade that culminates in guanylate cyclase activation, cyclic GMP generation and smooth muscle relaxation (Murad *et al.*, 1978; Ignarro *et al.*, 1981; 1984). Organic nitrate vasodilators, such as glyceryl trinitrate, are denitrated and the liberated inorganic nitrite converted to NO which directly, or via formation of S-nitrosothiols, stimulates guanylate cyclase. Sustained vascular exposure to glyceryl trinitrate has been shown to be associated with reduced denitration of glyceryl trinitrate (Brien *et al.*, 1986) and probably impaired NO formation (Henry *et al.*, 1989), but capacity for guanylate cyclase activation itself was, at most, only marginally reduced (Keith *et al.*, 1982; Schroder *et al.*, 1988; Henry *et al.*, 1989). On the other hand, sustained exposure of the vasculature to SNAC or NO has not previously been described, but in the current study it was associated with marked attenuation of responses to glyceryl trinitrate, SNAC and NO, while responses to a guanylate cyclase-independent vasodilator such as theophylline were not reduced. These results were consistent with sustained vascular exposure to SNAC or NO producing impaired guanylate cyclase activity, as has previously been reported for NO actions on soluble guanylate cyclase *in vitro* (Braughler, 1983). These findings were of particular interest for several reasons. Firstly, if NO or an S-nitrosothiol compound is an active intermediate of glyceryl trinitrate-induced vasodilatation, then the current studies indicate that the development of tolerance by glyceryl trinitrate at the proximal sites in the bioconversion cascade may act to protect guanylate cyclase from continuous exposure to high

concentrations of NO, S-nitrosothiols or both, which might otherwise impair its activity. Furthermore, these findings indicate that tolerance developed at the more distal sites in the bioconversion cascade, such as guanylate cyclase activation, will impair vascular responsiveness to a greater range of vasodilators than will glyceryl trinitrate-induced tolerance and thus, may be potentially more difficult to circumvent with agents that share this cascade of events. Secondly, the results may have some implications as regards the possible identity of EDRF as NO (Palmer *et al.*, 1987; Vanhoutte, 1987; Ignarro *et al.*, 1988), which mediates the vasodilator actions of the calcium ionophore A23187 (Zawadzki *et al.*, 1980; Furchgott, 1984). The current experiments indicated that pre-exposure of coronary artery rings to NO markedly attenuated subsequent vascular responsiveness to NO, while responsiveness to A23187 was, at most, marginally impaired. Furthermore, vascular responses to NO, more so than to A23187, were more attenuated by sustained vascular exposure to NO than by sustained exposure to glyceryl trinitrate. At present the reasons for these differences are unclear. On face value, these differences between the vasodilator actions of NO and A23187 in the face of tolerance induced by NO or glyceryl trinitrate are not entirely consistent with NO being the mediator of A23187-induced, endothelium-dependent relaxation and possibly support findings by other investigators suggesting that EDRF can be distinguished from NO (Shikano *et al.*, 1987; Dusting *et al.*, 1988; Loeb & Peach, 1988). However, other mechanisms may be involved and at present the observed differences cannot be taken as unequivocal evidence that the relaxant actions of A23187 were not mediated by release of EDRF as NO. For example, we cannot be certain that the extent of tolerance induced by the superfusion methods is the same throughout the coronary artery ring preparation, or that the NO released from the vascular endothelium by the action of A23187 and the NO added exogenously to the organ bath, stimulate identical sites to induce relaxation of the coronary arteries. The observed differences could be explained if a lesser degree of tolerance was induced at particular sites in the coronary artery that were relatively more important for mediating relaxation responses to the NO released by the action of A23187.

Finally, there are potentially important implications as regards the therapeutic application of S-nitrosothiols or of co-administered glyceryl trinitrate and N-acetylcysteine (May *et al.*, 1987; Packer *et al.*, 1987; Horowitz *et al.*, 1988) in efforts to optimize long-term vasodilator and anti-platelet effects. While several studies indicate that short-term co-administration of glyceryl trinitrate and N-acetylcysteine may partially reverse nitrate tolerance (May *et al.*, 1987; Packer *et al.*, 1987; Fung *et al.*, 1988), there is little clinical experience to date with long-term co-administration of these agents. Although it is possible that administration of sulphydryl compounds such as N-acetylcysteine will initially potentiate the haemodynamic effects of glyceryl trinitrate by generating S-nitrosothiol compounds (Fung *et al.*, 1988), the results of the current study indicate that during long-term vascular exposure to S-nitrosothiols the initial beneficial effects may not be maintained and indeed vascular responses to a wide range of NO-containing vasodilators may be markedly attenuated.

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Inhibition of N-methyl-D-aspartate- and kainic acid-induced neurotransmitter release by ω-conotoxin GVIA

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1 The role of voltage-sensitive calcium channels (VSCC) in N-methyl-D-aspartate (NMDA)- and kainic acid (KA)-evoked neurotransmitter release from rat cortical and hippocampal brain slices was evaluated by determining the effects of ω-conotoxin GVIA, an inhibitor of neuronal L- and N-type VSCC, and PN 200-110, a selective inhibitor of L-type VSCC.

2 Selective antagonists of the NMDA receptor ionophore complex, Mg^{2+}, CPP and MK-801, inhibited NMDA- but not KA-evoked release of [^{3}H]-noradrenaline from hippocampal and cortical brain slices. This suggests that cortical and hippocampal receptors are similar and that NMDA and KA act at distinct excitatory amino acid receptor subtypes.

3 [^{3}H]-noradrenaline release induced by both NMDA and KA was similarly inhibited (approximately 30%) by ω-conotoxin GVIA. In contrast, PN 200-110 had no significant effect, although there was a tendency towards inhibition.

4 The results suggest that although NMDA- and KA-receptors are pharmacologically distinct, the N-type, but not the L-type, VSCC plays a small but significant role in neurotransmitter release induced by both NMDA and KA. It remains to be determined whether the N-type VSCC are involved in the physiological and/or pathological manifestations of excitatory amino acid receptor stimulation.

Introduction

The multiplicity of excitatory amino acid (EAA) receptors has been well established (Watkins & Evans, 1981; Cotman & Iversen, 1987). Three receptor subtypes have been designated according to the stimulatory effects of relatively selective agonists: N-methyl-D-aspartate (NMDA), kainic acid (KA), and quisqualate. Stimulation of these receptors causes calcium-dependent excitatory effects reflected as membrane depolarization and evoked neurotransmitter release (Lehman *et al.*, 1985; Snell & Johnson, 1986). Glycine has been shown to enhance selectively the responsiveness of the NMDA-receptor by interacting with a strychnine-insensitive glycine site associated with the NMDA receptor ionophore complex (Johnson & Ascher, 1987; Kleckner & Dingledine, 1988). Results from electrophysiological studies suggested that receptor-coupled ion channels but not voltage-sensitive calcium channels (VSCC) contribute to EAA-induced gating of ion fluxes (MacDermott *et al.*, 1986). This was consistent with the studies of Pastuszko *et al.* (1984) and Lazarewicz *et al.* (1986) which suggested that KA- and glutamate-evoked $^{45}Ca^{2+}$ influx into synaptosomes was independent of VSCC. In contrast, Dingledine (1983) proposed that the depolarizing action of NMDA in the hippocampus involves the activation of a voltage-sensitive calcium conductance which could be partially inhibited by verapamil and D-600. Based on results of $^{45}Ca^{2+}$ influx studies, Riveros & Orrego (1986) have also suggested that EAA, as a result of their neuronal depolarizing action, may lead to the opening of VSCC. Thus, it may be that EAA indirectly activate VSCC subsequent to membrane depolarization caused by ion flux through EAA-gated channels.

Although the excitatory effects of EAA have been shown to be Ca^{2+}-dependent, it has not been consistently demonstrated that VSCC may serve as a source of Ca^{2+} to couple EAA receptor activation with a tissue response. Accordingly, the objective of the present study was to characterize pharmacologically EAA-receptor-mediated neurotransmitter

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release from brain slice preparations and to evaluate the relative contribution of VSCC to these excitator responses. [^{3}H]-noradrenaline release evoked by NMDA and KA was pharmacologically characterized by using selective NMDA-receptor antagonists, Mg^{2+} (Mayer *et al.*, 1984), MK-801 (Wong *et al.*, 1986) and CPP (Davies *et al.*, 1986). The role of VSCC in EAA-evoked [^{3}H]-noradrenaline release was examined by determining the effects of ω-conotoxin GVIA (ω-CT), an inhibitor of neuronal L- and N-type VSCC, and PN 200-110, a selective inhibitor of L-type VSCC (reviewed by Miller, 1987). In order to determine potential regional differences of EAA receptor responses, the evoked release of [^{3}H]-noradrenaline was characterized in both cortical and hippocampal brain slices. A preliminary account of this work has been published (Keith *et al.*, 1988).

Methods

Release of [^{3}H]-noradrenaline from cortical and hippocampal brain slices

Male Sprague-Dawley rats (250–300 g) were killed by decapitation and brains were rapidly removed. Hippocampal and cortical brain slices (0.25 × 0.25 mm) were prepared with a McIlwain tissue slicer (Brinkman Instruments, Westbury, N.Y., U.S.A.). The tissue slices were suspended in a Krebs-Ringer bicarbonate buffer, pH 7.4, of the following composition (mM): NaCl 134, KCl 5, $MgSO_4$ 1.2, KH_2PO_4 1.25, $CaCl_2$ 2, $NaHCO_3$ 25, glucose 10, ascorbic acid 0.17 and NaEDTA 0.03, that had been equilibrated with a mixture of 95% O_2 and 5% CO_2. The slices were incubated with 0.1 μM [^{3}H]-noradrenaline (11.3 Ci mmol^{-1}) in Krebs-Ringer solution that contained 1 μM pargyline to prevent metabolic degradation of [^{3}H]-noradrenaline by monoamine oxidase. After the incubation, slices were washed three times with approximately 50 ml of buffer. Two hundred μl aliquots of slices (equivalent to 7 mg wet weight of cortical or hippocampal tissue) were transferred into each of 20 chambers of a superfusion apparatus and were superfused at a rate of 1 ml 6 min^{-1} with Krebs-Ringer solution that contained 1 μM pargyline to block monoamine oxidase and 1 μM desipramine to prevent neuronal uptake. Following a 20 min washout period, 6 min fractions were collected. Brain slices were exposed to a single 6 min stimulus of either NMDA or KA at 56 min from the start of the superfusion. When indicated compounds were added to the superfusion fluid 18 min prior to NMDA or KA exposure and remained in the medium for the duration of exposure to NMDA or KA. NMDA-evoked neurotransmitter release was routinely performed in the absence of Mg^{2+} since previous studies have shown that physiological concentrations of Mg^{2+} inhibit NMDA-evoked neurotransmitter release (Lehman & Scatton, 1982; Jones *et al.*, 1987). KA-evoked release was routinely performed in the presence of 1.2 mM Mg^{2+}. At the conclusion of the experiment, the slices from each chamber were sonicated. The radioactivity present in the collected fractions and the tissue slices was estimated by liquid scintillation spectroscopy.

The percentage fractional efflux of tritium was estimated as the fraction of the amount of radioactivity in the superfusate fraction relative to the total amount of radioactivity in the slice at that particular point in time, multiplied by 100. The total amount of evoked tritium release by NMDA or KA was estimated by summing the percentage fractional release values over baseline for the four fractions following exposure to NMDA or KA. The percentage fractional release of tritium in the fraction before superfusion with NMDA or KA was used as an estimate of baseline release.

Statistical analyses

The data are expressed as means ± standard errors. Statistically significant differences between two means were determined by Student's *t* test for unpaired observations. Significant differences between three or more means were determined by one-way analysis of variance and the Bonferroni modified *t* test as described by Wallenstein *et al.* (1980).

Materials

(+)-MK-801 maleate ((+)-5-methyl-10,11-dihydro-5H-dibenzo(a,d)cyclohept-5,10-imine maleate) and (±)-CPP hydrate (3-(±)-(2-carboxypiperazin-4-yl)-1-phosphonic acid) were purchased from Research Biochemicals, Inc., Natick, MA, U.S.A.. N-methyl-D-aspartic acid, kainic acid, glycine, ascorbic acid, pargyline hydrochloride, and desipramine were purchased from Sigma Chemical Co., St. Louis, MO. ω-Conotoxin GVIA was purchased from Peninsula Laboratories, Inc., Belmont, CA, U.S.A. PN 200-110 (isopropyl - 4 - (2,1,3 - benzoxadiazol - 4 - yl) - 1,4 - dihydro-2,6-dimethyl-5-methoxy-carbonylpyridine-3-carboxylate) was a generous gift from Sandoz Ltd., Basle, Switzerland. [^{3}H]-noradrenaline hydrochloride was purchased from NEN Research Products, Boston, MA, U.S.A.

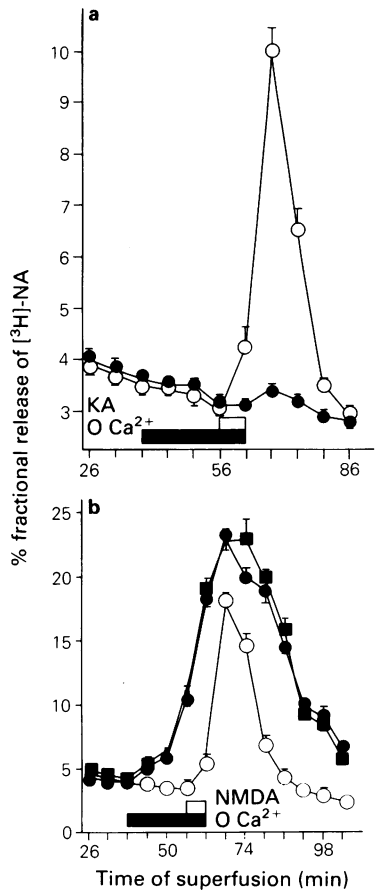

Figure 1 The effects of removal of extracellular Ca^{2+} on (a) kainic acid (KA)- and (b) N-methyl-D-aspartate (NMDA)-evoked release of [^{3}H]-noradrenaline ([^{3}H]-NA) from hippocampal brain slices. (a) Fractional release of [^{3}H]-noradrenaline from hippocampal brain slices evoked by 1 mM KA in the presence (○) or absence (●) of extracellular Ca^{2+}. Brain slices were exposed to a zero Ca^{2+} buffer (1 mM EGTA) 18 min before exposure to KA. $n = 8$. (b) Fractional release of [^{3}H]-noradrenaline from hippocampal brain slices evoked by 300 μM NMDA in the presence (○) or absence (●) of extracellular Ca^{2+}. Brain slices were exposed to a zero Ca^{2+} buffer (1 mM EGTA) 18 min before exposure to NMDA. Note that zero Ca^{2+} caused the release of [^{3}H]-noradrenaline, and when NMDA was *not* added to the superfusion buffer (■) the zero Ca^{2+}-induced release was virtually identical to that observed when NMDA was added to the zero Ca^{2+} buffer (●). Selected standard error bars are shown for clarity. $n = 8$.

Results

NMDA and KA evoked [^{3}H]-noradrenaline release from both cortical and hippocampal brain slices in a concentration-dependent fashion. The potencies of NMDA and KA were similar in both tissues. The EC_{50} values for NMDA were 65 μM and 50 μM, and the EC_{50} values for KA were 125 μM and 150 μM in the cortex and hippocampus, respectively. However, the maximum evoked release caused by both amino acids was approximately two times greater in hippocampal than in cortical tissues. The maximum total evoked release values for NMDA were 19.0 ± 0.9% and 30.2 ± 2.6%, and for KA 5.9 ± 0.5% and 13.2 ± 1.3% in the cortex and hippocampus, respectively ($n = 8$). When Ca^{2+} was omitted from the

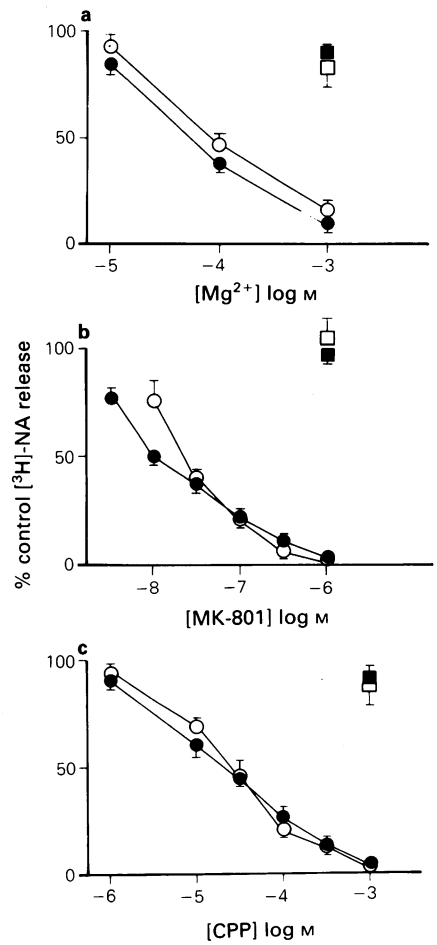

Figure 2 The effects of Mg^{2+} (a), MK-801 (b) and CPP (c) on 300 μM N-methyl-D-aspartate (NMDA)- and 1 mM kainic acid (KA)-evoked release of [^{3}H]-noradrenaline ([^{3}H]-NA) from cortical and hippocampal brain slices. NMDA, cortex (○); NMDA, hippocampus (●); KA, cortex (□); KA, hippocampus (■). $n = 4$–8. Bars represent s.e.mean values.

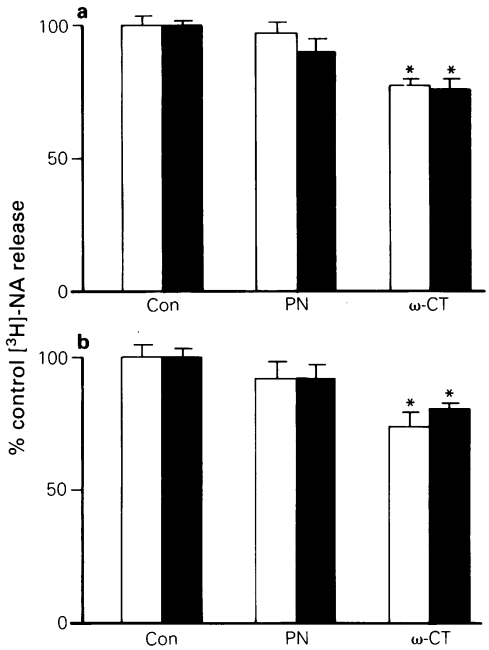

Figure 3 The effects of 1 μM PN 200-110 (PN) and 0.1 μM ω-conotoxin GVIA (ω-CT) on the total release of [^{3}H]-noradrenaline ([^{3}H]-NA) evoked by (a) 300 μM N-methyl-D-aspartate (NMDA) and (b) 1 mM kainic acid (KA) from cortical (open columns) and hippocampal (solid columns) brain slices. The results were expressed as a percentage of control evoked release values. Control NMDA-evoked release values were 33.4% and 18.9% in hippocampal and cortical slices, respectively. Control KA-evoked release values were 11.6% and 4.4% in hippocampal and cortical slices, respectively. *$P < 0.05$ compared to control. $n = 10$–30. Bars represent s.e.mean values.

superfusion buffer (in the presence of 1 mM EGTA), the evoked release caused by 1 mM KA was inhibited by greater than 95% in hippocampal brain slices (Figure 1a). Thus, KA-evoked release was clearly dependent upon the presence of extracellular Ca^{2+}. In contrast, it was not possible to determine whether NMDA-evoked release was Ca^{2+}-dependent because spontaneous, NMDA-independent neurotransmitter release occurred in the absence of both Mg^{2+} and Ca^{2+} in the superfusion solution (Figure 1b). These results are similar to those obtained by Jones *et al.* (1987) and probably reflect the dependence of membrane stability on the presence of divalent cations (Frankenhaeuser & Hodgkin, 1957; Shanes, 1958). The effects of removal of extracellular Ca^{2+} on NMDA- and KA-evoked responses in cortical slices resulted in virtually identical results to those obtained for hippocampal slices (not shown).

NMDA-evoked release of [^{3}H]-noradrenaline was inhibited by Mg^{2+} (Figure 2a), the noncompetitive antagonist, MK-801 (Figure 2b) and the competitive antagonist, CPP (Figure 2c) in both hippocampal and cortical brain slices with comparable potencies. These same agents had no significant effect on KA-evoked neurotransmitter release (Figure 2).

The contribution of VSCC to excitatory amino acid evoked neurotransmitter release was estimated by measuring 300 μM NMDA- and 1 mM KA-evoked release in the absence and presence of PN 200-110, a selective inhibitor of L-type VSCC or ω-CT, an inhibitor of neuronal L-type and N-type VSCC (Figure 3). PN 200-110 (1 μM) had no significant effect on either NMDA-evoked (Figure 3a) or KA-evoked (Figure 3b) release of [^{3}H]-noradrenaline from either hippocampal or cortical brain slices ($P > 0.05$), although there was a tendency towards inhibition. ω-CT (0.1 μM) caused a small but significant inhibition (20–30%; $P < 0.05$) of both NMDA and KA-evoked release of [^{3}H]-noradrenaline in hippocampal and cortical brain slices (Figure 3). The concentrations of PN 200-110 and ω-CT used in these experiments are at least four orders of magnitude higher than the affinity values obtained from radioligand binding studies (Lee *et al.*, 1984; Abe *et al.*, 1986; Wagner *et al.*, 1988; Feigenbaum *et al.*, 1988; Keith *et al.*, 1989), and thus represent saturating concentrations relative to their binding site affinities.

Discussion

The potential role that VSCC may play in mediating EAA-evoked responses is unclear. By promoting Na^+ and/or Ca^{2+} influx through receptor gated ion channels, EAA cause membrane depolarization which could serve as a trigger to open VSCC, and thus allow additional influx of Ca^{2+} to couple the response. Dingledine (1983) has demonstrated that NMDA-evoked electrophysiological responses are partially inhibited by the L-type VSCC antagonists, verapamil and D-600. Riveros & Orrego (1986) have suggested that EAA stimulate $^{45}Ca^{2+}$ influx through cadmium-sensitive but dihydropyridine-insensitive VSCC. However, information from several recent studies suggest that EAA-evoked Ca^{2+} influx is not mediated by the activation of VSCC (Pastuszko *et al.*, 1984; MacDermott *et al.*, 1986; Lazarewicz *et al.*, 1986). In the present study, the role of VSCC in coupling EAA-evoked responses was evaluated by measuring the effects of the dihydropyridine L-channel antagonist PN 200-110 and the neuronal L- and N-channel antagonist ω-CT on evoked neurotransmitter release.

The profiles of NMDA- and KA-evoked release of [^{3}H]-noradrenaline from cortical and hippocampal

brain slices were similar with the exception that the evoked release caused by both amino acids was approximately two times greater in hippocampal brain slices. The NMDA receptor/ionophore antagonists Mg^{2+}, MK-801 and CPP inhibited NMDA-evoked [^{3}H]-noradrenaline release in both cortical and hippocampal brain slices, but had no effect on KA-evoked release (Figure 2). These observations confirm that NMDA- and KA-evoked neurotransmitter release is mediated by actions at distinct EAA-receptor subtypes. These conclusions are similar to those of previous investigations on hippocampal brain slices (Lehman & Scatton, 1982; Jones *et al.*, 1987; Schmidt & Taylor, 1988; Lalies *et al.*, 1988). The results of the present investigation suggest that the EAA-receptors mediating the evoked release of [^{3}H]-noradrenaline are similar in both cortical and hippocampal tissues.

[^{3}H]-noradrenaline release evoked by both NMDA and KA from cortical and hippocampal brain slices was similarly inhibited by 0.1 μM ω-CT (approximately 30%, Figure 3), whereas 1 μM PN 200-110 had no significant effect. At identical concentrations, ω-CT and PN 200-110 inhibited K^+-evoked [^{3}H]-noradrenaline release from cortical brain slices by 55% and 17%, respectively (Keith *et al.*, 1989). If one assumes that the inhibitory effects of ω-CT are due to inhibition of both L- and N-type VSCC, and that the inhibitory effects of PN 200-110 are due to inhibition of only L-type VSCC (Miller, 1987), then the results of the present study suggest that N-type, but not L-type, VSCC play a small but significant role in [^{3}H]-noradrenaline release evoked by both NMDA and KA. Thus, although pharmacologically distinct, NMDA- and KA-receptors appear to utilize either directly or indirectly ω-CT-sensitive VSCC to a similar extent in mediating neurotransmitter release. Alternatively, the results of the present study could be accounted for by the direct inhibition of EAA-gated ion channels by ω-CT; however, no experimental evidence to date supports this possibility.

The fact that ω-CT maximally inhibits EAA-evoked neurotransmitter release by only 20–30% suggests that the majority of Ca^{2+} influx that couples release is being mediated by EAA-gated ionophores. The proposal that the NMDA-gated ionophore can allow Ca^{2+} influx has been well documented (MacDermott *et al.*, 1986; Ascher & Nowak, 1986; 1988; Mayer & Westbrook, 1987). Although the KA-linked ionophore is generally considered to be selective for monovalent cations (reviewed by MacDermott & Dale, 1987), a recent study using fura-2 signals in cultured neurones suggests that KA evokes calcium influx through VSCC as well as through another pathway which is probably the KA-gated ionophore (Murphy & Miller, 1989). Thus, our studies suggest that the KA-gated ionophore is more important than VSCC in coupling neurotransmitter release. The inability to demonstrate an L-channel component to NMDA-evoked neurotransmitter release is in contrast to the demonstration by Dingledine (1983) that the L-type inhibitors verapamil and D-600 (at 1–3 mM) partially inhibited NMDA-evoked depolarizations. The reason for this difference is unclear. Thus, although the results of the present investigation suggest that N-type VSCC play a role in EAA-evoked neurotransmitter release, it remains to be determined whether N-type VSCC contribute to the physiological and/or pathological manifestations of EAA-receptor stimulation.

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Effect of palytoxin on the calcium current and the mechanical activity of frog heart muscle

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1 The effect of palytoxin (PTX) on the Ca current (I_{Ca}) and the mechanical activity of frog atrial fibres was studied by use of the double sucrose gap voltage clamp technique.

2 In normal Ringer solution, PTX transiently increased the electrically-evoked peak tension which then decreased while a major contracture developed. PTX slowed the time course of the relaxation phase of the evoked tension.

3 Evidence is presented which suggests that the toxin also increased the entry of Ca and Sr via the Na-Ca exchange mechanism. It also induced the development of a Ca-dependent outward current which was inhibited by Sr.

4 In Na-free solution, PTX increased I_{Ca} and shifted the reversal potential for Ca towards more negative membrane potentials, thus suggesting that the internal Ca concentration had increased. Current-voltage, tension-voltage, time to peak-voltage and inactivation time constant-membrane potential curves were all shifted towards more negative membrane potentials in the presence of PTX.

5 These effects of PTX are similar to those caused by the increase in internal Ca concentration induced by Na ionophores by way of voltage-dependent Ca influx of the Na-Ca exchange mechanism.

Introduction

Palytoxin (PTX), a toxin, isolated from marine coelenterates (Moore & Scheuer, 1971), causes an irreversible sodium-dependent, tetrodotoxin (TTX)-insensitive depolarization of excitable membranes (Sauviat & Pichon, 1977; Dubois & Cohen, 1977; Weidmann, 1977; Pichon, 1982, Tessereaux *et al.*, 1983; Sauviat *et al.*, 1987). In cardiac membranes, PTX induced a cation channel (Sauviat *et al.*, 1987; Muramatsu *et al.*, 1988) which is practically impermeable to Ca ions (Ikeda *et al.*, 1988). Yet PTX is known to increase Ca^{45} uptake and induce a tonic contracture in heart (Rayner *et al.*, 1975) and vascular smooth muscle (Ito *et al.*, 1977; Ozaki *et al.*, 1983). In guinea-pig taenia coli Ozaki *et al.* (1987) suggested that PTX increased Ca influx through voltage-dependent Ca channels to cause contraction and showed that, after prolonged exposure to PTX, muscle tension was uncoupled from Ca influx. In the present work the action of PTX on the voltage-dependent Ca current (I_{Ca}) and mechanical activity of frog heart muscle has been investigated. A preliminary report of part of this work has already been published (Sauviat, 1987).

Methods

Current (clamp) and voltage clamp experiments were performed at 5–8°C on fine atrial trabeculae (75–150 μm in diameter, 2–4 mm in length) isolated from the heart of *Rana esculenta* using the double sucrose gap technique with vaseline seals (Rougier *et al.*, 1968). The limitation of the voltage clamp method, as applied to a multicellular preparation, as well as the experimental procedure have been discussed previously (Sauviat, 1980; Sauviat & Suchaud, 1981; Sauviat *et al.*, 1987; Gautier *et al.*, 1987; Pater & Sauviat, 1988). Starting from the holding potential, the potential of the test node was displaced in rectangular steps at a rate of 0.2 Hz; positive potentials correspond to depolarization. The inward Ca

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current (I_{Ca}) was measured as net inward current if not otherwise specified. In current-voltage relationships (I–V curves), outward currents correspond to positive currents and depolarizations to positive potentials applied from the holding potential (HP). The apparent reversal potential for Ca ions (V_{Ca}) was determined from I–V curves as follows. I–V curves were successively drawn for peak I_{Ca} in (i) the control solution; (ii) PTX; (iii) PTX and Cd (1 mM). Since 1 mM Cd inhibits I_{Ca}, the point of intersection of I–V curves drawn in the absence and in the presence of Cd corresponds to V_{Ca} (Gautier *et al.*, 1987; Pater & Sauviat, 1988). Mechanical activity was recorded by means of a transducer system (Pixie 8101) applied to the fibre by means of a thin glass lever under two experimental conditions. Firstly, a single frog atrial fibre was tied at both ends with natural silk thread with one end fixed to the bottom of the chamber and the other one to the glass lever. Then the preparation was mounted in a fast flow chamber which allowed rapid solution changes (less than 1 s) and was electrically driven by square pulses delivered at 0.2 Hz through bipolar platinum electrodes (earth isolated using an optoelectrometric coupling device). Secondly, under voltage clamp the tension was recorded in the test gap. In tension-membrane potential curves (T–V curves), an increase in muscle tension corresponds to an upward deflection. The time constants of the inactivation phase of I_{Ca} (τ_f) and of the relaxation phase of tension (τ_{relax}) were calculated by semi-logarithmic plots of the respective amplitudes versus time. The time constant of the tail current (τ_{tail}) following the onset of the capacitive current when the membrane was stepped back to the holding potential was measured by semi-logarithmic plot of the tail current amplitude versus time. The amplitude of the tail current was estimated at the intercept on the ordinate axis. The series resistance (R_s) of the preparation was determined at the beginning and at the end of each experiment. We have considered only experiments in which R_s did not change.

It should be noted that each figure is representative of six to seven experiments unless otherwise stated. Calculations are expressed as mean values ± s.e.; *n* corresponds to the number of fibres tested. Transmembrane potentials and currents as well as mechanical activity were displayed on an oscilloscope (Tektronix 5110 or 565) and recorded on the mass storage of a desk computer (Hewlett Packard 9826) by means of an 8 bit data acquisition system (Datalab) and plotted on an XY plotter (Hewlett Packard 7470) or photographed for further analysis.

The composition of the Ringer solution was (mM): NaCl 110.5, KCl 2.5, $CaCl_2$ 2. The pH of the solution was maintained at 7.3 with HEPES buffer (5 mM). In some experiments Sr was used as $SrCl_2$. Since in solution containing Na, PTX is known to depolarize the membrane in frog atrial fibres (Sauviat *et al.*, 1987), the effects of the toxin on I_{Ca} were analyzed in Na-free solution where NaCl was replaced with mannitol. This Na-free solution also contained 4 amino-pyridine (4-AP; 10 mM), tetraethylammonium (TEA; 10 mM) and tetrodotoxin (TTX, 0.57×10^{-6} M) to block outward K currents and Na currents respectively, K conductances were reduced by charging fibres with Cs ions (Mentrard *et al.*, 1984) which also depress ion accumulation and depletion phenomena (Sauviat *et al.*, 1987).

Palytoxin was kindly provided by Professor Scheuer from the department of Chemistry of the University of Hawai. The toxin was dissolved in distilled water at a concentration of 3.7×10^{-5} M and kept at 4°C. The stock solution was diluted just before use.

Results

Effect of palytoxin on mechanical activity

The addition of PTX to a frog atrial fibre in normal Ringer solution evoked a biphasic response. First it increased the amplitude of the stimulated tension (the first eight pulses in Figure 1b) while PTX slightly reduced basal tension. Then PTX induced a strong and almost irreversible contracture of the fibres while the amplitude of stimulated contractions decreased markedly (Figure 1a) and then relaxed more slowly. Under voltage clamp conditions in the presence of TTX, PTX also induced a marked contracture (Figure 1c). Figure 1c also shows that PTX increased the peak tension associated with a depolarizing pulse (400 ms duration, applied from a −80 mV holding potential) to −30 mV and reduced it when the fibre was depolarized to +20 mV. However, at both potentials, the duration of the relaxation phase was increased; at +20 mV, τ_{relax} increased from 205 ms in control solution to 650 ms in the presence of PTX. Figure 1d shows the T–V curve. In spite of the large contracture which developed, the amplitude of the peak tension was markedly increased in the potential range −80 to −10 mV.

Effect of palytoxin on membrane currents

In voltage clamp conditions with Ringer solution containing TTX Figure 2a shows that PTX produced at least four effects: (a) the development of an inward resting current at −80 mV; (b) a reduction of the peak Ca current magnitude at 0 mV; (c) the development of an outward current at 0 mV; (d) an increase of the amplitude of the tail current recorded

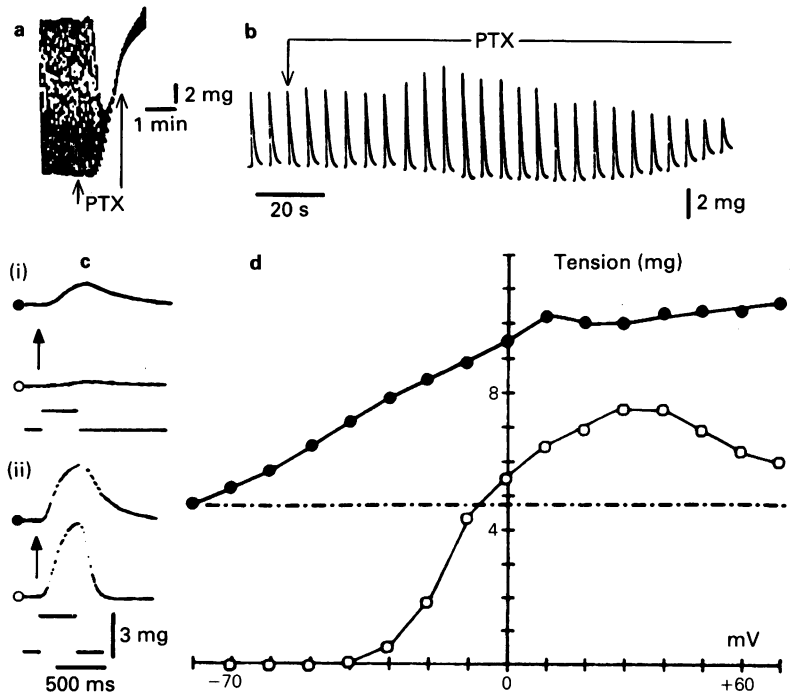

Figure 1 Effects of palytoxin (PTX, 3.7×10^{-7} M) on the peak tension evoked in frog atrial muscle fibres. (a) PTX produced an irreversible contracture in the fibre with a reduction in amplitude of peak stimulated tension. (b) The onset of the effect of PTX is shown on a faster time scale to show that PTX transiently increased the peak stimulated tension and relaxed basal muscle tension before the development of the contracture. (a) and (b): normal Ringer solution. (c) Under voltage clamp conditions, PTX (3.7×10^{-8} M): (i) increased the amplitude of the peak tension (upper traces) recorded at −30 mV and (ii) reduced it under +20 mV. (○) Ringer solution containing tetrodotoxin (TTX); (●) control solution containing PTX. The duration of the relaxation phase was lengthened and a large contracture developed at both membrane potentials. Arrows indicate the shift of the base line of the tension associated with the contracture induced by the toxin. Lower traces corresponded to 400 ms depolarizing steps applied from a −80 mV holding potential. (d) Peak tension-voltage relationships plotted before (○) and after (●) PTX (3.7×10^{-8} M) was added to the Ringer solution containing TTX. The dashed line (– · –) indicates the shift in base line tension due to the PTX-induced contracture. Holding potential was −80 mV.

when the potential was stepped back to the holding potential, associated with a lengthening of its time course. Table 1 presents the data relative to the effect of PTX on membrane currents. When Ca was replaced by Sr in the Ringer solution containing TTX an inward current (I_{Sr}) was recorded which suggests that Sr passes through the calcium channel (Chesnais *et al.*, 1971). In Sr solution (Figure 2b), PTX also induced an inward resting current at −80 mV, decreased the amplitude of the peak I_{Sr} at 0 mV while the outward current moderately increased and increased the magnitude and lengthened the time course of the tail current. Results obtained from five experiments are summarized in Table 1. In the solution containing Sr, the amplitudes of the tail current and τ_{tail} were increased by 60% and 88% respectively, compared with the Ca solution. In the presence of PTX, τ_{tail} was increased by about 175% in Ca and 135% in Sr solution.

Effect of palytoxin on Ca currents and the phasic tension

Ca currents were recorded from fibres bathed in a Na-free solution (which also contained Cs, TEA, 4A-P and TTX) to eliminate the PTX-induced Na current (Sauviat *et al.*, 1987). Figure 3a shows that, under these conditions, PTX did not alter either the resting membrane current or the leak current (I_{leak}). I_{leak}, estimated as the current measured at the end of a 20 mV hyperpolarizing pulse applied from a holding potential of −80 mV, was $0.019 \pm 0.002\,\mu A$ in the absence and $0.022 \pm 0.003\,\mu A$ ($n = 8$) in the presence of PTX. The

Table 1 Effect of palytoxin (PTX, 3.7×10^{-8} M) on membrane currents in frog heart

	Ca-containing solution		*Sr-containing solution*	
	Control	*PTX*	*Control*	*PTX*
Resting current induced by PTX (μA)	—	0.30 ± 0.05	—	0.53 ± 0.09
Inward Ca channel current (μA)	0.16 ± 0.06	0.11 ± 0.06	0.32 ± 0.10	0.10 ± 0.01
Outward current (μA)	0.15 ± 0.02	0.72 ± 0.05	0.13 ± 0.02	0.74 ± 1.13
Tail current amplitude (μA)	0.05 ± 0.01	0.15 ± 0.02	0.08 ± 0.01	0.13 ± 0.05
τ_{tail} (ms)	49.9 ± 13.9	137.1 ± 24.3	93.7 ± 15.6	220.6 ± 32.2

The resting current induced by PTX was measured at a holding potential of −80 mV. Owing to the resting current change induced by the toxin application, the inward current (elicited by a 0 mV depolarizing step applied from a −80 mV holding potential) was measured according to the method proposed by Horachkova & Vassort (1976). The outward current was measured at the end of the depolarizing steps (see Figure 2) in comparison with the corresponding resting current. The amplitude of the tail current and its time constant (τ_{tail}) were measured as described in methods. Mean ± s.e. of 5 fibres.

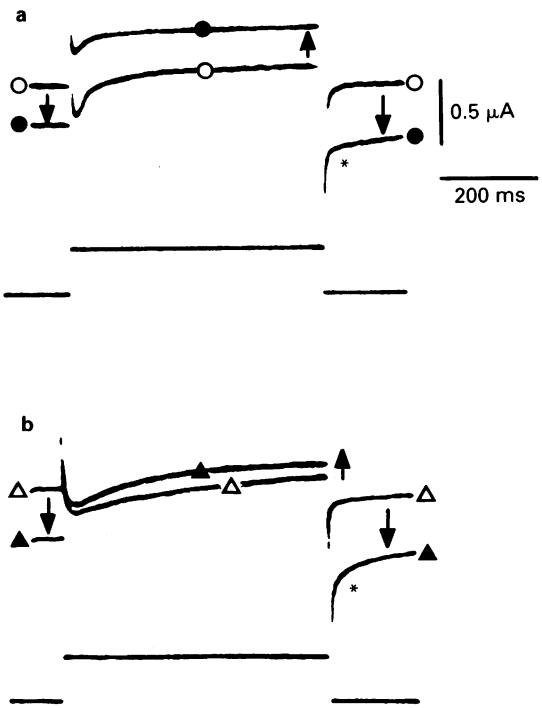

Figure 2 Effect of palytoxin (PTX) in voltage clamp experiments. Currents were evoked by voltage steps to 0 mV from −80 mV (lower traces) before (open symbols) and after (filled symbols) 3 min application of solution containing PTX (3.7×10^{-8} M) and tetrodotoxin (TTX). (a) Currents recorded in solution containing Ca. (b) Currents recorded in a Ca-free solution containing Sr. The arrows indicate changes in membrane currents induced by the toxin. (★) indicates the inward tail current induced by PTX when the potential was changed back to the holding potential. Pulse duration 523 ms in (a) and 538 ms in (b). The calibration bars in (a) also apply to (b).

membrane resistance (R_m calculated as the ratio between I_{leak} and the membrane potential) was 136 ± 14 kΩ (control) and 132 ± 17 kΩ (PTX) ($n = 8$) respectively. After 3 to 5 min exposure, PTX increased the magnitude of I_{Ca} recorded at negative membrane potentials (−40 to −20 mV in Figure 3a) and reduced it at more positive potentials. Current-voltage relationships show that PTX first shifted the entire I–V curve towards more negative membrane potentials (Figure 3b). The apparent reversal potential for Ca (V_{Ca}) was shifted by 12.5 mV towards a less positive membrane potential. After longer exposure to PTX (more than 10 min) the amplitude of I_{Ca} declined while the minimum of I–V curve remained unchanged and the shift in V_{Ca} towards more negative membrane potentials was enhanced (Figure 3b). V_{Ca} was $+63 \pm 1.8$ mV ($n = 5$) in the absence and $+45 \pm 5.2$ mV ($n = 5$) in the presence of PTX. The effect of PTX on the activation (d) and inactivation (f) parameters controlling the Ca conductance was determined. The time required for I_{Ca} to reach its maximum value (t_{max}) was used as an index of the activation parameter (MacDonald *et al.*, 1986) and measured as a function of the membrane potential. Figure 3c shows that PTX accelerated t_{max} in the voltage range −40 to −10 mV while t_{max} was unchanged at more positive membrane potentials. The time constant τ_f of the decay phase of I_{Ca} was plotted as a function of the membrane potential (Figure 3d). The τ_f-V curve was shifted on the membrane potential axis towards more negative membrane potentials by PTX. Figure 4a shows that, in the voltage range −30 to −10 mV, PTX increased both the amplitude of I_{Ca} and the phasic tension. The time constant of the relaxation phase was unchanged by the toxin; τ_{relax} was 2.15 s before, and 2.2 s after PTX application. I–V and T–V curves plotted in Figure 4b show that, in the presence of toxin, both curves are shifted towards more negative membrane potentials. The minimum of I–V curves

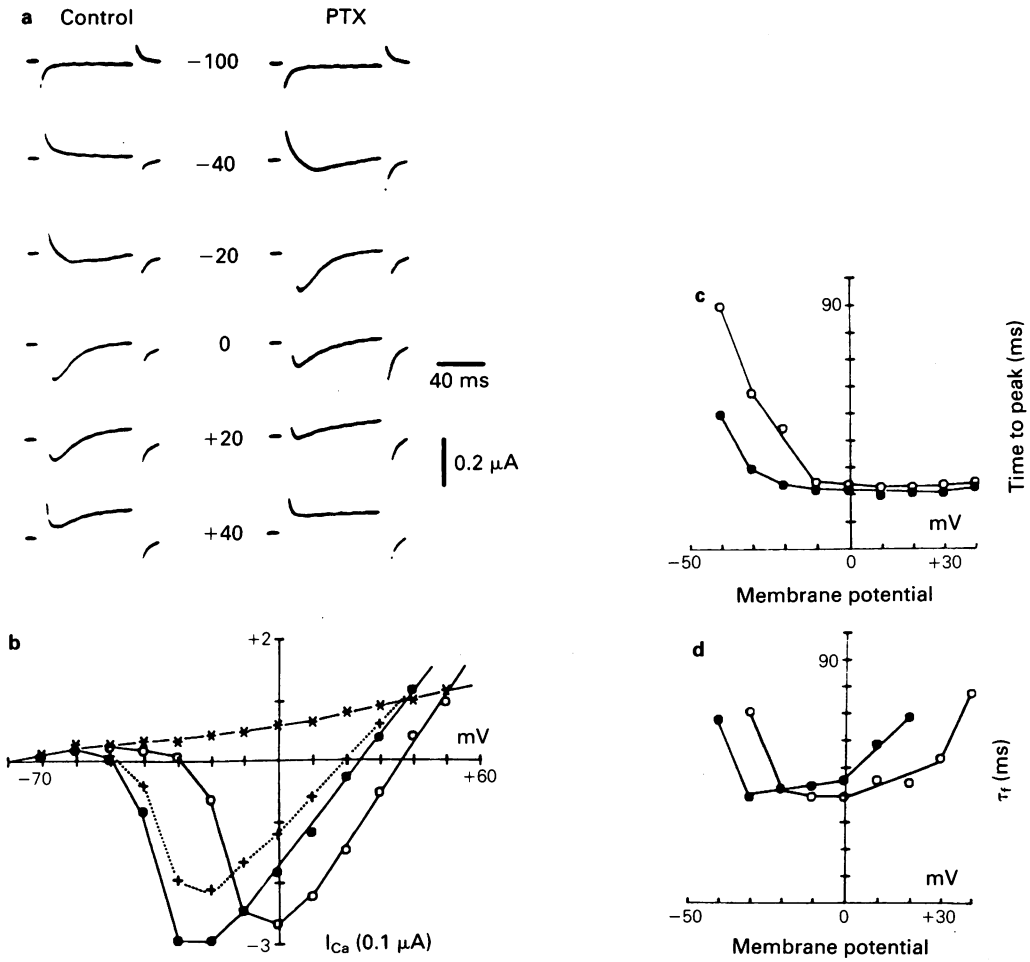

Figure 3 Effect of palytoxin (PTX, 3.7×10^{-8} M) on the Ca current (I_{Ca}) recorded in frog atrial muscle fibres in a Na-free control solution containing Cs, tetraethylammonium, 4-aminopyridine, and tetrodotoxin (see methods): (○) control currents and (●) currents after PTX. (a) Membrane current responses evoked by stepping from a holding potential of −80 mV to the membrane potentials indicated, in mV, between each pair of traces. Pulse duration was 96 ms. (b) Current-voltage relationships plotted for peak I_{Ca} in control solution (○), after 5 min (●) and 10 min (+ dotted line) of PTX application. The curve (*) was recorded 3 min after Cd (1 mM) was added to the solution containing the toxin. (c) Effect of PTX on the time to peak inward current-membrane potential curve. (d) Effect of PTX on the time constant of the inactivation phase of I_{Ca} (τ_f)-membrane potential relationship. In (a), (b), (c) and (d) the holding potential was −80 mV.

and the maximum of T–V curves are shifted by 17.8 ± 1.7 mV ($n = 6$) and 16.7 ± 5.5 mV ($n = 6$) respectively.

Discussion

The predominant effect of palytoxin reported in the present work was a shift of the Ca current-voltage and the phasic tension-voltage curves towards more negative membrane potentials which occurred at the same time as the development of a contracture.

The first point to be discussed concerns the effect of the toxin on the Ca current (I_{Ca}). In Na-free media, PTX increased the amplitude I_{Ca} by shifting the I–V curve towards a more negative membrane potential. Three alternative explanations might be put forward to explain this effect. First, a change in

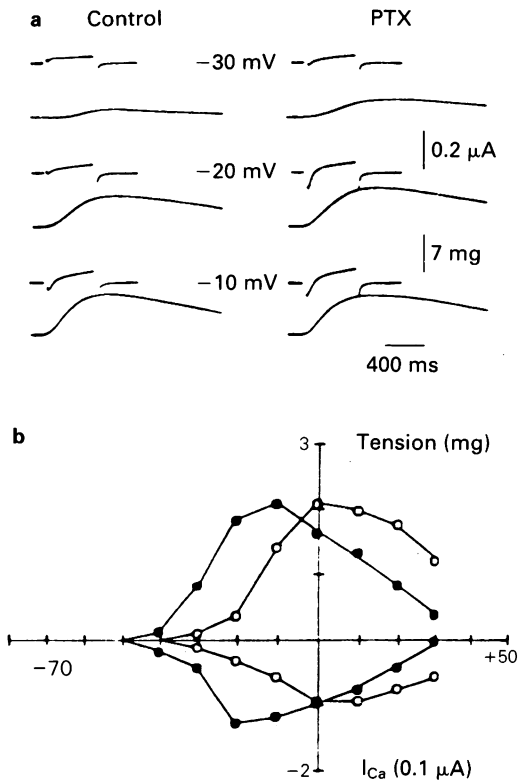

Figure 4 Effect of palytoxin (PTX) on the Ca current (I_{Ca}) and the phasic tension recorded in a Na-free control solution containing Cs, tetraethylammonium, 4-amino pyridine and tetrodotoxin. Control (○) and PTX (3.7×10^{-8} M) (●). (a) Current (upper traces) and tension (lower traces) responses elicited by stepping from a −80 mV holding potential to the membrane potentials indicated between each pair of traces (pulse duration 760 ms). (b) Current-voltage (lower curves) and tension-voltage (upper curves) relationships plotted in the control solution before (○) and 5 min after PTX treatment (●): holding potential = −80 mV.

the activation of I_{Ca} could be induced by PTX as suggested by the shift of t_{max}–V curve. Shift of I–V curves of the voltage-dependent Na current have been reported for batrachotoxin (Huang *et al.*, 1982; Mozhayeva *et al.*, 1986) and aconitine (Campbell, 1982). Both these substances are known to modify activation of the Na current. However, no acceleration of the time to peak of I_{Ca} was recorded in the present work. Secondly, another simple explanation of the reported shift could be that two populations of Ca channels exist. One may be recorded under normal conditions and the other may open at unusually negative membrane potentials which is activated by PTX. This might suggest that PTX activated T-type Ca channels (McCleskey *et al.*, 1986). However, no T-type Ca channels have yet been observed in frog atrial fibres (Pater & Sauviat, 1988). The third possibility which could account for the shift of I–V curve by PTX is that Ca might accumulate inside the fibre and decrease the potential through the membrane by screening internal negative charges. The shift of the apparent reversal potential of Ca towards more negative membrane potentials supports this idea since according to Mentrard *et al.* (1984), when frog atrial fibres were Ca loaded in Na-free media, Ca I–V curves were shifted towards more negative potentials. Moreover, Fischmeister & Horackova (1983) found that variations in V_{Ca} due to Ca accumulation did not significantly affect the inactivation of I_{Ca}. PTX displaced I–V, τ_f–V curves towards more negative membrane potentials as would be expected during Ca loading. While τ_f values were unchanged, the τ_f curves were shifted along the voltage axis. However, Ca overload might be linked to Ca entry via the channel formed by PTX (Ikeda *et al.*, 1988). Although it has not been shown in frog atrial fibres, PTX might also be able to induce Ca entry through the PTX formed channel.

In frog heart the quantity of Ca entering the cell upon depolarization was linearly related to the peak phasic tension (Horackova & Vassort, 1976). This point is clearly observed in Na-free solution where I–V and T–V curves are shifted by the same amount on the voltage axis in the presence of PTX. The tonic component of the tension is dependent on Ca entry via Na-Ca exchange (Horackova & Vassort, 1979) which may also be the major mechanism leading to relaxation (Roulet *et al.*, 1979; Vassort *et al.*, 1979). The effect of PTX on tonic tension and the relaxation phase is probably linked to activation of Na-Ca exchange. A fundamental feature of Na-Ca exchange is that, an elevation in internal Na (Na_i) will promote an exchange of Na_i for external Ca (Ca_0). Na ionophores such as ionomycin (Sheu *et al.*, 1986) and monensin (Hume & Uehara, 1986; Barcenas-Ruiz *et al.*, 1987) have recently been used as tools to depolarize the membrane by increasing the internal Na concentration ($[Na]_i$) and to study the role of Na-Ca exchange in contributing to the depolarization-induced increase in internal Ca concentration ($[Ca]_i$) in rat, guinea-pig and frog heart muscle cells. An increase in $[Ca]_i$ evoked in this manner led to several changes in the electrical and mechanical activity of heart muscle: (i) a reduction of the transmembrane Ca gradient: (ii) a decrease in transmembrane potential by the screening of internal negative charges by Ca; (iii) a decrease in I_{Ca} and phasic tension; (iv) a lengthening of the relaxation phase and the development of a tonic contracture. Although no change in $[Na]_i$ has been clearly

recorded in frog heart (Sauviat *et al.*, 1987) or in squid giant axon (Pichon, 1982), Na ions enter the cells via a PTX-formed channel. The peak tension-voltage curve plotted in solution containing Na in the presence of PTX (Figure 1d) is similar to the relationship observed in Na-loaded fibres by Vassort (1973) and Hume & Uehara (1986). It indicates Na loading which leads to Ca accumulation inside the fibre. PTX is also shown to increase the magnitude and the time constant of the tail current of I_{Ca} in solution containing Na (Figure 2a) while the tail current was unaltered in Na-free solution (Figure 3a). This observation is similar to results reported on monensin Na-loaded frog atrial cells (Hume & Uehara, 1986) in which the tail current change was identified as a Ca current generated by the Na-Ca exchange under depolarization. The present work also shows that Sr ions can substitute for Ca in the Na-Ca exchange mechanism as suggested by Potreau *et al.* (1987).

Another important observation concerns the development of an outward current to depolarizing steps in the presence of Na in the control solution. This current is activated in the presence of Ca and is not present when Ca is replaced by Sr. It might be a Ca-dependent K current (Siegelbaum & Tsien, 1980). In frog heart muscle, Sr as well as Ca, is able to enter the cells via the slow channel (Chesnais *et al.*, 1971), but cannot activate the Ca-dependent K current (Potreau, 1982). In the present work, although Sr enters the fibre via both the slow channel and the Na-Ca exchange, the development of the outward current is restricted in Sr solution. One consequence of Ca entry and accumulation induced by PTX is that, a large Ca-dependent outward current developed in addition to the well known depolarizing action of the toxin. Such a current might account for the shortening of the action potential observed in the presence of PTX by Sauviat *et al.* (1987).

In conclusion, the effect of PTX on the slow channel and tension is the direct consequence of an increase in the internal Ca content which is produced by activation of Na-Ca exchange which is stimulated by the PTX-evoked influx of Na ions. The present study also shows that PTX might be a powerful tool to analyse the Na-Ca exchange properties.

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5-Hydroxytryptamine is a possible neurotransmitter of the non-cholinergic excitatory nerves in the longitudinal muscle of rainbow trout stomach (*Salmo gairdneri*)

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1 The neurotransmitter of the non-cholinergic excitatory nerves in the rainbow trout stomach was identified on the basis of the pharmacological properties of the contractile responses to transmural stimulation (TMS) and nicotine.

2 TMS caused tetrodotoxin-sensitive contractions of rainbow trout stomach strips in a frequency-dependent manner (0.5–50 Hz). Atropine (1 μM) significantly decreased the contractile response to low-frequency stimulation (0.5–2 Hz), but did not affect that to high-frequency stimulation (3–20 Hz).

3 The atropine-resistant contractile response to TMS (20 Hz) was unaffected by hexamethonium (100 μM), phentolamine (5.4 μM), pyrilamine (1 μM), naloxone (1 μM) or substance P-induced desensitization.

4 5-Hydroxytryptamine (5-HT, 3 nM–3 μM) caused atropine-resistant contractions in a concentration-dependent manner. In the presence of atropine, methysergide (1 μM) decreased the contractile responses to TMS and 5-HT.

5 Nicotine (3 μM–500 μM) induced atropine-resistant contractions that were completely abolished by tetrodotoxin or hexamethonium. Also methysergide inhibited the contractile responses to nicotine.

6 An acid extract of rainbow trout stomach exhibited atropine-resistant contractions that were decreased by methysergide, in both rainbow trout stomach and guinea-pig ileum longitudinal smooth muscle preparations.

7 The present results indicate that, in longitudinal muscle strips of the rainbow trout stomach, 5-HT is one of the mediators (neurotransmitters) of the non-cholinergic excitatory contractions induced by TMS and nicotine.

Introduction

The regulation of the motility of the gastrointestinal tract by intrinsic nerves has been well studied in mammals, and on the basis of the pharmacological properties of the nerve-mediated mechanical responses, three types of intrinsic nerve (cholinergic excitatory, non-cholinergic excitatory and non-adrenergic inhibitory nerves) have been demonstrated in the gastrointestinal wall of mammals. The neurotransmitters of non-cholinergic and non-adrenergic nerves have been examined, and adenosine 5'-triphosphate (ATP), 5-hydroxytryptamine (5-HT), substance P and vasoactive intestinal polypeptide are considered to be possible neurotransmitters of non-adrenergic, non-cholinergic excitatory and inhibitory nerves (Burnstock, 1979; Costa & Furness, 1979; Franco *et al.*, 1979; Matušák & Bauer, 1986; De Beurme & Lefebvre, 1987).

Compared with mammals, only a little is known about the intrinsic innervation of the fish gastrointestinal tract. However, as in mammals, the presence of three types of intrinsic nerves (cholinergic excitatory, non-cholinergic excitatory

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and non-adrenergic inhibitory nerves) has been demonstrated (Stevenson & Grove, 1977; Burnstock, 1979; Grove & Campbell, 1979; Kitazawa *et al.*, 1988).

Non-cholinergic excitatory innervation has been found in some species of fish, such as goldfish (Burnstock *et al.*, 1972), dogfish (Nilsson & Holmgren, 1983), rainbow trout (Campbell & Gannon, 1976; Holmgren & Nilsson, 1981), ray (Young, 1980a), angler (Young, 1980b) and carp (Kitazawa *et al.*, 1988). On the basis of the similarity of the contractile responses to non-cholinergic nerve stimulation and contractile agents, ATP (Burnstock *et al.*, 1972; Young, 1980b), catecholamines (Campbell & Gannon, 1976; Nilsson & Holmgren, 1983), 5-hydroxytryptamine (5-HT) (Young, 1980a) and substance P (Kitazawa *et al.*, 1988) were considered as possible neurotransmitters of the non-cholinergic excitatory nerves in fish.

While the mechanical response to transmural stimulation (TMS) of rainbow trout stomach strips was being investigated *in vitro*, an atropine-resistant contractile response induced by TMS was observed (Kitazawa *et al.*, 1986). However, the substance mediating the non-cholinergic response has not, as yet, been identified. Physiological experiments showed that catecholamines, 5-HT, ATP and several neuropeptides, including substance P, affect the mechanical activity of the smooth muscle layer of the rainbow trout stomach (Holmgren, 1983; Kitazawa *et al.*, 1986).

The present study was carried out to identify the neurotransmitter of the non-cholinergic excitatory nerves in the rainbow trout stomach. The pharmacological properties of the atropine-resistant contractile response to TMS were clarified and the smooth muscle contracting substance from rainbow trout stomach, that causes a response that mimics atropine-resistant contraction, was extracted.

Methods

Tissue bath studies

Rainbow trout (*Salmo gairdneri*) of either sex (100 g–200 g body weight) were used in the present experiments. Each trout was stunned by a blow on the head and then bled. The abdominal cavity was opened by means of a midline incision and the stomach removed. The stomach was cut open and washed several times with a fresh nutrient solution. Longitudinal muscle strips (30 mm long and 2 mm wide) were prepared from the cardiac region of the stomach and then suspended vertically in an organ bath (4 ml or 8 ml). The organ bath had previously been filled with the warmed nutrient solution (20 ± 1°C) and equilibrated with a 95% O_2 + 5% CO_2 gas mixture. The composition of the nutrient solution was as follows (mM): NaCl 100.8, KCl 3.35, $CaCl_2$ 2.52, KH_2PO_4 1.11, $MgSO_4$ 1.17, $NaHCO_3$ 25 and glucose 11.1. Each stomach strip was connected to a force-displacement transducer (Nihon Kohden, TB-612T) that was coupled to an ink-writing polygraph to record the isometric contraction. The muscle preparations were initially subjected to a tension of 1.5 g and allowed to equilibrate for approximately 1 h.

Neuronal components present in the stomach wall were stimulated transmurally by means of two platinum ring electrodes placed at the top and bottom, respectively, of the organ bath. A pulse generator (Nihon Kohden, SEN-3201) was used to deliver rectangular pulses of various frequencies (supramaximal voltage, 1 ms duration) for periods of 10 s at 5 min intervals.

Extraction and bioassay

Rainbow trout stomach tissue (100 g) was minced with fine scissors and then placed in a glass tube containing 5 volumes of 1 N acetic acid and 0.02 N HCl, boiled for 10 min, cooled and finally homogenized. The tissue homogenate was centrifuged (10,000 *g* for 30 min at 4°C) and the supernatant collected. By means of ultrafiltration on a filter membrane (UK-10; Advantec Toyo, Japan), proteins and peptides with molecular weights of greater than 10,000 were removed. The filtered extract was defatted by washing with petroleum ether (3–4 times), lyophilized and then stored frozen (−20°C) until used. Rainbow trout stomach strips and guinea-pig ileum longitudinal smooth muscle (GPILM) were used to bioassay the smooth muscle contracting activity in the extracts. GPILM was prepared and suspended vertically in an organ bath (4 ml) containing an aerated Tyrode solution (35°C). The Tyrode solution had the following composition (mM): NaCl 136.9, KCl 2.7, NaH_2PO_4 0.4, $CaCl_2$ 1.8, $MgCl_2$ 2.1, $NaHCO_3$ 11.9 and glucose 5.6. The mechanical activity of the two smooth muscle preparations was measured with an isotonic transducer (Nihon Kohden, TD-112S) or a force-displacement transducer (Nihon Kohden, TB-612T) coupled to an ink-writing oscillograph. Each preparation was initially subjected to a tension of 1.5 g and equilibrated for 1 h. The lyophilized extract was dissolved in distilled water to a concentration equivalent to 2 g tissue wet weight per ml and then insoluble materials in the extract were removed by centrifugation (10,000 *g* for 30 min at 4°C). The reconstituted extract and drugs were usually added to the organ bath directly with a micropipette in a volume of less than 1% (40 μl) of

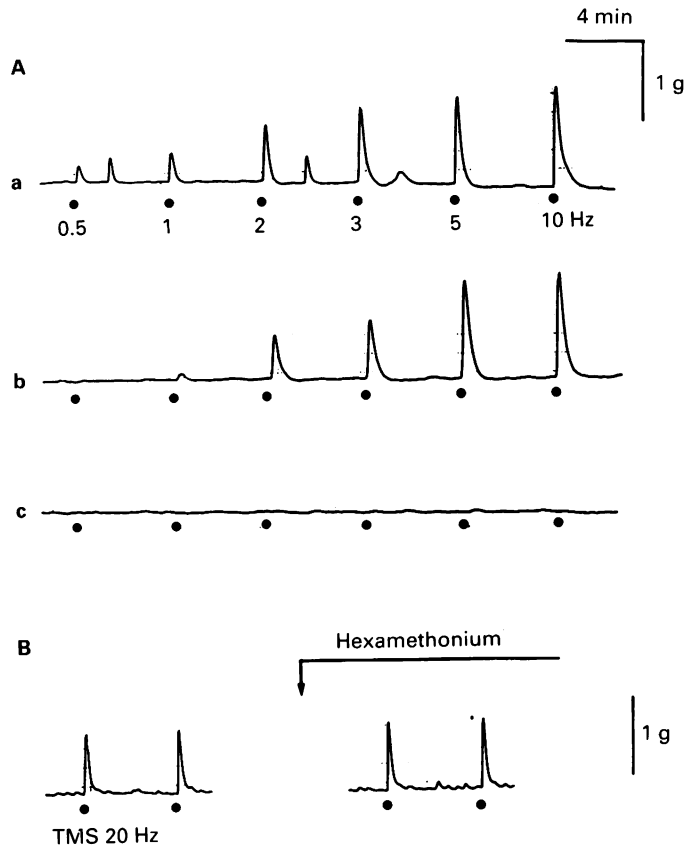

Figure 1 Contractile responses induced by transmural stimulation of longitudinal muscle strips of rainbow trout stomach. (A) Effects of atropine and tetrodotoxin on the contractile response to transmural stimulation (for periods of 10 s at 5 min intervals). The traces show the responses to various stimulation frequencies in normal Krebs solution (a), and Krebs solution containing atropine (1 μM) (b), and atropine (1 μM) plus tetrodotoxin (780 nM) (c), respectively. (B) Effect of hexamethonium (100 μM) on the atropine-resistant response to transmural stimulation (TMS, 20 Hz). The stomach strips were pretreated with atropine (1 μM).

that of the bath. The extract and drugs were washed out from the bath by replacing the bath solution with fresh nutrient medium.

Drugs

The following drugs were used: acetylcholine chloride (Wako), adrenaline bitartrate (Tokyo Kasei), ATP (Sigma), atropine sulphate (Tokyo Kasei), hexamethonium chloride (Wako), histamine hydrochloride (Wako), 5-hydroxytryptamine creatinine sulphate complex (Sigma), methysergide hydrogen maleate (Sandoz), methionine enkephalin (Peptide Institute Inc.), naloxone hydrochloride (Sigma), nicotine bitartrate (Wako), noradrenaline bitartrate (Wako), phentolamine mesylate (Ciba), prazosin hydrochloride (Tokyo Kasei), pyrilamine hydrochloride (Sigma), substance P (Peptide Institute Inc.), spantide (Peptide Institute Inc.), tetrodotoxin (Sankyo) and yohimbine hydrochloride (Tokyo Kasei). All drugs were dissolved in distilled water and added to the organ bath directly with a micropipette.

Statistical analysis

All values in the text and figures are means $\pm$ s.e.mean. Differences were evaluated by means of Student's t test, $P < 0.05$ being regarded as significant.

Results

Effect of TMS on longitudinal muscle strips of rainbow trout stomach

TMS applied for periods of 10 s at 5–7 min intervals caused a contraction of longitudinal muscle strips of rainbow trout stomach. The magnitude of the response increased in parallel with the stimulation frequency (Figure 1Aa). This contractile response was mediated by excitation of the intramural nerves, because tetrodotoxin (780 nM) completely abolished the response to TMS. To analyse the neurogenic component involved in the response to TMS, the effect of pretreatment with atropine was examined. Atropine (1 μM) had an inhibitory effect on the response to TMS. However, the inhibitory effect of atropine decreased with increasing stimulation frequency (Figure 1Ab). The inhibition by atropine at each stimulation frequency (from 0.5 to 20 Hz) was as follows: 85% at 0.5 Hz, 66% at 1 Hz, 45% at 2 Hz, 38% at 3 Hz, 13% at 5 Hz, 8% at 10 Hz and 1% at 20 Hz. The atropine-resistant response to TMS was completely abolished by tetrodotoxin (780 nM, Figure 1Ac), suggesting a non-cholinergic excitatory innervation of the rainbow trout stomach. In the normal Krebs solution, the contractile response to TMS was observed during stimulation. However, in the presence of atropine, the contractile response to TMS appeared after cessation of the stimulation (rebound-like response) in most of the preparations, but a preceding relaxation was not observed.

In the following experiment, to investigate the pharmacological properties of the non-cholinergic response to TMS, rainbow trout stomach strips were pretreated with atropine (1 μM) and the stimulation frequency was fixed at 20 Hz. At this frequency, the majority of the response to TMS was a non-cholinergic component. The non-cholinergic response to TMS was unaffected by the ganglion blocking agent, hexamethonium (100 μM) (Figure 1B).

To identify the neurotransmitter of the non-cholinergic nerves, initially, the response of rainbow trout stomach strips to some bioactive materials was investigated in atropine (1 μM)-treated preparations. Noradrenaline, methionine enkephalin, substance P and 5-HT, added to the organ bath, caused a contractile response. On the other hand, histamine did not cause any mechanical response at a fairly high concentration (100 μM) and pyrilamine had no inhibitory effect on the response to TMS (Table 1). Therefore, histamine was excluded as the neurotransmitter of the atropine-resistant contraction. Next, an attempt was made to determine which of noradrenaline, methionine enkephalin, substance P or 5-HT mediated the atropine-resistant contractile response to TMS.

Table 1 Effects of tetrodotoxin, atropine, phentolamine, pyrilamine, methysergide, naloxone and substance P-induced desensitization on the contractile response of rainbow trout stomach induced by high frequency transmural stimulation (TMS)

Drugs		*Contraction*[1] (%)	
Control (TMS, 20 Hz)		100	
Tetrodotoxin	(780 nM)	0 ± 0	(6)
Atropine	(1 μM)	99.8 ± 16.5	(5)
Phentolamine	(5.4 μM)	112.0 ± 5.7	(7)
Pyrilamine	(1 μM)	94.5 ± 4.5	(4)
Methysergide	(1 μM)	22.4 ± 10.2	(6)
Naloxone	(1 μM)	98.0 ± 16.3	(4)
SP-desensitization[2]		110.0 ± 6.5	(5)

Values are means ± s.e. The numbers in parentheses represent the number of preparations studied.
Rainbow trout stomach strips were electrically stimulated at 20 Hz for 10 s.
[1] The contraction is expressed as a % of the control response to TMS in the normal nutrient solution.
[2] Stomach strips were pretreated with substance P (1 μM) for 10 min and then transmural stimulation was performed.

Noradrenaline (10 nM–10 μM) caused a transient atropine-resistant contraction, the ED_{50} value was 340 ± 40 nM ($n = 5$). This response to noradrenaline was selectively decreased by phentolamine (5.4 μM) and yohimbine (1 μM), but not by prazosin (1 μM), indicating that the excitatory response to noradrenaline was mediated by α-adrenoceptors, as previously observed (Kitazawa *et al.*, 1986). The α-adrenoceptor antagonist, phentolamine (5.4 μM), had no inhibitory effect on the atropine-resistant contraction caused by TMS (Table 1).

Methionine enkephalin (10 nM–10 μM) caused concentration-dependent contractions of the stomach smooth muscle. Naloxone (1 μM) selectively decreased the responses to methionine enkephalin, but did not affect the atropine-resistant responses to TMS (Table 1).

Substance P (1 nM–300 nM) caused non-sustained atropine-resistant contractions of the stomach strips. The ED_{50} value was 20 ± 5 nM ($n = 4$). An interval of approximately 40 min was required to obtain a reproducible response with a high concentration of substance P (100 nM–300 nM). The contraction caused by substance P was followed by fading of the response, in spite of the continued presence of this peptide. Since substance P receptor antagonists did not decrease the response to substance P in rainbow trout stomach (Holmgren *et al.*, 1985), substance

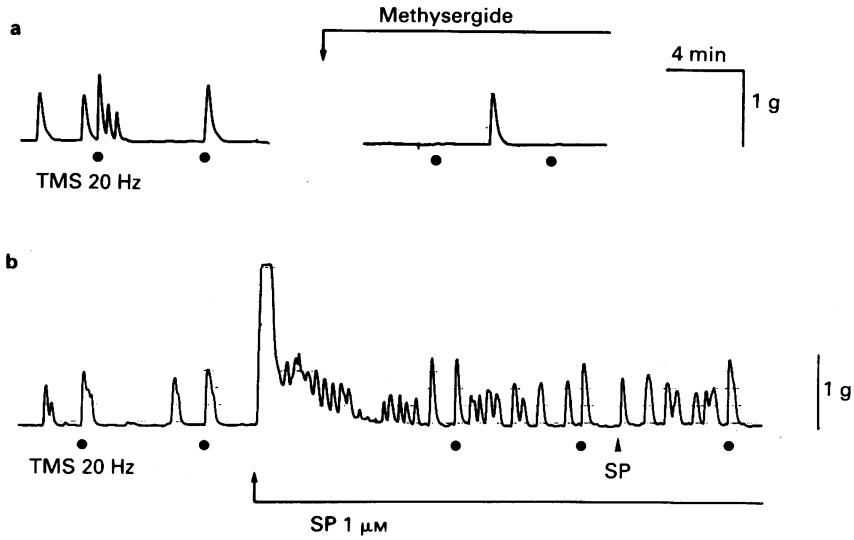

Figure 2 Effects of (a) methysergide (1 μM) and (b) continuous pretreatment with substance P (1 μM) on the atropine-resistant response to transmural stimulation (TMS 20 Hz) of longitudinal muscle strips of rainbow trout stomach. (b) The responses to substance P (SP, 1 μM, ▲) and transmural stimulation (TMS 20 Hz, ●) were examined in the continuous presence of substance P. In (a) and (b), the stomach strips were pretreated with atropine (1 μM).

P-induced desensitization was used in the present experiment. The response to substance P (1 μM) was markedly decreased after continuous exposure to this peptide (1 μM for 10 min). In such substance P-treated preparations, the contractile response to TMS was not decreased (Table 1 and Figure 2b).

5-HT (3 nM–3 μM) caused concentration-dependent contractions of the stomach strips through an action on methysergide-sensitive 5-HT receptors in the presence of atropine (Figure 3). The ED_{50} value was 95 ± 14 nM (n = 5). As shown in Figure 2a and Table 1, pretreatment with methysergide (1 μM) markedly

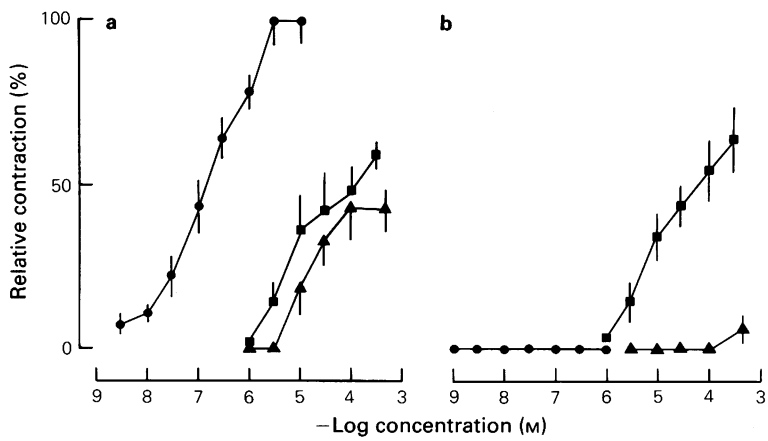

Figure 3 Relationships between the concentrations of ATP, 5-hydroxytryptamine and nicotine, and their excitatory effects on longitudinal muscle strips isolated from rainbow trout stomach. Concentration-response curves for nicotine (▲), ATP (■) and 5-hydroxytryptamine (●) in the presence of (a) atropine (1 μM), and (b) atropine (1 μM) plus methysergide (1 μM). Abscissa scale: concentration of drugs (−log M). Ordinate scale: amplitude of the contraction expressed as a percentage of that induced by 50 mM KCl. Points represent the means of over five experiments with s.e.means shown by vertical lines.

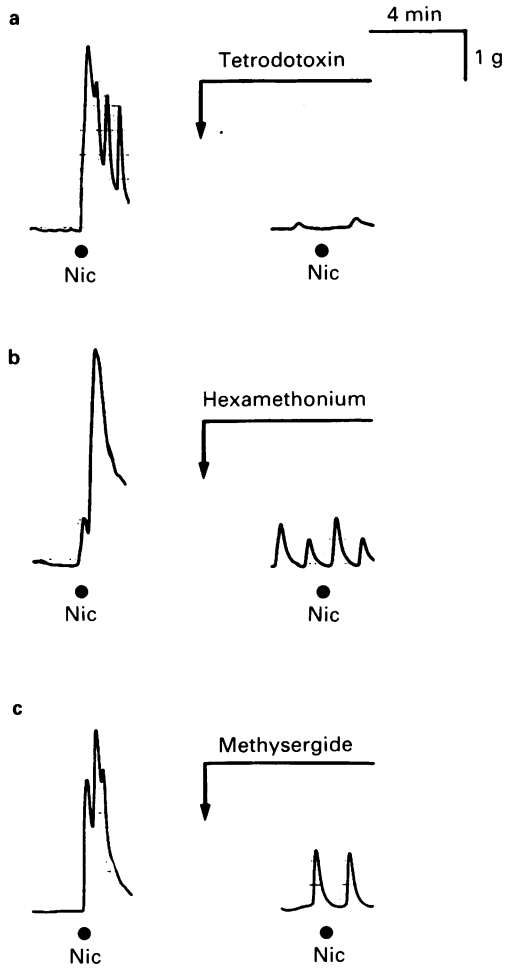

Figure 4 Atropine-resistant contractile responses to nicotine (Nic, 300 μM) of longitudinal muscle strips of rainbow trout stomach and the effect of pretreatment with tetrodotoxin (780 nM, a), hexamethonium (100 μM, b) and methysergide (1 μM, c). All the stomach strips were pretreated with atropine (1 μM).

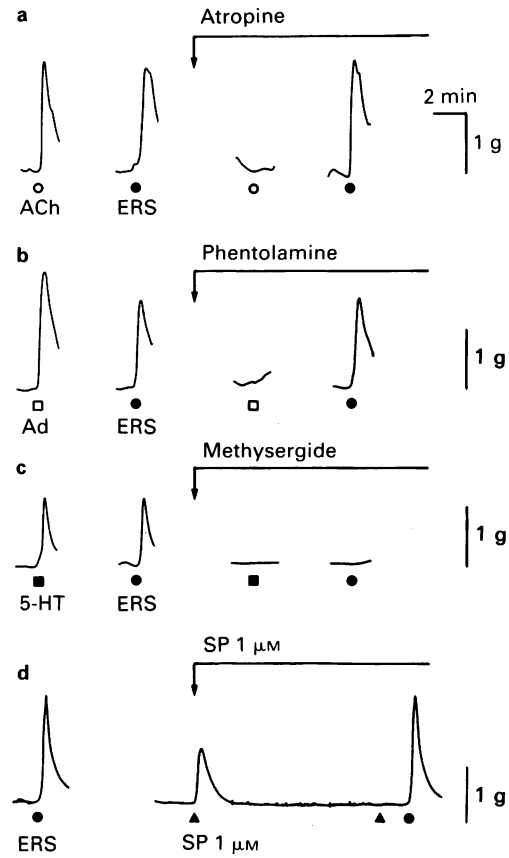

Figure 5 Smooth muscle contracting activity of an acid extract of rainbow trout stomach in longitudinal smooth muscle of rainbow trout stomach. Effects of atropine, phentolamine, methysergide and substance P-induced desensitization on the contracting activity of the extract (ERS, 15 mg tissue wet weight per ml, ●). (a) Atropine (1 μM), acetylcholine (ACh, 340 nM, ○); (b) phentolamine (5.4 μM), adrenaline (Ad, 1 μM, □); (c) methysergide (1 μM), 5-hydroxytryptamine (5-HT, 140 nM, ■); (d) substance P (SP, 1 μM, ▲) treatment for 10 min.

decreased the atropine-resistant contractile response to TMS (20 Hz). Methysergide itself did not affect the basal tonus or the spontaneous contractile activity of the stomach smooth muscle strips.

Effect of nicotine

Nicotine (3 μM–500 μM), added to the organ bath at 30 min intervals, caused non-sustained concentration-dependent contractions of the stomach strips. Atropine (1 μM) partly decreased the contractile response to nicotine, 40–50% of the initial response (non-cholinergic component) remained. The atropine-resistant response to nicotine was markedly decreased by hexamethonium (100 μM) or tetrodotoxin (780 nM), suggesting that nicotine was acting on nicotinic cholinoceptors on the postganglionic non-cholinergic nerves (Figure 4a and b). Methysergide (1 μM) completely abolished the atropine-resistant contractile response to nicotine (Figures 3 and 4c). The relative amplitude of the atropine-resistant contraction caused by nicotine (300 μM) in the presence of methysergide (1 μM) was $4.0 \pm 2.4\%$ ($n = 6$) of the control level.

Table 2 Pharmacological properties of the smooth muscle contracting activity of the acid extract of rainbow trout stomach

	Contraction[1] (%)					
	Tetrodotoxin (780 nM)	*Atropine* (1 μM)	*Phentolamine* (5.4 μM)	*Naloxone* (1 μM)	*Pyrilamine* (1 μM)	*Methysergide* (1 μM)
Rainbow trout stomach						
Extract (15 mg tissue wet weight ml^{-1})	97 ± 5 (5)	98 ± 4.5 (6)	106 ± 1.4 (4)	117 ± 7.4 (4)	95 ± 5.5 (5)	7 ± 3.1 (5)
Acetylcholine (340 nM)	102 ± 4 (4)	0 ± 0 (6)	—	—	—	100 ± 5.2 (6)
5-Hydroxytryptamine (140 nM)	95 ± 6 (5)	101 ± 2.1 (6)	—	—	—	−7 ± 2.4 (5)
Guinea-pig ileum						
Extract (10 mg tissue wet weight ml^{-1})	96 ± 3.6 (5)	67 ± 4.7 (6)	—	—	51 ± 16.5 (5)	59 ± 4.7 (5)

Values are means ± s.e. The numbers in parentheses represent the number of preparations studied. —: Not determined.
Smooth muscle contracting activity of the extract was bioassayed on both rainbow trout stomach strips and guinea-pig ileum longitudinal smooth muscle.
[1] The contraction is expressed as a % of the initial response in the normal nutrient solution.

Contractile response to ATP

ATP (1 μM–300 μM) caused a contraction of the stomach smooth muscle in spite of the presence of atropine (1 μM) (Figure 3); the ED_{50} value was 14 ± 4 μM ($n = 4$). Tetrodotoxin (780 nM) did not affect the response to ATP, indicating that ATP acted directly on the smooth muscle cells. Pretreatment with methysergide (1 μM) did not affect the concentration-response curve to ATP (Figure 3); the ED_{50} value for ATP in the presence of methysergide was 10 ± 3 μM ($n = 4$).

Effect of an acid extract of rainbow trout stomach

The smooth muscle excitatory activity of an acid extract obtained from rainbow trout stomach (ERS) was bioassayed in rainbow trout stomach strips. In some experiments, GPILM was used.

Rainbow trout stomach ERS caused contraction of rainbow trout stomach strips in a concentration-dependent manner (0.1 mg–20 mg tissue wet weight per ml). ERS was considered to act on the smooth muscle directly to cause the contractile response, because tetrodotoxin (780 nM) did not affect the contraction induced by ERS. The excitatory response to ERS was not decreased by atropine, phentolamine, pyrilamine or naloxone (Figure 5a and b, and Table 2). On the other hand, methysergide (1 μM) markedly decreased the responses to 5-HT and ERS (15 mg tissue wet weight per ml) (Figure 5c), but the contraction induced by acetylcholine (340 nM), of a similar amplitude, was unaffected by methysergide (Table 2). Continuous pretreatment with substance P (1 μM) decreased the contractile response to this peptide, but the ERS-induced contraction was unaffected (Figure 5d).

Guinea-pig ileum longitudinal muscle ERS also caused concentration-dependent contractions of the GPILM. The contractile response to ERS was rapid in onset, the maximum tension was reached within 10 s and the response was reproducible on repeated exposure for 2 min at 15 min intervals. The contractile response to ERS in the GPILM was significantly decreased by atropine (1 μM), pyrilamine (1 μM) and methysergide (1 μM), respectively (Figure 6 and Table 2). These results indicate that part of the smooth muscle contracting activity of ERS in GPILM is due to acetylcholine, histamine and 5-HT. In the presence of atropine, methysergide and pyrilamine, ERS still caused a contraction of the GPILM. In the presence of these three antagonists, substance P also induced an excitatory response (Figure 6d). Substance P-induced desensitization (500 nM for 10 min) markedly decreased the response to both ERS (10 mg tissue wet weight per ml) and substance P (0.4 nM) (Figure 6d). The substance P receptor antagonist, spantide (10 μM for 5 min), also decreased the contractile response to both ERS and substance P (0.4 nM).

Discussion

The results of the present experiments confirm the previous observations that TMS causes

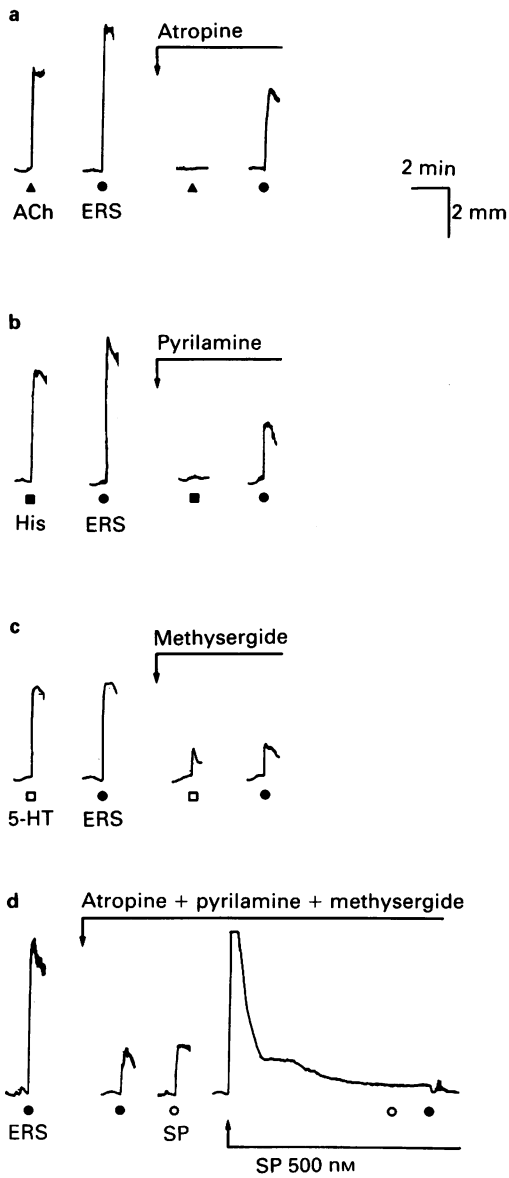

Figure 6 Smooth muscle contracting activity of an acid extract of rainbow trout stomach in guinea-pig ileum longitudinal smooth muscle. Effects of atropine, pyrilamine, methysergide and substance P-induced desensitization on the contracting activity of the extract (ERS, 10 mg tissue wet weight per ml, ●). (a) Atropine (1 μM), acetylcholine (ACh, 34 nM, ▲); (b) pyrilamine (1 μM), histamine (His, 45 nM, ■); (c) methysergide (1 μM), 5-hydroxytryptamine (5-HT, 1.5 μM, □). (d) In the presence of atropine (1 μM), pyrilamine (1 μM) and methysergide (1 μM), substance P-induced desensitization (500 nM for 10 min) decreased the responses to the extract (ERS) and substance P (SP, 0.4 nM, ○).

tetrodotoxin-sensitive frequency-dependent contractions of the rainbow trout stomach strips (Holmgren & Nilsson, 1981; Kitazawa *et al.*, 1986). Atropine significantly decreased the response to low frequency stimulation, but did not decrease that to high-frequency stimulation, and, therefore, it was suggested that low frequency stimulation mainly excited cholinergic excitatory nerves and high frequency stimulation excited non-cholinergic excitatory nerves. The atropine-resistant response induced by TMS was not decreased by hexamethonium, and nicotine, a ganglion stimulant, also caused tetrodotoxin-sensitive atropine-resistant contractions of the longitudinal muscle of rainbow trout stomach, indicating that the non-cholinergic nerves are of postganglionic origin. Although Holmgren & Nilsson (1981) demonstrated the presence of non-adrenergic, non-cholinergic (NANC) inhibitory nerves in the rainbow trout stomach, TMS and nicotine were not observed to have any inhibitory effect (relaxation) in the present experiments. Therefore, NANC inhibitory innervation of the rainbow trout stomach could not be confirmed.

Adrenergic stimulation (splanchnic nerve and catecholamine) causes a contractile response of the trout gastrointestinal smooth muscle (Burnstock, 1958; Campbell & Gannon, 1976; Kitazawa *et al.*, 1986). However, since the atropine-resistant response to TMS was unaffected by phentolamine, adrenergic nerves were not thought to be involved in the non-cholinergic response to TMS in the rainbow trout stomach, as previously observed (Holmgren & Nilsson, 1981).

From the present results, it is assumed that 5-HT is the neurotransmitter released from the non-cholinergic nerves in the rainbow trout stomach. The following evidence supports this assumption, (1) 5-HT caused a contraction of rainbow trout stomach strips directly, despite the presence of atropine, and the ED_{50} value was lower than that for acetylcholine (190 nM; Kitazawa *et al.*, 1986), and (2) the non-cholinergic excitatory responses induced by TMS and nicotine were markedly decreased by the 5-HT receptor antagonist, methysergide. Recently, Holmgren *et al.* (1985) demonstrated that the main action of exogenous substance P on the stomach wall of the rainbow trout was indirect, i.e., through activation of NANC excitatory nerves. The response to substance P was decreased by methysergide, so it was suggested that NANC nerves release 5-HT on excitation by substance P (Holmgren *et al.*, 1985). This functional observation supports the results of the present study.

ERS exhibited smooth muscle contracting activity in the rainbow trout stomach. This smooth muscle excitatory activity of ERS was unaffected by tetrodotoxin, atropine, phentolamine, pyrilamine and

naloxone, indicating that it was unlikely that acetylcholine, catecholamines, histamine or enkephalin mediated the contracting activity of ERS. Methysergide almost completely abolished the excitatory activity of ERS in the rainbow trout stomach, without affecting the response to acetylcholine. This result indicates that the main smooth muscle contracting activity induced by ERS in rainbow trout smooth muscle is due to 5-HT and suggests an important physiological role of 5-HT as an excitatory mediator in the motility of rainbow trout stomach. Endogenous 5-HT in the gastrointestinal tract is stored in both enterochromaffin cells of the mucosa and the enteric nervous system (Costa & Furness, 1979). Since morphological studies have already demonstrated the presence of 5-HT nerve fibres and nerve cell bodies in the myenteric plexus and smooth muscle layer, but not in the mucosa of the rainbow trout stomach (Anderson, 1983; Holmgren *et al.*, 1985), 5-HT present in ERS originates from enteric 5-HT nerves in the rainbow trout stomach. The presence of 5-HT nerves supports the conclusion of the present study, that 5-HT is one of the possible neurotransmitters of the non-cholinergic excitatory nerves stimulated by TMS and nicotine.

For the gastrointestinal tract of some fish, ATP has been proposed as the neurotransmitter of non-cholinergic excitatory nerves, since ATP induced a contractile response that mimicked that of non-cholinergic nerve stimulation (Burnstock *et al.*, 1972; Young, 1980b). In the present study, ATP was found to induce a distinct contraction of rainbow trout stomach strips in spite of the presence of atropine. Therefore, the possibility that ATP is involved in the non-cholinergic contraction of the rainbow trout stomach cannot be completely excluded. Further work is obviously needed to clarify the physiological role of ATP in the motility of rainbow trout stomach. However, as the contractile response to ATP was not affected by tetrodotoxin or methysergide, ATP did not stimulate 5-HT nerves, but acted directly on the smooth muscle cells.

In addition to rainbow trout stomach, GPILM was used to bioassay the smooth muscle contracting activity of ERS. GPILM showed a higher sensitivity to acetylcholine, histamine and substance P than the rainbow trout stomach. The smooth muscle contracting activity of ERS was decreased not only by methysergide, but also by atropine, pyrilamine, spantide and substance P-induced desensitization. Therefore, in addition to 5-HT, acetylcholine, histamine and substance P were determined to be present in ERS as smooth muscle excitatory substances. Acetylcholine has been found to be located mainly in the cholinergic nerves of the gastrointestinal tract (Paton & Aboo Zar, 1968), which supports the cholinergic innervation indicated by the physiological studies involving TMS and nicotine. The presence of histamine in ERS was suggested, but since it had no mechanical activity on the rainbow trout stomach at a relative high concentration, the physiological role of histamine could not be determined in the present study. Substance P has been considered as a possible neurotransmitter of the non-cholinergic excitatory nerves in the gastrointestinal tract of some mammals (Franco *et al.*, 1979; Leander *et al.*, 1981; Wali, 1985; Matušák & Bauer, 1986). Recent immunohistochemical studies demonstrated that substance P-like peptide-containing nerves are usually present in the gastrointestinal tract of several species of teleost fish, including the rainbow trout (Holmgren *et al.*, 1982; Bjenning & Holmgren, 1988). This histochemical evidence suggested the possibility of a similar neurotransmitter function (neurotransmitter of non-cholinergic excitatory nerves) of this peptide in fish. In fact, a substance P-like peptide mediated the non-cholinergic excitatory response of the carp intestinal bulb (Kitazawa *et al.*, 1988). In the present study, since a substance P-like peptide was extracted from the rainbow trout stomach, innervation by substance P-like peptide-containing nerves in this tissue was suggested. However, as substance P-induced desensitization did not decrease the non-cholinergic response to TMS, involvement of a substance P-like peptide in the non-cholinergic response to TMS in the rainbow trout stomach strips was excluded. Substance P causes the contraction of rainbow trout stomach smooth muscle indirectly, through the excitation of non-cholinergic nerves that release 5-HT (Holmgren *et al.*, 1985). Therefore, it is thought that substance P is not the final mediator of the non-cholinergic response, but that it acts as a neurotransmitter for the intrinsic non-cholinergic excitatory nerves (5-HT nerves).

In conclusion, these results show that: (1) 5-HT causes contraction of rainbow trout stomach strips, (2) the non-cholinergic responses induced by TMS and nicotine are decreased by methysergide, and (3) an acid extract of the rainbow trout stomach contains 5-HT. From this evidence, it is thought that 5-HT is one of the possible neurotransmitters of the non-cholinergic excitatory nerves in the rainbow trout stomach.

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Protein kinase C regulates the tonic but not the phasic component of contraction in guinea-pig ileum

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1 We have investigated the effect of phorbol esters and the down-regulation of protein kinase C on contraction of guinea-pig ileum longitudinal smooth muscle to carbachol and high K^+.

2 Phorbol 12,13-dibutyrate (PDBu) enhanced the phasic component and inhibited or enhanced, respectively, the tonic component of contraction to carbachol and high K^+. In contrast, 4α-phorbol, which does not activate protein kinase C, had no effect on these responses.

3 Exposure to phorbol 12-myristate 13-acetate (PMA; 1 μM) for up to 8 h induced a time-dependent loss of [^{3}H]-PDBu binding sites, consistent with the down-regulation of protein kinase C by this treatment.

4 The phasic component of contraction to carbachol or high K^+ was unaffected following the down-regulation of protein kinase C. The tonic component of contraction to carbachol was markedly enhanced by this treatment while that to high K^+ was partially suppressed.

5 These data suggest that although the activation of protein kinase C can lead to potentiation of the phasic component of contraction to carbachol or high K^+, this appears to have little physiological significance since the response is not altered in tissues in which protein kinase C has been down-regulated. On the other hand, protein kinase C may limit the tonic contraction to carbachol but potentiate that to high K^+.

Introduction

It is well established that receptor-stimulated hydrolysis of membrane inositol phospholipids generates at least two second messengers, inositol 1,4,5-trisphosphate (InsP3) and 1,2-diacylglycerol, which stimulate the release of intracellular Ca^{2+} and activate protein kinase C, respectively (for review, see Berridge, 1987). A great deal of evidence is now available to suggest that this pathway plays an important role in initiating contraction in smooth muscle (for review, see Watson & Godfrey, 1988). For example, in porcine coronary artery stimulated with acetylcholine (Ito *et al.*, 1979) or rabbit mesenteric artery stimulated with low concentrations of noradrenaline (Itoh *et al.*, 1983), the majority of the Ca^{2+} required for contraction is derived from intracellular stores and the formation of InsP3 is believed to have an essential role in stimulating this release (Suematsu *et al.*, 1984; Hashimoto *et al.*, 1986). Moreover, even in smooth muscles such as the guinea-pig ileum, in which the majority of Ca^{2+} utilised for contraction enters from the extracellular medium through voltage-operated channels, it has been hypothesized that the release of intracellular Ca^{2+} by InsP3 has an essentail role in mediating the subsequent entry of Ca^{2+} (Watson *et al.*, 1988).

In contrast, the role of protein kinase C in smooth muscle is less certain. In some, but not all smooth muscles, prolonged exposure to phorbol esters, which are potent stimulants of protein kinase C (Nishizuka, 1984), induces a slowly developing contraction which starts after several minutes and peaks at times often in excess of one hour (for review see Watson & Godfrey, 1988). In some instances this response appears to be dependent on the mobilisation of extracellular Ca^{2+} while in others it appears to be Ca^{2+}-independent. In other smooth muscles, however, the activation of protein kinase C has been reported to have no effect on tension or to induce relaxation. For example, in guinea-pig ileum longitudinal smooth muscle and trachea, phorbol esters have been reported to stimulate relaxation

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(Baraban *et al.*, 1985; Menkes *et al.*, 1986), although other workers have failed to confirm this finding in guinea-pig ileum (Salmon *et al.*, 1986).

These studies, however, have provided little direct evidence concerning the role of protein kinase C during contraction induced by receptor or non-receptor mechanisms. In the present paper, therefore, we have addressed this question through the investigation of the effect of phorbol esters and of the down-regulation of protein kinase C (induced by long-term exposure to phorbol esters) on contractions to carbachol and high K^+ in guinea-pig ileum longitudinal smooth muscle. If protein kinase C has an important role during contraction to these stimuli, then direct stimulation and down regulation should have opposite effects on the tension developed.

Methods

Tissue preparation

Hartley-strain guinea-pigs of either sex, weighing 300–500 g, were killed by a blow on the head. The whole length of the small intestine except for the duodenum and the ileocaecal region was quickly removed into pre-gassed (O_2/CO_2, 19:1) Krebs-Henseleit solution at room temperature. Longitudinal smooth muscle was carefully removed by gentle stroking with moist cotton wool (Sasaguri & Watson, 1988). Cells were isolated and dispersed with collagenase as described previously (Sasaguri & Watson, 1988). In brief, tissue slices were incubated in HEPES-buffered solution in the absence of Mg^{2+} or Ca^{2+} for 60 min before addition of collagenase (2.5 mg ml^{-1}), bovine serum albumin (1 mg ml^{-1}) and soyabean trypsin inhibitor (0.5 mg ml^{-1}). The digestion was continued for 60 min with gentle mixing every 10 min. The preparation was filtered through nylon mesh (250 × 250 μm) and cells obtained by centrifugation.

Recording of contractions

Longitudinal muscle strips (approximately 10 mm length with no tension) were set up in 3 or 5 ml glass organ baths to measure auxotonic contractions (1.5 g tension). Experiments were performed after 1 h equilibration in HEPES-buffered solution gassed with 100% O_2 at 37°C. All contraction data, if not otherwise indicated, are shown as percentages of the response to 1 μM carbachol recorded at the beginning of each experiment.

Assay of inositol phosphates

This was carried out on cross-chopped slices, prelabelled with [^{3}H]-inositol (5 μCi ml^{-1}), as described previously (Sasaguri & Watson, 1988) except for the following modification. The incubation was started by an application of stimulant dissolved in 5 μl of HEPES-buffered solution to cross-chopped tissue slices (50 μl aliquot of gravity-packed slices) suspended in 245 μl of the same solution containing 10.2 mM LiCl, which had been warmed for 2 min at 37°C. Incubation time is shown in the legends. LiCl was included in order to block inositol monophosphatase (Hallcher & Sherman, 1981). The reaction was stopped by addition of $CHCl_3$: methanol: HCl (100:200:2; v/v/v) and total inositol phosphates (inositol mono-, bis- and trisphosphates) were measured by Dowex-anion exchange chromatography.

Binding studies with phorbol ester and down-regulation of protein kinase C

Collagenase-dispersed cells (3 × 10^5 ml^{-1}) were incubated in HEPES-buffered solution (0.5 ml) containing an indicated concentration of [^{3}H]-PDBu at 37°C for 10 min. Non-specific binding was determined in the presence of a 100 fold higher concentration of unlabelled PDBu. Incubations were terminated by rapid filtration through GF/C filters, which were then washed three times with ice-cold HEPES-buffered solution (3 × 5 ml). Radioactivity on the filters was determined by liquid scintillation counting.

To cause the down-regulation of protein kinase C, tissue strips were incubated in 10 ml of HEPES-buffered solution containing 1 μM phorbol 12-myristate 13-acetate (PMA) for up to 8 h with oxygenation at 37°C. At various times, the tissue strips were removed, washed with buffer, and cells isolated and dispersed with collagenase as described above. The total time for the preparation of cells was approximately 150 min, thus allowing time for the washout of PMA before the binding of [^{3}H]-PDBu was measured.

Solutions

The following solutions were used: Krebs Henseleit (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 10. The solution was bubbled with O_2 and CO_2 (19:1); the pH was 7.4 at room temperature. HEPES-buffered (mM): HEPES/Tris buffer 5; NaCl 135, KCl 4, $MgCl_2$ 2, $CaCl_2$ 2, KH_2PO_4 1, glucose 12. The solution was bubbled with 100% O_2; the pH was 7.4 at 37°C. HEPES/Tris buffer was made by adding Tris to approximately 0.2 M HEPES to adjust pH to 7.4 at 37°C followed by dilution to 5 mM. High-K^+: Potassium concentration was elevated by the addition of KCl without replacement of NaCl, because lowering

the extracellular NaCl concentration stimulates inositol phospholipid hydrolysis by an unknown mechanism (Sasaguri & Watson, 1988).

Chemicals

[20(N)-^{3}H]-phorbol 12,13-dibutyrate ([^{3}H]-PDBu; 23 Ci mmol^{-1}, dissolved in acetonitrile: water [40:60, v/v]) was from Amersham International. Phorbol 12,13-dibutyrate (PDBu), phorbol 12-myristate 13-acetate (PMA), 4-α-phorbol, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), nifedipine, hydroxymethylaminomethane (Tris), ethyleneglycol-*bis*-(β-aminoethylether) N, N'-tetraacetic acid (EGTA), soyabean trypsin inhibitor (typeI-S), and bovine serum albumin (RIA grade) were all purchased from Sigma, carbamylcholine chloride (carbachol) was from BDH. Collagenase extracted from *Clostridium histolyticum* (0.219 μmol min^{-1} mg^{-1} protein) was from Worthington Enzymes.

Statistics

The results are expressed as the mean ± s.e.mean of the number of observations (n). The statistical significance was assessed by Student's t test for paired or unpaired values. P values less than 0.05 were considered to be significant. Binding curves were analysed by nonlinear regression using an iterative curve-fitting programme (GraphPAD, ISI Software).

Results

Effect of phorbol ester on contractions to carbachol or high K$^+$

The contractile response of guinea-pig ileum longitudinal smooth muscle to carbachol or high K$^+$ consists of two distinct phases, a rapid, transient component (phasic contraction) and a more slowly developing, often sustained component (tonic contraction). Representative traces of contraction to carbachol or high K$^+$ are shown later on (Figures 3 and 4).

In order to investigate the role of protein kinase C during the phasic component of contractions to carbachol or high K$^+$, a phorbol ester, PDBu, was applied 2 min before the stimuli. On its own, PDBu had no effect on the resting tension, although spontaneous oscillatory movements were slightly increased at concentrations above 100 nM (not shown). PDBu enhanced the phasic component of contraction to both stimuli with an EC_{50} value of approximately 10–30 nM and a maximal effect occurring at 300 nM (Figure 1). This concentration-

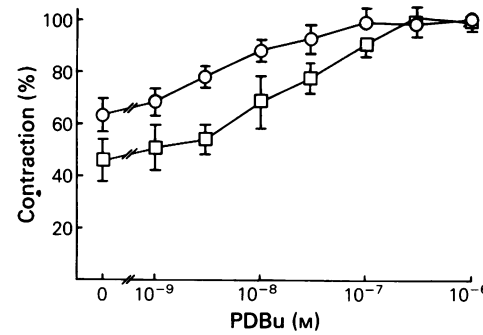

Figure 1 Dose-response curves for phorbol 12,13-dibutyrate (PDBu) potentiation of the phasic component of contraction to carbachol or high K$^+$. Approximately 20 min after recording the control contractions, PDBu was added 2 min before the addition of carbachol or KCl: (○) 0.3 μM carbachol, (□) 25 mM KCl. Vertical bars show s.e.mean of four independent experiments.

range is about 3 times higher than that at which PDBu induces maximal activation of protein kinase C in platelets (Lapetina, 1984; Nishizuka, 1984). Enhancement by PDBu (300 nM) was observed at all sub-maximally effective concentrations tested for both stimuli resulting in an approximately two fold shift to the left in their dose-response curves (Figure 2). In contrast, 4α-phorbol (1 μM), which does not acitvate protein kinase C, had no effect on the phasic component of contraction to carbachol or high K$^+$ (not shown). Under no conditions did phorbol esters inhibit the phasic component of contraction, in marked contrast to the results of Baraban *et al.* (1984).

In order to look at the effect of PDBu on the tonic component of contraction to carbachol or high K$^+$, PDBu was added 1 min after the application of these stimuli. As shown in Figure 3, PDBu (1 μM) inhibited the tonic component induced by lower concentrations (30–300 nM) of carbachol but markedly potentiated oscillatory contractions. In contrast PDBu caused a small potentiation of the tonic component of contraction to high K$^+$ except at a concentration of 112 mM (Figure 4). 4α-Phorbol (1 μM) had no effect on the tonic component of contraction to either stimuli.

Effect of down-regulation of phorbol ester binding site on contractions to carbachol or high K$^+$

To investigate the physiological role of protein kinase C in contraction to carbachol or high K$^+$, an attempt was made to deplete the tissues of this

enzyme by a long-term exposure to a high concentration of phorbol ester. This method has been used to reduce both amount and activity of protein kinase C in a variety of cell types including NG115-401L neuronal cells (Mattingly *et al.*, 1987).

Binding of [^{3}H]-PDBu (1 nM) to dispersed cells reached saturation by 10 min (not shown). The binding of [^{3}H]-PDBu was specific and appeared to bind to a single site as indicated by the linear Scatchard plot shown in Figure 5. The K_d value estimated from the saturation plot (Figure 5a) was 31.9 ± 4.9 nM; the density of binding sites 468 ± 22 fmol per 1.5 × 10^5 cells and the Hill coefficient 0.81 ± 0.12.

PMA was used to cause the down-regulation of protein kinase C, because PDBu is unable to cause complete down-regulation of this enzyme in some cell types (Mattingly *et al.*, 1987). Treatment with a high concentration of PMA (1 μM) caused a time-dependent reduction in specific [^{3}H]-PDBu-binding;

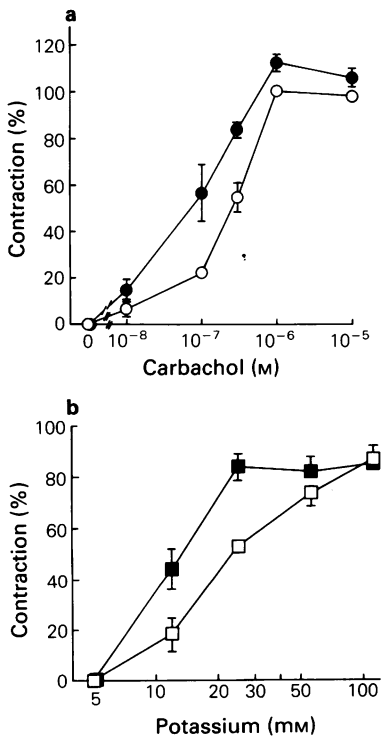

Figure 2 Effect of phorbol 12,13-dibutyrate (PDBu) on the dose-response curve for the phasic component of contraction to carbachol or high-K^+. The methodology is the same as in Figure 1. (a) Carbachol-induced contractions; (○) control; (●) PDBu (300 nM). (b) KCl-induced contractions; (□) control; (■) PDBu (300 nM). Vertical bars show s.e.mean of four independent experiments.

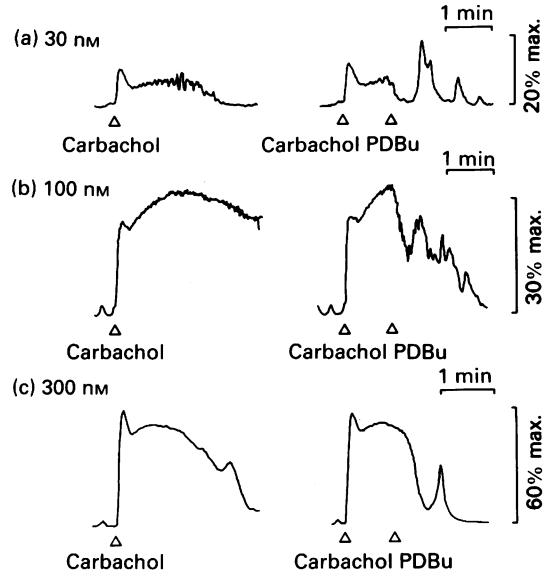

Figure 3 Effect of phorbol 12,13-dibutyrate (PDBu) on the tonic component of contraction to carbachol. Control traces, shown on the left, were recorded 20 min before the experimental traces, shown on the right, in which PDBu (1 μM) was added 1 min after carbachol. The traces are representative of four independent experiments.

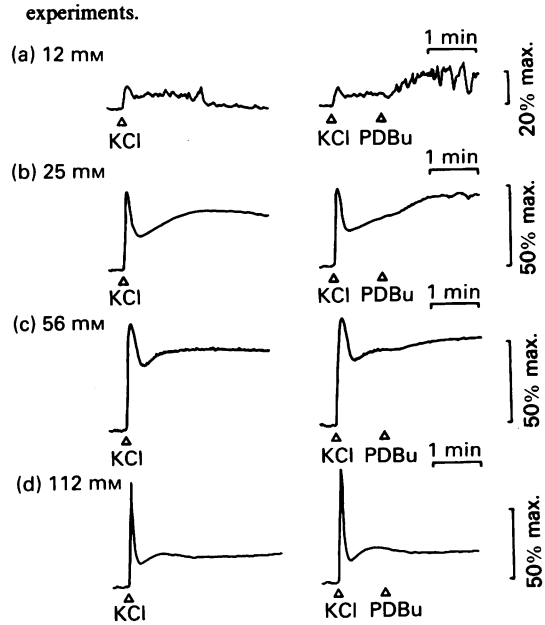

Figure 4 Effect of phorbol 12,13-dibutyrate (PDBu) on the tonic component of contraction to high K^+. Control traces, shown on the left, were recorded 20 min before the experimental traces, shown on the right, in which PDBu (1 μM) was added 1 min after KCl. The traces are representative of four independent experiments.

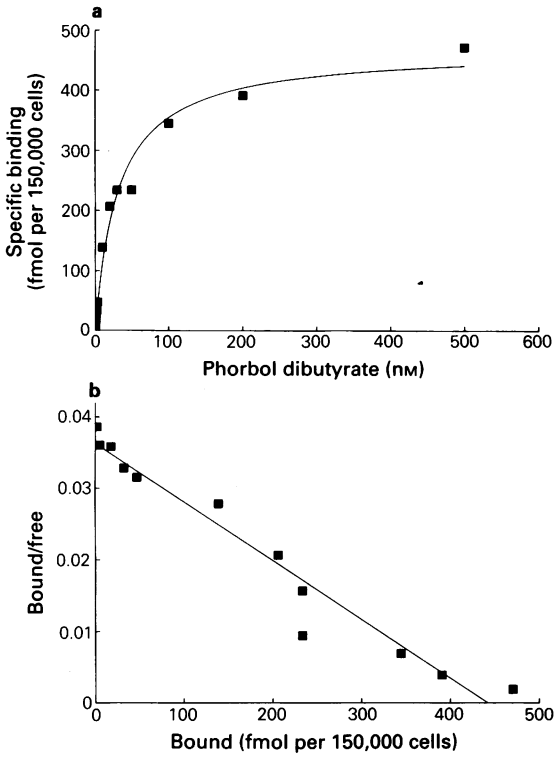

Figure 5 Specific binding of [^{3}H]-phorbol 12,13-dibutyrate (PDBu) to dispersed muscle cells prepared by collagenase digestion. (a) Dependence of the binding (per 1.5×10^5 cells) on concentration of [^{3}H]-PDBu. Vertical bars indicate s.e.mean of the three separate experiments in triplicate. (b) Scatchard analysis of the specific binding of [^{3}H]-PDBu. The line has been fitted by linear regression.

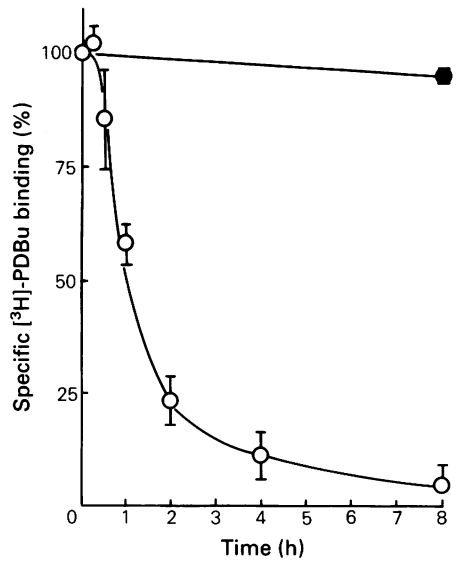

Figure 6 Time-course of down-regulation of the binding of 10 nM [^{3}H]-phorbol 12,13-dibutyrate (PDBu) elicited by treatment with phorbol 12-acetate, 13-myristate (PMA); 100% represents the specific binding (5976 ± 774 d.p.m.) before treatment with PMA. (○) Incubated with PMA (1 μM); (●) incubated without PMA for 8 h. Tissues were incubated in buffer for 150 min following exposure to PMA before the binding of [^{3}H]-PDBu was investigated. Vertical bars indicate s.e.mean from three independent experiments performed in quadruplicate.

this reduction was greater than 95% following an 8 h incubation with PMA (Figure 6).

Exposure of the tissue to PMA for up to 8 h had no effect on the resting tension (not shown). In contrast, and in agreement with the results with PDBu, there was an initial potentiation of the phasic component of contraction to both carbachol (1 μM) and K^+ (56 mM) following short-term exposure to PMA which slowly returned to control levels as the incubation with PMA proceeded (Figures 7 and 8). After exposure to PMA for 6 h the phasic contraction to these two stimuli was the same as in control tissues (Figure 7 and 8). The slow relaxation of the tonic component of contraction to carbachol observed in control tissues slowly disappeared with increased exposure to PMA and, after 2 h, began to increase (Figure 7). In contrast, the tonic component induced by high-K^+ (especially the initial steep rise of the tonic component) decreased as the incubation with PMA progressed and, after 8 h, the contraction was only 50% of that in control tissues (Figure 8).

Effect of phorbol ester on the formation of inositol phosphates

Since the present observations indicate that activation of protein kinase C inhibits the tonic component of the contractions induced by carbachol, we investigated whether this effect results from inhibition of the formation of inositol phosphates. As shown in Table 1, PDBu had little effect on the formation of inositol phosphates induced by carbachol and high K^+, although this concentration is maximally effective at potentiating the phasic contraction to these stimuli. Similarly, in tissues in which protein kinase C had been down-regulated by incubation with PMA, carbachol formation of inositol phosphates was not changed significantly (Table 2).

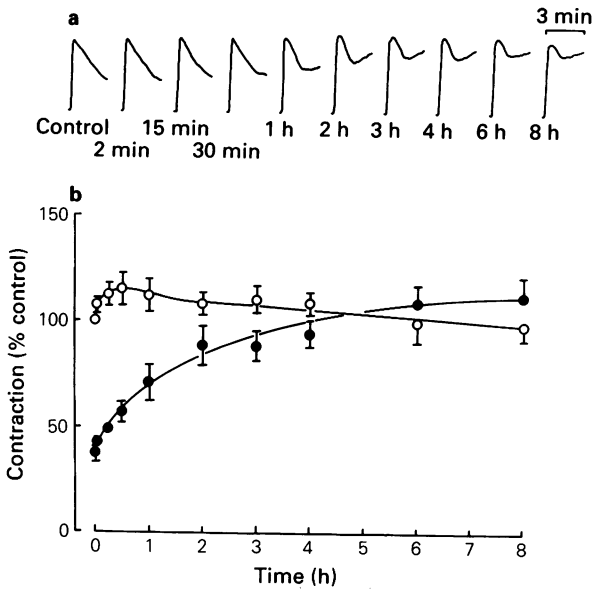

Figure 7 Effect of down-regulation of protein kinase C on contraction to carbachol. Longitudinal smooth strips were exposed to phorbol 12-acetate 13-myristate (PMA) for the times indicated and carbachol (1 μM)-induced contractions were recorded in the continued presence of 1 μM PMA. (a) A representative series of contractions obtained from a single tissue; (b) (○) phasic components; (●) tonic components measured 3 min after the application of carbachol. Data are expressed as percentages of the peak phasic component of contraction to 1 μM-carbachol obtained before application of PMA. Vertical bars indicate s.e.mean of four independent experiments.

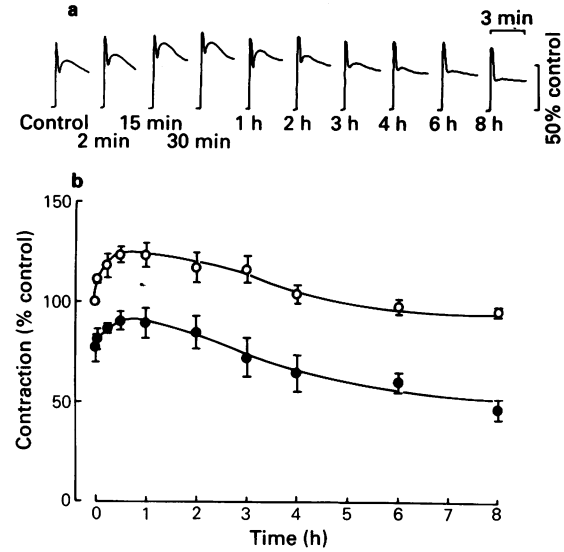

Figure 8 Effect of down-regulation of protein kinase C on contraction to high K^+. Longitudinal smooth strips were exposed to phorbol 12-acetate 13-myristate (PMA) for the times indicated and KCl (56 mM)-induced contractions were recorded in the continued presence of 1 μM PMA. (a) A representative series of contractions obtained from a single tissue; (b) (○) phasic component; (●) tonic component measured at 1 min after the application of KCl. Data are expressed as percentages of the phasic component of the contraction to 56 mM KCl obtained before application of PMA. Vertical bars indicate s.e.mean of four independent experiments.

Table 1 Effect of phorbol 12,13-dibutyrate (PDBu) on the formation of inositol phosphates by carbachol and high K^+

	Inositol phosphates (% of basal)
Basal	100.0 ± 3.7
Carbachol (300 nM)	130.0 ± 5.6
Carbachol (300 nM) + PDBu (1 μM)	131.6 ± 6.2
Carbachol (3 μM)	162.7 ± 4.9
Carbachol (3 μM) + PDBu (1 μM)	155.5 ± 6.5
KCl (112 mM)	199.1 ± 14.3
KCl (112 mM) + PDBu (1 μM)	207.3 ± 12.8

Tissue slices were pre-labelled with [^{3}H]-inositol as described in the methods and incubated with carbachol or high K^+ with or without phorbol dibutyrate for 3 min in the presence of LiCl (10 mM). Results are shown as means ± s.e.mean of percentage of basal (basal d.p.m. was 1079 ± 40) from four experiments performed in quadruplicate. * $P < 0.05$ compared with carbachol alone.

Table 2 Effect of down-regulation of protein kinase C on the formation of inositol phosphates by carbachol and high K^+

	Inositol phosphates (% *of control*) *Vehicle-treated*	*PMA-treated*
Basal	100.0 ± 4.7	90.9 ± 3.3 (100 ± 4)
Carbachol (1 μM)	111.9 ± 3.6	104.0 ± 1.7 (114 ± 2)
Carbachol (10 μM)	162.7 ± 6.9	154.7 ± 2.2 (170 ± 2)
K^+ (56 mM)	135.3 ± 2.6	129.2 ± 7.6 (142 ± 8)

Tissue strips were incubated in the absence or presence of PMA (1 μM) for 4 h at 37°C and then labelled with [^{3}H]-inositol as described in the methods. Other conditions are as described in Table 1. The results are shown as % mean ± s.e.mean of the basal value (d.p.m. = 403.4 ± 19.0) from vehicle-treated tissue from three experiments performed in quadruplicate; the values in parentheses show the results presented as % of basal from PMA-treated tissues.

Discussion

Role of the phosphoinositide pathway in contractions induced by carbachol

Little is known about the possible roles played by protein kinase C in the sequence of events leading to contraction following the activation of muscarinic receptors. In the present study we have used two approaches in an attempt to address this question. Firstly, we have shown that the activation of protein kinase C by phorbol esters potentiates the phasic component of the carbachol-induced contraction but inhibits the tonic component (when the phorbol ester is given after the decline of the phasic component) whilst increasing oscillatory movements. Secondly, we have shown that the down-regulation of protein kinase C has no significant effect on the phasic component of contraction but produces a marked potentiation of the tonic component. These observations can be reconciled in the following manner. Although the activation of protein kinase C can potentiate the phasic component of contraction, this appears to be of little physiological significance as this component was not significantly reduced in the down-regulated tissues. In contrast, the tonic component was inhibited following the application of PDBu but potentiated in the down-regulated tissues indicating that protein kinase C may have a negative feedback role during this component of contraction.

This negative feedback action of protein kinase C could be mediated through an inhibition of phospholipase C, as observed in vascular smooth muscle (Itoh *et al.*, 1988). However, we were unable to observe a similar inhibitory action of PDBu against the formation of inositol phosphates by carbachol at a concentration of 1 μM. Further, carbachol formation of inositol phosphates was not altered significantly following the down-regulation of protein kinase C. These data indicate that the activation of phospholipase C by carbachol is not under feedback control from protein kinase C.

Role of the phosphoinositide pathway in contractions induced by high K^+

Consistent with the results obtained for carbachol, phorbol esters also potentiated the phasic component of contraction to high K^+ but this response was not significantly changed following the down-regulation of protein kinase C. These data indicate that protein kinase C is unlikely to be involved in this component of the contractile response to K^+. This conclusion is in agreement with our earlier observation that the activation of phospholipase C by high K^+ is a delayed response mediated by the entry of Ca^{2+} through voltage-operated channels (Sasaguri & Watson, 1988).

On the other hand, and in contrast with the results obtained for carbachol, phorbol esters caused a small potentiation of the tonic component of contraction to high-K^+ and the down-regulation of protein kinase C inhibited this component. These results indicate that protein kinase C may be involved in the maintenance of the tonic contraction to high K^+. The contrast between these results and those obtained for carbachol is presumably due to the fundamentally different mechanisms through which these stimuli initiate contraction. Carbachol interacts with a cell-surface receptor coupled to a G-protein and high K^+ directly induces membrane depolarisation.

Potentiation of contraction by protein kinase C

The results obtained during this study show that under certain conditions the activation of protein kinase C appears to lead to an increase of tension. Phorbol esters potentiate the phasic component of contraction to both carbachol and high K^+ and also potentiate oscillatory movements when added either on their own or during the tonic component of contraction to carbachol. In addition protein kinase C appears to contribute positively to the tonic component of contraction to high K^+.

As described in the introduction and recently reviewed by Watson & Godfrey (1988), phorbol esters have been shown to potentiate contraction in a number of smooth muscles through both Ca^{2+}-dependent and independent mechanisms. The molecular basis of the potentiation observed in the present study is not known and measurements of intracellular Ca^{2+} and studies on skinned smooth muscle preparations are required to address this question. For example, in skinned muscles of the porcine coronary artery, the activation of protein kinase C by PMA increases tension under conditions in which the Ca^{2+} is buffered to resting cytoplasmic levels (Miller *et al.*, 1986; Itoh *et al.*, 1988).

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Relations between structure and nicotine-like activity: X-ray crystal structure analysis of (−)-cytisine and (−)-lobeline hydrochloride and a comparison with (−)-nicotine and other nicotine-like compounds

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1 Although (−)-cytisine is a rigid structure, it occurs in the crystal in two distinct but very similar conformations in which the pyridone ring is tilted relative to the charged nitrogen atom at much the same angle as the pyridine ring is in (−)-nicotine hydrogen iodide. The carbonyl group in the pyridone ring of (−)-cytisine, however, is on the side of the ring opposite to the pyridine nitrogen in (−)-nicotine.

2 The pK_a of (−)-lobeline HCl at 25°C is 8.6 (approx), indicating that (−)-lobeline is at least 90% in the protonated form at physiological pH (7.6). It is probably the phenyl 2-keto-ethyl part of (−)-lobeline, rather than the phenyl 2-hydroxy-ethyl part, which interacts with the receptor.

3 The combination within one molecule of a charged ('onium') nitrogen atom lying out of the plane of, and some distance (4.5–6.5 Å) from, an aromatic ring is common to many compounds with nicotine-like activity (e.g. nicotine, cytisine, choline phenyl ether bromide, dimethyl-phenyl-piperazinium (DMPP) iodide, coryneine iodide and *m*-hydroxyphenylpropyl trimethyl ammonium iodide). In some molecules the aromatic ring can be replaced by an unsaturated group, such as carbonyl (e.g. acetylcholine) or double-bonds (e.g. anatoxin).

4 Activity at nicotinic receptors appears to involve interactions between the positively charged nitrogen atom and a negatively charged group, probably close to cysteine residues 192 and 193 in the receptor. It is suggested that rather than specific groups in the molecule also being involved, activity at nicotinic receptors depends on interactions between a flat part of the drug containing double-bonds, or systems of double bonds, and a planar area in the receptor, possibly tyrosine or phenylalanine residues.

Introduction

The nicotine-like properties of the alkaloid (−)-cytisine have long been known (Dale & Laidlaw, 1912; Zachowski, 1938; Barlow & McLeod, 1969) and are of particular interest in the discussion of molecular interactions between agonists and nicotinic receptors, because (−)-cytisine should be a relatively rigid structure. This has been important in the ideas of Beers & Reich (1970) which led them to suggest that, as well as involving a charged 'onium' nitrogen, activity also involved a group, such as carbonyl, capable of forming a hydrogen bond with a donor group in the receptor. From modelling studies on the structures of nicotinic agonists, including cytisine, Sheridan *et al.* (1986) have claimed to identify a pharmacophore 'triangle', comprising 'the cationic center (A), an electronegative atom (B) and an atom (C) which forms a dipole with B'. In view of the significance which has been attached to the activity and structure of (−)-cytisine, it is important to make an X-ray structure analysis to determine its precise molecular parameters in the crystal.

An X-ray structure determination of the alkaloid (−)-lobeline has also been made. This is likewise classified as a nicotine-like agonist (Dixon, 1924). It is listed as a ganglion-stimulant (Dale & Rang, 1987) but interest in it has mainly been concerned with the marked reflex stimulation in respiration which it produced by an action on chemoreceptors in the

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aortic and carotid bodies (Dallemagne & Heymans, 1955). Reflex respiratory stimulation is also seen with nicotine and, to a lesser extent, with cytisine (Barlow & McLeod, 1969), but the effects of lobeline appear to have been so marked as to have justified its extensive use clinically (it has been included in the pharmacopoeias of many countries).

(−)-Lobeline is potentially a flexible molecule with no obvious structural resemblance to nicotine. Although the unit cell dimensions of lobeline HCl H_2O were described by Eeles (1953), a full X-ray structure analysis was not made. The pK_a of lobeline has also been measured as it could not be found in the literature. Both cytisine and lobeline are currently of interest because of their affinity for receptors in rat brain (Wonnacott, 1987) and ideas of how agonists interact with nicotinic receptors have been greatly helped by progress which has been made on the structure and amino acid sequence of the nicotinic receptor mostly from *Torpedo californica* (Noda *et al.* 1982) and *Torpedo marmorata* (Changeux *et al.*, 1984). It is now possible to try to identify and model putative binding sites using, for example, antibodies and peptide fragments from the receptor (Watters & Maeliecke, 1983; McCormick & Atassi, 1984; Smart *et al.*, 1984; Criado *et al.*, 1986; Kao & Karlin, 1986; Oblas *et al.*, 1986; Mulac-Jerevic & Atassi, 1986; Abramson *et al.*, 1988; Dennis *et al.*, 1988; Gotti *et al.*, 1988; Moskovitz & Gershoni, 1988).

Methods

Measurement of pK_a

Electrometric titrations were made as described by Armstrong & Barlow (1976), with a Metrohm E500 digital pH meter and an EA121 combination glass electrode, with 0.1 M sodium hydroxide added by means of an E412 Dosimat. The temperature was 25.0 ± 0.2°C. The experiments involved the addition of known amounts of alkali to a known amount of lobeline hydrochloride and measuring the pH. For each value of added alkali and pH the thermodynamic pK_a was calculated as described by Armstrong & Barlow (1976: the computer program used is listed by Barlow, 1983).

Lobeline base is not very soluble in water. Some titrations were made with 0.015 mmoles and a volume of 25 ml (giving an initial concentration of 0.06 mM) and at this dilution the base stayed in solution, but the results may not be accurate because of the need to weigh small amounts of lobeline and to use small volumes of alkali. Experiments were also made with larger quantities in 20 ml aqueous ethanol (50% by volume): with 0.2 mmoles in this medium the titration could proceed well beyond half-neutralization and with 0.1 mmoles the base remained in solution even when fully neutralized.

Crystal structure determinations

Crystal data for (−)-*cytisine* (I) $C_{11}H_{14}N_2O$, M_r = 190.3, orthorhombic, $P2_12_12_1$ (No:19), a = 7.175(2), b = 9.973(3), c = 26.639(9) Å, U = 1906(1) Å^3, Z = 8, D_x = 1.33 g cm^{-3}, Mo K_α X-radiation (λ = 0.71073 Å), μ = 0.81 cm^{-1}, $F(000)$ = 816, T = 298 K, $R(R_w)$ = 0.0325 (0.0303) for 1782 reflections with $I > 3\sigma(I)$.

Crystal data for lobeline hydrochloride (II) $C_{22}H_{28}O_2N^+ \cdot Cl^- \cdot H_2O$, M_r = 373.9, orthorhombic, $P2_12_12_1$ (No:19), a = 8.152(2), b = 14.370(4), c = 17.915(5) Å, U = 2099(1) Å^3, Z = 4, D_x = 1.24 g cm^{-3}, Mo K_α X-radiation (λ = 0.71073 Å), μ = 2.00 cm^{-1}, $F(000)$ = 840, T = 298 K, $R(R_w)$ = 0.0498 (0.0540) for 1515 reflections with $I > 3\sigma(I)$.

For both structure determinations diffracted intensity data were collected on Nicolet four-circle automated diffractometers by $\theta/2\theta$ variable speed scans. Table 1 gives the data collection parameters and details of data reduction, structure solution and refinement. Structure solution was by direct methods and Fourier difference synthesis. No corrections were applied for absorption or extinction. Structure I was refined by blocked-cascade least-squares on a Data General Desktop minicomputer with the *SHELXTL* package (Sheldrick, 1981), and structure II by full-matrix least-squares on a Digital MicroVAX II computer with the *SHELXTLplus* package (Nicolet Instrument Corp., 1988). All non-hydrogen atoms were refined with anisotropic thermal parameters. The phenyl rings in II were refined as regular hexagons (C—C 1.395 Å). Non-hydroxyl hydrogen atoms were incorporated at geometrically idealised positions (C—H 0.96Å, fixed U_{iso} of 1.2xU_{eq} of C) and refined by a riding model. Hydroxyl hydrogens in II were located from diffrence Fourier maps (low-angle, $\sin\theta/\lambda < 0.25$) and their position and isotropic thermal parameter were fixed during refinement. In compound I the piperidine N—H could not be located from a Fourier difference synthesis and so both *endo-* and *exo-* N—H positions were generated geometrically. Refinement of *endo-*/*exo-* site occupancy resulted in 90% of the *exo-* hydrogen occupancy. Residual peaks and troughs in the final ΔF maps were <0.26 e Å^{-3} for both structures. The function minimized during refinement was $\Sigma w(F_0 - |F_c|)^2$, where $w^{-1} = [\sigma^2(F) + gF^2]$ and values of g are given in Table 1. Atomic scattering factors and corrections for anomalous dispersion were taken from *International Tables for X-ray Crystallography, Vol. IV.*

Table 1 Data collection parameters, structure solution and refinement details for (−)-cytisine and (−)-lobeline hydrochloride monohydrate

Compound	*(−)-Cytisine*	*Lobeline hydrochloride monohydrate*
Crystal colour	Yellow	Colourless
Habit	Needle	Needle
Size (mm)	0.2 × 0.3 × 0.8	0.3 × 0.25 × 0.1
Diffractometer	R3m/V	$P2_1$
Unit cell determn.		
No. of reflns.	18	15
2θ range (°)	20–25	14–18
Scan range (°2θ)	2.9–50.0	2.9–52.5
Range of *hkl*	−2/8, −2/11, −2/31	0/10, 0/17, 0/22
Scan type	$\theta/2\theta$	$\theta/2\theta$
Scan speeds ($\cdot 2\theta\,\text{min}^{-1}$)		
Min.	2.00	1.50
Max.	29.30	29.30
No. of standard reflns.	3	3
Frequency of stds.	100	50
Variation/decay (%)	3	3
Data collected	3107	2512
Independent data	2479	2477
R_{int}	0.013	0.008
Solution method	Direct/ΔF	Direct/ΔF
Program system	*SHELXTL*	*SHELXTL*plus
Refinement method*	bcls	fmls
Refined data	1782	1515
$I > n\sigma(I)$: n	3	3
R	0.0325	0.0498
R_w	0.0303	0.0540
$w^{-1} = [\sigma^2(F) + gF^2]$;		
g-value	0.0005	0.002
Goodness-of-fit	1.135	1.080
Data:parameter ratio	7	6
Max. Δ/σ (final)	0.035	0.001
Max. $\Delta\rho$ final ($e\text{Å}^{-3}$)		
positive	0.19	0.26
negative	0.24	0.18
Hydrogen atoms:		
Location	Geometric	OH by ΔF, others geometric
Refinement	Riding (90% piperidine N–H *exo*-)	OH fixed, others riding

* bcls = blocked-cascade least-squares, fmls = full-matrix least-squares.

Materials

(−)-Cytisine (Sigma) was crystallized from acetone (Ing, 1931). (−)-Lobeline hydrochloride (Aldrich) was crystallized from aqueous ethanol (approx 20% water by volume).

Results

Final fractional atomic coordinates are given in Tables 2 and 3. The analysis shows two distinct conformers of cytisine in the asymmetric unit but these are very similar (Figure 1). The piperidine ring is fixed at right angles to a pyridone ring by a bond which forms another saturated six-membered ring.

In lobeline hydrochloride the nitrogen atom is again part of a piperidine ring but, like nicotine, it is a tertiary base and has an attached methyl group as well as a proton. The piperidine ring has two flexible chains linking a benzene ring to the carbon atom adjacent ot the nitrogen. In one the hydroxyl group and the benzene ring are almost coplanar (the C—C—C—OH torsion angle is −9°): in the other the keto group and the benzene ring are not coplanar (the C—C—C=O torsion angle is 38°). The two

Table 2 Atomic coordinates ($\times 10^4$) for (−)-cytisine

Atom	*x*	*y*	*z*
N(1)	5212(3)	3669(2)	624(1)
C(2)	3364(3)	3341(2)	569(1)
C(3)	2705(3)	2217(2)	803(1)
C(4)	3875(4)	1410(3)	1090(1)
C(5)	5692(4)	1748(3)	1146(1)
C(6)	6451(3)	2911(3)	918(1)
O(6)	8106(2)	3262(2)	947(1)
C(7)	6084(3)	4819(3)	359(1)
C(8)	4794(3)	5631(3)	24(1)
C(9)	3283(3)	4742(3)	−197(1)
C(10)	2159(3)	4211(3)	244(1)
C(11)	3881(4)	6789(2)	301(1)
N(12)	2745(3)	6291(2)	717(1)
C(13)	1297(3)	5384(3)	534(1)
N(1′)	8925(3)	9395(2)	1927(1)
C(2′)	7219(3)	9357(2)	2156(1)
C(3′)	5851(4)	8582(3)	1959(1)
C(4′)	6157(4)	7841(3)	1522(1)
C(5′)	7818(4)	7890(3)	1294(1)
C(6′)	9314(4)	8672(3)	1487(1)
O(6′)	10891(3)	8736(2)	1299(1)
C(7′)	10479(3)	10224(3)	2109(1)
C(8′)	10138(3)	10924(3)	2607(1)
C(9′)	8150(3)	11404(3)	2631(1)
C(10′)	6941(4)	10160(3)	2631(1)
C(11′)	10573(4)	10052(3)	3058(1)
N(12′)	9330(3)	8893(2)	3075(1)
C(13′)	7399(4)	9303(3)	3091(1)
H(3)	1414	1980	769
H(4)	3396	616	1247
H(5)	6485	1186	1346
H(7A)	6590	5413	608
H(7B)	7075	4476	153
H(8)	5557	5996	−238
H(9A)	2497	5254	−417
H(9B)	3832	4013	−380
H(10)	1153	3661	126
H(11A)	3102	7279	73
H(11B)	4830	7372	431
H(12A)	3529	5818	949
H(12B)	2169	7036	884
H(13A)	606	5041	814
H(13B)	473	5868	315
H(3′)	4659	8542	2122
H(4′)	5176	7298	1384
H(5′)	8007	7383	992
H(7′A)	11550	9656	2146
H(7′B)	10731	10898	1861
H(8′)	10980	11671	2621
H(9′A)	7950	11911	2933
H(9′B)	7868	11953	2345
H(10′)	5657	10430	2645
H(11C)	10415	10571	3359
H(11D)	11839	9747	3036
H(12C)	9606	8375	3370
H(12D)	9531	8354	2782
H(13C)	6616	8523	3094
H(13D)	7182	9818	3390

Table 3 Atomic coordinates ($\times 10^4$) for lobeline hydrochloride monohydrate

Atom	*x*	*y*	*z*
Cl(1)	974(2)	6193(1)	2386(1)
C(1)	1058(6)	3261(3)	1855(3)
O(1)	1676(5)	2325(3)	1820(2)
C(12)	−1022(5)	4316(2)	1262(2)
C(13)	−2123	4535	693
C(14)	−2408	3900	118
C(15)	−1591	3047	112
C(16)	−490	2828	682
C(11)	−205	3463	1257
C(2)	2531(6)	3903(3)	1787(3)
C(3)	3632(6)	3984(3)	2474(3)
C(4)	4936(7)	4725(4)	2380(3)
C(5)	5961(8)	4860(4)	3084(3)
C(6)	6630(7)	3923(4)	3359(3)
C(7)	5313(7)	3209(3)	3444(3)
N(1)	4400(5)	3060(3)	2714(2)
C(10)	5436(8)	2628(4)	2110(3)
C(8)	5901(7)	2277(3)	3756(3)
C(9)	6048(7)	2273(4)	4595(3)
O(9)	5835(7)	2976(3)	4955(2)
C(22)	5535(4)	1191(2)	5631(2)
C(23)	5802	357	6011
C(24)	6908	−294	5727
C(25)	7746	−111	5065
C(26)	7478	723	4685
C(21)	6373	1374	4969
O(1′)	7107(6)	3138(3)	6502(2)
H(1)	493	3354	2319
H(1′)	802	2021	1908
H(12)	−826	4752	1658
H(13)	−2685	5122	697
H(14)	−3165	4051	−274
H(15)	−1787	2611	−283
H(16)	72	2241	678
H(2A)	3195	3682	1382
H(2B)	2129	4514	1672
H(3)	2913	4172	2871
H(4A)	5649	4549	1978
H(4B)	4408	5304	2261
H(5A)	6863	5267	2975
H(5B)	5291	5131	3467
H(6A)	7146	4016	3835
H(6B)	7426	3700	3007
H(7)	4574	3461	3809
H(1A)	3548	2610	2797
H(10A)	6037	2118	2322
H(10B)	6190	3069	1900
H(10C)	4721	2400	1725
H(8A)	6959	2141	3546
H(8B)	5136	1802	3610
H(22)	4774	1639	5827
H(23)	5225	231	6467
H(24)	7091	−868	5988
H(25)	8506	−560	4869
H(26)	8055	848	4229
H(11′)	6898	2587	6363
H(12′)	7034	3574	6184

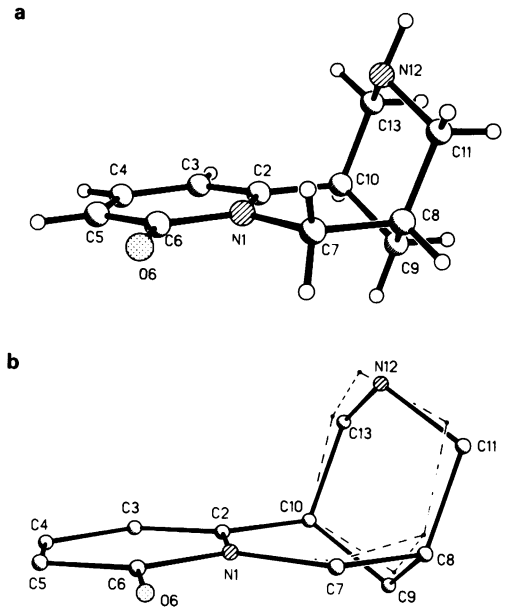


Figure 1 (a) The structure of (−)-cytisine showing the numbering: the basic nitrogen is at the top right and the pyridone ring on the left. (b) The two conformations of cytisine in the crystal superimposed.

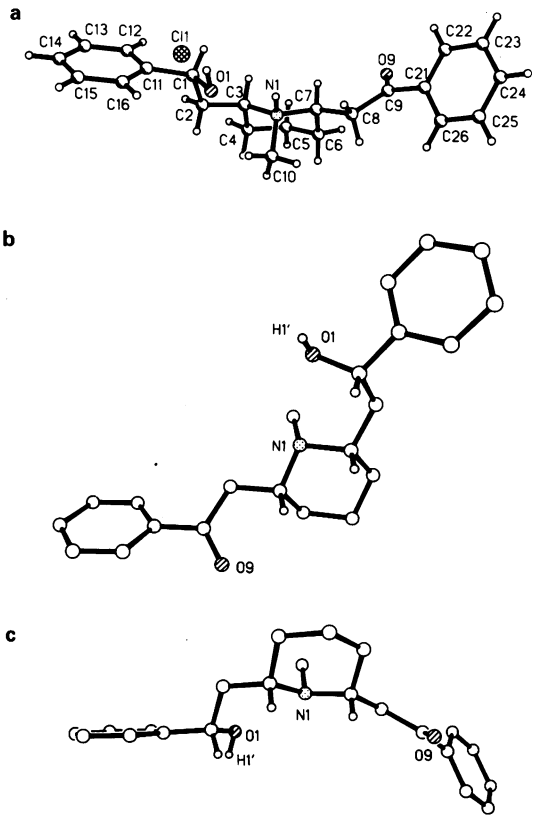


Figure 2 The structure of (−)-lobeline showing: (a) the numbering, (b) the keto-ethyl part and (c) the 2-hydroxyethyl part. Note the different positions of the nitrogen atom relative to the keto and hydroxyl groups.

links differ in that the keto group is orientated away from the nitrogen atom whereas the hydroxyl group is directed more towards the nitrogen (Figure 2).

Estimates (duplicates) of the pK_a of lobeline HCl in 50% aqueous ethanol were 8.53, 8.52 (2.5 mM), 8.60, 8.62 (5 mM) and 8.64, 8.75, (10 mM); in water, 8.62, 8.77 (0.6 mM). At physiological pH lobeline is likely to be at least 90% protonated. (−)-Lobeline is a stronger base at 25°C than nicotine (pK_as, 3.10 and 8.01; Barlow & Hamilton, 1962a) or cytisine, (pK_a 7.92; Barlow & McLeod, 1969). In part, at least, the greater basicity of lobeline can be ascribed to its nitrogen atom being less sterically hindered than the corresponding atom in cytisine or nicotine. From the effects of pH on the activity of nicotine it is known that it is the monoprotonated form which is biologically active (Barlow & Hamilton, 1962a). Similar experiments have shown that it is the protonated form of cytisine (and its mono-methyl analogue, caulophylline) which is active (Barlow & McLeod, 1969).

Although this study has been made with cytisine base, rather than with the protonated form, it seems highly unlikely that with such a locked cyclic molecule there can be much difference between the structures of the charged and uncharged forms.

Discussion

A comparison of the structures of (−)-cytisine and (−)-nicotine HI (Barlow *et al.*, 1986) is shown in Figure 3a, where arrowed atoms lie in the same plane. In cytisine and nicotine the aromatic ring is tilted to a similar extent. In Figure 3b the nicotine molecule has been moved so that the onium nitrogen atoms (point A in Sheridan's triangle) are superimposed. A horizontal rotation of −30° shows both rings edge-on (Figure 3c), but the pyridine nitrogen of nicotine and the amide carbonyl group of cytisine (point B in Sheridan's triangle) are on opposite sides of the ring. In theory the pyridine ring in nicotine could rotate and point B could be in a similar position in the two molecules but rotation is likely to be hindered. In the crystal structure of nicotine mono hydrogen iodide (the form which predominates at

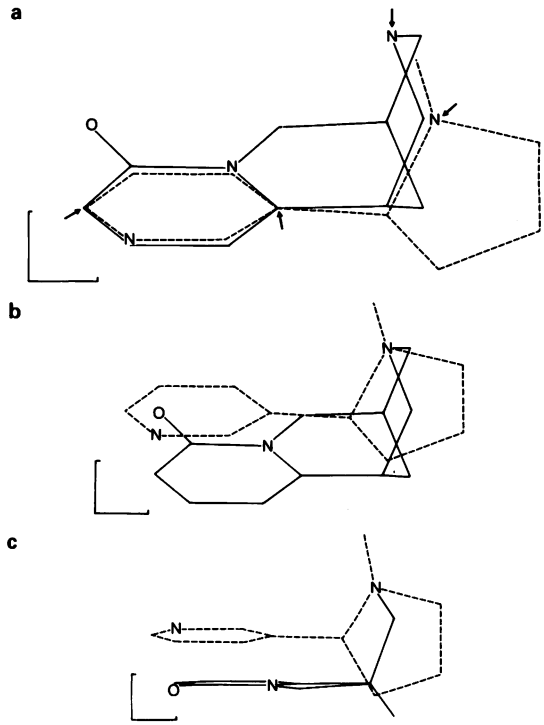


Figure 3 A comparison of cytisine and (−)-nicotine hydrogen iodide. The scale shows 1 Å (100 pm). In (a) the molecules have been aligned so that the ends of the aromatic rings (indicated by the arrows) form the X-axis and have a common origin at the righthand end of the ring. The basic nitrogen atoms (also indicated by the arrows) are set in the vertical plane and are some distance apart (1.35 Å). In (b) the nicotine molecule has been moved so that the basic nitrogen atoms are superimposed. In (c) a rotation of −30° about the X-axis has been made: this shows how closely parallel the rings are, relative to the basic nitrogen atom. In cytisine the pyridone ring is viewed edge-on and in nicotine it is only slightly different.

(−)-Cytisine is about 1.6 times as active as (−)-nicotine on the frog rectus and about 4 times as active as (−)-nicotine on guinea-pig ileum (ganglionic effects; Barlow & McLeod, 1969). It is from 2 to 6 times as active as (−)-nicotine in binding experiments with rat brain (Wonnacott, 1987).

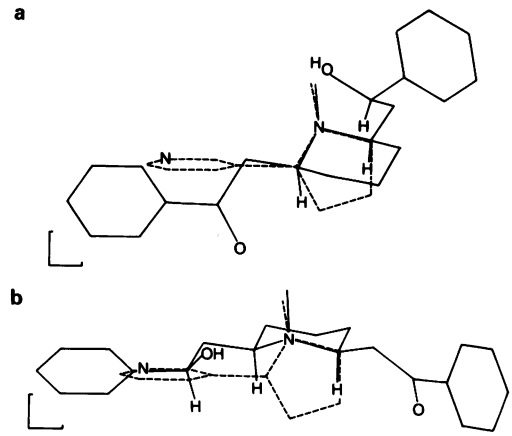
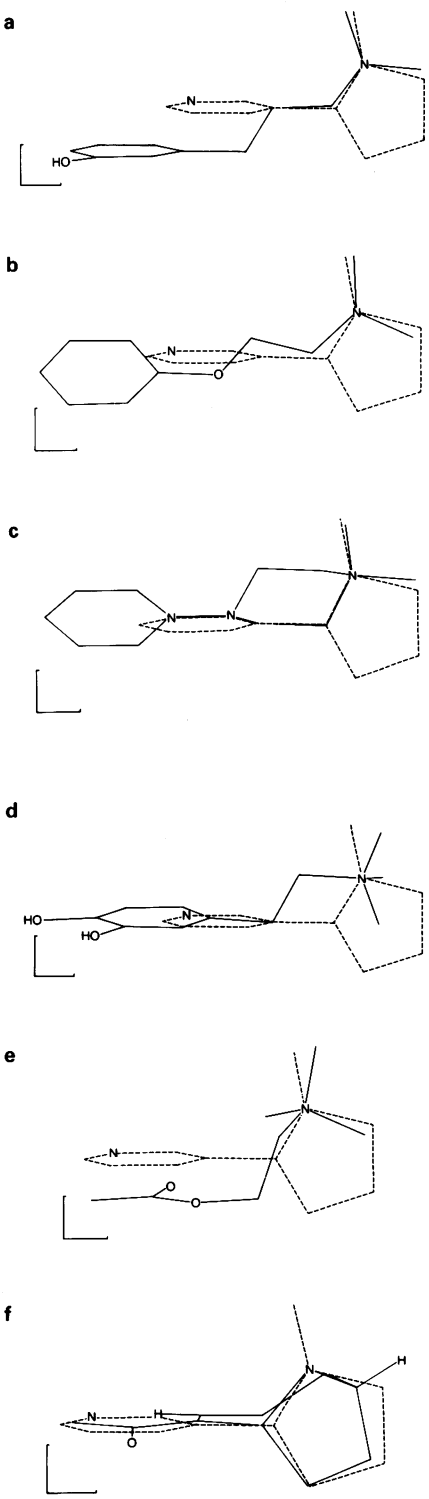


Figure 4 A comparison of lobeline with (−)-nicotine hydrogen iodide. The scale shows 1 Å (100 pm). The same procedure is followed as in Figure 3 but in (a) the phenyl ring of the 2-keto-ethyl part was superimposed on the pyridine ring of nicotine, with a common origin at the righthand end of the aromatic ring and the basic nitrogen atoms set in the plane of the paper. The origin was then moved so that these atoms are superimposed (cf. Figure 3b) and then a rotation of −30° about the X-axis has been made. In (b) the 2-hydroxy-ethyl part was superimposed on the pyridine ring, the origin moved and the rotation made.

Lobeline appeared to have less than 1% of the activity of nicotine on the frog rectus (Barlow & Veale, unpublished), on guinea-pig ileum it appeared to be about 10 times as active as nicotine but also blocked the preparation (possibly by blocking muscarinic receptors on the smooth muscle; Barlow & Franks, 1971). It is from 0.06 to 0.2 times as active as nicotine in binding experiments with rat brain (Wonnacott, 1987).

physiological pH) the two nitrogen atoms are orientated away from each other: the torsion angle about the bond linking the pyridine and pyrrolidine rings ((N)C—C—C—N(Me)) is 119°.

With lobeline either the 2-phenyl-2-ketoethyl part (Figure 4a) or the 2-phenyl-2-hydroxyethyl part (Figure 4b) can be compared with nicotine. In Figure 4a the phenyl ring of lobeline is almost at right angles to the pyridine ring of nicotine but in solution rotation may be possible in the lobeline molecule. The same applies to the phenyl and pyridine rings in Figure 4b but the onium groups in the two molecules are very differently placed. As the molecules are arranged, access to the pyrrolidine nitrogen in nicotine must be from the top whereas access to the nitrogen in lobeline must be from behind. There are also pharmacological reasons for believing that the phenyl 2-hydroxyethyl part is not likely to be involved. A 2-hydroxy group greatly reduces nicotine-like activity: the trimethylammonium derivative of adrenaline has feeble nicotine-like activity compared with coryneine (the trimethylammonium derivative of dopamine: Cuthbert, 1964) and 2-hydroxy phenethyl trimethyl ammonium bromide has negligible activity compared with phenethyl trimethylammonium bromide (Barlow & Gonzalez, 1986). In lobeline the hydroxyl group is actually 3



carbon atoms from the nitrogen but the groups are arranged less than 3 Å apart.

The X-ray crystal structures of many nicotine-like compounds have been obtained and Figure 5 shows a number of active compounds with their onium atoms superimposed on that of nicotine and presented as in Figure 3c and Figure 4. Their potency relative to nicotine is also indicated in the legend. Although many of these compounds are flexible and the conformation in the crystal may not indicate possible conformations in solution, there are structural similarities. As drawn, the compounds have the charged nitrogen atoms superimposed and the unsaturated ring lying to the left. The position and rotation of the ring varies from compound to compound but it is possible that they could all interact with some planar group in the receptor. With compounds such as *m*-hydroxyphenyl-propyl trimethylammonium iodide (Figure 5a) and coryneine (Figure 5d) the benzene rings are twisted at the same angle as in nicotine mono HI (and in cytisine). In choline phenyl ether bromide (Figure 5b) and dimethylphenylpiperazinium (DMPP) iodide (Figure 5c) the benzene ring is tilted differently, but rotation should be possible about the bond linking the benzene ring to the next atom (oxygen or nitrogen). With compounds lacking the aromatic ring, such as acetylcholine (Figure 5e) or anatoxin (Figure 5f), the comparison with nicotine can be made in many ways but it is still possible to observe a flat unsaturated part of the molecule in a position comparable to the aromatic ring.

It is possible to fit many of the structures (but not the hydroxyethyl part of lobeline) to the pharmacophore triangle described by Sheridan *et al.* (1986), but it is necessary to assume that in some molecules there is a conformational change (Table 4), e.g. with

Figure 5 A comparison of (−)-nicotine hydrogen iodide with: (a) *m*-hydroxyphenylpropyl trimethylammonium bromide (Barlow *et al.*, 1989). Activity F = 50, G = 1.25. (b) Choline phenyl ether (Celikel *et al.*, 1980). Activity F = 1.3, G = 1.25. (c) 1,1-Dimethyl-4-phenylpiperazinium iodide (DMPP: Chothia & Pauling, 1978). Activity G = 1 (but blocks muscarinic receptors), B = 0.02–0.2. (d) Coryneine iodide (Barlow *et al.*, 1989). Activity F = 12, G = 0.8. (e) Acetylcholine bromide (Svinning & Sorum, 1975). (f) Anatoxin (Huber, 1972; Koskinen & Rapoport, 1985): the structure shown is N-acetyl-anatoxin with the acetyl group removed.

The scale shows 1 Å (100 pm). The approximate potency relative to (−)-nicotine on the frog rectus is indicated by F (Barlow *et al.*, 1969), on guinea-pig ileum for effects at parasympathetic ganglia by G (Barlow & Franks, 1971; Barlow *et al.*, 1974) and in binding to rat brain by B (Wonnacott, 1987, with activities calculated from IC_{50} values given in Table 2).

Table 4 Comparison of the pharmacophore triangle with crystal structures: ± 0.3 represents the 'superposition tolerance'

	Atoms			*Distance* (Å)		
Compound	*A*	*B*	*C*	*AB*	*AC*	*BC*
Sheridan *et al.* (1986)				4.8 ± 0.3	4.0 ± 0.3	1.2
Nicotine HI	N	N	X*	4.74	3.78	1.4
Cytisine	N	=O	C(=O)	4.93	4.33	1.24
				4.87	4.24	1.24
Lobeline HCl						
Keto	N	=O	C(=O)	4.18	3.80	1.21
(CHOH−)	N	O(H)	C(OH)	2.93	3.14	1.44
Coryneine	N	*m*−O	*m*−C	6.83	5.94	1.36
(Rotated)				4.86	4.33	1.36
m-Hydroxyphenylpropyl trimethylammonium						
	N	*m*−O	*m*−C	8.39	7.35	1.34
(Rotated)				4.3	4.0	1.34

* Centroid of the pyridine ring (c.f. Sheridan *et al.*, 1986). The pharmacophore triangle used was that described by Sheridan *et al.* (1986).

coryneine iodide and *m*-hydroxyphenylpropyl trimethyl ammonium iodide, as with nicotine, the phenyl rings must be rotated. However, attempts to identifiy particular groups, other than the onium nitrogen atom, which are associated with activity have always been accompanied by argument (see, for instance, Hey, 1952; Barlow & Hamilton, 1962b; Barlow & Franks, 1973; Barlow *et al.*, 1969; 1974). While it is possible, for instance, that the carbonyl group in acetylcholine or 4-ketopentyl trimethylammonium can interact with a hydrogen donor group in the receptor, as postulated by Beers & Reich (1970), it is difficult to identify specific hydrogen acceptor groups in some of the other active compounds shown in Figure 5. Even if it is argued that *m*-hydroxyphenylpropyl trimethylammonium could act as a hydrogen acceptor as well as a hydrogen donor, the unsubstituted compound, phenylpropyl trimethylammonium has considerable activity on the frog rectus, being more active than choline phenyl ether (Barlow & Franks, 1973).

With the elucidation of the primary sequence of nicotinic receptor binding subunits it has been possible to try to identify particular groups which may be involved in the actions of agonists. The idea (Noda *et al.*, 1982) that the aspartate residue (138) interacts with the onium group of agonists now seems likely to be incorrect and some group in the region near the cysteine residues (192 and 193) seems much more probable. These groups are involved in the binding of α-bungarotoxin (Ralston *et al.*, 1987; Gotti *et al.*, 1988) and lophotoxin (Abramson *et al.*, 1988) and are labelled by a photo-affinity ligand (Dennis *et al.*, 1988). The adjacent tyrosine residue (190) is also labelled and there are aspartate residues nearby (195 and 200), though these are not among the major labelled products.

Although the precise geometry of the binding site is not yet known there is a fair indication of the groups likely to be involved (and the results obtained in this work at least provide coordinates for ligands which may be used in molecular modelling with the relevant part of the nicotinic receptor). These groups are consistent with the idea that agonist activity at nicotinic receptors may be associated with the charged nitrogen atom and an area of planarity such as an aromatic ring, rather than specific points such as in Sheridan's 'triangle'. This was suggested by Barlow *et al.* (1989) when describing the X-ray crystal structures of several phenolic quaternary ammonium salts with nicotine-like activity, some of which are included in Figure 5. The planar groups in the binding site could be tyrosine or phenylalanine and the idea that agonist activity involves a point and an area (rather than distinct points) may also explain why the enantiomeric potency ratio of nicotine is very variable.

Although natural (−)-nicotine is more active than (+)-nicotine in many tests, the potency ratio varies from 3:1 for neuromuscular block of the cat tibialis anterior muscle to 42:1 at parasympathetic ganglia in guinea-pig ileum (Barlow & Hamilton, 1965). Higher ratios have been obtained in binding experiments for sites in brain (88:1, Wonnacott, 1986; 177:1, Wonnacott, 1987). Indeed, from the existence of distinct groups of neuromuscular blocking and ganglion-blocking drugs it has long been known that the nicotinic receptors at these sites must be different.

Interactions involving a point and a flat area

would have no asymmetry if the point lies vertically above the area, but if it lies to either side of the vertical (as does the pyrrolidine nitrogen relative to the pyridine ring in nicotine in Figure 3a) there is asymmetry. The enantiomeric potency ratio of nicotine would, therefore, depend on the position of the onium binding group relative to the flat binding area (possibly tyrosine or phenylalanine), which could well differ from one type of receptor to another. The results obtained with cytisine and, to a lesser extent, with lobeline support the idea that the activity of agonists at nicotinic receptors, as well as involving the onium group, may also involve interactions between flat areas in drug and receptor. In addition to hydrophobic effects, binding could involve pi electrons in aromatic systems in compounds like nicotine, cytisine, *m*-hydroxyphenylpropyl trimethylammonium, coryneine and leptodactyline (not included in Figure 5), or double bonds or systems of double bonds in compounds such as acetylcholine, anatoxin, and 4-ketopentyl trimethyl ammonium iodide and unsaturated esters of choline such as murexine (not included in Figure 5: review, Barlow, 1964) and *iso*-arecolone methiodide (Spivak *et al.*, 1986; Waters *et al.*, 1988).

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Thapsigargin, a new calcium-dependent epithelial anion secretagogue

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1 Thapsigargin, a sesquiterpene lactone, was shown to cause electrogenic anion secretion in monolayers of human colonic epithelial cells, an effect which was crucially dependent upon calcium and did not involve eicosanoid formation.

2 To measure the secretory effect calcium needed to be present in the external bathing solution. By means of Fura-2 fluorescence measurements thapsigargin was shown to raise Ca_i by around 250 nM when the bathing solution contained calcium. In the nominal absence of external calcium thapsigargin raised Ca_i by only 60 nM, but from a lower basal value. This was insufficient to cause secretion.

3 Effects of other calcium-dependent secretagogues (e.g. lysylbradykinin) were inhibited in the presence of thapsigargin, whereas kinin responses were potentiated if the peptide was added following a stimulus which increases cyclic AMP.

4 From the data given here and the known behaviour of colonic epithelia it is concluded that thapsigargin increases Ca_i by a non-ionophoric mechanism by release from internal stores. Calcium-stimulated calcium influx then follows resulting in the opening of basolateral K channels, increasing the electrochemical gradient for chloride efflux, or alternatively by activating anion channels in the apical membrane. It is concluded that thapsigargin is a potentially important tool for examining epithelial mechanisms.

Introduction

Thapsigargin is a sesquiterpene lactone from the roots of *Thapsia garganica L.* (Christensen *et al.*, 1982). This agent allows the induction of hormone-independent calcium transients which both bypasses the receptor and does not require the production of inositol phosphates or activation of protein kinase C (Hanley *et al.*, 1988a,b). In platelets (Thastrup *et al.*, 1987) and rat peritoneal macrophages (Ohuchi *et al.*, 1988) thapsigargin produces a sustained increase in Ca_i apparently due to the mobilisation of intracellular stores coupled with an increase in calcium influx. This is to be contrasted with the transient effects of thapsigargin on Ca_i in the neural cell line NG115-401L (Jackson *et al.*, 1988) even in the presence of external calcium.

Intracellular mediators of epithelial chloride secretion are, classically, adenosine 3′:5′-cyclic monophosphate (cyclic AMP) and Ca^{2+} (Frizzell, 1977; Smith *et al.*, 1982). Agents such as carbachol, histamine and kinins apparently mediate their chloride secretory activity by raising Ca_i, at least this is one of the consequences of their interactions with receptors.

The calcium ionophore A23187 also promotes epithelial anion secretion (Cuthbert *et al.*, 1987) but its effect depends upon external calcium. This agent is not useful in detecting intracellular calcium stores which can be mobilised, or showing whether mobilisation modifies the responses to other calcium requiring secretagogues. A further problem which sometimes arises with A23187 is its propensity to cause the release of eicosanoids (Smith & McCabe, 1984; Erlij *et al.*, 1986). Consequent stimulation of adenylate cyclase by prostaglandins may mean A23187 constitutes a mixed signal, which employs cyclic AMP as well as Ca^{2+} messengers.

Here it is shown that thapsigargin promotes sustained anion secretion in human colonic epithelial

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monolayers, an effect which depends crucially on the elevation of Ca_i, initially, at least from an intracellular store.

Methods

Monolayer culture of HCA-7 and HCA-7 Colony 29 cells

Kirkland (1985) described the derivation of a human colonic adenocarcinoma cell line, HCA-7, derived from a resected tumour. HCA-7 Colony 29 is a similar cell line derived from HCA-7 cells on the basis of a slightly different morphology when grown on plastic surfaces. Methods for growing these cells on pervious supports to form epithelial monolayers have been described fully before (Cuthbert *et al.*, 1985; 1987). Cell monolayers (0.2 cm^2) were mounted in Ussing chambers and voltage clamped at zero potential. The resulting short circuit currents were displayed continuously on chart recorders, again as described before (Cuthbert *et al.*, 1987). The epithelial monolayers were bathed on each side with Krebs Henseleit solution (KHS) 20 ml, gassed with 95% O_2: 5% CO_2 and maintained at 37°C. KHS had the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11.1. To prevent precipitation when La^{3+} was used the KHS was modified as follows (mM): NaCl 147, KCl 5.9, $CaCl_2$ 2.5, $MgCl_2$ 1.2, Tris 10, HEPES 10, glucose 11.1 and sucrose 15. This solution had a pH of 7.5 when bubbled with 100% O_2 at 37°C. Sucrose was used to adjust the osmolarity to that of KHS (290 mOs l^{-1}). To reduce the calcium concentration of KHS to nominally zero a solution of EGTA, 35.5 mM, neutralised with NaOH to pH 7.5 was used. This solution was added to KHS to produce a final concentration of 2.5 mM which effectively removes ionised calcium without changing the sodium concentration. Readdition of $CaCl_2$ to give a concentration of 2.5 mM restores the ionised calcium to normal. This procedure can be used *in situ* without the need to wash EGTA away from the delicate cultures.

Measurements of Ca_i in adenocarcinoma cell suspensions

HCA-7 and HCA-7 Colony 29 cells were grown to confluence in 25 cm^2 culture flasks in Dulbecco's Eagles Medium (DMEM) with glucose (4.5 g l^{-1}), 10% foetal calf serum, kanamycin (100 μg ml^{-1}), amphotericin (2.5 μg ml^{-1}) and sodium pyruvate (110 mg l^{-1}). Cells were removed from the flasks and disaggregated by several treatments with trypsin 0.05% (w/v) in versene (Glasgow formula) washed by centrifugation in DMEM and finally suspended in a solution of the following composition (mM): NaCl 137, KCl 5.4, $CaCl_2$ 1.0, KH_2PO_4 0.4, $MgSO_4$ 0.3, HEPES 10, glucose 1.2 and bovine serum albumin (0.1%). The final suspending solution contained Fura 2-AM, 2 μM. The cells were incubated at room temperature (20–22°C) for 15 min and at 37°C for 90 min with occasional gentle agitation.

To measure Ca_i aliquots of the cell suspension (1–2 $\times$ 10^6 cells) were rapidly washed twice in the above solution with a microfuge, the cell pellet being finally suspended in 2.5 ml of buffer. When it was desired to suspend the cells in a solution nominally free of calcium the buffer was made without calcium addition and the cells washed in this medium. Suspensions were placed in a stirred, heated (37°C) quartz cuvette in a LS-5B Perkin Elmer Spectrofluorimeter and the emission fluorescence at 510 nm measured at excitation wavelengths of 340 and 380 nm, cycled every 15 s. The estimated value of Ca_i was calculated from the formula

$$Ca_i = K_d \frac{Sf}{Sb} \frac{R - Rmin}{Rmax - R}$$

(Poenie *et al.*, 1985)

where R is the ratio of the fluorescence at 340 nm to that at 380 nm, K_d = 224 nm and where the values Sf/Sb, Rmin and Rmax were determined separately by standard solutions of Fura-2 containing either Ca^{2+}, 12 mM or EGTA, 10 mM.

Statistical treatments

Student's *t* test, either paired or unpaired as appropriate, was used to assess significance, a value of $P < 0.05$ being considered significant.

Results

Effects of thapsigargin on anion secretion in HCA-7 monolayers

When applied to the apical surface of HCA-7 monolayers thapsigargin produced an immediate increase in short circuit current (SCC) which was maintained for at least 30 min, the longest time for which observations were made. No effect was seen when the sesquiterpene was added to the basolateral side, but this may have been because the agent adsorbed to the Millipore filter upon which the cells were grown. The limited availability of thapsigargin did not allow significantly higher concentrations than 250 nM to be added to the basolateral side in the hope of saturating possible adsorption sites. Therefore, throughout these experiments we have used concentrations of

either 170 nM or 240 nM, applied apically. These concentrations have been shown to be maximally effective in other systems (Thastrup *et al.*, 1989). In 13 separate experiments the response was 50.5 ± 5.7 nEq $20\,min^{-1}$ $0.2\,cm^{-2}$ to thapsigargin, 170 nM, most of the variation being due to differing responses between batches of cultured epithelia. Equivalent amounts of solvent (dimethylsulphoxide) to those used to dissolve thapsigargin were without effect on SCC.

The nature of the basal SCC in HCA-7 monolayers is unknown and is unaffected by a variety of blocking agents (lanthanum ions, piretanide, acetazolamide and EGTA) which we shall describe, subsequently, as affecting the responses to thapsigargin.

All secretagogues previously examined in HCA-7 monolayers cause anion secretion (Cuthbert *et al.*, 1987), the cells behaving as crypt rather than villous cells. This was true too of stimulation by thapsigargin as the induced current was partially sensitive to both acetazolamide and piretanide at maximally effective concentrations. These two blockers acted additively to give about 80% inhibition of SCC, and the order of addition was immaterial. These results suggest that both chloride and bicarbonate ions are secreted electrogenically (Table 1). The nature of the residual thapsigargin-induced current is unknown. Some chloride secretagogues, notably kinins, have epithelial effects which are partially mediated by eicosanoid formation. It was found for a number of epithelial preparations that piroxicam, 5 μM, essentially abolishes prostaglandin formation. With thapsigargin, 170 nM, the response was 19.8 ± 3.8 nEq $25\,min^{-1}$ $0.2\,cm^{-2}$ in five preparations. In four preparations from the same batch preincubated for 20 min with piroxicam, 5 μM, the response was 20.7 ± 2.6 nEq $25\,min^{-1}$ $0.2\,cm^{-2}$. Therefore no evidence was obtained that eicosanoids are involved in the actions of thapsigargin.

Table 1 Effects of piretanide and acetazolamide on thapsigargin-induced short circuit current (SCC) in HCA-7 monolayers

Blocker	*% Inhibition of SCC*
Piretanide	51.0 ± 4.3 (5)
Acetazolamide	32.8 ± 1.5 (5)

Piretanide (200 μM) was added first in three experiments and acetazolamide (0.5 mM) first in two experiments. Acetazolamide was added to both sides of the preparations and piretanide to the basolateral side only. Thapsigargin was used at 170 nM; 16.2 ± 5.7% of the thapsigargin-induced current was unaffected by either inhibitor. Numbers in parentheses indicate number of measurements.

Indirect evidence for the involvement of calcium in the action of thapsigargin

Lanthanum ions, added basolaterally, immediately abolished the thapsigargin-induced current, but without affecting basal SCC (Figure 1). In the continued presence of lanthanum, forskolin, an agent acting through an increase in cellular cyclic AMP, was able to restore the SCC to the level attained with thapsigargin. Lanthanum ions added before any other agent caused a transient increase in SCC, as seen in Figure 1, but with no lasting effect on basal SCC.

The sustained SCC promoted by thapsigargin was dependent upon calcium in the bathing solution. When EGTA was added in a sufficient quantity to chelate free Ca^{2+} the SCC fell to approximately the basal level, which was obtained before the addition of thapsigargin. When the Ca^{2+} concentration was restored to normal the SCC returned to the original plateau value following a transient increase to a higher value (Figure 2). Mean values for a series of similar experiments are given in Table 2. The pattern of responses to thapsigargin in the presence and absence of calcium was compared to the effect of an identical protocol upon the responses to forskolin (Figure 2). With forskolin, removal or readdition of Ca^{2+} had only a minor effect on the plateau responses; Table 2 gives mean values for a series of similar observations.

To explore further the relationship between thapsigargin and the generation of an intracellular calcium signal, the effects of the terpene on two other calcium requiring secretagogues, lysylbradykinin (LBk) and A23187, were investigated. For the experi-

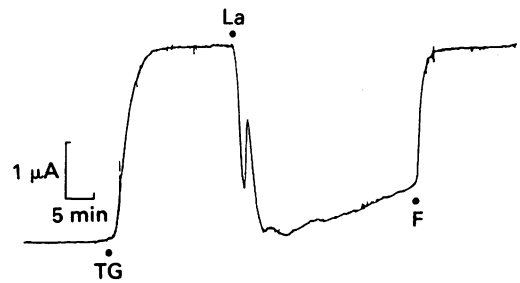

Figure 1 Short circuit current (SCC) responses in HCA-7 monolayers (0.2 cm^2) to thapsigargin (TG, 170 nM) applied apically. When the SCC had stabilized La^{3+} (1.0 mM) was added to the basolateral side and, sometime later, forskolin, F, 10 μM added apically. Modified KHS was used to prevent precipitation when La^{3+} was added. Horizontal line indicates zero SCC.

Table 2 Effect of Ca^{2+} removal and readmission on the plateau short circuit current (SCC) response to forskolin and thapsigargin in HCA-7 monolayers

	ΔSCC plateau	*ΔEGTA*	*ΔCa^{2+}*	n
Thapsigargin (170 nM)	9.8 ± 1.4	−6.7 ± 0.9*	6.8 ± 1.1*	4
Forskolin (10 μM)	12.8 ± 1.5	−0.6 ± 0.2	−0.2 ± 0.2	5

All values are given as $\mu A\,cm^{-2}$. The current changes following addition of EGTA, 2.5 mM, or restoration of Ca^{2+} to the solutions bathing both sides of the tissue were measured 5 min after the solution change. The asterisked values are significantly different ($P < 0.001$) from the corresponding values obtained when forskolin, added apically, was used as the secretagogue.

ments with A23187 we chose to use HCA-7 Colony 29 monolayers, another cell line derived from the same adenocarcinoma. We did this to see if thapsigargin had similar effects on another related cell line. With pairs of HCA-7 Colony 29 monolayers the effect of A23187, 1 μM, alone or during a plateau response to thapsigargin was examined (Figure 3, Table 3). The response to ionophore was significantly attenuated ($P < 0.005$) in the presence of terpene, a situation which would obtain if both agents acted through a final common pathway.

Lysylbradykinin causes anion secretion in HCA-7 monolayers acting at either basolateral or apical receptors (Cuthbert *et al.*, 1985). Responses at one surface do not preclude or attenuate responses at the contralateral surface. This enabled a crossover design to be adopted in pairs of preparations. In one, apical LBk was followed by thapsigargin and then basolateral LBk. In the other preparation the kinin was added in the reverse order. One such experiment is illustrated in Figure 4, and Table 4 gives the results from 4/5 pairs of experiments. The effects of LBk on SCC are inhibited after thapsigargin, highly significantly so with basolaterally applied LBk, again indicating a possible common final effector mechanism. An alternative explanation for the last two sets of experiments is that the actions of LBk and A23187 after thapsigargin are curtailed by a limitation on the maximal transporting capacity of the tissue. However, if forskolin was used instead of thapsigargin then the actions of LBk were significantly increased. One example is illustrated in Figure 4 and statistical data are provided in Table 4.

Effects of thapsigargin on Ca_i

Thapsigargin (170 nM) was added to Fura-2 loaded cells, irradiated alternatively at 340 nm and 380 nm and the fluorescence emission measured at 510 nm. The results of two single experiments are given in Figure 5, where Ca_i rose from an estimated value

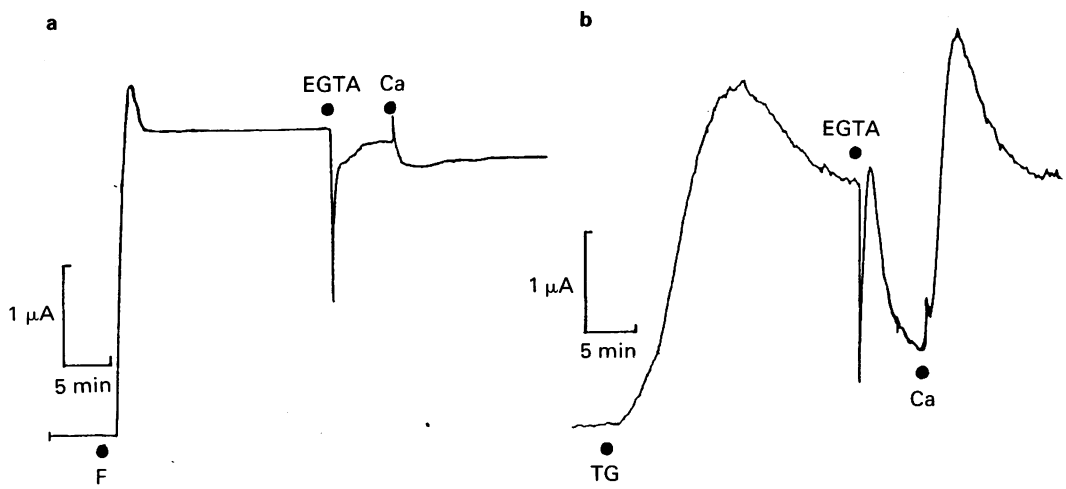

Figure 2 Responses to two HCA-7 monolayers, each 0.2 cm^2, to (a) forskolin (F, 10 μM added apically) and (b) thapsigargin (TG, 170 nM). After responses reached a plateau EGTA, 2.5 mM, was added (neutralised with NaOH to pH 7.4) to remove all ionised calcium from the bathing fluid on both sides of the tissue. Later sufficient $CaCl_2$ was added to restore the ionised concentration to 2.5 $mEq\,l^{-1}$. Horizontal lines indicate zero short circuit current.

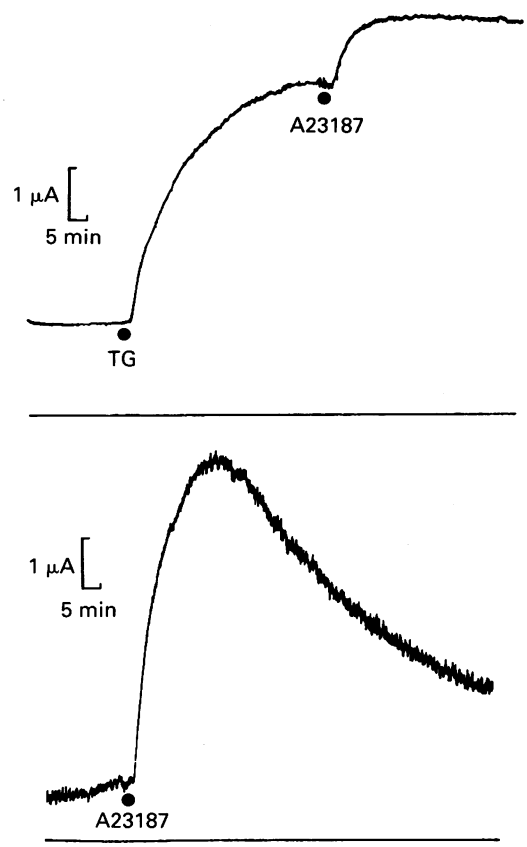

Figure 3 Responses to A23187 (1 μM) alone and after thapsigargin (TG, 170 nM) in Colony 29 monolayers (each 0.2 cm^2). Horizontal lines indicate zero short circuit current. Both secretagogues were added to the apical solution.

Table 3 Effects of thapsigargin (170 nM) on the responses to A23187 (1 μM) in HCA-7 Colony 29 monolayers

Thapsigargin	*A23187*	*A23187 after thapsigargin*
25.2 ± 5.8 (6)	59.4 ± 12.8 (6)	7.7 ± 2.3 (6)

Responses are measured as nEq 0.2 cm^{-2} 25 min^{-1}. Numbers in parentheses indicate number of observations. By use of a two tailed *t* test the responses to A23187 were reduced significantly ($P < 0.005$) after thapsigargin. Note that with A23187 and thapsigargin together the response is less than with A23187 alone. Both thapsigargin and A23187 were added apically.

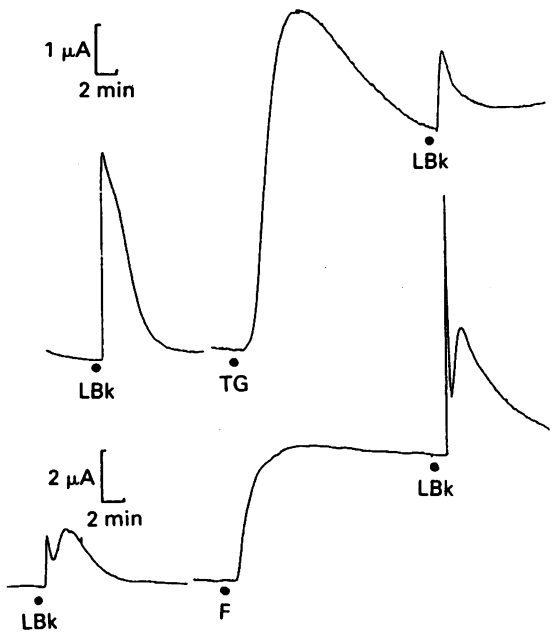

Figure 4 Montage of four experiments with HCA-7 monolayers using the crossover design described in the text. The left hand upper trace shows the response to lysylbradykinin (LBk) (0.1 μM) applied apically. Subsequently thapsigargin (TG) (250 nM) was added and during the plateau, LBk, 0.1 μM, basolaterally. This part of the experiment is not shown. The right hand upper trace is from a monolayer from the same batch. Initially, LBk, 0.1 μM was applied basolaterally (not shown), then TG followed by LBk apically. Thus the responses to apically applied LBk, before and after TG can be compared in the figure. The pair of traces shown at the bottom of the diagram were from a different batch of cultures. Shown are the responses to LBk, 0.1 μM (basolaterally) applied either before or after forskolin F, 10 μM. As both the size and duration of the responses were affected we have integrated the area under each response during the first eight minutes, i.e. approximately the duration of the normal responses. These areas were converted to nEq. Statistical details from a series of crossover experiments are given in Table 4.

around 100 nM to 400 nM within two minutes. Thereafter the Ca_i concentration remained elevated and fairly constant for 15 min. Similar experiments were carried out in solutions with nominally zero calcium. Thapsigargin still caused a maintained increase in Ca_i but the level attained was similar to the basal level found with calcium containing solutions. After 15 min addition of calcium, 1 mM, caused a further increase in Ca_i to a value comparable with that

Table 4 Effect of lysylbradykinin (LBk, 0.1 μM) before and during the plateau phase response to thapsigargin (240 nM) or to forskolin (10 μM)

	Control (nEq)	*After thapsigargin* (nEq)	n
LBk (Basolateral)	9.5 ± 0.8 (4)	3.6 ± 0.7 (4)	$P < 0.001$
LBk (Apical)	3.3 ± 0.6 (5)	1.6 ± 0.3 (5)	$P < 0.05$
	Control (nEq)	*After forskolin* (nEq)	
LBk (Basolateral)	5.3 ± 1.0 (11)	13.9 ± 1.3 (8)	$P < 0.001$
LBk (Apical)	2.5 ± 0.1 (8)	12.3 ± 0.8 (11)	$P < 0.001$

Numbers in parentheses indicate the number of observations. Both forskolin and thapsigargin were added apically.

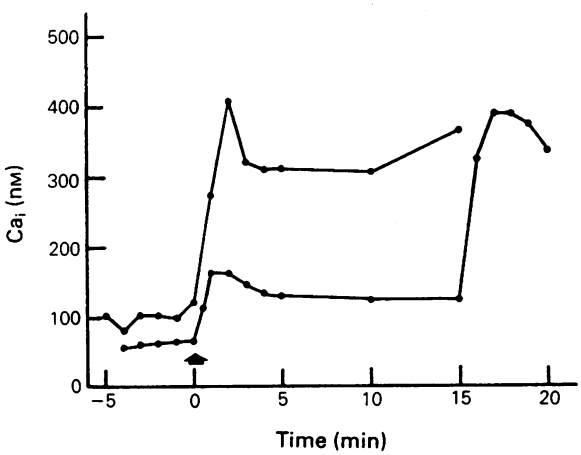

Figure 5 Estimated values of intracellular calcium in two separate experiments in isolated HCA-7 colonocytes. Thapsigargin (170 nM) was added at the times indicated by the arrow. The upper graph shows results from an experiment carried out with 1 mM calcium in the bathing fluid. The lower graph shows results from an experiment carried out at nominally zero calcium concentration. In this experiment calcium, 1 mM, was added at 15 min.

obtained in the first experiments. The data from nine separate experiments are given in Table 5.

Discussion

A large variety of secretagogues have been shown to cause anion secretion in intestinal epithelia. In terms of mechanisms the clearest examples have come from experiments on cultured epithelial monolayers where only a single cell type is present. For example T84, Caco2, HT 29 and HCA-7 monolayers all behave as colonic crypts, secreting anions in response to secretagogues. It has been shown that secretagogues can be divided into two classes. First, those that increase cyclic AMP such as forskolin, vasoactive intestinal polypeptide, prostaglandin E_2 and a second group which raise Ca_i, examples being carbachol, LBk and A23187.

An earlier study demonstrating that thapsigargin raised Ca_i by a non-ionophoric mechanism so bypassing receptor events at the cell surface, rather as forskolin bypasses receptors linked to adenylate cyclase (Thastrup *et al.*, 1989), made the sesquiterpene a possible tool for investigating epithelial mechanisms. The experiments described here show

Table 5 Estimated values of Ca_i (nM) in Fura-2 loaded HCA-7 cells

Time (min)	*Ca external 1.0 mM*	n	P	*Ca external 0 mM*	n	P
−5	199 ± 50	6	NS	82	2	NS
0	266 ± 48	6		87 ± 13	3	
1	430 ± 102	6	<0.05	147 ± 21	3	NS
5	452 ± 97	6	<0.01	145 ± 15	3	<0.05
10	398 ± 70	6	<0.01	142 ± 10	3	<0.01
15	433 ± 70	6	<0.01	152 ± 16	3	<0.01
16				358 ± 24	3	<0.01
20				376 ± 47	3	<0.05

Values of Ca_i at zero time were compared with those at other times by means of a two-tailed, paired *t* test. Thapsigargin (170 nM) was added at zero time and calcium (1 mM) was added at 15 min. The two sets of experiments were made with either Ca (1 mM) or nominally zero Ca in the external bathing solution. In the nominally zero calcium experiments the observations at 16 and 20 min were made after Ca (1 mM) was added.

for the first time that thapsigargin causes anion secretion in epithelial monolayers. The inhibitory actions of piretanide and acetazolamide on the SCC increase caused by the terpene indicated that both electrogenic chloride and bicarbonate secretion are stimulated.

Considering the functional responses to thapsigargin, it is clear that the effects on anion secretion are dependent upon calcium. The inhibitory effect of lanthanum ions and of calcium chelation on the responses to thapsigargin not only indicates calcium-dependence, but a dependence upon external calcium. It must be emphasized that neither lanthanum nor calcium removal reduced SCC to zero but simply removed the SCC increase caused by thapsigargin. It seems unlikely that either eicosanoids or cyclic AMP are implicated in the responses to thapsigargin. The responses to thapsigargin were unaffected by piroxicam and calcium chelation had no effect on the response to forskolin, unlike that to thapsigargin. Thus epithelia differ from the behaviour of rat peritoneal macrophages where eicosanoid formation has been demonstrated (Ohuchi *et al.*, 1987).

During the plateau phase of the thapsigargin-induced current the responses to LBk and to A23187 were attenuated; indeed the sum of the responses to thapsigargin plus A23187 was less than that to A23187 given alone (Table 3). It is quite likely that two agents employing the same intracellular signal will produce less than an additive response. However, it is more difficult to understand how both agents acting sequentially can produce a smaller response than one agent acting alone. It is possible that the rate of change of Ca_i is important or alternatively, thapsigargin may desensitize the calcium response pathways that generate the response. The potentiation of LBk by forskolin reflects the synergism between Ca_i and cyclic AMP signals which has been found for a number of other systems (Cartwright *et al.*, 1985). Our data with forskolin show, at least for LBk that the attenuation with thapsigargin was not due to limitations of the maximal transporting capacity of the tissue because of the significant potentiation seen with forskolin. The results with LBk are similar to those obtained with NG115-401L neuronal cells where thapsigargin pretreatment has been shown to inhibit the effects of bradykinin (Jackson *et al.*, 1988).

The evidence discussed thus far for the involvement of Ca_i in the response to thapsigargin is indirect. Direct evidence that Ca_i was elevated in colonocytes was obtained from Fura 2 fluorescence. Ca_i rose by around 250 nM within a minute or so of adding thapsigargin and was maintained for at least 15 min. In a further set of experiments HCA-7 cells were suspended in a medium nominally free of calcium ions. Thapsigargin was still able to cause an increase in Ca_i, but in this situation the plateau value was only 150 nM. Indeed the basal values were around 90 nM, lower than those found in the control condition. It was shown that calcium removal with EGTA abolished the effect of thapsigargin, suggesting that Ca_i values of around 150 nM are insufficient to trigger anion secretion. In an earlier study (Cuthbert, 1985) half-maximal chloride secretion was achieved in permeabilised cells bathed in a buffer containing 1 μM ionised calcium. Readmission of calcium to the bathing fluid, in these experiments, increased Ca_i to values comparable with those seen with thapsigargin in the presence of calcium.

Thus thapsigargin can raise Ca_i in the absence of external calcium, presumably from an intracellular store. Whether raised Ca_i then promotes calcium-mediated calcium entry, as has been proposed previously (Hanley *et al.*, 1988a; Thastrup *et al.*, 1989), or whether the terpene has a direct action on the membrane cannot be ascertained from these results. However, it is worth noting that Ca-activated Ca-entry has been proposed to be a direct consequence of Ca_i elevation (Putney, 1986) in accord with these results.

Both cyclic AMP and calcium-sensitive potassium channels have been described in the basolateral membranes of some colonocytes (Dharmasathaphorn & Pandol, 1986). When basolateral K-channels are activated the consequent hyperpolarisation of the apical membrane serves to promote anion exit. In some epithelia this may be as an important mechanism as the opening of Ca or cyclic AMP-sensitive apical chloride channels for the promotion of transepithelial anion secretion. Calcium removal with EGTA had no effect on the SCC in the presence of forskolin which suggests that, in this instance, cyclic AMP-sensitive K-channels were activated. This contrasts with the effect of calcium removal on thapsigargin responses, where presumably Ca-sensitive K channels are operating.

While it will be necessary to use other techniques to determine the apical and basolateral components of the thapsigargin responses, it is likely that this new agent will be an important tool for epithelial studies.

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Effects of potassium channel toxins from *Leiurus quinquestriatus hebraeus* venom on responses to cromakalim in rabbit blood vessels

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1 The effects of fractionated *Leiurus quinquestriatus hebraeus* venom on cromakalim-induced $^{86}Rb^+$ efflux in rabbit aortic smooth muscle were examined.

2 Crude venom (0.1–30 $\mu g\,ml^{-1}$) produced a concentration-dependent decrease of 1 μM cromakalim-induced $^{86}Rb^+$ response. The maximum blocking activity attainable was approximately 60%.

3 Fractionation of crude venom by gel permeation chromatography and subsequent chromatography on a cation ion-exchange column, produced two fractions (X and XI), active in the $^{86}Rb^+$ blocking assay.

4 Fraction XII contained charybdotoxin (~85% pure). After a final high performance liquid chromatography (h.p.l.c.) purification step, the purified toxin failed to inhibit the cromakalim-stimulated $^{86}Rb^+$ efflux although it was a potent inhibitor of A23187-induced K^+ flux in human erythrocytes and the large conductance calcium-activated potassium channel in rabbit portal vein smooth muscle.

5 Subsequent purification of fraction X by h.p.l.c. yielded a minor peak which contained $^{86}Rb^+$ blocking activity. This subfraction was also capable of inhibiting apamin-sensitive, angiotensin II-stimulated K^+ flux in guinea-pig hepatocytes.

6 It is concluded that the potassium channel opened by cromakalim in rabbit aortic smooth muscle is not blocked by charybdotoxin but by another distinct toxin in the venom of *Leiurus quinquestriatus hebraeus*.

Introduction

Potassium channels are involved in an unusually diverse range of biological activities. For example, some channels are sensitive to membrane depolarization while others are activated by neurotransmitters, intracellular messengers and adenosine 5'-triphosphate (ATP), as well as changes in cell volume (reviewed by Cook, 1988). Several different types of potassium channel often occur within the same cell.

The anti-hypertensive drug cromakalim (BRL 34915) produces an increase in potassium permeability in a variety of isolated smooth muscles (Cook 1988). Little is known of the properties of the potassium channel opened by cromakalim. However, it has been shown that the increase in potassium permeability produced by the drug is neither dependent on the entry of calcium through dihydropyridine-sensitive calcium channels, nor is it blocked by apamin (Weir & Weston, 1986; Quast 1987), a toxin which selectively blocks many calcium-activated potassium channels ($K_{(Ca)}$ channels) of low conductance. Preliminary reports have indicated that cromakalim increases the open time of large $K_{(Ca)}$ channels in both cultured aortic smooth muscle cells and in aortic smooth muscle membranes incorporated into planar lipid bilayers (Kusano *et al.*, 1987; Gelband *et al.*, 1988).

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In portal vein, the cromakalim-mediated increase in $^{86}Rb^+$ tracer efflux can be inhibited by the venom of the Israeli scorpion, *Leiurus quinquestriatus hebraeus* (Quast & Cook, 1988). This venom has been shown to contain at least two different toxins which block distinct $K_{(Ca)}$ channels (Castle & Strong, 1986). One is charybdotoxin, a selective inhibitor of high conductance $K_{(Ca)}$ channels in both skeletal and smooth muscle cells (Miller *et al.*, 1985; Beech *et al.*, 1987; Valdivia *et al.*, 1988; Talvenheimo *et al.*, 1988).

This present study demonstrates that the cromakalim-induced $^{86}Rb^+$ efflux in rabbit aorta, presumed to be indicative of the potassium channel 'opened' by cromakalim, is not blocked by charybdotoxin but by another component of the venom. A purification procedure, that allows the isolation and characterization of both of these two toxins is described.

A preliminary account of the findings has been presented to the British Pharmacology Society (Weir & Strong, 1988).

Methods

Fractionation of L. quinquestriatus hebraeus *venom*

Crude venom (200 mg batches) was placed in a 15 ml centrifuge tube and extracted for 20 min with deionised water (5 ml, 4°C) three times. The extract was centrifuged (45,000*g*, 20 min, 4°C) and the supernatant carefully removed from a viscous opaque gel. The gel was extracted three more times and the supernatants pooled (final volume, 15 ml). The combined supernatants were chromatographed at 4°C on a Sephadex G-50 (superfine) column (90 × 2.6 cm diameter) previously equilibrated with 50 mM ammonium formate buffer, pH 3.5. The column was eluted with equilibration buffer at 40 ml h^{-1} and the eluant was monitored at 278 nm. The peaks containing biological activity in the $^{86}Rb^+$ flux assay from two batches of crude venom were diluted with an equal volume of water and re-chromatographed on an S-Sepharose ion exchange column (10 × 1.6 cm diameter) equilibrated with 50 mM ammonium acetate buffer pH 6.0. After washing the column with equilibration buffer to remove unbound material, the column was eluted with a linear ammonium acetate salt gradient (50–600 mM, pH 6.0, total volume 800 ml) at 90 ml h^{-1}. Fractions were monitored at 278 nm. Fraction X was further chromatographed on a reverse phase column (Pharmacia ProRPC 5/2), equilibrated with 0.01 M sodium acetate/0.04 M acetic acid (buffer A). Bound material was eluted from the column with a linear gradient of 0–100% buffer B (50% aqueous methanol/0.05 M acetic acid, 1:1) at a flow rate of 1 ml min^{-1}. The column was monitored at 280 nm.

$^{86}Rb^+$ *efflux experiments*

Male mongrel rabbits (1.8–3.0 kg, supplied by Sandoz) were killed by a blow to the head. After the thoracic cavity was exposed, the aorta was removed. The aorta was cut into 36 equally sized rings and then each ring was cut along its longitudinal axis. Each ring segment was then mounted on a gassing manifold as described previously by Hamilton *et al.* (1986). The tissues were allowed to equilibrate, firstly in Krebs-Henseleit solution (KHS) for 45 min and then in KHS containing $^{86}Rb^+$ (5 μCi ml^{-1}) for 2 h. During the following efflux experiment, samples were taken at 2 min intervals throughout the entire procedure (80 min). Initially, $^{86}Rb^+$ was allowed to efflux from the tissues into normal KHS for 10 min. Tissues were then exposed to KHS containing PN 200-110 (0.5 μM) (PN/KHS) for 20 min before the transfer to PN/KHS solution supplemented with the toxin under test. After 20 min in toxin solution, the tissues were challenged with cromakalim (1 μM) for a further 20 min. Finally, tissues were washed in PN/KHS for 10 min. Control tissues were treated similarly but exposed to vehicle (0.1% ethanol) alone. Radioactivity in the efflux samples was counted in the Cerenkov mode at 50% efficiency. The amount of radioactivity remaining in the tissue at the end of the experiment was determined by dissolving the tissue in 500 μl Lumasolve (Lumac) at 50°C overnight, 500 μl 1N HCl and 10 ml Optifluor (Packard) was added to the solubilised sample which was then counted in the ^{32}P channel at 100% efficiency. The efflux data were expressed as the first order rate constant (k) of $^{86}Rb^+$ efflux (min^{-1}) (Quast, 1987). Cromakalim-induced $^{86}Rb^+$ efflux was expressed as the area under the curve (AUC) in mg as determined by weighing (see also Quast, 1987).

Single channel recordings

Single smooth muscle cells were isolated from rabbit portal vein by incubating the tissue in a nominally calcium-free physiological medium containing papain, bovine serum albumin and dithiothreitol (Beech & Bolton, 1989) and were used within 12 h. Patch-clamp experiments were performed at room temperature on isolated outside-out patches (Hamill *et al.*, 1981) in quasi-physiological medium of the following composition (mM); bath (external) solution: Na^+ 126, K^+ 6, Ca^{2+} 1.7, Mg^{2+} 1.2, Cl^- 138, glucose 14, HEPES 10.5; pipette (internal) solution: K^+ 134, Mg^{2+} 1.2, Cl^- 136, EGTA (ethylene glycol *bis*-(2-aminoethylether) N,N,N',N'-tetraacetic acid) 0.8, glucose 14, HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) 10.5. Both solutions were titrated to pH 7.2 with NaOH. Analogue signals were recorded on FM tape (7.5 in s^{-1}) and

low pass filtered at 2 kHz (8 pole Butterworth filter: Barr and Stroud). The data were then digitized using a CED 401 A/D converter (Cambridge Electronic Design) in conjunction with a BBC microcomputer and displayed on a plotter.

Erythrocyte potassium efflux assay

Erythrocytes from freshly drawn human blood were separated from plasma, platelets and leucocytes by sedimentation in Dextran 70 (6%, w/v in saline) and resuspended to a haematocrit of 7% in a medium containing (mM): NaCl, 145, KCl 0.1, $MgCl_2$ 1, $CaCl_2$ 1, inosine 10, Tris HCl 10, pH 7.4. Net potassium fluxes were measured using a potassium-sensitive electrode placed in the cell suspension (Cook & Haylett 1985; Castle & Strong 1986). The venom fractions to be tested were pre-incubated with 2 ml of erythrocyte suspension for 2 min at 37°C prior to the addition of A23187 (final concentration 5 μM). Potassium loss during the subsequent 2 min was expressed as a percentage of the total cell potassium content. The latter was evaluated after each experiment by adding digitonin (final concentration 200 μM) to the cell suspension. Control experiments were performed concurrently, without venom fractions.

Hepatocyte potassium efflux assay

Hepatocytes were prepared from male Hartley guinea-pigs by collagenase digestion (Burgess *et al.*, 1981). Cells were incubated and experiments carried out at 37°C in Eagles MEM (Wellcome) supplemented with 2% bovine serum albumin and 10% new-born calf serum at a density of approx. 1×10^7 cells ml^{-1}. Net potassium fluxes were measured in a similar way to those described above for the erythrocyte assay, except that potassium release was stimulated with 100 nM angiotensin II, instead of A23187 (Cook & Haylett, 1985).

Amino acid sequence determination

Purified fraction XII was reduced with dithiothreitol and alkylated by pyridylethylation essentially as described by Tarr *et al.* (1983). The toxin was dissolved in 50 μl Tris buffer (50 mM, pH 8.0) containing 6 M guanidine hydrochloride. Dithiothreitol (200 μg) was added and the mixture incubated for 2 h at room temperature under an atmosphere of argon. Vinylpyridine (0.2 μl) dissolved in 0.8 μl isopropanol was added and the incubation continued for another 2 h. Reagents and toxin were separated by high performance liquid chromatography (h.p.l.c.) on a C_{18} column using a linear 0–100% acetonitrile gradient in aqueous 0.1% heptafluorobutyric acid. For Lys-C digestion, pyridylethylated toxin was dissolved in 50 μl Tris buffer (50 mM, pH 8) containing 4 M urea. Endoproteinase Lys-C (0.4 μg) was added and the mixture incubated for 4 h at 37°C. The digest was separated by h.p.l.c. on a C_{18} column using a linear 0–80% acetonitrile gradient in aqueous 0.1% heptafluorobutyric acid. For V8 protease digestion, pyridylethylated toxin was dissolved in 100 μl sodium phosphate buffer (50 mM, pH 7.8); 1 μg *Staphylococcus aureus* protease V8 was added and the solution incubated for 20 h at 37°C. The digest was separated on a C_4 column using the same conditions described for V8 peptides. N-terminal amino acid sequences were determined by automated Edman degradation in a gas-phase sequencer (Applied Biosystems 470A). After conversion of cleaved amino acids to the corresponding phenylhydantoin derivatives, the latter were subsequently analysed on line (Applied Biosystems 120A h.p.l.c. system).

Materials

Leiurus quinquestriatus hebraeus venom was purchased from Latoxan (Rosans, France). Cromakalim (BRL 34915; (±)-6-cyano-3,4-dihydro-2,2-dimethyl-*trans*-4-(2-oxo-1-pyrrolidyl)-2H-benzo[b]pyran-3-ol) and PN 200-110 (isradipine; 3,5-pyridinedicarboxylic acid, 4-(4-benzofurazanyl)-1,4-dihydro-2,6-dimethyl, 1-methylethylester) were synthesized at Sandoz, Basel. Stock solutions of cromakalim (1 mM) and PN 200-110 (1 mM) were prepared in 50% ethanol: 50% twice distilled water and further dilutions were made with a modified Krebs-Henseleit solution (KHS; Cook *et al.*, 1988a). Solutions of PN 200-110 were protected from light and all $^{86}Rb^+$ efflux experiments were done under sodium light. Chromatography resins were obtained from Pharmacia; A23187, collagenase (type IV), papain, (±)-dithiothreitol, angiotensin II and digitonin were obtained from Sigma. $^{86}Rb^+$ was purchased from New England Nuclear. All other chemicals were reagent grade and were obtained from Merck.

Results

Cromakalim (0.03–300 μM) produced a concentration-dependent increase in $^{86}Rb^+$ efflux from rabbit aortic tissue (Cook *et al.*, 1988b). In the presence of PN 220-110 (0.5 μM), there was no significant difference in either the shape of the curve or in the maximum response produced by cromakalim. From these results, 1 μM cromakalim was chosen for subsequent flux studies described in this paper, as it represented approximately an EC_{50}. PN200-110 was included in the wash solution as a protective

measure against unknown components in the venom which could themselves stimulate $^{86}Rb^+$ loss from the tissue and thus hamper the interpretation of the data (Quast & Cook, 1988).

Crude *Leiurus quinquestriatus hebraeus* venom (0.1–30 $\mu g\,ml^{-1}$) produced a concentration-dependent decrease of the rubidium response stimulated by 1 μM cromakalim (Figure 1). However the venom failed to inhibit completely the rubidium response induced by the drug. The maximum blocking activity observed was approximately 60% and with higher concentrations of venom a stable plateau was obtained and no further inhibition was observed.

Different batches of crude venom contained varying amounts of mucopolysaccharides which resulted in a viscous, stringy gel upon rehydration of the lyophilised venom at any concentration greater than 1 mg dry weight of venom per ml. After centrifugation at 45,000 *g* for 20 min the supernatant was often still too viscous for chromatography, causing many gel exclusion columns to dehydrate. This problem was overcome by acidification of the venom supernatant with formic acid and chromatography of the supernatant on a Sephadex G50 column equilibrated with 50 mM ammonium formate pH 3.5. Batches of crude venom (200 mg) processed in this manner (20 mg dry weight ml^{-1}) afforded one major peak (G50-C, molecular mass ~7000 kDa) which represented 65% of the A_{280}-absorbing material applied to the column and 94% of the cromakalim-induced $^{86}Rb^+$ flux blocking activity of crude venom (Figure 2). Peaks G50-A and B contained, amongst others, material of a mucopolysaccharide nature and peaks G50-D and E were non-proteinaceous U.V.-absorbing compounds (data not shown).

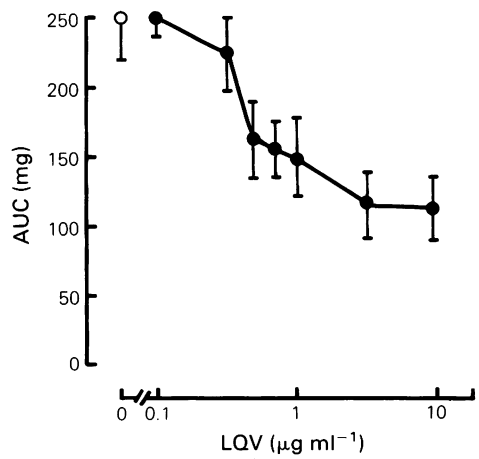


Figure 1 Inhibitory log concentration-effect curve for crude *Leiurus quinquestriatus hebraeus* venom (●) against 1 μM cromakalim in rabbit aorta. (○) Control response to cromakalim in the absence of venom. Ordinate scale: area under the curve (AUC) in mg as a measure of the stimulation of $^{86}Rb^+$ efflux; 100 mg corresponds to an increase in *k* (first order rate constant) by $0.2 \times 10^{-2}\,min^{-1}$ for 20 min. Abscissa scale: venom concentration, log scale. Data represent the means from four experiments (s.e.mean shown by vertical bars).

Fractions constituting peak G50-C were pooled from two batches of venom processed separately, diluted with an equal volume of water, and re-chromatographed on a cation exchange column of S-Sepharose equilibrated with 50 mM ammonium acetate pH 6.0. Of the A_{278}-absorbing material applied to the column, 18% was retained under these initial equilibration conditions. On application of a linear salt gradient, twelve distinct fractions were obtained (Figure 3). Each fraction was assayed for its ability to inhibit cromakalim-stimulated $^{86}Rb^+$ fluxes as before. As shown in Figure 3, most blocking activity was found in fractions X and XI. Fraction XII (containing charybdotoxin, see later), showed an insignificant ability to inhibit cromakalim-induced $^{86}Rb^+$ efflux. In contrast some fractions (most notably fraction IV), caused a *stimulation* of $^{86}Rb^+$ efflux.

Both fractions X and XI were potent inhibitors of the cromakalim-mediated increase in $^{86}Rb^+$ efflux, as indicated by their individual concentration-response curves (Figure 4). Unlike crude venom, both fractions X and XI produced more than 60% inhibition of the cromakalim-induced $^{86}Rb^+$ efflux. We were unable to test higher concentrations due to the small amounts of toxins available. Fraction X was re-chromatographed on a C_1/C_8 reverse phase

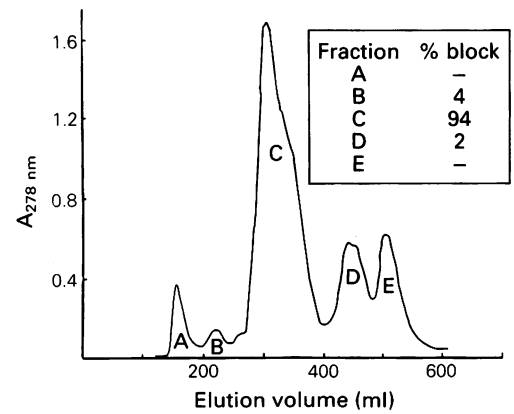


Figure 2 Sephadex G-50 chromatography of crude scorpion venom. Ordinate scale: absorbance at 280 nm. Abscissa scale: column elution volume (ml). Individual fractions making up the five major peaks were pooled separately and assayed for blocking activity in the cromakalim-induced $^{86}Rb^+$ flux assay. Activity was standardised to the total amount of A_{278} absorbance present in each fraction.

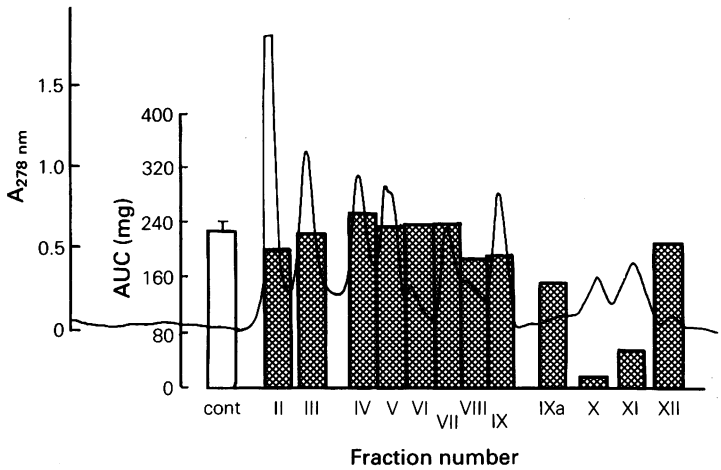


Figure 3 S-Sepharose ion exchange chromatography of fraction G-50-C (see Figure 2). Ordinate scale: absorbance at 280 nm. Abscissa scale: column elution volume. Individual fractions making up identified peaks II–XII were pooled separately and assayed for blocking activity in the cromakalim-stimulated $^{86}Rb^+$ efflux assay. Activity was standardized to the total amount of A_{278} absorbance present in each fraction.

column (Figure 5). Material bound under the starting conditions was eluted from the column with a linear gradient of acetic acid/methanol. Biological activity was located in one of the minor peaks, representing less than 2% of the total amount of A_{280} absorbing material applied to the column and less than 0.02% of total A_{280} absorbing material in the crude venom used at the start of the purification procedure. Fractions eluting from this reverse phase column were also assayed for their ability to block apamin-sensitive low conductance $K_{(Ca)}$ channels in hepatocytes, using an angiotensin II-stimulated potassium efflux assay. The only fraction that showed activity in this assay was the dominant fraction that inhibited chromakalim-induced $^{86}Rb^+$ fluxes (Figure 5).

Rechromatography of fraction XII on the same C_1/C_8 reverse-phase column, using an acetic acid/methanol gradient resulted in a single major peak (Figure 6, lower). This was identified as charybdotoxin by its elution characteristics both in this system (Smith *et al.*, 1986) and on a trifluoroacetic acid/acetonitrile solvent gradient, run on a C_{18} reverse phase column (Gimenez-Gallego *et al.*, 1988). Reduction and alkylation of purified peak XII, followed by enzymatic digestion with either Lys-C or V8 protease afforded the partial sequence shown (see below), which was identical to the C-terminal region of charybdotoxin (Gimenez-Gallego *et al.*, 1988).

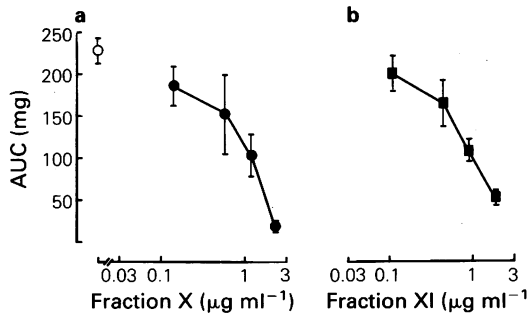


Figure 4 Inhibitory log-concentration-effect curve for fractions X (a, ●) and XI (b, ■) against cromakalim-stimulated $^{86}Rb^+$ efflux in rabbit aorta: (○) represents the control response to 1 μM cromakalim in the absence of any inhibitory fraction. Ordinate scale: area under the curve (AUC) in mg as a measure of the stimulation of $^{86}Rb^+$ efflux; 100 mg corresponds to an increase in k by 0.2×10^{-2} min^{-1} for 20 min. Abscissa scale: fraction concentration (μg ml^{-1}). Data represent the means from three experiments (s.e.mean shown by vertical bars).

Purified fraction XII proved to be a potent inhibitor of A23187-stimulated potassium efflux from human erythrocytes (Figure 6, trace A). In contrast, both fractions X (Figure 6, trace B) and XI were completely devoid of activity in the erythrocyte assay. Fractions X and XI also did not show any

(a) Z F T N V S C T T S K E C W S V C Q R L H N T S R G K C M N K K C R C Y S

(b) E C W S V C Q R L H N T S R G K C M N K K C R C Y S

(a) Charybdotoxin sequence, Gimenez-Gallego *et al.*, 1988; (b) Purified fraction XII, partial sequence.

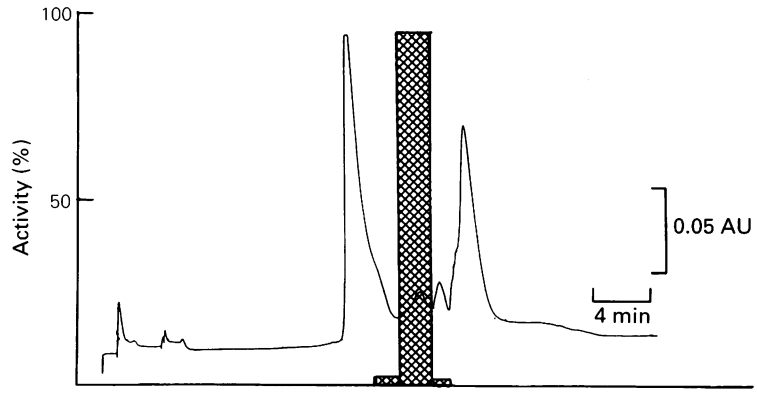


Figure 5 High performance liquid chromatography of fraction X (see Figure 3) on a Pharmacia C_1/C_8 reverse phase column. Ordinate scale: absorption at 280 nm. Abscissa scale: elution time (min). The column was equilibrated with solvent A and eluted with a linear gradient 0–100% solvent B at 1 ml min^{-1} (solvent A: 0.05 M sodium acetate buffer, pH 4.6; solvent B: methanol/100 mM acetic acid, (1/1)). Arrow indicates the start of the linear gradient. Biological activity in the $^{86}Rb^+$ flux assay is indicated by a hatched bar and expressed as a percentage of the total activity found in fraction X.

blocking activity against high conductance $K_{(Ca)}$ channels (see below). Interestingly, cromakalim failed to stimulate potassium efflux from the red blood cells (data not shown).

The activity of purified fraction XII at the single channel level was studied in a series of patch-clamp experiments using outside-out patches from rabbit portal vein smooth muscle cells. Care was taken to

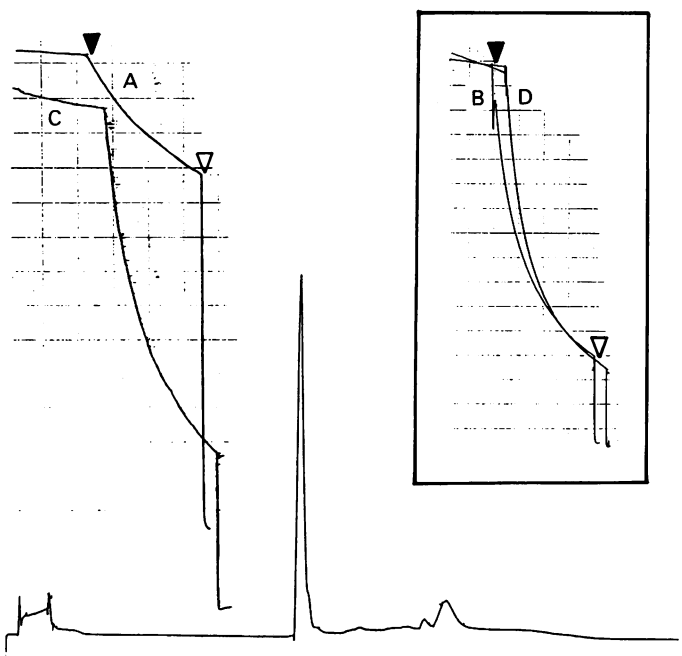
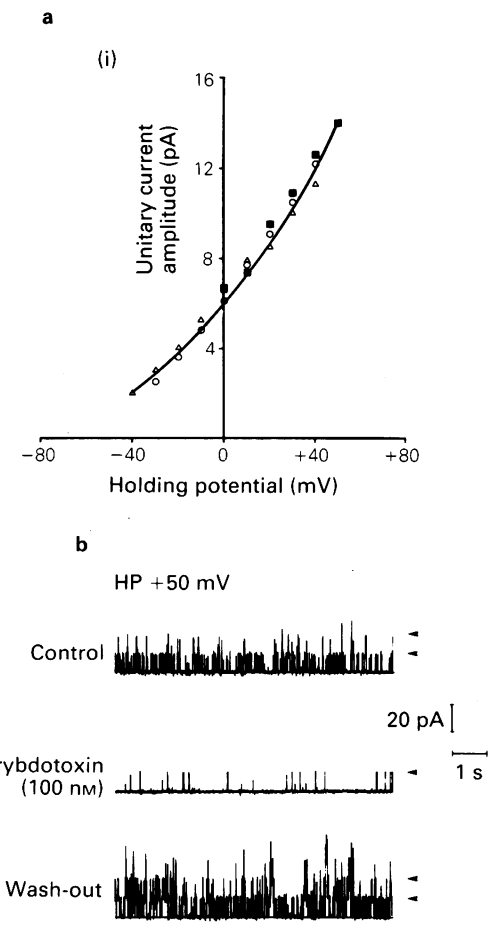


Figure 6 (Upper) Erythrocyte potassium flux assay. Experimental traces of (A) 100 nM (0.4 μg ml^{-1}) purified fraction XII (charybdotoxin); (B) 20 μg ml^{-1} fraction X (inset); (C,D) controls. Potassium flux was initiated by application of 5 μM A23187 (in the presence/absence of toxins) at the symbol (▼). Total cell potassium was determined at the end of each assay by adding 200 μM digitonin at the symbol (▽). (Lower) h.p.l.c. purification of fraction XII (charybdotoxin) on a C_1/C_8 reverse phase column, same conditions as in Figure 5.



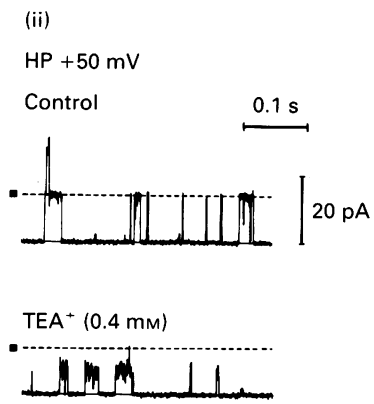


Figure 7 Effect of fraction XII (charybdotoxin) on high conductance $K_{(Ca)}$ channels in an outside-out patch from a rabbit portal vein smooth muscle cell. (a) Identification of the channel: (i) voltage-current relationship (■) for the unitary current amplitude of the channel. Other symbols (○, △) are for comparison with similar channels recorded from different patches under similar conditions. The line, which was drawn by eye, crosses the voltage axis close to the calculated potassium equilibrium potential (−78 mV). (ii) bath-applied tetraethylammonium (TEA^+) (0.4 mM) induced about a 50% decrease in unitary current – characteristic of open channel block. The signal was digitized at 4 kHz. (b) The effect of bath-applied purified fraction XII when the patch was held at +50 mV. The arrows mark the unitary current levels for the fully open state(s) of the $K_{(Ca)}$ channels and the continuous line marks the current when no $K_{(Ca)}$ channels were open. The block was reversible upon wash-out. The signal was digitized at 0.4 Hz.

characterize the channels appearing in these patches, before application of any toxic sample to the patch to ensure that they were indeed high conductance $K_{(Ca)}$ channels. The channels had a unitary conductance of ~90 pS (0 mV) with a physiological potassium gradient and were blocked by 0.4 mM TEA^+ (Figure 7a, right). The opening probability of the channels increased as the negativity at the intracellular side of the membrane was decreased (Figure 7b, left) and qualitative experiments with pipette solutions containing different amounts of free ionized calcium indicated that the opening probability of the channels also increased as the internal calcium concentration increased (data not shown). These experiments therefore provided strong evidence that the channels appearing in outside-out patches were high conductance $K_{(Ca)}$ channels as described by Inoue *et al.* (1985) for these cells.

In the presence of external, purified fraction XII (100 nM, assuming identity with charybdotoxin), the frequency of channel openings was markedly reduced as compared with the control (80–90%; Figure 7b). There was no appreciable effect on the unitary current of the high conductance $K_{(Ca)}$ channels or on the small channel in this patch (of unknown identity), which was observed to be open most of the time during the recording. The block was fully reversible on washout, when the opening probability of the channels appeared to be greater than in the controls. In support of the conclusion that charybdotoxin blocks single $K_{(Ca)}$ channels, the toxin also blocked spontaneous transient outward currents (STOCS), which represent $K_{(Ca)}$ currents in isolated smooth muscle cells (Benham & Bolton, 1986), at the same concentration (Beech & Bolton, 1989). Charybdotoxin affects neither the delayed rectifier potassium current (Beech & Bolton, 1989), the resting membrane resistance nor the cromakalim-induced potassium current of these smooth muscle cells (Beech and Bolton, unpublished).

Discussion

Leiurus quinquestriatus venom has been shown previously to contain toxins that block a remarkable number of different types of potassium channels. For example, large conductance $K_{(Ca)}$ channels in smooth muscle cells (Beech *et al.*, 1987; Kusano *et al.*, 1987) and skeletal muscle T-tubular vesicles (Miller *et al.*, 1985); apamin-sensitive low conductance channels in hepatocytes (Abia *et al.*, 1986; Castle & Strong, 1986); apamin-insensitive channels in thymocytes and Erlich cells (Abia *et al.*, 1986); intermediate conductance $K_{(Ca)}$ channels in erythrocytes (Abia *et al.*, 1986); Castle & Strong, 1986; Beech *et al.*, 1987), as well as the cromakalim-stimulated increase in potassium permeability in portal vein (Quast & Cook, 1988). The biological effects of crude venom on large conductance $K_{(Ca)}$ channels can be attributed to charybdotoxin, a peptide toxin first isolated by Miller *et al.* (1985) and recently sequenced (Gimenez-Gallego *et al.*, 1988). Although charybdotoxin has been shown subsequently to block a variety of large conductance $K_{(Ca)}$ channels (Figure 7; Guggino *et al.*, 1986; Beech *et al.*, 1987; Hoshi & Aldrich, 1987), it is becoming increasingly clear that the toxin is not completely selective. Charybdotoxin also blocks a 50 pS $K_{(Ca)}$ channel in *Aplysia* neurones (Hermann & Erxleben, 1987) as well as a voltage-sensitive potassium channel in lymphocytes (Lewis & Cahalan, 1988). In this present study we have confirmed that the toxin which we had previously tentatively identified as charybdotoxin (Castle & Strong, 1986), also blocks the human erythrocyte $K_{(Ca)}$ channel, which has been shown to have a single channel conductance value of ~20 pS (Hamill, 1981; Grygorczyk *et al.*, 1984). Since cromakalim failed to stimulate potassium efflux from erythrocytes, it is extremely unlikely that red cells possess cromakalim-sensitive potassium channels.

The results presented in this study unequivocally show that charybdotoxin does not affect the potassium channel opened by cromakalim (see also data by Talvenheimo *et al.* (1988), which implies a similar conclusion) and that the inhibitory effects of crude *L. quinquestriatus* venom on tracer efflux from aorta is due to another, distinct toxin species. This second toxin seems rather elusive. It appears to be present in the venom in extremely small amounts, in much smaller quantity than charybdotoxin. However, unlike charybdotoxin, we found that the biological activity of this second toxin is not very stable and becomes increasingly so during the final stages of purification; hence quantitation of the amount present may well be an underestimate. Many venoms contain toxic components that are stabilized in the crude venom through secondary non-covalent associations and we cannot exclude the possibility that the inhibitory effects of crude *L. quinquestriatus hebraeus* venom on cromakalim-stimulated tracer efflux might well be due to such a synergism. Purification schemes for fractionating the venom (with the aim of purifying charybdotoxin) often involve an ion exchange step at pH 9 or greater, sometimes approaching pH 11; these extremely basic conditions invariably reduce or destroy the biological activity of the second toxin. The present purification scheme has been designed to minimize this problem. The inability of crude venom to inhibit completely the cromakalim-stimulated $^{86}Rb^+$ efflux is intriguing. This could be explained either by the presence of stimulatory factors in the crude venom (Figure 3) or else by the fact that cromakalim is stimulating, in addition, another venom-insensitive potassium channel. This second possibility is unlikely in view of the fact that semi-purified fractions X and XII, in the concentrations used, can almost completely block efflux.

The most highly purified fraction containing the cromakalim-associated inhibitory activity also inhibited the apamin-sensitive potassium efflux from hepatocytes and we have previously shown that this component also inhibits [^{125}I]-monoiodoapamin binding to hepatocytes (Castle & Strong, 1986). As this paper was in its final stages of preparation, the complete purification and sequence of this apamin-binding toxin was published (Chicchi *et al.*, 1988). At present we suggest that this second toxin has a broad specificity for both apamin-sensitive, $K_{(Ca)}$ channels and those potassium channels that are opened by cromakalim, although we cannot rule out the possibility that these two activities will be shown subsequently to reside on distinct molecules.

The isolation of a voltage-dependent potassium channel gene from the *Shaker* mutant of *Drosophila* (Papazian *et al.*, 1987; Pongs *et al.*, 1988) and the subsequent use of *Drosophila* cDNA libraries to identify a potassium channel gene in mammalian brain (Tempel *et al.*, 1988; Baumann *et al.*, 1988) suggests many structural similarities between different types of potassium channel and therefore perhaps it is not surprising to find toxins that have high affinities for more than one potassium channel subtype.

Much of this work was done while P.N.S. was a visiting scientist at Sandoz, Basel and he especially thanks Drs U. Quast and N.S. Cook for the generous hospitality of their laboratories during his visit. We thank Drs U. Quast, N.S. Cook, D.G. Haylett, N.S. Castle and Professor D.H. Jenkinson for stimulating discussions and J. Dosogne, Y. Baumlin for technical assistance. We also thank the Wellcome Trust and the M.R.C. for financial support to D.J.B., P.N.S. and Professor T.B. Bolton.

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An electrophysiological comparison of a novel class Ic antiarrhythmic agent, NIK-244 (ethacizin) and flecainide in canine ventricular muscle

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1 Electrophysiological effects of NIK-244 (ethacizin), a novel class I antiarrhythmic drug, were compared with flecainide in canine ventricular muscle by use of conventional microelectrode techniques.

2 At concentrations of 10^{-6} M or higher, NIK-244 depressed the maximum rate of rise of depolarization ($\dot{V}_{max}$) significantly in a concentration-dependent manner. Also, the resting potential was depolarized at 10^{-5} M. NIK-244 did not have any effect on the other action potential parameters or on the effective refractory period.

3 Flecainide significantly decreased $\dot{V}_{max}$ at 3×10^{-6} M or higher and the resting potential was depolarized at 10^{-5} M. Like NIK-244, flecainide did not affect other action potential parameters.

4 NIK-244 and flecainide caused a use-dependent block of $\dot{V}_{max}$, and the rates of onset of inhibition at 3 Hz stimulation were 0.014 ± 0.002 AP^{-1} at 2×10^{-6} M NIK-244 and 0.021 ± 0.012 AP^{-1} at 10^{-5} M flecainide. Under the same conditions, the time constants of the recovery from use-dependent block were 27.1 ± 13.3 s and 12.2 ± 2.5 s for NIK-244 and flecainide, respectively.

5 These results suggest that NIK-244, like flecainide, should be classified as a slow kinetic drug and as Ic.

Introduction

NIK-244 (ethacizin), 2-(ethoxycarbonylamino)-10-{3-(diethylamino)propionyl}-phenothiazine hydrochloride, has been developed in the U.S.S.R. and is a new antiarrhythmic drug of the phenothiazine group (Rosenshtraukh *et al.*, 1979; 1986). NIK-244 is the diethylamino analogue of ethmozin, which had been developed earlier as an antiarrhythmic agent (Rosenshtraukh *et al.*, 1979). It has recently been reported that NIK-244 possesses an even more powerful antiarrhythmic action than ethmozin (Shugushev *et al.*, 1984; Rosenshtraukh *et al.*, 1986). Recent results in mammalian myocardium (Nesterenko & Rosenshtraukh, 1983) suggest that NIK-244 is a novel class I antiarrhythmic agent according to the classification of Vaughan Williams (1980). Hageman *et al.* (1986) showed that NIK-244 neither reduced nor enhanced reflex-induced changes in sympathetic or parasympathetic actions. Clinically it has also been found that NIK-244 prolonged PR and QRS intervals but had little effect on QT interval and refractory ventricular period, and that NIK-244 had high antiarrhythmic efficacy in patients with ventricular premature beats (Smetnev *et al.*, 1983; Rosenshtraukh *et al.*, 1986).

The aim of the present experiments was to investigate the effects of NIK-244 on canine ventricular muscle. It was compared with flecainide, a class 1c and a slow kinetic antiarrhythmic drug (Campbell, 1983a), since it appears from the results of Rosenshtraukh *et al.* (1986) and Smetnev *et al.* (1983) that NIK-244 may not affect the action potential duration. Furthermore, we sought to determine the rate of onset of the inhibition and the recovery from use-dependent block of $\dot{V}_{max}$ in order to obtain more electrophysiological information.

Methods

Eight mongrel dogs of either sex, weighing 7–10 kg, were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, intravenously). The details of the

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Table 1 Changes in action potential parameters induced by NIK-244 in canine ventricular muscle

	n	*Resting potential* (mV)	*Action potential amplitude* (mV)	$\dot{V}_{max}$ (Vs^{-1})	*Action potential durations* (ms) 50%	90%	*Effective refractory period* (ms)
Control	11	−87 ± 4	111 ± 10	244 ± 27	169 ± 27	223 ± 25	220 ± 35
10^{-8} M	11	−87 ± 4	110 ± 10	237 ± 35	171 ± 25	220 ± 23	222 ± 31
10^{-7} M	11	−86 ± 3	110 ± 9	229 ± 51	172 ± 22	223 ± 22	221 ± 31
10^{-6} M	11	−86 ± 3	106 ± 6	190 ± 31**	168 ± 29	224 ± 19	223 ± 26
10^{-5} M	4	−82 ± 5*	97 ± 26	145 ± 19**	164 ± 28	231 ± 13	219 ± 32

The values represent mean ± s.d. *n*: number of experiments. * $P < 0.05$; ** $P < 0.01$, with respect to control values.

methods have been described in recent papers (Satoh & Hashimoto, 1986; Satoh *et al.*, 1987). In brief, the heart was quickly excised, and 3 to 5 preparations were obtained from the right ventricle of each dog. The preparations were driven at 1 Hz by a stimulator (Dia Medical System, DPS-160B). The duration of the stimuli was 1–2 ms and the voltage was about 50% above the threshold. The action potential was recorded by a conventional glass microelectrode technique (the electrode resistance was 5–10 MΩ) on an oscilloscope (Nihon Kohden VC-10) and a thermal array recorder (Nihon Kohden, WS-641G), and photographed (Nihon Kohden RLG-6201). The refractory period was measured at the 11th pulse with shorter intervals by interrupting the constant stimulation interval of 1 s.

To examine the use-dependent block of $\dot{V}_{max}$, experiments of the kind represented in Figure 1 were performed. In drug-free solution, the control value of $\dot{V}_{max}$ was determined. Then, NIK-244 was administered, and the stimulation was stopped. Following a resting period of 90 s, repetitive stimulation was resumed at the same frequency as the control. For recovery of $\dot{V}_{max}$ inhibition, the stimulation at 3 Hz was stopped during exposure to drugs and the diastolic intervals were changed. The percentage of recovery from use-dependent block was estimated by an equation: $1 - (\dot{V}_{max})_{test}/(\dot{V}_{max})_{first}$, where $(\dot{V}_{max})_{test}$ is the value of $\dot{V}_{max}$ at stimulation after the diastolic interval, and $(\dot{V}_{max})_{first}$ is that at the first stimulation after rest during exposure to the drug.

Perfusion solution

The composition of modified Tyrode solution (mM) was as follows: NaCl 137, KCl 4, $MgCl_2$ 2.7, $NaHCO_3$ 11.9, NaH_2PO_4 0.45 and dextrose 5.5. The pH was adjusted to 7.4 with NaOH. The preparations were superfused in a bath with oxygenated (97% O_2 plus 3% CO_2) Tyrode solution. The temperature was maintained at 36°C.

Drugs

The following drugs were used; NIK-244 (ethacizin HCl), which was kindly supplied by Nikken Chemicals Ltd., and flecainide acetate (Ricker Laboratories). These drugs were administered cumulatively.

Statistical analysis

Values are given as mean ± s.d. and comparisons were by Student's paired *t* test, as appropriate. Probability levels of less than 0.05 were taken as indicating significant differences.

Results

Effects of NIK-244 and flecainide on the action potential

At concentrations from 10^{-8} to 10^{-5} M, NIK-244 inhibited the maximum rate of depolarization ($\dot{V}_{max}$) in a concentration-dependent manner (Table 1). The other parameters of the action potential were unaffected. NIK-244 depressed $\dot{V}_{max}$ significantly at 10^{-6} M or higher. At a concentration of 10^{-5} M, the resting potential was depolarized. The action potential durations (APD) at 50% and 90% repolarizations were not lengthened and the effective refractory period was not increased to any significant extent.

Flecainide was also examined to compare its effects with those of NIK-244. Like NIK-244, flecainide inhibited $\dot{V}_{max}$ in a concentration-dependent manner, but did not cause any other effects on the action potential parameters. At 3×10^{-6} M or higher concentrations, flecainide depressed $\dot{V}_{max}$ significantly (Table 2). Flecainide, 10^{-5} M, depressed the action potential amplitude and depolarized the resting potential; it did not affect the action potential duration or the effective refractory period.

These results show many similarities between NIK-244 and flecainide and indicate that NIK-244 is a class Ic antiarrhythmic drug that produces no change in APD.

Table 2 Changes in action potential parameters induced by flecainide in canine ventricular muscle

	n	*Resting potential* (mV)	*Action potential amplitude* (mV)	$\dot{V}_{max}$ (Vs^{-1})	*Action potential durations* (ms) 50%	90%	*Effective refractory period* (ms)
Control	9	−82 ± 6	99 ± 14	260 ± 9	178 ± 30	216 ± 29	231 ± 10
10^{-6} M	9	−80 ± 7	98 ± 12	231 ± 88	172 ± 38	219 ± 37	235 ± 13
3×10^{-6} M	9	−79 ± 9	98 ± 12	228 ± 33*	182 ± 34	226 ± 33	242 ± 14
10^{-5} M	8	−73 ± 11*	91 ± 15*	200 ± 41**	187 ± 30	228 ± 29	253 ± 16

The values represent means ± s.d. *n*: number of experiments. * $P < 0.05$, ** $P < 0.01$, with respect to control values.

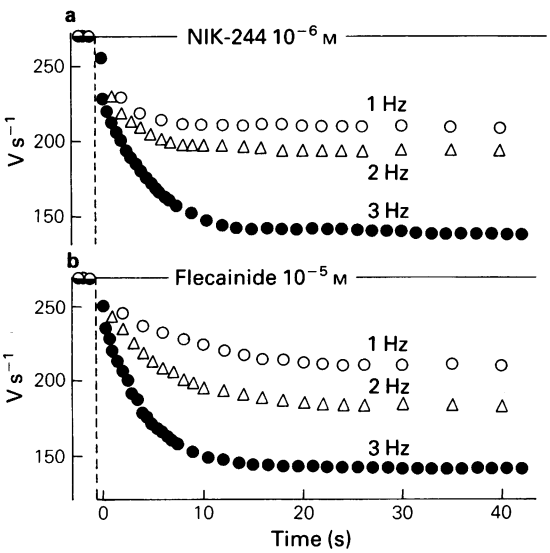

Figure 1 Time courses of inhibition of $\dot{V}_{max}$ at different stimulation frequencies in the presence of NIK-244 (a) and flecainide (b). Both drugs depressed $\dot{V}_{max}$ in a use-dependent manner. The ordinate scale shows magnitude of $\dot{V}_{max}$, and the abscissa scale represents before and during drug administration at times (0–40 s) after the onset of stimulation.

Use-dependent block of $\dot{V}_{max}$

As shown in Figure 1, the $\dot{V}_{max}$ at the first action potential was decreased (a resting block), and declined during stimulation to a new steady-state (a use- or a frequency-dependent block). NIK-244 inhibited $\dot{V}_{max}$ frequency-dependently. The decline curve at 1 Hz was unaffected at 10^{-7} M, but in the presence of 10^{-6} M NIK-244 it became steeper (Figure 1a). After the rest, $\dot{V}_{max}$ of the first action potential was 258 $V s^{-1}$. The resting block was 4.4% ($\dot{V}_{max}$ was 270 $V s^{-1}$ in drug-free solution). At 1 Hz $\dot{V}_{max}$ was 213 $V s^{-1}$; at 2 Hz, 193 $V s^{-1}$; and at 3 Hz, 141 $V s^{-1}$ as a new steady-state.

Application of 10^{-5} M flecainide also caused a marked use-dependent block (Figure 1b). Flecainide depressed $\dot{V}_{max}$ from 269 $V s^{-1}$ in drug-free solution to 235 $V s^{-1}$ at 1 Hz, to 183 $V s^{-1}$ at 2 Hz and to 139 $V s^{-1}$ at 3 Hz. The resting block was 7.1%.

These findings indicate that NIK-244, like flecainide, is an antiarrhythmic drug with slow rate of onset of $\dot{V}_{max}$ inhibition.

Rate of onset of $\dot{V}_{max}$ *inhibition*

Campbell (1983a, b) showed that antiarrhythmic drugs can be classified by the rate of onset of blockade in the presence of concentrations which caused

Table 3 The rate of onset of inhibition of the maximum rate of depolarization at different frequencies of stimulation by NIK-244 and flecainide

	n	1	2	3 Hz
NIK-244				
10^{-6} M	11	0.011 ± 0.028	0.011 ± 0.026	0.012 ± 0.009
2×10^{-6} M	3	0.011 ± 0.018	0.012 ± 0.022	0.014 ± 0.002
10^{-5} M	3	0.015 ± 0.016	0.021 ± 0.011	0.020 ± 0.019
Flecainide				
10^{-6} M	5	0.010 ± 0.024	0.011 ± 0.021	0.014 ± 0.026
3×10^{-6} M	4	0.012 ± 0.014	0.012 ± 0.011	0.014 ± 0.014
10^{-5} M	13	0.015 ± 0.009	0.018 ± 0.003	0.021 ± 0.012

Values represent the means ± s.d. *n*: number of experiments.
The unit of values is AP^{-1}.

about 50% of the use-dependent depression of $\dot{V}_{max}$ at an interstimulus interval of 300 ms (approximately equivalent to 3 Hz). Thus, in the present experiments, using concentrations of 10^{-6} M (47.8% inhibition) and 2×10^{-6} M (56.3% inhibition) of NIK-244, the rates of onset of $\dot{V}_{max}$ inhibition were $0.012 \pm 0.009\,AP^{-1}$ ($n = 11$) and $0.014 \pm 0.002\,AP^{-1}$ ($n = 3$), respectively. When stimulation started, the upstroke velocities declined in an exponential fashion, action potential by action potential, as shown in Figure 1. The logarithms of the decremental losses of $\dot{V}_{max}$ of the action potentials during the train were plotted against pulse number. Thus, the unit of AP^{-1} represents the reciprocal of the number of action potentials which corresponds to the time constant for the decay of $\dot{V}_{max}$. The results obtained at different frequencies and concentrations are summarized in Table 3.

On the other hand, the $\dot{V}_{max}$ was inhibited by 37% and 58% in the presence of flecainide 3×10^{-6} M and 10^{-5} M, respectively. At 3 Hz, the rates of onset of $\dot{V}_{max}$ inhibition were $0.014 \pm 0.014\,AP^{-1}$ ($n = 4$) in 3×10^{-6} M and $0.021 \pm 0.012\,AP^{-1}$ ($n = 13$) in 10^{-5} M flecainide. The rates of onset of $\dot{V}_{max}$ inhibition were examined at different frequencies and in the presence of various concentrations (Table 3).

These results indicate that NIK-244 had slow kinetics of $\dot{V}_{max}$ inhibition, like flecainide.

Recovery of $\dot{V}_{max}$ from use-dependent block

Figure 2 illustrates a typical example, in which $\dot{V}_{max}$ of the premature action potential is plotted against diastolic interval (abscissa scale). In the absence of drug, the recovery of $\dot{V}_{max}$ following the basic driven action potential was complete within 50 ms and was well fitted by a monoexponential function with a time constant (τ) of 22 ± 4 ms ($n = 8$). In the presence of 10^{-6} M and 2×10^{-6} M NIK-244, the time constants for the recovery were 26.8 ± 17.2 s ($n = 4$) and 27.1 ± 13.3 s ($n = 3$), respectively. In contrast, at 10^{-5} M flecainide, it was 12.2 ± 2.5 s ($n = 3$). Note that the time constant for NIK-244 was much slower, almost twice that of flecainide.

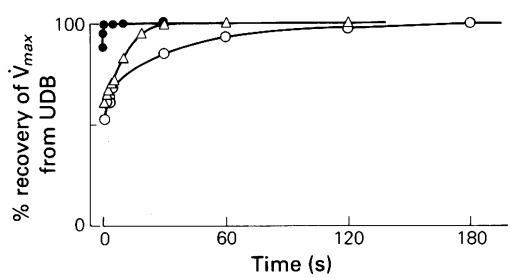

Figure 2 Recovery of $\dot{V}_{max}$ from use-dependent block (UDB). The percentage recovery was plotted against the diastolic interval. (●) Control, the time constant (τ) was 25 ms in drug-free solution; (△) flecainide 10^{-5} M, $\tau = 10.3$ s; (○) NIK-244 2×10^{-6} M, $\tau = 25.7$ s.

These results show that NIK-244 is an antiarrhythmic agent with a much slower recovery from use-dependent block than flecainide.

Discussion

Class I antiarrhythmic drugs (local anaesthetic type) are effective in the treatment of certain kinds of cardiac arrhythmias. These drugs depress the fast Na channel responsible for action potential generation. Hondeghem & Katzung (1977) and Hille (1977) in recent reviews have emphasized that the rapid upstroke phase of cardiac action potentials is blocked in a use-dependent manner during exposure to an antiarrhythmic drug (modulated receptor hypothesis). Rested sodium channels have a much lower affinity for antiarrhythmic drugs than depolarized (activated or inactivated) channels. In addition, the more depolarized the holding potential is, the slower is the rate of recovery from block.

Usually it is considered that the classifications of Class I antiarrhythmic drugs by effects on APD and by the rate of onset of $\dot{V}_{max}$ inhibition and recovery from use-dependent block are similar, but both were quite different. The former is divided into further three subclasses; antiarrhythmic drugs causing the prolongation of APD are in subclass Ia, those causing the shortening of APD are in Ib, and drugs with no change in APD are in Ic. On the other hand, there are marked differences among drugs in the speed at which $\dot{V}_{max}$ falls to a new plateau level and then recovers to an initial level during exposure to an antiarrhythmic drug. Therefore, class I antiarrhythmic drugs have been divided into three distinct subclasses by differences on their interaction with fast Na^+ channel (binding and dissociation kinetics); i.e., fast, intermediate and slow kinetic drugs (Harrison *et al.*, 1981; Campbell, 1983a).

Action potential duration

In the present experiments, neither NIK-244 nor flecainide caused any significant effects on APD or on the effective refractory period, although with both drugs minor prolongations were observed. This is consistent with the results of Smetnev *et al.* (1983) and Rosenshtraukh *et al.* (1986), who have already shown that intravenous NIK-244 causes no significant change in ventricular refractory periods and QT interval. Like flecainide, therefore, NIK-244 should be considered a class Ic antiarrhythmic drug.

NIK-244 decreases the slow inward current as well as the fast inward current in frog atrial trabeculae

and canine Purkinje fibres (Rosenshtraukh *et al.*, 1979; Chazov *et al.*, 1984; Urthaler *et al.*, 1986). Since NIK-244 had no effect on APD it may also decrease time-dependent outward current. Voltage-clamp experiments are required to ascertain this possible mechanism of action.

Block development and recovery of $\dot{V}_{max}$

In the present experiments, NIK-244 did not cause any effect on APD but inhibited $\dot{V}_{max}$, like flecainide. Comparing NIK-244 and flecainide, the rate of onset of use-dependent block was similar, being $0.014\,AP^{-1}$ and $0.021\,AP^{-1}$, respectively. The time constant of recovery for NIK-244 was approximately twice that of flecainide. These values of the rate of onset of $\dot{V}_{max}$ inhibition and the recovery for flecainide are consistent with those reported previously (Campbell, 1983a, b), although animal species are different. According to Harrison *et al.* (1981) and Campbell (1983b), the values for the fast kinetic drugs are more than $0.277\,AP^{-1}$, those for the intermediate ones in the range from 0.113 to $0.055\,AP^{-1}$, and those for the slow ones less than $0.029\,AP^{-1}$. In the present experiments, the rates of onset of $\dot{V}_{max}$ inhibition for both NIK-244 and flecainide were less than $0.029\,AP^{-1}$. We, therefore, conclude that NIK-244 should be classified as an antiarrhythmic drug with slow kinetics of action. This is confirmed by the fact that NIK-244 slowed the recovery time from use-dependent block to twice that of flecainide.

There are many factors that determine the binding or the dissociation rates for the fast Na channel. Courtney (1980) and Campbell (1983b) showed that drugs with higher lipid solubility may produce a faster and greater block, whereas drugs with lower molecular weight may leave the channel more quickly during each diastolic interval of the action potentials. On the other hand, Hille (1977) reported that the rate of block development correlated well with lipid solubility but not with molecular weight or size of the drug. The molecular weight of NIK-244 is 449.99, and is relatively large, indicating that NIK-244 might have slow kinetic characteristics. More recently, Courtney (1988) has shown that drugs having quite small dimensions (X, Y and Z) can gain access to the receptors during maintained depolarization (inactivated state), despite poor lipid distribution capabilities. In NIK-244, X = 3–6; Y = 6.5; Z = 15 Å, which are quite different from the smaller dimensions of lidocaine with fast kinetics. Extensive studies are required to elucidate the mechanism of the kinetics more clearly.

In conclusion, the present results show many similarities between NIK-244 and flecainide, such as no change in APD and effective refractory period, the slow rate of onset of $\dot{V}_{max}$ inhibition, and the slow kinetics of recovery from use-dependent block. It is, therefore, suggested that the new antiarrhythmic agent, NIK-244 (ethacizin), like flecainide, has slow kinetics of action.

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Effects of the putative antagonists phaclofen and δ-aminovaleric acid on $GABA_B$ receptor biochemistry

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1 Phaclofen and δ-aminovaleric acid (δ-AVA) have been reported to be antagonists at γ-aminobutyric $acid_B$ ($GABA_B$) receptors. Phaclofen, δ-AVA and related compounds were examined for potency and specificity at $GABA_B$ and $GABA_A$ receptors in rat cortical membranes labelled with [^{3}H]-(−)-baclofen and [^{3}H]-muscimol, respectively. Additionally phaclofen and δ-AVA were examined in two functional tests of central $GABA_B$ activity in rat cortical slices, namely the inhibition of forskolin-stimulated cyclic AMP accumulation, and the potentiation of isoprenaline-stimulated cyclic AMP accumulation.

2 δ-AVA (IC_{50} = 11.7 μM) was 20 fold more potent than phaclofen (IC_{50} = 229 μM) on $GABA_B$ receptor binding. All compounds possessing a phosphonic acid group, including phaclofen, which were active at $GABA_B$ receptors were inactive at $GABA_A$ receptors, while δ-AVA was equally potent at both receptors. Several compounds exhibited Hill coefficients of less than unity in displacing [^{3}H]-(−)-baclofen binding.

3 (−)-Baclofen inhibited forskolin-stimulated cyclic AMP accumulation (IC_{50} = 7.9 μM) but this effect was not stereospecific. Phaclofen (1 mM) was inactive against this inhibition but produced a potentiation of the forskolin effect. δ-AVA (1 mM) failed to antagonize the effect of baclofen; rather it mimicked baclofen.

4 (−)-Baclofen (10 μM) potentiated isoprenaline-stimulated cyclic AMP accumulation, an effect antagonized by phaclofen (1 mM). δ-AVA (1 mM) may be a weak antagonist but also potentiated basal cyclic AMP accumulation.

5 We conclude that neither δ-AVA nor phaclofen are potent specific $GABA_B$ receptor antagonists.

Introduction

Receptors for the inhibitory neurotransmitter γ-aminobutyric acid (GABA) are divided into two subtypes, $GABA_A$ and $GABA_B$, on the basis of their pharmacological specificity (Hill & Bowery, 1981). The $GABA_A$ receptor is a well characterized complex of binding sites for GABA, the benzodiazepines, and barbiturates together with an associated chloride ion channel, (see for example Olsen, 1981). Potent $GABA_A$ agonists include GABA, muscimol and isoguvacine while bicuculline and its methohalide salts are potent antagonists (Curtis *et al.*, 1970). Baclofen is a potent $GABA_B$ agonist with no activity at $GABA_A$ receptors. However there is a lack of potent and specific $GABA_B$ receptor antagonists. δ-Aminovaleric acid (δ-AVA) has been shown to antagonize the action of (−)-baclofen in the periphery (Muhyaddin *et al.*, 1982) and more recently in the CNS (Watling & Bristow, 1986), although δ-AVA is equally potent at $GABA_A$ and $GABA_B$ receptors (Bowery *et al.*, 1983). The phosphonic acid derivative of baclofen, phaclofen, has also been reported to antagonize the actions of baclofen in the periphery (Kerr *et al.*, 1987) and this compound has recently been reported to antagonize central $GABA_B$ receptors (Dutar & Nicoll, 1988). We describe here the use of filtration binding assays with [^{3}H]-(−)-baclofen and [^{3}H]-muscimol to look at the potency of an extensive range of compounds, including δ-AVA, phaclofen and related compounds at $GABA_B$ receptors and their specificity for $GABA_B$ compared with $GABA_A$ receptors. However, such ligand binding assays do not provide any information on whether compounds active at $GABA_B$ receptors are agonists or antagonists; such a differentiation can only be made by use of functional assays of $GABA_B$ receptor activity. We have therefore also examined the actions of δ-AVA and phaclofen on two reported actions of (−)-baclofen. These are the inhibition by (−)-baclofen of forskolin-stimulated cyclic AMP accumulation (Hill &

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Dolphin, 1984; Wojcik & Neff, 1984; Hill, 1985; Karbon & Enna, 1985; Xu & Wojcik, 1986) and the potentiation by (−)-baclofen of the stimulation of cyclic AMP production by agonists such as noradrenaline, isoprenaline and vasoactive intestinal peptide (VIP) (Hill & Dolphin, 1984; Karbon & Enna, 1985; Watling & Bristow, 1986).

A preliminary account of some of this work has been presented to the British Pharmacological Society (Robinson *et al.*, 1988).

Methods

Animals

In all experiments male Lister Hooded rats (200–250 g) were used, obtained from Harlan Olac, Bicester.

Radioligand binding assays

A frozen osmotically-shocked membrane preparation from rat cortex was used for both [^{3}H]-(−)-baclofen and [^{3}H]-muscimol binding assays.

Frozen cortex was homogenized in 6 volumes ice-cold buffer (10 mM Tris-HCl, pH 7.4, containing 0.32 M sucrose), using a Potter Elvehjem homogeniser fitted with a teflon pestle. The homogenate was centrifuged at 500 *g* for 10 min. The resulting supernatant was stored on ice and the pellet rehomogenized and centrifuged as above. This was repeated so that three pooled supernatants gave a total suspension of 18 volumes g^{-1} tissue wet weight. The suspension was centrifuged at 30,000 *g* for 30 min at 4°C and the resulting crude synaptosomal membrane pellet resuspended in 60 volumes ice cold distilled water using an Ultra Turrax homogenizer. The membrane suspension was given a second osmotic shock by use of centrifugation and resuspension in disilled water as before. Membrane pellets were harvested by centrifugation at 30,000 *g* for 12 min at 4°C and stored at −20°C for [^{3}H]-(−)-baclofen binding. For [^{3}H]-muscimol binding, following freezing at −20°C overnight, membrane pellets were thawed at room temperature, resuspended in 90 volumes distilled H_2O using an Ultra-Turrax homogenizer and harvested by centrifugation at 30,000 *g* for 12 min at 4°C. All membrane pellets were stored at −20°C for up to 2 months prior to their use in binding assays.

The activity of ligands at the $GABA_B$ receptor was assayed by a [^{3}H]-baclofen equilibrium binding assay as described by Hill & Bowery (1981). The binding procedure was novel, however, in that binding was terminated by filtration rather than centrifugation. Frozen membrane pellets were thawed, resuspended in assay buffer (50 mM Tris-HCl, pH 7.4 containing $CaCl_2$, 2.5 mM) using an Ultra-Turrax homogenizer and incubated at room temperature for 45 min. The membranes were harvested by centrifugation at 30,000 *g* for 12 min at 20°C, and then washed in assay buffer a further three times, with 15 min incubations between each centrifugation. Final equilibrated membrane pellets were resuspended in assay buffer (1–2 mg protein ml^{-1}) and assayed immediately. The binding assay was performed in 96 well microtitre plates in a total incubation volume of 200 μl, and routinely utilized 30 nM [^{3}H]-baclofen. Specific binding was defined with an excess of GABA (100 μM). Binding was initiated by the addition of tissue (approximately 0.25 mg protein) to the microtitre well. Following 10 min incubation at 25°C the binding was terminated by filtration through glass fibre filters using a Skatron semi-automatic cell harvester. Filters were rapidly washed with approximately 1 ml ice cold assay buffer in a 2 s wash time. Following drying, the radioactivity on the filters was measured by liquid scintillation counting.

The activity of ligands at the $GABA_A$ receptor was assayed by a [^{3}H]-muscimol equilibrium filtration binding assay (Williams & Risley, 1979). Frozen membrane pellets were thawed, resuspended in ice cold assay buffer (50 mM Tris-HCl, pH 7.4) by use of an Ultra-Turrax homogenizer and centrifuged at 30,000 *g* for 10 min at 4°C. The resulting pellet was resuspended in assay buffer (1–1.5 mg protein ml^{-1}) and used immediately in the binding assay. The assay was essentially as described above for [^{3}H]-(−)-baclofen binding, but 10 nM [^{3}H]-muscimol and approximately 0.2 mg protein were used and the assay was incubated on ice for 10 min. Following filtration, the filters were rapidly washed with approximately 4.5 ml ice cold assay buffer in a 7 s wash time.

Forskolin- and isoprenaline-stimulated cyclic AMP accumulation

Modulation by baclofen of forskolin- and isoprenaline-stimulated cyclic AMP accumulation was measured essentially as described by Hill (1985). Slices (300 × 300 μm, 45°) of cerebral cortex were cut with a McIlwain tissue chopper and were washed for 90 min at 37°C in Krebs-Ringer bicarbonate buffer (pH 7.4, containing (mM): NaCl 118.5, KH_2PO_4 1.18, $MgSO_4$ 1.18, $NaHCO_3$ 25, $CaCl_2$ 2.54 and glucose 11) and continually gassed with O_2/CO_2 (95/5%). The buffer was changed every 30 min. Aliquots (50 μl) of packed slices were preincubated with (−)-baclofen (and test drugs where appropriate) for 15 min at 37°C before the addition of buffer, forskolin or isoprenaline to give a final incubation volume of 1 ml. Slices were routinely incubated with 0.5 μM forskolin

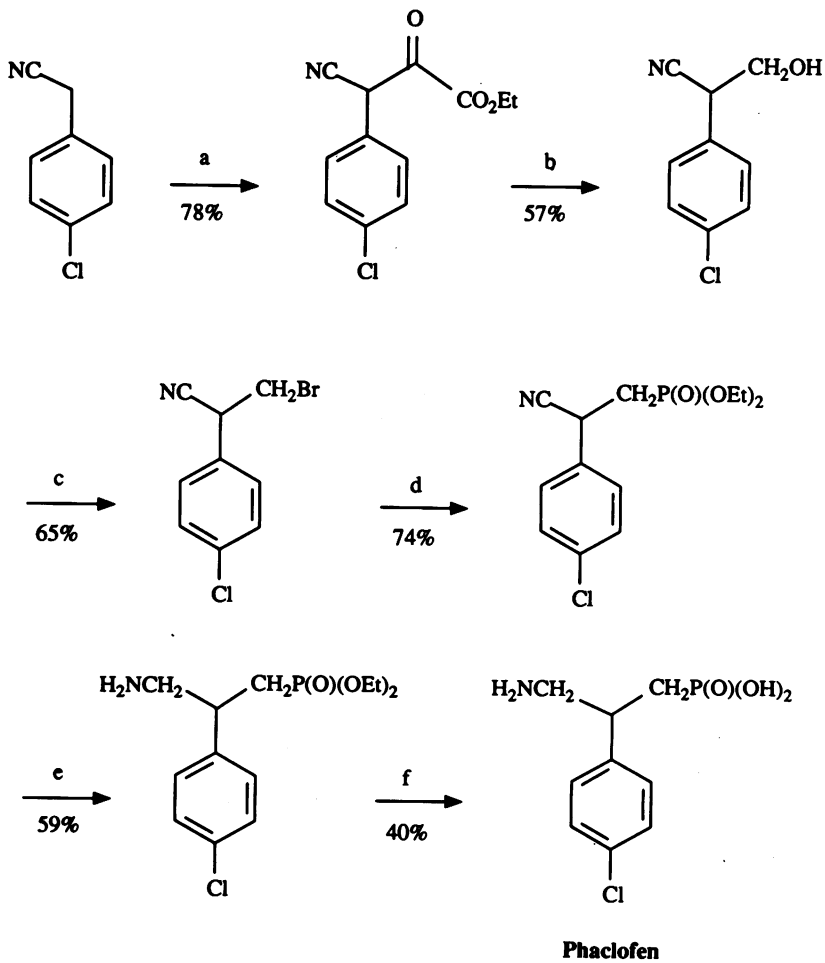

Figure 1 Synthesis of phaclofen. Reagents were (a) NaOEt, diethyloxalate; (b) Na_2CO_3, HCHO; (c) PBr_3; (d) $P(OEt)_3$; (e) PtO_2, $H_2(g)$; (f) 6N HCl.

for 20 min or with 1 μM isoprenaline for 10 min. In the experiments in which forskolin was used, the buffers contained the phosphodiesterase inhibitor, isobutylmethylxanthine (IBMX) during the 15 min pre-incubation and 20 min incubation with forskolin. Slices were rapidly sedimented by centrifugation, the supernatants removed, and 20 μl concentrated HCl (10 M) added. After at least 30 min at room temperature, the slices were neutralised with 236 μl 1 M NaOH and sedimented by centrifugation. The supernatant was removed and the cyclic AMP content measured with a cyclic AMP assay kit, based on the protein binding assay of Brown *et al.* (1971). The residual slices were solubilized in 1 M NaOH containing SDS (0.7% w/v) and Na_2CO_3 (2% w/v) prior to analysis of the protein. Data are expressed as pmol cyclic AMP accumulated mg^{-1} protein and were analysed by a one-way analysis of variance followed by a protected one-tailed t test.

Protein estimation

Protein concentration of membrane suspensions and solubilized tissue slices was determined by a phenol reagent technique (Dawson & Heatlie, 1984) with bovine serum albumin used as standard.

Synthesis of phaclofen

Phaclofen (3-amino-2-(4-chlorophenyl) propylphosphonic acid) was conveniently synthesized by the route shown in Figure 1. No attempt was made to

Table 1 Potencies (pIC_{50}) and Hill coefficients (n_H) of compounds in $GABA_B$ and $GABA_A$ binding assays

Compound	$GABA_B$ pIC_{50}	$GABA_B$ n_H	$GABA_A$ pIC_{50}
GABA	7.59 ± 0.09	0.67 ± 0.05	7.00
(−)-Baclofen	7.17 ± 0.15	0.77 ± 0.11	i.a
SL 75102	7.12 ± 0.26	0.61 ± 0.08	6.42
3-Hydroxy-GABA	5.95	1.0	6.16
(+)-Baclofen	5.17 ± 0.08	0.94 ± 0.04	i.a
Muscimol	5.68 ± 0.18	0.54 ± 0.05	8.05
3-Aminopropane sulphonic acid	5.60 ± 0.11	0.60 ± 0.04	7.31
4-Aminobutyl phosphonic acid	5.14 ± 0.13	0.67 ± 0.03	i.a
3-Phenyl-GABA	5.11 ± 0.10	0.75 ± 0.12	i.a
3-Aminopropyl phosphonic acid	5.00 ± 0.12	0.89 ± 0.09	i.a
δ-Aminovaleric acid	4.93 ± 0.15	0.62 ± 0.17	5.14
2(3-Chlorophenyl)GABA	4.75 ± 0.11	0.81 ± 0.02	i.a
δ-Aminolevulinic acid	4.50 ± 0.07	1.32 ± 0.17	3.46
4-Guanidinobutyric acid	4.33	0.77	4.31
6-Aminohexanoic acid	4.17 ± 0.02	0.83 ± 0.08	i.a
Kojic acid	3.87	1.19	i.a
Phaclofen	3.64 ± 0.17	0.77 ± 0.17	*i.a
Isoguvacine	i.a	—	7.27
4-Imidazole acetic acid	i.a	—	6.10
Bicuculline methiodide	i.a	—	4.70

Data are means ± s.e.mean, where applicable from 2–16 experiments using triplicate assays. i.a, less than 10% displacement at 100 μM; *i.a, less than 10% displacement at 1 mM. 4-Aminopyridine, 7-aminoheptanoic acid, 2-(4-chlorophenyl)ethylamine, 3-guanidinoproprionic acid, kynurenic acid, L-kynurenine, L-kynurenine sulphate, D,L-pipecolic acid and progabide all displaced less than 10% [^{3}H]-(−)-baclofen binding at 100 μM.

optimise the reported yields. This route has the advantage of avoiding the photochemical step that is an integral part of the published method of synthesis of phaclofen (Chiefari *et al.*, 1987).

Drugs

Cyclic AMP ^{3}H radioassay kits were supplied by Amersham International plc (Aylesbury). [^{3}H]-(−)-baclofen (39 Ci mmol^{-1}) and [^{3}H]-muscimol (17.5 Ci mmol^{-1}) were obtained from New England Nuclear, Du Pont (UK) Ltd. 4-Imidazole acetic acid and 3-hydroxy-GABA were supplied by Aldrich Chemical Co, and isoguvacine by Research Biochemicals Inc., U.S.A. SL75102 (4-([(4-chlorophenyl) (5-fluoro-2-hydroxy phenyl) methylene] amino butyric acid) and progabide were gifts from Synthelabo, Paris, France and (+)- and (−)-isomers of baclofen were gifts from Ciba Geigy Ltd., Switzerland. All other fine chemicals were obtained from Sigma Chemical Co., Poole.

Results

GABA receptor binding assays

The binding of [^{3}H]-(−)-baclofen to rat cortical membranes was measured by a filtration binding method. In initial experiments it was observed that single point binding data were comparable using the centrifugation and filtration assay. However, when filtration was used the specific binding represented a much greater percentage of the total binding than when centrifugation was used (67 ± 8%, $n = 11$ instead of 21 ± 6%, $n = 5$, both given as mean ± s.e.mean). The binding of [^{3}H]-(−)-baclofen (10–150 nM) was saturable and Scatchard analysis revealed a dissociation constant (K_D) of 73 nM and B_{max} of 0.34 pmol mg^{-1} protein. The K_D was 99 nM when derived by kinetic analysis (data not shown).

An extensive range of compounds was tested for activity in displacing 30 nM [^{3}H]-(−)-baclofen binding. The potencies and Hill coefficients of active compounds were derived by Hill analysis of the displacement curve (Table 1). Compounds which exhibited activity at $GABA_B$ receptors were further analysed in [^{3}H]-muscimol binding. The potencies of compounds in displacing [^{3}H]-muscimol (10 nM) are also presented in Table 1.

Forskolin-stimulated cyclic AMP accumulation

Forskolin stimulated the production of cyclic AMP in slices of rat cortex in a concentration-dependent manner, saturating at 1.5 μM (data not shown); 0.5 μM forskolin was used in all subsequent experiments.

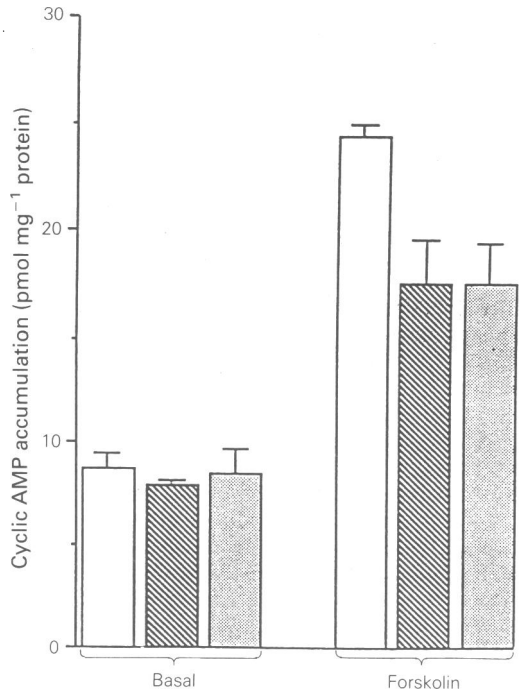

Figure 2 Effects of baclofen isomers on basal and forskolin-stimulated (0.5 μM) cyclic AMP accumulation in slices of rat cerebral cortex. (−)-Baclofen (hatched columns) and (+)-baclofen (stippled columns) were present at 10 μM; control (open columns). Data are mean from three experiments using triplicate assays; s.e.mean shown by vertical bars.

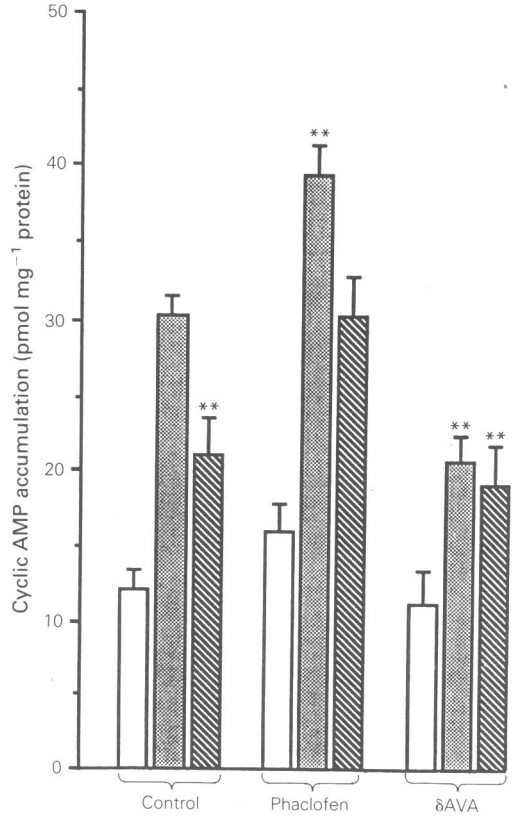

Figure 3 Effects of phaclofen and δ-aminovaleric acid (δ-AVA) on the inhibition by (−)-baclofen of forskolin-stimulated cyclic AMP production in slices of rat cerebral cortex. Phaclofen and δ-AVA (both at 1 mM) were tested as indicated on basal (open columns), forskolin-stimulated (0.5 μM, stippled columns) and forskolin-stimulated cyclic AMP accumulation in the presence of 10 μM (−)-baclofen (hatched columns). Data are mean from 4–8 experiments using triplicate assays; s.e.mean shown by vertical bars. Basal cyclic AMP accumulation in the presence of phaclofen or δ-AVA was not significantly different from the control value; $P > 0.05$. Forskolin-stimulated cyclic AMP accumulation in the presence of phaclofen, δ-AVA and/or (−)-baclofen was tested for significant difference from the control forskolin-stimulated value: ** $P < 0.01$.

Neither (−)- or (+)-baclofen, at a concentration of 10 μM, had any effect on basal cyclic AMP accumulation (in the absence of forskolin) but at 10 μM both isomers produced 40% inhibition of forskolin-stimulated cyclic AMP accumulation (Figure 2). The inhibition by baclofen of forskolin-stimulated cyclic AMP accumulation was not stereoselective as (−)-baclofen and (+)-baclofen were equipotent (IC_{50} values (μM): (−)-baclofen, 7.9 ± 4.0, (+)-baclofen, 8.0 ± 4.0, $n = 3$ in triplicate assays). At high concentration (1 mM), GABA mimicked (−)-baclofen in inhibiting forskolin-stimulated cyclic AMP accumulation. The effect of the $GABA_A$ agonist, isoguvacine was inconsistent (data not shown).

At a concentration of 1 mM, phaclofen and δ-AVA were without effect on basal cyclic AMP accumulation (in the presence of IBMX, Figure 3). δ-AVA mimicked (−)-baclofen in inhibiting forskolin-stimulated cyclic AMP accumulation and did not show any significant reversal of the inhibition produced by (−)-baclofen (Figure 3).

Phaclofen appeared to reverse the inhibition by (−)-baclofen of forskolin-stimulated cyclic AMP production but this was an indirect result of potentiation by phaclofen of the forskolin-stimulated cyclic AMP (Figure 3).

Isoprenaline-stimulated cyclic AMP accumulation

Isoprenaline (1 μM) doubled basal cyclic AMP accumulation in slices of rat cortex and this effect was potentiated by 10 μM (−)-baclofen (Figure 4). Phaclofen (1 mM) had no significant effect on cyclic AMP

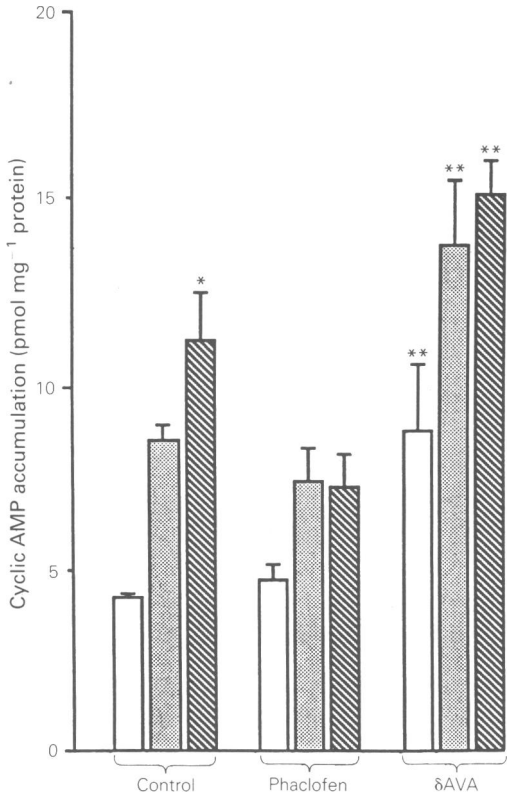

Figure 4 Effects of phaclofen and δ-aminovaleric acid (δ-AVA) on the potentiation by (−)-baclofen of isoprenaline-stimulated cyclic AMP production in slices of rat cerebral cortex. Phaclofen and δ-AVA (both at 1 mM) were tested, as indicated, on basal (open columns), isoprenaline-stimulated (1 μM, stippled columns) and isoprenaline-stimulated cyclic AMP accumulation in the presence of 10 μM (−)-baclofen (hatched columns). Data are mean from 4–8 experiments using triplicate assays; s.e.mean shown by vertical bars. Basal cyclic AMP accumulation in the presence of phaclofen or δ-AVA was tested for significant difference from the control basal value. Isoprenaline-stimulated cyclic AMP accumulation in the presence of phaclofen, δ-AVA and/or (−)-baclofen was tested for significant difference from the control isoprenaline-stimulated value; * $0.05 > P > 0.01$; ** $P < 0.01$.

accumulation in the absence or presence of isoprenaline but completely reversed the potentiation observed with (−)-baclofen. At 1 mM, δ-AVA more than doubled basal cyclic AMP accumulation and therefore showed an apparent potentiation of isoprenaline-stimulated cyclic AMP accumulation. In the presence of δ-AVA, (−)-baclofen showed a smaller potentiation of isoprenaline-stimulated cyclic AMP accumulation than control (Figure 4).

Discussion

The preparation of membranes and the assay used to measure $GABA_B$ binding were essentially the same as originally reported by Hill & Bowery (1981) who used both [^{3}H]-GABA and [^{3}H]-(−)-baclofen. However the methods described here differ in that membranes were prepared from rat cortex rather than whole brain, and the binding assay was terminated by filtration rather than centrifugation of the membranes. This change did not lead to a marked difference in single point binding of [^{3}H]-baclofen and B_{max} and K_D values were in the range reported by others (Drew *et al.*, 1984; Karbon *et al.*, 1983; Mennini *et al.*, 1986), although the B_{max} value was lower than that reported by Bowery *et al.* (1983). The use of filtration is quicker and more convenient than centrifugation and offers the major advantage of a greatly increased percentage specific binding signal.

With respect to the compounds presented in Table 1 which have previously been tested in $GABA_B$ binding, the pharmacology described is in close agreement with Bowery *et al.* (1983), both in the order of potency and absolute potency in displacing [^{3}H]-(−)-baclofen. The only discrepancy is that (+)-baclofen is 10 fold more potent in our $GABA_B$ assay than in that of Bowery *et al.* (1983).

The parallel study of compounds in both $GABA_A$ and $GABA_B$ binding assays (Table 1) enables some conclusions on their receptor subtype specificity to be made. SL 75012, 3-hydroxy-GABA, muscimol, 3-aminopropyl sulphonic acid and δ-AVA all lack $GABA_B$ specificity as previously reported (Lloyd *et al.*, 1982; Bowery *et al.*, 1983; Falch *et al.*, 1986). All compounds active in $GABA_B$ binding possessing a phenyl ring were inactive in $GABA_A$ binding, and all the phosphonic acid derivatives active in $GABA_B$ binding were inactive in $GABA_A$ binding. This provides a basis for the $GABA_B$ specificity of the putative antagonist phaclofen, and although phaclofen was 20 fold less potent than δ-AVA in displacing [^{3}H]-(−)-baclofen binding (Table 1), it was specific.

When discussing potencies of compounds in the $GABA_B$ binding assays, what must be borne in mind is that it is based on agonist binding and may represent binding to a high affinity agonist state. Consequently, the potency of antagonists in displacing [^{3}H]-(−)-baclofen binding may not indicate absolute potency.

Forskolin stimulated the accumulation of cyclic AMP in slices of rat cortex and this was inhibited by (−)-baclofen with a maximal inhibition of 40% (Figure 2), in agreement with previous workers (Hill & Dolphin, 1984; Hill, 1985; Karbon & Enna, 1985). Any effect of (−)-baclofen which is via $GABA_B$ receptors should be stereoselective with respect to the (−)-isomer (Hill & Bowery, 1981). One group of

workers (Hill & Dolphin, 1984; Hill 1985) has shown that in the presence of IBMX, in slices of rat cortex, (−)-baclofen was at least 100 fold more potent than (+)-baclofen on forskolin stimulated cyclic AMP accumulation. We found that (−)-baclofen exhibited the same potency in inhibiting forskolin-stimulated cyclic AMP accumulation as reported by Hill (1985) but we were unable to demonstrate stereospecificity although this was demonstrated in both binding (Table 1) and in other studies on baclofen (Stirling *et al.*, 1989). Because of the lack of stereoselectivity and the fact that phaclofen does not antagonize we cannot therefore conclude that the action of baclofen on forskolin stimulated cyclic AMP accumulation is via $GABA_B$ receptors.

To date, no pharmacology has been published for the baclofen/forskolin interaction. δ-AVA appeared to act as an agonist mimicking baclofen by inhibiting forskolin-stimulated cyclic AMP accumulation (Figure 3). It failed to antagonize the inhibition by (−)-baclofen. In contrast, phaclofen potentiated the effect of forskolin, and did not affect the (−)-baclofen inhibition of this activity.

Agonists, such as noradrenaline, isoprenaline and VIP, stimulate cyclic AMP production in brain slices, and (−)-baclofen augments this effect (Hill & Dolphin, 1984; Hill, 1985; Karbon & Enna, 1985; Watling & Bristow, 1986). Watling & Bristow (1986) reported that δ-AVA (1 mM) antagonized the potentiation by (−)-baclofen of VIP-stimulated cyclic AMP accumulation in slices of rat cortex. Our data clearly demonstrate that at 1 mM, phaclofen is an antagonist at the $GABA_B$ receptors responsible for modulating isoprenaline-stimulated cyclic AMP accumulation (Figure 4). δ-AVA may also have acted as an antagonist in this system and partially attenuated the (−)-baclofen stimulation but this is difficult to conclude given the raised basal cyclic AMP levels produced by addition of δ-AVA alone. In conclusion, phaclofen, and possibly δ-AVA are antagonists at $GABA_B$ receptors modulating agonist-stimulated cyclic AMP production.

The evidence that δ-AVA was a $GABA_B$ antagonist came from rat anoccocygeus muscle (Muhyaddin *et al.*, 1982) and guinea-pig ileum (Sawynok, 1986), although there has been some dispute as to whether the latter effect has a $GABA_A$ component (Allan & Dickenson, 1986; Schwarz *et al.*, 1988). δ-AVA also antagonized the $GABA_B$ receptor responsible for the (−)-baclofen-induced reduction of population spikes in hippocampal slices (Nakahiro *et al.*, 1985) and reversed baclofen-induced hypothermia and analgesia (Hunter *et al.*, 1988). While δ-AVA may antagonize the $GABA_B$ receptors augmenting agonist-stimulated cyclic AMP accumulation in brain slices (Watling & Bristow, 1986), we have previously shown that it is an agonist at the $GABA_B$ receptor modulating K^+-stimulated $[Ca^{2+}]_i$ in synaptosomes (Stirling *et al.*, 1989) and it also acted as an agonist in the forskolin system.

Phaclofen, first described as an antagonist at peripheral $GABA_B$ receptors (Kerr *et al.*, 1987), has been shown to be an antagonist at postsynaptic $GABA_B$ receptors modulating K^+-conductances in hippocampal slices (Dutar & Nicoll, 1988) and at $GABA_B$ receptors in the locus coeruleus (Olpe *et al.*, 1988). While we have shown that phaclofen antagonizes the potentiation by (−)-baclofen of isoprenaline-stimulated cyclic AMP accumulation, it appeared as an agonist at $GABA_B$ receptors modulating synaptosomal K^+-stimulated $[Ca^{2+}]_i$ (Stirling *et al.*, 1989) and has no effect on either the (−)-baclofen/forskolin interaction or on most of the behavioural tests we have used (Hunter *et al.*, 1988).

What is clear from the available data on putative $GABA_B$ antagonists is that they do not always behave in the same manner in different systems, possibly because of receptor heterogeneity. It is striking that several of the compounds examined (Table 1) exhibit Hill coefficients markedly less than unity, supporting this concept. One tentative division could be pre- and postsynaptic $GABA_B$ receptors. Phaclofen is an antagonist at postsynaptic but not presynaptic $GABA_B$ receptors in the hippocampus (Dutar & Nicoll, 1988; Nicoll & Dutar, 1988). Furthermore phaclofen and δ-AVA are agonists at the $GABA_B$ receptors modulating synaptosomal K^+-stimulated $[Ca^{2+}]_i$ which are presumably presynaptic (Stirling *et al.*, 1989). It is not known whether the $GABA_B$ receptors augmenting isoprenaline-stimulated cyclic AMP accumulation are pre- or postsynaptic, but as phaclofen, and probably δ-AVA, are antagonists, we suggest that they are postsynaptic even though phaclofen has been claimed to antagonize presynaptic rather than postsynaptic $GABA_B$ receptors in the spinal cord (Kerr *et al.*, 1987), and the peripheral $GABA_B$ receptors antagonized by δ-AVA are presynaptic (Muhyaddin *et al.*, 1982).

In conclusion, more potent and specific antagonists are necessary before we can fully characterize $GABA_B$ receptors and define their roles in modulation of the nervous system.

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GABA receptors on the somatic muscle cells of the parasitic nematode, *Ascaris suum*: stereoselectivity indicates similarity to a $GABA_A$-type agonist recognition site

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1 The γ-aminobutyric acid (GABA) receptors on the somatic muscle cells of *Ascaris*, which mediate muscle cell hyperpolarization and relaxation, have been characterized by use of intracellular recording techniques.

2 These receptors are like mammalian $GABA_A$-receptors in that the response is mediated by an increase conductance to chloride ions. The $GABA_A$-mimetic, muscimol, has a relative potency of 0.40 ± 0.02 ($n = 3$) compared to GABA.

3 The stereoselectivity of the GABA receptor on *Ascaris* is identical to that for the mammalian $GABA_A$-receptor, as determined from the relative potency of three pairs of enantiomers of structural analogues of GABA.

4 The most potent agonist is (S)-(+)-dihydromuscimol which is 7.53 ± 0.98 ($n = 5$) times more potent than GABA.

5 The *Ascaris* GABA receptor is not significantly blocked, at concentrations below 100 μM by the potent, competitive $GABA_A$-receptor antagonist, SR95531.

6 The *Ascaris* GABA receptor does not recognise agents that are known to block the GABA gated chloride channel in mammalian preparations such as *t*-butylbicyclophosphorothionate (TBPS, 10 μM, $n = 2$) or the insecticide dieldrin (100 μM, $n = 3$).

7 GABAergic responses in *Ascaris* are not potentiated by pentobarbitone (100 μM, $n = 3$) or flurazepam (100 μM, $n = 3$).

8 The potencies of various GABA-mimetics in the *Ascaris* preparation have been compared with their potency at displacing $GABA_A$-receptor binding in mammalian brain. Excluding the sulphonic acid derivatives of GABA, the correlation coefficient (r) between the potencies of compounds in the two systems is 0.74 ($P < 0.01$). The significance of this correlation is discussed.

9 The pharmacology of the *Ascaris* GABA receptor is discussed in relation to other invertebrate systems and the mammalian subclassification of GABA receptors.

Introduction

Invertebrate γ-aminobutyric acid (GABA) receptors do not readily fit into the mammalian subclassification of $GABA_A$- and $GABA_B$-subtypes (see Walker & Holden-Dye, 1989 for review) as described for the mammalian system by Bowery *et al.*, 1980. Thus, whilst many of them are similar to the mammalian $GABA_A$-receptor subtype in that both responses are mediated by the opening of a chloride ionophore (Fatt & Katz, 1953; Boistel & Fatt, 1958; Takeuchi & Takeuchi, 1967; Motokizawa *et al.*, 1969; Pitman & Kerkut, 1970; Curtis & Johnston, 1974; Krnjević, 1974; Bokisch & Walker, 1986; Pinnock *et al.*, 1988), they are consistently insensitive or only very weakly activated by the sulphonic acid

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GABA analogues, such as 3-aminopropane sulphonic acid or piperidine-4-sulphonic acid in insect (Roberts *et al.*, 1981; Sattelle *et al.*, 1988), arthropod (Roberts *et al.*, 1981), gastropod (Vehovsky *et al.*, 1989), and nematode (Holden-Dye *et al.*, 1988) preparations. This is in marked contrast to the potent agonist action of these compounds at $GABA_A$-receptors in mammalian and amphibian preparations (Curtis & Watkins, 1961; Campochiaro *et al.*, 1977; Nistri & Constanti, 1979).

Another difference between invertebrate and mammalian $GABA_A$-receptors is that apart from a few exceptions (Walker *et al.*, 1971; Piggott *et al.*, 1977; Yarowsky & Carpenter, 1978; Waldrop *et al.*, 1987), these receptors are generally only weakly (Smart & Constanti 1986), or not blocked (Scott & Duce 1987; Sattelle *et al.*, 1988; Benson 1988a; Holden-Dye *et al.*, 1988) by the definitive, competitive $GABA_A$-receptor antagonist, bicuculline. Nevertheless, micromolar concentrations of the noncompetitive antagonist picrotoxin, which acts at the level of the chloride channel in mammalian preparations (Olsen, 1981; Barker, 1985), is sufficient to block GABA-mediated responses in most invertebrates (Piggott *et al.*, 1977; Hori *et al.*, 1978; Constanti, 1978; Walker & Roberts, 1982; Sattelle *et al.*, 1988). In arthropods the interaction of picrotoxin with the receptor channel complex may differ slightly from the interaction in mammalian systems in that the block may not be truly noncompetitive (Constanti, 1978).

$GABA_A$-mediated responses in mammalian preparations are potentiated by benzodiazepines and barbiturates (Olsen, 1981 for review). There is some evidence for such an interaction at insect GABA receptors (Beadle *et al.*, 1986; Robinson *et al.*, 1986; Lees *et al.*, 1987). Whether or not other invertebrate GABA systems will also exhibit such modulation has yet to be determined.

The GABA receptor described in this study mediates muscle cell hyperpolarization and relaxation in the parasitic nematode *Ascaris suum*. GABA is apparently the endogenous ligand for this receptor as, using GABA-specific antisera, GABA-immunoreactivity has been localized in the inhibitory motorneurones and activation of these neurones elicits inhibitory postsynaptic potentials in the muscle cells (Johnson & Stretton, 1987). Other inhibitory amino acids, (glycine, β-alanine and taurine) are without effect on the *Ascaris* muscle cell (Holden-Dye *et al.*, 1988). The GABA receptor on *Ascaris* muscle cells is neither bicuculline- nor picrotoxin-sensitive (Wann, 1987; Holden-Dye *et al.*, 1988). Studies using various GABA receptor agonists, however, have indicated that the agonist recognition site may resemble the mammalian $GABA_A$-receptor in some respects. Thus, it is activated by isoguvacine and muscimol (Holden-Dye *et al.*, 1988). A further similarity is indicated by the observation that the agonists show positive cooperativity at the *Ascaris* GABA receptor (Holden-Dye *et al.*, 1988), as they do at receptors in mammalian preparations (Nistri & Constanti, 1979). Furthermore, although the GABA response is not blocked by picrotoxin, which is known to act at the GABA-operated anion channel in mammalian preparations, the response to GABA in *Ascaris* muscle is accompanied by an increase in conductance of the membrane for chloride ions (Martin 1980; Holden-Dye & Walker, 1989).

The *Ascaris* GABA receptor is not sensitive to the $GABA_B$-receptor agonist, baclofen (Holden-Dye *et al.*, 1988). Indeed, there is no evidence to date of a $GABA_B$-type receptor in invertebrate preparations.

In this study we have further characterized the *Ascaris* GABA receptor. We have tested for antagonism by a potent, competitive $GABA_A$-antagonist SR95531, by noncompetitive $GABA_A$-receptor antagonists and for modulation by known modulators of GABA-receptors in mammalian preparations. In particular, we have used stereoisomers of structural analogues of GABA to test more rigorously the notion that the agonist recognition site at GABA receptors in the invertebrate *Ascaris* resembles that of the mammalian $GABA_A$-receptor.

Methods

Ascaris were obtained from the local abattoir on a weekly basis and maintained in the laboratory. An anterior section of the worm, approximately 2 cm long, was excised and slit along one lateral line to enable the strip to be laid out flat. The intestine was removed revealing the muscle bag cells. The section was securely pinned out, cuticle side down, in a perspex perfusion chamber and continuously perfused with artificial perienteric fluid (APF), 32–34°C, the composition of which was as follows: (mM) NaCl 67, Na acetate 67, KCl 3, $CaCl_2$ 3, $MgCl_2$ 15.7, Tris base 5, pH 7.6 with acetic acid. The cells were impaled with two potassium acetate (4 M)-filled glass microelectrodes with a resistance of 10–30 MΩ. Conventional electrophysiological recording techniques were employed using an Axoclamp 2A system. Transmembrane potentials were monitored. Input conductance of the cells was estimated by injecting current pulses, (0.1 Hz, 500 ms pulse width, 10–35 nA) through the second microelectrode and recording the resulting electrotonic potentials. Generally speaking there is little rectification in the hyperpolarizing direction so this method provides an estimate of the input conductance (Figure 1). Drugs were applied via a fine bore plastic tube (0.8 mm internal diameter)

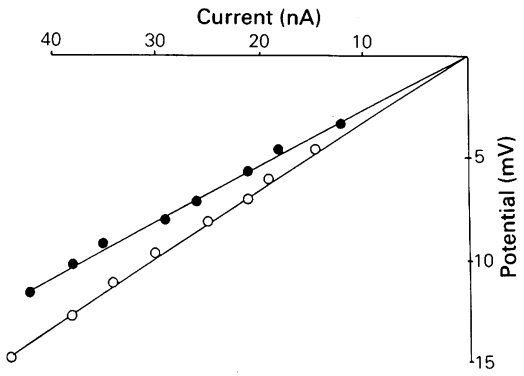


Figure 1 The current-voltage relationship for an *Ascaris* muscle cell. The cell was impaled with two microelectrodes. Hyperpolarizing current pulses, 10–40 nA, 500 ms pulse width, 0.1 Hz, were passed through one microelectrode and the electrotonic potential in the cell was monitored with the second electrode. The plots in the absence (○) and presence (●) of GABA are linear indicating that the cell did not rectify in the hyperpolarizing direction.

directed at the cell from which a recording was being made. Drug solutions were separated by air bubbles in the perfusion tubing which escaped through a small hole in the tubing immediately prior to entry to the bath. This method of application allows rapid and complete change of the composition of the perfusion stream over the cell under study (Slater & Carpenter, 1984). Studies with dye indicate that the perfusion stream bathes the cell under study and then swirls around the chamber. The dye is apparently evenly distributed in the perfusion chamber after 40 s. Drugs were applied at a rate of 8–10 ml min^{-1} for a period of up to 45 s (enabling the maximum response to develop) followed by a 5 min wash. Each drug was applied in ascending concentrations. With this technique, the conductance increase elicited by a given concentration of GABA is stable to multiple applications. Relative potencies of compounds compared to GABA were determined by fitting the dose-response curves by eye and taking the ratio of the concentration of the drug to the concentration of GABA that elicit equivalent responses in the same cell from a parallel portion of the graphs. Statistical significance was determined by Student's paired t test. Linear regression analysis was performed using the Biosoft programme, LINEFIT (Barlow, 1983). Maximum responses and EC_{50} values were obtained by fitting the dose-response data to saturation curves using the programme Fit&Hill (Barlow, 1983). The stereoisomers of the structural GABA analogues, (S)-(−)-4-methyl-trans-aminocrotonic acid (4-me-TACA), (R)-(+)-4-me-TACA, (S)-(+)-3-OH-GABA, (R)-(−)-3-OH-GABA, (S)-(+)-dihydromuscimol (DHM) and (R)-(−)-DHM were prepared as described previously (Krogsgaard-Larsen *et al.*, 1985). GABA was obtained from BDH, *t*-butylbicyclophosphorothionate (TBPS) from Research Biochemicals Inc., flurazepam from Roussel Laboratories. We are grateful to Q. Sanofi Recherche for the gift of SR95531 (2-[carboxy-3'-propyl]-3-amino-6-paramethoxy-phenyl-pyridazinium-Br). Water insoluble compounds such as dieldrin were dissolved in dimethylsulphoxide (DMSO) and diluted in artificial perienteric fluid (APF) such that the final concentration of DMSO was 1%. Other drugs and chemicals were supplied by Sigma.

Results

The resting membrane potential of the *Ascaris* muscle cells was 29.6 ± 0.6 mV with a resting input conductance of 2.53 ± 0.15 μS ($n = 28$). The responses obtained to GABA agree well with those that we have found previously (Holden-Dye *et al.*, 1988). The *Ascaris* muscle cells responded reproducibly to GABA with the threshold for the hyperpolarization between 5–10 μM GABA and the maximum response, of between 10–20 mV depending upon the resting membrane potential, developing at around 30 μM GABA (Figure 2). The maximum conductance change varies between cells in the range 1–3 μS, however the dose-response relationship is consistent, with an EC_{50} of 26.3 ± 3.5 μM ($n = 17$), as determined from 17 cells in which a clear maximum was obtained. Uptake is unlikely to affect significantly the potency of GABA or GABAmimetics in this preparation as it has been shown previously that the GABA uptake inhibitor, nipecotic acid, does not affect the potency of GABA (Hewitt, 1987). This does not necessarily mean that an uptake mechanism does not exist for GABA in these cells. It is most likely that the perfusion rate would overcome the effects of an uptake system in this study. In three paired experiments the relative potency of muscimol compared to GABA was determined (Figure 3). The mean relative potency compared to GABA was 0.40 ± 0.02 ($n = 3$). The slope of the dose-response curve is very similar to that for GABA indicating that muscimol interacts with the receptor in a similar manner to GABA, i.e. it exhibits positive cooperativity.

SR95531 did not significantly block the GABA response at 100 μM (Figure 4), although at 500 μM about 40% blockade was evident. This effect was completely reversible.

Dieldrin (100 μM) did not block the conductance increase elicited by GABA in *Ascaris* muscle cells (Figure 5). This agent was applied at least 10 min

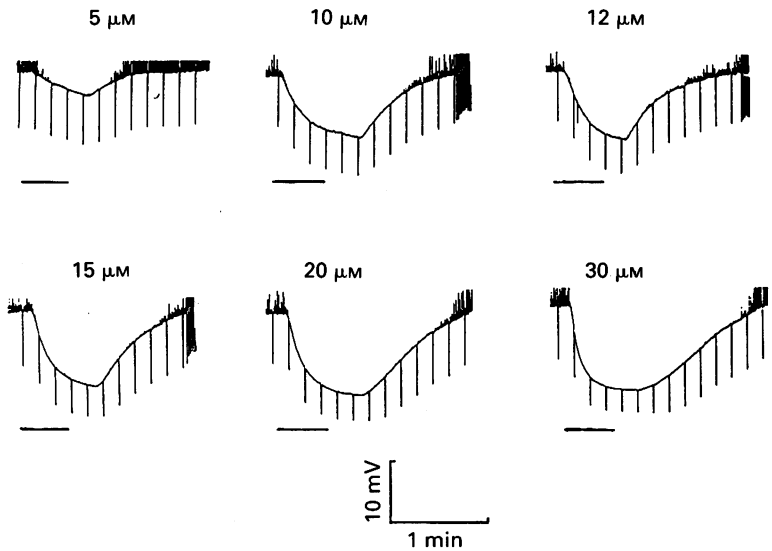


Figure 2 Hyperpolarization and increase in input conductance of the *Ascaris* somatic muscle cell to locally applied GABA. The duration of GABA application is indicated by the bar. The traces illustrate the responses obtained to increasing concentrations of GABA (as indicated). The resting membrane potential of this cell was −31 mV, resting input conductance 2.5 μS. Downward deflections are the electrotonic potentials resulting from the injection of 20 nA, 0.1 Hz, 500 ms pulse width.

before the addition of GABA and during the GABA application.

There was no evidence for a significant enhancement of the GABA response by either pentobarbitone (100 μM) or by flurazepam (100 μM; Figure 6). In one experiment the cell was exposed to 100 μM flurazepam for 60 min and the GABA response was not enhanced.

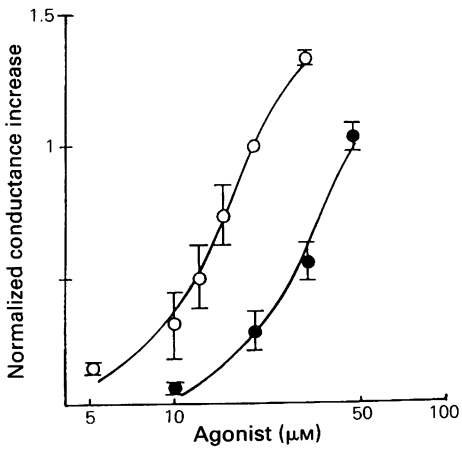
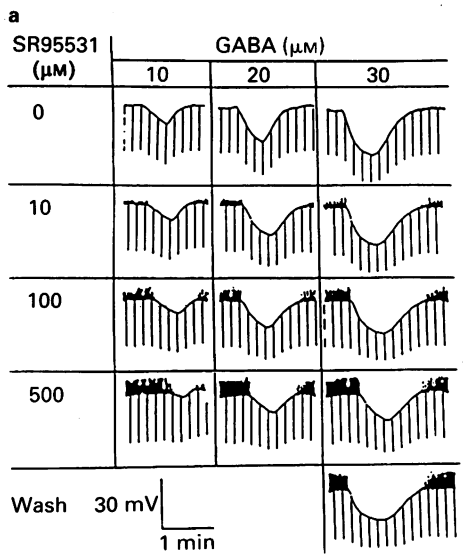


Figure 3 The increase in input conductance in *Ascaris* muscle cell evoked by bath applied GABA (○) and muscimol (●). The conductance increase was normalized with respect to the response obtained with 20 μM GABA ($n = 3$).

In a series of 5 experiments the relative potencies of the stereoisomers of the structural analogues of GABA compared to GABA were determined. All the compounds studied were GABAmimetic in that they elicited a membrane hyperpolarization and an increase in input conductance (Figure 7), though with varying degrees of potency (Table 1). The isomers of DHM and 3-OH-GABA were all apparently full agonists as shown by the fact that their dose-response curves are parallel to that for GABA (Figure 8). The isomers of 4-me-TACA may also be full agonists but their low potency meant that this could not be determined in this preparation. The degree of stereoselectivity for the compounds was most marked for the DHM compounds, with the (S)-(+)-isomer being nearly 10 times more potent than the (R)-(−)-isomer. (R)-(−)-3-OH-GABA was only 2 times more potent than the (S)-(+)-isomer of this compound; however, this difference in potency, though small was statistically significant ($P < 0.01$, $n = 5$, paired Student's t test) indicating that the receptor does truly distinguish between these compounds. (S)-(+)-DHM was by far the most potent compound at the GABA receptor in this preparation and is the most potent compound at this site that we have found to date. The (S)-(−)-isomer of 4-me-TACA was only weakly effective in eliciting a response, being approximately 20 times less potent than GABA. Threshold responses for the compound (R)-(+)-4-me-TACA were above 1 mM making



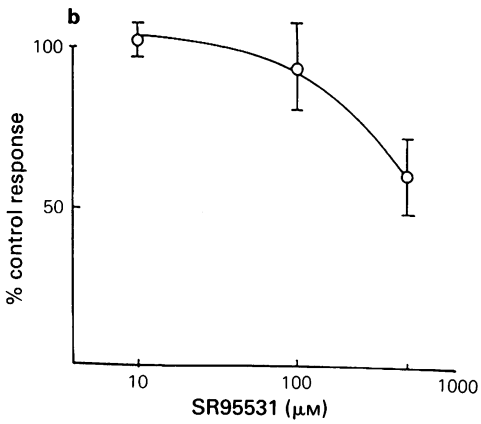


Figure 4 Weak antagonism of the GABA response by SR95531. (a) Typical responses to GABA (10, 20 and 30 μM) before and after the addition of SR95531. (SR95531 was applied by local perfusion for 2 min before the GABA application and with GABA). The downward deflections are elicited by injection of current (20 nA, 500 ms, 0.1 Hz) and allow determination of the input conductance. The resting membrane potential of this cell was −30 mV and the resting input conductance was 2.5 μS. Note that the GABA-induced increase in input conductance is only noticeably depressed at 500 μM SR95531. The wash was for 5 min and resulted in complete recovery of the conductance response to GABA. (b) The inhibition of the response to GABA by SR95531 in 3 separate preparations (with s.e.mean shown by vertical bars). The conductance increase to 30 μM GABA in the presence of SR95531 is expressed as a percentage of the conductance increase to 30 μM GABA in the absence of SR95531.

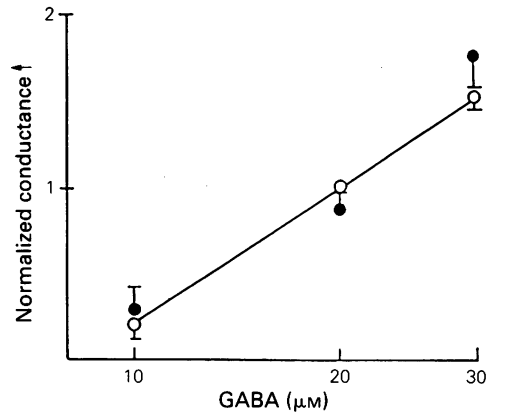


Figure 5 Inability of a non-competitive $GABA_A$-receptor antagonist to block the GABA response in *Ascaris*. Normalized conductance increase to GABA in the absence (○) and presence (●) of 100 μM dieldrin ($n = 3$, vertical bars show s.e.mean).

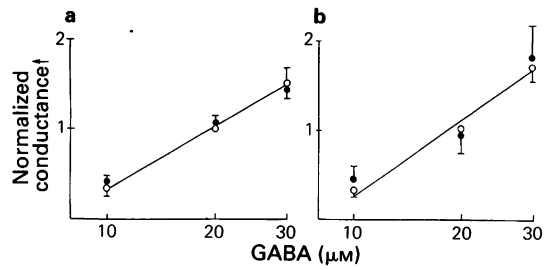


Figure 6 Lack of modulation of the GABA response by benzodiazepines and barbiturates. (a) Normalized conductance increase to GABA in the absence of (○) and after at least 10 min pre-exposure to (●) 100 μM pentobarbitone ($n = 3$, vertical bars show s.e.mean) (b) Normalized conductance increase to GABA in the absence (○) of and after at least 10 min pre-exposure to (●) 100 μM flurazepam ($n = 3$).

impossible an accurate determination of its relative potency compared to GABA.

Discussion and conclusions

The *Ascaris* muscle bag cells responded to locally applied GABA with an increase in membrane conductance accompanied by a hyperpolarization. This response was mimicked by the $GABA_A$-receptor agonist, muscimol. However, the receptor showed little similarity to the $GABA_A$-receptor in terms of blockade by competitive or noncompetitive antagonists, or in terms of modulation by benzodiazepines

Table 1 The potencies of structural analogues of γ-aminobutyric acid (GABA) at eliciting an increase in input conductance at the *Ascaris* GABA receptor were determined in 5 preparations: relative potencies of the compounds compared to GABA were determined from equipotent concentrations of the drug and GABA on parallel portions of the dose-response curve

Drug	*Relative potency*
(S)-(+)-dihydromuscimol	7.53 ± 0.98
GABA	1
(R)-(−)-dihydromuscimol	0.85 ± 0.08
(R)-(−)-3-OH-GABA	0.25 ± 0.02*
(S)-(+)-3-OH-GABA	0.13 ± 0.02
(S)-(−)-4-methyl-TACA	0.007 ± 0.001
(R)-(+)-4-methyl-TACA	No effect at 1 mM.

A relative potency of less than 1 indicates the compound is less potent than GABA (*$P < 0.01$ compared to (S)-(+)-3-OH-GABA). Each value is the mean ± s.e.mean, $n = 5$.

and barbiturates. We have previously reported a lack of sensitivity of the *Ascaris* GABA receptor to bicuculline and to other competitive $GABA_A$-receptor antagonists (Holden-Dye *et al.*, 1988). Recently it has been suggested that the pyridazinyl-GABA derivative, SR95103, is a competitive antagonist at the *Ascaris* GABA receptor (Duittoz & Martin, 1989). This substance belongs to a class of compounds that were developed as selective, competitive, potent $GABA_A$-receptor antagonists (Wermuth & Bizière, 1986). In binding studies it was demonstrated that SR95103 and SR95331, another pyridazinyl-GABA derivative, were approximately 20 and 200 times more potent than bicuculline, respectively (Wermuth & Bizière, 1986). Similar results were noted in mammalian electrophysiological studies (Desarmenien *et al.*, 1987). Thus, the lack of effect of SR95531, the most potent pyridazinyl derivative available, at the *Ascaris* GABA receptor unless high micromolar concentrations are used, indicates that this class of compounds does not recognise the *Ascaris* GABA receptor. TBPS and dieldrin are agents that are known to inhibit GABA-stimulated chloride flux in vertebrate preparations (Squires *et al.*, 1983; Gant *et al.*, 1987). Both are reasonably potent in these preparations with an effect occurring in the sub-micromolar range. Binding sites for TBPS, sensitive to dieldrin, have been identified in insect ganglia (Lunt *et al.*, 1988). These compounds are believed to act in a noncompetitive manner, exerting their blocking action at the channel site rather than at the receptor. In *Ascaris*, however, neither of these agents had a

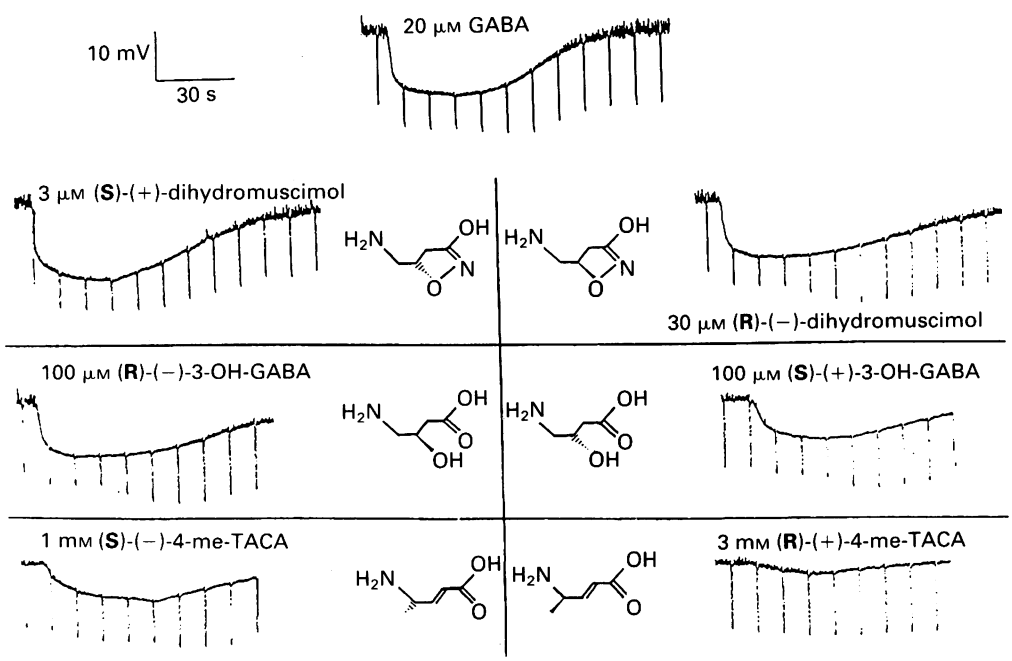


Figure 7 The GABA-mimetic activity of stereoisomers of structural analogues of GABA illustrating the structures of the compounds tested. The drugs were applied by local perfusion at the concentration indicated, for 30 s.

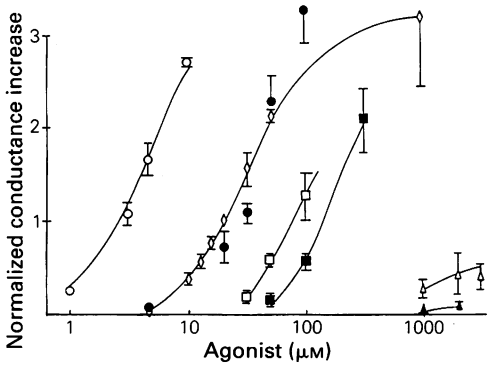


Figure 8 Dose-response relationships for stereoisomers of structural analogues of GABA. The conductance increases are normalized with respect to the response for 20 μM GABA. GABA (◇); (S)-(+)-DHM (○); (R)-(−)-DHM (●); (R)-(−)-3-OH-GABA (□); (S)-(+)-3-OH-GABA (■); (S)-(−)-4-me-TACA (△); (R)-(+)-4-me-TACA (▲). ($n = 3$–6, vertical bars show s.e.mean).

significant effect on the GABA response (Figure 5; Holden-Dye *et al.*, 1988). The tissue was pre-exposed to the compounds 10 min before the addition of GABA so it is unlikely that access was a problem. We could find no evidence that the *Ascaris* GABA receptor is coupled to either a benzodiazepine or a pentobarbitone modulatory site. In insects it has been found that 10 μM pentobarbitone or 10 μM flurazepam enhance the GABA response by up to 70% (Beadle *et al.*, 1986). Neither of these compounds had an effect in *Ascaris* at concentrations up to 100 μM (Figure 6). Thus, the GABA gated anion channel in *Ascaris* is pharmacologically distinct from that coupled to the mammalian $GABA_A$-receptor and also different from the GABA receptor in insect ganglia. The *Ascaris* GABA receptor is apparently novel in its lack of recognition of typical competitive and noncompetitive antagonists for $GABA_A$- receptors whilst being insensitive to baclofen and coupled to a chloride ionophore.

GABA, in common with a number of endogenous transmitter agents, is a flexible molecule able to assume a number of different conformations only one of which may be suitable for receptor activation (Krogsgaard-Larsen, 1988). The receptor in *Ascaris* is similar to other GABA receptors in preferring the extended conformation of GABA, corresponding to the compound trans-aminocrotonic acid (Holden-Dye *et al.*, 1988). The structural analogues of GABA that were tested in this study were GABA-mimetic and revealed further stereoselectivity at this GABA receptor. The degree of stereoselectivity seems to depend on the relative rigidity of the molecule. Thus, the most inflexible molecule, DHM, exhibits the greatest degree of stereoselectivity with the S-isomer being 10 times more potent than the R-isomer. There is only a slight degree of stereoselectivity between the enantiomers of 3-OH-GABA. Presumably, molecules such as 3-OH-GABA can overcome, to a certain extent, the steric hindrance imposed by the substitution of an unfavourable group by movement around the unfixed bonds. Both hydroxyl group substitution at the 3 position, and methyl group substitution at the 4 position decreased the efficacy of the compounds at the GABA receptor. The substitution of a methyl group seems to be particularly detrimental for GABA-mimetic activity at this site as 4-me-TACA was particularly weak at eliciting a response. The receptor, however, still distinguishes to a small extent between the two isomers of this compound. The stereoselectivity of the receptor for DHM and 4-me-TACA was the same as that found for the displacement of $GABA_A$-receptor binding in mammalian brain (Krogsgaard-Larsen *et al.*, 1988). The stereoselectivity for 3-OH-GABA, at the $GABA_A$-binding site (Krogsgaard-Larsen *et al.*, 1988) is the opposite of what we found in *Ascaris*. On spinal neurones, however, the (R)-(−)-enantiomer of 3-OH-GABA is marginally more potent than the (S)-(+)-enantiomer (Krogsgaard-Larsen *et al.*, 1985), corresponding exactly to the stereoselectivity that we observed at the *Ascaris* GABA receptor.

It has been established that the $GABA_A$-receptor not only has rigid requirements for the structural configuration of GABAergic compounds but also the degree of delocalization of both the positive and negative charges on the molecule is a crucial determinant of GABA-mimetic activity (Steward *et al.*, 1975). The most potent compound we have studied to date at the *Ascaris* muscle GABA receptor is DHM. This compound is also remarkably potent at gastropod neurones and at mammalian $GABA_A$-binding sites and neurones (Roberts *et al.*, 1981; Krogsgaard-Larsen *et al.*, 1985). The S-isomer of this compound is nearly 8 times more potent in *Ascaris* than GABA itself and on this basis we propose that it most accurately reflects the active structural and electronic configuration of GABA at the receptor. This molecule may provide a lead for the development of novel, competitive antagonists at the *Ascaris* GABA receptor if slight adjustments can be made to the molecule such that it retains its high affinity for the GABA receptor but its efficacy is decreased.

The results presented above clearly indicate that despite the lack of recognition of typical $GABA_A$-receptor antagonists by the receptor in *Ascaris* the receptor is sensitive to the $GABA_A$-agonist, muscimol. It has also previously been shown that the GABA-mimetic and anthel-

mintic agent piperazine acts as a weak GABA agonist in *Ascaris* (Martin, 1982). The sulphonic acid derivatives of GABA are without any effect in *Ascaris*. We have tested 3-aminopropane sulphonic acid and piperidine-4-sulphonic acid (P4S) both of which are without effect up to 1 mM (Holden-Dye *et al.*, 1988), despite the fact that these compounds have been shown to be extremely potent $GABA_A$-mimetic agents in electrophysiological studies in mammalian and amphibian preparations (Nistri & Constanti, 1979 for review). Sulphonic acid derivatives, however, are universally ineffective at GABA receptors in invertebrates and will not activate gastropod, arthropod or insect GABA receptors (see Introduction). In mammalian brain too, there is some circumstantial evidence to suggest that the way in which the receptor recognises the sulphonic acids and GABA differs. For example, the order of potency of compounds at displacing GABA binding is of the same relative order as that for displacing P4S binding, however the absolute potency is different by an order of magnitude (Krogsgaard-Larsen, 1981; Falch & Krogsgaard-Larsen, 1982). Furthermore, the sulphonic acids exhibit differences from GABA in the way in which they are coupled to the modulatory sites for barbiturates and benzodiazepines. P4S is a very weak stimulator of diazepam binding compared to GABA (Olsen, 1981; Olsen & Snowman, 1982; Krogsgaard-Larsen, 1988) and in contrast the binding of P4S is remarkably sensitive to stimulation by barbiturates (Olsen, 1981). On the basis of this evidence Krogsgaard-Larsen (1988) has suggested that sulphonic acid derivatives interact with the GABA receptor in a fundamentally different fashion from GABA itself.

The correlation between the ability of agents to stimulate the *Ascaris* GABA receptor and to displace $GABA_A$-receptor binding in mammalian brain is illustrated in Figure 9. Although we are comparing a system that measures affinity and efficacy with one that indicates the affinity of compounds only (binding) it can be seen that the relative order of potency of the compounds is roughly comparable in the two systems. Indeed, if we are justified in omitting the sulphonic acid derivatives from the calculations, on the basis of the evidence outlined above, then the two systems compare well with a significant ($P < 0.01$, positive ($r = 0.74$) correlation.

We conclude, therefore, that the GABA receptor on the somatic muscle cells of the parasitic nematode *Ascaris* is very similar to the mammalian $GABA_A$-receptor with regard to its activation by agonists. This would imply that the essential features of the receptor required for agonist recognition have been conserved through the course of evolution. The receptor does not recognise the typical $GABA_A$-competitive antagonists, however, indicating

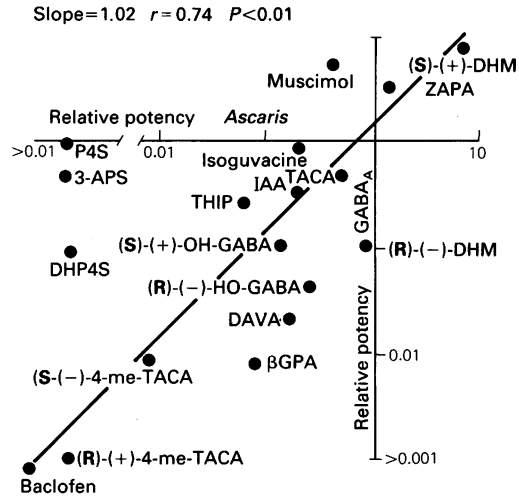


Figure 9 Correlation between the potency of compounds at displacing $GABA_A$-receptor binding (Krogsgaard-Larsen, 1988) or at GABA-mimetic activity in guinea-pig ileum [DAVA (Dickenson *et al.*, 1988), ZAPA (Allan *et al.*, 1986)] with their potency on *Ascaris* muscle cells (Holden-Dye & Walker, 1988; Holden-Dye *et al.*, 1988). The potency was determined as the relative potency compared to GABA in each system. The sulphonic acid derivatives were excluded from the calculation of the correlation coefficient; see text for details. (DHP4S, dihydropiperidine-sulphonic acid; 3-APS, 3-aminopropanesulphonic acid; IAA, imidazole acetic acid; DAVA, δ-aminovaleric acid; βGPA, β-guanidopropionic acid; ZAPA, (Z)-3-[amino-iminomethyl)thio]-2-propenoic acid hydrochloride).

that sites for antagonist binding at the receptor are different from those in the mammalian system. The chloride ionophore that mediates the GABA response in *Ascaris* must also be substantially different from the $GABA_A$-gated chloride channel in that none of the non-competitive antagonists for $GABA_A$-receptors was effective in *Ascaris*. Neither does the *Ascaris* GABA receptor exhibit the regulatory binding sites that are generally associated with the $GABA_A$-receptor. A similar receptor for GABA has recently been described in *Limulus* heart (Benson, 1988b). We have recently discovered that a diphenylamine-2-carboxylate derivative, 5-nitro-2-(3-phenylpropylamino)benzoic acid, which is known to block chloride transport in renal tubules (Wangemann *et al.*, 1986) is a noncompetitive antagonist at *Ascaris* GABA receptors (Colquhoun *et al.*, 1989). The possibility that there is a class of GABA receptor common to some invertebrate species with novel sites for antagonist recognition has important implications for pesticide and anthelmintic research.

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Properties of the cromakalim-induced potassium conductance in smooth muscle cells isolated from the rabbit portal vein

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1 Single smooth muscle cells were isolated freshly from the rabbit portal vein and membrane currents were recorded by the whole-cell or excised patch configurations of the patch-clamp technique at room temperature.

2 Cromakalim (Ckm, 10 μM) induced a potassium current (I_{Ckm}) that showed no pronounced voltage-dependence and had low current noise.

3 This current, I_{Ckm}, was inhibited by (in order of potency): phencyclidine > quinidine > 4-aminopyridine > tetraethylammonium ions (TEA). These drugs inhibited the delayed rectifier current, I_{dK}, which is activated by depolarization of the cell, with the same order of potency.

4 Large conductance calcium-activated potassium channels (L_{KCa}) in isolated membrane patches were blocked by (in order of potency) quinidine > TEA $\approx$ phencyclidine. 4-Aminopyridine was ineffective. A similar order of potency was found for block of spontaneous transient outward currents thought to represent bursts of openings of L_{KCa} channels.

5 The low current noise of I_{Ckm} at positive potentials, and its susceptibility to inhibitors indicated that it was not carried by L_{KCa} channels, and that it may be carried by channels which underlie I_{dK}. It was observed that when I_{Ckm} was activated, I_{dK} was reduced. However, in two experiments, I_{Ckm} was much more susceptible to glibenclamide than I_{dK}; possible reasons for this are discussed.

Introduction

Cromakalim (Ckm; BRL 34915) belongs to a group of drugs that appear to have a novel mechanism of action and are presently termed K^+-channel openers (Weston & Abbott, 1987; Cook, 1988). It is active on cardiac muscle (Osterrieder, 1988) and on neurones (Alzheimer & ten Bruggencate, 1988) but appears to be most potent on smooth muscle. Although Ckm has a number of potential clinical applications, emphasis has been placed on its possible use as a peripherally acting anti-hypertensive (Vandenburg *et al.*, 1986), where it seems to act directly on the vascular muscle, independently of the endothelium (Southerton *et al.*, 1987). The decrease in muscle tone has been proposed to result from a hyperpolarization caused by an increase in the K^+-conductance of the smooth muscle cell membrane (Hamilton *et al.*, 1986). However, other mechanisms may contribute to the action of Ckm, because the spontaneous electrical and phasic mechanical activity of smooth muscle (e.g. Weir & Weston, 1986b; Quast, 1987) can be inhibited without hyperpolarization occurring (see also Cook, 1988).

This study has investigated the mechanism underlying the hyperpolarization by evaluating the effects of Ckm on the membrane currents of single rabbit portal vein smooth muscle cells. Ckm was found to increase the membrane K^+-conductance. The voltage-dependence of this conductance was examined and the hypothesis assessed that Ckm acts to increase the open-time of a population of K^+-channels identifiable in these cells by other means. Preliminary findings have been communicated (Beech & Bolton, 1987a,b).

Methods

Cell dispersion

Adult, New Zealand White rabbits (2.5–3 kg) were killed by injection of a lethal dose of sodium pentobarbitone. The main branch of the portal-mesenteric

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vein was removed and dissected free of fat and connective tissue. It was cut into small pieces ($\sim 2 \times 3$ mm) and six were incubated in low-Ca^{2+} (20–30 μM) physiological salt solution (PSS) for 10 min (36°C) and then re-suspended in a mixture of papain (5 mg ml), DL-dithiothreitol (DTT, 3–5 mM) and bovine serum albumin (BSA, 2–4 mg ml^{-1}) in the low-Ca^{2+} solution for 20–30 min at 36°C. Tissue segments were then removed from the mixture and mildly agitated in the low-Ca^{2+} PSS. The isolated cells were centrifuged (100 g for 1.5 min), the pellet re-suspended in PSS containing 0.8 mM $CaCl_2$ and the suspension stored on glass cover-slips at 4°C. Cells were used between 2 and 12 h after isolation.

Current recording and analysis

Recordings were made by using the whole-cell or excised-patch configurations of the patch-clamp technique (Hamill *et al.*, 1981). Patch pipettes were made of borosilicate glass (external diameter 1.4–1.6 mm, internal diameter 0.6–0.8 mm) and had resistances of 1–4 MΩ (whole-cell recording; access resistance = 5–10 MΩ) or 5–10 MΩ (excised-patch recording). A List EPC-7 current-voltage converter was used. Data were recorded on FM-tape (Racal) and were either displayed on a chart recorder (Gould 2400S) or digitized off-line by means of a CED 1401 analogue-to-digital interface (Cambridge Electronic Design), stored on floppy diskettes and then displayed by a graph plotter (7470A Hewlett Packard). Digital records were analysed by a BBC microcomputer in conjunction with the CED 1401. All currents were low-pass filtered at 1 kHz with an 8-pole Bessel filter (Barr & Stroud; attenuation rate = 48 dB/octave). Functions were fitted by the iterative algorithm of Marquardt (1963), with the criterion of minimizing the unweighted sum of the squares of the deviations. Leakage current (whole-cell) was subtracted by an analogue circuit. Twenty mV hyperpolarizing steps (each 0.5 s) were applied 1 s after each depolarizing step and the inward current required was inverted, multiplied by the appropriate factor and subtracted from the current elicited by the depolarizing step.

Solutions and reagents

The PSS used in the bath had the following composition (mM): Na^+ 126, K^+ 6, Ca^{2+} 1.7, Mg^{2+} 1.2, Cl^- 137.8, glucose 14, HEPES 10.5 and was titrated to pH 7.2 with NaOH. When the ionic composition of this solution was altered, the osmolarity was maintained by adjusting the NaCl concentration. Recording pipette (=intracellular) solution had the following composition (mM): K^+ 133.5, Mg^{2+} 1.2, Cl^- 136, EGTA 0.8, glucose 14, HEPES 10.5 and was titrated to pH 7.2 with NaOH. The Ca^{2+} concentration of this solution was estimated to be 1 nM (when 10 nM Ca^{2+} was required 0.07 mM $CaCl_2$ was added). For some experiments GTP ($\sim$0.2 mM) and/or ATP (1 mM) were included in the pipette solution in an attempt to improve the responsiveness of the cells to Ckm. PSS containing 4-aminopyridine (4-AP, >1 mM) was alkaline and was titrated to pH 7.2 with HCl. PSS containing phencyclidine (100 μM) was acidic and was titrated with NaOH to pH 7.2. Experiments were performed at room temperature (20–23°C).

Papain (type 4), DL-dithiothreitol (DTT), bovine serum albumin (BSA), tetraethylammonium chloride (TEA), 4-aminopyridine (4-AP), quinine, procaine, phencyclidine hydrochloride, glibenclamide, guanosine 5'-triphosphate (GTP), adenosine 5'-triphosphate (ATP), ethylene glycol *bis*-(b-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) were all obtained from Sigma. Quinidine hydrochloride (BDH) and (±)-6-cyano-3,4-dihydro-2,2 dimethyl-trans-4-(2-oxo-1-pyrollidyl)-2H-benzo[b]pyrano-3-ol (cromakalim, Beecham) were also used. Charybdotoxin from *Leiurus quinquestriatus* venom (for the separation procedure see Castle & Strong, 1986) and apamin were generous gifts from Dr P.N. Strong.

Drugs were bath applied by an infusion-withdrawal system. The bath volume was $\sim$0.5 ml and exchange was judged to be complete within 15–20 s. All responses were assessed after complete equilibration of drugs.

Results

Patch-clamp recording in the 'current-clamp' mode enabled an estimation of the resting membrane potential of single cells which had known ionic gradients, to be made. For the example shown (Figure 1A), the ionic gradients were physiological (see Methods) and the potential fluctuated around −35 mV. Application of Ckm (10 μM) increased the potential to about −70 mV and this was maintained in the presence of Ckm. The response reached a maximum slowly, after about 4–5 min, and a decrease in voltage noise occurred concomitant with the hyperpolarization. Increasing the concentration beyond 10 μM had very little further effect; 1 μM Ckm produced only a small hyperpolarization (2–5 mV).

Voltage-clamp of single cells using low resistance pipettes permitted the recording of current across the cell membrane when the ionic gradients were known. Figure 1B shows current, relative to zero current (i.e.

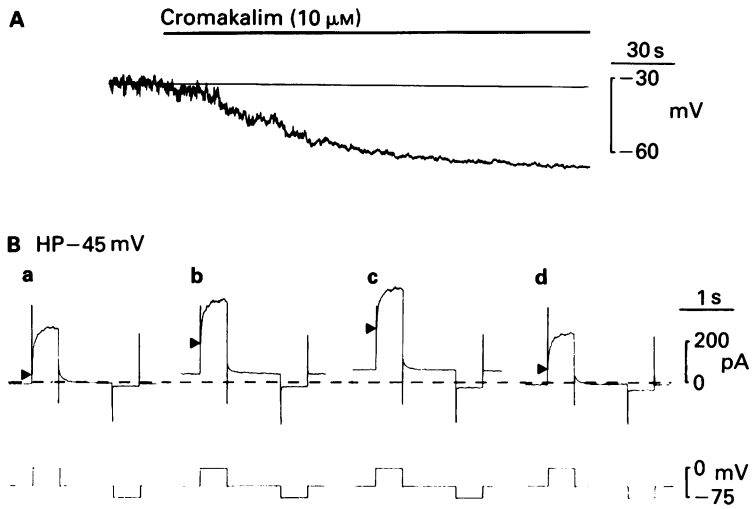

Figure 1 Effects of bath applied cromakalim (Ckm; 10 μM) on membrane potential and currents recorded from rabbit portal vein single smooth muscle cells bathed in physiological salt solution (PSS). (A) Pipette current was maintained constant and membrane potential recorded. Ckm increased the membrane potential by about 35 mV after 4 min. (B) Voltage-clamped cell, where the broken line represents zero holding current. The cell was held at −45 mV and stepped to 0 and −75 mV, each for 0.5 s every 5 s. Records show the control (a), 3 min (b) and 5 min (c) after the application of Ckm, and after repeated washing (d). The outward current induced by Ckm (I_{Ckm}) was associated with an increase in membrane conductance (g_{Ckm}). Quasi-instantaneous current is marked (▶).

when the holding potential (HP) was the resting membrane potential) recorded from a cell clamped at −45 mV and bathed in PSS. The step to 0 mV elicited a capacity current (outward), followed by a quasi-instantaneous current (the difference between the current at the holding potential and the current level to which the capacity current decays before voltage-gated currents develop) and then a time-dependent outward current. The step to −75 mV induced a capacity current (inward) followed by a time-independent inward current. Application of Ckm (10 μM) generated an outward current (I_{Ckm}) with respect to the control holding current which averaged 43.7 ± 5.3 pA (mean ± s.e.mean, $n = 12$; HP −40 mV, K^+-equilibrium potential (E_K) at −78 mV). I_{Ckm} was always associated with an increase in the quasi-instantaneous current, indicating an increased membrane conductance (g_{Ckm}). The fact that the current was outward and was carried by K^+ (see below) suggested that the effect was not due to a decrease in the seal resistance. Figure 1B shows that the current required to clamp the cell at −75 mV was similar for both the control and after equilibration with Ckm, suggesting that I_{Ckm} reversed near this potential, that is, near E_K (at −78 mV). The effect of Ckm on the quasi-instantaneous current reversed slowly after extensive washing (Figure 1Bd).

Reversal potential of I_{Ckm}

To determine the ion selectivity of g_{Ckm} the potential at which I_{Ckm} reversed was estimated. Cells were equilibrated for 10 min after establishing the whole-cell configuration in an attempt to minimize the effect of any slowly shifting junction potential. To find the reversal potential the currents required to step the cell for 100 ms to various potentials more negative inside than the holding potential were measured and plotted against voltage both in the absence of Ckm and after I_{Ckm} had developed fully (see Figure 2a). The voltage at which the two lines intersected gave the reversal potential for I_{Ckm}.

For the cell illustrated in Figure 2a, E_K was at −58 mV and I_{Ckm} reversed close to this at −59 mV (−55 ± 2 mV, $n = 3$). For the cell illustrated in Figure 2b, E_K was at −78 mV and for the plot the control current was subtracted so that only I_{Ckm} is shown. At a holding potential of −50 mV, I_{Ckm} reversed close to E_K at −77 mV (−73 ± 3 mV, $n = 5$), and at a holding potential of +30 mV (1 min was allowed for inactivation of voltage-gated channels: see below) the reversal potential was similar. These results suggested that g_{Ckm} was K^+-selective. The small discrepancy between the mean reversal potential for I_{Ckm} and E_K can probably be attributed to a junction potential, especially since spontaneous

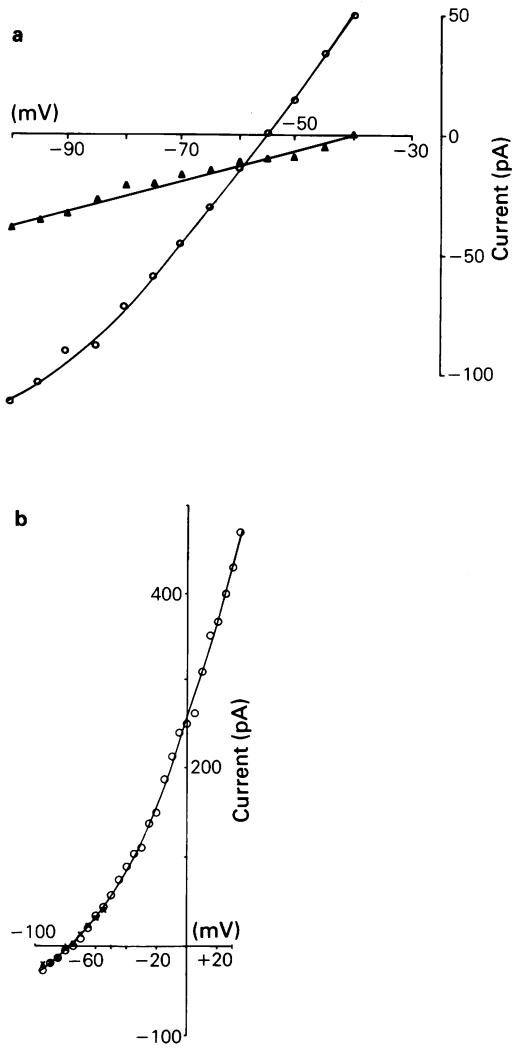

Figure 2 Measurement of the reversal potential for the cromakalim (Ckm)-induced potassium current (I_{Ckm}). Abscissae: transmembrane potential (mV). Ordinate scale: inward (−) and outward (+) transmembrane current. (a) The K^+ equilibrium potential (E_K) was at −58 mV (bath K^+ concentration = 13 mM) and the cell was held at −40 mV. The points represent the currents required to hyperpolarize the cell from the holding potential plotted relative to zero current; control (▲), 10 μM Ckm (○). (b) I_{Ckm} measured in two experiments on one cell which was bathed in PSS (E_K at −78 mV). Two holding potentials were used; −50 mV (×) and +30 mV (○) and each was established for 1 min before the hyperpolarizing steps were applied (method as for a). Current in the absence of Ckm was subtracted from current with 10 μM Ckm present and the line was drawn by eye. Positive current is outward, negative current is inward according to the usual convention.

transient outward currents (see below) were found to reverse at the same potential as I_{Ckm} (Beech, 1988).

The relationship between current and potential (Figure 2b) was represented by a shallow curve indicating that g_{Ckm} showed little voltage-dependence. The curved nature of the voltage/current relationship may reflect the equivalent relationship for the single channel currents underlying the response, as mild outward rectification is commonly observed for single K^+-channels recorded from the excised patch when exposed to an asymmetrical K^+-gradient (e.g. see Benham *et al.*, 1986).

Effects of K^+ channel inhibitors on I_{Ckm}

The effects of inhibitors on I_{Ckm} were determined by applying increasing concentrations of each inhibitor once I_{Ckm} had been established. Inhibition was measured as the change in holding current relative to the control level and to I_{Ckm} in the absence of inhibitor. For these experiments, cells were held at −40 mV to establish the largest driving force without activating I_{dK} (the delayed outward rectifier K^+-current of these cells: see below). Changes in the control holding current induced by the inhibitors were assessed in separate cells ($n = 3$–4): small inward shifts were found for quinidine, 4-AP and TEA and points shown in Figure 7a have been corrected for these effects.

The inhibitory effects of phencyclidine, quinidine, 4-AP and TEA on I_{Ckm} are illustrated in Figures 3 and 7a. Phencyclidine ($n = 3$) blocked I_{Ckm} completely at 100 μM, 50% block occurred at about 4 μM and the effect was fully reversible after wash-out (Figure 3a). Quinidine ($n = 3$) induced complete block at 100 μM and 50% block at about 10 μM (Figure 3b). 4-AP ($n = 4$) at 0.2 mM caused about 50% block but higher concentrations produced further block in some cells, whilst increasing I_{Ckm} in others (Figure 3c). TEA ($n = 5$) produced complete block at 40 mM and 50% block at about 7 mM (Figure 3d). I_{Ckm} was inhibited by quinine (0.1–0.4 mM, $n = 1$) or procaine (5 mM, $n = 2$), but was unaffected by charybdotoxin (100 nM, $n = 3$). The experiments indicated that the inhibitors had the following order of potency against I_{Ckm}; phencyclidine > quinidine = (quinine) > 4-AP > (procaine) > TEA (parentheses indicate when the concentration for 50% block was not determined).

Effects of inhibitors on large conductance Ca^{2+}-activated K^+ (L_{KCa}) channels

L_{KCa} channels are of large unitary conductance and have been characterized for a number of smooth muscle types (Inoue *et al.*, 1985; Benham *et al.*, 1986). In this study, unitary currents were recorded

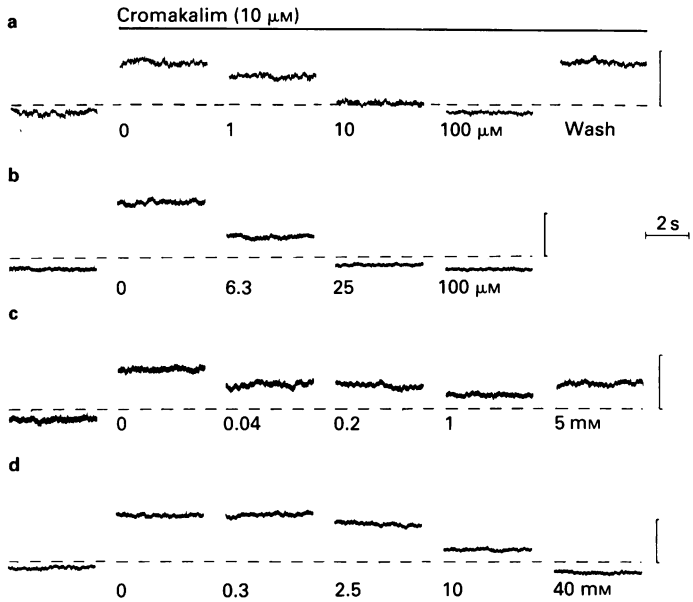

Figure 3 The effects of phencyclidine (a), quinidine (b), 4-aminopyridine (c) or tetraethylammonium (d) on holding current (HP −40 mV) in the presence of 10 μM cromakalim (Ckm). The broken lines represent zero holding current, the records on the left indicate the control current levels and all others are in the presence of 10 μM Ckm. The calibration bars represent 50 pA and upward deflection outward current.

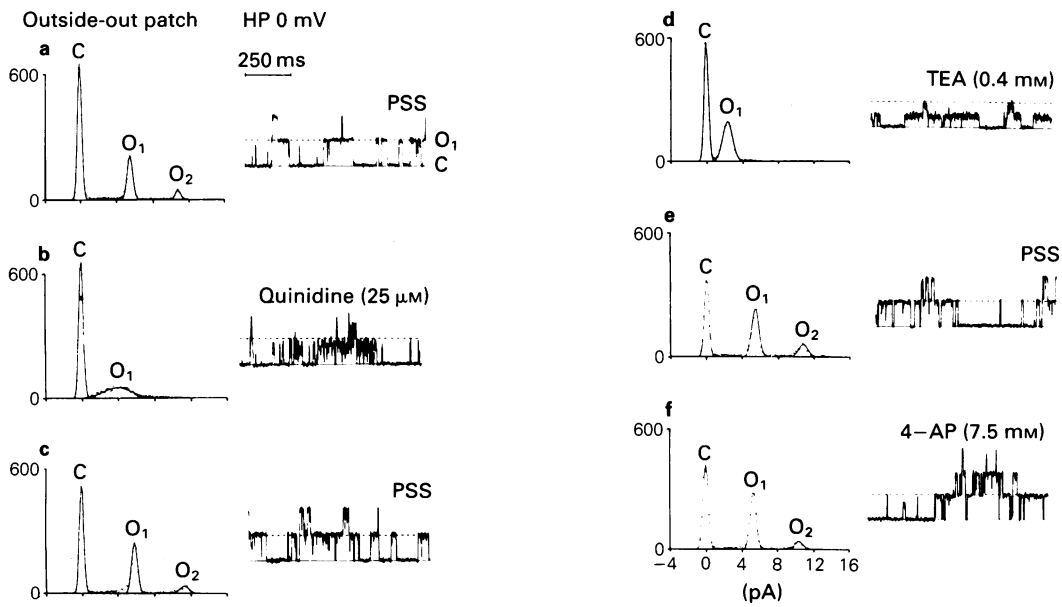

Figure 4 Effects of bath applied quinidine (b), tetraethylammonium (TEA, d) or 4-aminopyridine (4-AP, f) on large conductance Ca^{2+}-activated K^+ (L_{KCa}) channels in outside-out membrane patches exposed to a physiological K^+ gradient. The plots on the left show current amplitude histograms for 4 s of record (~4000 points) fitted by Gaussian functions. Signals were captured at 1 kHz and the bin size was 0.16 pA. Shown on the right are actual records (1 s) for the same experiments, where the continuous line indicates the current level when no channel openings were observed (C) and the broken line the first unitary current level (O_1) in the absence of any drug. PSS indicates the absence/wash-out of drug.

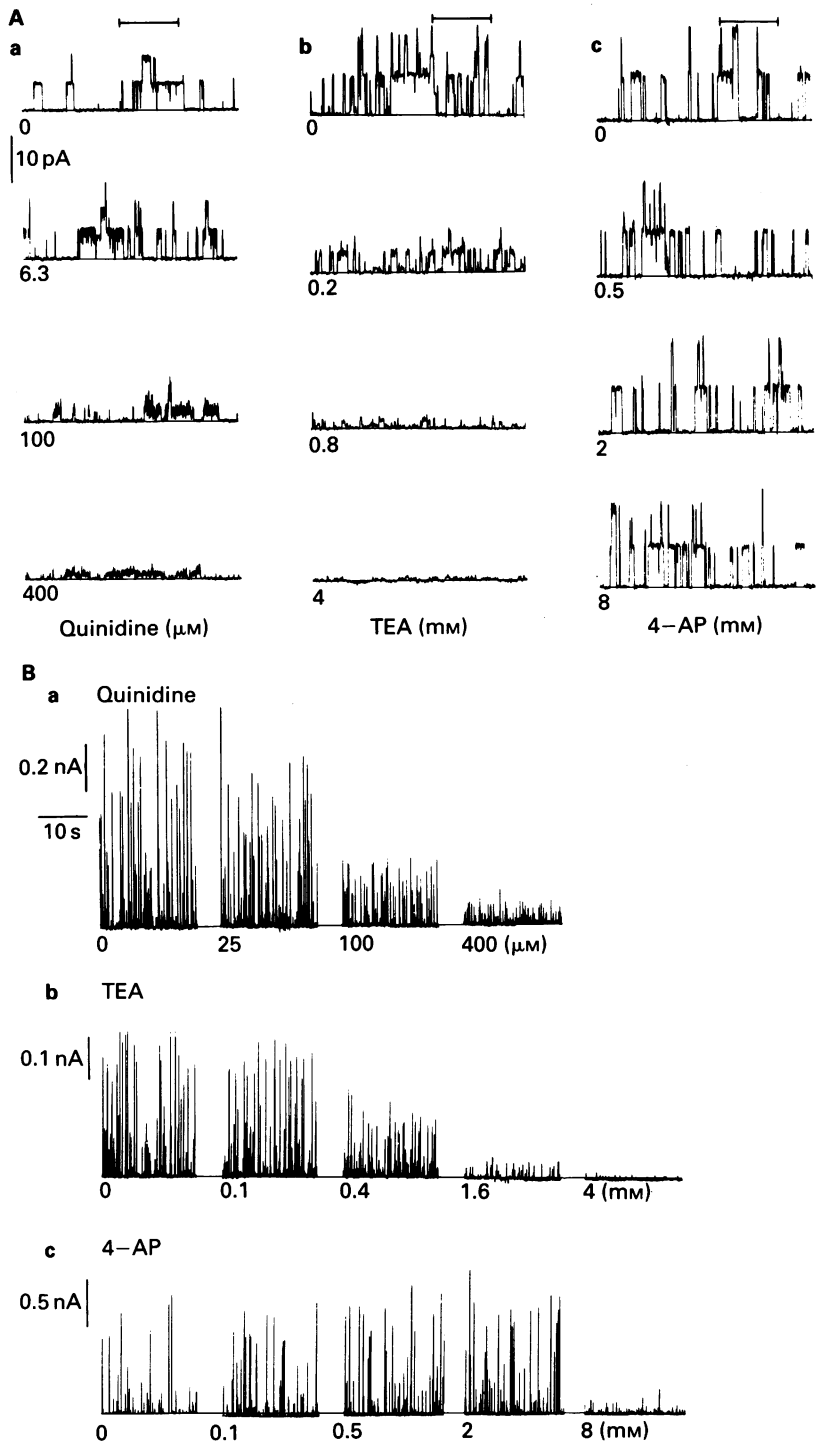

Figure 5 Effects of quinidine (a), tetraethylammonium (TEA, b) or 4-aminopyridine (4-AP, c) on large conductance Ca^{2+}-activated K^+ (L_{Kca}) channels (outside-out patch) and on spontaneous transient outward currents (STOCs) (whole-cell). (A) Unitary currents through L_{KCa} channels (physiological K^+-gradient). Upward deflection indicates outward current and continuous lines mark the current level when no channels were open. The Ca^{2+}-concentration in the pipette was 1 nM (a,c) or 10 nM (b), patches were held at 0 mV (a), +20 mV (b) and +35 mV (c), and the time calibration bars represent 500 ms (a,b) and 125 ms (c). (B) STOCs recorded from single cells bathed in PSS; holding potentials were −10 mV (a,c) and −35 mV (b). Quinidine (a) or TEA (b) each reduced STOCs in a concentration-dependent manner, whilst 4-AP (c) had no effect at concentrations up to 2 mM but caused a marked reduction in STOC amplitude at 8 mM.

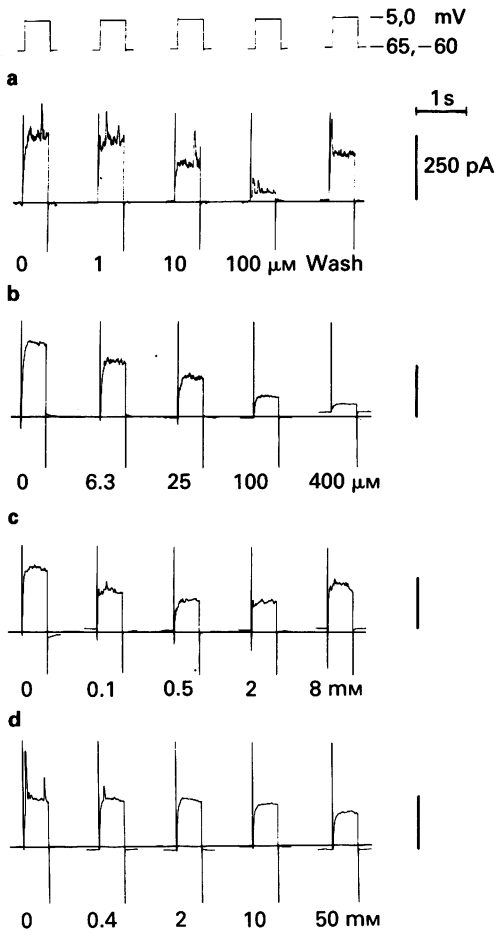

Figure 6 The effects of phencyclidine (a), quinidine (b), 4-aminopyridine (4-AP) (c) or tetraethylammonium (TEA) (d) on whole-cell current (I_{dK}) activated upon depolarization to 0 mV (a; HP −60 mV) or −5 mV (b,c, d; HP −65 mV) for cells bathed in PSS. The steps were of 0.5 s duration and all vertical calibration bars represent 250 pA. Scaled leakage current was subtracted. Control currents (0) are shown on the left, upward deflection indicates outward current and continuous lines mark the control current at the holding potential. The time-dependent outward current measured at the end of the depolarizing step was taken to represent I_{dK}.

through these channels in the excised outside-out patch. As described by Inoue *et al.* (1985), the channels have a conductance of about 100 pS at 0 mV when exposed to a physiological K^+-gradient, are activated upon depolarization and have an opening probability dependent on the internal Ca^{2+}-concentration. It was necessary to buffer Ca^{2+} to < 10 nM to ensure a low probability of opening, for it was common to observe many active L_{KCa} channels (> 10) in each patch. Patches were clamped between −20 and +35 mV so that only 2 to 3 unitary current levels were observed. The effects of K^+ channel inhibitors were assessed by measuring the decrease in unitary current with increasing concentration of inhibitor bath-applied to the outside of the membrane, on the assumption that open-channel block (see Hille, 1984) was the major effect.

Figure 4 shows an assessment of the effects of quinidine (b), TEA (d) or 4-AP (f) on L_{KCa} channels in the outside-out patch. Current amplitude histograms were constructed and each peak was fitted by a Gaussian (Normal) function of the form;

$$f = f_{max}\{\exp((i - a)^2/2\sigma^2)\},$$

where f is the number of points in each bin (the frequency), i the current amplitude, a the mean of the curve and σ^2 the variance about the mean. Quinidine or TEA, but not 4-AP produced a leftward shift of the peak(s) for the open state(s), indicating a decrease in unitary current, and increased σ^2, which describes the noise in the open state. No change in the opening probability of the channels in the presence of any of the inhibitors was indicated. This suggested that the block by TEA or quinidine could be described by a decrease in the mean unitary current and that 4-AP neither decreased the unitary current nor modified the gating of these channels.

Quinidine ($n = 3$) and TEA ($n = 3$–4) each reduced the unitary current in a concentration-dependent manner (Figure 5A), 50% reduction occurring at about 90 and 300 μM respectively (Figure 7b). 4-AP ($n = 3$) (up to 8 mM) bath-applied to the outside-out (Figures 4 and 5Ac) or inside-out patch had no effect when applied to either side of the membrane. Phencyclidine (≤ 10 μM) in one experiment had little effect on the channels but induced some open channel block at 100 μM.

Effects of inhibitors on spontaneous transient outward currents

Spontaneous transient outward currents (STOCs) are thought to result from the opening of K^+ channels activated by a spontaneous, cyclical release of Ca^{2+} from stores within the cell (Benham & Bolton, 1986). To test the postulate that the channels opened were L_{KCa} channels, the effects of K^+ channel inhibitors on STOCs were assessed to enable a comparison to be made with the single L_{KCa} channel data. Effects of STOCs were evaluated by measuring the sum of their amplitudes over a 1 min period. Quinidine ($n = 3$) and TEA ($n = 13$) caused a 50% reduction at about 80 μM and 0.5 mM respectively

(Figure 5Ba,b), but phencyclidine (up to 100 μM) had little effect in 3 experiments (see Figure 6a) suggesting that STOCs were carried by L_{KCa} channels. Complete block of STOCs by 4 mM TEA indicated that the small conductance Ca^{2+}-activated K^+ channels described by Inoue *et al.* (1985) play little role in the generation of STOCs. 4-AP ($n = 4$) at a high concentration (≥ 5 mM) tended to inhibit the STOCs of some cells (Figure 5Bc). However, this seemed to result from an effect on the Ca^{2+}-stores and not from a direct effect on the L_{KCa} channels (Beech, 1988).

Effects of inhibitors on current carried by delayed rectifier K^+ channels (I_{dK})

When the EGTA concentration in the pipette was ≥ 0.8 mM the time- and voltage-dependent outward current (I_{dK}) elicited upon stepping cells to potentials negative of 0 mV has been shown to be carried principally by delayed rectifier-like K^+-channels, which are of small conductance (Beech & Bolton, 1989b). Preliminary experiments (Beech & Bolton, 1987a) indicated that these channels have a pharmacological profile unlike that of the L_{KCa} channels. Experiments were performed in PSS, so both Ca^{2+}- and K^+-currents were expected to be activated upon depolarization. However, the net current was principally I_{dK} at the end of the 0.5 s test pulse, since the Ca^{2+}-current is almost completely inactivated at this time (Ohya *et al.*, 1988). ATP was not included in the pipette solution as it accentuates the slow Ca^{2+}-current component of smooth muscle cells (Ohya *et al.*, 1987). Cl^--current did not interfere since currents were assessed with a test potential near 0 mV, the Cl^--equilibrium potential. The current measured at the end of the 0.5 s pulse was, therefore, taken to represent I_{dK}.

Phencyclidine ($n = 3$) reduced I_{dK} with almost complete block at 100 μM, 50% block at about 30 μM and recovery on wash-out (Figure 6a and Figure 7c). Quinidine ($n = 3$) also reduced I_{dK} with almost complete block at 400 μM (Figure 6b) and 50% inhibition at about 30 μM. TEA ($n = 4$) at 50 mM produced only a 40% reduction of I_{dK} (Figure 6d). In three cells studied 4-AP reduced I_{dK} at concentrations up to 2 mM, inducing 50% block at about 0.2 mM (Figures 6c and 7c). Figure 6c shows an increase in transient net inward current in the presence of 4-AP, but this probably reflects a net effect due to reduced outward current. However, high concentrations of 4-AP (≥ 5 mM) tended to augment both outward and inward currents. Charybdotoxin (100 nM) (Beech & Bolton, 1989b) or apamin (100 nM) had no effect on I_{dK}. Thus, I_{dK} and I_{Ckm} showed the same rank order of susceptibility to the four inhibitors, phencyclidine

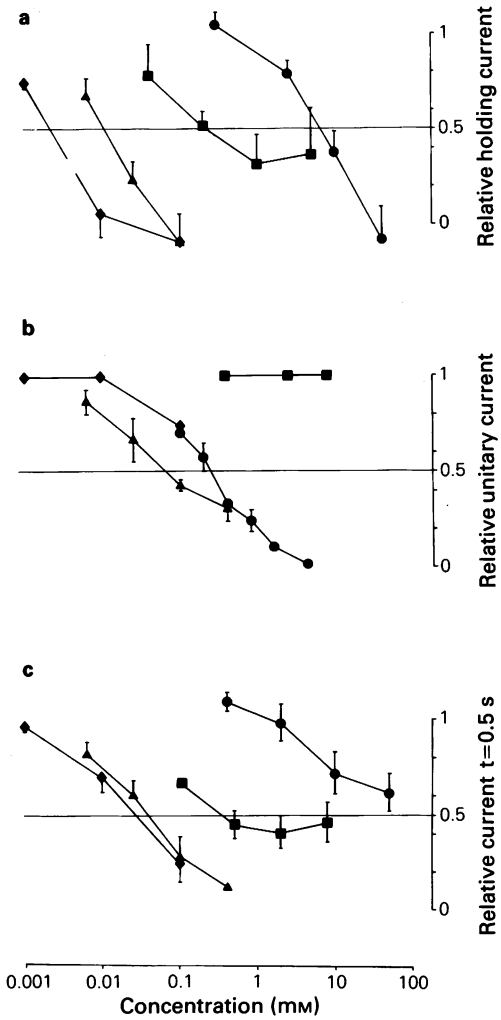

Figure 7 Pharmacological profiles for whole-cell cromakalim-induced potassium current (I_{Ckm}, a), the large conductance Ca^{2+}-activated K^+ (L_{KCa}) single channel current (b) and for whole-cell delayed rectifier current (I_{dK}, c). The points represent the means of 3 to 5 experiments where standard error bars are drawn. The inhibitors: phencyclidine (◆), quinidine (▲), 4-aminopyridine (■) or tetraethylammonium (●) were bath applied and their concentration plotted on a $\log_{10}$ scale. (a) I_{Ckm}, the ordinate scale represents the holding current, where 1 is I_{Ckm} and 0 the current level before the application of Ckm. (b) L_{KCa} single channel currents. The ordinate scale represents unitary current where 1 is the current through the fully open channel and 0 that through the fully closed channel without drug. (c) I_{dK}, the ordinate scale represents scaled leakage subtracted outward current elicited upon stepping to 0 mV, where 1 is the current before drug application and 0 the current at the holding potential (−60 or −65 mV).

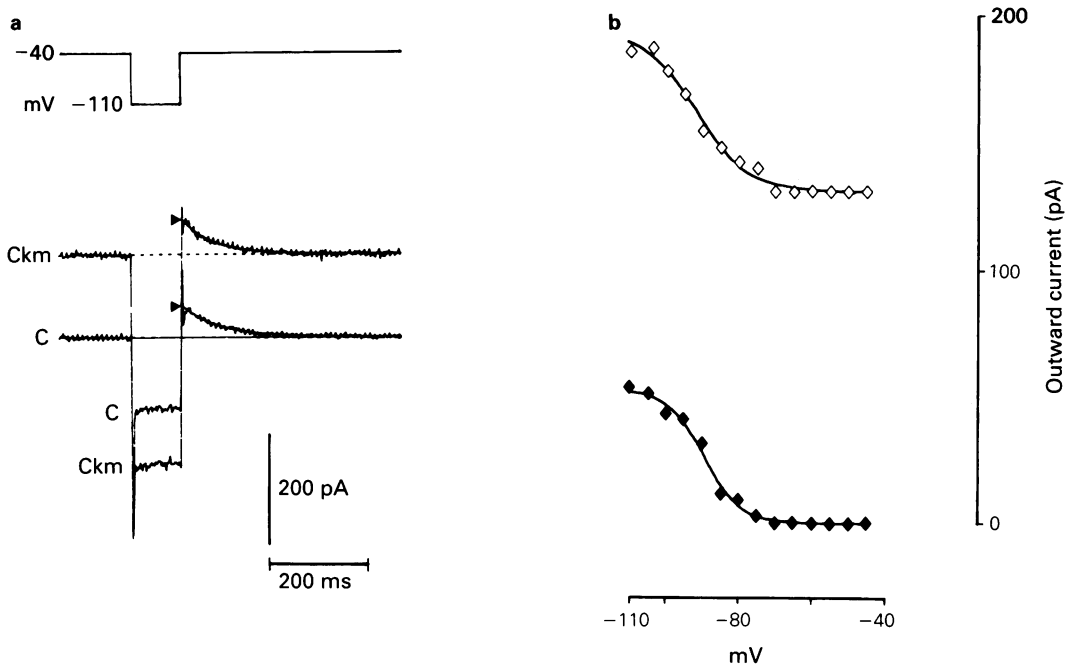

Figure 8 The lack of effect of 10 μM cromakalim (Ckm) on the transient voltage-dependent outward current (I_{fo}). (a) Current recorded from a cell bathed in PSS, showing the effect of a 100 ms step to −110 mV from the HP of −40 mV; for the control (C) and during the maximum response to Ckm. Records are digital (2 KHz). The horizontal lines (solid for C, broken for Ckm) mark the current level for the −40 mV HP before the step was applied (zero current in b is measured from the solid line). The distance between each of these lines and the appropriate, arrowed current level gives the amplitude of I_{fo} at time zero for the step back to −40 mV. The time-dependent decay of I_{fo} was fitted by a single exponential (shown). (b) The same cell, the inactivation curve for I_{fo} was plotted for 100 ms hyperpolarizing steps from −40 mV; for the control (C; ◆) and in the presence of Ckm (◇). Points were fitted by a Boltzmann equation (see text), indicating that I_{fo} was 50% inactivated at −87.3 mV for the control and at −92 mV in the presence of Ckm.

> quinidine > 4-AP > TEA, and neither were susceptible to block by apamin or charybdotoxin at concentrations which block other potassium currents.

Lack of effect of cromakalim on the transient, voltage-dependent outward current

The transient voltage-dependent outward current (I_{fo}; Beech, 1988; Beech & Bolton, 1988, 1989a) is an outward current, carried principally by K^+, that has fast kinetics and is activated upon depolarization from very negative HPs, usually negative to −50 mV. It closely resembles the A-current of neuronal cells (reviewed by Rogawski, 1985), which is thought to regulate spike frequency. The effect of Ckm on I_{fo} was investigated because preliminary experiments have suggested that I_{fo} shows a similar susceptibility to K^+-channel inhibitors as does I_{dK} (and I_{Ckm}). I_{fo} was blocked by bath applied phencyclidine or 4-AP but not by TEA, apamin (100 nM) or charybdotoxin (100 nM) (Beech & Bolton, 1989a). In addition, if Ckm changed the voltage-dependence of I_{fo} this might change spike frequency in the whole smooth muscle tissue, perhaps explaining why Ckm can sometimes inhibit spontaneous spike activity at concentrations that produce no appreciable hyperpolarization (see Cook, 1988).

Ckm (10 μM) in 3 experiments had little effect on the amplitude, kinetics or inactivation curve of I_{fo} (Figure 8). In addition, I_{Ckm} was 2.5 × larger than I_{fo} at −40 mV. Thus, although I_{fo} was probably not maximally activated upon stepping to −40 mV it was reasonable to presume that if I_{Ckm} was carried by the same channels then a reduction in the amplitude of I_{fo} would occur, but in fact it was slightly increased. It should be noted that the inactivation

curve for I_{f_0} is not in its steady-state position on the voltage axis as the hyperpolarizing steps used were short (100 ms). When the channels were given sufficient time for steady-state to be achieved at each potential the current was 50% available at about −78 mV (Beech & Bolton, 1989a).

Effect of cromakalim on I_{dK}

Because I_{dK} and I_{Ckm} had a similar susceptibility to a number of blocking agents, it seemed possible that Ckm may act on I_{dK} or a component of it. A depolarizing pulse from a negative holding potential caused activation of I_{dK} which increased to a peak and then declined; if the pulse was sufficiently long (several seconds) I_{dK} was almost completely inactivated. Holding for 20 s at 0 mV for example inactivated I_{dK} almost completely leaving only 150 pA of non-inactivating current as shown in Figure 9b (●) where the points were fitted by a curve described by a Boltzmann equation.

$$I = (I_{max} - I_{min})/\{1 + \exp((V - V_h)/V_s)\} + I_{min},$$

where V_h is the potential at which half of the channels were available for activation, V_s the slope factor, I the current available and V the conditioning voltage. Examples of actual records from two conditioning potentials are shown in Figure 9a. Ckm did not induce any appreciable shift in the threshold for activation of I_{dK}.

The application of cromakalim produced outward current at the conditioning potential if this was positive to E_K (Figure 9a, the −90 mV conditioning potential is close to E_K and little I_{Ckm} is apparent). Stepping to the test potential (+40 mV) in the presence of cromakalim produced a peak current which was a little larger than in its absence if the conditioning potential was −90 mV (Figure 9a and b), but if the conditioning potential was 0 mV then peak outward current was substantially increased (by about 150 pA in Figure 9a,b). Measurements were made at the peak of the outward current shortly after the beginning of the test pulse (actually the outward current record is almost flat during the test pulse upon stepping from 0 mV in Figure 9a). At the end of the test pulse (4 s) cromakalim also increased outward current more if the conditioning potential was 0 mV rather than −90 mV (Figure 9a), although the effect of varying the conditioning potential was less (Figure 9b). The increase in quasi-instantaneous current produced by Ckm was similar in size to the effect of Ckm on outward current, measured either at peak or the end of the pulse, if I_{dK} was inactivated by holding for 20 s at 0 mV before the test pulse (Figure 9). These results show that when Ckm activated outward current, I_{dK} was reduced and that when I_{dK}

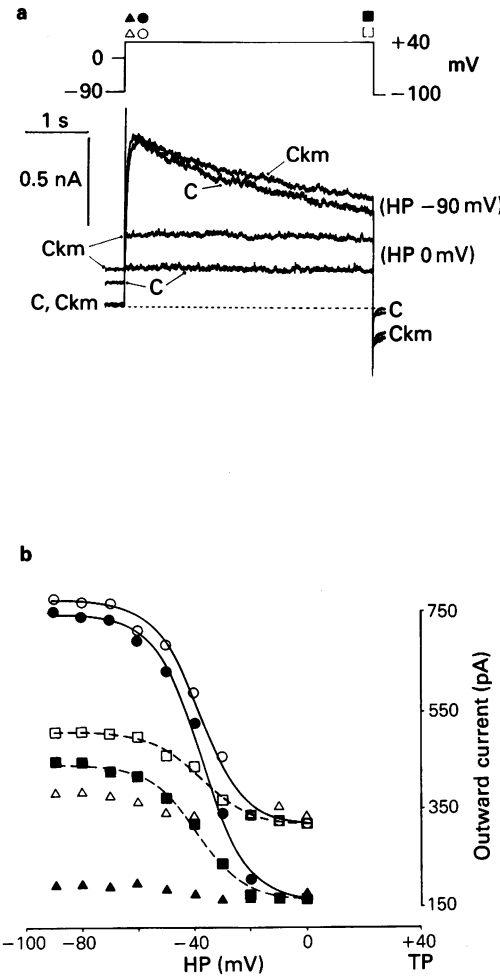

Figure 9 The effect of cromakalim (Ckm, 10 μM) on the whole cell delayed rectifier current (I_{dK}). Currents were recorded from cells bathed in PSS. (a) Effects of two different 20 s conditioning voltage steps (from −100 mV to −90 mV and from −100 mV to 0 mV) on current elicited upon stepping to +40 mV for 4 s, for the control (C) and in the presence of Ckm. The illustrated traces commence shortly before the step to +40 mV. The broken line represents the current level at the −100 mV HP (current above this line being referred to as outward, see b). Leakage and capacity currents were not subtracted. Records are digital (0.7 kHz). (b) The same experiment (HP −100 mV), outward current at +40 mV (see above) plotted against conditioning voltage. Quasi-instantaneous current at time ≈ 3 ms (control, ▲; Ckm, △), peak current (control, ●; Ckm, ○) and that after 4 s (control, ■; Ckm, □) are shown (see a). Points were fitted by the Boltzmann equation (see text). V_h values were −37 and −38 mV, and V_s 8.1 and 8.3 mV for peak current, before and in the presence of Ckm, respectively.

was inactivated by holding at 0 mV, Ckm had a larger effect. This type of result would be expected if Ckm and depolarization acted on the same population of K-channels. Thus, if Ckm has already opened a proportion of these channels, a smaller proportion will be available to be opened by a depolarizing step.

Figure 9a is of additional interest as steps to +40 mV have been shown to activate L_{KCa} channels (Beech & Bolton, 1989b). These channels do not inactivate and they contribute the major noise component because their unitary conductance is large. The unitary current amplitude (i) and probability of opening (p) of a channel population are related to the mean macroscopic current (I) and its variance (σ^2) by the relationship, $\sigma^2 = i(1 - p)I$ (see Hille, 1984). Thus if the probability of opening is low the effect of increasing i is to increase noise (σ^2). However, for the experiment shown (Figure 9), Ckm decreased the variance about a single exponential fitted to the current decay at +40 mV from about 4.5× that about the mean at −90 mV to about 4×. This suggested that Ckm had not increased the opening probability of L_{KCa} channels in this experiment. It is unlikely that Ckm blocks the channels underlying I_{dK} because the total outward current elicited upon depolarization (i.e. including leakage current) was never observed to be less in the presence of Ckm (10–100 μM) than it was in the control.

Discussion

These results suggest that cromakalim induced opening of a population of channels, which allowed a potassium current to flow in these smooth muscle cells; normally this would cause hyperpolarization of the cells. The current, I_{Ckm}, showed no pronounced voltage-dependence and was highly selective for K^+. The sensitivity of this current to various inhibitors was very similar to that of the Ckm-induced $^{86}Rb^+$/$^{42}K^+$-efflux (Quast, 1987; Coldwell & Howlett, 1987; Weir & Strong, 1988; Quast & Cook, 1988) or relaxation (Allen *et al.*, 1986; Wilson, 1987) demonstrated in smooth muscle tissues, suggesting that I_{Ckm} and Ckm-induced $^{86}Rb/^{42}K$ efflux and relaxation have a common underlying mechanism.

Several types of K^+-channel have been proposed to exist in smooth muscle but certain of these channels do not appear to be involved in the generation of I_{Ckm}. Firstly, it seems reasonable to exclude an apamin-sensitive K^+-channel in the action of Ckm (Weir & Weston, 1986a; Allen *et al.*, 1986; Coldwell & Howlett, 1987). Secondly, as phencyclidine did not affect the resting K^+-conductance in the present experiments, the K^+-channel type which underlies this would seem not to generate I_{Ckm}. The channels underlying the M-current (Sims *et al.*, 1985) are unlikely to be involved as they are not inhibited by 4-AP (Brown & Adams, 1980). The single voltage-independent K^+-channel (K_M) described by Inoue *et al.* (1986) was shown to close if extracellular Ca^{2+} was reduced to <10 μM, an effect opposite to that obtained for the Ckm-induced $^{86}Rb^+$-efflux (Howlett & Coldwell, 1987). Although there are insufficient data available to assess the possible role of the slow, potential sensitive K^+-channels (Benham & Bolton, 1983) or the small conductance Ca^{2+}-activated K^+ channels (Inoue *et al.*, 1985) of smooth muscle cells, preliminary experiments with excised inside- or outside-out patches have not revealed such channels to be opened by Ckm (unpublished observation, Beech & Bolton). Ckm was found not to affect I_{fo}, which is a transient and rapidly inactivating potassium current available only at strongly negative potentials (Figure 8).

A number of studies have indicated that Ckm increases the open-time of the large conductance of Ca^{2+}-activated K^+-channels (L_{KCa}): Kusano *et al.* (1987) observed an increase in open-time for on-cell patch recordings; Gelband *et al.* (1988) a decrease in the time constant of the long closed state for Ckm (0.05 μM) applied to the outside of the membrane; and Trieschmann *et al.* (1988) an increase in opening probability resulting from a decrease in the closed time for Ckm (1 μM) applied to the inside of the membrane. The present experiments do not support a significant action on L_{KCa} channels, principally on the basis of the susceptibility of I_{Ckm} to inhibitors and the low noise of I_{Ckm} which positively excludes these large conductance (about 100 pS) channels. Large conductance Ca^{2+}-activated K^+-channels, and spontaneous transient outward currents (STOCs) showed a different inhibitor sensitivity to I_{Ckm}, in particular, they were insensitive to 4-AP, much more sensitive to TEA and blocked by charybdotoxin (Beech *et al.*, 1987; Kusano *et al.*, 1987). Ckm-induced $^{86}Rb^+$-efflux (Weir & Strong, 1988) and I_{Ckm} were not.

A possible explanation for the current evoked by Ckm is that this drug short-circuits the voltage-gate of delayed rectifier K^+-channels which carry I_{dK}, so that an increased open-time is maintained independent of the membrane potential or the state of the channels. Thus, Ckm may open the delayed rectifier K^+-channels from either their resting or inactivated state, as I_{Ckm} could be induced at all potentials examined (−100 to +40 mV). The evidence for this came from experiments which showed that I_{dK} was reduced when I_{Ckm} was activated. I_{Ckm} also showed a similar sensitivity to that of I_{dK} to several inhibitors. If I_{Ckm} has some explanation other than the above, then the time-dependent change in the magnitude of

I_{Ckm} when I_{dK} is evoked by a depolarizing pulse needs to be explained. Other substances have been described which act in a way analogous to that suggested for Ckm: maitotoxin induces a sustained inward Ca^{2+}-current and a concomitant decrease in the normal voltage-activated Ca^{2+}-current (Kobayashi *et al.*, 1987), and veratridine holds Na^+-channels in an open-state (transition only from the normal open-state) such that the voltage-activated Na^+-current is reduced (Sutro, 1986).

Although the rank order of potencies of various inhibitors were the same for I_{Ckm} and I_{dK}, there were minor differences in potency of individual inhibitors on I_{Ckm} and on I_{dK} which could be attributed to the method of analysis. For example, tachyphylaxis of the Ckm-induced response, a decrease in the seal resistance (not compensated for by the control experiments) or a voltage-dependence of the block could have contributed to making I_{Ckm} appear more sensitive to inhibition than I_{dK}. There was, however, a marked discrepancy when comparing the sensitivities of the currents to glibenclamide in two experiments, I_{Ckm} being much more strongly affected (Figure 10). This observation requires further investigation. The effects of glibenclamide may indicate that Ckm channels are similar to the ATP-regulated K^+-channels of cardiac cells (Noma, 1983) or pancreatic β-cells (Cook & Hales, 1984), which are also inhibited by this substance (Sturgess *et al.*, 1985), albeit at a lower concentration (e.g. see Fosset *et al.*, 1988).

However, we and others have not identified ATP-regulated K^+-channels in smooth muscle cells from portal vein or small intestine (Ohya *et al.*, 1987; Beech, 1988), although they have been claimed to be present in mesenteric artery (Davies *et al.*, 1989). The current-voltage relationship of I_{Ckm} was unaffected by 1 mM ATP intracellularly (Beech & Bolton, unpublished) and, moreover, ATP regulated K^+-channels rectify inwardly whereas I_{Ckm} rectifies outwardly. Several other observations are difficult to reconcile with the idea that ATP-regulated K^+-channels underlie I_{Ckm}. The channels described by Davies *et al.* (1989) had a conductance of 135 pS which is the same as that of L_{KCa} channels and 2–3 times larger than that of any other ATP-sensitive channel (Ashcroft, 1988). Any current through these 135 pS channels is likely to be extremely noisy; for example, in Figure 10 the extra 500 pA of current produced by Ckm would represent the opening (on average) of 37 channels each carrying 13.5 pA. Random fluctuations in the number of open channels would produce a conspicuously noisy current. However, we found that Ckm reduced current noise (Figure 9) rather than increased it, which suggests that the unitary current carried by channels opened by Ckm might be small. I_{dK} channels have a conductance of about 5 pS (Beech & Bolton, 1989b). In contrast, the current carried by L_{KCa} channels in single portal vein cells is extremely noisy at positive potentials and this current, and the current noise, is drastically reduced by TEA or charybdotoxin (Beech & Bolton, 1989b). However, glibenclamide had no appreciable effect on current noise (Figure 10) which argues strongly against the idea that a large conductance channel similar in size to the L_{KCa} channel underlies the Ckm-induced current. Also, Ashford *et al.* (1988) have shown that Ckm has no effect on

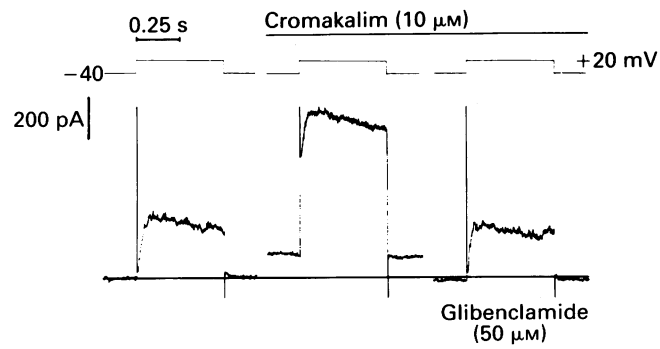

Figure 10 Effect of glibenclamide (50 μM) on the outward current to cromakalim (10 μM). Potential dependent outward current was elicited by stepping from the holding potential of −40 mV to +20 mV. Cromakalim was applied and evoked an outward current (upward displacement of holding current) and outward current was much larger upon stepping to +20 mV. Glibenclamide reversed the effects of cromakalim with only a small effect on the potential-dependent component of current evoked by the +20 mV test step; this current is largely the delayed rectifier current (I_{dK}).

single, diazoxide-activatable, ATP-regulated K^+-channels from an insulin-secreting cell-line. Thus it is more likely that block of I_{Ckm} by glibenclamide results from competition at the Ckm-binding site rather than the blockade of the channel carrying the current.

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Cromakalim-induced relaxation of guinea-pig isolated trachealis: antagonism by glibenclamide and by phentolamine

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1 Tested against the spontaneous tone of guinea-pig isolated trachealis, cromakalim (0.1–100 μM), isoprenaline (1 nM–1 μM) and theophylline (1 μM–1 mM) each produced concentration-dependent relaxation.

2 Glibenclamide (0.1–10 μM) did not itself alter the spontaneous tone of the trachea nor did it modify the relaxant actions of isoprenaline or theophylline. In contrast, glibenclamide (0.1 and 1 μM) caused a concentration-dependent rightward shift of the log concentration-effect curve of cromakalim. Glibenclamide (10 μM) reduced the slope of the log concentration-effect curve of cromakalim and moved the foot of the curve back towards the control position.

3 Phentolamine (1, 10 and 100 μM) did not itself alter the spontaneous tone of the trachea nor did it modify the relaxant actions of isoprenaline or theophylline. In contrast phentolamine caused concentration-dependent depression of the log concentration-effect curve of cromakalim.

4 Neither prazosin (1 μM) nor yohimbine (10 μM) modified the spontaneous tone of the trachea. Prazosin and yohimbine each failed to antagonise the effects of cromakalim, isoprenaline and theophylline.

5 Intracellular electrophysiological recording showed that glibenclamide (1 μM) and phentolamine (100 μM) caused minor change in the resting membrane potential of trachealis cells. Slow wave activity was slightly depressed by these agents. In contrast tetraethylammonium (TEA; 8 mM) caused marked depolarisation, and promoted the conversion of slow waves into regenerative action potentials. These electrical changes were accompanied by tonic tension development.

6 Phentolamine (100 μM) and glibenclamide (1 μM) reduced and reversed both the relaxation and the hyperpolarisation induced by cromakalim (10 μM).

7 It is concluded that glibenclamide and phentolamine each provide selective antagonism of the relaxant action of cromakalim in guinea-pig trachealis. These agents also inhibit the plasmalemmal hyperpolarisation induced by cromakalim. The effect of phentolamine is unrelated to the blockade of α_1- or α_2-adrenoceptors. If either glibenclamide or phentolamine act to block the K^+ channels opened by cromakalim, then such channels are not identical to those which endow the trachealis plasmalemma with its powerful rectifying behaviour.

Introduction

The K^+ channel opening drug, cromakalim, causes concentration-dependent suppression of the spontaneous tone of guinea-pig isolated trachealis (Allen *et al.*, 1986; Arch *et al.*, 1988). This effect is associated both with the stimulation of $^{86}Rb^+$ efflux and with hyperpolarisation of the trachealis plasmalemma to a value close to the K^+ equilibrium potential (Allen *et al.*, 1986). K^+ channel blocking agents such as procaine and tetraethylammonium (TEA) inhibit cromakalim-induced hyperpolarisation and relaxation (Allen *et al.*, 1986) but since these agents are known to block several types of K^+ channel (Yamanaka *et al.*, 1985; Cook, 1988), they do not serve to identify the K^+ channel opened by cromakalim. In guinea-pig trachealis this channel is not sensitive to apamin (Allen *et al.*, 1986).

It has recently been shown (Quast & Cook, 1988; Wilson *et al.*, 1988; Newgreen *et al.*, 1989) that glibenclamide can antagonise cromakalim-induced relaxation of vascular smooth muscle. Furthermore,

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McPherson & Angus (1989) have shown that phentolamine can antagonise the effects of cromakalim on vascular smooth muscle by an action unrelated to the blockade of α-adrenoceptors. Observations of this kind prompted the experiments of the present study, in which we have sought to determine whether glibenclamide and phentolamine can provide selective antagonism of the effects of cromakalim on trachealis muscle. It was reasoned that such experiments might shed more light on the nature of the K^+ channel opened by cromakalim in this tissue. A preliminary account of this work has been communicated to the British Pharmacological Society (Murray & Small, 1989).

Methods

Guinea-pigs (350–550 g) of either sex were killed by stunning and bleeding. Tracheae were excised, cleaned of adhering adipose and connective tissue and opened by cutting longitudinally through the cartilage rings diametrically opposite the trachealis.

Tissue bath studies of mechanical activity

Small segments of trachea were set-up for the isometric recording of tension changes essentially as described by Foster *et al.* (1983). At the outset of each experiment tissues were subjected to an imposed tension of 1.25 g. Approximately 20 min later, aminophylline (1 mM) was added in order to determine the recorder pen position at zero tone. The aminophylline was then washed from the tissues and when tone subsequently reached its peak value, study of relaxant drugs commenced.

Cumulative concentration-effect curves were constructed for all relaxant drugs examined. Three fold, concentration increments were used for cromakalim, and ten fold increments for isoprenaline and theophylline. Each concentration increment was allowed 8 min, 4 min and 5 min tissue contact respectively for these drugs.

Pilot experiments revealed that some tissue desensitization to cromakalim occurred when cumulative concentration-effect curves for this agent were repeatedly constructed on the tissue. Accordingly, desensitization to cromakalim was minimised by interposing a concentration-effect curve for theophylline between successive cromakalim curves. Following construction of initial log concentration-effect curves for cromakalim and theophylline, test tissues were exposed to an antagonist (glibenclamide, phentolamine, prazosin or yohimbine). Forty min later, concentration-effect curves for cromakalim and theophylline were reconstructed in the presence of the antagonist. Isoprenaline was studied in a similar fashion except that concentration-effect curves for theophylline were not interposed between successive isoprenaline curves. In all experiments time-matched control tissues were treated identically to test tissues but were not exposed to the antagonist under investigation.

Intracellular electrophysiological recording

Simultaneous recording of intracellular electrical activity and mechanical changes of a contiguous segment of trachea was performed with the technique and tissue holder described by Dixon & Small (1983). Accordingly, part of the trachealis was immobilized to permit long-term intracellular electrical recording, while mechanical activity of contiguous muscle bundles was measured under an initial imposed tension of 1.25 g. The recording microelectrodes were filled with 3 M KCl and were of resistance greater than 40 MΩ.

Three types of electrophysiological experiments were performed. In the first type of experiment, a trachealis cell was impaled and, after a lapse of several minutes to check the stability of the electrical record, glibenclamide (1 μM), phentolamine (100 μM) or tetraethylammonium (TEA, 8 mM) was added to the Krebs solution superfusing the tissue. The effects of each of these agents on electrical and mechanical activity were monitored for a minimum of 10 min.

The second type of experiment was conducted either in normal Krebs solution or in Krebs solution containing glibenclamide (1 μM) or phentolamine (100 μM). Following cellular impalement, cromakalim (10 μM) was added to the superfusing solution and the effects of cromakalim (in the absence and presence of glibenclamide or phentolamine) on electrical and mechanical activity were monitored for a minimum of 4 min.

The third type of electrophysiological experiment was conducted in normal Krebs solution. Following cellular impalement, cromakalim (10 μM) was added to the superfusing solution. Eight min later, glibenclamide (1 μM) or phentolamine (100 μM) was added to the superfusate. The effects of the drug combination on the electrical and mechanical activity were monitored for as long as the microelectrode tip remained within the impaled cell.

Drugs, solutions and statistical analysis of results

Drug concentrations are expressed in terms of the molar concentration of the active species. The following drugs were used: aminophylline (BDH), cromakalim (Beecham Research Laboratories), glibenclamide (Hoechst), (−)-isoprenaline hydro-

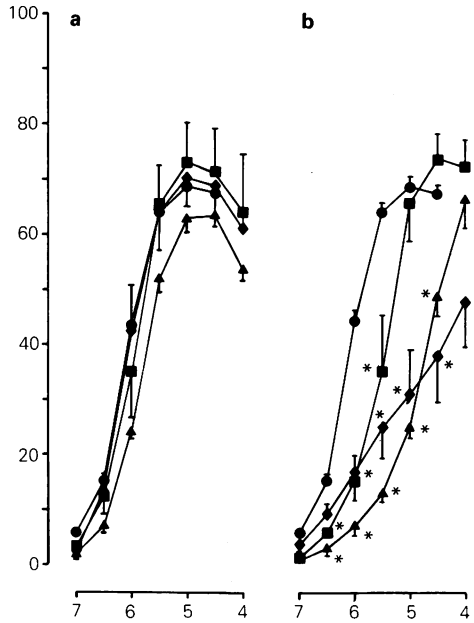

Figure 1 Cromakalim-induced relaxation of guinea-pig isolated trachealis: antagonism by glibenclamide. Abscissae: molar concentration of cromakalim on a log scale. Ordinate scale: relaxation expressed as a percentage of the maximal relaxation induced by aminophylline. (a) Time-matched control tissues. (b) Test tissues. In each case (●) indicates the pooled, initial log concentration-effect curve for cromakalim. Subsequent log concentration-effect curves were constructed in the absence (a, time-matched controls) or presence (b, test tissues) of glibenclamide 0.1 μM (■), 1 μM (▲) or 10 μM (◆). Data points are the means of values from at least six tissues. Vertical lines show s.e.mean. *indicates a significant ($P < 0.05$) difference from the corresponding point in the time-matched control tissues.

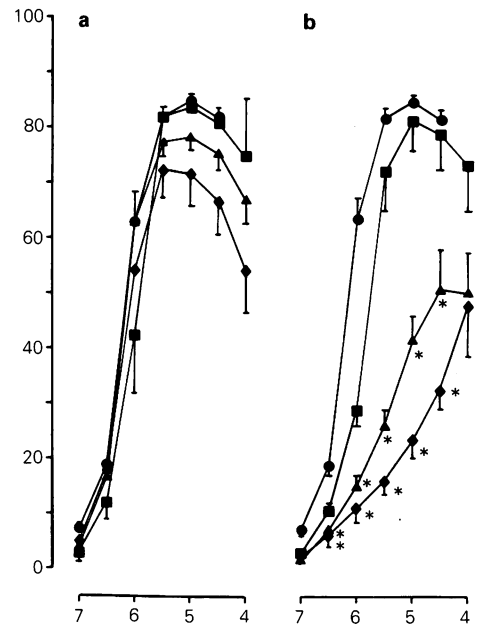

Figure 2 Cromakalim-induced relaxation of guinea-pig isolated trachealis: antagonism by phentolamine. Abscissae: molar concentration of cromakalim on a log scale. Ordinate scale: relaxation expressed as a percentage of the maximal relaxation induced by aminophylline. (a) Time-matched control tissues. (b) Test tissues. In each case (●) indicates the pooled, initial log concentration-effect curve for cromakalim. Subsequent log concentration-effect curves were constructed in the absence (a, time-matched controls) or presence (b, test tissues) of phentolamine 1 μM (■), 10 μM (▲) or 100 μM (◆). Data points are the means of values from at least six tissues. Vertical lines show s.e.mean. *indicates a significant ($P < 0.05$) difference from the corresponding point in the time-matched control tissues.

chloride (Sigma), phentolamine mesylate (Ciba), prazosin hydrochloride (Pfizer), tetraethylammonium bromide (Sigma), theophylline (Sigma), yohimbine hydrochloride (Sigma). While stock solutions of most drugs were prepared in distilled water, those of cromakalim were prepared in 70% v/v ethanol and those of glibenclamide and phentolamine in absolute ethanol. A stock solution of isoprenaline was prepared in 0.1 M HCl and dilutions of this agent were prepared in distilled water containing 0.57 mM ascorbic acid as an antioxidant.

The Krebs solution used in all the experiments had the following composition (mM): Na^+ 143.5, K^+ 5.9, Ca^{2+} 2.6, Mg^{2+} 1.2, Cl^- 127.6, HCO_3^- 25, SO_4^{2-} 1.2, $H_2PO_4^-$ 1.2 and glucose 11.1.

The significance of differences between means was assessed by a two-tailed, unpaired t test. The null hypothesis was rejected when $P < 0.05$.

Results

Tissue bath studies of mechanical activity

Cromakalim (0.1–100 μM; Figures 1, 2, 3), isoprenaline (1 nM–1 μM) and theophylline (1 μM–1 mM) each produced concentration-dependent suppression of the spontaneous tone of the trachea. As observed previously (Small *et al.*, 1989), the maximal relaxant effects of isoprenaline and theophylline were identical to that of aminophylline. In contrast the maximal relaxant effect of cromakalim was approximately 70–80% (Figures 1, 2, 3) of that of aminophylline.

Glibenclamide (0.1–10 μM) caused little, if any change in the tone of the trachea (Figure 4) and did not modify the relaxant actions of either isoprenaline or theophylline. Mean (±s.e.mean; $n > 6$) pD_2 values in the absence and in the presence of 10 μM

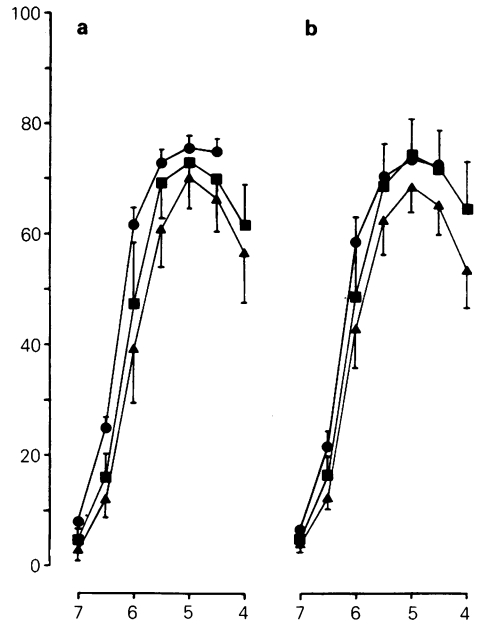

Figure 3 Cromakalim-induced relaxation of guinea-pig isolated trachealis: the failure of prazosin (1 μM) or yohimbine (10 μM) to provide antagonism. Abscissae: molar concentration of cromakalim on a log scale: Ordinate scale: relaxation expressed as a percentage of the maximal relaxation induced by aminophylline. (a) Effects of prazosin (1 μM). (b) Effects of yohimbine (10 μM). In each case (●) indicates the pooled, initial log concentration-effect curve for cromakalim. Subsequent log concentration-effect curves were constructed in the presence of the putative antagonist ((■); test tissues) or its vehicle ((▲); time-matched control tissues). Data represent the means of values from at least six tissues. Vertical lines show s.e.mean. Note that prazosin (1 μM) and yohimbine (10 μM) each failed to antagonise cromakalim.

glibenclamide were 7.90 ± 0.12 and 8.13 ± 0.08 respectively for isoprenaline and 4.08 ± 0.07 and 4.23 ± 0.12 respectively for theophylline. In contrast, glibenclamide antagonised the action of cromakalim. Glibenclamide (0.1 and 1 μM) caused a concentration-dependent rightward shift of the log concentration-effect curve for cromakalim without changing the slope or maximal effect. However, glibenclamide (10 μM) reduced the slope of the log concentration-effect curve of cromakalim and moved the curve leftwards back towards the pre-glibenclamide position (Figure 1).

Phentolamine (1–100 μM) also caused little, if any, change in the tone of the trachea (Figure 4) and did not modify the relaxant action of either isoprenaline or theophylline. Mean ($n > 6$) pD_2 values in the absence and in the presence of 100 μM phentolamine were 8.31 ± 0.06 and 8.37 ± 0.09 respectively for isoprenaline and 4.29 ± 0.05 and 4.27 ± 0.06 respectively for theophylline. In contrast, phentolamine antagonised the action of cromakalim. Phentolamine (1 μM) caused a small rightward shift in the log concentration-effect curve for cromakalim without depressing the maximal response. At a higher concentration (10 μM and 100 μM) phentolamine reduced the slope of the log concentration-effect curve for cromakalim and reduced the maximal response (Figure 2).

Neither prazosin (0.01–1.0 μM) nor yohimbine (1–10 μM) caused a change in the tone of the trachea, or modified the relaxant actions of isoprenaline or theophylline. Furthermore, neither prazosin (1 μM) nor yohimbine (10 μM) modified the relaxant action of cromakalim (Figure 3).

Intracellular electrophysiological recordings

The effects of glibenclamide, phentolamine and TEA on the spontaneous electrical and mechanical activity of the trachea were each examined on a minimum of six different tissues. Glibenclamide (1 μM) or phentolamine (100 μM) added to the Krebs solution superfusing the trachea produced little or no change in mechanical tone. Monitored over a 10 min period, these agents caused some suppression of spontaneous slow wave activity. Glibenclamide did not significantly alter the resting membrane potential but phentolamine always evoked a slight depolarisation (Table 1 and Figure 4). In contrast, TEA (8 mM) caused marked depolarisation of the trachealis cells, increased or initiated slow wave activity and promoted the conversion of slow waves into regenerative action potentials. These electrical changes were accompanied by tonic tension development (Table 1 and Figure 4).

As we have previously demonstrated (Allen *et al.*, 1986), treatment of the trachealis muscle with cromakalim (10 μM) suppressed spontaneous slow waves, hyperpolarised the trachealis cells and relaxed the tissue (Figure 5). When the same concentration of cromakalim was tested in tissues equilibrated with Krebs solution containing glibenclamide (1 μM) or phentolamine (100 μM), the hyperpolarisation (Figure 5 and Table 2) and the relaxation induced by cromakalim were both reduced. That glibenclamide and phentolamine could antagonise both the hyperpolarisation and the relaxation induced by cromakalim was also demonstrated in experiments where the tissues were first exposed to cromakalim and then glibenclamide, or phentolamine was added in an attempt to reverse the effects of the K^+ channel opener.

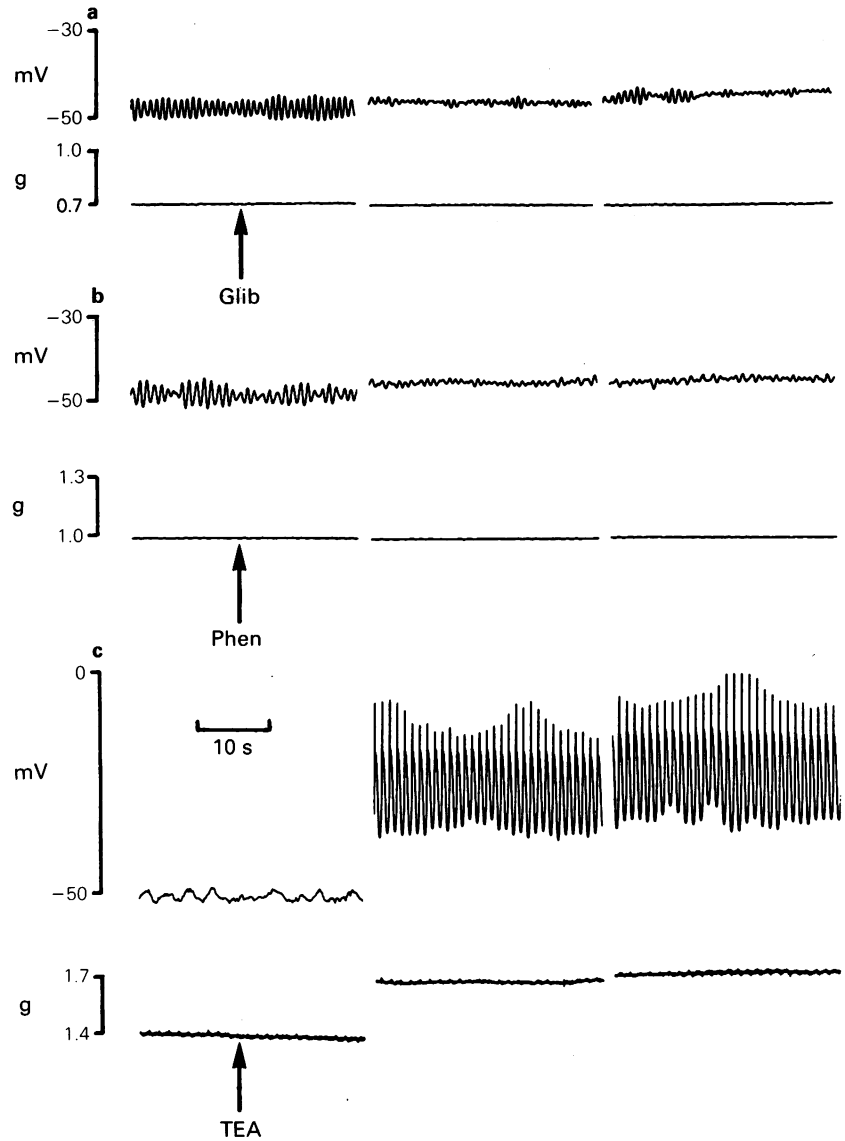

Figure 4 Guinea-pig isolated trachealis: the effects of some antagonists of cromakalim on electrical and mechanical activity. In each row of recordings the upper trace represents membrane potential changes recorded from a single cell, while the lower trace represents the mechanical activity of a contiguous segment of trachea. In each row the left hand panel indicates control activity and tissue exposure to the indicated drug commences at the arrow. The centre and right hand panels show activity recorded 5 and 10 min (respectively) after tissue exposure to the relevant drug. Note (in a and b, respectively) that glibenclamide (Glib) (1 μM) and phentolamine (Phen) (100 μM) caused some suppression of spontaneous slow wave activity and very minor depolarisation. These electrical changes were accompanied by little or no change in mechanical tone. In contrast (c) tetraethylammonium (TEA) (8 mM) evoked marked depolarisation, promoted the discharge of regenerative action potentials and caused tonic tension development.

The electrophysiological experiments involving tracheal exposure to cromakalim (10 μM) and the subsequent addition either of glibenclamide (1 μM) or of phentolamine (100 μM) involved maintaining a recording of membrane potential for a total of 30–40 min. During this period marked relaxation (induced by cromakalim) and subsequent recovery occurred. Accordingly such experiments proved

Table 1 The effects of glibenclamide (1 μM), phentolamine (100 μM) and tetraethylammonium (TEA, 8 mM) on the resting membrane potential and mechanical tone of guinea-pig isolated trachea

Treatment	*Membrane potential change* (mV)	*Tension change* (mg)	*Number of tissues examined*
Glibenclamide 1 μM	−1.0 ± 1.4	0.0 ± 13.0	11
Phentolamine 100 μM	−4.3 ± 0.5*	0.0 ± 31.0	11
TEA 8 mM	−25 ± 3.8*	+750 ± 130*	6

Data represent mean ± s.e.mean changes observed 10 min after tissue exposure to the drug. − indicates depolarisation or relaxation. + indicates hyperpolarisation or contraction. * indicates a change significantly ($P < 0.05$) different from zero.

extremely difficult to complete. The microelectrode often became dislodged from the impaled cell before the effects of glibenclamide or phentolamine had fully developed. Nevertheless, on all occasions where this experiment was attempted, it was clear that glibenclamide (1 μM) or phentolamine (100 μM) reversed both the electrical and mechanical effects of cromakalim (10 μM). The records presented in Figure 6 clearly illustrate this and are representative of the few occasions when the microelectrode impalement of a single cell was maintained for the full duration of the experiment.

Control experiments revealed that tissue exposure to cromakalim (10 μM) for periods in excess of 8 min led to some spontaneous reversal of electrical and mechanical responses. However, this proceeded more slowly than that induced by either glibenclamide or phentolamine. In view of the difficulties of maintaining long-term recordings of membrane potential, quantitative comparison of spontaneous and drug-induced reversal of responses to cromakalim was made in experiments where mechanical activity alone was measured. At the time (20 min) when glibenclamide (1 μM) and phentolamine (100 μM) had caused reversal of 79.9 ± 5.7% and 116.1 ± 3.7% respectively, a spontaneous reversal of 19.1 ± 3.9% was observed.

Discussion

The antagonism of cromakalim by glibenclamide

There have been several studies (Quast & Cook, 1988; Wilson *et al.*, 1988; Newgreen *et al.*, 1989) showing that glibenclamide is able to antagonise cromakalim acting on vascular smooth muscle. That the action of glibenclamide exhibits some selectivity is suggested by its ability, in rat thoracic aorta, to antagonise the relaxant action of cromakalim but not that of sodium nitroprusside (Newgreen *et al.*, 1989). The results of the present study show that glibenclamide can antagonise both the relaxant action of cromakalim in trachealis muscle and also its ability to hyperpolarise the trachealis cells. Furthermore, the selectivity of the action of glibenclamide is confirmed by its failure to antagonise the tracheal relaxant actions of isoprenaline or theophylline.

Glibenclamide (0.1–20 nM) increases insulin secretion by blocking ATP-dependent K^+ channels in

Table 2 The effects of pretreatment with glibenclamide (1 μM) or phentolamine (100 μM) on the tracheal hyperpolarisation induced by cromakalim (10 μM)

Treatment	*Hyperpolarisation* (mV) *induced at the stated time after the administration of cromakalim* 4 min	8 min	*Number of tissues examined*
Cromakalim 10 μM	19.3 ± 2.2	20.0 ± 2.1	8
Glibenclamide 1 μM + cromakalim 10 μM	7.3 ± 2.0*	11.0 ± 2.6*	6
Phentolamine 100 μM + cromakalim 10 μM	3.3 ± 1.3*	1.0 ± 2.3*	4

Data represent mean ± s.e.mean of values from at least 4 tissues. * indicates a significant ($P < 0.05$) difference from the corresponding value for cromakalim alone.

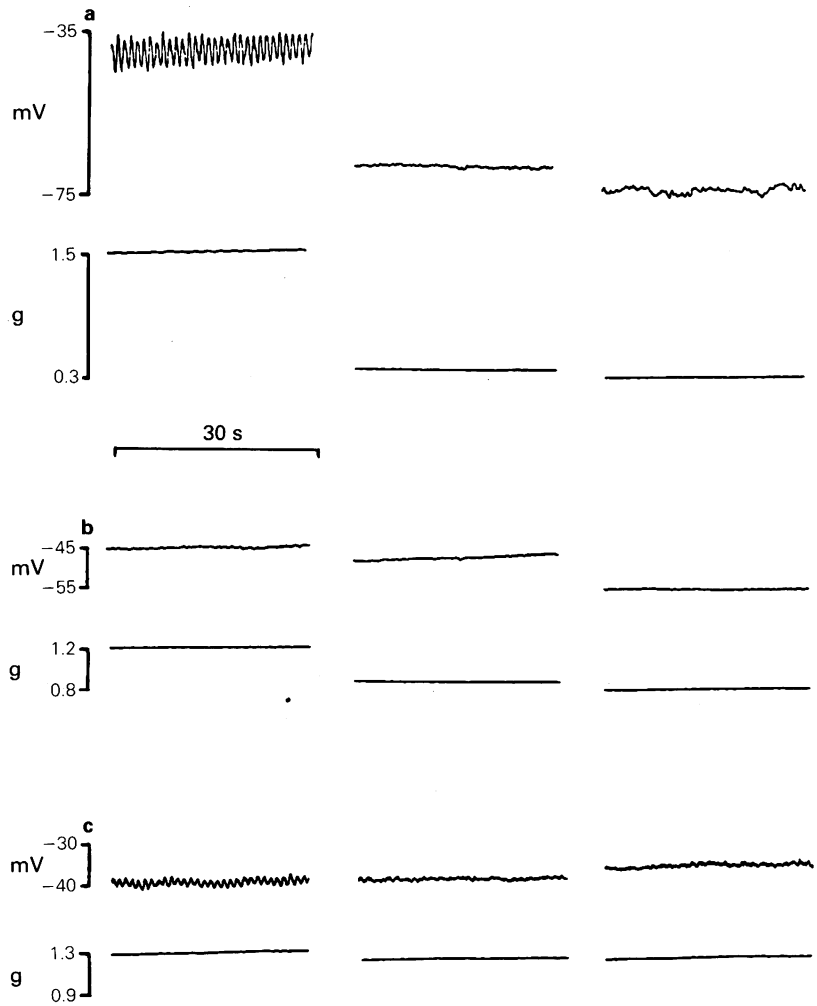

Figure 5 Guinea-pig isolated trachealis: the electrical and mechanical effects of cromakalim (10 μM) and their antagonism by tissue pretreatment with glibenclamide or phentolamine. In each row of recordings the upper trace represents membrane potential changes recorded from a single cell, while the lower trace represents the mechanical activity of a contiguous segment of trachea. In each row the left hand panel indicates control activity. The centre and right hand panels indicate activity recorded 4 and 8 min respectively after tissue exposure to cromakalim (10 μM). (a) No antagonist present. (b and c) Glibenclamide (1 μM) and phentolamine (100 μM), respectively, present throughout. Note that glibenclamide and phentolamine each reduced the ability of cromakalim to hyperpolarise and relax the trachealis muscle.

RINm5F insulinoma cells (Schmid-Antomarchi *et al.*, 1987). This has prompted the suggestion (Newgreen *et al.*, 1989) that glibenclamide may block the K^+ channel opened by cromakalim in smooth muscle. However, the high concentrations (0.1–10 μM) of glibenclamide required for antagonism of cromakalim in smooth muscle indicate that the cromakalim-opened K^+ channel in smooth muscle may not be identical to the ATP-dependent K^+ channel of pancreatic β-cells.

If glibenclamide indeed acts to block the K^+ channel opened by cromakalim in guinea-pig trachealis, then its failure to cause significant membrane potential change (Figure 4 and Table 1) suggests that the cromakalim-sensitive K^+ channel is not open under normal circumstances and does not play an important role in determining the resting membrane potential. A similar suggestion has been made with regard to the action of glibenclamide in vascular smooth muscle (Quast & Cook, 1988).

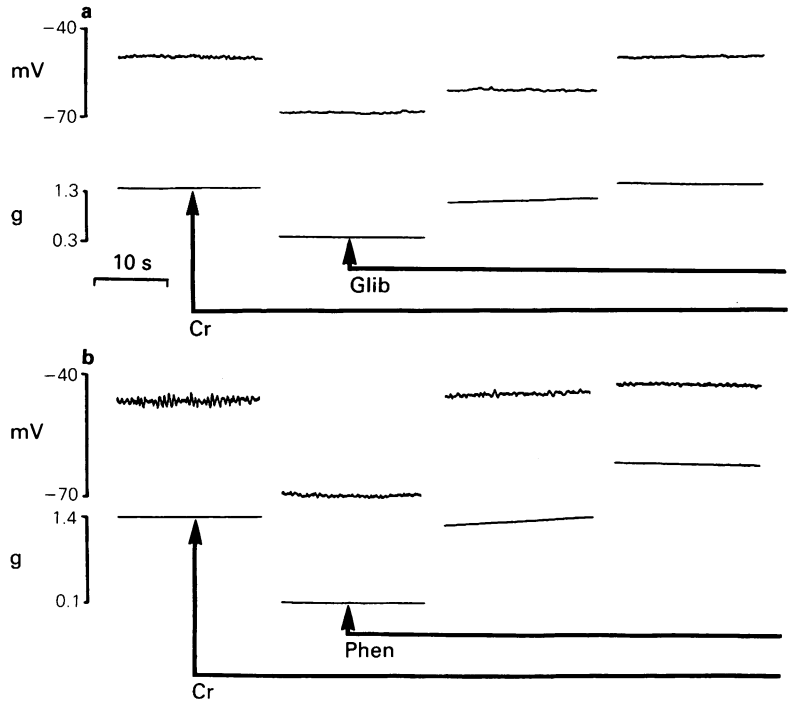

Figure 6 The electrical and mechanical effects of cromakalim (10 μM) in guinea-pig isolated trachealis: their reversal by glibenclamide or phentolamine. In each row of recordings the upper trace represents membrane potential changes recorded from a single cell, while the lower trace represents the mechanical activity of a contiguous segment of trachea. In each row the left hand panel indicates control activity and tissue exposure to cromakalim (Cr) (10 μM) commences at the arrow. Subsequent panels show activity recorded 8 min after tissue exposure to cromakalim (when glibenclamide (Glib, 1 μM; a) or phentolamine (Phen, 100 μM; b) was added at the arrow) and 18 and 28 min after tissue exposure to cromakalim. Note that, 20 min after the addition of glibenclamide or phentolamine, the membrane potential changes and tension changes evoked by cromakalim were effectively reversed towards the pre-cromakalim levels.

The antagonism of cromakalim by phentolamine

In vascular smooth muscle from the dog and other species, McPherson & Angus (1989) showed that phentolamine antagonised both the relaxant activity of cromakalim and its ability to hyperpolarise the tissue. The action of phentolamine was selective in that other vasorelaxants were not antagonised. Furthermore, since cromakalim was not antagonised by prazosin, rauwolscine or phenoxybenzamine, the action of phentolamine seemed unrelated to α-adrenoceptor blockade. Results of the present study suggest that phentolamine provides similar antagonism of cromakalim acting on trachealis muscle. The antagonism was selective in that phentolamine antagonised neither isoprenaline nor theophylline. The antagonism also seemed unrelated to the blockade of either α_1- or α_2-adrenoceptors since neither prazosin nor yohimbine antagonised cromakalim.

Tested on the trachealis, phentolamine (1 μM) caused some rightward shift in the cromakalim log concentration-effect curve. Higher concentrations of phentolamine depressed the curve and reduced the maximal effect of cromakalim (Figure 2). Similar changes in the cromakalim log concentration-effect curve were obtained in experiments with vascular smooth muscle (McPherson & Angus, 1989).

McPherson & Angus (1989) suggested that phentolamine might antagonise cromakalim by blocking the K^+ channel opened by cromakalim in smooth muscle. If this suggestion is correct, then the failure of phentolamine markedly to depolarise the trachealis (Figure 4 and Table 1) reinforces the evidence provided by experiments with glibenclamide, that the K^+ channel opened by cromakalim is not open under normal circumstances and does not play an important role in determining the resting membrane potential.

Does cromakalim open the K^+ channel responsible for the strong rectifying behaviour of the trachealis plasmalemma?

It has been known for almost two decades that the plasmalemma of trachealis cells is endowed with such strong rectifying properties that regenerative action potentials are not discharged spontaneously and cannot be evoked in response to the passage of cathodal current. Early evidence that this rectifying behaviour was linked to the activity of plasmalemmal K^+ channels included the observation that TEA (a K^+ channel inhibitor) could induce action potential discharge in trachealis muscle (Small & Foster, 1988). Measurement of electrotonic potentials evoked by anodal and cathodal current pulses in canine trachealis (Kannan *et al.*, 1983) has directly revealed the ability of TEA to reduce tracheal rectification. Furthermore, patch clamp recording from canine airways smooth muscle has revealed the existence of Ca^{2+}- and voltage-dependent K^+ channels in the plasmalemma. The high unitary conductance (270 pS), voltage-dependency and susceptibility of these channels to blockade by TEA, all suggest their involvement in the rectifying behaviour of the plasmalemma (McCann & Welsh, 1986).

Since TEA can, to some extent, inhibit the hyperpolarisation and relaxation of guinea-pig trachealis induced by cromakalim (Allen *et al.*, 1986), it might be argued that cromakalim opens the K^+ channels which endow the plasmalemma with its rectifying properties. However, TEA can block several different types of K^+ channel (Cook, 1988) and cannot therefore serve to identify the K^+ channel opened by cromakalim.

In the present study, glibenclamide and phentolamine each inhibited the mechanical and electrical effects of cromakalim. However, neither of these agents was able to mimic the effects of TEA in inducing the discharge of regenerative action potentials. This strongly indicates that glibenclamide and phentolamine do not reduce the rectifying behaviour of the trachealis plasmalemma.

As discussed above, the way in which glibenclamide and phentolamine interfere with the action of cromakalim in smooth muscle is currently poorly understood. These agents could prevent cromakalim binding to its site of action. They could interfere with biochemical processes linking the cromakalim site of action to the K^+ channel opened by cromakalim. Alternatively, as suggested by Newgreen *et al.* (1989) and McPherson & Angus (1989), they could act simply to block the relevant K^+ channel.

If it can be assumed that either of these agents block the K^+ channel opened by cromakalim, then the present results suggest that cromakalim does not act to open the large Ca^{2+}- and voltage-dependent K^+ channels which endow the trachealis plasmalemma with its strong rectifying properties.

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Effects of extracellular adenine nucleotides on the electrical, ionic and secretory events in mouse pancreatic β-cells

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1 The mechanisms whereby extracellular adenine nucleotides modulate pancreatic β-cell function were studied with mouse islets stimulated by 15 mM glucose.

2 Adenosine 5'-triphosphate (ATP) and adenosine 5'-diphosphate (ADP) (100 μM) inhibited insulin release, ^{45}Ca efflux and ^{86}Rb efflux from islet cells, and decreased electrical activity in β-cells. These changes were rapid but small and transient.

3 α,β-Methylene ADP caused a rapid and sustained inhibition of insulin release, ^{45}Ca efflux and ^{86}Rb efflux from islet cells. It also produced a slight hyperpolarization of the β-cell membrane, with sustained modification of the pattern but only transient decrease of the intensity of the electrical activity. In the absence of extracellular Ca^{2+}, α,β-methylene ADP increased ^{45}Ca and ^{86}Rb efflux without changing insulin release. Most effects of α,β-methylene ATP were qualitatively similar but quantitatively smaller than those of the ADP-analogue.

4 Adenylylimido-diphosphate (AMP-PNP) slightly increased ^{45}Ca and ^{86}Rb efflux and potentiated insulin release in the presence of extracellular Ca^{2+}. However, its effects on electrical activity in β-cells were qualitatively similar to those of the α,β-methylene analogues.

5 The small effects of ATP and ADP could result from their degradation into adenosine. α,β-Methylene ADP appears to increase K^+ permeability of the β-cell membrane and to produce a second, intracellular, effect which largely contributes to the inhibition of insulin release. Another recognition site, with higher affinity for triphosphate derivatives, could mediate the small stimulatory effects of AMP-PNP.

Introduction

Since the pioneer work of Burnstock (1972), it has been widely accepted that micromolar concentrations of extracellular adenosine 5'-triphosphate (ATP) and other nucleotides can influence numerous biological processes by acting on specific membrane receptors (Burnstock & Brown, 1981; Gordon, 1986; White, 1988).

It has long been reported that high concentrations of exogenous ATP increase insulin release *in vitro* (R-Candela *et al.*, 1963; Sussman *et al.*, 1969; Loubatières *et al.*, 1972; Feldman & Jackson, 1974). However, little attention was paid to these observations until Loubatières-Mariani *et al.* (1979) suggested that membrane purinoceptors could control β-cell function. This proposal was based on the evidence that micromolar concentrations of purine nucleotides markedly potentiate glucose-induced insulin release from the perfused rat pancreas. Subsequent functional studies by the same laboratory showed that the purinoceptor present on rat β-cells is of the P_{2y}-type (Chapal & Loubatières-Mariani, 1981a, b; Bertrand *et al.*, 1987), according to the classification of Burnstock & Kennedy (1985).

Recently, it has been proposed that ATP released by nerve endings could act in synergism with acetylcholine to increase insulin secretion (Bertrand *et al.*, 1986). It has also been speculated that ATP, which is co-secreted with insulin (Leitner *et al.*, 1975), could exert a positive feedback control of insulin release (Gylfe & Hellman, 1987; Blachier & Malaisse, 1988; Loubatières-Mariani & Chapal, 1988).

In the present work, mouse islets were used to investigate the possible mechanisms whereby ATP,

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ADP and some structural analogues affect insulin release. We focused on the changes in ionic fluxes and membrane potential, as they are critically involved in the regulation of β-cell function (Henquin & Meissner, 1984; Matthews, 1985; Petersen & Findlay, 1987).

Methods

All experiments were performed with islets of fed female NMRI mice (25–30 g), killed by decapitation. For electrophysiological experiments, a piece of pancreas was fixed in a small perifusion chamber, and the membrane potential of single β-cells was continuously recorded with microelectrodes (Meissner & Schmelz, 1974). β-Cells were identified by the typical electrical activity that they display in the presence of 10–15 mM glucose. For all other experiments, islets were isolated after collagenase digestion of the pancreas. For measurement of the efflux of ^{45}Ca or ^{86}Rb (used as tracer for K), the islets were first loaded with the tracers for 90 min (Garrino & Henquin, 1988). They were then transferred to a dynamic system of perifusion permitting monitoring of the efflux (Henquin, 1979). During the experiments of ^{86}Rb efflux, a portion of each effluent fraction was drawn for measurement of immunoreactive insulin; rat insulin was used as a standard (Henquin & Lambert, 1975).

The medium used had the following ionic composition (mM): NaCl 120, KCl 4.8, $CaCl_2$ 2.5, $MgCl_2$ 1.2, and $NaHCO_3$ 24. It was gassed with 94% O_2/6% CO_2 to maintain a pH 7.4, and was supplemented with 1 mg ml^{-1} bovine serum albumin fraction V (Boehringer, Mannheim, Germany), except for electrophysiological recordings. Ca^{2+}-free solutions were prepared by replacing $CaCl_2$ by $MgCl_2$.

The sodium salts of ATP and ADP were obtained from Boehringer (Mannheim, Germany). The sodium salt of α,β-methylene ADP, and the lithium salts of α,β-methylene ATP and adenylylimido-diphosphate (AMP-PNP) were purchased from Sigma Chemical Co (St Louis, MO, U.S.A.).

Electrophysiological experiments are illustrated by recordings, that are representative of the indicated number of experiments, performed with different mice. Certain changes in membrane potential were quantified manually on paper recordings. These measurements and results of ionic fluxes and insulin release are presented as means ± s.e.means.

Results

Effects of ATP and ADP

Addition of 100 μM ATP or ADP to a medium containing 15 mM glucose and 2.5 mM Ca^{2+} caused a small but rapid decrease in ^{86}Rb efflux and insulin release from perifused islets (Figure 1). These inhibitory effects were transient. At steady state, neither ^{86}Rb efflux nor insulin release was significantly

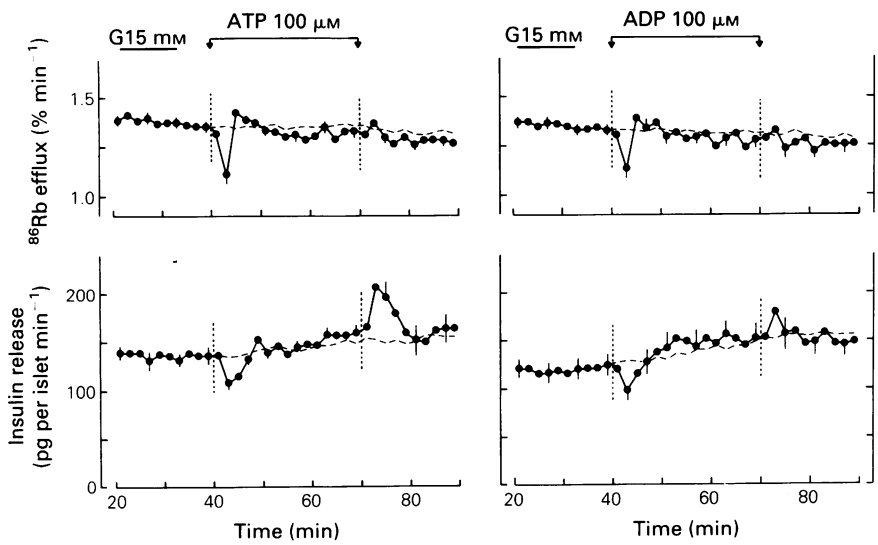

Figure 1 Effects of ATP and ADP on ^{86}Rb efflux and insulin release from mouse islets perifused with a medium containing 15 mM glucose (G). Control experiments without test substance are shown by broken lines. Values are means of 5 experiments; vertical lines show s.e.mean.

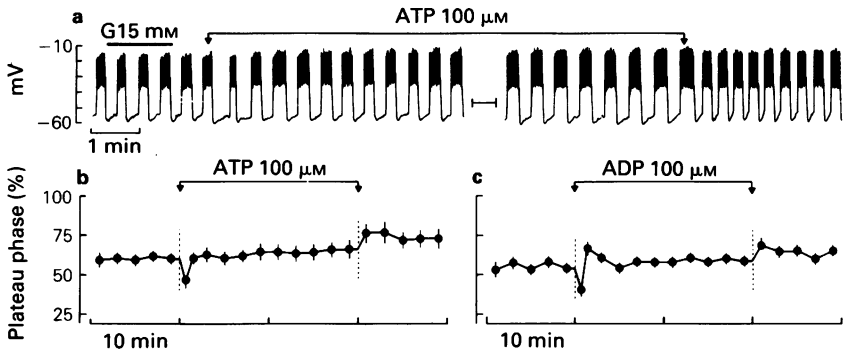

Figure 2 Effects of ATP and ADP on the membrane potential of mouse β-cells perifused with a medium containing 15 mM glucose (G). (a) This recording is representative of results obtained in 6 cells. The lower panels show the percentage of time spent at the plateau potential (with spike activity) by 6 cells stimulated with ATP (b) or ADP (c). Values are means and vertical lines show s.e.mean.

affected by the nucleotides. Removal of ATP was, however, consistently followed by a short-lived rebound of insulin release. This off-response was less marked and less consistently seen after withdrawal of ADP.

When mouse islets are perifused with 15 mM glucose, β-cells exhibit typical electrical activity consisting of repetitive slow waves of the membrane potential with bursts of rapid spikes superimposed on the plateau potential (Figure 2a). ATP consistently produced an initial decrease in this electrical activity, which was due to the shortening of one or two slow waves and the lengthening of one or two intervals. This was reflected by a decrease in the fraction of plateau phase during the first minute following addition of ATP (Figure 2b). Qualitatively and quantitatively similar changes were observed after addition of ADP to the medium (Figure 2c). These small changes are not artefacts simply produced by the change of solution, since they did not occur when the perifusion medium was alternately derived from two control solutions containing 15 mM glucose only (not shown). At steady state, the electrical activity remained marginally modified by ATP and ADP. The frequency of slow waves was slightly decreased because of a small lengthening of slow waves (Table 1). As these changes were rather small, the fraction of plateau phase was not significantly affected (Figure 2b and c). Removal of the nucleotides was, however, consistently followed by a small increase ($P < 0.05$) in electrical activity.

Table 1 Effects of ATP, ADP and analogues on the slow waves of membrane potential triggered by 15 mM glucose in mouse β-cells

Test substance (100 μM)	*Slow waves* (%)	*Intervals* (%)	*Frequency* (%)
ATP	120 ± 9	106 ± 9	90 ± 6
ADP	122 ± 6*	105 ± 7	87 ± 3*
α,β-methylene ADP	156 ± 8*	184 ± 13**	60 ± 3*
α,β-methylene ATP	165 ± 20*	135 ± 15	70 ± 8*
AMP-PNP	135 ± 6**	134 ± 24	76 ± 4**

In each experiment, slow waves and intervals were measured between 6 and 10 min after addition of the test substance. These values are expressed as a percentage of control values, measured in the same cell, during the last four min preceding the addition. For all cells, these control values were ($n = 27$): slow waves 13.0 ± 0.9 s; intervals 11.3 ± 0.9 s; frequency 2.7 ± 0.2 per min. AMP-PNP = adenylimido-diphosphate. Values are means ± s.e.mean for 5–6 cells. *$P < 0.05$, **$P < 0.01$ vs controls (by paired t test).

Effects of ATP and ADP analogues

In the presence of 15 mM glucose and 2.5 mM Ca^{2+}, α,β-methylene ADP caused a sustained and monophasic decrease in ^{45}Ca efflux and insulin release (Figure 3). The concomitant inhibition of ^{86}Rb efflux was biphasic: the initial marked fall was followed by a subsequent increase of the efflux rate to values slightly lower than in controls. In the absence of extracellular Ca, α,β-methylene ADP slightly increased ^{45}Ca efflux, more markedly accelerated ^{86}Rb efflux, and was without effect on basal insulin release (Figure 3). All these changes were reversible upon removal of α,β-methylene ADP. The analogue also modified glucose-induced electrical activity in β-cells. The membrane rapidly hyperpolarized by a few mV, while slow waves became shorter and intervals

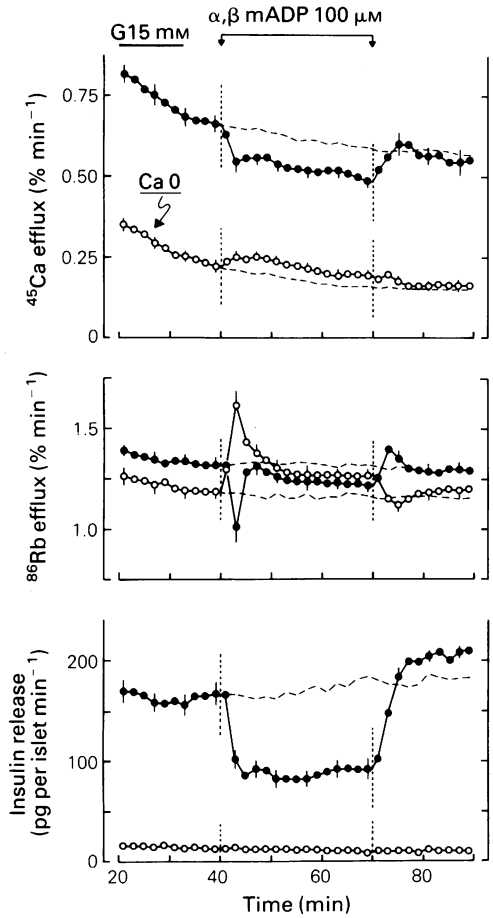

Figure 3 Effects of α,β-methylene ADP (α,β mADP) on ^{45}Ca or ^{86}Rb efflux and on insulin release from mouse islets perifused with a medium containing 15 mM glucose (G). The experiments were performed in the presence of 2.5 mM Ca^{2+} (●) or in the absence of added Ca^{2+} (see Methods) (○). Control experiments without addition of test substance are shown by broken lines. Values are means of 4–5 experiments; vertical lines show s.e.mean.

longer for 2–3 min (Figure 4a). At steady-state, the slope of the depolarization during the intervals was not so steep as in the absence of α,β-methylene ADP. However, the lengthening of the intervals was largely compensated for by a lengthening of the slow waves (Table 1). This explains why a significant inhibition of the plateau phase was only observed during the first minutes (Figure 4d). Yet, removal of the ADP analogue was followed by a consistent increase in activity with progressive resumption of the control pattern. The effects of α,β-methylene ADP were not prevented by blockade of α_2-adrenoceptors with 10 μM yohimbine (data not shown).

The changes in ^{45}Ca or ^{86}Rb efflux and in insulin release brought about by α,β-methylene ATP were qualitatively similar to those produced by α,β-methylene ADP (Figure 5), but were of smaller amplitude. The ATP analogue did not produce a rapid inhibition of the electrical activity (Figure 4b and e), but modified the characteristics of slow waves and intervals in a manner similar to that of the ADP analogue (Table 1).

Different results were obtained with AMP-PNP. This analogue slightly increased ^{45}Ca and ^{86}Rb efflux, and potentiated insulin release (Figure 6). It also caused a decrease in the frequency of slow waves, that was due to a lengthening of both slow waves and intervals (Table 1) and, hence, not accompanied by a significant change in overall electrical activity (Figure 4c and f). In the absence of extracellular Ca, 100 μM AMP-PNP had no effect on basal insulin release but still stimulated ^{86}Rb efflux; it had no consistent effect on ^{45}Ca efflux, a small initial increase being only occasionally observed (data not shown).

Since α,β-methylene ATP and AMP-PNP were used as lithium salts, control experiments were carried out to ascertain that lithium was not responsible for some of the effects. A low concentration (0.4–1 mM) of LiCl was without effect on insulin release, ^{86}Rb efflux and membrane potential (data not shown).

Discussion

The most unexpected observation of this study is that the response of mouse β-cells to ATP, ADP and their analogues is almost opposite to that of rat β-cells. Whereas all agents tested here increased insulin release from the perfused rat pancreas (Loubatières-Mariani *et al.*, 1979; Chapal & Loubatières-Mariani, 1981), only AMP-PNP produced a similar, small effect in mouse islets. The other agents rather caused a transient or sustained inhibition. This discrepancy is unlikely to be due to the isolation of the islets *per se*, since ATP, α,β-methylene ATP and α,β-methylene ADP have been found to increase insulin release in rat isolated islets (Petit *et al.*, 1987; Blachier & Malaisse, 1988). We are not aware of such a marked species-dependency of a tissue-response to purinoceptor stimulation. It is germane, however, to point out that activation of A_1-adenosine receptors causes a considerably smaller and shorter inhibition of insulin release in mouse islets (Petit *et al.*, 1988) than in rat islets (Campbell & Taylor, 1982; Bertrand *et al.*, 1989).

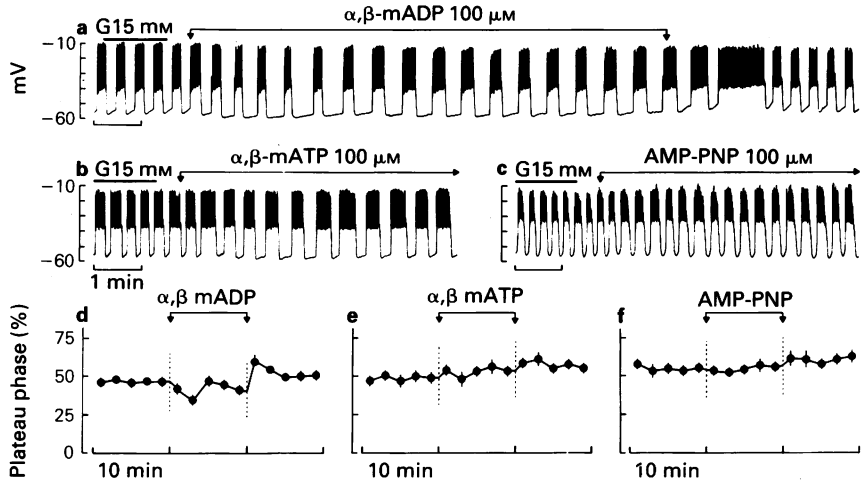

Figure 4 Effects of α,β-methylene ADP (α,β-MADP), α,β-methylene ATP (α,β mATP) and of adenylylimidodiphosphate (AMP-PNP) on the membrane potential of mouse β-cells perifused with a medium containing 15 mM glucose (G). Recordings (a), (b) and (c) are representative of results obtained in 5 cells each. The lower panels show the percentage of time spent at the plateau potential (with spike activity) in these cells. Values are means and vertical lines show s.e.mean.

A second unexpected finding is that the various putative agonists of P_2-purinoceptors that we used produced distinct effects. This may, however, be explained by their different stability.

The small effects of ATP and ADP on ^{86}Rb efflux, insulin release and β-cell membrane potential are strikingly similar to those produced by a low concentration (50 μM) of adenosine and by the adenosine analogue, N^6-(−)-phenylisopropyladenosine (Petit *et al.*, 1988). The most plausible interpretation, therefore, is that ATP and ADP are rapidly metabolized by ectonucleotidases (Gordon, 1986) and that adenosine acts on A_1-purinoceptors.

The sustained inhibitory effects of α,β-methylene ATP and α,β-methylene ADP, which cannot be metabolized to adenosine, strongly suggest the existence of a second inhibitory purinoceptor on mouse β-cells. As AMP-PNP did not mimic the effects of the methylene-analogues, one conservative hypothesis is that this second receptor has a preference for diphosphate derivatives, and recognizes α,β-methylene ATP only after partial hydrolysis. This latter would be consistent with the apparent fragility of ATP discussed above.

Finally, the small increase in insulin release produced by AMP-PNP could be best explained by a stimulatory purinoceptor with higher affinity for triphosphate derivatives. The fact that AMP-PNP did not cause the same rapid inhibitions as ATP and ADP might be explained if the analogue is not hydrolyzed to AMP (and then adenosine) because of the absence of ectopyrophosphatases on islet cells.

Obviously, our suggestion that mouse β-cells possess 3 recognition sites of purine derivatives will require further support from studies specifically designed to test this hypothesis. Except for the A_1-purinoceptor, for the existence of which separate evidence has been obtained (Petit *et al.*, 1988), it would be premature to name these sites according to the established nomenclature of purinoceptors (Burnstock & Kennedy, 1985; Gordon, 1986).

The causal links between glucose-induced electrical activity in β-cells and insulin release are well established (Henquin & Meissner, 1984). Adenine nucleotides partially dissociate these two events. A sustained inhibition of insulin release was produced by α,β-methylene ADP and α,β-methylene ATP, although the overall electrical activity was not or was only marginally decreased. A similar phenomenon was previously observed upon activation of α_2-adrenoceptors (Cook & Perara, 1982). It is striking that the reduction in slow wave frequency brought about by adrenaline was also associated with an increase in the duration of both slow waves and intervals. Moreover, the effects of adrenaline on ^{45}Ca and ^{86}Rb efflux were also similar to those produced by α,β-methylene ADP, at least in the presence of extracellular Ca^{2+} (Tamagawa & Henquin, 1983). Since the effects of the nucleotide persisted in the presence of yohimbine, one can exclude the hypothesis that it worked through α_2-adrenoceptors. The emerging conclusion is that α,β-methylene ADP, like adrenaline (through α_2-adrenoceptors), triggers an intracellular event that contributes to the inhibi-

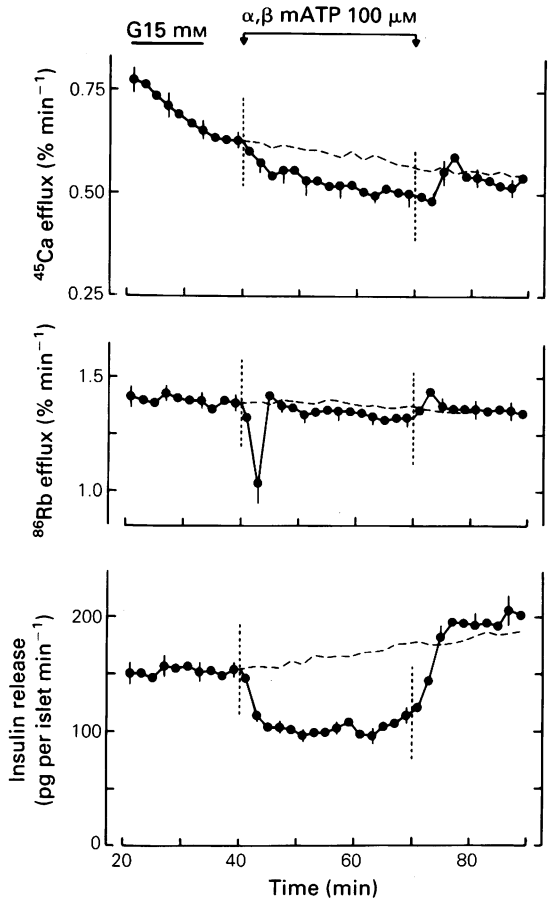

Figure 5 Effects of α,β-methylene ATP (α,β mATP) on ^{45}Ca or ^{86}Rb efflux and on insulin release from mouse islets perifused with a medium containing 15 mM glucose (G). The experiments were performed in the presence of 2.5 mM Ca^{2+}. Control experiments without addition of test substance are shown by broken lines. Values are means of 4–5 experiments; vertical lines show s.e.mean.

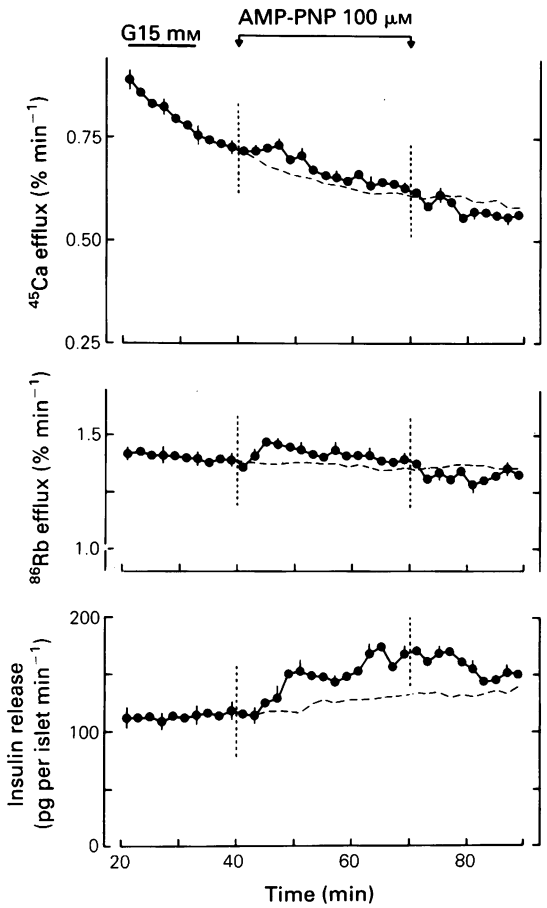

Figure 6 Effects of adenylylimido-diphosphate (AMP-PNP) on ^{45}Ca or ^{86}Rb efflux and on insulin release from mouse islets perifused with a medium containing 15 mM glucose (G). The experiments were performed in the presence of 2.5 mM Ca^{2+}. Control experiments without addition of test substance are shown by broken lines. Values are means of 4–5 experiments, vertical lines show s.e.mean.

tion of insulin release. Consistent with this conclusion is the observation that AMP-PNP affected electrical activity in a way qualitatively similar to that of α,β-methylene analogues, but increased insulin release. Evidence is indeed accumulating, which suggests that the membrane potential controls the triggering signal (Ca^{2+} influx), but that the cell response (insulin release) may be independently amplified or attenuated (Henquin *et al.*, 1987).

One important difference between the effects of α,β-methylene ADP and adrenaline is that the ADP analogue accelerated ^{86}Rb efflux in the absence of Ca^{2+}, whereas adrenaline decreased it (Tamagawa & Henquin, 1983). We consider this as good, albeit indirect, evidence that α,β-methylene ADP increases K^+ permeability in β-cells. The rapid fall in ^{86}Rb efflux that it produced in the presence of Ca^{2+} is not inconsistent with this idea. Thus, the initial hyperpolarization could result from the activation of one type of K^+ channel (ATP-sensitive?) by the nucleotide, and immediately attenuate the activation of other types of K^+ channels (voltage- and Ca^{2+}-dependent). The net effect is then a transient decrease in the efflux of ^{86}Rb. On the other hand, when Ca^{2+} influx does not occur (in Ca^{2+}-free solutions), the genuine action of the nucleotide to activate Ca^{2+}-

independent K^+ channels may be expressed by an acceleration of ^{86}Rb efflux. Activation of P_{2y}-purinoceptors leads to an increase in K^+ permeability in certain other tissues (Gallacher, 1982; Gordon & Martin, 1983).

In some tissues, activation of P_2 purinoceptors causes mobilization of intracellular Ca^{2+} (Charest *et al.*, 1985; Dubyak, 1986). This appears to be the case also in mouse islets, since α,β-methylene ADP slightly accelerated ^{45}Ca efflux in the absence of extracellular Ca. A similar conclusion was reached previously from experiments using ob/ob mouse islets, in which, however, ATP analogues triggered a more marked but less sustained increase in ^{45}Ca efflux (Gylfe & Hellman, 1987). That no insulin release occurred simultaneously is not surprising given the small magnitude of this Ca mobilization. The relationship between insulin release and Ca mobilization is non-linear (Garcia *et al.*, 1988).

In conclusion, only artificial adenine nucleotides have marked and sustained effects on insulin release by mouse islets. If one assumes that ATP and ADP released by nerve terminals within the islets or secreted with insulin exert similar effects to those of exogenously added nucleotides, the previous speculation that ATP participates in a positive feed-back control of β-cells does not hold for the mouse. Ectonucleotidases are thought to play an important role in the modulation of certain nerve cells by degrading locally released ATP to adenosine (Richardson *et al.*, 1987). Further experiments are needed to determine whether this is also the case in β-cells.

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Identification of a single α_1-adrenoceptor corresponding to the α_{1A}-subtype in rat submaxillary gland

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1 The α_1-adrenoceptors present in membranes of rat liver, cortex and submaxillary gland were labelled with [^{3}H]-prazosin and the affinity of 15 ligands for these receptors was determined.

2 In saturation studies, [^{3}H]-prazosin bound with high affinity (K_d = 30–39 pM) to a single population of sites in all three preparations.

3 In competition studies using rat cortex, evidence for heterogeneity of the α_1-adrenoceptor binding sites was obtained. Displacement isotherms for amidephrine, benoxathian, oxymetazoline, phentolamine and WB 4101 were biphasic and were consistent with the presence of both α_{1A}- and α_{1B}-adrenoceptor subtypes as described by Morrow & Creese (1986) and Han *et al.* (1987).

4 The rat liver and submaxillary gland membrane preparations both possessed homogeneous populations of α_1-adrenoceptors. However, there were pharmacological differences between the receptors in these two preparations. Rat submaxillary gland α_1-adrenoceptors displayed high affinity for amidephrine, benoxathian, oxymetazoline, phentolamine and WB 4101 and therefore appeared to represent α_{1A}-adrenoceptors. Rat liver α_1-adrenoceptors possessed lower affinity for these ligands (6–65 fold) suggesting that these receptors were of the α_{1B}-subtype.

5 Spiperone exhibited 12.9 fold higher affinity for rat liver α_{1B}-adrenoceptors than for rat submaxillary gland α_{1A}-adrenoceptor and may therefore represent the first α_{1B}-adrenoceptor selective ligand.

Introduction

The concept of α_1-adrenoceptor subtypes has arisen from both ligand binding and functional studies (See review by Minneman, 1988) although at present there is no consensus as to the number of subtypes or their definition (McGrath & Wilson, 1988).

In functional studies Rufollo *et al.* (1982), have provided evidence that there is heterogeneity of postjunctional α_1-adrenoceptors in mammalian aortae, although in these studies the existence of postsynaptic α_2-adrenoceptors may have complicated interpretation of the results (Minneman, 1988). McGrath (1982) initially proposed that postsynaptic α_1-adrenoceptors were heterogeneous and that α_{1a} and α_{1b}-adrenoceptors existed. More recently, however, McGrath & Wilson (1988) have questioned the validity of subdividing α_1-adrenoceptors. Indirect evidence for the presence of α_1-adrenoceptor subtypes has been suggested by the observation that there are considerable differences in the pA_2 values of antagonists for α_1-adrenoceptors of various tissues. Thus, depending on the preparation and agonist chosen, the reported pA_2 values for a single antagonist often extend over 2 log orders (Drew, 1985). Docherty (1987) has, however, suggested an alternative explanation for variations in antagonist pA_2 values.

In contrast to the difficulty in subclassifying the α_1-adrenoceptors identified in functional studies, in radioligand binding studies there is now relatively strong support for the concept of α_1-adrenoceptor subtypes. In rat cerebral cortex, both phentolamine and WB 4101 have been shown to produce biphasic displacement isotherms (Morrow *et al.*, 1985; Han *et al.*, 1987). Morrow & Creese (1986) have suggested that this phenomenon is due to the presence of α_{1A}- and α_{1B}-adrenoceptor subtypes.

The recent demonstration by Han *et al.* (1987), that the α_1-adrenoceptors of rat vas deferens, which

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mediate contractile responses through a calcium entry blocker-sensitive mechanism, display the pharmacology of the α_{1A}-adrenoceptor identified in binding studies, while the α_1-adrenoceptors of rat spleen, which mediate contractile responses through a calcium entry blocker-insensitive mechanism, display the pharmacology of the α_{1B}-adrenoceptor, provides additional support for the existence of α_1-adrenoceptor subtypes.

In receptor binding studies on the α_1-adrenoceptor, while rat liver and spleen α_1-adrenoceptors have been shown to be exclusively of the α_{1B}-adrenoceptor subtype (Han *et al.*, 1987), there have been no preparations described to date in which α_1-adrenoceptors are exclusively of the α_{1A}-adrenoceptor subtype. In the present study we have conducted a detailed ligand binding characterization of the α_1-adrenoceptors present in rat cortex, liver and submaxillary gland and find that the rat submaxillary gland α_1-adrenoceptors are exclusively of the α_{1A}-adrenoceptor subtype.

Methods

Membrane preparation

All membrane preparation procedures were conducted at 4°C and used ice cold buffers. Livers, cerebral cortices and submaxillary glands from adult Sprague-Dawley rats were obtained from Pel Freeze (Arkansas, U.S.A.).

Rat cortices were homogenized in a polytron P10 tissue disrupter (setting 10; two 10 s bursts) and an EDTA-washed membrane fraction was prepared as described by Cheung *et al.* (1982). Briefly, the cortical homogenate was centrifuged at 43,000 *g* for 12 min at 4°C and the crude membrane pellet obtained was washed by resuspending in homogenizing buffer and centrifuging at 43,000 *g* for 12 min. The pellet from this step was washed twice, in a similar manner, but with ice cold 50 mM Tris, 0.5 mM EDTA assay buffer (pH 7.4 at 4°C). The final pellet was resuspended in assay buffer (pH 7.4 at 25°C) and stored under liquid nitrogen until required.

The livers and submaxillary glands were separately homogenized for 30 s in 5 volumes (w/v) of 50 mM Tris, 5 mM EDTA homogenizing buffer (pH 7.4 at 4°C) in a Waring Blender at maximum setting. The homogenates obtained were further homogenized in a Polytron P10 tissue disrupter (setting 10; two 10 s bursts) and filtered through a double layer of cheesecloth. The homogenates were centrifuged at 500 *g* for 10 min and the supernatants reserved. The pellets were resuspended in 2 volumes of homogenizing buffer and rehomogenized in the polytron P10 tissue disrupter and centrifuged at 500 *g* for 10 min. The supernatants from this step were combined with the original supernatants and membrane fractions were prepared exactly as described above for the rat cortex.

Ligand binding assays

Membranes, [^{3}H]-prazosin and competing drugs were incubated in a final volume of 1 ml of assay buffer (pH 7.4 at 25°C) for 1 h at 25°C. In competition studies the final assay concentration of radioligand was 0.03–0.07 nM while in saturation studies the concentration of [^{3}H]-prazosin was varied between 0.005 and 2 nM. In all experiments non-specific binding was defined by use of 10 μM phentolamine. Incubations were terminated by vacuum filtration over 0.1% PEI pretreated glass fibre filters using a Brandel cell harvester. The filters were washed for 10 s with ice cold 0.1 M NaCl and radioactivity retained on the filters was determined by liquid scintillation spectrometry.

Protein assay

Protein was measured by the dye binding method (Bio-Rad) with bovine gamma globulin used as standard.

Data analysis

Competition and saturation binding data were analysed as described previously using iterative curve fitting techniques (Michel & Whiting, 1988). IC_{50} values were converted to K_i values by use of the Cheng-Prusoff approximation (1973).

Materials

[^{3}H]-prazosin (specific activity 82 Ci mmol^{-1}) was obtained from New England Nuclear. Phentolamine was obtained from Ciba-Geigy. Prazosin was obtained from Pfizer. Benoxathian, spiperone, spiroxatrine and WB 4101 (2-(2,6-dimethoxyphenoxyethyl)aminoethyl-1,4-benzodioxane) were obtained from Research Biochemical Inc. Amidephrine was synthesized by Dr R. Clark, IOC, Syntex. All other chemicals and reagents were obtained from Sigma chemical company.

Results

Kinetic studies

In rat cortex, liver and submaxillary gland, [^{3}H]-prazosin binding achieved equilibrium within 15–30 min at a concentration of 0.03 nM (the lowest con-

Table 1 Affinity of ligands for α_1-adrenoceptors of rat cortex, liver and submaxillary gland

Ligand	*Submaxillary gland* pK_i/nH	*Cortex* pK_i/nH	*Liver* pK_i/nH	*Selectivity ratio**
Amidephrine	6.25 (0.02)	5.20 (0.10)	4.74 (0.04)	32.4
	0.81 (0.01)	0.62 (0.02)	0.99 (0.03)	
Benoxathian	9.67 (0.04)	8.67 (0.04)	8.35 (0.06)	20.9
	0.97 (0.08)	0.69 (0.01)	1.02 (0.05)	
Chlorpromazine	9.03 (0.14)	ND	9.46 (0.11)	0.4
	1.11 (0.11)		1.03 (0.04)	
Clonidine	6.90 (0.10)	6.45 (0.13)	6.32 (0.05)	3.8
	0.96 (0.06)	0.79 (0.08)	1.04 (0.07)	
Corynanthine	7.47 (0.02)	ND	7.28 (0.02)	1.5
	0.98 (0.06)		0.90 (0.02)	
Idazoxan	6.54 (0.16)	6.38 (0.08)	6.29 (0.05)	1.8
	1.01 (0.06)	0.85 (0.09)	1.00 (0.02)	
Imiloxan	5.14 (0.06)	ND	5.17 (0.17)	0.9
	0.80 (0.02)		0.80 (0.03)	
Noradrenaline	6.59 (0.05)	6.09 (0.10)	6.53 (0.10)	1.1
	0.95 (0.02)	0.66 (0.08)	0.88 (0.02)	
Oxymetazoline	8.49 (0.04)	7.34 (0.13)	6.68 (0.05)	64.6
	0.82 (0.03)	0.54 (0.02)	1.02 (0.04)	
Phentolamine	8.85 (0.13)	8.33 (0.10)	8.07 (0.05)	6.0
	0.94 (0.01)	0.72 (0.02)	1.07 (0.05)	
Rauwolscine	6.33 (0.15)	6.29 (0.06)	6.63 (0.05)	0.5
	1.01 (0.08)	0.90 (0.06)	0.99 (0.02)	
Spiperone	8.14 (0.01)	ND	9.25 (0.04)	0.08
	0.98 (0.01)		1.01 (0.02)	
Spiroxatrine	7.41 (0.10)	ND	7.54 (0.10)	0.7
	1.06 (0.08)		1.01 (0.08)	
WB 4101	9.98 (0.08)	9.19 (0.17)	8.78 (0.05)	15.8
	0.85 (0.03)	0.69 (0.07)	1.04 (0.04)	
Xylometazoline	7.87 (0.08)	ND	6.58 (0.07)	19.5
	0.96 (0.08)		1.08 (0.09)	

Values are the mean (±s.e.mean) of the Hill coefficient (nH) or the negative logarithm of the K_i (pK_i). * Selectivity ratio is the antilog (pK_i submaxillary gland − pK_i liver). ND = not determined.

centration used in competition studies) and was stable for up to 2 h of further incubation.

In more detailed kinetic studies carried out with rat cortical and liver membranes, the rate constants for association were 3.35×10^8 and $5.73 \times 10^8 \text{ min}^{-1} \text{ M}^{-1}$, respectively. Dissociation of the radioligand from these preparations was slow with half lives of 29 min and 38 min in rat cortex and rat liver, respectively. Both the association and dissociation data were consistent with the presence of only a single population of binding sites. The kinetically derived K_d values for rat cortex and rat liver were 0.07 and 0.03 nM, respectively.

Saturation binding studies

The binding of [^{3}H]-prazosin in rat cortex, liver and submaxillary gland was saturable and of high affinity. Binding was consistent with the presence of a single population of sites in each of the three preparations examined. In rat cortex, liver and submaxillary gland K_d values of 59 ± 8.2 pM, 31 ± 3.5 pM and 39 ± 2.7 pM, respectively, were obtained. The values in rat liver and cortex were in good agreement with the kinetically derived K_d values obtained above. In the rat cortex, liver and submaxillary gland, B_{max} values were 70 ± 7 fmol mg^{-1} protein, 192 ± 10 fmol mg^{-1} protein and 193 ± 9 fmol mg^{-1} protein, respectively.

Competition studies

Fifteen ligands were evaluated for their affinity at α_1-adrenoceptors present in rat cortex, liver and submaxillary gland. The data are presented in Table 1 and representative competition curves for amidephrine, oxymetazoline and WB 4101 are shown in Figure 1. In rat liver and rat submaxillary gland the nH values for all compounds were close to unity. Amidephrine, benoxathian, oxymetazoline, phentolamine and WB4101 displayed 6–65 fold higher affinity in rat submaxillary gland than in rat liver. In

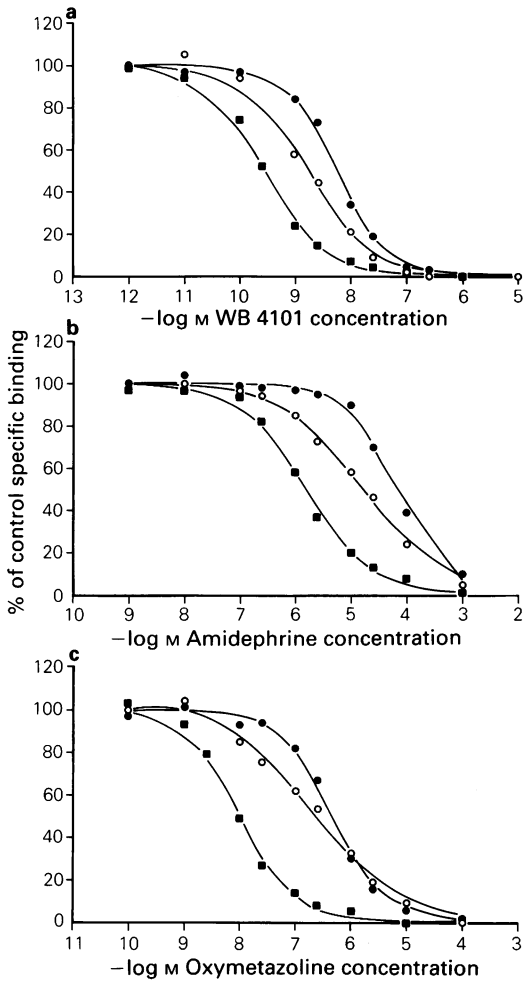

Figure 1 Competition of WB 4101 (a), amidephrine (b) and oxymetazoline (c) for [^{3}H]-prazosin binding sites present in rat cortex (○), liver (●) and submaxillary gland (■) membranes. In rat cortex, liver and submaxillary gland, total binding was 1230, 1401 and 980 d.p.m, respectively, while non-specific binding was 54, 43 and 65 d.p.m, respectively.

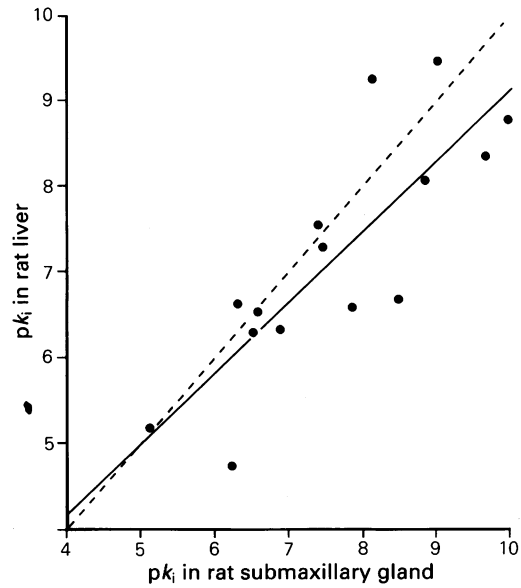

Figure 2 Correlation between ligand affinity estimates obtained at the α_1-adrenoceptors of rat liver and rat submaxillary gland labelled with [^{3}H]-prazosin. The dashed line represents the line of identity, while the solid line represents the regression line. The data were best described by the expression $y = 0.826x + 0.868$ ($r = 0.82$).

contrast, spiperone displayed 12.9 fold higher affinity in rat liver than in rat submaxillary gland. In Figure 2 affinity data obtained in the rat liver and rat submaxillary gland are compared. This analysis reveals that the correlation between affinity estimates at the two α_1-adrenoceptor binding sites was poor ($r = 0.82$).

In rat cortex the nH values for amidephrine, benoxathian, oxymetazoline, phentolamine and WB 4101 were significantly less than unity and the data could be better described by assuming the presence of two populations of [^{3}H]-prazosin binding sites for which these compounds displayed affinities that differed 19–58 fold (Table 2). The two populations of sites were present in approximately equal proportions at the concentration of [^{3}H]-prazosin used in this study and consequently it was difficult to determine which of the two sites corresponded to the α_{1A}- and α_{1B}-adrenoceptors previously described. Consequently, in Table 2 the sites displaying high affinity for the compounds were termed site 1 while the remaining low affinity sites were termed site 2.

Comparing the affinity estimates of amidephrine, benoxathian, oxymetazoline, phentolamine and WB 4101 at the two sites identified in rat cortex with the affinity data obtained in rat submaxillary gland and liver, it would appear that the affinity estimates of these compounds for site 1 in rat cortex were in good agreement with the affinity estimates obtained in rat submaxillary gland, while affinity estimates for site 2 in rat cortex were in good agreement with the affinity estimates obtained in rat liver.

Even though the nH values of displacement curves for noradrenaline, clonidine and idazoxan were less than unity in rat cortex, it should be noted that these data could not be consistently fitted to a two site model of binding.

Table 2 Binding parameters of ligands at the α_1-adrenoceptor binding sites of rat cortex

		2 Site model	
	Hill model	*Site 1*	*Site 2*
	pK_i	pK_i	pK_i
Ligand	nH	(% of sites)	(% of sites)
Amidephrine	5.20 (0.01)	5.97 (0.13)	4.49 (0.06)
	0.62 (0.02)	52.6 (5.20)	47.4 (5.20)
Clonidine	6.45 (0.13)	ND	
	0.79 (0.08)		
Noradrenaline	6.09 (0.10)	ND	
	0.66 (0.08)		
Oxymetazoline	7.34 (0.13)	8.32 (0.05)	6.55 (0.05)
	0.54 (0.02)	48.0 (5.40)	52.0 (5.40)
Benoxathian	8.67 (0.04)	9.69 (0.25)	8.26 (0.09)
	0.69 (0.01)	42.3 (3.30)	56.7 (3.30)
Idazoxan	6.38 (0.08)	ND	
	0.85 (0.09)		
Phentolamine	8.33 (0.10)	8.98 (0.14)	7.69 (0.17)
	0.72 (0.02)	58.9 (3.20)	41.1 (3.20)
Prazosin	9.91 (0.14)	ND	
	0.94 (0.05)		
Rauwolscine	6.29 (0.06)	ND	
	0.90 (0.06)		
WB 4101	9.19 (0.17)	9.82 (0.16)	8.53 (0.09)
	0.69 (0.07)	54.5 (3.80)	45.5 (3.80)

The values shown are the mean (±s.e.mean; $n = 4$–6) for the negative logarithm of the *Ki* (pK_i) and the hill coefficient (nH). In rat cortex the data were better described by assuming [^{3}H]-prazosin to label two populations of binding sites which amidephrine, benoxathian, oxymetazoline, phentolamine and WB 4101 could differentiate. ND = 2 site fit not possible.

Discussion

The results of the present study add additional support to the concept of α_1-adrenoceptor subtypes. The data obtained in the present study substantiate previous studies (Morrow & Creese, 1986; Han *et al.*, 1987) which have shown that benoxathian, phentolamine and WB 4101 differentiate two populations of binding sites in rat cortex. In the present study, the two populations of binding sites, which presumably represented the α_{1A}- and α_{1B}-adrenoceptors, were present in approximately equal proportions. Thus, it was difficult to determine unequivocally which of the sites, designated as site 1 and site 2, corresponded to the α_{1A}- and α_{1B}-adrenoceptor.

In addition to benoxathian, phentolamine and WB 4101, the agonist ligands amidephrine, oxymetazoline and xylometazoline also identified two populations of sites in rat cortex. As with the data obtained by use of the antagonists, the two sites discriminated by the agonists were present in equal proportions.

In order to determine unequivocally the selectivity of the agonist and antagonist compounds for the putative α_{1A}- and α_{1B}-adrenoceptor subtypes, they were further evaluated in a range of other tissues in an attempt to determine if preparations which displayed homogeneous populations of the two putative binding sites could be identified. Of the tissues examined, the rat liver and rat submaxillary gland appeared to be the most interesting for detailed study.

Han *et al.* (1987) have previously shown that rat liver α_1-adrenoceptors labelled with [^{125}I]-BE2254 are homogeneous and that they correspond to the α_{1B}-adrenoceptor subtype. The data obtained in the present study in which a range of 15 ligands was used support this contention since all compounds tested appeared to interact with only a single population of α_1-adrenoceptors. Furthermore, affinity estimates for benoxathian, phentolamine and WB 4101 in rat liver were very similar to their affinity for the site 2 α_1-adrenoceptors of rat cortex. Since benoxathian, phentolamine and WB 4101 display low affinity for the α_{1B}-adrenoceptor (Han *et al.*, 1987) it would appear that the low affinity component of binding in rat cortex, site 2, represented the α_{1B}-adrenoceptor. The data obtained with the agonists, amidephrine and oxymetazoline, also support this contention. By inference these results would indicate that site 1 α_1-adrenoceptors of rat cortex correspond to α_{1A}-adrenoceptors.

Rat submaxillary gland α_1-adrenoceptors have not been examined in detail before. Morrow & Creese (1986) have suggested that both α_{1A}- and α_{1B}-adrenoceptors are present in this tissue since the ratio of the affinity estimates for prazosin and phentolamine obtained in this tissue by Elverdin *et al.* (1984) is relatively high. The results of the present study would indicate, however, that rat submaxillary gland α_1-adrenoceptors were homogeneous, and that these α_1-adrenoceptors differed from those present in rat liver. The observation that affinity estimates for amidephrine, benoxathian, oxymetazoline, phentolamine and WB 4101 in rat submaxillary gland were almost identical to those obtained at site 1 in rat cortex (α_{1A}) would suggest that rat submaxillary gland α_1-adrenoceptors were therefore of the α_{1A}-subtype.

In this respect it is interesting to note that in rat submandibular gland Bylund *et al.* (1982) have shown that phentolamine and WB 4101 display high affinity for [^{3}H]-prazosin binding sites. Based upon these findings Morrow & Creese (1986) have proposed that these receptors are of the α_{1A}-adrenoceptor subtype.

It is clear from the present study that while the sub-classification of α_1-adrenoceptors into α_{1A}- and α_{1B}-adrenoceptor subtypes based upon their relative affinities for phentolamine and prazosin can be useful (Morrow & Creese, 1986), a more rigorous pharmacological classification would be desirable.

Such a classification requires the availability of selective ligands for the putative receptor subtypes. Previous studies have shown that benoxathian (an analogue of WB 4101), phentolamine and WB 4101 can discriminate between α_1-adrenoceptor subtypes (Han *et al.*, 1987). In addition, 5 methyl-urapidil has also been reported as a selective α_{1A}-adrenoceptor ligand (Gross *et al.*, 1988). The results of the present study suggest that the α_1-adrenoceptor agonists amidephrine, oxymetazoline and xylometazoline (a close analogue of oxymetazoline) can also discriminate α_{1A}- from α_{1B}-adrenoceptors. While interpretation of affinity estimates for these compounds is complicated by their agonist properties, the failure of guanine-nucleotides or sodium ions to modulate either their affinity or their selectivity for submaxillary gland over liver α_1-adrenoceptors (unpublished observations), would suggest that the selectivity of the agonists was not due to the presence of affinity states of the α_1-adrenoceptor. In this respect it is also relevant to note that neither noradrenaline (Table 1) nor phenylephrine (unpublished observations) was able to discriminate between the rat liver and rat submaxillary gland α_1-adrenoceptors.

With respect to the ability of oxymetazoline to discriminate between α_{1A}- and α_{1B}-adrenoceptors, this property may explain the previous finding that oxymetazoline revealed heterogeneity of [^{125}I]-BE 2254 binding to rat cortical membranes (Minneman, 1983). Indeed, Minneman (1988) has recently indicated that oxymetazoline is capable of differentiating between α_1-adrenoceptor subtypes.

At present, we are not aware of any α_{1B}-adrenoceptor selective ligands. In the present study spiperone possessed 12.9 fold higher affinity for the rat liver α_{1B}-adrenoceptor than for the rat submaxillary gland α_{1A}-adrenoceptor and may therefore represent the first α_{1B}-selective ligand. It should be noted that this compound is not a specific α_1-adrenoceptor ligand since it also displays high affinity for several other receptors, most notably the dopamine D_2-receptor and the 5-HT_2-receptors.

Specificity of action is also a problem with respect to the α_{1A}-adrenoceptor subtype selective ligands. Thus, WB 4101 is also a 5-HT_{1A} ligand (Morrow & Creese, 1986) and possesses high affinity for α_{2A}-adrenoceptors (Cheung *et al.*, 1982). Phentolamine displays high affinity for α_2-adrenoceptors of rat submaxillary gland (unpublished observations) while oxymetazoline is a potent ligand at α_2-adrenoceptor subtypes (Bylund, 1985). The availability of a receptor binding assay for both the α_{1A}- and α_{1B}-adrenoceptor subtypes should aid in the development of the more selective and specific ligands which will be required for functional evaluation of the physiological role of the putative α_1-adrenoceptor subtypes.

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Differences between the α_2-adrenoceptor in rat submaxillary gland and the α_{2A}- and α_{2B}-adrenoceptor subtypes

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1 The α_2-adrenoceptors of rat submaxillary gland, labelled with [^{3}H]-rauwolscine, were characterized by use of a range of subtype selective ligands and were compared to rabbit spleen α_{2A}-adrenoceptors and rat kidney α_{2B}-adrenoceptors.

2 In rat submaxillary gland, [^{3}H]-rauwolscine labelled an apparently homogeneous population of binding sites with relatively low affinity (K_d = 11.65 nM) compared to the affinity in rat kidney (K_d = 2.18 nM) and rabbit spleen (K_d = 4.64 nM).

3 In competition studies using 16 ligands the α_2-adrenoceptors in rat submaxillary gland appeared to differ from both the α_{2A}-adrenoceptor of rabbit spleen (r = 0.62) and also the α_{2B}-adrenoceptor of rat kidney (r = 0.28).

4 The affinity data obtained with benoxathian, imiloxan and WB 4101 indicated the presence of an α_{2B}-adrenoceptor in rat submaxillary gland. However, data for chlorpromazine, oxymetazoline, spiroxatrine and xylometazoline indicated that submaxillary gland α_2-adrenoceptors were of the α_{2A} subtype. The affinity estimate for prazosin in rat submaxillary gland was intermediate between its affinity at the α_{2A}- and α_{2B}-adrenoceptors while affinity estimates for idazoxan and phentolamine in rat submaxillary gland were greater than those obtained at either the α_{2A}- or α_{2B}-adrenoceptor.

5 These data indicate that rat submaxillary gland α_2-adrenoceptors differ from the α_{2A}- and α_{2B}-adrenoceptors found in rabbit spleen and rat kidney, respectively.

Introduction

Recent studies on the ligand binding properties and molecular biology of the α_2-adrenoceptor have indicated that α_2-adrenoceptor subtypes exist. Regan *et al.* (1988) have cloned two genes that encode α_2-adrenoceptors and have shown that these two receptors can be differentiated on the basis of their affinity for a range of ligands. These molecular biological studies complement the data obtained from ligand binding studies conducted in several laboratories (Nahorski *et al.*, 1985; Bylund, 1985; 1988) which have demonstrated that α_2-adrenoceptors can be separated into α_{2A} and α_{2B} adrenoceptor subtypes depending upon their affinity for a range of ligands. According to the present classification scheme, α_{2A}-adrenoceptors display high affinity for oxymetazoline and WB 4101 and low affinity for prazosin and chlorpromazine, while α_{2B}-adrenoceptors display the converse selectivity for these agents (Bylund, 1988).

We have previously demonstrated that rabbit spleen and rat kidney possess α_2-adrenoceptors that display the pharmacology of the α_{2A}- and α_{2B}-adrenoceptor, respectively (Michel *et al.*, 1989). In the present study we extend our characterization of the rabbit spleen and rat kidney α_2-adrenoceptors and report on the characterization of an α_2-adrenoceptor subtype present in rat submaxillary gland that displays significant differences from the α_{2A}- and α_{2B}-adrenoceptors described to date.

Methods

Membrane preparation

All membrane preparation procedures were conducted at 4°C and used ice cold buffers. Kidneys and

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submaxillary glands from adult Sprague-Dawley rats and spleens from adult New Zealand White rabbits were obtained from Pel-Freeze (Arkansas, U.S.A.). Tissues were homogenized for 30 s in 3 volumes (w/v) of 50 mM Tris, 5 mM EDTA homogenizing buffer (pH 7.4 at 4°C) in a Waring Blender at maximum setting. The homogenates obtained were further homogenized in a Polytron P10 tissue disrupter (setting 10; two 10 s bursts) and filtered through a double layer of cheesecloth. The homogenates were centrifuged at 500 *g* for 10 min and the supernatants reserved. The pellets were resuspended in 2 volumes of homogenizing buffer and rehomogenized in the polytron P10 tissue disrupter and centrifuged at 500 *g* for 10 min. The supernatants from this step were combined with the original supernatants and centrifuged for 12 min at 43,000 *g*. The crude membrane pellets obtained were washed by resuspending in homogenizing buffer and centrifuging at 43,000 *g* for 12 min. The pellets from this step were washed twice, in a similar manner, but with ice cold 50 mM Tris, 0.5 mM EDTA assay buffer (pH 7.4 at 4°C). The final pellets were resuspended in assay buffer (pH 7.4 at 25°C) and stored under liquid nitrogen until required.

Ligand binding assays

Membranes, [^{3}H]-rauwolscine and competing drugs were incubated in a final volume of 0.25 ml of assay buffer (pH 7.4 at 25°C) for 45 min at room temperature (22°C). In competition studies the final assay concentration of radioligand was 1–2 nM while in saturation studies the concentration of [^{3}H]-rauwolscine was varied between 0.1 and 105 nM. In all experiments non-specific binding was defined by use of 10 μM phentolamine. Incubations were terminated by vacuum filtration over 0.1% polyethyleneimine pretreated glass fibre filters using a Brandel cell harvester. The filters were washed for 10 s with ice cold 0.1 M NaCl and radioactivity retained on the filters was determined by liquid scintillation spectrometry.

Protein was determined by the dye binding method (Bio-Rad) with bovine gamma globulin as standard.

Data analysis

Competition and saturation binding data were analysed as described previously by use of iterative curve fitting techniques (Michel & Whiting, 1988). IC_{50} values were converted to K_i values using the Cheng-Prusoff approximation (Cheng & Prusoff, 1973).

Materials

[^{3}H]-rauwolscine (Specific activity 82 Ci mmol^{-1}) was obtained from New England Nuclear. Chlorpromazine, noradrenaline and oxymetazoline were obtained from Sigma chemical company as were all chemicals and reagents used. Phentolamine was obtained from Ciba-Geigy while prazosin was obtained from Pfizer. Benoxathian, spiroxatrine and WB 4101 (2-(2,6-dimethoxyphenoxyethyl) aminomethyl-1,4-benzodioxane) were obtained from Research Biochemical Inc. Idazoxan, imiloxan, UK 14304 (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline bitartrate) and SKF 104078 (6-chloro-9-[(3-methyl-2-butenyl)oxyl-3-methyl-1H-2,3,4,5-tetrahydro-3-benzazepine) were synthesized by Dr R. Clark, IOC, Syntex.

Results

Saturation studies

Results of saturation binding studies conducted in rat kidney, rat submaxillary and rabbit spleen membranes are presented graphically in Figure 1. In rat submaxillary gland, [^{3}H]-rauwolscine labelled a single population of binding sites ($B_{max} = 137.5 \pm 12.2$ fmol mg^{-1} protein; $n = 4$) with low affinity ($K_d = 11.65 \pm 1.0$ nM). The K_d value obtained in this preparation was significantly higher than the K_d values obtained in either rat kidney ($K_d = 2.18 \pm 0.46$ nM; $n = 4$) or rabbit spleen ($K_d = 4.64 \pm 0.78$ nM; $n = 4$).

Competition studies

Affinity estimates for a range of compounds at α_2-adrenoceptors of rat submaxillary gland were determined in competition studies. These results are presented in Table 1 along with affinity estimates obtained in both rat kidney and rabbit spleen. Results in rat kidney and rabbit spleen for several of the ligands have been described previously (Michel *et al.*, 1989). Representative displacement isotherms for idazoxan, spiroxatrine and WB 4101 in the three tissues are shown in Figure 2.

Displacement isotherms for most compounds displayed Hill coefficients that were close to unity and the data could only be described assuming the presence of a single population of binding sites. For WB 4101 and oxymetazoline in rabbit spleen, and for oxymetazoline in rat kidney, Hill coefficients were less than unity and we have previously shown that these data are better described by assuming the presence of two populations of binding sites (Michel *et al.*, 1989). WB 4101 and oxymetazoline displayed

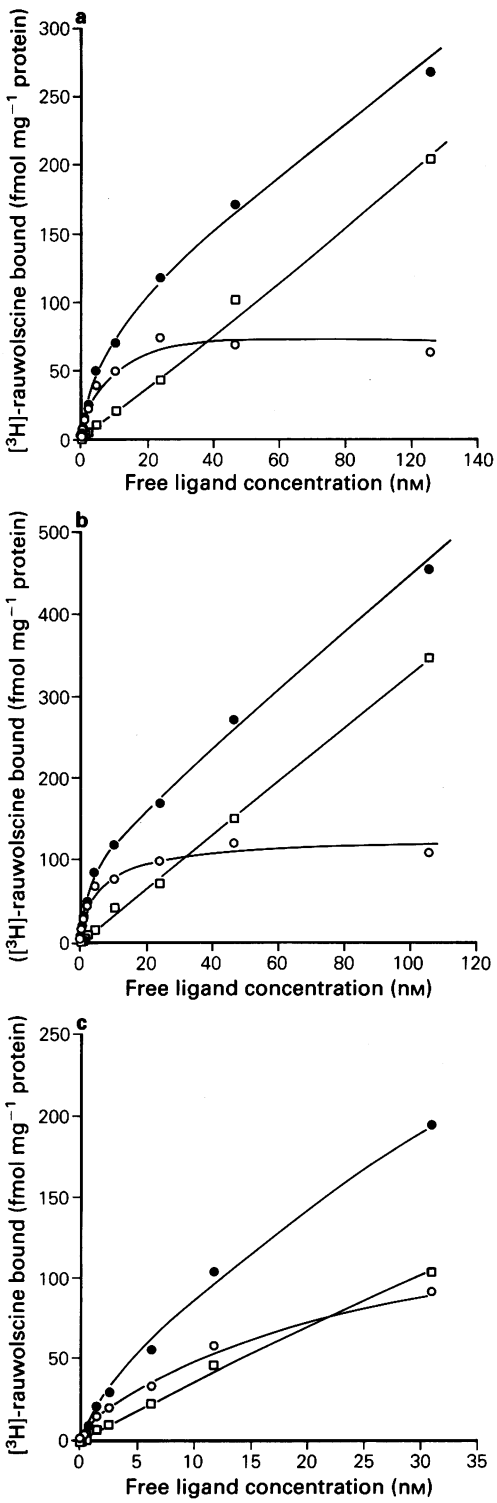

Figure 1 Saturation binding of [^{3}H]-rauwolscine in rabbit spleen (a), rat kidney (b) and rat submaxillary gland (c). The figures show total (●), specific (○) and nonspecific binding (□) of [^{3}H]-rauwolscine plotted as a function of the free ligand concentration. In the rabbit spleen, rat kidney and rat submaxillary gland, protein was present at 214, 198 and 138 μg, respectively, B_{max} values were 132, 141 and 140 fmol mg^{-1} protein, respectively, and K_d values were 4.6, 2.8, and 12.7 nM, respectively. In all cases the lines were drawn by hand and do not correspond to the best fit regression line.

pK_i values of 8.71 and 8.60, respectively, for 70–75% of the α_2-adrenoceptors labelled using [^{3}H]-rauwolscine in rabbit spleen.

The α_2-adrenoceptor of rat submaxillary gland displayed relatively high affinity for imiloxan with the pK_i value being similar to that obtained at the α_{2B}-adrenoceptor of rat kidney. For benoxathian and WB 4101, affinity estimates in rat submaxillary gland were also similar to those obtained at the α_{2B}-adrenoceptor of the rat kidney.

For oxymetazoline, spiroxatrine and xylometazoline, affinity estimates in rat submaxillary gland were similar to those obtained at the α_{2A}-adrenoceptor in the rabbit spleen.

Finally, for idazoxan and phentolamine, affinity estimates at the rat submaxillary gland α_2-adrenoceptor were significantly higher than those obtained at either the α_{2A}- or α_{2B}-adrenoceptors. For prazosin, the affinity estimate in rat submaxillary gland was intermediate between affinity estimates obtained at the α_{2A}- and α_{2B}-adrenoceptor.

Correlation between affinity estimates obtained in rat kidney, rat submaxillary gland and rabbit spleen

In Figures 3, 4 and 5 correlations between affinity estimates obtained in the three preparations are presented. As can be seen from Figure 3, there was a poor correlation ($r = 0.066$) between affinity estimates obtained in rat kidney and rabbit spleen. Furthermore, 7 of the compounds examined displayed greater than 10 fold selectivity for one or the other of the α_2-adrenoceptors compared (Table 1, column A).

Rat submaxillary gland α_2-adrenoceptors differed substantially ($r = 0.276$) from the α_{2B}-adrenoceptor of rat kidney (Figure 4) with 4 compounds displaying greater than 10 fold selectivity for either the rat kidney α_{2B}-adrenoceptor or rat submaxillary gland α_2-adrenoceptor (Table 1 column C).

The rat submaxillary gland α_2-adrenoceptor displayed some similarity to the rabbit spleen α_2-adrenoceptor ($r = 0.62$) although 4 of the compounds displayed greater than a 10 fold difference in affinity between these two α_2-adrenoceptors (Figure 5; Table 1, column B).

Table 1 Affinity of ligands for α_2-adrenoceptors of rabbit spleen, rat kidney and rat submaxillary gland

Ligand	*Rabbit spleen* pK_i/nH	*Rat kidney* pK_i/nH	*Rat submaxillary* pK_i/nH	*Selectivity ratio* A	B	C
Benoxathian	7.65 (0.10)	6.37 (0.01)	6.50 (0.04)	0.05	14.1	0.74
	0.88 (0.07)	1.12 (0.05)	0.99 (0.04)			
Chlorpromazine	6.06 (0.02)	7.28 (0.03)	5.98 (0.15)	16.6	1.2	20.0
	1.01 (0.05)	1.06 (0.03)	1.02 (0.07)			
Clonidine	6.81 (0.12)	7.24 (0.04)	7.20 (0.06)	2.7	0.41	1.1
	0.83 (0.04)	0.83 (0.04)	0.83 (0.02)			
Corynanthine	6.36 (0.03)	6.53 (0.01)	5.81 (0.05)	1.5	3.5	5.2
	1.02 (0.02)	0.99 (0.08)	0.87 (0.01)			
Idazoxan	7.12 (0.02)	7.29 (0.02)	8.25 (0.07)	1.5	0.07	0.11
	0.96 (0.11)	0.83 (0.06)	0.79 (0.10)			
Imiloxan	5.52 (0.03)	7.26 (0.03)	7.01 (0.03)	55.0	0.03	1.8
	0.92 (0.05)	0.97 (0.02)	0.96 (0.12)			
Noradrenaline	6.37 (0.10)	6.98 (0.03)	6.22 (0.01)	4.1	1.4	5.8
	0.81 (0.05)	0.78 (0.04)	0.70 (0.04)			
Oxymetazoline	8.11 (0.04)	6.60 (0.02)	8.06 (0.04)	0.03	1.1	0.03
	0.74 (0.04)	0.71 (0.02)	0.78 (0.07)			
Phentolamine	7.78 (0.06)	7.97 (0.09)	8.47 (0.09)	1.5	0.20	0.32
	0.92 (0.04)	1.02 (0.04)	0.88 (0.06)			
Prazosin	5.33 (0.08)	7.22 (0.07)	6.34 (0.06)	77.6	0.10	7.6
	0.93 (0.08)	0.96 (0.04)	1.05 (0.04)			
Rauwolscine	8.06 (0.10)	8.46 (0.10)	7.73 (0.01)	2.5	2.1	5.4
	0.92 (0.11)	0.86 (0.07)	0.89 (0.02)			
SKF 104078	7.00 (0.04)	6.96 (0.05)	6.51 (0.16)	0.91	3.1	2.8
	1.03 (0.06)	1.22 (0.03)	0.97 (0.04)			
Spiroxatrine	7.03 (0.18)	8.47 (0.13)	6.60 (0.10)	27.5	2.7	74.1
	0.90 (0.06)	0.85 (0.04)	0.94 (0.04)			
UK 14304	7.46 (0.08)	6.61 (0.01)	6.80 (0.02)	0.14	4.6	0.6
	0.76 (0.04)	0.86 (0.07)	0.87 (0.12)			
WB 4101	8.17 (0.09)	7.46 (0.04)	7.22 (0.09)	0.19	8.9	1.7
	0.67 (0.04)	1.02 (0.06)	0.96 (0.03)			
Xylometazoline	7.98 (0.06)	6.60 (0.03)	7.70 (0.04)	0.04	1.9	0.08
	0.84 (0.17)	0.76 (0.04)	0.78 (0.02)			

Values are the mean (±s.e.mean) of the Hill coefficient (nH) or the negative logarithm of the affinity constant (pK_i) from between 3 and 9 experiments. A = Antilog (pK_i rat kidney − pK_i rabbit spleen). B = Antilog (pK_i rabbit spleen − pK_i rat submaxillary gland). C = Antilog (pK_i rat kidney − pK_i rat submaxillary gland).

Discussion

The main finding of the present study was that the α_2-adrenoceptor found in rat submaxillary gland differed from the α_{2A}- and α_{2B}-adrenoceptor subtypes already identified in ligand binding studies (Nahorski *et al.*, 1985; Bylund, 1985). The evidence for this was based upon paradoxical differences between ligand affinities obtained in rat submaxillary gland and those obtained in rabbit spleen and rat kidney.

In saturation studies, [^{3}H]-rauwolscine bound with low affinity to a homogeneous population of binding sites in rat submaxillary gland. The demonstration that the K_d value obtained in this preparation was significantly higher than the K_d obtained in either rabbit spleen or rat kidney would suggest that rat submaxillary gland α_2-adrenoceptors differ from the α_{2A}- and α_{2B}-adrenoceptor subtypes identified in rabbit spleen and rat kidney. More definitive evidence for differences between the rat submaxillary gland α_2-adrenoceptor and the α_{2A}- and α_{2B}-adrenoceptor subtypes was obtained in competition studies.

In rat submaxillary gland, the affinity estimates obtained for imiloxan, benoxathian and WB 4101 (ligands which can clearly distinguish the α_{2A}- and α_{2B}-adrenoceptor subtypes) were similar to their respective affinities for the rat kidney α_{2B}-adrenoceptor. While these data would suggest that the rat submaxillary gland α_2-adrenoceptor was of the α_{2B}-subtype, affinity estimates for the subtype selective ligands oxymetazoline, xylometazoline and chlorpromazine indicated the presence of an α_{2A}-adrenoceptor.

To complicate further the classification of rat sub-

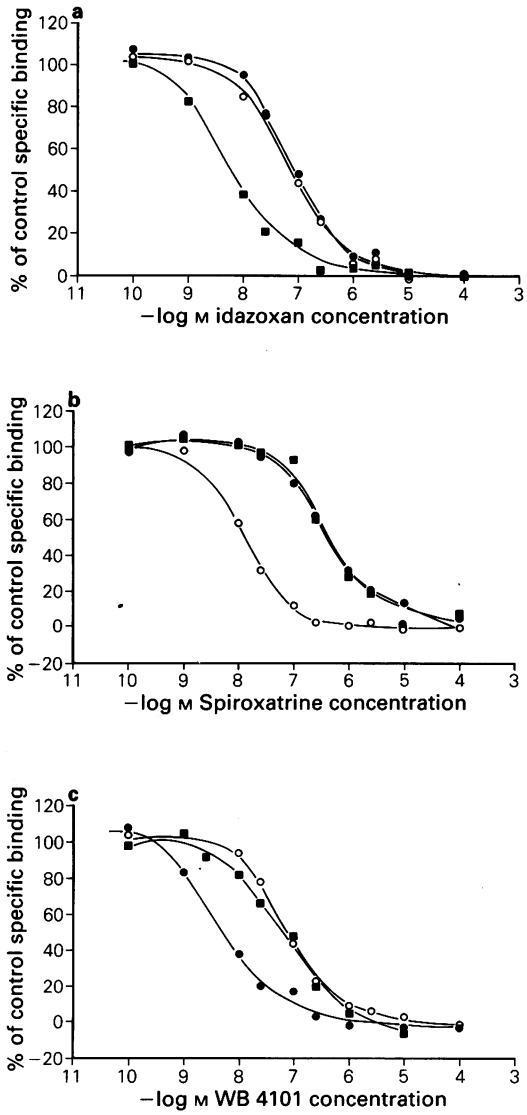

Figure 2 Displacement of [^{3}H]-rauwolscine binding from rabbit spleen (●), rat kidney (○) and rat submaxillary gland (■) by either idazoxan, spiroxatrine or WB 4101. Data for idazoxan (a); data for spiroxatrine (b); data for WB 4101 (c). In all cases membranes, [^{3}H]-rauwolscine (1–2 nM), and various concentrations of the competitors were incubated for 45 min at 22°C before separating bound and free ligand by vacuum filtration. The data are from a single representative experiment. In rabbit spleen, total binding was 1109 d.p.m and nonspecific binding (NSB) was 245 d.p.m. In rat kidney total binding was 1287 d.p.m and NSB was 267 d.p.m. In rat submaxillary gland total binding was 980 d.p.m and NSB was 375 d.p.m. In all cases the lines were drawn by hand and do not correspond to the best fit regression line.

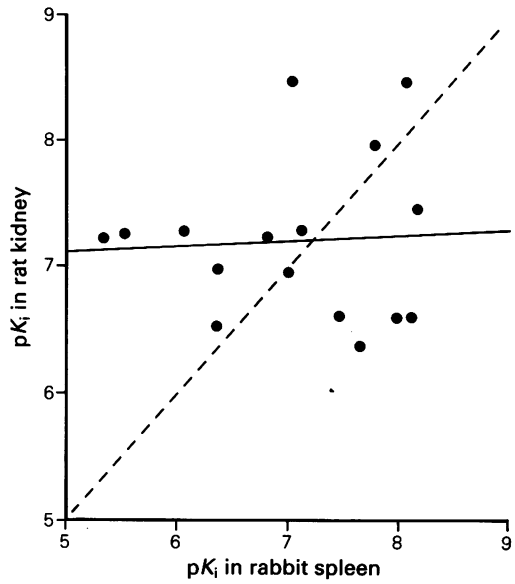

Figure 3 Correlation between ligand affinity estimates obtained at the α_2-adrenoceptors of rat kidney and rabbit spleen labelled with [^{3}H]-rauwolscine. The dashed line represents the line of identity, while the solid line represents the regression line. The data were best described by the expression $y = 0.0461x + 6.881$ ($r = 0.066$).

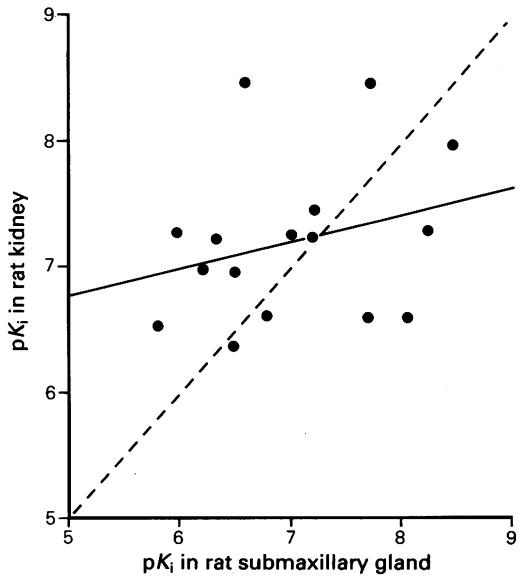

Figure 4 Correlation between ligand affinity estimates obtained at the α_2-adrenoceptors of rat kidney and rat submaxillary gland labelled with [^{3}H]-rauwolscine. The dashed line represents the line of identity, while the solid line represents the regression line. The data were best described by the expression $y = 0.216x + 5.690$ ($r = 0.276$).

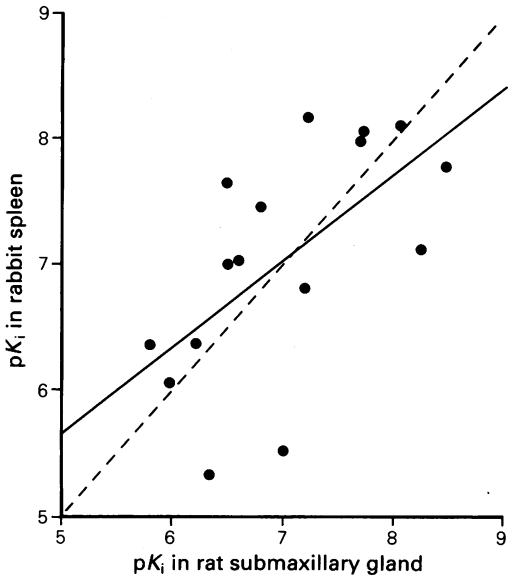

Figure 5 Correlation between ligand affinity estimates obtained at the α_2-adrenoceptors of rabbit spleen and rat submaxillary gland labelled with [^{3}H]-rauwolscine. The dashed line represents the line of identity, while the solid line represents the regression line. The data were best described by the expression $y = 0.691x + 2.199$ ($r = 0.62$).

maxillary gland α_2-adrenoceptors, the affinity estimate for prazosin in this tissue was intermediate between that obtained at the α_{2A}- and α_{2B}- adrenoceptor while for phentolamine and idazoxan, affinity estimates in rat submaxillary gland exceeded those obtained at both the α_{2A}- and α_{2B}-adrenoceptor.

While the simplest explanation for these differences is that the rat submaxillary gland α_2-adrenoceptor differs from the rabbit spleen α_{2A}- and the rat kidney α_{2B}-adrenoceptor, several alternatives should be considered briefly.

It is possible that during the membrane preparation procedure modifications to the receptors could have occurred. This appears to be unlikely since such a problem has not been widely reported for any other neurotransmitter receptor system. Furthermore, with respect to the α_2-adrenoceptors in rat cortex and human platelet Kawahara & Bylund (1985) have shown that such a phenomenon does not occur.

The anomalous binding affinities obtained in rat submaxillary gland could have been due to the presence of a heterogeneous mixture of α_{2A}- and α_{2B}-adrenoceptors in this preparation or even to the presence of receptor heterogeneity in the other two tissues.

Heterogeneity of the α_2-adrenoceptors present in rat submaxillary gland seemed unlikely for several reasons. Firstly, the K_d of [^{3}H]-rauwolscine was higher in rat submaxillary gland than in either rabbit spleen or rat kidney indicating that rat submaxillary gland did not contain a heterogeneous mixture of the α_2-adrenoceptor subtypes found in rat kidney or rabbit spleen. The additional finding that idazoxan and phentolamine were more potent in rat submaxillary gland than in rabbit spleen or rat kidney would also support this contention. While the intermediate affinity of prazosin for rat submaxillary gland α_2-adrenoceptors may indicate possible heterogeneity of the α_2-adrenoceptors in this preparation, the finding that Hill coefficients for prazosin in all three preparations were close to unity would tend to rule out this possibility. Furthermore, ligands such as spiroxatrine, imiloxan and chlorpromazine which display greater than 10 fold selectivity for either the α_{2A}- or the α_{2B}-adrenoceptor, did not display an intermediate affinity for rat submaxillary gland α_2-adrenoceptors as would be expected if this preparation did in fact contain a heterogeneous mixture of α_{2A}- and α_{2B}-adrenoceptor subtypes.

These findings do not exclude the possibility that the rat kidney or rabbit spleen could contain a heterogeneous population of binding sites. Certainly, the data obtained with oxymetazoline appear to indicate heterogeneity of binding in rat kidney and rabbit spleen. Thus, in rabbit spleen two populations of [^{3}H]-rauwolscine site that differed in affinity for oxymetazoline by 30–100 fold (Michel *et al.*, 1989) could be identified. While this may indicate heterogeneity of α_2-adrenoceptors in rabbit spleen several other observations would tend to argue against this.

Most notably, chlorpromazine, imiloxan and prazosin which were equally as selective as oxymetazoline (in terms of their affinity for rat kidney as opposed to rabbit spleen α_2-adrenoceptors) did not identify similar heterogeneity in rabbit spleen to that identified by oxymetazoline. Furthermore, benoxathian, which was as selective as oxymetazoline for α_{2A}- as opposed to α_{2B}-adrenoceptors, did not identify the same heterogeneity in rabbit spleen.

The reason for the apparent heterogeneity of binding identified by oxymetazoline is unknown at present. It should be noted that it was probably not due to affinity states of the α_2-adrenoceptors since the non-hydrolyzable guanaline triphosphate (GTP) analogue, Gpp(NH)p, did not modulate the binding of oxymetazoline to the three α_2-adrenoceptors examined in the present study (unpublished observations).

On the basis of these findings, and with the possible exception of the data obtained with oxymetazoline, it appears unlikely that tissue-related factors, receptor heterogeneity or agonist affinity states could be easily invoked to account for the differences in the

pharmacology of the α_2-adrenoceptors in rat kidney, rat submaxillary gland and rabbit spleen. It therefore appears that the three tissues examined express different α_2-adrenoceptors.

In this respect it is interesting to note that additional evidence for three subtypes of the α_2-adrenoceptor has also been presented. In ligand binding studies Bylund (1988) has recently reported that the α_2-adrenoceptor present in the opposum kidney OK cell line (Murphy & Bylund, 1988) differ from the α_{2A}- and α_{2B}-adrenoceptor subtypes and may therefore represent an α_{2C}-adrenoceptor. Furthermore, and as mentioned by Bylund (1988), studies on the molecular biology of the α_2-adrenoceptor by Kobilka *et al.* (1987) have identified three regions of hybridization in southern blots probed at reduced stringency using a DNA probe directed against the α_2-adrenoceptor of human platelet. Two of these regions have been identified as corresponding to genes that encode α_2-adrenoceptors. While the third region of hybridization may represent a third α_2-adrenoceptor, it should be noted that since a DNA probe for the β-adrenoceptor identified the 5-HT_{1a}-receptor (Fargin *et al.*, 1988), it may also be possible that the third putative α_2-adrenoceptor gene localized to chromosome 2 does not represent an α_2-adrenoceptor.

Comparison of the three receptor binding sites identified in the present study with the pharmacology of the α_2-adrenoceptor gene products and the three binding sites identified by Bylund (1988) indicates several features of similarity and several disparities.

In a previous study (Michel *et al.*, 1989) we have shown that the rabbit spleen and rat kidney α_2-adrenoceptors correspond closely to the α_{2A}- and α_{2B}-adrenoceptor subtypes, respectively, identified by Bylund (1988). The rat submaxillary gland α_2-adrenoceptor does not, however, appear to correspond to the α_{2C}-adrenoceptor present in the OK cells. Firstly, the affinity of rauwolscine and WB 4101 for the α_{2C}-adrenoceptor (Murphy & Bylund, 1987) is higher than that at the α_{2A}- or α_{2B}-adrenoceptor (Bylund *et al.*, 1988) whereas in our study, the rat submaxillary gland α_2-adrenoceptors displayed the lowest affinity for these ligands. In addition, prazosin and chlorpromazine displayed high affinity for both the α_{2B}- (Bylund *et al.*, 1988) and α_{2C}-adrenoceptors (Murphy & Bylund, 1988) whereas they displayed lower affinity for the rat submaxillary gland α_2-adrenoceptor than for the α_{2B}-adrenoceptor in our study.

The cloned α_2-adrenoceptors identified by Regan *et al.* (1988) were initially thought to correspond to the α_{2A}- and α_{2B}-adrenoceptor subtypes. However, this has been questioned by Bylund (1988) who has suggested that while the α_2-adrenoceptor of chromosome 10 (α_2-C10) appears to correspond to the α_{2A}-adrenoceptor, the pharmacology of the α_2-adrenoceptor of chromosome 4 (α_2-C4) differs from that of the α_{2A}-, α_{2B}- or α_{2C}-adrenoceptors. The results from the present study also indicate difficulties in comparing ligand binding data on the α_{2A}- and α_{2B}-adrenoceptors to binding data obtained using the cloned α_2-C4 and α_2-C10 adrenoceptors. Thus, imiloxan, which was an α_{2B}-adrenoceptor selective ligand in our study, did not differentiate between α_2-C4 and α_2-C10 (Regan *et al.*, 1988). Furthermore, WB 4101 which displays up to a 10 fold *higher* affinity for α_{2A}- as opposed to α_{2B}-adrenoceptors (Cheung *et al.*, 1982; Bylund *et al.*, 1988) displayed an 8 fold *lower* affinity for the α_2-C10 adrenoceptor than for the α_2-C4 adrenoceptor.

Clearly, further study is required to correlate definitively the α_2-adrenoceptors identified in ligand binding studies with those identified from molecular biological studies. In addition, further study is required to identify functional correlates for the increasing number of α_2-adrenoceptor binding sites that are being characterized in ligand binding studies.

This is especially relevant since, on the basis of existing reports, at least 6 α_2-adrenoceptor subtypes could be present. The α_{2A}- and α_{2B}-adrenoceptor subtypes identified in ligand binding studies are now widely accepted (Bylund, 1988). The α_2-adrenoceptors of the rat submaxillary gland identified in the present study and the α_{2C}-adrenoceptors of the OK cell line (Murphy & Bylund, 1988) also appear to represent two further distinct α_2-adrenoceptor subtypes. Given that the cloned α_2-C4 adrenoceptor may differ from either the α_{2A}-, α_{2B}- or α_{2C}-adrenoceptors identified to date (Bylund, 1988), the α_2-C4 adrenoceptor may represent a fifth subtype of α_2-adrenoceptor. Finally, since SKF 104078 distinguishes between pre- and postsynaptic α_2-adrenoceptors (Ruffolo *et al.*, 1988) and a ligand binding correlate for this receptor has not been identified to date, presynaptic α_2-adrenoceptors may form a possible sixth subtype of the α_2-adrenoceptor.

While such extensive heterogeneity is not without precedent [for instance, 5 muscarinic receptor genes have been identified by Bonner *et al.* (1988) despite the fact that only three functional and ligand binding sites were previously thought to exist (Hammer *et al.*, 1987)], the situation with regard to α_2-adrenoceptor subtypes is complicated by the heavy reliance on ligand binding data. In this respect the identification of additional subtype selective ligands such as imiloxan (Michel *et al.*, 1989) and spiroxatrine in the present study should aid in identifying functional correlates for the α_2-adrenoceptor subtypes identified in ligand binding sites.

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In favour of the vesicular hypothesis: neurochemical evidence that vesamicol (AH5183) inhibits stimulation-evoked release of acetylcholine from neuromuscular junction

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1 The effects of optical isomers of vesamicol (2-(4-phenylpiperidino) cyclohexanol), an inhibitor of acetylcholine (ACh) storage, on stimulation-evoked release of [^{3}H]-acetylcholine ([^{3}H]-ACh) from the neuromuscular junction have been studied in the region of the mouse hemidiaphragm which contains the motor endplates, and which can easily be loaded with [^{3}H]-choline. This method made it possible to detect exclusively the Ca_o-dependent release of [^{3}H]-ACh in response to stimulation, and therefore to test the vesicular hypothesis.

2 (−)-Vesamicol was approximately 20 times more potent than (+)-vesamicol in reducing stimulation-evoked release of [^{3}H]-ACh.

3 4-Aminopyridine, a potassium channel blocker, enhanced the release of ACh in response to stimulation, but failed to increase release from hemidiaphragm which had been pretreated with (−)-vesamicol.

4 The fact that (−)-vesamicol inhibited the release of [^{3}H]-ACh in response to electrical stimulation only when it was administered prior to the loading of the tissue with [^{3}H]-choline, and had no effect when the stores had already been filled with labelled [^{3}H]-ACh indicates that the stimulation-evoked release of [^{3}H]-ACh is of vesicular origin and (−)-vesamicol has no effect on the release process. This is the first neurochemical evidence for the vesicular origin of stimulation-evoked release of ACh from the neuromuscular junction.

Introduction

Recent evidence indicates that vesicles acquire acetylcholine (ACh) by a transport process (Anderson *et al.*, 1983; cf. Tucek, 1984) and vesamicol (2-(4-phenylpiperidino) cyclohexanol; AH5183) selectively inhibits the transport of ACh into synaptic vesicles (Marshall, 1970; cf. Marshall & Parsons, 1987). Whitton *et al.* (1986) provided evidence on the isolated unparalysed phrenic nerve-hemidiaphragm preparation of the mouse that vesamicol diminished the miniature endplate potential (m.e.p.p.) amplitude and that the reduction was not due to postjunctional block but was rather due to a reduction in quantal size. Provided that ACh released in quanta at the neuromuscular junction (Del Castillo & Kata, 1954) emanates from terminal synaptic vesicles, vesamicol should inhibit the evoked release of freshly synthesized ACh.

It was of interest to determine whether vesamicol reduced ACh release by blocking the release process or by blocking the refilling of vesicular stores with newly synthesized ACh formed in the cytoplasm. If vesamicol blocks the transport of newly synthesized cytoplasmic ACh into the vesicular fraction, and this fraction is responsible for signal transmission at the neuromuscular junction, then vesamicol might be expected to block the refilling of vesicular stores with newly synthesized ACh. Vesamicol should then block the release of [^{3}H]-ACh and the contraction

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of the muscle in response to electrical stimulation, but not block the uptake of [^{3}H]-choline and the subsequent transformation of choline into ACh.

To test these possibilities, a recently developed radioisotope method (Foldes *et al.*, 1984; Somogyi *et al.*, 1987) was used to measure the evoked, Ca^{2+}-dependent release of freshly synthesized ACh without the use of cholinesterase inhibition from a neuromuscular junction that had been loaded with [^{3}H]-choline.

Methods

Preparation of the innervated region of hemidiaphragm

Male Swiss-Webster mice, weighing 25–30 g were exposed to halothane and after the loss of the righting reflex they were decapitated. The middle portions of both hemidiaphragms (about 2–3 mm to the right and to the left of the insertions of the phrenic nerves), together with the attached rib segments, were suspended in organ baths of 1 ml. The rib end of the preparation was attached to the bottom of the bath and its tendon to an FT03 force displacement transducer. The organ bath was filled with Krebs solution (NaCl 113, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 0.9, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 11.5 mmol l^{-1}). The bath temperature was maintained at 37°C. The pH of the solution when aerated with 95% O_2:5% CO_2 was 7.38–7.42.

Incubation

The release of ACh was determined in 1 ml organ baths. The preparations were loaded with [^{3}H]-choline (4 μCi ml^{-1}) for 40 min. During this time the preparations were stimulated at 1 Hz to facilitate the incorporation of [^{3}H]-choline into the [^{3}H]-ACh pool of the motor nerve terminal. At the end of this period, the radioactivity of the muscles, consisting partly of [^{3}H]-choline and partly of [^{3}H]-ACh, was $3.61 \pm 0.4 \times 10^5$ Bq g^{-1} ($n = 10$). To remove excess [^{3}H]-choline the preparations were washed three times and then were transferred to another bath and superfused with modified Krebs solution at the rate of 2 ml min^{-1} for 60 min. Subsequently, hemicholinium-3 (5×10^{-5} mol l^{-1}) was added to the solution to prevent reuptake of [^{3}H]-choline and the perfusion rate was reduced to 0.5 ml min^{-1}.

Collection

During superfusion at the rate of 0.5 ml min^{-1}, 3 min (1.5 ml) effluents were collected continuously with a fraction collector into scintillation vials throughout the experiment. The preparations were stimulated twice with 0.8 s trains of 50 Hz, rectangular wave, supramaximal stimuli of 0.2 ms duration every 10 s for 3 min, starting at the 7th (S_1) and 37th (S_2) min of the collection period.

Calculation

In the mouse phrenic nerve-hemidiaphragm preparation, as in many other preparations (Kilbinger & Wessler, 1980; Vizi *et al.*, 1984), electrical stimulation increases only the release of [^{3}H]-ACh while the output of [^{3}H]-choline remains unchanged (Wessler & Steinlein, 1987; Vizi *et al.*, 1987). Accordingly, the evoked release of [^{3}H]-ACh was calculated from the difference between the expected resting and the actual measured outflow of radioactivity caused by 3 min electrical stimulation. Resting release, assumed to follow first-order kinetics, was determined by 'least square' fitting to an exponential curve of the Bq g^{-1} concentrations of two 1.5 ml effluents collected before and after stimulation. The expected spontaneous outflow was obtained by interpolation between the fitted points.

The radioactivity of the samples was measured in a LKB Scintillation Beta Spectrometer. There was considerable variation from one preparation to the other in the amount of radioactivity (Bq g^{-1}) released during the stimulation periods. Therefore fractional release of radioactivity was calculated as a percentage of the total content of tritium in the tissue at the beginning of each collection period. The total content of tritium in the tissues was obtained by summing the radioactivity found in the tissues at the end of the experiments with the radioactivity released during the course of experiments. The stimulation-evoked release was expressed as a percentage of the tritium in the tissue after subtraction of the presumed basal release. A computer programme was used for calculation. The ratios of the radioactivity released by the two consecutive stimulation periods, S_2/S_1, were constant.

Stimulation

Trains of 40 supramaximal rectangular wave stimuli were applied at 50 Hz with a Grass S-88 stimulator every 10 s. The stimulus duration was 0.2 ms. A pattern of short trains (0.8 s) of high frequency stimulation (50 Hz) was selected because, in mammals, voluntary muscle movements are elicited with short neuronal impulse bursts of 15–60 Hz (Zierler, 1974).

Statistical analysis

The statistical significance of the results was determined by one-way analysis of variance of the

Table 1 Effect of vesamicol on the release of radioactivity from hemidiaphragm loaded with [^{3}H]-choline

Present in the bath	*Evoked release (S_1) of ACh* $Bq\,g^{-1}$	*FR (%)*	S_2/S_1
Control	1784 ± 145 (5)	1.71 ± 0.13 (5)	0.91 ± 0.07 (5)
(−)Vesamicol, 0.5 μM throughout the expt.	1140 ± 110* (4)	1.05 ± 0.12* (4)	0.84 ± 0.06 (4)
(−)Vesamicol, 10 μM throughout the expts.	371 ± 50* (5)	0.26 ± 0.03* (5)	0.83 ± 0.10 (5)
(+)Vesamicol, 10 μM throughout the expts.	1193 ± 110* (4)	1.10 ± 0.11* (4)	0.90 ± 0.07 (4)
(−)Vesamicol, 10 μM during loading	850 ± 41* (4)	0.68 ± 0.05* (4)	0.84 ± 0.06 (4)

Phrenic nerve was stimulated twice (S_1 and S_2), 30 min elapsed between the two stimulations. Stimulation: 0.8 s train of 50 Hz, supramaximal stimuli of 0.2 ms every 10 s for 3 min.
*Significant difference ($P < 0.01$) from control.
Number of experiments is in parentheses. Note (−)-vesamicol has much greater effect than (+)-vesamicol.

logarithmically transformed data, followed by Dunn's test. $P < 0.05$ was considered significant. Results are presented as mean values ± s.e.mean.

Drugs

Vesamicol (2-(4-phenylpiperidino)cyclohexanol, AH5183) was a gift from Dr S. Parsons, (Univ. of California, Santa Barbara, U.S.A.) and the optical isomers were purchased from RBI (MA, U.S.A.). Hemicholinium-3 was purchased from Sigma Chemical Co.

Results

The content of radioactivity after 40 min incubation with [^{3}H]-choline followed by 90 min washout was 360750 ± 44150 $Bq\,g^{-1}$ ($n = 10$). The resting release was 2044 ± 180 $Bq\,g^{-1}$ in 3 min ($n = 10$). When the fractional release was calculated, this value proved to be 1.96 ± 0.14% of the radioactivity present in the tissue at the beginning of the consecutive stimulations of 3 min duration. Release evoked by the first stimulation was 1784 ± 145 $Bq\,g^{-1}$ (1.71 ± 0.12%, $n = 5$), and the S_2/S_1 ratio was 0.91 ± 0.07 ($n = 5$).

When (−)-vesamicol was added to the bathing fluid between S_1 and S_2 in the concentration range 1–50 μM, it did not affect the S_2/S_1 ratio, i.e. the stimulation evoked release. The resting release, however, was slightly reduced when 50 μM (−)-vesamicol was added to the organ bath; R_2/R_1 was reduced from 0.91 ± 0.08 to 0.65 ± 0.04 ($n = 5$-5, $P < 0.01$). At a concentration of 10 μM vesamicol had no effect.

When the tissue was exposed for 20 min to 10 μM (−)-vesamicol and subsequently loaded with [^{3}H]-choline in the presence of (−)-vesamicol, the uptake of radioactivity was 395500 ± 46840 $Bq\,g^{-1}$ ($n = 4$). In further experiments (−)-vesamicol was kept in the perfusion fluid throughout the experiments. While the resting release was unchanged (1.80 ± 0.21% in 3 min), the stimulation evoked release was much smaller (371 ± 50 $Bq\,g^{-1}$, 0.26 ± 0.03%, $n = 5$) from (−)-vesamicol treated hemidiaphragm, than from control tissue (Table 1). The effect of (−)-vesamicol was concentration-dependent. At 0.5 μM it was less effective, the release evoked by stimulation being 1.05 ± 0.12% of the total radioactivity ($n = 4$), but the reduction was still significant ($P < 0.01$). (+)-Vesamicol kept in the perfusion fluid throughout the experiment was much less effective in reducing the evoked release of ACh (Table 1). Racemic vesamicol (10 μM) also reduced the release of ACh (920 ± 160 $Bq\,g^{-1}$, $n = 3$) in response to stimulation (50 Hz, 18 trains of 40 stimuli). 4-Aminopyridine (4-AP) increased the release of ACh as indicated by the increase of S_2/S_1 from 0.88 ± 0.07 to 1.87 ± 0.11. However, when (−)-vesamicol was kept in the perfusion fluid throughout the experiments, 4-AP did not enhance the release (Table 2).

Discussion

Shortly after Fatt & Katz (1952) first described m.e.p.ps, vesicles were revealed within the nerve terminals by electronmicroscopy (de Robertis & Bennett, 1955) and neurochemical evidence was also obtained (Whittaker *et al.*, 1964) for ACh storage in vesicles. It was therefore natural to ascribe the quantal nature of ACh release to the vesicles. Since then many results have been published that are compatible with the idea that the ACh involved in signal transmission at many neuroeffector junctions is released from vesicles by a process of exocytosis. Nevertheless, there is some evidence that quantal release may originate from cytoplasmic stores of ACh (Tauc *et al.*, 1974; Israel *et al.*, 1979; Dunant, 1986).

Recently a sensitive radiochemical method was developed (Foldes *et al.*, 1984; Somogyi *et al.*, 1987)

Table 2 Effect of 4-aminopyridine on the release of radioactivity from mouse hemidiaphragm loaded with [^{3}H]-choline

Drugs	*Release of ACh* S_2/S_1
Control	0.88 ± 0.07 (7)
4-Aminopyridine, 50 μM	1.87 ± 0.11* (4)
(−)-Vesamicol treated + 4-Aminopyridine, 50 μM	0.96 ± 0.12 (4)

Phrenic nerve was stimulated twice (S_1 and S_2), 30 min elapsed between the two stimulations. Drugs were added to the perfusion fluid 20 min prior to the second stimulation. (−)-Vesamicol (10 μM) was present in the perfusion fluid throughout the experiments.
*Significant difference from control ($P < 0.01$).
Note that (−)-vesamicol treatment prevents the effect of 4-aminopyridine.

that measures selectively the Ca-dependent, stimulation-evoked quantally released, freshly synthesized ACh from mouse phrenic nerve-hemidiaphragm *in vitro* without cholinesterase inhibition. In response to stimulation, ACh is released, therefore any radioactivity released above resting release represents the release of freshly synthesized ACh (Vizi & Somogyi, 1989). With this method the effect of vesamicol, a compound able to block the uptake of freshly formed ACh by synaptic vesicles (Anderson *et al.*, 1983; cf. Marshall & Parsons, 1987) was studied on stimulation-evoked release of ACh from mouse hemidiaphragm previously loaded with [^{3}H]-choline.

In the present study, (−)-vesamicol did not reduce the uptake of [^{3}H]-choline, but drastically reduced the release of radioactivity in response to stimulation only in those cases when the compound was loaded prior to and during [^{3}H]-choline loading, and was left in the organ bath throughout the experiment. When it was added after loading with [^{3}H]-choline, between S_1 and S_2 stimulations, the compound had no effect on the stimulation-evoked release of ACh, indicating that it has no effect on the release process. It also failed to affect resting release, which is Ca_o^{2+}-independent and mainly released in non-quantal form (Vizi & Vyskocil, 1979).

The finding that (−)-vesamicol was about 20 times more active than (+)-vesamicol suggests that the effect of the compound is stereoselective. This is in good agreement with the observation of Estrella *et al.* (1988), who studied the optical isomers of vesamicol on neuromuscular transmission in rat diaphragm and found that the (−)-form is more active. The inhibition of potassium channel conductances by 4-AP did not reverse the inhibition of ACh release when (−)-vesamicol was present in the fluid.

The results obtained in the present study indicate that vesamicol reduces the electrically evoked release of [^{3}H]-ACh from the neuromuscular junction, a result consistent with the earlier observation that it reduces the evoked release of ACh from CNS tissue (Ricny & Collier, 1986; Suszkiw & Toth, 1986), as it has been shown to do at peripheral autonomic synapses (Collier *et al.*, 1986; Cabeza & Collier, 1988). These data suggest that it blocks the uptake of [^{3}H]-ACh into synaptic vesicles.

The neurochemical evidence in the present study is in favour of the concept that ACh released by nerve stimulation originates from vesicles at least at this neuromuscular junction.

In the experiments of Whitton *et al.* (1986) using mouse hemidiaphragm preparations, vesamicol reduced the quantal size to a smaller extent than it reduced stimulation-evoked ACh release in our experiments. This point needs some explanation. The major difference between the two techniques is that in our experiments only the release of freshly formed ACh is measured, while in the experiments by Whitton *et al.* (1986) the release of endogenous ACh was recorded indirectly by an action on the post-junctional membrane. In the latter case the action of vesamicol, both on twitches (Marshall, 1970) and on electrophysiologically recorded events (Van der Kloot, 1986; Whitton *et al.*, 1986) is very slow, requires higher concentrations and is highly dependent on the amount of ACh depleted and not replaced. The reason for the quantitative difference in the neurochemical and electrophysiological studies may be the large margin of safety of ACh release at the neuromuscular junction (Paton & Waud, 1967): 6–10 times more ACh is released by stimulation than is required for signal transmission.

Thus, vesamicol only inhibited the release of ACh if the drug was administered prior to the loading of the [^{3}H]-ACh into the preparation. It had little or no effect on the release of ACh which was previously loaded into vesicles. In addition, it had no effect on the release mechanism, as indicated by its ineffectiveness on the S_2/S_1 ratio when it was administered just before the second stimulation period.

While in our experiments vesamicol treatment did not reduce resting release of ACh, Vyskocil (1985) observed a hyperpolarization in the mouse diaphragm following vesamicol administration, an effect attributed to the reduction of non-quantal release of ACh. This type of release is Ca_o^{2+}-independent, its rate depends on the activity of Na/K-activated ATPase (Vizi & Vyskocil, 1979; Vyskocil, 1985) and is of cytoplasmic origin. The finding that the presence of vesamicol was capable of inhibiting the release of Ca_o^{2+}-dependent, stimulation-evoked release of ACh, suggests that this type of release is of vesicular origin.

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Differential cardiovascular and respiratory responses to central administration of selective opioid agonists in conscious rabbits: correlation with receptor distribution

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1 The effects of intracerebroventricular (i.c.v.) and intracisternal (i.c.) administration of a range of doses (0.01, 0.1 and 1.0 nmol kg^{-1}) of specific μ- δ- and κ-opioid agonists on cardiovascular and respiratory function and on plasma catecholamines have been studied in conscious rabbits. The distribution of μ- δ- and κ-opioid receptors was localized in rabbit brain by *in vitro* autoradiography.

2 The μ-agonist [D-Ala^2, MePhe^4-Gly^5-ol]enkephalin (DAGOL) given i.c.v. caused a large rise in plasma noradrenaline and adrenaline, hypertension accompanied by an initial bradycardia followed by tachycardia, respiratory depression and sedation. After i.c. administration there were similar changes in heart rate (HR) and respiration, but no significant changes in mean arterial pressure (MAP) or plasma catecholamines.

3 The δ-agonist [D-Pen^{2,5}]enkephalin (DPDPE) increased MAP and HR after both i.c.v. and i.c. administration, caused a small increase in noradrenaline but had no effect on adrenaline and did not alter respiration rate or blood gases. After i.c.v. DPDPE the rabbits became more alert and active.

4 The κ-agonist U69593 given i.c.v. or i.c. had no effect on MAP or HR. After i.c.v. U69593, $PaCO_2$ fell, but there were no other respiratory effects. The responses to dynorphin 1-13, an endogenous κ-agonist, were similar to those of U69593.

5 The opioid antagonist naloxone (30 nmol kg^{-1}) given intravenously (i.v.) blocked the effects of i.c.v. DAGOL (1 nmol kg^{-1}). A 100 fold higher dose of i.v. naloxone (3 μmol kg^{-1}) was required to abolish the effects of i.c.v. DPDPE (1 nmol kg^{-1}).

6 Autoradiographic studies demonstrated a high density of μ- and δ-opioid receptors in hypothalamic sites. In the brainstem μ-receptors were demonstrated in the nucleus tractus solitarius (NTS) and δ-receptors in the dorsal motor nucleus of the vagus. κ-Receptors were not detected in either the hypothalamus or brainstem.

7 These findings demonstrate that DAGOL increases sympatho-adrenal outflow, probably by stimulation of hypothalamic μ-receptors. The effects on HR are probably partly through a baroreflex and partly through an action of DAGOL on μ-receptors in the dorsal motor nucleus of the vagus. DPDPE probably acts on δ-receptors in the NTS to increase MAP and HR. Respiratory depression resulted from stimulation of μ-receptors in the brainstem with no evidence of δ- or κ-receptors being involved.

Introduction

A potential role for endogenous opioid peptides in central cardiovascular control has been indicated by the demonstration that opioid peptides and their receptors are present in areas of brain with established roles in the regulation of cardiovascular function (Hokfelt *et al.*, 1977; Goodman *et al.*, 1980). Further evidence came from studies in which central administration of opioid peptides was shown to alter

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sympathetic outflow (Van Loon et al., 1981; Pfeiffer et al., 1983) or to influence baroreflex mechanisms (Schaz et al., 1980). In conscious rabbits we have demonstrated that the predominantly μ-opiate agonist morphine acts centrally to increase sympatho-adrenal outflow and produce hypertension (May et al., 1988; 1989a). Similar responses have been described in conscious rats after intracerebroventricular (i.c.v.) injection or hypothalamic microinjections of the selective μ-opioid agonist [D-Ala^2, MePhe^4, Gly^5-ol] enkephalin (DAGOL) (Pfeiffer et al., 1982; 1983). The cardiovascular effects resulting from stimulation of central δ- and κ-receptors are less clear because of the relative lack of specificity of the agonists that have been available.

In the present study the cardiovascular and respiratory effects of the μ-opioid agonist DAGOL have been compared with the effects of the δ-opioid agonist (D-Pen^{2,5})enkephalin (DPDPE) and the κ-opioid agonist, U69593. The specificity of DPDPE and U69593 for their respective receptor subtypes is an order of magnitude greater than that of other agonists selective for these receptors (Mosberg et al., 1983; Lahti et al., 1985). These selective agonists have been given i.c.v. and intracisternally (i.c.) to differentiate between periventricular and brainstem sites of action. Conscious animals have been used to avoid interference from anaesthetic agents. The possible sites of action have been investigated further by use of *in vitro* autoradiography to localize μ-, δ- and κ-opioid receptor distribution in rabbit brain with the same selective agonists.

Methods

Autoradiography

The *in vitro* autoradiographic technique was essentially that described by Young & Kuhar (1979), modified according to the protocol described by Dashwood et al. (1988). Rabbits were anaesthetized with alphaxalone 0.9%/alphadolone acetate 0.3% (Saffan, Glaxovet) (1 ml i.v. followed by 0.25 ml i.v. as required). They were perfused with 1.0 litre ice cold 100 mM phosphate buffer, pH 7.4, containing 0.32 M sucrose and then lightly fixed with 1.0 litre 0.1% formaldehyde solution in phosphate sucrose buffer (Dashwood et al., 1985). The brains were removed and frozen at -70°C. Frozen sections (20 μm) of brainstem and forebrain were cut serially in a cryostat at -20°C and thaw-mounted onto gelatinised microscope slides. The presence of opioid receptors was established by use of the appropriate tritiated ligands (Gillan & Kosterlitz, 1982; Lahti et al., 1985; Gulya et al., 1985; Dashwood et al., 1985; 1986; 1988). All sections were preincubated in 170 mM Tris HCl buffer, pH 7.4, containing 100 mM NaCl to reduce levels of endogenous transmitter. Generalised opiate binding was established by incubating sections for 60 min at 4°C in 170 mM Tris/100 mM NaCl buffer, pH 7.4, containing 4 nM [^{3}H]-naloxone. The degree of binding to non-specific sites was established by incubating in the presence of 1 μM unlabelled naloxone or morphine. Binding sites of opioid subtypes were identified by incubating in 50 mM Tris/HCl, pH 7.4, containing 5 mM $CaCl_2$, 2 mg ml^{-1} bovine serum albumin, and 20 μg ml^{-1} bacitracin containing 4 nM [^{3}H]-DPDPE, [^{3}H]-DAGOL or [^{3}H]-ethylketocyclazocine (EKC) for 60 min at 22°C. Binding of [^{3}H]-EKC to δ- and μ-sites was prevented by incubating sections in the presence of 100 nM concentrations of DPDPE and DAGOL respectively. Binding to κ-sites was further investigated by incubating sections in 5 nM [^{3}H]-U69593 in 50 mM HEPES buffer, pH 7.4, containing 10 mM $MgCl_2$. The degree of non-specific binding of [^{3}H]-DAGOL, [^{3}H]-DPDPE, [^{3}H]-EKC and [^{3}H]-dynorphin was established by incubation in the presence of 1 and 10 μM unlabelled naloxone. After incubation, sections were washed (three times for 10 min) in buffer minus bacitracin and blown dry in a stream of cold air. Following drying the slide-mounted sections were affixed to 24 × 30 cm X-ray cassettes with double sided tape and apposed to tritium-sensitive film (Hyperfilm ^{3}H, Amersham) for 4 to 12 weeks at 4°C. After this exposure period the films were developed in Kodak D19 developer (undiluted for 5 min at 22°C), fixed in Ilfospeed fixer (diluted 1 to 4 with distilled water for 5 min at 22°C), then rinsed in running tap water for at least 20 min before allowing the films to dry. Autoradiographs were visually examined and, where appropriate, photographed.

In vivo *experiments*

Experiments were carried out on male Sandy Half-Lop rabbits (National Institute for Medical Research, Mill Hill, London) weighing between 2.1 and 3.3 kg; they were allowed free access to food (RHM R14, Labsure Animal Diets) and water.

Surgical procedures

For injection into the right lateral ventricle rabbits were anaesthetized with Saffan (1 ml i.v. followed by 0.25 ml as required) and the cannulae (Harvard Apparatus) were implanted aseptically. Coordinates were 1.0 mm caudal to bregma, 3.0 mm lateral to midline and 8.5 mm below the dura. The opioid agonists were injected i.c.v. in a volume of 25 μl using a Hamilton syringe with a 25 G needle that protruded 1 mm from the cannula tip. The injection was

flushed in with 25 μl of saline with a needle that reached half way down the cannula shaft. For injection into the cisterna magna the catheter described by Head *et al.* (1983) was modified as previously described (May *et al.*, 1989b) and inserted under Saffan anaesthesia (1 ml i.v. followed by 0.25 ml i.v. as required). Injections were given in 25 μl of saline using a Hamilton syringe with a 30 G needle. The injection was flushed in with 20 μl of saline; the dead volume of the catheter was 10 μl.

The injection site was confirmed at the end of a series of experiments by injection of 25 μl of bromophenol blue (1%) either i.c.v. or i.c. followed by the appropriate volume of saline. The animals were killed, the brains removed and the distribution of dye examined.

Experimental protocol

Animals were not used for at least 7 days post operation. Before each experiment cannulae were inserted into the central artery and marginal vein of the ear under 1% lignocaine local anaesthesia. The arterial cannula was connected to a pressure transducer (Bell and Howell) and BP and HR were recorded on a Devices polygraph. During the experiment the rabbits were lightly restrained as previously described (May *et al.*, 1988). Intervals of at least 7 days were allowed between studies.

All agonists were filtered through a sterile 0.2 μm filter before injection from a sterilized Hamilton syringe. After a 30 min control period increasing doses (0.01, 0.1 and 1.0 nmol kg^{-1}) of the agonists were given at 1 h intervals either i.c.v. or i.c. in conscious rabbits. For dynorphin an additional dose of 5.0 nmol kg^{-1} was also given. Naloxone was given via the marginal vein of the ear 15 min after DAGOL and 10 min after DPDPE.

Blood collection

Blood (2 ml) collected from the arterial cannula into fluoride oxalate tubes was centrifuged and glucose was measured in the plasma by a glucose oxidase method using a Chem-Lab analyser. Catecholamines were measured in plasma obtained from blood (2.5 ml) collected into cooled tubes containing 50 μl of EGTA (0.095%)/glutathione (0.06%) and kept on ice until centrifugation at 4°C and then stored at −70°C. Catecholamines were separated by high performance liquid chromatography (h.p.l.c.) and detected by electrochemical detection (May *et al.*, 1988). Immediately following blood collection an equal volume of 0.9% saline was infused i.v. Arterial blood (100 μl) was collected into a capillary tube for the immediate measurement of blood gases (Instrument Laboratory 230 blood gas analyser).

Drugs and chemicals

DAGOL and DPDPE were purchased from Bachem, porcine dynorphin 1-13 from Cambridge Research Biochemicals, 5α, 7α, 8β-(−)-N-methyl-N-(7-(1-pyrrolidinyl)-1 oxaspiro(4,5)dec-8-yl) benzeneacetamide (U69593) from Amersham International and naloxone HCl from Sigma and Endo Labs. DAGOL, DPDPE and naloxone were dissolved in sterile non-pyrogenic isotonic saline (saline). U69593 (5 mg) was dissolved in 35% ethanol in saline (2 ml) and dynorphin (1 mg) was dissolved in 0.002 M acetic acid (1 ml) and further dilutions of both agonists were made in saline.

[^{3}H]-naloxone (42–60 Ci $mmol^{-1}$), [^{3}H]-DPDPE (26–41 Ci $mmol^{-1}$), [^{3}H]-DAGOL (58 Ci $mmol^{-1}$), [^{3}H]-U69593 (40 Ci $mmol^{-1}$) were purchased from Amersham International and [^{3}H]-EKC (24 Ci $mmol^{-1}$) from New England Nuclear.

Statistical analysis

Data are presented as means ± s.e.mean. Analysis was performed on the differences from the control values using the SAS statistical programme. The control values for mean arterial pressure (MAP) and HR consisted of the mean of the six readings taken during the control period. A logarithmic transformation was performed on the values for plasma glucose, noradrenaline and adrenaline before analysis. The data were subjected to analysis of variance and where the null hypothesis was rejected Bonferroni's comparisons were performed on the means; $P < 0.05$ was considered significant.

Results

Autoradiography

In rabbit forebrain and medulla oblongata (shown diagrammatically in Figure 1) there was a striking variation in the distribution of opioid receptor subtypes. With all ligands the degree of non-specific binding was similar to that shown for [^{3}H]-naloxone (Figures 2a and 3a) and in every case it was less than 30% of the total binding. In rabbit forebrain the presence of [^{3}H]-naloxone binding in areas surrounding the 3rd ventricle demonstrated the presence of opioid binding sites in the hypothalamus (Figure 2a). There was also a high density of [^{3}H]-DAGOL and [^{3}H]-DPDPE binding in hypothalamic sites (Figures 2e and 2f) but no binding of [^{3}H]-U69593 (Figure 2d) demonstrating the presence of μ- and δ-opioid binding sites, but the absence of κ-binding sites in the hypothalamus. The low density of [^{3}H]-EKC binding in the hypothalamus (Figure 2c) may

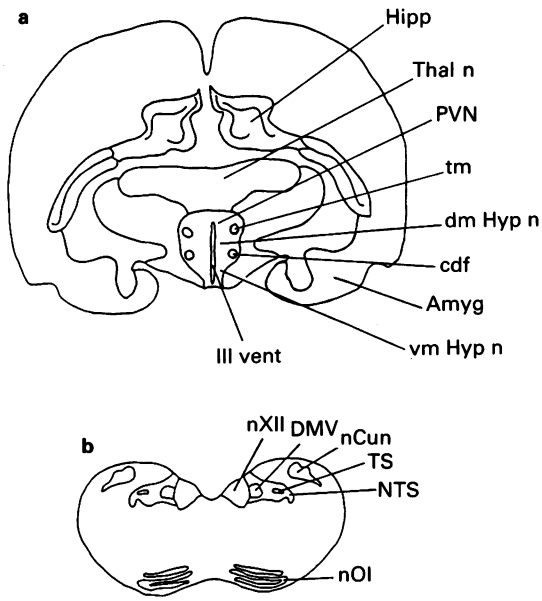

Figure 1 Line drawings of rabbit forebrain (a), and medulla oblongata (b). Abbreviations: (a) Hipp, hippocampus; Thal n, thalamic nuclei; PVN, paraventricular nucleus; tm, tractus mamillothalamicus; dm Hyp n, dorsomedial hypothalamic nuclei; cdf, columna decendens fornicis; Amygd, amygdala; vm Hyp n, ventromedial hypothalamic nuclei; III vent, third ventricle. (b) nXII, hypoglossal nucleus; DMV, dorsomedial nucleus of the vagus; nCun, cuneate nucleus; TS, tractus solitarius; NTS, nucleus of the tractus solitarius; nOl olivary nuclei.

reflect some small degree of binding to μ- or δ-receptors or to a subtype of κ-receptors to which [^{3}H]-U69593 does not bind. There were low levels of [^{3}H]-DAGOL binding in the central amygdaloid nucleus with high levels in the basolateral and medial amygdaloid nuclei (Figure 2e); there was binding of [^{3}H]-DPDPE in all these nuclei (Figure 2f) but there was no binding of [^{3}H]-U69593 (Figure 2d). In addition, there was a high density of μ-binding sites in ventral and dorsal thalamic nuclei and of κ-binding sites in dorsal and lateral thalamic nuclei.

In the brainstem [^{3}H]-DAGOL binding demonstrated the presence of μ-binding sites in the hypoglossal nucleus and the dorsal motor nucleus of the vagus (Figure 3e), whereas [^{3}H]-DPDPE binding indicated the presence of δ-binding sites in the nucleus tractus solitarius (NTS) (Figure 3f). An absence of κ-binding sites in the brainstem is indicated by the lack of [^{3}H]-U69593 or [^{3}H]-EKC binding (Figures 3c and d).

Cardiovascular effects

The μ-opioid agonist DAGOL given i.c.v. caused a dose-related increase in MAP and a fall in HR in six conscious rabbits (Figure 4). There were no changes after the lowest dose, but after 0.1 and 1.0 nmol kg^{-1} there were significant increases in MAP ($P < 0.05$) and falls in HR ($P < 0.05$) within 5 min. At 120 min after the highest dose, MAP had returned to control but HR was significantly elevated above control ($P < 0.05$). Following i.c. administration of the same doses of DAGOL ($n = 5$) there was a trend for MAP to increase and HR to fall but these changes did not reach statistical significance.

The δ-receptor agonist, DPDPE, given i.c.v. to six rabbits caused a dose-related increase in BP which was accompanied by a rise in HR (Figure 5). Following the highest dose (1.0 nmol kg^{-1}) there were significant increases in MAP ($P < 0.05$) and HR ($P < 0.05$) reaching a peak after 15 min. Both MAP and HR fell over the next hour but then increased so that 120 min after the highest dose they were both significantly elevated above control ($P < 0.05$). The same doses of DPDPE given i.c. ($n = 6$) caused similar changes in MAP and HR (Figure 5).

The κ-agonist, U69593, given i.c.v. ($n = 6$) or i.c. ($n = 5$) had no significant effects on MAP or HR at any of the doses used. To confirm that the lack of cardiovascular response to U69593 was not a result of its binding to a subpopulation of κ-receptors, the effects of dynorphin 1-13, an endogenous κ-agonist were examined. Dynorphin given i.c.v. ($n = 4$) or i.c. ($n = 4$) to conscious rabbits had no effect on MAP but after the two highest doses there was a gradual increase in HR which began after 0.1 nmol kg^{-1} and reached 67 ± 20 beats min^{-1} above control ($P < 0.05$) at 60 min after 5.0 nmol kg^{-1}, i.c.

Effects on plasma catecholamines and plasma glucose

Samples for plasma catecholamines were taken at the end of the control period, immediately before the highest dose (60 min after the middle dose) and then at 5, 30, and 60 min after the highest dose.

Following i.c.v. DAGOL (1.0 nmol kg^{-1}) plasma noradrenaline increased 4 fold ($P < 0.05$) and plasma adrenaline 120 fold ($P < 0.05$) and they remained significantly elevated after 60 min (Table 1). After i.c. DAGOL there were no significant increases in noradrenaline or adrenaline. The changes in plasma glucose after i.c.v. and i.c. DAGOL correlated with the changes in adrenaline (Table 1).

Following DPDPE (1.0 nmol kg^{-1}) there was a trend for noradrenaline to increase after i.c.v. and i.c. administration which was significant 60 min after i.c. administration (Table 1). There were no significant increases in plasma adrenaline or glucose.

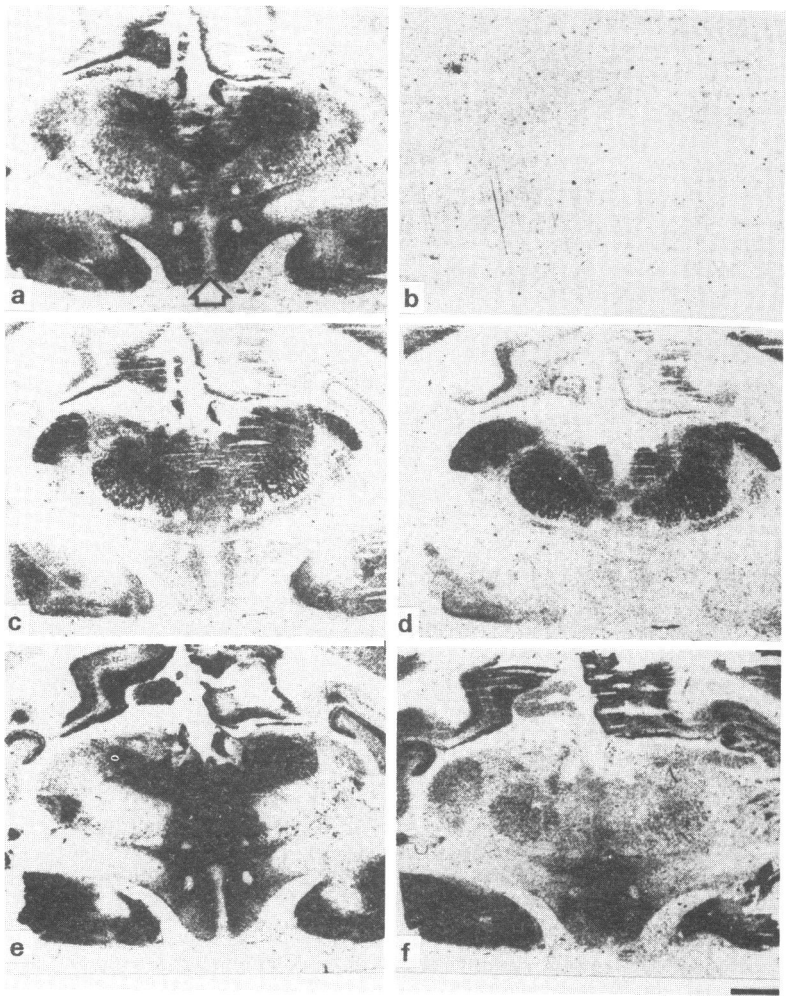

Figure 2 Distribution of opioid receptor subtypes in rabbit forebrain. Consecutive 20 μm sections were incubated with [^{3}H]-naloxone (a), [^{3}H]-naloxone + 1 μM unlabelled naloxone (b), [^{3}H]-ethylketocyclazocine ([^{3}H]-EKC) (c), [^{3}H]-U69593 (d), [^{3}H]-[D-Ala^2, MePhe^4-Gly^5-ol]enkephalin ([^{3}H]-DAGOL) (e) and [^{3}H]-[D-Pen^{2,5}]enkephalin ([^{3}H]-DPDPE) (f). The darker the image the greater the density of receptors. Arrow indicates 3rd ventricle. Scale bar = 2 mm.

U69593 caused no significant changes in noradrenaline after any of the doses given i.c.v. or i.c. There was a small increase in adrenaline of $1.1 \pm 0.5\,\text{nmol}\,l^{-1}$ ($P < 0.05$) at 5 min, but this was not accompanied by a change in plasma glucose. Dynorphin had no effect on plasma noradrenaline or adrenaline levels after i.c.v. or i.c. administration of any of the doses. Plasma glucose was significantly increased ($P < 0.05$) at 30 and 60 min after dynorphin ($5\,\text{nmol}\,l^{-1}$) given i.c.v. (1.1 ± 0.3 and $1.6 \pm 0.2\,\text{mmol}\,l^{-1}$, respectively) and i.c. (1.6 ± 0.7 and $2.0 \pm 0.3\,\text{mmol}\,l^{-1}$, respectively).

Respiratory changes

DAGOL given i.c.v. ($n = 6$) or i.c. ($n = 5$) had no significant effects on respiration rate. After i.c.v. and i.c. DAGOL there was a dose-related increase in $P_{a}CO_2$ and fall in $P_{a}O_2$ (Figure 6). These changes had not developed 5 min after administration when the cardiovascular responses were greatest, but developed slowly over 30 min and began to return to control after 60–90 min. In contrast, DPDPE given i.c.v. ($n = 6$) or i.c. ($n = 6$) had no effects on respiration rate or blood gases. After i.c.v. U69593 ($n = 6$),

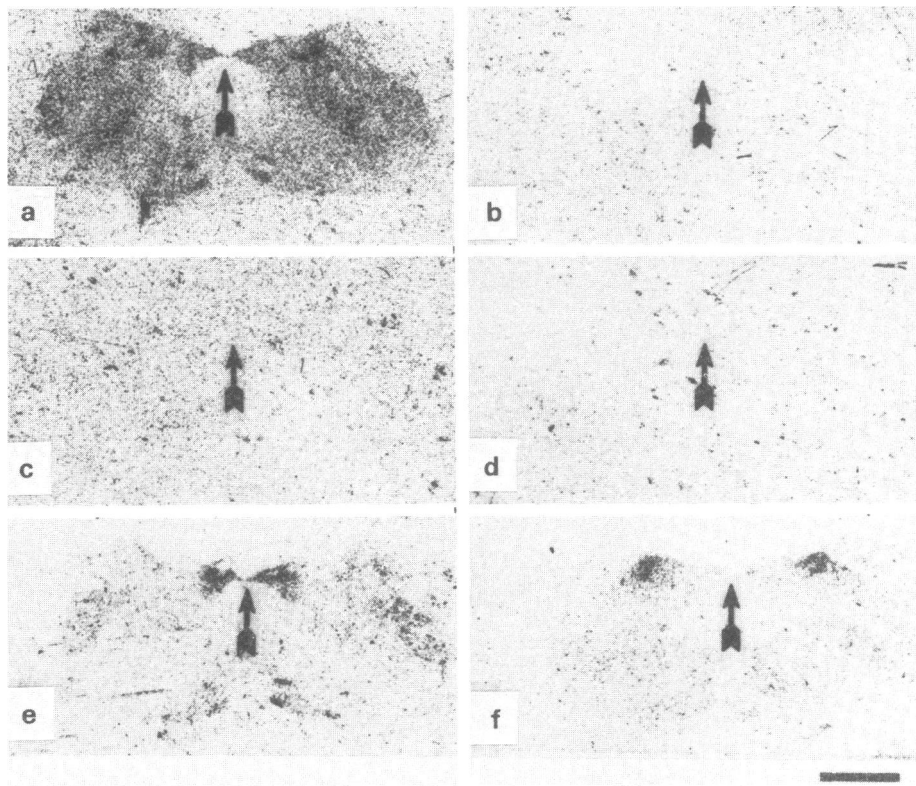

Figure 3 Distribution of opioid receptor subtypes in rabbit medulla oblongata. Consecutive 20 μm sections were incubated with [^{3}H]-naloxone (a), [^{3}H]-naloxone + 1 μM unlabelled naloxone (b), [^{3}H]-EKC (c), [^{3}H]-U69593 (d), [^{3}H]-DAGOL (e) and [^{3}H]-DPDPE (f). The darker the image the greater the density of receptors. Arrow indicates floor of 4th ventricle. Scale bar = 2 mm. Abbreviations as in Figure 2.

$Paco_2$ fell from 30.2 ± 0.7 to 24.9 ± 1.2 mmHg ($P < 0.05$) and remained significantly reduced for 60 min. There were similar falls in $Paco_2$ after i.c.v. dynorphin ($n = 4$). At 15 min after dynorphin, 1.0 nmol kg^{-1}, $Paco_2$ had fallen from 35.4 ± 0.9 to 30.2 ± 1.3 mmHg ($P < 0.05$) and by 60 min after 5.0 nmol kg^{-1}, $Paco_2$ had fallen to 27.5 ± 1.7 mmHg ($P < 0.05$). Neither U69593 nor dynorphin given i.c.v. altered respiration rate or Pao_2. There were no changes in respiration rate or blood gases after any of the doses of i.c. U69593 ($n = 5$) or i.c. dynorphin ($n = 4$).

Behaviour

DAGOL given i.c.v. had a dose-related sedative effect; the rabbits were mildly sedated after the middle dose and heavily sedated for 1–2 h after the highest dose. There was only a mild degree of sedation after the highest i.c. dose. Following i.c.v. but not i.c. DPDPE, the rabbits became more alert and active and started grooming. There were no behavioural changes after U69593 or dynorphin given i.c.v. or i.c.

Effect of i.v. naloxone on responses to i.c.v. DAGOL and i.c.v. DPDPE

DAGOL (1.0 nmol kg^{-1}) administered i.c.v. to six conscious rabbits caused similar changes in MAP, HR, plasma catecholamines and respiration to those seen previously. At 15 min after DAGOL, i.v. administration of naloxone (30 nmol kg^{-1}) caused a significant fall in MAP ($P < 0.05$) and increase in HR ($P < 0.05$) within 5 min (Figure 7); i.v. naloxone (3 nmol kg^{-1}) had no effect. The increase in plasma adrenaline of 22.7 ± 3.0 nmol l^{-1}, 15 min after DAGOL was significantly reduced ($P < 0.05$) within 5 min of naloxone to 4.2 ± 2.1 nmol l^{-1}, whereas the increase in noradrenaline of 3.3 ± 0.4 nmol l^{-1} was not significantly reduced at 5 min after naloxone (1.5 ± 0.3 nmol l^{-1}). In contrast, in the absence of

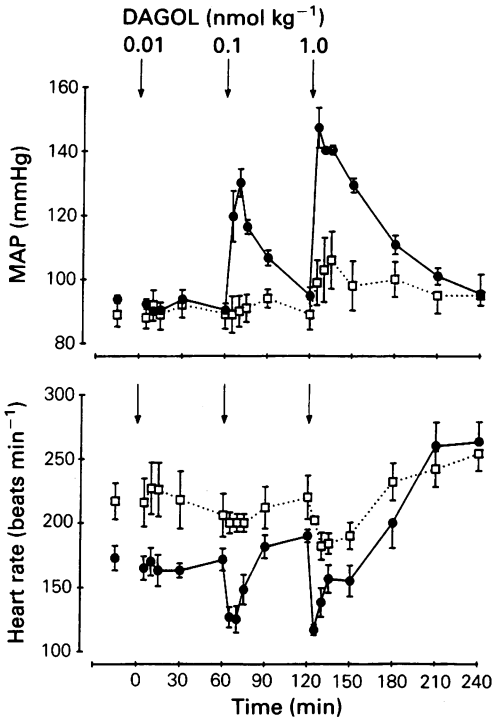

Figure 4 Cardiovascular effects of i.c.v. and i.c. DAGOL in conscious rabbits. After a 30 min control period, three doses of DAGOL (0.01, 0.1 and 1.0 nmol kg^{-1}) were given at 0, 60 and 120 min respectively, either i.c.v. (●) or i.c. (□). The control value is the mean of six readings taken during the control period. Each point represents the mean (i.c.v. $n = 6$, i.c. $n = 5$), with s.e.mean shown by vertical bars.

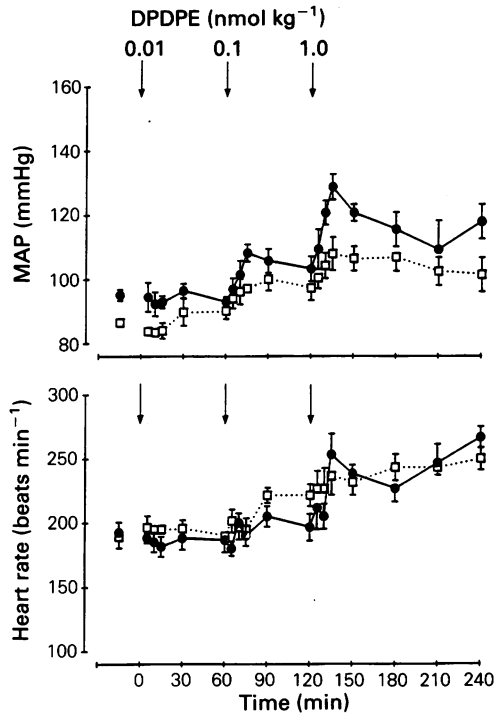

Figure 5 Cardiovascular effects of i.c.v. and i.c. DPDPE in conscious rabbits. After a 30 min control period three doses of DPDPE (0.01, 0.1 and 1.0 nmol kg^{-1}) were given at 0, 60 and 120 min respectively, either i.c.v. (●) or i.c. (□). The control value is the mean of six readings taken during the control period. Each point represents the mean (i.c.v. $n = 6$, i.c. $n = 6$), with s.e.mean shown by vertical bars.

naloxone plasma catecholamines did not fall until 60 min after DAGOL (Table 1). Within 5 min of naloxone the DAGOL-induced respiratory depression was attenuated. Respiration rate increased from 69 ± 8 to 112 ± 29 breaths min^{-1}, $Paco_2$ fell from 44.3 ± 2.5 to 37.8 ± 2.1 mmHg and Pao_2 increased from 72.2 ± 4.7 to 76.5 ± 3.1 mmHg. In the absence of naloxone, respiratory depression increased until 30 min after DAGOL and only began to return to control levels after 90 min (Figure 6). Naloxone also abolished the sedative effect of DAGOL.

DPDPE (1 nmol kg^{-1}) was given i.c.v. followed after 10 min by either i.v. saline (300 μl kg^{-1}) or i.v. naloxone (3 μmol kg^{-1}) ($n = 6$). DPDPE caused a similar increase in MAP and HR to that in the earlier dose-response study. After i.v. naloxone (3 μmol kg^{-1}), MAP and HR returned to control within 10 min; at all times after naloxone MAP was significantly ($P < 0.05$) lower than in the same group of rabbits when saline was given after DPDPE (Figure 7). Naloxone (300 nmol kg^{-1}) injected i.v. had no effect on any of the responses to i.c.v. DPDPE. The effects of naloxone on noradrenaline were unclear as the initial increases after DPDPE were different. Plasma noradrenaline was 0.8 ± 0.2 nmol l^{-1} above control 10 min after DPDPE and 5 min after naloxone (3 μmol kg^{-1}) it was 0.5 ± 0.3 nmol l^{-1} above control. When saline was given after DPDPE the initial rise in noradrenaline was 1.7 ± 0.7 nmol l^{-1} and 5 min after saline it was 1.6 ± 0.4 nmol l^{-1}. The increased activity and alertness caused by DPDPE was abolished by naloxone.

Discussion

The present study demonstrates that in conscious rabbits the μ-receptor agonist DAGOL given i.c.v.

Table 1 Changes in plasma noradrenaline, adrenaline and glucose after i.c.v. and i.c. [D-Ala^2, MePhe^4-Gly^5-ol] enkephalin (DAGOL) in conscious rabbits

			i.c.v.			i.c.		
Treatment	*Dose* (nmol kg^{-1})	*Time* (min)	*Noradrenaline* (nmol l^{-1})	*Adrenaline* (nmol l^{-1})	*Glucose* (mmol l^{-1})	*Noradrenaline* (nmol l^{-1})	*Adrenaline* (nmol l^{-1})	*Glucose* (mmol l^{-1})
Control			1.05 ± 0.14	0.22 ± 0.05	6.36 ± 0.25	1.53 ± 0.33	0.44 ± 0.15	6.78 ± 0.12
DAGOL	0.1	60	1.07 ± 0.11	0.52 ± 0.13	7.03 ± 0.16	1.68 ± 0.59	0.40 ± 0.10	7.02 ± 0.27
DAGOL	1.0	5	4.00* ± 0.29	26.54* ± 2.79		2.48 ± 0.84	2.78 ± 1.19	
DAGOL	1.0	30	4.60* ± 0.73	25.43* ± 5.81	16.66* ± 0.74	2.41 ± 0.77	1.95 ± 0.88	8.53* ± 0.67
DAGOL	1.0	60	3.26* ± 0.25	14.18* ± 1.59	5.34* ± 1.58	2.03 ± 0.48	0.84 ± 0.39	7.81 ± 0.52

After a 30 min control period DAGOL (0.01, 0.1 and 1.0 nmol kg^{-1}) was given at 60 min intervals. Samples were collected at the end of the control period, at 60 min after 0.1 nmol kg^{-1} and at 5, 30 and 60 min after 1.0 nmol kg^{-1}. Values are means ± s.e.mean (i.c.v. $n = 6$, i.c. $n = 5$).
* $P < 0.05$ for comparisons with control.

caused hypertension which was probably due to α-adrenoceptor-mediated vasoconstriction resulting from the high circulating catecholamine levels. A similar mechanism has been shown following intravenous administration of the μ-opioid agonist morphine in conscious rabbits (May *et al.*, 1988; 1989a),

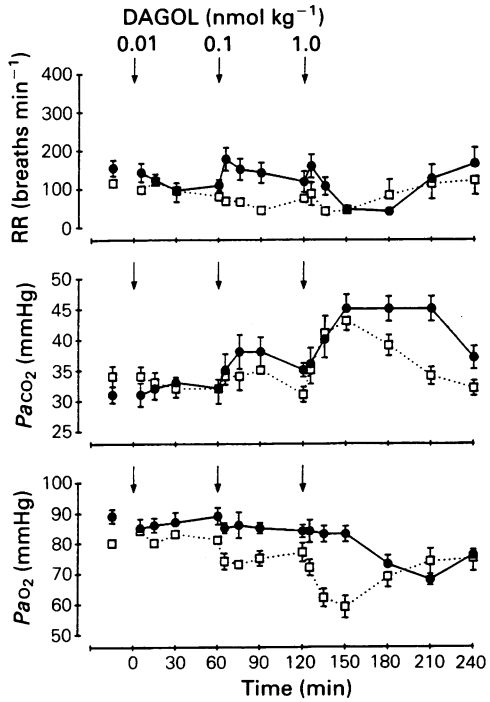

Figure 6 Respiratory effects of i.c.v. and i.c. DAGOL in conscious rabbits. After a 30 min control period three doses of DAGOL (0.01, 0.1 and 1.0 nmol kg^{-1}) were given at 0, 60 and 120 min respectively, either i.c.v. (●) or i.c. (□) (RR = respiration rate). Each point represents the mean (i.c.v. $n = 6$, i.c. $n = 5$), with s.e.mean shown by vertical bars.

and after i.c.v. administration of DAGOL in conscious rats (Pfeiffer *et al.*, 1983). The present findings indicate that the effect of DAGOL is due to an action on periventricular sites as it occurred after i.c.v. but not i.c. administration. Our demonstration of μ-receptors in the hypothalamus, which would be reached rapidly as drugs pass from the lateral into the 3rd ventricle, indicates that this may be a site of action. This is supported by the finding that microinjections of DAGOL into the anterior hypothalamus of conscious rats increased sympatho-adrenal outflow and produced hypertension (Pfeiffer *et al.*, 1982). The accompanying bradycardia probably resulted from a baroreflex-mediated increase in vagal tone. The delayed tachycardia which occurred after the highest dose suggests attenuation of the baroreflex mechanisms at this time, probably by an action on the brainstem as it occurred after i.c. as well as i.c.v. administration. This may result from an action on μ-receptors in the dorsal motor nucleus of the vagus. The hypertension following i.c.v. DAGOL is not secondary to the respiratory depression as there was no increase in BP after i.c. DAGOL when there were similar changes in blood gases to those after i.c.v. DAGOL. This conclusion is supported by the fact that hypertension preceded the onset of respiratory depression.

The δ-receptor agonist DPDPE also caused hypertension but, unlike DAGOL, it was accompanied by a parallel increase in HR. The similar changes after i.c.v. and i.c. administration suggest an action on δ-receptors demonstrated by autoradiography in the NTS rather than on those in the hypothalamus. DPDPE did not induce a generalized increase in sympatho-adrenal outflow but evoked a selective release of noradrenaline with no change in adrenaline. The hypertension following DPDPE may, therefore, result from vasoconstriction in selective vascular beds, and from an increase in cardiac output as the rise in MAP paralleled the rise in HR.

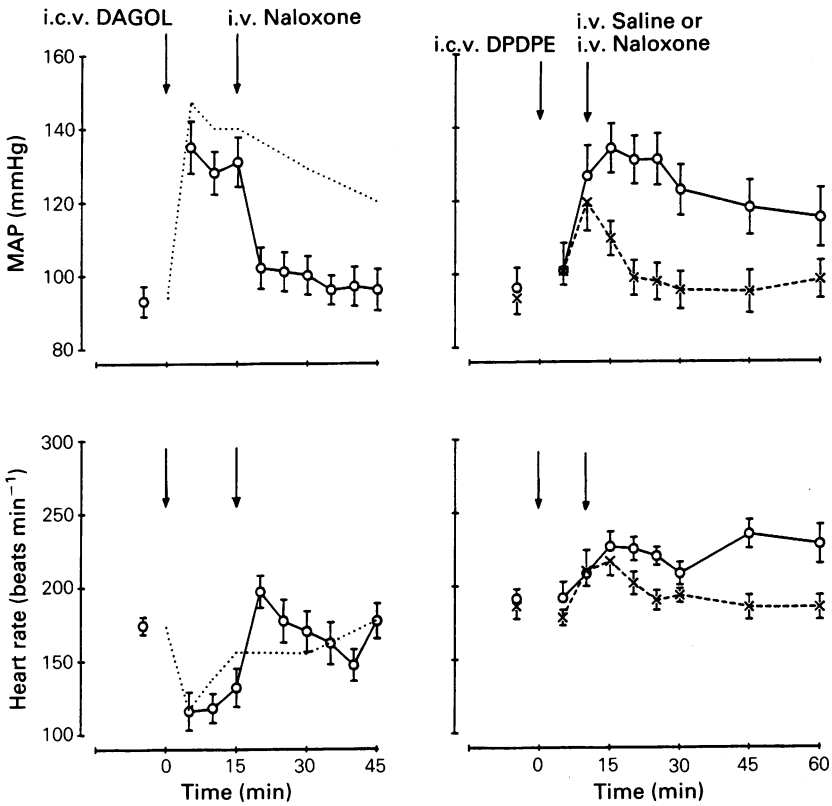

Figure 7 Effect of i.v. naloxone on the cardiovascular responses to i.c.v. DAGOL and DPDPE in conscious rabbits. Left panels: after a 30 min control period, DAGOL (1.0 nmol kg^{-1}) was given i.c.v. at 0 min followed by i.v. naloxone (30 nmol kg^{-1}) at 15 min. For comparison the dotted line represents the changes in MAP and HR seen after i.c.v. DAGOL (1.0 nmol kg^{-1}) taken from Figure 4. Each point represents the mean ($n = 6$) with s.e.mean shown by vertical bars. Right panels: after a 30 min control period DPDPE (1.0 nmol kg^{-1}) was given i.c.v. at 0 min followed either by i.v. naloxone (3 μmol kg^{-1}) (×) or i.v. saline (300 μl kg^{-1}) (○) at 10 min. Each point represents the mean ($n = 6$) with s.e.mean shown by vertical bars.

Table 2 Changes in plasma noradrenaline, adrenaline and glucose after i.c.v. and i.c. [D-Pen^{2,5}]enkephalin (DPDPE) in conscious rabbits

			i.c.v.			i.c.		
Treatment	*Dose* (nmol kg^{-1})	*Time* (min)	*Noradrenaline* (nmol l^{-1})	*Adrenaline* (nmol l^{-1})	*Glucose* (mmol l^{-1})	*Noradrenaline* (nmol l^{-1})	*Adrenaline* (nmol l^{-1})	*Glucose* (mmol l^{-1})
Control			1.11 ± 0.20	0.25 ± 0.06	6.65 ± 0.16	1.52 ± 0.28	0.33 ± 0.17	7.30 ± 0.42
DPDPE	0.1	60	1.33 ± 0.34	0.19 ± 0.04	6.95 ± 0.22	1.47 ± 0.24	0.46 ± 0.36	7.67 ± 0.16
DPDPE	1.0	5	1.56 ± 0.23	0.16 ± 0.05		1.84 ± 0.27	0.27 ± 0.15	
DPDPE	1.0	30	2.00 ± 0.53	0.23 ± 0.04	7.56* ± 0.29	1.70 ± 0.14	0.40 ± 0.29	7.87 ± 0.25
DPDPE	1.0	60	1.32 ± 0.11	0.38 ± 0.12	7.81* ± 0.29	2.34* ± 0.14	0.66 ± 0.52	8.53 ± 0.31

After a 30 min control period DPDPE (0.01, 0.1 and 1.0 nmol kg^{-1}) was given at 60 min intervals. Samples were collected at the end of the control period, at 60 min after 0.1 nmol kg^{-1} and at 5, 30 and 60 min after 1.0 nmol kg^{-1}. Values are means ± s.e.mean (i.c.v. and i.c. $n = 6$).

* $P < 0.05$ for comparisons with control.

The changes in MAP and HR after DPDPE are similar to those caused by i.c.v. and i.c. administration of leucine-enkephalin in conscious rats (Schaz *et al.*, 1980) suggesting that these effects of leucine-enkephalin resulted from stimulation of δ-opioid receptors. Comparison with other studies is difficult as [D-Ala^2, D-Leu^5]enkephalin has frequently been used as a δ-selective ligand but, as stated by the authors, it also has significant effects at μ-receptors (Pfeiffer *et al.*, 1982).

It is unlikely that the different responses to DAGOL and DPDPE result from different tissue distribution of the agonists in the brain leading to stimulation of separate populations of μ-receptors for two reasons. Firstly both agonists are highly specific particularly at the relatively low doses used. Secondly, the effects of DAGOL were inhibited by low doses of the μ-selective antagonist naloxone, whereas much higher doses were required to antagonize the effects of DPDPE, consistent with an action on δ-receptors.

The κ-receptor agonists U69593 and dynorphin had no effects on MAP but at the highest doses they caused a small elevation of HR. In contrast, a fall in HR has been shown in anesthetized rats after microinjection of dynorphin into the NTS and nucleus ambiguus (Hassen *et al.*, 1984), and microinjection of the selective κ-agonist U50488 into the dorsal motor nucleus of the vagus also reduced HR (Hassen, 1985). These differences from the present findings may result from the higher doses used, the use of anaesthetics or the different sites of administration. The similar effects after dynorphin and U69593 in the rabbit indicate that U69593 acts on the same population of κ-receptors as this endogenous κ-agonist. These findings correlate with the autoradiographic studies which were unable to demonstrate κ-binding using U69593 in rabbit hypothalamus or brainstem. The low degree of binding demonstrated in the hypothalamus using EKC may be a result of binding to μ- or δ-receptors, since only low concentrations of unlabelled DAGOL and DPDPE were included with the [^{3}H]-EKC incubation solutions.

These studies have also enabled investigation of the receptor subtype involved in opiate-induced respiratory depression which is of importance as it is a major side effect of narcotic analgesics. The respiratory depressant effects of morphine depend on stimulation of μ_2-receptors (Ling *et al.*, 1983), but a role for δ-receptors in opiate-induced respiratory depression has also been suggested (Pazos & Florez, 1984) and κ-agonists have been shown to reduce respiratory rate and tidal volume (Hassen *et al.*, 1984). Our results, using more specific agonists in conscious animals, suggest that opioid-induced respiratory depression is mediated entirely by μ-receptors. There is no evidence indicating a role for δ- or κ-receptors as DPDPE had no effect on respiration rate or blood gases and the κ-agonists reduced $PaCO_2$ after i.c.v. administration. This is also supported by the finding that the respiratory effects of DAGOL are blocked by low doses of naloxone which would not be expected to inhibit effects at δ- or κ-receptors. The respiratory depression occurred after i.c.v. and i.c. administration indicating that the receptor sites involved are probably in the brainstem.

These studies have demonstrated striking differences in the localisation of opioid receptor subtypes in hypothalamic and brainstem nuclei associated with cardiovascular control. Taken together with the specific functional effects of the agonists these findings are further evidence of role for opioid peptides in the central regulation of cardiovascular function. The hypertensive action of DAGOL and DPDPE indicates that the endogenous μ- and δ-ligands may be involved in the pathogenesis of hypertension. The ability of naloxone to reduce BP in spontaneously hypertensive rats (Wexler, 1984) and to prevent the increase in BP following renal artery constriction (Szilagyi *et al.*, 1986) supports a role for opioid peptides in hypertension. Naloxone also attenuates the hypertension in response to stress (Naranjo *et al.*, 1986) indicating that opioids may mediate stress-induced cardiovascular changes. In contrast, the ability of naloxone to reduce the hypotension following various forms of shock (Holaday, 1983) indicates that in this situation opioid peptides are acting to reduce BP. Thus opioid peptides have multiple effects on the cardiovascular system depending on the receptor subtype stimulated, the site of administration and the state of the animal. Further studies are required to determine the physiological role of endogenous opioid peptides in cardiovascular control and their possible involvement in the cardiovascular responses to stress.

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Effects of receptor-selective neurokinin agonists and a neurokinin antagonist on the electrical activity of spinal cord neurones in culture

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1 Rat spinal cord neurones grown in tissue culture were used to examine the electrophysiological effects of the neurokin in (NK)-selective agonists (pGlu^6, Pro^9) substance P(6–11) (septide; NK_1, 10^{-6} M) and (pGlu^5, MePhe^8, MeGly^9)SP(1–7) (DiMe-C7; NK_3, 10^{-6} M). In addition, the effect of the neurokinin antagonist (D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11})SP (10^{-5} M) on the neurokinin-evoked responses was investigated.

2 Neurokinin-evoked responses consisted of an increase in neuronal activity with or without long-lasting (mean: 50 s) depolarizations of the membrane potential of up to 25 mV. The latter also occurred in the presence of tetrodotoxin (10^{-7} M) (direct response).

3 In a number of spinal cord neurones ($n = 17$) only septide induced a membrane depolarization while DiMe-C7 elicited no response. On the other hand, in 2 neurones a response was exclusively evoked by DiMe-C7.

4 The neurokinin antagonist (D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11})SP had no effect of its own but blocked the septide- and DiMe-C7-induced depolarizations. It had no effect on the glutamate (10^{-5} M)-evoked depolarization.

5 It is concluded that by the use of neurokinin receptor-selective agonists, subpopulations of spinal cord neurones in primary dissociated cell culture can be differentiated which express the NK_1 or the NK_3 receptor. Cells expressing only the NK_1 receptor outnumber those expressing only the NK_3 receptor subtype. Both receptors can be blocked by the neurokinin antagonist (D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11})SP.

Introduction

Neurokinins are a group of small, structurally related peptides characterized by a common C-terminal pentapeptide Phe-X-Gly-Leu-Met-NH_2. The neurokinins present in the mammalian central nervous system are substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) (Kimura *et al.*, 1983; Maggio *et al.*, 1983). These neurokinins have been shown to interact with different receptor subtypes: NK_1 (SP), NK_2 (NKA) and NK_3 (NKB) (for review see Quirion & Dam, 1988).

Neurokinins have been identified in central terminals of certain small-diameter primary afferent fibres (Hökfelt *et al.*, 1975) and neurokinin receptors have been localized in the spinal cord (for review see Quirion & Dam, 1988). Several lines of evidence suggest that neurokinins play a role as neuromediators in the spinal processing of nociceptive information (Jessel, 1981; Piercey *et al.*, 1986). Neurokinins have also been shown to be expressed in primary cultures of sensory and spinal cord neurones of rats (Neale *et al.*, 1982; Adler *et al.*, 1984; Wienrich & Reuss, 1985). SP in particular has been shown to modulate electrical activity of cultured spinal cord neurones (Vincent & Barker, 1979; Hösli *et al.*, 1981; Nowak & MacDonald, 1982).

However, the study of the physiological effects of neurokinins with respect to neurokinin receptor subtypes is hampered by the fact that the endogenous neurokinins are preferential—but not selective agonists. Therefore, neurokinin agonists have been synthesized with high affinity for only one receptor subtype: (pGlu^6, Pro^9)SP(6–11) (septide) is a selective agonist for the NK_1 receptor (Laufer *et al.*, 1986). (pGlu^5, MePhe^8, MeGly^9)SP(1–7) (DiMe-C7)

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is a selective agonist for the NK_3 receptor (Sandberg *et al.*, 1982; Lee *et al.*, 1982).

The present study was undertaken to investigate the effects of two selective neurokinin receptor agonists on the electrical activity of spinal cord neurones in primary dissociated cell culture and to study the effect of a putative neurokinin antagonist (D-Arg^1, D-Pro^2, D-$Trp^{7,9}$, Leu^{11})SP (Rosell *et al.*, 1983) in this system.

We demonstrated that spinal cord neurones can be detected in culture which express only the NK_1 receptor. They outnumber those which express only the NK_3 receptor. The neurokinin antagonist (D-Arg^1, D-Pro^2, D-$Trp^{7,9}$, Leu^{11})SP blocks both, NK_1- and NK_3-induced responses.

Preliminary accounts of this work have been presented to the German Physiological Society (Wienrich *et al.*, 1987a, 1988b).

Methods

Cell culture

Cell cultures were prepared in a manner similar to that described by Ransom *et al.* (1977). Briefly, spinal cords with attached dorsal root ganglia (DRG) were dissected from 15 day-old Wistar rat embryos (Wi-AF/Han-EMD(SPF)). The tissue was minced, trypsin (Difco)-dissociated (0.05%) for 5 min, and, after trituration transferred to collagen-coated Petriperm dishes equipped with collagen-coated cover slips. Beginning on day 4, 5'-fluoro-2'-deoxyuridine (FdU) plus uridine (final concentrations 10 and 25 $\mu g\,ml^{-1}$, respectively) were added to the medium for 2–3 consecutive days to control the proliferation of non-neuronal cells. The medium contained nerve-growth factor (NGF, 20 $ng\,ml^{-1}$) and foetal calf- and horse serum (10 and 5%, respectively); no antibiotics were added. Cultures were used for recording after 25 days *in vitro*. At this stage electrical activity was fully developed (Jackson *et al.*, 1982). The experiments were exclusively performed on spinal cord neurones which were distinguished from DRG neurones on morphological and electrophysiological grounds (Peacock *et al.*, 1973; Ransom *et al.*, 1977).

Recording

Glass microelectrodes with a tip diameter $< 1\,\mu m$, filled with 3 M potassium acetate (50–70 megohm) were used for intracellular recording. Cells were transferred to a perfusion chamber on the stage of an inverted microscope (Zeiss) and penetrated with the aid of a step motor-driven micromanipulator (Sonnhof *et al.*, 1982). Membrane potential was continuously monitored throughout the experiment. Signals were amplified and displayed using conventional electrophysiological equipment. For signal recording and documentation, a Pascal programme package developed by Sontheimer *et al.* (1988) was used.

Experimental protocol

Peptides were applied by bath perfusion when the recording was stable. Perfusion rate was 5 $ml\,min^{-1}$ and 95% of the bath was exchanged within 5 to 10 s. Experiments were performed at room temperature. Septide and DiMe-C7 were diluted from stock solutions (10^{-3} M) to yield final concentrations ranging from 10^{-8} M to 10^{-5} M after 10 or 30 s application. Concentrations of peptides in the bath were assessed with h.p.l.c. at the beginning of the experimental series. Most experiments were performed with final concentrations of 10^{-6} M for both agonists, with a time interval of 5 min between each consecutive application. Both agonists were applied to each neurone studied. In some experiments glutamate (10^{-5} M to 10^{-3} M for 10 s) was used as an excitatory agonist and applied in the same way as the neurokinin agonists. The neurokinin antagonist (10^{-5} M final concentration) was applied for 20 s, 5 s before the agonist application. After washout for 10 min, the agonist application was repeated. The same experimental procedure was then performed in the presence of tetrodotoxin (TTX, 10^{-7} M).

Solutions and drugs

Cells were cultured in Dulbecco-modified Eagle's medium (Gibco-Europe), supplemented with 4×10^{-3} M L-glutamine (Boehringer Mannheim), 10% foetal calf-, 5% horse serum (both Gibco Europe) and nerve-growth factor (2.5 S NGF, 20 $ng\,ml^{-1}$, Calbiochem).

A recording solution of the following composition (mM) was used: NaCl 116, KCl 5.3, NaH_2PO_4 1.0, $MgSO_4$ 0.8, $CaCl_2$ 1.8, glucose 21.5, HEPES 10.0, pH 7.4, adjusted at room temperature with 1 N NaOH.

The drugs used were: FdU and uridine (Serva); tetrodotoxin (TTX, Sigma); DiMe-C7, septide and (D-Arg^1, D-Pro^2, D-$Trp^{7,9}$ Leu^{11})SP (Bachem); Na-L-glutamate (E. Merck). All drugs were dissolved in distilled water (10^{-3} M stock solution), and stored at −20°C. All further dilutions were made with recording solution. Stability of peptides in solution was routinely assessed with h.p.l.c.

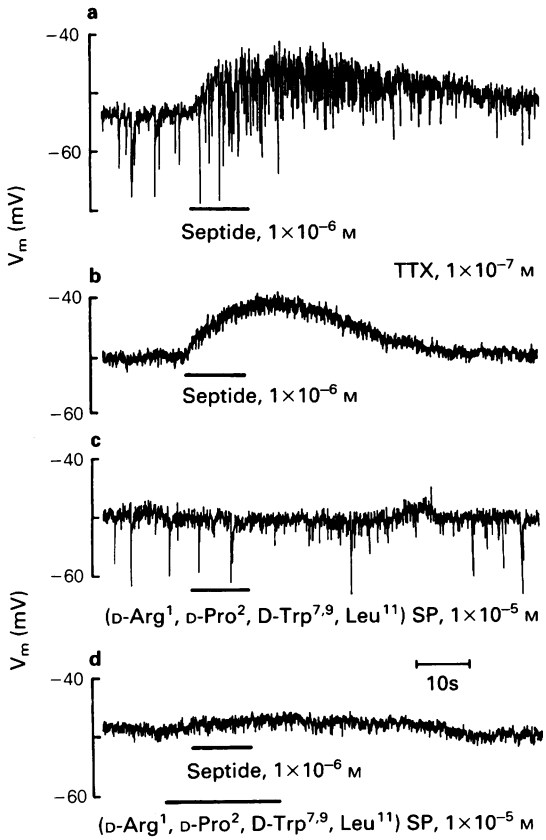

Figure 1 Action of the neurokinin receptor-selective agonist septide on the electrical activity of a single spinal cord neurone in culture and the effect of the putative neurokinin antagonist (D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}) substance P(SP). During recording of membrane potential (V_m), septide (10^{-6} M) was applied for 10 s (bar) and elicited an increase in neuronal activity and a membrane depolarization (a). The latter was also observed when action potential-induced transmitter release was blocked by tetrodotoxin (TTX, 10^{-7} M) (b). The antagonist applied on its own (10 s, 10^{-5} M, bar) produced no change in electrical activity (c). When the antagonist (10^{-5} M) was applied for 20 s (bar), beginning 5 s before septide (10^{-6} M) was applied for 10 s, the septide-induced change in electrical activity was blocked in the absence (not shown) or presence of TTX (d).

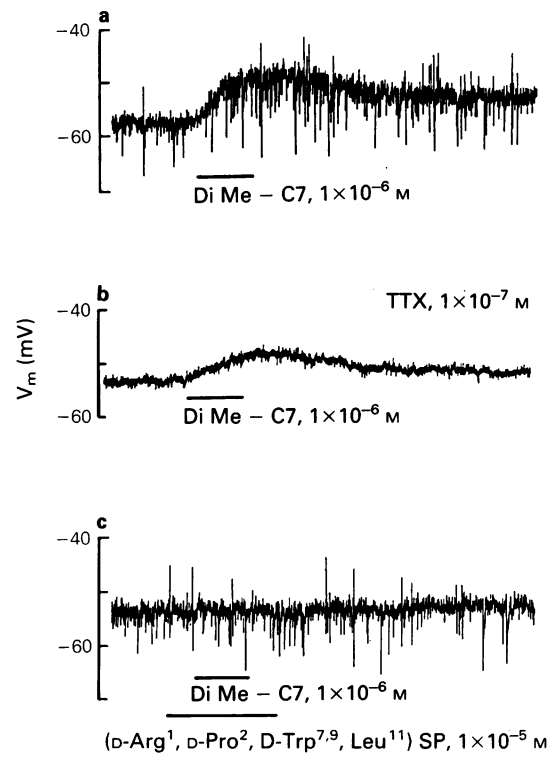

Figure 2 Action of the neurokinin receptor-selective agonist DiMe-C7 on the electrical activity of a single spinal cord neurone in culture and the effect of the neurokinin antagonist (D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}) substance P(SP). DiMe-C7 and the antagonist were applied as described in Figure 1. DiMe-C7 (10^{-6} M) elicited an increase in neuronal activity and a membrane depolarization (a). The latter effect was also observed in the presence of tetrodotoxin (TTX, 10^{-7} M) (b). In the presence of the antagonist (10^{-5} M), the DiMe-C7-induced depolarization was blocked (c).

Results

Effects of septide and DiMe-C7 on membrane potential

The effects of the selective neurokinin receptor agonists septide and DiMe-C7 on the membrane potential of cultured spinal cord neurones were tested. Neurones had average resting potentials of −50 to −60 mV. Cells with resting potentials more positive than −50 mV were discarded. Septide and DiMe-C7 (both 10^{-6} M) evoked responses in 62 out of 90 neurones studied. The most prominent effect was an increase in neuronal activity (synaptic potentials and/or action potentials; Figures 1, 2 and 3), which was often accompanied by a membrane depolarization lasting from 19 to 95 s (mean: 50 s). The amplitudes of the membrane depolarizations evoked by septide ranged from 6 mV to 24 mV (mean: 15 mV; Figure 1), while those evoked by DiMe-C7 were slightly smaller, ranging from 5 mV to 13 mV (mean: 9 mV; Figure 2). The amplitudes of the

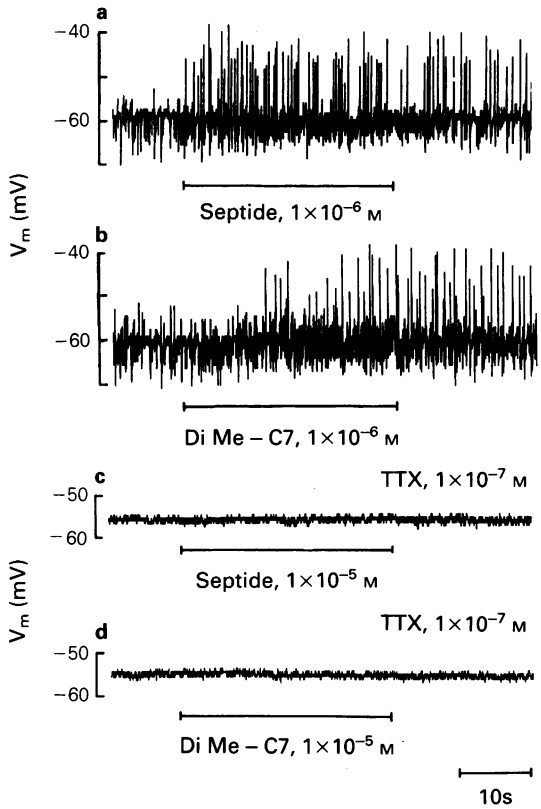

Figure 3 Effects of septide (a, c) and DiMe-C7 (b, d) on the electrical activity of a single spinal cord neurone in culture. Septide and DiMe-C7, applied for 30 s at 10^{-6} M, both induced an increase in neuronal activity without any change in membrane potential (V_m) (a, b). In the presence of tetrodotoxin (TTX, 10^{-7} M), both agonists were ineffective (c, d).

neurokinin-induced depolarizations increased in size with concentration (not shown). When TTX (10^{-7} M) was added to the bath in order to block action potential-induced transmitter release (Narahashi *et al.*, 1964), neurokinin-evoked membrane depolarization persisted (Figures 1 and 2), and neurokinin-induced increases in neuronal activity were no longer observed (Figures 1, 2 and 3).

Since septide and DiMe-C7 are selective neurokinin agonists, it was of particular interest to see whether neurones fell into groups with regard to their responses to either septide or DiMe-C7, thereby indicating a differential expression of neurokinin receptor subtypes in cultured spinal cord neurones. Of those neurones which responded to neurokinin application with an increase in neuronal activity and a membrane depolarization (31 out of 62), 12 were responsive to both, septide and DiMe-C7. Seventeen neurones responded to septide but not to DiMe-C7, while only 2 cells responded exclusively to DiMe-C7. These results suggest that spinal cord neurones in culture are not uniform with regard to the expression of neurokinin receptor subtypes.

Effect of a neurokinin antagonist on depolarizing responses to neurokinin agonists and glutamate

The putative neurokinin antagonist (DArg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11})SP was tested for its effect on agonist-induced responses. The antagonist (10^{-5} M) did not induce any agonist effects (Figure 1), but blocked the membrane depolarizations evoked by septide ($n = 16$; Figure 1) and DiMe-C7 ($n = 7$; Figure 2) in the presence (Figure 1) or absence (Figure 2) of TTX. After a washout period of 10 min, the agonist-evoked response showed an 80% recovery (not shown). Desensitization to agonists was excluded by two repetitive agonist applications yielding identical responses.

Glutamate evoked excitatory responses of up to 20 mV ($n = 32$) in the presence (Figure 4) or absence of TTX. These responses were not affected by the neurokinin antagonist ($n = 3$; Figure 4).

Discussion

Characterization of neurokinin receptor subtypes on cultured spinal cord neurones

The present results demonstrate that neurokinin agonists evoke two types of excitatory response in cultured spinal cord neurones, namely increases in neuronal activity with or without accompanying depolarizations of the membrane potential. The neurokinin-induced membrane depolarizations were always evident in the presence of TTX. This points to a direct effect of neurokinins on the cell under study since in the presence of TTX the cell does not receive synaptic input from action potential-induced transmitter release. On the other hand, neurokinin-evoked increases in neuronal activity without any change in membrane potential suggest an indirect effect, with agonists acting on neurones with synaptic input to the cell under study (Figure 3). This would explain the lack of response to septide and DiMe-C7 in the presence of TTX. Thus, spinal cord neurones with direct and indirect neurokinin responses can be detected in culture.

The present data suggest the expression of NK_1 and/or NK_3 receptor subtypes on cultured spinal cord neurones since both receptor-selective agonists elicit responses. The number of cells responding to septide only exceeded the number of cells responding

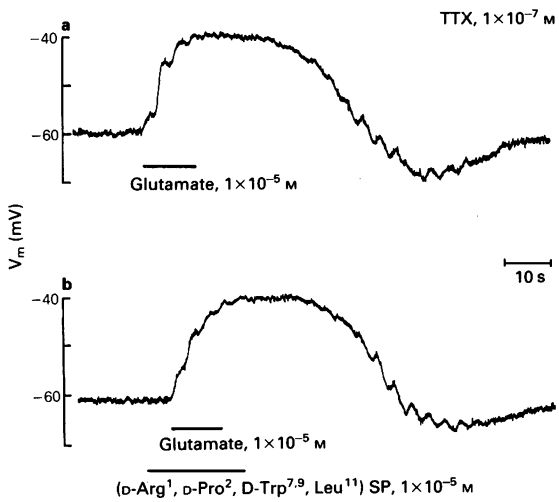

Figure 4 Lack of effect of the neurokinin antagonist (D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}) substance P(SP) (10^{-5} M) on the glutamate (10^{-5} M)-induced depolarization recorded from a single spinal cord neurone in culture in the presence of tetrodotoxin (TTX, 10^{-7} M). The mode of application for glutamate alone (a) and glutamate in the presence of the antagonist (b) was the same as described in Figures 1 and 2. The concentration of glutamate in the bath was not assessed by h.p.l.c. Therefore, due to the perfusion system, the actual concentration of glutamate applied to the cell might be slightly smaller than 10^{-5} M. Glutamate was routinely tested at 10^{-5} M since relatively few cells responded to glutamate at lower nominal concentrations.

exclusively to DiMe-C7 (17 vs 2), thereby indicating a larger number of cells expressing only the NK_1 receptor compared to cells expressing only the NK_3 receptor in our culture system. This would be in agreement with high levels of NK_1 binding sites in various laminae and nuclei of the dorsal and ventral horns throughout the spinal cord, while NK_3 sites are restricted to the superficial laminae of the dorsal horn (Ninkovic *et al.*, 1984; Glowinski *et al.*, 1987; Yashpal *et al.*, 1988). Therefore, NK_1 binding sites probably outnumber NK_3 sites in the spinal cord. Moreover, successful recording with intracellular microelectrodes depends on the use of comparatively large cells; it is therefore likely that in this study cell selection was biased towards ventral horn neurones, possibly motoneurones which are the largest neurones in the spinal cord (Ransom *et al.*, 1977). These technical aspects would favour a selection of neurones with NK_1 binding sites being predominant in the ventral horn.

The presence of neurokinin receptors on cultured spinal cord neurones has already been demonstrated (Vincent & Barker, 1979; Hösli *et al.*, 1981; Nowak & MacDonald, 1982), however, with no attempt to discriminate neurokinin receptor subtypes. By use of cross-desensitization- and electrophysiological techniques, the presence of NK_1 and NK_3 receptor subtypes in the rat spinal cord has recently been suggested (Wienrich *et al.*, 1987b). The functional implications of the presence of NK_1 and NK_3 receptors in the spinal cord remains to be elucidated.

Effect of the neurokinin antagonist (D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11})SP

The present study shows that (D-Arg^1,D-Pro^2,D-Trp^{7,9}, Leu^{11})SP blocks both the septide- and DiMe-C7-induced depolarizations in cultured spinal cord neurones. This is in agreement with the action of (D-Arg^1,D-Pro^2,D-Trp^{7,9}, Leu^{11})SP in the isolated spinal cord preparation of the newborn rat (Yanagisawa *et al.*, 1982; Matsuto *et al.*, 1984; Akagi *et al.*, 1985; Wienrich *et al.*, 1988a). Even though in spinal cord culture the discrimination of different neurokinin receptor subtypes by the antagonist is poor, the action of (D-Arg^1,D-Pro^2,D-Trp^{7,9}, Leu^{11})SP appears to be neurokinin receptor-specific. The antagonist was shown to have no effect on responses evoked by a number of different mediators in a variety of smooth muscle and nervous tissue preparations (Leander *et al.*, 1983; Konishi *et al.*, 1983; Akagi *et al.*, 1985), the latter including the lack of effect of (D-Arg^1,D-Pro^2,D-Trp^{7,9}, Leu^{11})SP on glutamate-induced excitations in spinal cord neurones shown in the present study. However, the antagonist blocks a variety of bombesin-induced responses (Porreca *et al.*, 1985; Moody *et al.*, 1987).

The data of the present study demonstrate differential expression of NK_1 and NK_3 receptor subtypes on cultured spinal cord neurones, the activation of which can be blocked by a neurokinin receptor antagonist.

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Mediators of burn-induced neuromuscular changes in mice

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1 Muscle paresis and aberrant pharmacological responses are two important pathophysiological changes that have been observed at the neuromuscular junction following thermal injury. By use of the mouse model of 20%, 30% and 50% total body surface area (BSA) burn, we examined the significance of intracellular mediators, adenosine 3′ : 5′-cyclic monophosphate (cyclic AMP) and prostaglandin E_2 (PGE_2) in perturbing the physiological function of tension development and the pharmacological response to (+)-tubocurarine (+)-Tc at day 21 post-burn.

2 Cyclic AMP levels increased with the size of burn. The relationship between mean cyclic AMP levels and burn size was significant ($R^2 = 0.96$, $r = 0.98$). Significant ($P < 0.05$) reductions in tension development (g) were observed for the 30% and 50% BSA burn group compared to controls (30.3 ± 8.3 and 34.1 ± 5.9 vs 59.1 ± 1.0, respectively). Tension alterations were associated with increased cyclic AMP levels; the relationship between increased cyclic AMP levels and tension decrease was significant ($R^2 = 0.82$, $r = 0.91$). The dose of (+)-Tc required to inhibit twitch tension increased in proportion to burn size and was statistically significant in the 50% BSA burn group compared to controls (0.3320 ± 0.09 vs 0.1093 ± 0.11 mg kg^{-1}, $P < 0.05$). The alterations in the effective dose of (+)-Tc were significantly correlated to increases in cyclic AMP levels ($R^2 = 0.70$, $r = 0.83$). Although PGE_2 levels were elevated in the 30% and 50% burn groups, no relation was seen to either tension or (+)-Tc doses.

3 These studies, therefore, support the hypothesis that cyclic AMP plays a significant role in physiological and pharmacological responses in skeletal muscle following thermal injury.

Introduction

Major thermal injury produces an array of systemic alterations, such as increased metabolic rates and immunological unresponsiveness (Herndon *et al.*, 1978; Demling, 1985). Pathological changes in skeletal muscle include increased fatiguability, muscle weakness and aberrant responses to neuromuscular (NM) blocking agents (Martyn *et al.*, 1980; 1986; Helm *et al.*, 1985; Martyn, 1986). NM dysfunction has been confirmed electromyographically by the presence of decreased nerve conduction velocity, positive sharp waves, and fibrillation potentials (Helm *et al.*, 1985; Mills *et al.*, 1986). These functional changes in muscle are thought to be responsible for the skeletal muscle immobilization and hypoventilation observed clinically in burn patients. Numerous clinical studies have documented a rightward shift in the dose-response curves for non-depolarizing NM relaxants, including (+)-tubocurarine ((+)-Tc) (Martyn *et al.*, 1980; 1986; Martyn, 1986). The mechanisms by which these systemic effects of burn injury are brought about have yet to be characterized.

Previous insight into NM function and the effects of drugs on NM transmission has implicated, among others, cyclic nucleotides like adenosine 3′:5′-cyclic monophosphate (cyclic AMP) and guanosine 3′:5′-cyclic monophosphate (cyclic GMP) (Standaert & Dretchen, 1981). Disruption in the normal cycling of these intracellular mediators may perturb NM dysfunction. In addition, prostaglandin E_2 (PGE_2) has been implicated in both the modulation of acetylcholine release (Shore *et al.*, 1987) and the antagonism of twitch depression in skeletal muscle produced by (+)-Tc (Hill & Wong, 1980). Therefore, the altered drug response and physiological function in skeletal muscle following burn injury may be mediated by changes in the cyclic nucleotide and/or prostaglandin system in the nerve and/or muscle,

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because thermal injury cases increases in concentrations of both catecholamines and/or cyclic AMP (Aikawa *et al.*, 1975) and prostaglandins (Demling, 1985).

Since alterations in NM function are related to both time after and magnitude of burn injury (Martyn *et al.*, 1986; Tomera *et al.*, 1988), precise characterization of the effects can be made only when these variables are controlled. The purpose of this study, which utilizes the mouse burn model, is to examine the role of the mediators cyclic AMP and PGE_2, in altering maximal development of tension and responses to (+)-Tc in the gastrocnemius muscle. These systemic changes were evaluated at 21 days following burns of 20%, 30% and 50% total body surface area (BSA).

Abstracts of this work were presented at the 37th Annual Fall Meeting of the American Physiological Society, New Orleans LA, October 5–9, 1986; and the Massachusetts General Hospital Research Symposium XI, Boston MA, January 15, 1987.

Methods

Animals

Male CF_1 mice, 9–10 months old (Charles River Breeding Laboratories, Wilmington, MA), were permitted to adapt to ambient conditions of $22 \pm 2°C$ for at least 1 week and given standard laboratory chow and drinking water *ad libitum*. Prior to inducing burn, the mice were deeply anaesthetized with pentobarbitone ($40\,mg\,ml^{-1}$, i.p., Lemmon Company, Sellersville, PA) and then shaved. Animals were determined to be anaesthetized when compression of their toes with haemostats did not result in a reflex withdrawal response.

Murine model of burn injury

This model was previously described for studies relative to hypermetabolism, immunosuppression and, more recently, to NM changes (Tomera *et al.*, 1988). All investigations involving mice were conducted in compliance with guidelines for experimental procedures set forth in the NIH Guide for the Care and Use of Laboratory Animals (Department of Health, Education and Welfare, No. 80-23, Office of Science and Health Reports, DRR/NIH, Bethesda, MD). The project was also reviewed and approved by the Animal Studies Committee of the Shriners Burns Institute and the Massachusetts General Hospital.

Anaesthetized mice were shaved in an area corresponding to either 20%, 30% or 50% of their total BSA; this was calculated for individual mice as $A = K(Wt)^{2/3}$, where A is area, Wt is weight, and K is a proportionality constant, equal to 12.3 for mice (Schildt & Nilsson, 1970). By use of a template, specific dorsal and ventral BSA were immersed in 95°C water for 8 s and immediately removed. The thermally injured area did not involve the legs. This scalding procedure produces a full-thickness third-degree burn injury with sharp margins. Applications of 1% silver sulphadiazine (Silvadene Cream, Marian Laboratories, Inc., St. Louis, MO) were made to areas of burn injury, followed by gauze dressings. All animals that underwent burn injury were resuscitated with 0.9% saline ($4\,mg\,kg^{-1}$ per % burn, i.p.). Control mice did not suffer burn injury in any way but were treated in a similar fashion by immersion in lukewarm water.

No further anaesthesia was administered during the recovery period. Mice were placed under an incandescent lamp to maintain warmth while recovering in an ambient environmental temperature of 26°C. The animals were then given 0.9% saline as drinking water for 1 week after burn injury.

Neuromuscular pharmacodynamics

Development of tension and responses to (+)-Tc in the gastrocnemius muscle were examined on day 21 post-burn by monitoring the indirectly (nerve) stimulated twitch response. Controls were anaesthetized with $40\,mg\,kg^{-1}$ (i.p.) pentobarbitone; in contrast, the burn-injured groups were given only $20\,mg\,kg^{-1}$ (i.p.) due to their increased sensitivity to this anaesthetic, regardless of the size of burn injury. All animals underwent tracheotomy to control respiration. The gastrocnemius muscle, surgically exposed and subjected to an optimal preload tension of 5 g, evoked maximal contraction (E_{max}) when a supramaximal electrical stimulus was applied to the sciatic nerve. A Grass S88 stimulator (Grass Medical Instruments, Quincy, MA) was used to administer supramaximal stimuli, 0.2 ms duration at 0.15 Hz. The force of contraction was recorded through a Grass FT 03C force displacement transducer.

Assurance of the appropriate depth of anaesthesia was ascertained when the left leg of the mouse was placed into a metal clamp, facilitating the vertical orientation of the leg for the partial dissection of the gastrocnemius muscle. Insertion of the foot in the clamp before administration of (+)-Tc caused it to be compressed in order to maintain the stability of the preparation. Appropriate anaesthetic depth was achieved by monitoring the parameter of the reflex withdrawal response upon clamping and throughout surgical preparation. In control studies anaesthetized mice subjected to this technique showed no reflex withdrawal response for periods as long as one hour. Thus, the potentially problematic area of the use of

NM blocking drugs in preventing animal response to nociceptive stimuli was averted.

Muscle twitch tension was permitted to stabilize for at least 10 min, after which cumulative doses of (+)-Tc (tubocurarine chloride injection, E.R. Squibb and Sons, Inc., Princeton, NJ) were given via the tail vein with a 27 gauge needle until twitch tension was suppressed by 95–99%. The response to each cumulative dose was plotted as % suppression of the control response (E_{max}) by the calculation: % suppression = actual twitch height/control twitch height × 100.

Linear regression analyses performed on the dose-response data were used to estimate effective dose (ED) values. Data of response (log((1 − y)/y)) vs (+)-Tc (log dose) underwent log-probit analysis (Litchfield & Wilcoxon, 1949). Regression analyses were performed by means of the RS1 program of a Digital computer (Professional 350, Digital Equipment Corporation, Maynard, MA). The ED values of the competitive antagonist (+)-Tc, at which 50% and 95% of the maximal contraction were attentuated, were referred to as ED_{50} and ED_{95}. These dose-tension response curves provided a quantitative means of studying antagonist ((+)-Tc) /cholinoceptor interaction at the NM junction in normal and pathological states (Condoris, 1971).

Cyclic nucleotide determinations

Following the twitch inhibition studies, a sample of gastrocnemius muscle was taken for analysis of cyclic nucleotide levels; the animals were then killed. Specimens were immediately quick-frozen (−70°C) with liquid nitrogen. Levels of cyclic AMP in gastrocnemius muscle were measured by use of ^{125}I radioimmunoassay (RIA) kits (Dupont-New England Nuclear (NEN) Research Products, Boston, MA). On the day of the assay, the previously frozen skeletal muscle segments were powdered, thawed in trichloroacetic acid (6% TCA) and homogenized to a tissue slurry. After centrifugation the supernatant was cleaned with water-saturated ether, dried and redissolved in sodium acetate buffer; each sample underwent RIA in duplicate (Steiner *et al.*, 1972).

The resulting pellet was dissolved in NaOH (1 N) and assayed for protein content (Lowry *et al.*, 1951). Recovery of cyclic AMP from the TCA extract as monitored by adding known amounts of ^{3}H-cyclic nucleotide (4000 c.p.m. [^{3}H]-cyclic AMP) to separate tissue slurries and passing their supernatant through a column (Dowex 50W-X8, H^+, 0.6 × 5.0 cm, Bio-Rad Laboratories, Richmond, CA). Recoveries for cyclic AMP in gastrocnemius muscle were >99%. All cyclic AMP levels were expressed as pmol mg^{-1} protein. For both kits intra- and inter-assay variation was not greater than 4% and 6%, respectively. Only one kit was used for all assay determinations. The sensitivity of the kit was 0.2 pmol ml^{-1}.

Determination of prostaglandin E_2

The extraction and purification of PGE_2 was accomplished by a modification of previously described procedures (Green *et al.*, 1978). In brief, frozen gastrocnemius muscle sections were thawed in sodium acetate buffer containing indomethacin (10 μg ml^{-1}). After homogenization, the proteins were denatured by the addition of 5 volumes of 80% ethanol. The mixture sat for 30 min and then the precipitate was then passed through filter paper (Whatman No. 1 Qualitative, American Scientific Products, Bedford, MA). The resulting clear solution contained compounds soluble in 80% ethanol. Most of the ethanol solvent was evaporated in an analytical evaporator (Organomation, South Berlin, MA). The resulting aqueous solution was diluted with water to reduce the concentration of residual ethanol.

The pH was adjusted with 0.1 N NaOH to a range of 8.0–8.5, which brought the prostaglandins to a fully dissociated form (water-soluble 'soaps'). The non-polar compounds (e.g. triglycerides) were extracted with petrol ether (Malinkrodt Corp., Paris, KY) four times, with a total of 15 ml each time. The petrol ether (top layer) was transferred to large test tubes (14.4 × 2 cm) and removed by evaporation. The residual water phase was acidified to pH 3.0–3.5 (HCl), which converted prostaglandins and fatty acids to the water-insoluble non-dissociated form. Prostaglandins were then extracted with ethyl acetate (Malinkrodt Corp.). The acidified water phase was extracted 3 times with the organic solvent. Subsequently, the combined organic phases were cleaned with small volumes of distilled water until the pH of the wash water was the same as that of the distilled water. After evaporation of the organic phase, the residue was diluted with an assay buffer of the following composition: 0.9% NaCl, 0.01 M EDTA, 0.3% bovine γ-globulin, 0.005% Triton X-100, 0.05 M sodium azide, 0.0255 M $NaH_2PO_4 \cdot H_2O$, 0.0245 M $NaH_2PO_4 \cdot 7H_2O$; pH 6.8. When similarly acidified plasma samples (pH 3.5–4.0) were processed with the PGE_2 ^{125}I-RIA kit as described in the manufacturer's specifications (Dupont-NEN Research Products, Boston, MA), the procedure consistently yielded >90% recovery of [^{3}H]-PGE_2.

Each sample was reconstituted with standard assay buffer (0.1 ml) and assayed in duplicate for 24 h. Results from the standards were used to reconstruct a dose-response curve from which the unkowns were read by interpolation. Protein precipitates recovered from initial filtration were dissolved in 1 N NaOH and assayed by the Lowry method. All

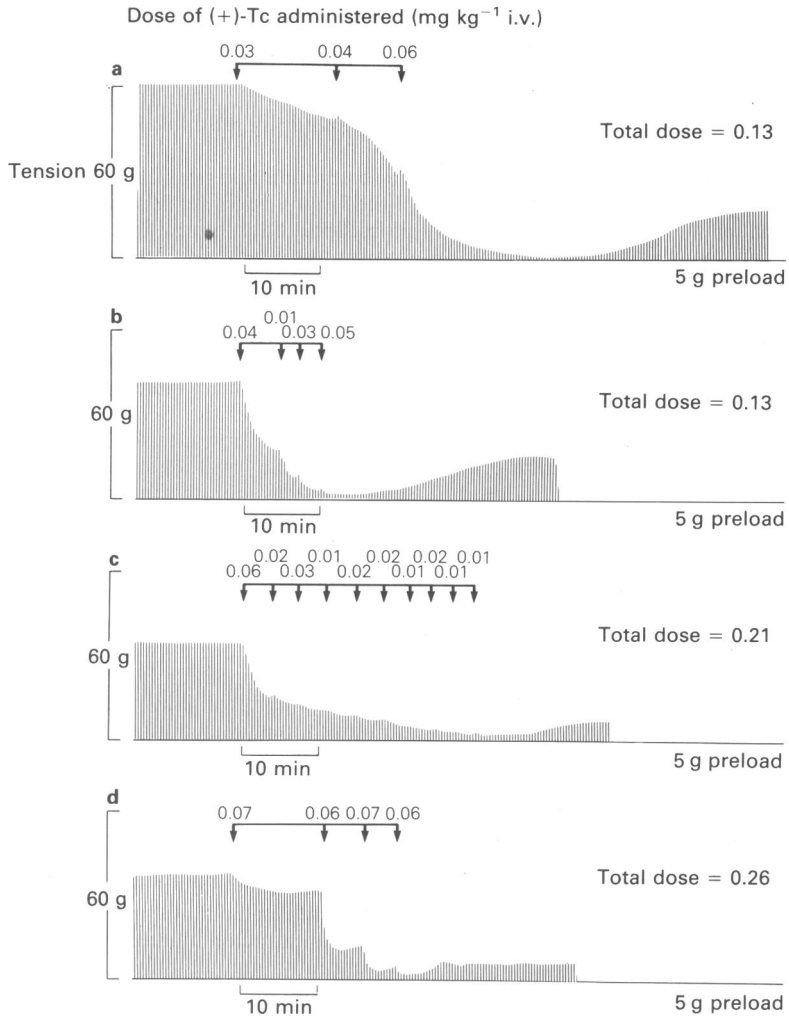

Figure 1 Representative traces of the twitch responses of gastrocnemius following thermal injury of (b) 20%, (c) 30% and (d) 50% total body surface area (BSA) compared with controls (a). Maximal contractile responses (E_{max}) for the 30% and 50% groups were significantly depressed compared to controls. Although the 20% group showed a depressed E_{max}, statistical significance was not achieved. Points of (+)-tubocurarine ((+)-Tc) injection (↓) via the tail vein allowed doses to be administered until 95–99% suppression was achieved. These points do not correspond directly to equipotent doses of (+)-Tc. Percentages refer to the % of thermally injured BSA.

results were expressed as fmol mg^{-1} protein. Intra-assay and inter-assay reproducibility using the recommended protocols were no greater than 6% and 18%, respectively. However, all PGE_2 determinations were made with one assay kit, the sensitivity of which was 0.13 pg PGE_2 0.1 ml^{-1}.

Statistical analyses

Data are presented as mean ± s.e.mean. For each of the variables (E_{max}, ED_{50}, ED_{95}, cyclic AMP and PGE_2) the statistical treatment of the data used one-way analysis of variance (ANOVA) to compare the means of the four groups (Hochberg, 1974; Ury, 1976). Because of the disparity in variability between the groups, PGE_2 was analysed on a logarithmic scale. To preserve the overall significance level during multiple testing (six pairwise comparisons among four means), it was necessary to apply a multiple comparison procedure. The GT2 method was appropriate because of the inequality among the sample sizes for the four groups (Hochberg, 1974;

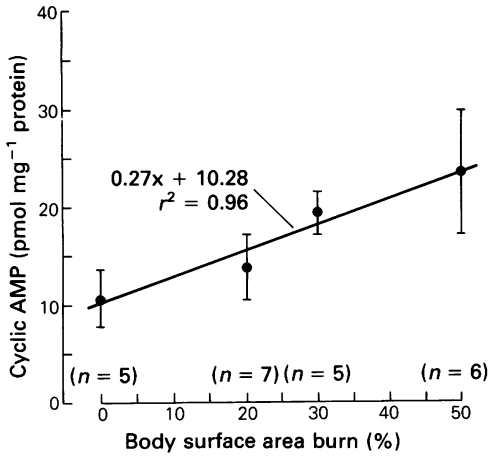

Figure 2 Levels of cyclic nucleotides within gastrocnemius muscles of mice (i.e., controls, 20%, 30% and 50% BSA). Each point represents the mean and vertical lines show s.e.mean, where n = number of observations = number of animals. Each sample was assayed in duplicate. There was a significant ($P < 0.001$) correlation between increase in cyclic AMP and increase in burn size ($R^2 = 0.96$, $r = 0.98$).

Ury, 1976). Linear regression analysis was used to examine relationships between ED vs. cyclic AMP and tension vs. cyclic AMP. For this evaluation the RS1 programme of a Digitial Pro 350 computer was used. The square of the correlation coefficients (R^2) is defined to be the coefficient of determination (Alder & Roessler, 1975). The proportion of the total variation in Y that can be explained by the linear relationship existing between X and Y. When multiplied by 100, the proportion is converted to %.

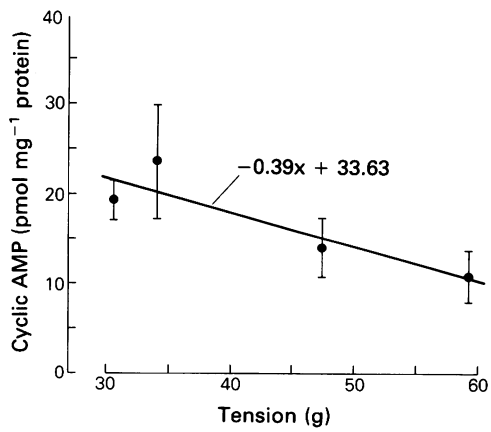

Figure 3 Relationship between cyclic AMP levels and tension of gastrocnemius muscles of mice with different sizes of burn. With an increase in cyclic AMP levels the maximal tension development decreased ($R^2 = 0.82$, $r = 0.91$). Each point represents the mean and vertical lines show s.e.mean.

Results

Tension responses

Representative twitch responses, before and after administration of (+)-Tc, are depicted for all experimental groups in Figure 1. These typical trace recordings show a decrease in maximal tension development (E_{max}) in the injured groups even before administration of (+)-Tc. The groups with larger burns showed significant ($P < 0.05$) reductions in maximal tension development (30% burn, 30.30 ± 8.32 g; 50% burn, 34.10 ± 5.89 g) at the common preload of 5 g compared with tension in controls (59.14 ± 1.03 g). When (+)-Tc was administered the peak effect following each dose was observed within 3 min. ED_{95} values (mg kg^{-1}) for the 50% burn group were increased 3 fold compared to controls (0.332 ± 0.09 vs. 0.109 ± 0.01, respectively, $P < 0.05$). That is, the NM junction of the 50% burn group demonstrated hyposensitivity to the neuromuscular effects of (+)-Tc, despite the decrease in maximal tension developed before the administration of (+)-Tc. The animals in the 50% burn group also lost weight ($P < 0.01$) at 21 days (41.45 ± 1.1 g) compared to their pre-burn weight (45.0 ± 0.7 g), whereas the other burn groups did not. The ED_{95} values in the 20% (0.110 ± 0.01) and 30% (0.189 ± 0.06) burn groups were similar to controls.

Cyclic nucleotides in skeletal muscle

Figure 2 shows the relationship between burn size and cyclic AMP in muscle; the mean cyclic AMP levels increased in proportion to the size of burn. The relationship between mean cyclic AMP levels (pmol mg^{-1} protein) and BSA burn (%) was significantly different from zero and is described by the equation cyclic AMP = 0.27% BSA + 10.28 with an $R^2 = 0.96$ and $r = 0.98$.

The increase in cyclic AMP levels was associated with a simultaneous decrease in tension. The decrease in tension was statistically significant ($P < 0.05$) for the 30% and 50% groups compared to controls (30.30 ± 8.32, 34 ± 5.89% vs. 59.14 ± 1.03%, respectively). The relationship between the mean values of cyclic AMP (pmol mg^{-1} protein) and tension (g) was significantly different

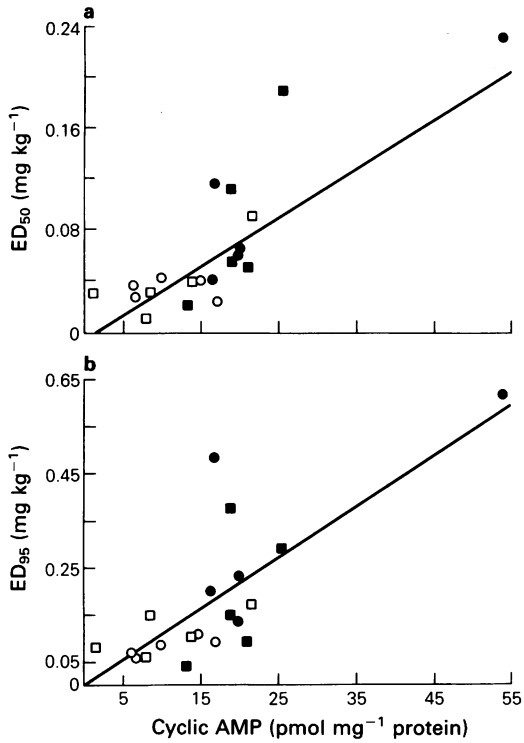

Figure 4 Relationships between cyclic AMP levels (pmol mg^{-1} protein) and either (a) ED_{50} or (b) ED_{95} for inhibition of gastrocnemius muscle tension by (+)-tubocurarine, before (○, control) and after burn injury (□, 20%; ●, 30%; ■, 50% BSA). A direct correlation was observed between the two variables: (a) $y = -0.01 + 0.004 \times R^2 = 0.70$, $r = 0.84$, $P < 0.001$, $n = 20$; (b) $y = 0.011 \times + 2.395e - 4$, $R^2 = 0.58$, $r = 0.76$, $P < 0.001$, $n = 20$.

from zero and was described by the equation cyclic AMP $= -0.3898 \cdot$ tension $+ 33.6304$ with an $R^2 = 0.82$ and $r = 0.91$ (Figure 3). Similarly direct relationships were observed between effective dose (ED) values for inhibition of gastrocnemius tension by (+)-Tc and cyclic AMP levels (Figure 4). The correlation coefficients between ED_{50} and ED_{95} were $r = 0.83$ ($R^2 = 0.70$, $P < 0.001$) and $r = 0.76$ ($R^2 = 0.58$, $P < 0.0001$), respectively.

PGE_2 levels in muscle

Elevated ($P < 0.05$) levels of PGE_2 (fmol mg^{-1} protein) were found in the 30% (187.57 ± 21.46) and 50% (86.49 ± 4.08) groups compared with controls (59.92 ± 5.43) (Figure 5). The difference in the means between the 30% and 50% groups were also significant ($P < 0.001$). No significant increase was observed in the 20% group. There was no relationship between changes in tension or ED values to changes in PGE_2 levels.

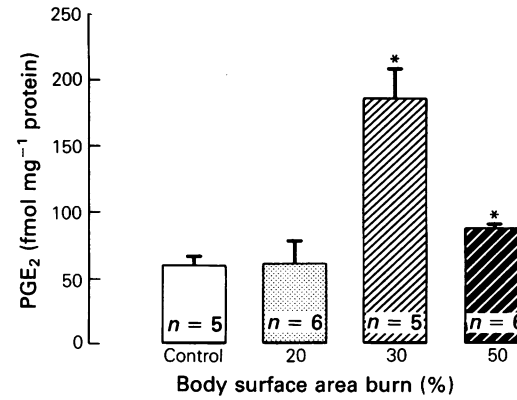

Figure 5 Prostaglandin E_2 (PGE_2) levels in gastrocnemius muscle of mice subjected to proportional increases in thermal injury. All levels were measured subsequent to inhibition of electrical stimulation by (+)-tubocuranine represent mean and vertical bars show s.e.mean, where n = number of observations = number of animals. Each sample was assayed in duplicate. Asterisks indicate statistically significant (*$P < 0.001$) differences compared to controls. The 30% and 50% groups differed significantly from each other ($P < 0.001$).

Discussion

Previous studies have documented the skeletal muscle disorders of patients with burns, including weakness and signs of denervation (Helm *et al.*, 1985; Mills *et al.*, 1986). Thermally injured patients are also hyposensitive to the effects of non-depolarizing muscle relaxants such as (+)-Tc (Martyn *et al.*, 1980; Helm *et al.*, 1985; 1986; Martyn, 1986). Following thermal injury, patients require doses and serum concentrations 3 to 5 fold greater than normal for equivalent paralysis. The mechanisms, perturbing altered skeletal muscle function and cholinoceptor sensitivity have not been completely elucidated (Martyn, 1986; Kim *et al.*, 1988). Therefore, the murine model of burn injury was evaluated to replicate the changes that occur at the NM junction, as well as to suggest causative mechanisms. Except for an increased mortality compared to rats (Herndon *et al.*, 1978), the murine model of burn trauma proved versatile for NM junction evaluation and provided useful information about the short-term (3 week) effects manifested by

varying sizes of BSA. Specifically, differences in NM pharmacology, evaluated by changes in ED for (+)-Tc, demonstrated altered action of NM relaxants at the motor endplate in the presence of increasing burn size. In the 30% and 50% burn groups, a significant decrease in maximum developed tension (muscle weakness) was also observed in the gastrocnemius prior to the administration of (+)-Tc. These effects, therefore, are consistent with clinical observations (Helm *et al.*, 1985; Martyn *et al.*, 1980; 1986; Martyn, 1986; Mills *et al.*, 1986).

Levels of cyclic nucleotides were measured to evaluate their roles in the rightward shifts of the dose-response curves to (+)-Tc and in the decrease in muscle tension following thermal injury. The significant correlation between increases in ED values and cyclic AMP levels (Figure 4) suggests a role for cyclic nucleotides in the shift of the dose-response curve. This confirms previous work (Standaert & Dretchen, 1981) in non-burned situations that cyclic nucleotides play an important role in the release of acetylcholine and in the antagonism of drugs such as (+)-Tc. The molecular mechanism by which cyclic nucleotides alter pharmacological response has not been defined in the present experiments, but it may be related to the role of cyclic AMP in: (1) turning on messenger RNA and increasing cholinoceptor numbers (Fontaine *et al.*, 1986; Langet & Changeux, 1987); and (2) serving as an intracellular index of the oxidative capacity of various fuels in skeletal muscles (Freeksen *et al.*, 1986).

There was a direct correlation ($R^2 = 0.96$, $r = 0.98$) between burn size and cyclic AMP levels (Figure 2). The increased cyclic AMP levels were associated with decreases in tension ($R^2 = 0.86$, $r = 0.93$, Figure 3); that is, 86% of variability in tension is explained by changes in cyclic AMP. This is best illustrated by the larger burn injuries (30–50% BSA), whose active tension was less than 35 g while showing the highest cyclic AMP values (approximately 20–25 pmol mg^{-1} protein). Again, these changes may be related to an increase in the number of cholinoceptors or the denervation-like state which is associated with an increase in receptor number (Fambrough, 1979; Kim *et al.*, 1988). Alternatively, cyclic AMP is a key compound involved in the mediation of numerous systemic effects initiated by burn trauma (Tomera *et al.*, 1988). Further elucidation of the mechanisms implicating cyclic nucleotides may be characterized by the use of specific agonists and antagonists that decrease cyclic AMP levels in muscle.

The observed systemic changes are important because the gastrocnemius NM junction was distant from the area of thermal injury and the muscle itself did not suffer direct thermal trauma. Neural or circulating factors appear to provoke these changes in skeletal muscle distant from the thermal injury site. Furthermore, our observations indicate that the ability of these animals to move did not appear to be compromised as a consequence of their trauma; areas of thermal injury were predetermined so as not to impede limb mobility. This contrasts with the muscle weakness and resistance (hyposensitivity) to non-depolarizing relaxants observed following immobilization of a limb or disuse atrophy (Wilmore *et al.*, 1974; Gronert *et al.*, 1984). However, in our study the animals or muscles that lost weight were the ones which showed hyposensitivity to (+)-Tc. Normally one would expect a weaker muscle to be extremely sensitive to (+)-Tc; the reason for the paradoxical relationship between weight loss or tension and hyposensitivity (resistance) to (+)-Tc effects is unclear. However, Greengard and coworkers have shown that the phosphorylation of the nicotinic cholinoceptor by cyclic AMP-protein kinase (PK) regulates receptor desensitization both *in vitro* and in cultured muscle cells (Huganir *et al.*, 1986). In purified, reconstituted receptors, phosphorylation on the gamma (γ) and delta (δ) subunits by cyclic AMP-PK increases by 7 to 8 fold the rate of rapid desensitization. How much, if any, of the 2.5 fold elevation in cyclic AMP we observed may contribute to the 3 fold shift in the ED_{95} of (+)-Tc of gastrocnemii from thermally injured mice requires clarification.

A point of clarification is required to discern whether differences in ED values are the result of differences in pentobarbitone dose between burn and control groups. A dose of 20 mg kg^{-1} was used in all burn groups, including 20% and 30% burn groups. However, the 20% and 30% groups showed no shift in ED values, while the 50% group did. It is noteworthy that good documentation is lacking to indicate an interaction between muscle relaxation by (+)-Tc and anaesthetic depth during pentobarbitone anaesthesia (Miller & Savarese, 1986).

Cyclic nucleotide levels were measured in muscle sections distal from the point of entry of the nerve. We were unable to distinguish cyclic AMP levels within nerve and muscle. However, because nerve entry is localized to one area of muscle, these levels are primarily representative of cyclic nucleotides within skeletal muscle. The increase of cyclic AMP levels in proportion to burn size (Figure 2) may have been due to a greater release of endogenous catecholamines as a consequence of thermal trauma. Catecholamines were not measured in our model, but persistent increases in the levels of catecholamines are known to occur in burn-injured patients and animals (Wilmore *et al.*, 1974; Aikawa *et al.*, 1975).

Levels of cyclic AMP within NM preparations similar to ours are thought to be the gross result of 3

parts: that contributed by the excitation-secretion system of motor nerve endings (Standaert & Dretchen, 1981); that reflecting net activities of skeletal muscle adenylate cyclase and phosphodiesterase (Arch & Newsholme, 1976); and that reflecting cyclic AMP content within whole blood. Within motor nerves cyclic AMP can have a biphasic effect on NM transmission. Phosphodiesterase inhibition by theophylline can cause a concentration-dependent inhibition or facilitation of excitation-secretion (Standaert & Dretchen, 1981; Fuke *et al.*, 1987). However, the latter is usually observed at very high (toxic) concentrations. Also, MDL 12,330A evokes prejunctional enhancement and postjunctional depression of NM transmission (Silinsky & Vogel, 1986). Catecholamines can affect cyclic AMP by stimulation of β-receptors which postjunctionally inhibits NM transmission. However, they can also enhance NM transmission (Kuba, 1971). Therefore, at the NM junction cyclic AMP can sometimes have opposing effects. Directly within mouse gastrocnemius muscle harvested from mice preparations *in situ*, adrenaline (25 nmol kg^{-1} body wt, i.v.) caused cyclic AMP levels (nmol g^{-1} wet wt) to be increased 2 fold (i.e., basal 1.95; stimulated 4.8) (Arch & Newsholme, 1976). A similar finding was made in rat gastrocnemius when adrenaline (50 nmol kg^{-1} body wt, directly injected into heart) caused cyclic AMP levels to be increased 3 fold (i.e., basal 0.69; stimulated 1.82). Therefore, the 2.5 fold increase observed in larger BSA burn mice is in good agreement with effects of systemically administered exogenous catecholamines in non-burned mice. We realize that the shifts are not as great compared to other tissues. However, the modulating role of cyclic AMP is also quite different in skeletal muscle. Nevertheless, as stated above, the observed cyclic AMP levels can account for the 86% of the tension variability in burn trauma. Also, our changes concur with a 2 to 3 fold increase in adenylate cyclase (derived from mouse gastrocnemius muscle) activity after being maximally stimulated with sodium fluoride. Contributions from whole blood in our estimates would be minimal, as (a) the sample section of muscle was rinsed free (2 s) of all blood and immediately frozen in liquid nitrogen; and (b) cyclic AMP is poorly soluble in liquid and would not permeate the cell membrane while in the circulation.

PGE_2 was measured within skeletal muscle distant from the site of burn injury as an index of burn trauma for the following reasons. First, the prostaglandin E series is known to influence tissue levels of cyclic AMP. Currently, this series is thought to activate membrane-bound adenylate cyclase activity within cardiac muscle by stimulation of a receptor other than the β-adrenoceptor (Levy & Klein, 1973). Also relative to neurotransmission (Martyn, 1986) is the finding that prostaglandins may modulate the release of the neurotransmitter acetylcholine at the NM junction (Hill & Wong, 1980; Shore *et al.*, 1987). Specifically, antagonism of (+)-Tc blockade was observed during infusion of PGE_2 or PGF_2 (Hill & Wong, 1980). In our thermal injury model, levels of PGE_2 were found to be greater in muscles from animals with burn injuries of 30% and 50% BSA compared to controls or mice with 20% BSA (Figure 5). However, a consistent, direct relationship does not exist between NM function and PGE_2 changes.

In conclusion, the present study evaluated the effects of graded increases in size of burns on NM function and pharmacology. We tested the hypothesis that cyclic nucleotides and PGE_2 play a role in NM dysfunction. A relationship based on the correlation coefficient is suggested to exist between NM changes and cyclic nucleotide levels but not to PGE_2 changes. Nevertheless, even in our early use of this model, the potential for a thorough pharmacological evaluation of the various mechanisms triggered by burn injury was demonstrated. The murine model is a useful approach to understanding the distant effects of burn injury at the skeletal muscle level, which in turn may lead to faster patient rehabilitation.

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Capsaicin evokes secretion of nasal fluid and depletes substance P and calcitonin gene-related peptide from the nasal mucosa in the rat

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1 The secretion of nasal fluid was studied in anaesthetized rats after topical application of capsaicin, and of calcitonin gene-related peptide (CGRP) alone or CGRP in combination with substance P (SP). The flow of nasal fluid was stimulated and the secretions collected by a filter paper technique. The concentrations of SP and CGRP in nasal biopsies were determined after topical or systemic administration of capsaicin.

2 Capsaicin (single dose administration) stimulated nasal secretion in a dose-dependent manner. The effect was inhibited by hexamethonium, lignocaine, or by the tachykinin antagonist (D-Pro^2, D-Trp^{7,9})-SP, but not by atropine, or by a combination of the histamine H_1-receptor antagonist chlorpheniramine and the H_2-receptor antagonist ranitidine.

3 When applied cumulatively, capsaicin rapidly produced desensitization. The concentrations of SP and CGRP in the nasal mucosa were reduced by capsaicin 6 days after topical or s.c. administration but not 15 min after topical application of desensitizing doses.

4 CGRP did not stimulate the secretion of nasal fluid and did not alter SP-evoked nasal secretion.

5 The inhibition by hexamethonium of the capsaicin-evoked nasal secretion suggests the involvement of ganglionic reflexes. In addition, the inhibition of the response to capsaicin by (D-Pro^2,D-Trp^{7,9})-SP and lidocaine and the depletion of SP and CGRP after capsaicin indicate the involvement of tachykinin-mediated axon reflexes.

Introduction

Capsaicin activates sensory nerve endings in the nose and the paranasal sinuses, thereby stimulating protective reflexes involved in sneezing and in increased vasopermeability (Lundblad *et al.*, 1984) and stimulated mucociliary activity (Lindberg & Mercke, 1986).

The sensory nerve endings of the nasal mucosa harbour substance P (SP) (Lundblad *et al.*, 1983; Uddman *et al.*, 1983) and calcitonin gene-related peptide (CGRP) (Sundler *et al.*, 1985). Capsaicin is known to evoke the release of SP (Jessell *et al.*, 1978) and CGRP (Franco-Cereceda *et al.*, 1987) from such nerve fibres. SP stimulates the secretion of nasal fluid (Petersson, 1989) whereas CGRP does not (Geppetti *et al.*, 1988).

In this study, the effect of topically applied capsaicin and CGRP or CGRP combined with SP on nasal secretion in the rat, and the effect of topically or systemically given capsaicin on the SP and CGRP content in the nasal mucosa were studied.

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Methods

Animals

Male Sprague-Dawley rats (300–450 g) were anaesthetized with pentobarbitone (Mebumal Vet, ACO, Sweden), 80 mg kg^{-1} i.p. After tracheotomy, they were placed in a supine position and allowed to breathe spontaneously through a tracheotomy cannula.

Nasal challenge

A filter paper technique was used for the challenge and collection of nasal fluid (Petersson, 1989). Test

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solutions of capsaicin, CGRP and SP were applied in drops of 2.5 μl to strips of filter paper (Munktell No. 150, Stora Kopparberg, Sweden). The strips were 40 by 5 mm with a narrow end, 13 by 1 mm; they were covered with adhesive film on one side. After being dried and weighed, the strips were inserted into the nasal cavities and exchanged every 5 min. Upon removal, the strips were reweighed and the weight gain (i.e. the weight of the nasal secretions) calculated. The experiments began 15 min after tracheotomy with application of three non-impregnated paper strips in sequence in both nasal cavities unless otherwise stated. The fluid collected during the third 5 min interval, i.e. the secretion immediately preceding the capsaicin challenge, was taken as the baseline secretion. The contralateral cavity continued to receive non-impregnated paper strips. Capsaicin was applied as single or cumulative doses. CGRP and SP were applied in cumulative doses. In one series of experiments a fixed dose of CGRP was administered in each paper strip with cumulatively increasing doses of SP.

Tissue preparations after capsaicin pretreatment

Nasal biopsies were taken from rats 15 min after (a) the cumulative administration of capsaicin (total dose 2360 nmol), 6 days after (b) a single dose of capsaicin (1630 nmol) topically applied via filter paper strips, after (c) drug-free paper strips (control), and after systemically given (d) capsaicin, 50 mg kg^{-1} s.c., dissolved in ethanol, Tween 80 and saline (vehicle) or after (e) vehicle alone. The rats in groups (b)–(e) were non-tracheotomized and anaesthetized with pentobarbitone, 40 mg kg^{-1}, both when challenged and before they were killed by an intracardial air embolus. In order to counteract respiratory impairment caused by capsaicin, the rats were pretreated with terbutaline, 0.1 mg kg^{-1} s.c., and theophyllamine, 10 mg kg^{-1} i.p. (Lundberg *et al.*, 1983) before the capsaicin injections. Four of seven rats given capsaicin s.c. and five of six rats given a single dose of capsaicin survived. Lundberg *et al.* (1983) found a survival rate of 60–80% after systemic capsaicin treatment. The nasal mucosa which had been exposed to filter paper or the nasal mucosa in systemically treated animals was removed from both sides. After being weighed, the tissue specimens were rapidly frozen on dry ice and stored at −80°C. After being boiled in 0.5 M acetic acid (1–2 ml per 10 mg tissue) for 15 min, the specimens were homogenized in a Polytron (1–2 min). After centrifugation at 2000 *g* for 15 min the supernatants were lyophilized. For radioimmunoassay the freeze-dried material was dissolved in 1.5 ml 0.05 M phosphate buffer, pH 7.4, with human serum albumin (HSA), 2.5 g l^{-1} (Hoechst, Behring, Maburg, F.R.G.), and centrifuged at 2000 *g*.

Radioimmunoassay

For the radioimmunoassay (RIA) of SP and CGRP the samples were analysed in serial dilutions. Each extract was assayed in duplicate at two dilutions with an individual control for binding in the absence of antibody. SP-like immunoreactivity (LI) was measured as described by Brodin *et al.* (1985). The antiserum SP 2 was used in a final dilution of 1:125,000 and [^{125}I]-(Tyr^{8})-SP was used as tracer. The sensitivity was 10 pmol l^{-1} and the interassay variation 9% in the range 20–90 pmol l^{-1}. The antiserum does not detect any known tachykinin besides SP.

CGRP-LI was measured as described by Grunditz *et al.* (1986). The antiserum K-8429 was used in a final dilution of 1:75,000 and [^{125}I]-(Tyr^{0})-CGRP was used as a tracer. The sensitivity was 20 pmol l^{-1} and the interassay variation $<$15% in the range 20–200 pmol l^{-1}. There is no cross reaction with human calcitonin, katacalcin or calcitonin C-terminal adjacent peptide.

Drugs and solutions

Test solutions for topical application: capsaicin (Fluka AG, Buchs, Switzerland) dissolved in 95% ethanol to 658 mM and diluted (1:3) to 0.066 mM; CGRP (Sigma, St. Louis, MO) in distilled water containing 0.1% BSA to 4 mM and diluted (1:10) to 40 μM and SP (Sigma) in 0.1% BSA containing water to 40 mM and diluted (1:10) to 40 μM. Capsaicin for systemic pretreatment was initially dissolved to a concentration of 100 mg ml^{-1} in 10% ethanol and 10% Tween 80. Further dilutions were then made in saline giving a stock solution of 10 mg kg^{-1}. The following receptor antagonists were given i.p. 15 min before the experiment: atropine sulphate (ACO, Sweden), 1 mg kg^{-1}; hexamethonium chloride, 30 mg kg^{-1}; the H_1-receptor antagonist chlorpheniramine maleate (Sigma), 10 mg kg^{-1}, and the H_2-receptor antagonist ranitidine hydrochloride (Glaxo, U.K.), 4.0 mg kg^{-1}. The tachykinin antagonist, (D-Pro^{2},D-Trp^{7,9})-SP (Ferring, Malmö, Sweden), 26 mM, in 0.1% BSA was diluted to 13 mM and to 6.6 mM and applied to filter paper strips; strips impregnated with doses of 17, 33 and 66 nmol respectively (applied at 5 min intervals), giving a total dose of 116 nmol applied during 15 min, were applied to one nasal cavity. A local anaesthetic agent, 4% lignocaine hydrochloride (Sigma), 20 μl, was applied to one nasal cavity by means of a microsyringe 15 min before the experiment. Terbutaline was obtained from Draco (Sweden) and theophyllamine from ACO (Sweden).

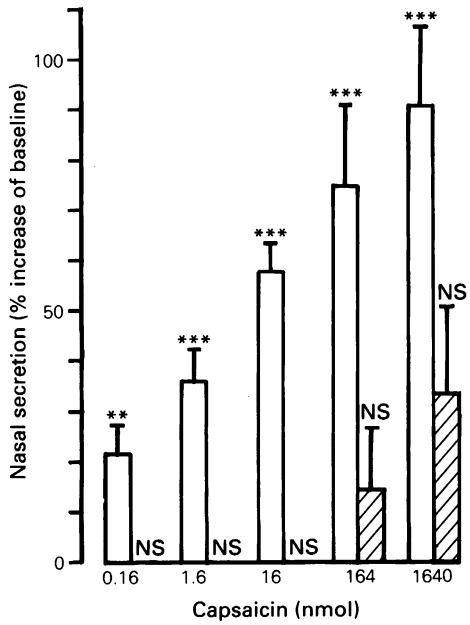

Figure 1 Nasal fluid secretion (expressed as % increase of baseline) evoked by single doses of topically applied capsaicin (dose-response curve, $n = 8$ at each dose). Secretions from the stimulated nasal cavity is indicated by open columns and that from the contralateral cavity by hatched columns. Secretion on the contralateral side did not increase. Values in this and subsequent figures are expressed as means with vertical lines indicating s.e.mean. Asterisks indicate significant increase compared to baseline. NS $P > 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Statistical analysis

The secretion rate was expressed as weight of nasal fluid per unit time (mg per 5 min), or as % increase over baseline. The concentrations of SP- and

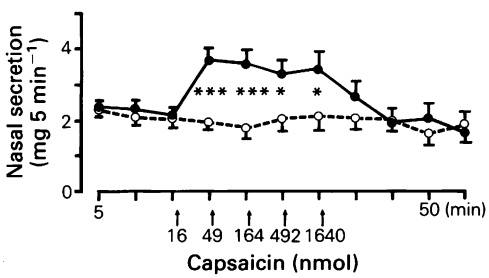

Figure 2 Nasal fluid secretion (mg 5 min^{-1}) evoked by cumulatively increasing doses of capsaicin (topical application) ($n = 14$). Secretion was stimulated by the first dose (16 nmol) but not further stimulated by subsequent doses. Capsaicin-stimulated (●) and control (○) nasal sides are indicated. * $P < 0.01$, *** $P < 0.001$.

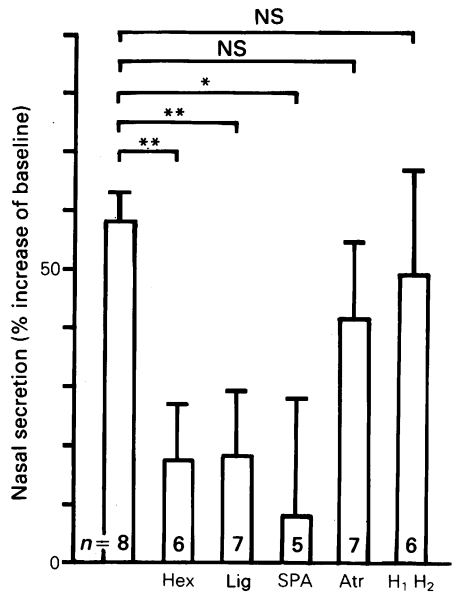

Figure 3 Nasal fluid secretion (% increase of baseline) in response to capsaicin (16 nmol) before and after pretreatment with hexamethonium (Hex), lignocaine (Lig), (D-Pro^2,D-Trp^{7,9})-substance P (SPA), atropine (Atr), chlorpheniramine plus ranitidine (H_1 H_2). Asterisks indicate statistical significance for comparisons between groups. NS $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

CGRP-LI were expressed as pmol g^{-1} tissue wet weight. Different experimental groups were compared by use of unpaired t test. Two-way analysis of variance was used to compare paired observations, i.e. differences between experimental (ipsilateral) and control (contralateral) sides and between repeated observations on the same side. Because of the small number of animals non-parametric tests (Mann-U-Whitney and Friedman) were performed with similar significances as the parametric tests. P values <0.05 were considered as significant.

Results

Capsaicin stimulated the secretion of nasal fluid in a dose-dependent manner when administered as a single dose (Figure 1). There was no effect on the contralateral side. After each dose the secretion returned to the baseline level within 5 min (not shown). When given in a cumulative fashion, capsaicin stimulated secretion initially; subsequent doses produced progressively smaller responses (Figure 2).

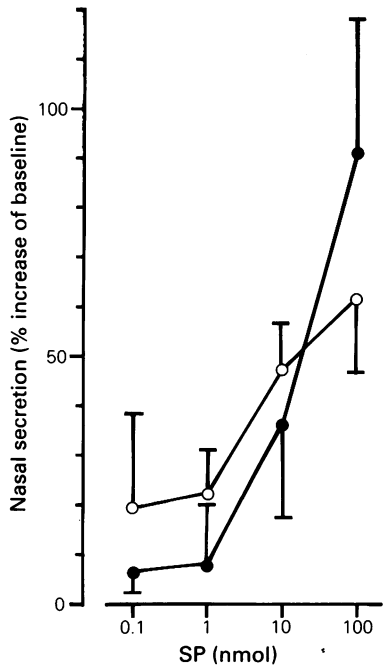

Figure 4 Nasal fluid secretion (% increase of baseline) evoked by cumulatively increasing doses of substance P (SP) (●, $P < 0.01$, $n = 5$). The addition of calcitonin gene-related peptide (1 nmol, ○, $n = 5$) did not change the SP-evoked increase of secretion.

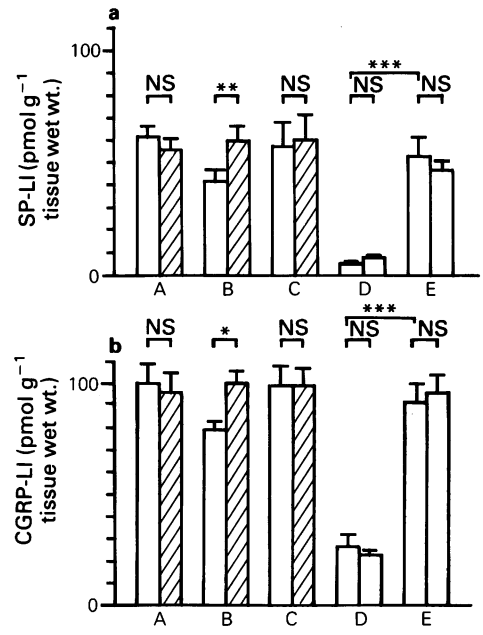

Figure 5 The concentrations (pmol g^{-1} tissue wet weight) of (a) substance P (SP)- and (b) calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) in rat nasal mucosa 15 min after (A) topical capsaicin cumulatively given (2360 nmol in all; $n = 14$, see Figure 2), 6 days after (B) topical capsaicin (1640 nmol as a single dose, $n = 5$), after (C) filter paper strip (control, $n = 4$), and after s.c. (D) capsaicin, 50 mg kg^{-1}, dissolved in ethanol and Tween 80 (vehicle) ($n = 4$) or (E) the vehicle (control, $n = 5$). Open columns indicate nasal side receiving capsaicin (group A and B) or drug-free filter paper strips (group C). Hatched columns indicate control side receiving drug-free strips (group A and B) or no strips (group C). In group D and E no strips were applied. NS $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

The increased secretion evoked by 16 nmol capsaicin was greatly reduced by hexamethonium, lignocaine, or (D-Pro^2,D-$Trp^{7,9}$)-SP, but not by atropine or by H_1- and H_2-receptor antagonists in combination (Figure 3). After 0.16 nmol capsaicin the response was abolished by lignocaine ($n = 4$, not shown). The antagonizing drugs did not affect the baseline secretion.

CGRP (0.01 to 10 nmol, $n = 5$) did not stimulate secretion (not shown), nor did CGRP (1 nmol) alter the secretory response to SP (Figure 4).

The concentrations of SP and CGRP in the nasal mucosa were reduced 6 days after topically or systemically given capsaicin but not 15 min after topical application of desensitizing doses of capsaicin (Figure 5).

Discussion

Capsaicin stimulated nasal fluid secretion in the rat in agreement with recent findings in man (Geppetti *et al.*, 1988). The fact that lignocaine inhibited the capsaicin-evoked response suggests that sensory nerve endings were activated. Analogously, Lundblad *et al.* (1984) found that local anaesthesia reduced capsaicin-evoked sneezings in the guinea-pig. In the same study hexamethonium failed to reduce the sneezing response. In contrast, the capsaicin-evoked nasal secretion in the present study was suppressed by hexamethonium, suggesting that the secretory response involves not only axon reflexes (engaging sensory nerve terminals) but also ganglionic reflexes. The capsaicin-evoked increase in mucociliary activity in the rabbit sinus mucosa was also reduced by hexamethonium (Lindberg & Mercke, 1986). Hence, different nervous mechanisms

seem to control the various protective reflexes in the nose. Hexamethonium is known to reduce the systemic blood pressure (e.g. Martling *et al.*, 1985), and we cannot exclude the possibility that a fall in blood pressure contributed to the hexamethonium-induced reduction of the capsaicin-evoked secretion. However, nasal fluid secretion evoked by exogenous SP was unaffected by hexamethonium (Petersson, 1989). We conclude that even though hexamethonium may cause some reduction in blood pressure, such an effect is unlikely to explain the marked effect of hexamethonium on the response to capsaicin.

The failure of capsaicin to evoke a contralateral response supports the involvement of local reflexes. Moreover, atropine failed to reduce the response to capsaicin, in agreement with the findings in man by Geppetti *et al.* (1988) but in contrast to those by Stjärne *et al.* (1989). In our studies, the possibility that secretion evoked by capsaicin via a central reflex was suppressed by the general anaesthetic cannot be excluded. The fact that the capsaicin-evoked secretion was resistant to atropine and reduced by hexamethonium suggests that a transmitter distinct from acetylcholine is involved and that a peripheral ganglion is included in the reflex. Vasoactive intestinal peptide (VIP), which occurs together with acetylcholine in neurones of the sphenopalatine ganglion (Uddman *et al.*, 1980) is an unlikely candidate, since VIP does not stimulate secretion of nasal fluid (Wells & Widdicombe, 1986). A ganglionic reflex involving SP might offer a better explanation.

SP, which stimulates nasal fluid secretion, is present in the sensory nerve fibres in the nasal mucosa (Lundblad *et al.*, 1983). In fact, SP is likely to be responsible for at least a part of the capsaicin-evoked secretion because capsaicin was shown to release SP from such fibres in the nasal mucosa, in agreement with the findings by Lundblad *et al.* (1985), and an antagonist of SP, (D-Pro^2,D-Trp^{7,9})-SP, inhibited the capsaicin-evoked secretion in the present study. Antagonists of SP also antagonize other tachykinins, such as neurokinin A (NKA) (Beding-Barnekow *et al.*, 1988). NKA occurs together with SP in sensory nerve fibres in the nasal mucosa (Sundler *et al.*, 1985) and may be responsible for some of the effect of capsaicin.

CGRP, which coexists with SP and NKA in many sensory neurones (Sundler *et al.*, 1985), is a potent vasodilator agent (Martling *et al.*, 1988). It interferes with the flare response to SP (Brain & Williams, 1985; 1988) and NKA (Gamse & Saria, 1985) in the skin, and with the effects of SP in the eye (Wahlestedt *et al.*, 1986). An involvement of CGRP in the capsaicin-evoked nasal secretion is conceivable but unlikely in view of the fact that CGRP did not stimulate nasal fluid secretion (see also Geppetti *et al.*, 1988), nor did CGRP change the increased secretion evoked by SP in the present work. CGRP also failed to evoke an increased mucociliary activity (Lindberg *et al.*, 1986).

Mast cells occur close to nerve terminals (Wiesner-Menzel *et al.*, 1981). Capsaicin releases SP (Gamse *et al.*, 1979) and CGRP (Franco-Cereceda *et al.*, 1987) from sensory nerve terminals, and SP (Johnson & Erdös, 1973) and CGRP (Piotrowski & Foreman, 1986) are known to liberate histamine from mast cells. However, the secretory response to capsaicin in the present study was not reduced by H_1- or H_2-receptor blockers. Hence, histamine is unlikely to mediate the response to capsaicin. This conclusion is supported by the observation that histamine does not stimulate secretion of nasal fluid in the rat (Petersson *et al.*, 1989).

Basal as well as stimulated nasal fluid is derived mainly from the nasal glands. However, plasma transudate contributes to an unknown degree. Capsaicin is known to evoke increased leakage of albumin across both the endothelial (vascular) and epithelial membranes in the nasal mucosa (Petersson & Svensjö, 1989). The volume of transudate contributing to the nasal fluid can be calculated by determining the amount of fluoresceinated albumin in the nasal secretions and in the plasma. The proportion of transudate in relation to the total amount of nasal fluid was not more than 10% before and 20% after challenge with capsaicin (Petersson & Svensjö, 1989).

Tachyphylaxis to capsaicin developed rapidly following the topical application of increasing doses of the drug. Interestingly, however, administration of capsaicin did not lower the mucosal concentrations of SP and CGRP 15 min after the application (and did not raise the concentrations of SP and CGRP in the nasal secretions (Petersson, unpublished observations)). The failure to demonstrate a rapid reduction of SP and CGRP in the nasal mucosa following administration of capsaicin is difficult to explain in view of the desensitization that resulted. The systemic dose range of capsaicin used to deplete SP in tissues of adult rats is 50–950 mg kg^{-1} s.c. (see Gamse *et al.*, 1981). When using 50 mg kg^{-1} s.c., Lundblad *et al.* (1983) found an almost total loss of SP immunoreactive nerve fibres (immunohistochemistry) in the rat nasal mucosa one week after treatment. In the present study not only SP but also CGRP was depleted one week after systemically given capsaicin. After a week topically applied capsaicin also reduced the concentration of SP—in agreement with Lundblad *et al.* (1985)—and of CGRP in the nasal mucosa. Thus, desensitization to capsaicin can be observed before SP and CGRP are depleted in accordance with the findings by Lembeck & Donnerer (1981), who demonstrated that the plasma extravasation in the skin was reduced 10 min after

the injection of capsaicin, whereas the content of SP was not reduced until after 300 min.

If SP and CGRP are mobilized in response to acute exposure to capsaicin in the present study, the amount may be too small to be detected but large enough to evoke secretion. The existence of different pools of SP and CGRP in sensory nerve fibres with different release kinetics has been suggested (Håkanson *et al.*, 1987). This might explain why capsaicin evokes tachykinin-mediated effects in the nasal mucosa while failing to deplete acutely SP and CGRP.

In conclusion, the capsaicin-evoked nasal secretion in the rat seems to reflect the activation of ganglionic and axon reflexes. Tachykinins are likely to be responsible for at least part of the capsaicin-evoked secretion.

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Effects of hypoxia, elevated K^+ and acidosis on the potency of verapamil, diltiazem and nifedipine in the guinea-pig isolated papillary muscle

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1 The effects of the structurally diverse calcium channel blockers verapamil, nifedipine and diltiazem were investigated on the force of contraction of guinea-pig, electrically stimulated papillary muscles *in vitro*.

2 Calcium channel blocking potency was assessed either as a direct negative inotropic effect or as the ability of the drugs to antagonise the positive inotropic effects of added calcium (Ca^{2+}). By either method, the rank order of potency was found to be nifedipine > verapamil > diltiazem.

3 Various factors which mimic some of the consequences of acute ischaemia *in vivo*, namely low pH, hypoxia and elevated K^+, in combination, but not singly, enhanced the negative inotropic potency of verapamil and to lesser extent that of diltiazem, but not that of nifedipine.

4 Whilst the various interventions, especially in combination, produced a profound negative inotropic effect themselves, this was not responsible *per se* for the potentiation of the negative inotropic effects of verapamil and diltiazem, since the negative inotropic effects of nifedipine and dinitrophenol were not potentiated under the 'ischaemic' conditions.

5 By use of antagonism of the positive inotropic action of exogenous Ca^{2+} as an alternative measure of potency, the differential influence of 'ischaemia' on calcium channel blocker potency was confirmed. The effect of verapamil was potentiated some 9 fold, that of diltiazem about 2 fold, and that of nifedipine was unchanged.

6 The differential effect of 'ischaemia' on the potencies of the calcium channel blockers was unexpected. Verapamil (but not nifedipine) is thought to bind to the calcium channel at a specific site not easily accessible from the extracellular space. 'Ischaemic' conditions may cause membranal perturbations which allow verapamil easier access to its binding site thus increasing its negative inotropic potency.

Introduction

The calcium channel blocking drugs which comprise several structurally diverse classes of drugs are potent modulators of the functional activity of vascular smooth muscle and cardiac muscle. Their therapeutic usefulness has been proven in the treatment of a wide range of cardiovascular diseases which include hypertension (Buhler & Kiowski, 1988), angina (Hugenholtz, 1988) and supraventricular tachycardia (see Singh, 1986). This therapeutic activity largely arises from the vasodilator and negative inotropic, negative dromotropic and negative chronotropic properties of the compounds.

Several studies have provided evidence that the potency of certain calcium channel blocking drugs as negative inotropic agents is enhanced in the ischaemic, compared with the normally perfused, myocardium of the anaesthetized dog (Smith *et al.*, 1976; Abrahamsson *et al.*, 1985). However, analysis of the direct cardiac action of drugs *in vivo* can be complicated by systemic effects of these same agents. Thus, for example, profound vasodilatation produced by calcium channel blocking drugs could influence myocardial contractility indirectly to varying degrees depending on the calcium channel blocker used. In an attempt to circumvent this problem Smith and coworkers (Briscoe & Smith, 1982; Smith & Briscoe, 1985) examined the calcium antagonistic potencies of several calcium channel blockers *in vitro* on cat, electrically stimulated papillary muscle both in a normal

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physiological salt solution and in one with an artificially lowered pH. With verapamil, for example, an 8 fold increase in Ca^{2+} antagonistic potency was observed under the acidotic conditions. Other studies have shown a potentiating effect of elevated extracellular K^+ upon calcium channel blocking potency in rat isolated perfused hearts (Curtis & Walker, 1986; 1988). However, acidosis and elevated K^+ represent only two of several cellular consequences of ischaemia (see Gettes, 1986), the other obvious consequence being hypoxia. If acidosis and elevated extracellular K^+ alone potentiate the negative inotropic potency of calcium channel blocking drugs, it is possible that a combination of these factors plus hypoxia may have a more profound potentiating effect and one more closely resembling that occurring during ischaemia *in vivo*.

In the present study it was therefore our aim to examine the effect of these various factors which occur during ischaemia *in vivo*, alone and in combination, upon the potency of calcium channel blocking drugs *in vitro*. To this end, the calcium channel blocking potencies of verapamil, diltiazem and nifedipine were assessed in guinea-pig isolated, electrically stimulated papillary muscles, both by measuring their direct negative inotropic effects in the presence of a fixed Ca^{2+} concentration and also by measuring the abilities of the drugs to antagonise the positive inotropic effect of exogenously administered Ca^{2+}.

Methods

Guinea-pig isolated papillary muscle preparation

Male guinea-pigs weighing between 250 g and 400 g were killed by cervical dislocation. The whole heart was rapidly removed and placed in a Petri dish containing a physiological salt solution of the following composition (mM): NaCl 118, $NaHCO_3$ 25, KCl 4.7, $MgSO_4$ 0.6, KH_2PO_4 1.2, glucose 11, $CaCl_2$ 1.3, vigorously bubbled with 95% O_2 plus 5% CO_2. Two papillary muscles were carefully dissected from the right ventricle. Individual papillary muscles were suspended vertically between platinum stimulating electrodes in glass tissue chambers (20 ml capacity) and each connected by a cotton thread to a force transducer (Dynamometer UF1). The tissue chambers contained physiological salt solution bubbled with 95% O_2 plus 5% CO_2 and maintained at 32°C. It has been shown previously (see Furchgott, 1972) that the viability of electrically stimulated papillary muscles is better maintained at this temperature than at 37°C. A resting tension of 0.5 g was applied to each muscle and the preparations were individually stimulated at a frequency of 1 Hz at 5 V, with square wave pulses of 5 ms duration (Glaxo Multi-Range Stimulator) during a 1 h equilibration period. Changes in developed tension were displayed on an 8-channel pen recorder (Ormed MX8). Readjustment of any deviation from the resting tension was made only during the equilibration period. Following the equilibration period, the voltage was reduced to zero and then gradually increased until full contraction of the preparation was elicited and then further increased by 10% (threshold voltage; usually approximately 1 V). The frequency of stimulation was also increased to 3 Hz. It was rarely necessary in an experiment to alter the stimulation voltage, although on some occasions if contraction failed (especially when external Ca^{2+} concentration was raised above 10 mM; see below) it was gradually increased until mechanical activity was fully restored. Following a further second equilibration period of 20 min the basal developed tension of the preparations was 144 ± 8 mg ($n = 138$). The pH, Po_2 and Pco_2 in a sample of the physiological salt solution from each chamber were also measured at this time by an ABL1-Acid Base Laboratory (Radiometer).

Experimental design—negative inotropic potency

In the first series of experiments, following the second equilibration period, one papillary muscle of each pair was exposed to one of the following drugs; verapamil (10 nM–10 μM), nifedipine (10 nM–10 μM), diltiazem (100 nM–10 μM) or dinitrophenol (0.1–10 μM). These were added cumulatively, usually in 3 fold increments in concentration, 30 min contact being allowed at each new concentration. The other papillary muscle of each pair received vehicle alone (control preparation) and was used only to correct the negative inotropic effects obtained from the preparation treated with each test drug, for any spontaneous change in developed tension which occurred over the course of drug administration. Developed tension in each preparation 30 min after the addition of each new concentration of drug was expressed as a percentage of the value immediately before drug addition and the following correction applied:–

$$\text{Drug-induced effect in drug treated preparation} = \frac{\text{\% developed tension remaining in test preparation 30 min after drug}}{\text{\% developed tension remaining in control preparation 30 min after vehicle}} \times 100$$

The corrected data for each drug-treated papillary muscle were plotted as percentage developed tension against concentration of calcium channel blocker. Negative inotropic potency was expressed as an IC_{50} value (the concentration of drug required to produce

a 50% decrease in developed tension), derived by an interpolation from this plot.

In vitro *'ischaemic' conditions*

The negative inotropic potency of verapamil was also tested under various conditions *in vitro* which mimic some of the consequences of ischaemia *in vivo*. Following the initial 1 h equilibration period the frequency of stimulation was increased to 3 Hz and voltage reduced to threshold (see above). Five minutes later the various interventions were applied either singly or in various combinations.

Partial depolarisation Extracellular K^+ concentration was raised from 5.9 to 9.2 mM by the addition of KCl to the physiological salt solution, substituted for NaCl so as to maintain osmolarity.

Acidosis pH was reduced from 7.4 to either 6.9 or 6.5 by reducing the $NaHCO_3$ concentration from 25 mM to 8 mM or 3 mM respectively (metabolic acidosis). NaCl concentration was adjusted accordingly to keep total Na^+ concentration constant. In other experiments (respiratory acidosis), pH was lowered to between 6.9 and 7.0 by bubbling with a gas mixture with increased CO_2 content (see below). In each case the pH fell, as dictated by the Henderson-Hasselbach equation.

Hypoxia Relatively hypoxic conditions were created by substituting the usual 95% O_2/5% CO_2 gas mixture for one comprising 55% O_2/40% N_2/5% CO_2. The CO_2 component was deliberately left unchanged so as not to interfere with pH. The one exception to this was the gas mixture for respiratory acidosis which contained 17.5% CO_2 as well as reduced O_2 (55%) with 27.5% N_2.

When the combination of 'ischaemic' conditions was applied, the physiological salt solution was changed first (metabolic acidosis, elevated K^+) followed 10 min later by the change in gas mixture (hypoxia). After application of these 'ischaemic' variables (which took up to 20 min in total and caused negative inotropic effects in their own right), a further equilibration period of 20 min was allowed before the first concentration of drug was added.

In further experiments, the negative inotropic potency of verapamil was measured in preparations electrically stimulated at (i) threshold voltage in the presence of propranolol (300 nM) which was present in the bathing solution throughout the entire experiment or (ii) supramaximal voltage (5 V) in the absence or presence of propranolol (300 nM). Initial experiments carried out with supramaximal voltage (5 V) in the absence of propranolol were abandoned due to marked and variable fade in the developed tension. A protocol was therefore devised in which voltage of stimulation was increased from threshold to 5 V for 5 min and then returned to threshold voltage. This increase in voltage caused a positive inotropic effect and the peak of this response was reproducible if the periods of high voltage stimulation were spaced at intervals of 30 min.

Following two control responses verapamil (or the appropriate amount of vehicle) was added in increasing concentration (10 nM–10 μM). The first concentration of verapamil was added to the preparation 5 min after the second control voltage increase and left to equilibrate for 25 min. At the end of this time the voltage was increased again and the effect of verapamil on the peak response to the voltage increase measured. The voltage was then reduced, the next concentration of verapamil added, and the procedure repeated. No change of the bathing medium was made at any time after addition of the first concentration of verapamil or vehicle.

Experimental design—antagonism of Ca^{2+}-induced positive inotropic effects

In further experiments, the potencies of the calcium channel blockers were assessed in an alternative way by measuring their abilities to displace Ca^{2+} concentration-response curves, which were obtained when cumulatively increasing concentrations of Ca^{2+} were added sequentially to the preparations. In each experiment four papillary muscles (from 2 or 3 animals) were prepared as before and following the equilibration period the frequency of stimulation was again increased to 3 Hz and the voltage reduced to threshold. The physiological salt solution was then replaced by one containing a reduced concentration of Ca^{2+} (0.6 mM) and after 20 min a cumulative Ca^{2+} concentration-response curve (1.0–9 mM) was constructed on each preparation and the resulting increase in developed tension reeorded. The bathing medium was then replaced every 5 min for at least 30 min until the developed tension had returned to the control value. After a further 30 min a second cumulative Ca^{2+} concentration-effect curve was constructed. Following this curve, three of the preparations were exposed to a different concentration of calcium channel blocking drug whilst the fourth preparation was exposed to vehicle alone. Thirty minutes later a third Ca^{2+} concentration-effect curve was constructed. For each individual preparation, total developed tension at each new Ca^{2+} concentration in the third concentration-effect curve was expressed as a percentage of the response to 9 mM Ca^{2+} in the second Ca^{2+} curve and results plotted graphically. Ca^{2+} concentration-effect curves in the presence of the calcium channel blocking drugs were

Table 1 The effect of the ischaemic interventions alone and in combination upon developed tension in guinea-pig papillary muscles

		pH	Po_2 (mmHg)	Pco_2 (mmHg)	*% change in developed tension*	*% recovery of initial developed tension in verapamil-untreated preparations*[a]
A	Normal ($n = 68$)	7.4 ±0.004	560 ±4	35 ±0.2	3 ± 2	—
B	Mild metabolic acidosis ($n = 10$)	6.9 ±0.006	526 ±6	33 ±1.4	5 ± 10	133 ± 22
C	Severe metabolic acidosis ($n = 10$)	6.5 ±0.01	543 ±5	32 ±0.3	−50 ± 8	118 ± 8
D	Hypoxia ($n = 12$)	7.4 ±0.003	354 ±3	36 ±0.3	−39 ± 4	83 ± 9
E	9.2 mM K^+ ($n = 8$)	7.4 ±0.004	580 ±7	36 ±0.7	−30 ± 5	78 ± 10
F	Mild metabolic acidosis + hypoxia ($n = 10$)	6.9 ±0.004	352 ±4	35 ±0.2	−50 ± 3	107 ± 12
G	Mild metabolic acidosis + hypoxia +9.2 mM K^+ ('ischaemia') ($n = 20$)	6.9 ±0.007	341 ±3	34 ±0.4	−70 ± 2	109 ± 8
H	Mild respiratory acidosis + hypoxia +9.2 mM K^+ ($n = 14$)	7.0 ±0.01	288 ±13	91 ±5.0	−88 ± 1	55 ± 6

[a] Values were recorded 20 min after return to normal conditions and represent values from only half of the preparations studied (i.e. those receiving vehicle).

usually parallel with the control curve. However, in some experiments the curves became non-parallel above the 50% response level. Responses were therefore compared, at the EC_{40} level where the curves were consistently parallel. Ca^{2+} concentration-ratios (CR) were calculated for each concentration of calcium channel blocker or its vehicle by dividing the EC_{40} value obtained in the presence of test drug or vehicle (third Ca^{2+} curve) by the EC_{40} value obtained from the corresponding curve obtained in the absence of drug (second Ca^{2+} curve). The CR values from the drug-treated preparations were divided by the CR value from the vehicle-treated preparation to correct for any spontaneous changes in sensitivity. For each experiment drug potency was then expressed graphically by plotting the $\log_{10}$ of these corrected CR values against calcium channel blocker concentration. CR_2 values were derived by interpolation from these plots and are defined as the concentration of drug required to displace the calcium concentration-response curve two fold. By use of this protocol, the potencies of verapamil, nifedipine and diltiazem were determined in both 'normal' and 'ischaemic' (metabolic acidosis, hypoxia and 9.2 mM K^+) conditions. Finally the CR_2 value for verapamil alone was also determined in conditions which comprised respiratory acidosis, hypoxia and 9.2 mM K^+.

Drugs used

The following compounds were dissolved and diluted in distilled H_2O before use: (±)-verapamil HCl (Sigma), diltiazem HCl (Glaxo Group Research), dinitrophenol (Sigma) and propranolol HCl (ICI). Nifedipine (Sigma) was dissolved in absolute ethanol at a concentration of 10 mM. Further dilutions of nifedipine (100 nM–1 μM) were prepared in distilled H_2O. All drug solutions were prepared freshly, immediately before use. Because of the light-sensitive nature of nifedipine, all experiments were carried out in subdued lighting conditions and the solutions protected from light and kept on ice awaiting use. All chemicals for the physiological salt solutions were of Analar grade and obtained from BDH

Ltd., England. All gas mixtures were obtained from BOC.

Expression of results and statistical analysis

Individual groups of IC_{50} and CR_2 values appeared to be log normally distributed for each group of experiments, as assessed by the Wilks-Shapiro test of normality. Because of this, statistical comparison of sets of individual IC_{50} or CR_2 values was performed by means of parametric statistics (unpaired Student's t test) on the $\log_{10}$ of these values. In addition because the data sets were log normally distributed, mean IC_{50} and CR_2 (Tables 2, 3 and 4) were expressed as geometric means with 95% confidence limits. All other grouped data (e.g. Table 1) were expressed as arithmetic mean values ± s.e.mean.

Since the Ca^{2+} antagonistic potency of each calcium channel blocking drug under 'normal' and 'ischaemic' conditions was only compared statistically at one point on the concentration-effect relationship (i.e. at the CR_2 level), a fuller statistical analysis which utilised all data points was undertaken. Thus all of the individual CR values for a particular compound, obtained over a series of experiments under 'normal' and 'ischaemic' conditions, were best fitted to two lines by means of a parallel line model. Statistical differences between these two lines were then determined by analysis of co-variance.

In all analyses the level of statistical significance was taken as $P < 0.05$.

Results

The direct negative inotropic effects of the various 'ischaemic' interventions on the force of contraction of guinea-pig electrically stimulated papillary muscles

The various modifications in pH, Po_2 and K^+ caused negative inotropic effects in their own right (Table 1). Combinations of modifications caused decreases in the force of contraction, which were approximately equal to the sum of the decreases in force of contraction caused by the individual interventions alone. Although the negative inotropic effects were relatively large (up to 70% of the starting developed tension in the case of the combination of pH 6.9, 9.2 mM K^+ and hypoxia), after 2.5 h of these conditions (henceforth referred to as 'ischaemia'), preparations which received only vehicle were largely able to recover and maintain (for at least 30 min) their initial developed tension when returned to 'normal' conditions at the end of each experiment (Table 1). The measured pH values were also very close to those predicted by the Henderson-Hasselbach equation. This suggested that the variously modified physiological salt solutions were fully saturated with the gas mixtures. The hypoxic gas mixture which contained N_2 substituted for some of the O_2, reduced the normal Po_2 value by about 40% (Table 1).

Verapamil-induced negative inotropic effects

Cumulative addition of verapamil (10 nM–10 μM) to the papillary muscles caused concentration-related decreases in the developed tension (Figure 1a). Under 'normal' conditions, the mean concentration of verapamil which caused a 50% reduction in developed tension (IC_{50}) was 107 nM ($n = 34$, Table 2). The singular interventions of metabolic acidosis (pH 6.9 or 6.5), elevated K^+ (9.2 mM K^+) or hypoxia

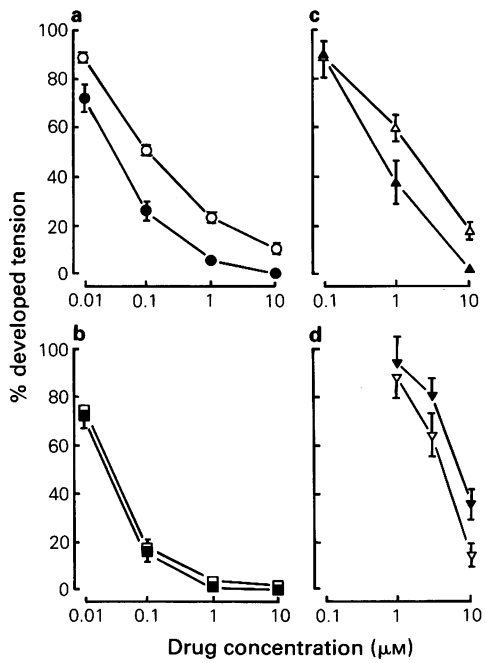

Figure 1 The negative inotropic effects of (a) verapamil, (b) nifedipine, (c) diltiazem and (d) dinitrophenol on guinea-pig electrically stimulated papillary muscles under 'normal' (open symbols) and 'ischaemic' (solid symbols) conditions. Preparations were maintained at 32°C and stimulated at 3 Hz with pulses of 5 ms duration at threshold voltage (approximatelly 1 V). Concentration-effect curves are the mean from n experiments (a, $n = 10$–34; b, $n = 5$; c, $n = 6$–7; d, $n = 7$–9); vertical lines show s.e.mean.

Table 2 The effect of the various ischaemic interventions upon the negative inotropic potency of verapamil in guinea-pig isolated papillary muscles

Experimental intervention	*Verapamil IC_{50} (nM)*	n
'Normal'	107 (80–142)	34
Mild metabolic acidosis (pH 6.9)	124 (54–295) NS	5
Severe metabolic acidosis (pH 6.5)	106 (40–282) NS	5
9.2 mM K^+	134 (36–501) NS	4
Hypoxia	101 (60–169) NS	6
Mild metabolic acidosis + hypoxia	75 (51–111) NS	5
Mild metabolic acidosis + hypoxia + 9.2 mM K^+ ('ischaemia')	28 (17–48) *	10

IC_{50} values are geometric mean values (95% confidence interval) from *n* experiments.
Individual log IC_{50} values for verapamil under each of the various conditions were compared to the log IC_{50} values of verapamil under the 'normal' conditions by unpaired Student's *t* test.
NS = not significant, * $P < 0.001$.

alone, did not significantly affect this IC_{50} value (Table 2). In addition, a combination of hypoxia plus metabolic acidosis (pH 6.9), which produced a greater negative inotropic effect than either intervention alone (Table 1), produced a small but not statistically significant decrease in the mean IC_{50} of verapamil (Table 2). However a further combination of metabolic acidosis (pH 6.9), elevated K^+ (9.2 mM) and hypoxia ('ischaemia') resulted in a significant reduction in the mean IC_{50} of verapamil to 28 nM (Table 2; Figure 1a). This effect represented a four fold potentiation of the negative inotropic effect of verapamil.

Effect of stimulation voltage upon the 'ischaemia'-induced potentiation of the negative inotropic effect of verapamil

Since papillary muscle preparations were stimulated at threshold voltage, it is possible that the 'ischaemia' induced a conduction failure which would have been observed as a negative inotropic effect. This 'ischaemia'-induced negative inotropic effect may have led to a potentiation of the negative inotropic action of verapamil. Experiments were therefore performed with papillary muscles under 'normal' and 'ischaemic' conditions at both threshold (~1 V) and supra-maximal (5 V) stimulation voltages. However, since at such supra-maximal voltages a significant release of endogenous catecholamine occurs (Blinks, 1966), a further series of studies was performed at both threshold and supra-maximal voltage in the additional presence of propranolol (300 nM). The mean IC_{50} values from these experiments are shown in Table 3. The negative inotropic potency of verapamil under either 'normal' or 'ischaemic' conditions varied between the four experimental protocols. For example, there was a significant reduction in the potency of verapamil

Table 3 The effect of raised stimulation voltage in the absence or presence of propranolol upon the negative inotropic potency of verapamil in guinea-pig isolated electrically stimulated papillary muscles under 'normal' and 'ischaemic' conditions

	'Normal' IC_{50} (nM)	n	*'Ischaemic' IC_{50} (nM)*	n	*Ratio N:I*
Threshold voltage	107 (80–142)	34	28 (17–48)**	10	3.8 (2.2–6.8)
Threshold voltage + propranolol (300 nM)	65 (33–128)	9	25 (17–37)*	10	2.6 (1.3–5.3)
Supramaximal voltage	338 (150–763)	11	85 (50–146)*	10	4.0 (1.6–10.1)
Supramaximal voltage + propranolol (300 nM)	174 (95–321)	17	41 (29–57)**	9	4.3 (1.8–9.9)

IC_{50} values are geometric mean (95% confidence interval) from *n* experiments. Individual $\log_{10}$ IC_{50} values for verapamil under 'ischaemic' conditions were compared with the corresponding $\log_{10}$ IC_{50} values of verapamil under 'normal' conditions by means of unpaired Student's *t* test; ** $P < 0.001$, * $P < 0.01$. Ratio N:I values represent the ratio of the 'normal': 'ischaemic' mean IC_{50} values. The 95% confidence interval for this single ratio represents the antilog of the confidence interval derived from unpaired *t* test on the $\log_{10}$ transformed IC_{50} values under 'normal' and 'ischaemic' conditions.

Table 4 The effect of 'ischaemia' upon the actions of verapamil, nifedipine, diltiazem and dinitrophenol determined in the guinea-pig isolated papillary muscle

	Direct negative inotropic effect					*Antagonism of positive inotropic effect of Ca^{2+}*				
	IC_{50}[a] (nM)					CR_2[b] (nM)				
Drug	*'Normal'*	n	*'Ischaemia'*	n	*Ratio N:I*	*'Normal'*	n	*'Ischaemia'*	n	*Ratio N:I*
Verapamil	107 (80–142)	34	28* (17–48)	10	3.8 (2.2–6.8)	467 (237–920)	5	50** (17–147)	4	9.3 (3.2–27.5)
								50[d]** (16–156)	4	9.3 (3.1–28.2)
Nifedipine	28 (22–35)	5	24[NS] (15–36)	5	1.2 (0.8–1.8)	34 (16–72)	5	29[NS] (9–95)	4	1.2 (0.4–3.9)
Diltiazem	1380 (516–3700)	6	570[NS] (270–1200)	7	2.4 (0.8–7.2)	2454 (162–37,200)	4	1080[NS] (448–2581)	4	2.3 (0.1–39.8)
Dinitrophenol[c]	2560 (1020–6450)	7	4760[NS] (2770–8200)	9	0.5 (0.2–1.4)	NT		NT		—

IC_{50} and CR_2 values are geometric means (95% confidence interval) from *n* experiments. Ratios N:I values represents the ratio of the 'normal': 'ischaemic' mean IC_{50} or CR_2 values. The 95% confidence interval for this single ratio represents the antilog of the confidence interval derived from unpaired *t* test on the $\log_{10}$ transformed IC_{50} or CR_2 values under 'normal' and 'ischaemic' conditions for each drug.

[a] Concentration that reduced the developed tension of papillary muscle by 50%.

[b] Concentration that produced a 2 fold rightward displacement of a Ca^{2+} positive inotropic concentration-effect curve.

[c] IC_{40} values shown since highest concentration of dinitrophenol used did not always produce 50% reduction in developed tension.

[d] Ischaemic conditions comprised of respiratory acidosis with hypoxia and elevated $[K^+]$ instead of metabolic acidosis, hypoxia and elevated $[K^+]$.

Individual log IC_{50} or CR_2 values for each drug under 'ischaemic' conditions were compared with the corresponding log values obtained under 'normal' conditions by means of unpaired Student's *t* test.

NS = not significantly different; * $P < 0.001$; ** $P < 0.0001$.

NT = not tested.

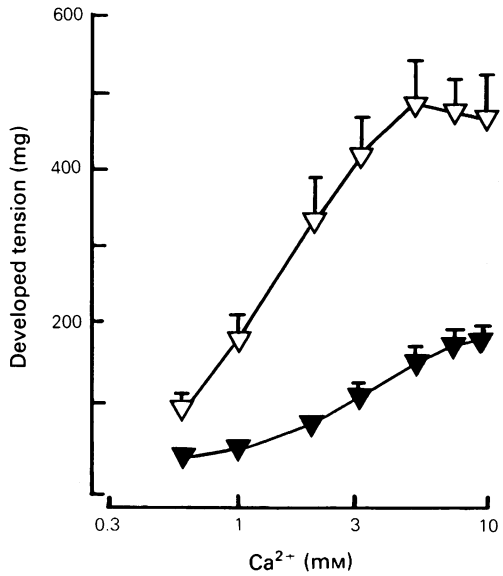

Figure 2 The effects of Ca^{2+} on developed tension of guinea-pig electrically stimulated papillary muscles. Ca^{2+} concentration-effect curves were constructed under either 'normal' (▽) or 'ischaemic' (▼) conditions. For stimulation parameters see Figure 1. Concentration-effect curves are the mean of 6 experiments in each case; vertical lines show s.e.mean.

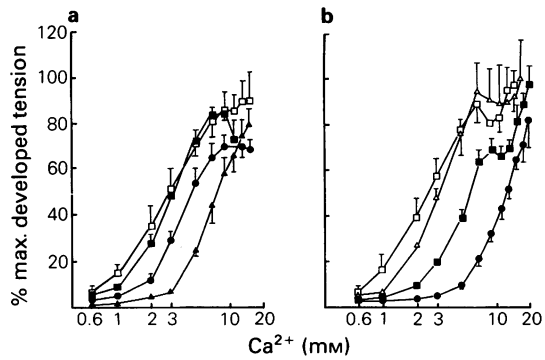

Figure 3 The effect of verapamil upon the positive inotropic effect of Ca^{2+} in guinea-pig isolated electrically stimulated papillary muscle. Curves shown represent the mean ($n = 4$–5) third Ca^{2+} concentration-effect curve performed on separate preparations. Responses were expressed as a percentage of the 9 mM Ca^{2+} response in the second curve (see Methods). Curves shown are those performed in the presence of vehicle (□) or (a) verapamil (100 nM, ■; 1000 nM, ● or 3000 nM, ▲) under 'normal' conditions or (b) verapamil (10 nM, △; 100 nM, ■ or 1000 nM, ●) under 'ischaemic' conditions. Vertical lines show s.e.mean.

($P < 0.01$) at 5 V compared to threshold voltage of stimulation under either 'normal' (338 vs 107 nM) or 'ischaemic' (85 vs 28 nM) conditions. A small but significant ($P < 0.05$) reduction in potency was also observed under 'normal' or 'ischaemic' conditions at 5 V compared with threshold voltages in the presence of propranolol. However, in all instances, the IC_{50} values under 'ischaemic' conditions were significantly ($P < 0.01$) less than the corresponding values under 'normal' conditions (Table 3). This potentiation of the negative inotropic effect of verapamil by 'ischaemia' was very reproducible between the various protocols (Table 3).

Nifedipine, diltiazem and dinitrophenol

Nifedipine (10 nM–10 μM) and diltiazem (0.1–10 μM) caused concentration-related negative inotropic effects in both 'normal' and 'ischaemic' conditions (Figure 1b and c). Unlike verapamil, the negative inotropic effect of nifedipine was unaltered by the 'ischaemic' conditions (Figure 1b, Table 4). Although the negative inotropic effect of diltiazem measured at the IC_{50} level was potentiated some 2–3 fold by the 'ischaemic' conditions this change did not achieve statistical significance ($P = 0.09$) (Figure 1c, Table 4).

The finding that the negative inotropic effect of the calcium channel blocking drugs was affected differentially was unexpected. However, since the combination of interventions constituting 'ischaemia' produced such a profound negative inotropic effect itself, it was conceivable that this effect itself was in some way responsible for the potentiation observed. As a test of specificity the negative inotropic effects of a non-calcium channel blocking drug, dinitrophenol, were examined under 'normal' and 'ischaemic' conditions. Over the concentration range 0.1–10 μM, this agent produced negative inotropic effects with an IC_{50} under 'normal' conditions of 2.6 μM. Interestingly, unlike verapamil, the negative inotropic effect of this compound was not potentiated by the 'ischaemic' conditions compared to 'normal' conditions (Figure 1d, Table 4).

Drug-induced antagonism of the positive inotropic effect of Ca^{2+} in 'normal' and 'ischaemic' conditions

Determination of direct negative inotropic potency was difficult because the residual developed tension after application of the 'ischaemic' conditions was relatively small. As an alternative procedure, the antagonistic effect of the three calcium channel blockers upon the positive inotropic effect of exogenously added Ca^{2+} was therefore investigated. Whilst Ca^{2+} (1.0–9 mM) caused a concentration-

related increase in developed tension in both 'normal' and 'ischaemic' conditions, which was highly reproducible in successive curves, the overall change in developed tension was smaller in 'ischaemic' than in 'normal' conditions for any given concentration of Ca^{2+} (Figure 2). Verapamil (0.1–3 μM) produced concentration-related rightward displacements of the Ca^{2+} concentration-response curve in 'normal' conditions (Figure 3a). However, the antagonistic activity of verapamil was greater under 'ischaemic' conditions (Figure 3b). This increase in potency is more clearly seen in Figure 4a which is a plot of verapamil concentration versus its effect (Ca^{2+} CR values) derived from the individual experiments shown in Figure 3a and b. From this plot the CR_2 (concentration to produce a two fold rightward displacement of the Ca^{2+} concentration-effect curve) for verapamil was found to be approximately 9 fold less under 'ischaemic' compared with 'normal' conditions (Table 4).

As an alternative to the metabolic acidosis used in the 'ischaemic' conditions, in a further series of experiments, the pH of the physiological salt solution was reduced by bubbling the solution with a gas mixture containing 17.5% CO_2, 55% O_2 and 27.5% N_2. Effects produced by these conditions are shown in Table 1 (H). Under these 'ischaemic' conditions with the lowered pH component represented by this respiratory acidosis, the mean CR_2 for verapamil was reduced to 50 nM (Table 4). This value was not significantly different from the CR_2 obtained for verapamil under the 'ischaemic' conditions incorporating metabolic acidosis and represented an approximate 9 fold potentiation compared with 'normal' conditions (Table 4).

Diltiazem (2–20 μM) and nifedipine (0.03–1 μM) also produced concentration-related rightward displacements of the Ca^{2+} concentration-response curves and the concentration-response relationships for both drugs under 'normal' and 'ischaemic' conditions are shown in Figure 4b and c. Under 'ischaemic' conditions the CR_2 value for diltiazem was decreased 2–3 fold whilst that of nifedipine was unaltered. Since statistical analysis of these data (Table 4) only considered a single point (i.e. the CR_2 value) on the concentration-effect curve for each drug, the whole of the CR data under 'normal' and 'ischaemic' conditions were analysed by means of an analysis of co-variance. This analysis confirmed the highly significant potentiation of verapamil ($P < 0.001$) and the lack of potentiation of nifedipine. However, by use of this more comprehensive analysis a significant potentiation ($P < 0.01$) of diltiazem was revealed.

Table 4 summarises the potencies of the test compounds in both 'normal' and 'ischaemic' conditions obtained by both methods of measuring Ca^{2+} antagonistic potency. A good correlation was

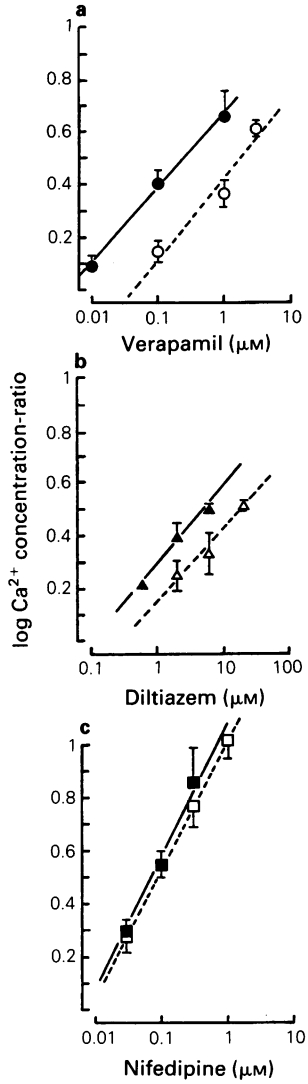

Figure 4 The concentration-effect relationship of (a) verapamil, (b) diltiazem, and (c) nifedipine, for antagonism of the positive inotropic effect of Ca^{2+} in guinea-pig isolated, electrically stimulated papillary muscle under 'normal' (open symbols) and 'ischaemic' (solid symbols) conditions. Data, which were taken from experiments such as those depicted in Figure 3, are expressed as log Ca^{2+} CRs versus concentration of antagonist (for calculation of CRs see Methods). Stimulation parameters as in Figure 1. Each point is the arithmetic mean of the $\log_{10}$ of each CR value from 4–6 experiments. These data were best fitted by a parallel line model. Statistical differences between these lines relating to data obtained under 'normal' and 'ischaemic' conditions for each drug were determined by analysis of co-variance. Statistical differences between drug potency in 'normal' and 'ischaemic' conditions were also assessed at the CR_2 level (see legend to Table 4). Vertical lines show s.e.mean.

observed between the two protocols, both in the rank order of calcium channel blocker potency in 'normal' conditions (nifedipine > verapamil > diltiazem) and for the differential sensitisation of drug potency induced by 'ischaemia' (verapamil > diltiazem > nifedipine $\simeq$ 0).

Discussion

In the present study, the negative inotropic activities of the calcium channel blocking drugs, verapamil, diltiazem and nifedipine were quantified in guinea-pig isolated, electrically driven papillary muscles by two methods. Regardless of the method used, either direct negative inotropic activity or antagonism of the positive inotropic effect of exogenous Ca^{2+}, the rank order of potency was nifedipine > verapamil > diltiazem. Under various experimental conditions, namely hypoxia, acidosis and elevated extracellular K^+, designed to mimic the consequences of ischaemia *in vivo*, the negative inotropic potency and Ca^{2+} antagonistic activity of verapamil were found to be significantly potentiated. However, this potentiation was observed only when a combination of these conditions was applied, not when they were applied singly. In addition, under this combination of conditions the activity of the three calcium channel blockers studied were differentially affected. In short, whilst the potencies of verapamil and diltiazem increased, that of nifedipine was unaltered.

Several previous *in vivo* studies have examined the effect of regional myocardial ischaemia upon the negative inotropic potency of calcium channel blocking drugs. Some studies have shown a potentiating effect of ischaemia on drug potency (Smith *et al.*, 1976; Abrahamsson *et al.*, 1985). However, others have failed to demonstrate such an effect (Sherman *et al.*, 1981; Romson *et al.*, 1986). There have also been various studies, which have utilised isolated perfused cardiac muscle, that have confirmed a potentiating effect of ischaemia upon calcium channel blocking potency (Ngai *et al.*, 1983; Konishi *et al.*, 1984). However, none of these studies has attempted to examine systematically the contributory factors occurring during ischaemia which lead to this potentiating effect. In the cat isolated papillary muscle, Briscoe & Smith (Briscoe & Smith, 1982; Smith & Briscoe, 1985) examined the effect of changes in the pH of the bathing medium from 7.4 to 6.0 upon the negative inotropic potency of a range of calcium channel blocking drugs. More recently, Curtis & Walker (1986; 1988) also studied the effect of increasing buffer K^+ on the negative inotropic potency of verapamil and nifedipine in rat isolated Langendorff perfused ventricles. However, in the ischaemic myocardium *in vivo*, changes in local pH and elevation in extracellular K^+ do not occur singly but do so in tandem. Therefore, in the present study, we attempted to mimic more comprehensively the resultant biochemical sequelae of ischaemia, and thus considered, the combined effects of acidosis elevated extracellular K^+ and reduced O_2 availability on the negative inotropic potency of the calcium channel blockers *in vitro*.

The level of hypoxia chosen was largely dictated by the negative inotropic effect produced by the reduction of Po_2 in the bathing solution. Under normal conditions the Po_2 was 500–600 mmHg, whereas during the conditions termed 'hypoxic' the value was reduced to approximately 350 mmHg. This solution is therefore not truly 'hypoxic', since the Po_2 of arterial blood is only 95 mmHg. However, since the papillary muscle preparation receives oxygen only by diffusion this reduction in Po_2 was enough to compromise functional performance. The choice of the type of acidosis also required some consideration, since there is some controversy as to whether myocardial ischaemia is associated with a metabolic or respiratory acidosis (Ichihara *et al.*, 1984; Kodama *et al.*, 1984). Both types of acidosis were therefore investigated upon the Ca^{2+} antagonist activity of verapamil. The level of acidosis and K^+ concentration chosen for the current experiments, 6.9–7.0 and 9.2 mM respectively, are close to levels measured in the myocardium *in vivo* shortly after total coronary occlusion (Weiss *et al.*, 1984; Hill & Gettes, 1980). We were, however, limited in the levels of acidosis and K^+ concentration that we could choose by the magnitude of the negative inotropic effects produced by the interventions themselves. This was especially the case when we were attempting to quantify the potencies of the calcium channel blocking drugs by measurement of their negative inotropic effects. It was also interesting to note that although the 'ischaemic' conditions used caused a sustained 70% decrease in the developed tension of the guinea-pig papillary muscle, those preparations which received only vehicle and were returned to 'normal' conditions at the end of the experiment (between 2–3 h later) generally regained and maintained their original developed tension (Table 1). This suggests that the 'ischaemic' conditions imposed were not severe enough to cause irreversible functional damage to the muscle.

In the present study it was surprising to find that the negative inotropic effect of verapamil was not significantly potentiated by either mild (pH 6.9) or severe (pH 6.5) acidosis alone or by elevated extracellular K^+. These results contrast with the findings of Smith & Briscoe (1985) and Curtis & Walker

(1986). In the case of the former study it may be significant that the pH of the bathing medium was reduced to as low as 6.0. In the case of the study of Curtis & Walker (1986), these authors showed that the greatest potentiation of the negative inotropic effect of verapamil occurred at K^+ concentrations up to 5.9 mM, the starting concentration in the present study. Above this concentration the degree of potentiation of verapamil decreased. This, along with the fact that these authors utilised a perfused cardiac muscle preparation from the rat, paced at a higher frequency than used in the present work, may explain the discrepancy between the two studies.

A further combination of hypoxia and 'mild' acidosis still failed to affect significantly the potency of verapamil although a slight trend was observed. In fact, it was only when a combination of mild acidosis, elevated K^+ and hypoxia was used that a significant potentiation of the negative inotropic effects of verapamil was observed. In addition, the nature of the acidosis used in this combination did not appear to be critical. Thus, both mild (pH 6.9–7.0), metabolic or respiratory acidosis potentiated equally the verapamil-induced antagonism of the positive inotropic effect of Ca^{2+}. Interestingly, the critical combination of conditions necessary to observe potentiation of verapamil's effects in the present study has also been found to be necessary to achieve electrophysiological changes in cardiac muscle *in vitro* similar to those seen in ischaemic muscle *in vivo*. Thus, in guinea-pig papillary muscle (Kodama *et al.*, 1984) or in porcine heart (Moréna *et al.*, 1980), a combination of elevated extracellular K^+, hypoxia and acidosis produced a much more pronounced effect upon the action potential than did the individual interventions themselves. The effects observed with the combination of interventions, namely a reduced maximum upstroke velocity and shortened action potential duration, also mirrored more precisely the type of cardiac action potential measured during coronary artery occlusion (Cinca *et al.*, 1980). Both this observation and those of the present study therefore emphasise the importance of considering all of the integral components present during ischaemia in cardiac muscle when attempting to mimic the situation *in vitro*.

A further unexpected finding of the present study, was the lack of potentiation of the Ca^{2+} antagonistic actions of nifedipine. A rank order of potentiation of verapamil > diltiazem > nifedipine also contrasts with that obtained by Smith & Briscoe (1985). Whilst differences in species and experimental conditions may have contributed to this inconsistency, it is interesting to note other findings of an identical order of potentiation of these three compounds to that obtained in the present study. In each of these studies, which concerned either anoxia-induced damage of rat myocytes (Miller *et al.*, 1985), necrotic changes in the myocardium of cardiomyopathic hamsters (Jasmin & Proschek, 1984) or veratrine-induced Ca^{2+} overload in rat myocytes (Ver Donck *et al.*, 1986), verapamil was more protective than diltiazem, whilst nifedipine (or a similar dihydropyridine, nisoldipine) afforded no protection at all. The similarity of these results with the rank order of 'ischaemia'-induced increase in potency of the same compounds in the present study may be coincidental, but is striking nevertheless.

The present study raises two obvious questions. Firstly, what is the mechanism by which the potency of verapamil is increased in the 'ischaemic' conditions and secondly, why is the potency of nifedipine not changed? Initially, upon observing the 'ischaemia'-induced potentiation of the negative inotropic effect of verapamil, we wondered whether conduction failure was occurring in the papillary muscles under the 'ischaemic' conditions and that it was this that was somehow producing the potentiation observed. However, in a series of experiments with increased stimulation strength to overcome any conduction failure, the 'ischaemia'-induced potentiation was consistently observed, so ruling out the involvement of such a mechanism. In these experiments, with increasing stimulation voltage a small but significant reduction was always observed in the negative inotropic potency of verapamil, when compared with the value obtained under the corresponding experimental conditions at threshold voltage. This could imply that at threshold voltage, under either 'normal' or 'ischaemic' conditions, part of the negative inotropic effect of verapamil was exerted through a negative dromotropic action. Under conditions of supramaximal stimulation such an effect would be overcome and hence the negative inotropic potency of verapamil reduced. However, from the results obtained at higher stimulation voltages this negative dromotropic action of verapamil would also appear to be unrelated to the 'ischaemia'-induced potentiation observed.

Further confirmatory evidence that conduction failure was not responsible for the potentiating effect of 'ischaemia' was the finding that neither nifedipine nor dinitrophenol were affected under these conditions. This observation would also seem to rule out the possibility that the negative inotropic effect induced by the 'ischaemic' interventions themselves, somehow facilitated the negative inotropic action of verapamil, since other negative inotropic agents would also be expected to be potentiated by such a mechanism. Thus, an alternative explanation must be sought for the differential potentiation of the calcium channel blocking drugs seen in the present study.

Calcium channel blocking drugs can be classified

according to their properties as either voltage-dependent or use (frequency)-dependent. The three compounds tested in the present study are all voltage-dependent (Kanaya *et al.*, 1983; see Chin, 1986): that is, as the membrane potential become less negative, calcium channel blocking potency is enhanced. If 'ischaemia' led to a partial membrane depolarisation in the cardiac cells, a generalised potentiation of the calcium channel blocking activity of all of the compounds would have been anticipated. However, this was not the case. In contrast, the so-called use- or frequency-dependency is more a property of verapamil than of nifedipine. One interpretation of this use/frequency-dependency is that a compound which has its binding site either within the cell or in the calcium channel itself requires channel opening to gain access to this site (Katz, 1985; Frank, 1986). There is now good evidence that the verapamil binding site associated with the calcium channel in the mammalian myocardium is on the inner surface of the cell membrane (Heschler *et al.*, 1982). As mentioned, dihydropyridines such as nifedipine, exhibit less use-dependency than verapamil (Lee & Tsien, 1983 see Curtis & Walker, 1988). One explanation for this is that, as neutral entities, they are highly lipid soluble and so should easily gain access to their binding site independent of channel opening (Kokubun & Reuter, 1984). The 'ischaemia'-induced potentiation of verapamil observed in the present study could suggest that more verapamil gains access to its binding site, possibly as a consequence of a perturbation of the cell membrane. Such a change would not be anticipated to affect the potency of nifedipine.

In conclusion, in an *in vitro* model of ischaemia, the present study has demonstrated a potentiation of the calcium channel blocking activity of verapamil thus, confirming previous findings *in vivo*. In contrast to other *in vitro* studies, the phenomenon appears to depend upon a critical combination of conditions and, interestingly, does not apply universally to all calcium channel blocking drugs. Elucidation of the mechanisms involved may yield important information regarding the future design of compounds with marked selectivity of action in the ischaemic over the normally perfused myocardium. Such selectivity may be of benefit in reducing myocardial infarct size or reducing the incidence of the arrhythmias of early myocardial ischaemia (Curtis & Walker, 1988).

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Endothelium-dependent relaxation and hyperpolarization of canine coronary artery smooth muscles in relation to the electrogenic Na-K pump

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1 In the smooth muscle cells of canine coronary artery, acetylcholine (ACh) produced a transient, endothelium-dependent hyperpolarization of the membrane. A similar hyperpolarization was also elicited by exposure to Krebs solution after incubation of the artery in K-free solution for 30 min.

2 A hyperpolarization of reproducible amplitude was generated when ACh was applied at intervals greater than 30 min. Repetitive application of ACh at 15 min intervals caused a successive reduction in the amplitude of hyperpolarization.

3 The reduction in the amplitude of relaxation during five successive applications of ACh at 15 min intervals was less than 10% of the first relaxation.

4 The ACh-induced hyperpolarization was blocked by atropine but not by ouabain, whereas the K-free induced hyperpolarization was blocked by ouabain. In low Na (Li-substituted) solution, ACh still induced a hyperpolarization but the K-free induced hyperpolarization was absent.

5 In coronary artery precontracted by high-K solution, ACh produced an endothelium-dependent relaxation, without membrane hyperpolarization. The associated relaxation was resistant to ouabain but sensitive to atropine.

6 It is concluded that in the canine coronary artery, the electrogenic Na-K pump does not contribute to the endothelium-dependent hyperpolarization or relaxation. The results are consistent with the release of two different inhibitory factors from the vascular endothelium.

Introduction

Arterial smooth muscles are relaxed by acetylcholine (ACh) in an endothelium-dependent manner, and the mediator may be an endothelium-derived relaxing factor (EDRF) (Furchgott & Zawadzki, 1980) and a hyperpolarizing factor (Chen *et al.*, 1988; Chen & Suzuki, 1989). The EDRF-induced relaxation is accompanied by an increase in the production of guanosine 3′:5′-cyclic monophosphate (cyclic GMP), and the actions of this factor are similar to those of the nitrovasodilators (Ignarro & Kadowitz, 1985). Nitric oxide is one of the possible candidates for EDRF (Palmer *et al.*, 1987), and the similarities (Ignarro *et al.*, 1987) and differences (Shikano & Berkowitz, 1987; Shikano *et al.*, 1987) between these two substances are now subject to much investigation.

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The endothelium-derived hyperpolarizing factor (EDHF) acts mainly transiently and its actions are resistant to methylene blue or haemoglobin which are inhibitors of the actions of EDRF (Martin *et al.*, 1985). In the rat aorta, EDHF is responsible for about 20–40% of the ACh-induced endothelium-dependent relaxation (Chen & Suzuki, 1989).

The endothelium-dependent hyperpolarization of smooth muscle membrane produced by ACh is mainly due to an increase in K-conductance in the rabbit saphenous (Komori & Suzuki, 1987a) and ear arteries (Suzuki, 1988) and in rat arteries (Chen *et al.*, 1988; Chen & Suzuki, 1989). However, in the canine coronary artery, Feletou & Vanhoutte (1988) reported that the endothelium-dependent hyperpolarization produced by ACh was sensitive to ouabain whereas the actions of EDRF were resistant to this drug. These data therefore suggested that the hyperpolarization was produced by an activation of the

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electrogenic Na-K pump in the smooth muscle membrane rather than an increase in K-conductance. These two contradictory results were obtained from experiments using different methods. In the former group, investigations were carried out in tissues with intact endothelial cells whereas the latter were performed using endothelium-free tissue in a cascade experiment, which makes direct comparison between the two results difficult.

In the present study, we have investigated the possible involvement of an electrogenic Na-K pump in the endothelium-dependent hyperpolarization produced by ACh, using preparation of canine coronary artery with intact endothelial cells. The electrogenic Na-K pump was inhibited by ouabain or Li ions (Kerkut & York, 1971).

Methods

Mongrel dogs of either sex, weighing 10–15 kg, were anaesthetized by i.v. injection of Na pentobarbitone (40 mg kg^{-1}), and exsanguinated from the femoral artery. The heart was excised and kept in Krebs solution at room temperature. The left descending coronary artery with an outer diameter of 0.5–1.0 mm (located mainly in the left ventricular wall) was dissected for a length of 0.5–0.7 cm, and cleaned by removing the connective tissues and heart muscles which surrounded the vessel.

For recording electrical responses of smooth muscle cells, the artery was cut open along its longitudinal axis and mounted in a recording chamber made of lucite plate, with a capacity of about 2 ml. At the bottom of the chamber was a silicon rubber plate (Shin-etsu kagaku, Tokyo, KE-66) on which the isolated vessel, endothelial layer uppermost, was immobilized with tiny pins, and superfused with warmed (35°C) Krebs solution at a flow rate of 2–3 ml min^{-1}.

Glass capillary microelectrodes were made from borosilicate glass tube (outer diameter, 1.2 mm with a core inside, Hilgenberg, West Germany), and filled with 3 M KCl. Their tip resistance ranged from 40–80 MΩ. A microelectrode was inserted into a smooth muscle cell through the endothelial layer, and the electrical responses thus recorded were displayed on a pen-writing recorder (Recticorder, Nihon-kohden RJG-4024). Mechanical responses of smooth muscles of the coronary artery were recorded separately under isometric conditions using a ring segment of the vessel as described previously (Suzuki & Casteels, 1979).

When necessary, the endothelial cells were removed mechanically by the method of Furchgott & Zawadzki (1980) in the case of microelectrode experiments, or by rubbing the internal surface of the vessel with a steel wire for mechanical experiments (Nagao & Suzuki, 1987).

The ionic composition of Krebs solution was as follows (mM): Na^+ 134.7, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.5, $H_2PO_4^-$ 1.2, HCO_3^- 15.5, Cl^- 137 and glucose 11.5. The solution was aerated with O_2 containing 3% CO_2, and the pH of the solution was at 7.2–7.4. The K-free solution was prepared by replacing KCl and KH_2PO_4 with NaCl and NaH_2PO_4, respectively. The high-K solution was prepared by replacing NaCl with KCl. The concentration of Na^+ was reduced to 15.5 mM by substituting NaCl with LiCl (Li-Krebs).

Drugs used were acetylcholine chloride, atropine sulphate (all from Sigma Chemical Co., St. Louis, MO.), and ouabain (g-strophanthin, Merck).

Experimental values are shown as means ± s.d., and statistical significance was determined by Student's t test ($P < 0.05$ was considered significant).

Results

Electrical properties of the ACh-induced hyperpolarization

The resting membrane potential of the canine coronary artery smooth muscles ranged between −50 and −60 mV, and the values recorded from individual cells were stable. Figure 1 shows that application of 10^{-5} M ACh transiently hyperpolarized the membrane and that the membrane potential reverted to the resting membrane potential level within 7–10 min (Figure 1a). When ACh was applied for 2 min, the hyperpolarized membrane was restored to the previous resting potential level within 2–3 min, after a transient depolarization of 3–5 mV (Figure 1b). The ACh-induced hyperpolarization was not seen in the presence of atropine (10^{-6} M) (Table 1) or after removal of the endothelial cells (Figure 1c).

Figure 1d shows the relationship between the amplitude of the ACh-induced peak hyperpolarization and the concentration of ACh. ACh ($> 10^{-7}$ M) produced a concentration-dependent hyperpolarization, with a maximum at 10^{-4} M ACh, only in tissues with intact endothelial cells.

Experiments were also carried out to determine the time required for recovery of the ACh-induced hyperpolarization. ACh (10^{-5} M) was applied twice at various intervals and the amplitude of the hyperpolarization produced by the second exposure to ACh relative to the first was measured. In every trial throughout these experiments, ACh was applied for 1 min, which allowed the peak hyperpolarization to develop. When ACh was applied at 5 min intervals,

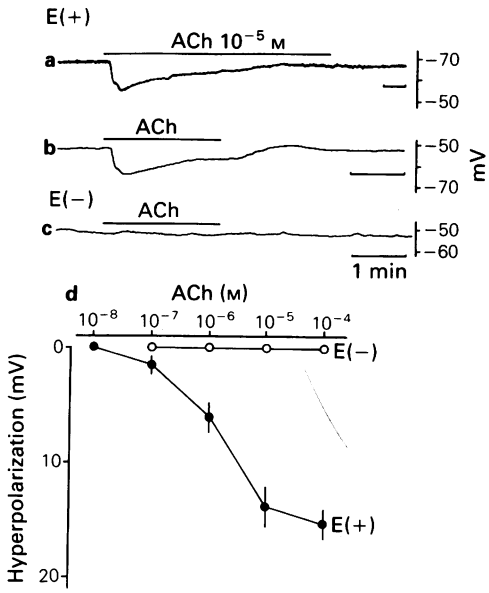

Figure 1 Electrical responses recorded from smooth muscle cells of the canine coronary artery during application of acetylcholine (ACh, 10^{-5} M) in tissues with intact endothelial cells (E (+), a and b) and with endothelial cells removed (E (−), c); (a) and (b) but not (c) were recorded from the same tissue. ACh was applied at the bar above each record. (d) Concentration-dependent hyperpolarizations produced by ACh in smooth muscle cells of canine coronary artery, in tissues with (E (+), ●) and without (E (−), ○) endothelial cells. Means with s.d. shown by vertical lines (n = 5–12).

the hyperpolarization produced by the second ACh challenge was decreased in amplitude and also the rate of potential change slowed (Figure 2a). As shown in Figure 2b, a reproducible amplitude of hyperpolarization was generated by ACh when the intervals between successive exposure were 30 min.

When ACh (10^{-5} M) was applied 5 times successively every 15 min, the amplitude of the resulting hyperpolarization decreased by 8–10% of the previous response, and the 5th response was about 70% of the first (Figure 2c).

Effects of inhibition of the electrogenic Na-K pump on the ACh-induced hyperpolarization

In tissues with intact endothelial cells, the membrane was also hyperpolarized transiently (10–15 min) by administration of Krebs solution after incubation in K-free solution for 30 min (Figure 3a and b). The amplitude of the ACh (10^{-5} M)- and K-free-induced hyperpolarizations obtained from five different tissues were 13.9 ± 1.6 mV ($n = 15$) and 14.2 ± 1.8 mV ($n = 10$), respectively.

Table 1 Membrane potential of smooth muscle cells in the canine coronary artery

Control (resting membrane potential)	-53.5 ± 1.6 mV ($n = 24$)
Endothelial cells removed	-52.9 ± 1.3 mV ($n = 10$)
Atropine 10^{-6} M	-52.8 ± 1.4 mV ($n = 13$)
K-free solution (30–40 min)	-48.4 ± 1.8 mV ($n = 10$)*
Ouabain 10^{-6} M (10–60 min)	-50.7 ± 1.3 mV ($n = 11$)*
Li-Krebs solution	-40.9 ± 1.4 mV ($n = 9$)*
24.9 mM $[K]_o$ solution	-47.6 ± 1.5 mV ($n = 10$)*

Values are means ± s.d.
(n = number of observations). * Significant difference from the control ($P < 0.05$).

Application of ouabain (10^{-6} M) for 30–60 min depolarized the membrane by 1–3 mV (Table 1). In tissues with an intact endothelium and in the presence of ouabain, ACh (10^{-5} M) produced a hyperpolarization of the membrane, the amplitude and configuration of which were identical to those seen in control conditions (Figure 3c). The mean peak amplitude of the hyperpolarization produced by ACh (10^{-5} M) in the presence of ouabain was 14.3 ± 1.7 mV ($n = 8$, $P > 0.05$). The K-free-induced hyperpolarization was, however, completely blocked by ouabain, or on some occasions, converted to a small transient (5–7 min) depolarization (1–3 mV), when Krebs solution was applied after incubation in K-free solution for 30–40 min (Figure 3d).

Substitution of about 88% of Na ion ($[Li]_o$ = 119.2 mM) depolarized the cell membrane by about 12 mV (Table 1). In this Li-Krebs solution, ACh (10^{-5} M) produced a transient hyperpolarization (Figure 3e), but exposure to Krebs solution after incubation in K-free solution did not (Figure 3e). Li-substitution reduced the magnitude of the ACh-induced hyperpolarization (9.5 ± 1.9 mV, $n = 8$, $P < 0.05$) and markedly accelerated its decay (compare Figure 3e with Figure 1a).

ACh-induced hyperpolarization in high $[K]_o$ solution

If the ACh-induced hyperpolarization is produced by an increase in membrane K-conductance, it should be decreased or absent in high $[K]_o$ solution. In rat arteries, the expected $[K]_o$ to block the ACh-induced hyperpolarization was 20–25 mM (Chen & Suzuki, 1989). Experiments were thus carried out to observe the effects of 24.9 mM $[K]_o$ solution on the

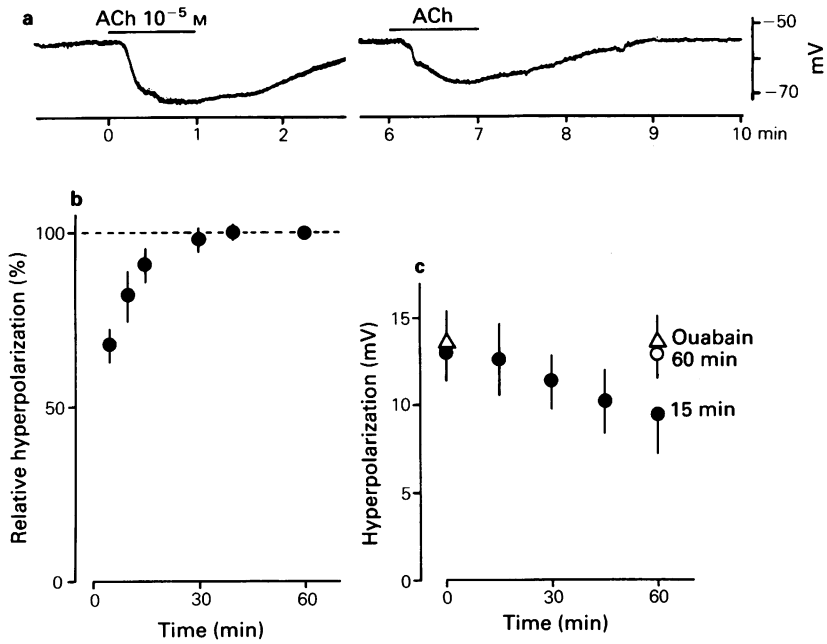

Figure 2 Tachyphylaxis of the acetylcholine (ACh)-induced hyperpolarization in smooth muscle of the canine coronary artery. (a) Hyperpolarizations produced by application of ACh (10^{-5} M, for 1 min) on two occasions separated by a 5 min interval (recordings were interrupted for about 3 min between two ACh applications). (b) Recovery of the ACh response from desensitization. Amplitude of the hyperpolarization produced by the second ACh was measured as a percentage of the first, and plotted against the time between the two applications of ACh. Means with s.d. shown by vertical lines ($n = 5$–7). (c) Tachyphylaxis of the ACh-induced hyperpolarization. Amplitude of the hyperpolarization produced by application of ACh (10^{-5} M) on 5 successive occasions each separated by a 15 min interval (●), or on 2 occasions separated by a 60 min interval in the absence (○) and presence of 10^{-6} M ouabain (△). Each ACh challenge was applied for 1 min, and the peak amplitude of the hyperpolarization was measured.

endothelium-dependent hyperpolarization produced by ACh in the canine coronary artery.

Increasing $[K]_o$ to 24.9 mM depolarized the smooth muscle membrane by about 6 mV (Table 1). Application of ACh (10^{-5} M) in the control condition produced hyperpolarization (Figure 4a, the mean amplitude being 13.8 ± 1.6 mV, $n = 6$). However, this hyperpolarization was decreased in amplitude or disappeared (Figure 4b) in high $[K]_o$ solution (mean amplitude, 0.2 ± 0.7 mV, $n = 5$).

These results strongly suggest that the ACh-induced hyperpolarization generated in the smooth muscle of canine coronary artery is mainly due to an increase in K-conductance of the membrane.

Mechanical properties of the ACh-induced relaxation

Increasing $[K]_o$ to 24.9 mM produced a contraction of smooth muscles in ring segment of the canine coronary artery. The tension elevated by the high-$[K]_o$ solution was 18 ± 4% ($n = 5$) of the maximum value produced by 118 mM $[K]_o$ solution, and a similar level of tension was maintained for up to 2 h by the high-$[K]_o$ solution. In tissues with intact endothelial cells, application of ACh ($> 10^{-8}$ M) in the high-$[K]_o$ solution inhibited the contraction in a concentration-dependent manner and reached a maximum value at 10^{-5} M ACh. This relaxation was not observed in tissues devoid of endothelial cells or in the presence of atropine (10^{-6} M).

Experiments were also carried out to observe the effects of repetitive application of ACh on the amplitude of relaxation. As demonstrated in Figure 5a, ACh (10^{-5} M) inhibited the high-$[K]_o$-induced contraction to about 30%, and the tension was restored to the initial level within 5–7 min of the removal of ACh.

When the amplitude of the ACh-induced relaxation was measured as a percentage of the initial value, successive applications of ACh at 15 min intervals caused some reduction of the inhibitory actions of ACh. The amplitude of relaxation produced by the 5th ACh challenge was about 90% of the first (Figure 5b). Two applications of ACh at 60 min

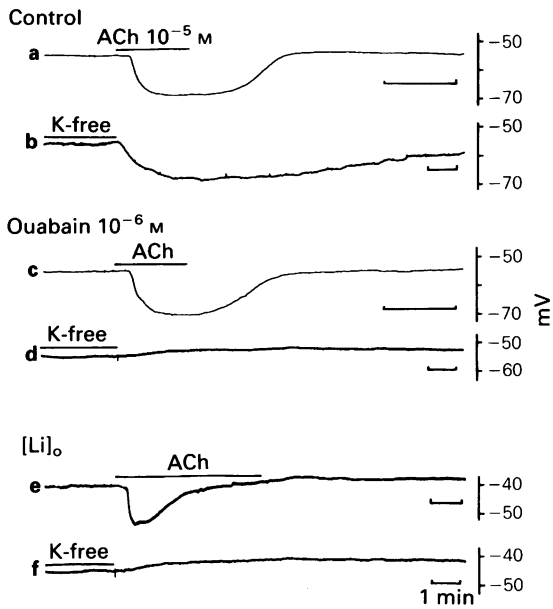

Figure 3 Membrane hyperpolarizations produced by acetylcholine (ACh, 10^{-5} M) (a, c and e) or administration of K ions after incubation in K-free solution for 30 min (b, d and f) in smooth muscle cells of the canine coronary artery. (a) and (b) Control: (c) and (d) in the presence of 10^{-6} M ouabain; (e) and (f) in low $[Na]_o$ solution substituted with Li (Li-Krebs).

intervals relaxed the muscle to the same extent ($P > 0.05$).

In the presence of ouabain (10^{-6} M), ACh still relaxed the muscles which had been precontracted with high-$[K]_o$ solution. The amplitude of the relaxation produced by 10^{-5} M ACh was

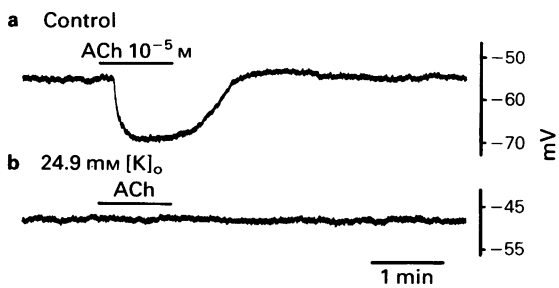

Figure 4 Effects of acetylcholine (ACh, 10^{-5} M) on membrane potential of smooth muscle cells from canine coronary artery, in high $[K]_o$ solution. (a) Control; (b) in 24.9 mM $[K]_o$ solution. ACh was applied for 1 min (bar). (a) and (b) are recordings from the same cell.

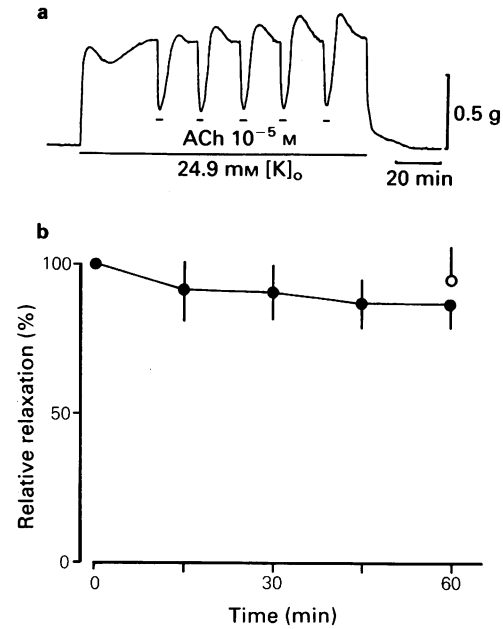

Figure 5 Acetylcholine (ACh)-induced relaxation in the canine coronary artery. (a) A ring segment of the artery was contracted by 24.9 mM $[K]_o$ solution, and exposed to ACh (10^{-5} M) for 3 min on 5 occasions at 15 min intervals. (b) The relaxations produced by application of 10^{-5} M ACh as described in (a) (●) or on 2 occasions separated by a 60 min interval (○) were observed in tissues contracted with 24.9 mM $[K]_o$ solution, and the amplitude of the maximum relaxation relative to the first was measured ($n = 5$).

$63.3 \pm 14.8\%$ ($n = 23$) of the high-$[K]_o$-induced contraction in the control and $54.8 \pm 8.7\%$ ($n = 15$) in the presence of ouabain (10^{-6} M), values which were not significantly different ($0.05 < P < 0.1$).

Discussion

The present experiments have demonstrated that in the smooth muscle cells of canine coronary artery, ACh produced a transient, endothelium-dependent hyperpolarization. These results confirmed observations in other arteries such as the pig coronary artery (Bény *et al.*, 1986), the rabbit saphenous (Komori & Suzuki, 1987a,b), ear (Suzuki, 1988) and aortic vessels (Bèny & Brunet, 1988), the guinea-pig mesenteric (Bolton *et al.*, 1984) and basilar arteries (Nishiye *et al.*, 1989), the rat aorta (Taylor *et al.*, 1988) and main pulmonary arteries (Chen *et al.*, 1988) and the canine mesenteric artery (Komori *et*

al., 1988). Cascade experiments demonstrated that in the canine coronary artery, the endothelium-dependent hyperpolarization by ACh was produced by an unknown substance which is liberated from the endothelial cells (Feletou & Vanhoutte, 1988). The resistance of the ACh-induced hyperpolarization to methylene blue or haemoglobin, an inhibitor of the action of EDRF (Martin *et al.*, 1985) also indicates that the ACh-induced hyperpolarization is not associated with the actions of EDRF (Chen *et al.*, 1988; Chen & Suzuki, 1989).

Tachyphylaxis was observed to the ACh-induced hyperpolarization, and over 30 min between successive exposure to ACh, was required to obtain a reproducible amplitude of hyperpolarization in the canine coronary artery. This period of time was longer than in rat aorta (about 5 min) but was comparable with the characteristics of the histamine-induced hyperpolarization in this artery (Chen & Suzuki, 1989). Although the detailed mechanisms remain to be determined, desensitization of ACh- or histamine-receptors on the endothelial cell membrane seems to contribute to the transient nature of the endothelium-dependent hyperpolarization. The sustained relaxation produced by ACh in the rabbit saphenous artery (Komori & Suzuki, 1987a) and aorta (Bèny & Brunet, 1988) indicates that the release of EDRF is maintained until ACh is removed, and such a property of EDRF is distinct from the transient nature of the hyperpolarization. The transient response of the smooth muscle membrane of the rat aorta to histamine is not due to depletion of EDHF or to desensitization of receptors for EDHF in the smooth muscle membrane (Chen & Suzuki, 1989).

In cascade experiments, the endothelium-dependent hyperpolarization observed in the canine coronary artery was blocked by ouabain (Feletou & Vanhoutte, 1988). However, the present experiments demonstrated that in this artery with intact endothelial cells, ACh produced a hyperpolarization in the presence of ouabain and the results are in agreement with those observed in the rabbit ear artery (Suzuki, 1988). Some arteries require very high concentrations of ouabain to inhibit the electrogenic Na-K pump and in the rat main pulmonary artery, 10^{-3} M ouabain was required to block the K-free-induced hyperpolarization (Suzuki & Twarog, 1982). In the canine coronary artery, 10^{-6} M ouabain was sufficient to block the K-free-induced hyperpolarization but it was insufficient to block the ACh-induced hyperpolarization. Therefore, the ACh-induced hyperpolarization is almost certainly generated by mechanisms other than activation of the electrogenic Na-K pump in this tissue. Reduction or disappearance of the ACh-induced hyperpolarization in high $[K]_o$ solution suggests that this hyperpolarization is accompanied by an increase in K-conductance of the membrane, and the results are thus consistent with the findings in other arteries (Kuriyama & Suzuki, 1978; Komori & Suzuki, 1987a; Chen & Suzuki, 1989). Direct evidence of an increase in K-conductance was obtained in ion-flux experiments (Chen *et al.*, 1988). It remains to be determined why the effects of ouabain on the ACh-induced hyperpolarization differ between the endothelium-intact and endothelium-free (cascade) tissues.

Li ions can permeate the Na-channel, but they cannot be extruded from the cell by the electrogenic Na-K pump (Kerkut & York, 1971). In Li-Krebs solution, ACh produced an endothelium-dependent hyperpolarization, but the K-free-induced hyperpolarization was abolished. These results again indicate that the endothelium-dependent hyperpolarization in the canine coronary artery does not involve activation of the electrogenic Na-K pump. However, the decay of the hyperpolarization was accelerated in the Li-Krebs solution. It is uncertain whether this shortening is due to the effects of Li ions, to a reduction of Na ion concentration, or to other mechanisms.

In the present study, ACh relaxed the high-K-induced contractions, in an endothelium-dependent manner and this relaxation was not altered by ouabain. Therefore, the electrogenic Na-K pump does not seem to contribute to the EDRF-induced relaxation of vascular smooth muscle (Feletou & Vanhoutte, 1988), and as such the mechanism involved may be different from the endothelium-dependent relaxation to arachidonic acid (Rubanyi & Vanhoutte, 1985).

In the canine coronary artery, repetitive application of ACh caused a successive, marked reduction in amplitude of the endothelium-dependent hyperpolarization, with only a slight reduction in the amplitude of the endothelium-dependent relaxation. These results further suggest that the relaxing and hyperpolarizing responses are generated by different factors, and as such are in agreement with our previous observations (Komori & Suzuki, 1987a,b; Chen *et al.*, 1988; Chen & Suzuki, 1989).

It is concluded that in the canine coronary artery, the endothelium-dependent hyperpolarization and relaxation of smooth muscle cells produced by ACh does not seem to involve activation of the electrogenic Na-K pump. Different time courses in the tachyphylaxis occurred between the hyperpolarization and relaxation suggesting that both EDRF and EDHF are involved in the action of ACh in this artery.

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Effects of putative activators of K^+ channels in mouse pancreatic β-cells

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1 The vasodilator and antihypertensive properties of pinacidil, cromakalim (BRL 34915), nicorandil and minoxidil sulphate may be due, at least in part, to their ability to open K^+ channels in vascular smooth muscles. In this study, mouse pancreatic islets were used to determine whether these drugs affect insulin release by acting on K^+ channels of β-cells. Their effects were compared to those of diazoxide.

2 Diazoxide caused a dose-dependent inhibition of insulin release by islets incubated with 15 mM glucose (93% at 100 μM). Pinacidil inhibited release by 36 and 72% at 100 and 500 μM, respectively. Cromakalim and nicorandil were less effective (35 and 25% inhibition at 500 μM). Minoxidil sulphate increased insulin release at 500 μM.

3 In the presence of 7 mM glucose and in the absence of Ca^{2+} (to avoid activation of Ca^{2+}-dependent K^+ channels), ^{86}Rb efflux from islet cells was increased by 100–500 μM pinacidil and 500 μM nicorandil, which were, however, less potent than diazoxide. Cromakalim was ineffective, whereas 500 μM minoxidil sulphate decreased the efflux rate. In the absence of glucose and presence of Ca^{2+}, 500 μM cromakalim and minoxidil sulphate inhibited ^{86}Rb efflux.

4 Like diazoxide, pinacidil (500 μM) abolished glucose-induced electrical activity in β-cells and hyperpolarized the membrane.

5 ATP-sensitive K^+ currents were studied in single β-cells by the whole cell patch-clamp technique. Pinacidil increased the current less than did diazoxide. In contrast, cromakalim and minoxidil sulphate decreased K^+-currents whilst nicorandil was without effect.

6 It is concluded that pinacidil, like diazoxide, inhibits insulin release from β-cells by opening ATP-sensitive K^+ channels, whereas the smaller inhibitory effects of cromakalim and nicorandil may involve actions other than on K^+ channels in these cells. Minoxidil sulphate potentiates glucose-induced insulin release, probably by inhibiting ATP-sensitive K^+ channels. However, all these effects of the vasodilators are only seen at high concentrations and are thus unlikely to occur *in vivo*.

Introduction

Regulation of insulin release by many secretagogues involves a coordinated control of K^+ and Ca^{2+} permeabilities of the β-cell membrane (Henquin, 1987; Petersen & Findlay, 1987). Studies with intracellular microelectrodes and measurements of ionic fluxes first showed that a decrease in K^+ permeability of the plasma membrane underlies glucose-induced depolarization to the threshold potential, at which voltage-dependent Ca^{2+} channels are activated (Sehlin & Täljedal, 1975; Henquin, 1978; Atwater *et al.*, 1978). Subsequent patch-clamp studies demonstrated that this decrease is due to closure of ATP-sensitive K^+ channels (Ashcroft *et al.*, 1984; Cook & Hales, 1984; Rorsman & Trube, 1985). Hypoglycaemic sulphonylureas, the most important pharmacological stimulators of insulin release, appear to induce a similar sequence of events by directly closing the same K^+ channels (Henquin, 1980; Henquin & Meissner, 1982; Sturgess *et al.*, 1985; Trube *et al.*, 1986). On the other hand, the hyperglycaemic sulphonamide, diazoxide, antagonizes the effects of glucose or tolbutamide by opening these ATP-sensitive K^+ channels (Henquin & Meissner,

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1982; Trube et al., 1986; Dunne et al., 1987; Zünkler et al., 1988).

A new class of drugs, able to relax smooth muscles apparently by opening K^+ channels, is currently arousing great interest because of their potential use as vasodilator and antihypertensive agents. The best characterized of these drugs are pinacidil, cromakalim (BRL 34915), nicorandil and minoxidil sulphate (Cook, 1988; Hamilton & Weston, 1989).

The objective of the present study was to determine whether these putative activators of K^+ channels affect pancreatic β-cell function. Their effects were compared to those of diazoxide. Our aims were first to gain more insight into the tissue-specificity of the action of the K^+ channel activators and, second, to predict whether alterations of glucose homeostasis could be expected in patients eventually treated with these drugs.

Methods

Measurements of insulin release, ^{86}Rb efflux and β-cell membrane potential

All experiments were performed with islets of fed female NMRI mice (25–30 g) killed by decapitation. For measurement of the membrane potential of β-cells with an intracellular microelectrode, a piece of pancreas was fixed in a small chamber and continuously perifused (Meissner & Schmelz, 1974). β-Cells were identified by the typical electrical activity that they display in the presence of a stimulatory concentration of glucose. For all other experiments, islets were isolated after collagenase digestion of the pancreas (Lacy & Kostianovsky, 1967). The effect of the various test agents on insulin release was measured with incubated islets. Immediately after isolation, the islets were preincubated for 45 min at 37°C, in a medium containing 15 mM glucose and 2.5 mM Ca^{2+}. Batches of 3 islets were then incubated for 60 min in 1 ml of medium containing 15 mM glucose, 2.5 mM Ca^{2+} and appropriate concentrations of the test agents. A portion of the medium was taken at the end of the incubation for measurement of insulin, with rat insulin used as standard (Henquin & Lambert, 1975). For measurement of the efflux of ^{86}Rb (used as tracer for K), the islets were first loaded with the tracer during 90 min in a medium containing 15 mM glucose and 2.5 mM Ca^{2+} (Garrino & Henquin, 1988). They were then transferred to a dynamic system of perifusion permitting monitoring of ^{86}Rb efflux (Henquin, 1978). The concentrations of glucose and Ca^{2+} used for these experiments are indicated in the legend to each figure.

The solutions used for measurements of insulin release, ^{86}Rb efflux and β-cell membrane potential had the following ionic composition (mM): NaCl 120, KCl 4.8, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 24. Ca^{2+}-free solutions were prepared by replacing $CaCl_2$ with $MgCl_2$; the concentration of total residual Ca^{2+} was less than 20 μM. All solutions were gassed with O_2/CO_2 (94:6) to maintain a pH of 7.4 and were supplemented with 1 mg ml^{-1} bovine serum albumin fraction V (Boehringer, Mannheim, Germany), except for electrophysiological recordings.

Patch clamp measurements in β-cells

Immediately after isolation, mouse islets were dispersed into single cells and clusters of cells, which were then cultured for 1–4 days in 35 mm plastic Petri dishes (Plant, 1988). Patch-clamp recordings were performed with an LM-EPC 7 patch-clamp amplifier (List Electronic, Darmstadt, F.R.G.). Pipettes with resistances of 4–6 MΩ were made of borosilicate glass (Dow Corning, Midland, MI, U.S.A.) and coated with Sylgard. Capacity currents were compensated using the patch-clamp amplifier. Series resistance compensation was not used in most experiments. When quantitative measurements were made, the currents and thus the voltage error were small. Experiments were performed in the plastic Petri dishes, reduced in volume to about 0.6 ml by a Plexiglas insert. The dish was continuously perfused at approximately 2 ml min^{-1} with solutions at room temperature (20–22°C). Only single cells were used for whole-cell recordings. β-Cells were recognised by the presence of single channel currents with an amplitude of approximately 5 pA at 0 mV (single channel conductance around 70 pS) in the cell-attached mode, and by the increase in current in the whole-cell mode during dialysis with low ATP (0.3 mM) pipette solutions. In the cell-attached mode, β-cells, identified as described above, respond to an increase in the bath glucose concentration from 1 to 15 mM with electrical activity.

ATP-sensitive K^+ currents were isolated from other membrane currents in β-cells in the whole-cell mode using a pipette solution with low free Ca^{2+} concentration (50 nM), and an appropriate pulse protocol. The membrane potential was held at −70 mV, close to the resting potential of the cell. Voltage steps of 100 ms duration to −80 mV and −60 mV, separated by a 100 ms gap, were applied at a frequency of 0.1 Hz. Pulses were generated by computer and the current responses digitized at 2.5 kHz stored on floppy disk (Hof, 1986). In some experiments currents were digitized (Sony PCM 501 ES) and stored on video tape. Currents were displayed on a pen recorder, or the holding current and maximum responses to pulses of +10 mV and −10 mV were measured from records stored on computer.

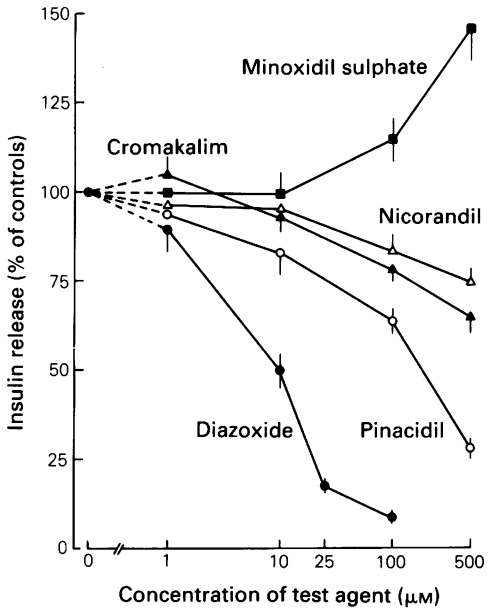

Figure 1 Effects of various concentrations of the test agents on insulin release from mouse islets incubated in the presence of 15 mM glucose and 2.5 mM Ca^{2+}: diazoxide (●), pinacidil (○), cromakalim (▲), nicorandil (△), minoxidil sulphate (■). Results are expressed as a percentage of control release measured within the same experiment. Insulin release by control islets averaged 7.77 ± 0.39 ng per islet 60 min^{-1} ($n = 74$). Values are means for 11–32 batches of islets; s.e.mean shown by vertical lines.

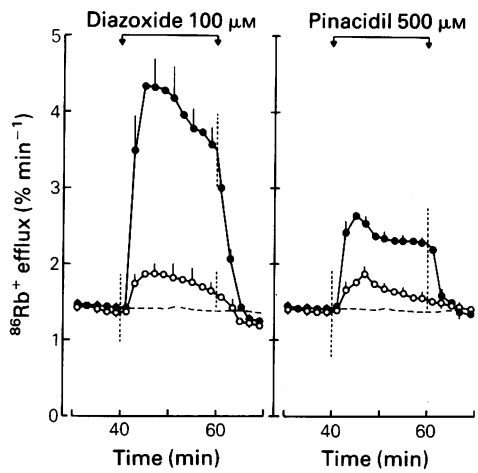

Figure 2 Effects of diazoxide and pinacidil on ^{86}Rb efflux from mouse islets perifused with a calcium-free medium containing 7 mM glucose. The experiments were performed in the absence (●) or in the presence (○) of 100 μM tolbutamide. Controls without addition of test substance are shown by the broken lines. Values are means for 4–5 experiments with s.e.mean shown by vertical lines.

Two types of solution were used for patch-clamp experiments. The bath solution contained (mM): NaCl 140, KCl 5.6, $CaCl_2$ 2.6, $MgCl_2$ 1.2, HEPES 10, and was titrated to pH 7.4 with NaOH. Pipettes were filled with a solution containing (mM): KCl 135, $MgCl_2$ 4, $CaCl_2$ 2, EGTA 10, Na_2ATP 0.3, HEPES 10. This solution was titrated to pH 7.15 with KOH.

Drugs

Diazoxide was provided by Schering Corp. (Bloomfield, NJ, U.S.A.). The other substances were obtained from the following sources: pinacidil [(±)-N-cyano-*N*-4-pyridyl-*N*-1,2,2-trimethylpropylguanidine, monohydrate] from Leo Pharmaceutical Products (Ballerup, Denmark); minoxidil (2,4-diamino-6-piperidinylpyridine 3-oxide) sulphate from The Upjohn Co. (Kalamazoo, MI, U.S.A.); cromakalim (BRL 34915) [(±)-6-cyano-3,4-dihydro-2,2-dimethyl-trans-4-(2-oxo-1-pyrollidyl)-2H-benzo[b]pyran-3-ol] from Beecham Pharmaceuticals (Harlow, UK) and nicorandil [N-(nitroxy-2-ethyl)pyridinecarboxamide-3] from Rhône–Poulenc (Vitry, France). $^{86}RbCl$ was purchased from the Radiochemical Centre (Amersham, Bucks, UK). All other reagents were from Merck A.G. (Darmstadt, Germany).

Stock solutions of diazoxide and pinacidil (40 mM) were prepared in NaOH, whereas stock solutions of cromakalim, nicorandil and minoxidil sulphate (500 mM) were prepared in dimethylsulphoxide. These stock solutions were used for up to five days with similar results. Aliquots of the stock solutions were added to the appropriate media just before use. Although without effect, dimethylsulphoxide alone was added to appropriate control solutions (1 μl ml^{-1}).

Presentation of results

Electrophysiological experiments are illustrated by recordings which are representative of the indicated number of experiments, performed with different mice. Other results are presented as means ± s.e.mean for the indicated number of experiments or batches of islets. The statistical significance of differences between means was assessed by Student's *t* test or by analysis of variance followed by Dunnett's multiple comparisons test. Differences were considered significant at $P < 0.05$.

Results

Effects on insulin release and ^{86}Rb efflux

Figure 1 shows the effects of the different test agents on insulin release by islets incubated in the presence of 15 mM glucose. Diazoxide caused a dose-dependent inhibition with almost complete suppression (93%) of secretion at 100 μM. Pinacidil also effectively inhibited release at 100 μM (36%) and 500 μM (72%). Cromakalim and nicorandil were clearly less effective causing only 35 and 25% inhibition, respectively, at 500 μM. On the other hand, minoxidil sulphate significantly potentiated insulin release at 500 μM (by 48%).

Addition of 100 μM diazoxide or 500 μM pinacidil to a Ca^{2+}-free medium containing a substimulatory concentration of glucose (7 mM) caused a marked and sustained increase in ^{86}Rb efflux from perifused islets (Figure 2). This effect was rapidly reversible on removal of either diazoxide or pinacidil and antagonized in the presence of 100 μM tolbutamide.

The maximum change in ^{86}Rb efflux induced by the different test agents is shown by Figure 3. The effect of pinacidil was significant at 100 and 500 μM, but was much smaller than that of diazoxide. Nicorandil only marginally accelerated the efflux rate at 500 μM. Cromakalim was ineffective. In contrast, minoxidil slightly decreased ^{86}Rb efflux at 500 μM.

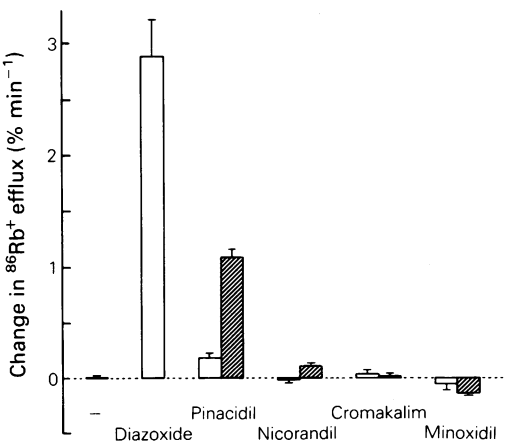

Figure 3 Maximum effect of the test agents on ^{86}Rb efflux from mouse islets perifused with a calcium-free medium containing 7 mM glucose. The results were obtained from experiments similar to those shown in Figure 2. Test agents were used at 100 μM (open columns) or 500 μM (hatched columns). The change in ^{86}Rb efflux is given as the difference between the actual efflux rate immediately before addition of the test agent (36–40 min) and after addition of the test agent (46–50 min). Values are means for 4–6 experiments with s.e.mean shown by vertical bars.

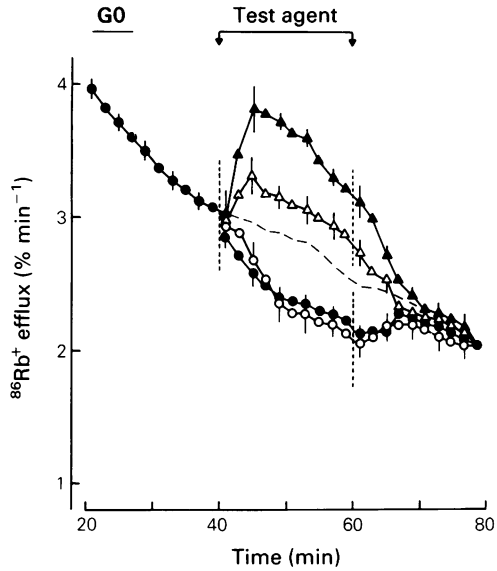

Figure 4 Effects of the test agents on ^{86}Rb efflux from mouse islets perifused with a glucose-free medium (G0) containing 2.5 mM $CaCl_2$. Diazoxide 100 μM (▲), pinacidil 500 μM (△), cromakalim 500 μM (○), minoxidil sulphate 500 μM (●). Controls without addition of test substance are shown by the broken line. Values are means for 4–5 experiments with s.e.mean shown by vertical lines.

The effect of the test agents on ^{86}Rb efflux from islet cells was also measured during perifusion of the islets with a medium containing calcium but no glucose. Under these conditions 500 μM minoxidil or cromakalim slightly decreased the efflux rate, whereas 100 μM diazoxide and 500 μM pinacidil increased it (Figure 4). Nicorandil at 500 μM was without effect (not shown). None of the drugs modified basal insulin release.

Effects on β-cell membrane potential

In the presence of a stimulatory concentration of glucose, β-cells display typical electrical activity (Figure 5). Diazoxide (100 μM) rapidly abolished this activity and hyperpolarized the membrane. Pinacidil (500 μM) produced a similar, but less rapid and marked effect. In one out of the four cells examined, the inhibition of electrical activity by 500 μM pinacidil was only partial, whereas it was always complete with 100 μM diazoxide. Moreover, the hyperpolarization by 100 μM diazoxide (17.0 ± 2.0 mV) brought the membrane potential to the same

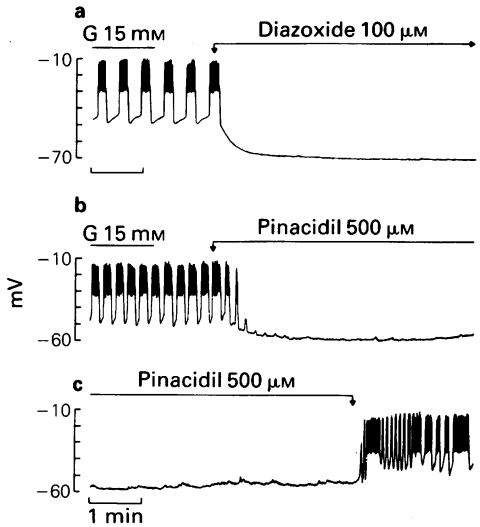

Figure 5 Effects of diazoxide and pinacidil on the membrane potential of single mouse β-cells perifused with a medium containing 15 mM glucose and 2.5 mM $CaCl_2$. The drugs were added at the indicated concentrations and for the periods shown by the arrows: (c) is the direct continuation of (b); (a) was obtained in another cell. These records are representative of results obtained in 4 similar experiments.

level as in the absence of glucose, whereas the hyperpolarization by pinacidil was smaller (7.8 ± 1.5 mV) and less sustained (Figure 5).

Effects on ATP-dependent K^+ currents

Following rupture of the patch membrane and dialysis with an intracellular medium containing an ATP-concentration lower than that in the intact cell, ATP-sensitive K^+ currents were activated as ATP diffused out of the cell (Figure 6a; Trube *et al.*, 1986). After reaching a maximum, which was maintained for a different time in each individual cell, the currents then declined as run-down of the conductance occurred. The efficacy of isolation of the ATP-sensitive K^+ current in these experiments is shown by the near complete block of the currents by 100 μM tolbutamide (Figure 6b).

Addition of diazoxide to the external solution reversibly increased the amplitude of the ATP-sensitive K^+ current (Figure 6), an effect dependent on the time at which the drug was applied. Diazoxide (100 μM) produced only a small increase in the current during the phase in which the current was fully activated, but its effectiveness increased as run-down occurred. Owing to this variability, quantitative estimates of the effects of diazoxide and other

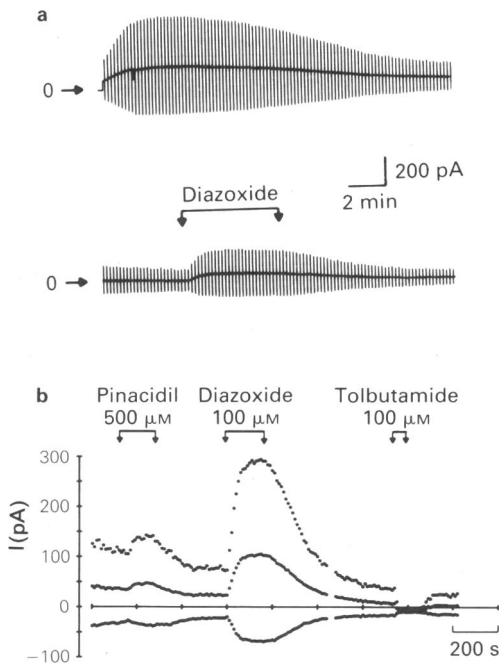

Figure 6 (a) Time-course of activation and run-down of ATP-sensitive K^+ currents, and the effect of 100 μM diazoxide, in a single β-cell. The upper trace shows a recording of currents from a β-cell, starting some seconds after dialysis of the cell with a pipette solution containing 0.3 mM ATP. Currents were recorded in response to pulses of ±10 mV from a holding potential of −70 mV. The lower record is a continuation of the upper record and shows the effect of diazoxide during the linear run-down phase. Zero current level is shown by the arrow. (b) Effects of pinacidil, diazoxide and tolbutamide on ATP-sensitive K^+ currents. The current values shown are the currents at the holding potential of −70 mV: middle trace; at −60 mV: upper trace; and at −80 mV: lower trace. Drugs were added at the indicated concentrations and for the periods shown by the bars.

drugs were first made when run-down had reached an approximately linear phase (Figure 6). Under such conditions, the effects of diazoxide in different cells were similar and could be seen with concentrations as low as 10 μM.

Of the other substances tested, only pinacidil was able to increase the ATP-sensitive K^+ current in a way similar to diazoxide. At 100 μM, the effect of pinacidil was only marginal and barely visible (data not shown). At 500 μM, however, pinacidil increased the current by 35 ± 3% (Figure 6b). Diazoxide applied immediately after complete washout of pinacidil increased the current by 257 ± 20%. In contrast, cromakalim and minoxidil sulphate, at 500 μM,

decreased the current through ATP-sensitive K^+ channels by 42% and 29% respectively (means, $n = 3$), while nicorandil was without effect (data not shown).

Discussion

The specific aim of this study was to determine whether drugs known to activate K^+ channels in smooth and cardiac muscle cells produce a similar effect in pancreatic β-cells and thereby alter insulin release. We therefore selected the most appropriate experimental conditions to detect even small effects of the drugs. Insulin release was measured in the presence of a stimulatory concentration of glucose which allows either stimulatory or inhibitory effects of a test substance to be detected. However, measurements of ^{86}Rb efflux under these conditions (15 mM glucose and 2.5 mM $CaCl_2$) are not adequate to demonstrate that a small inhibition of release, such as that produced by a low concentration of diazoxide (15 μM), may also be due to an increase in membrane K^+ permeability (unpublished data). This difficulty is due to the fact that activation of one type of K^+ channel (ATP-sensitive) and the concomitant change in membrane potential reduce the activation of other K^+ channels (voltage, and voltage and Ca^{2+}-sensitive). Thus ^{86}Rb efflux was measured in the presence of 7 mM glucose and in the absence of extracellular Ca^{2+}. Under these conditions, most ATP-sensitive K^+ channels are closed (Misler *et al.*, 1986; Ashcroft *et al.*, 1988) and the activation of Ca^{2+}-dependent K^+ channels is avoided. ^{86}Rb efflux was also measured in the absence of glucose, when a sufficient number (not all) of ATP-sensitive K^+ channels are open to hyperpolarize the β-cell membrane (Ashcroft *et al.*, 1988). ATP-sensitive K^+ currents were measured in the whole cell configuration as described by Trube *et al.* (1986), but the effect of the drugs was tested when the current was more stable (later phase of run-down).

Effects of diazoxide

The effects of diazoxide described here confirm those previously observed with the drug on ATP-sensitive K^+ channels (Trube *et al.*, 1986; Dunne *et al.*, 1987; Zünkler *et al.*, 1988), membrane potential, ^{86}Rb efflux and insulin release (Henquin & Meissner, 1982; Henquin *et al.*, 1982). There remains no doubt that diazoxide inhibits insulin release by the following sequence of events: the opening of ATP-sensitive K^+ channels leads to hyperpolarization of the β-cell membrane; this reduces activation of voltage-dependent Ca^{2+} channels and, hence, Ca^{2+} influx.

It has long been known that diazoxide exerts a hypotensive action through direct relaxation of vascular smooth muscles (Koch-Weser, 1976), an effect that may be due to the opening of K^+ channels in these tissues (Cook, 1988; Winquist *et al.*, 1989).

Effects of pinacidil

A recent brief report has shown that 500 μM pinacidil increases ^{86}Rb efflux and inhibits glucose-induced insulin release from rat islets (Lebrun *et al.*, 1988). Our results show that pinacidil mimics all effects of diazoxide. It activated ATP-sensitive K^+ currents, accelerated ^{86}Rb efflux (an effect counteracted by tolbutamide), hyperpolarized the β-cell membrane, suppressed the Ca^{2+}-dependent electrical activity and inhibited insulin release. However, these effects were only produced by a high concentration of pinacidil (100–500 μM) and remained smaller than those produced by 100 μM diazoxide. We, therefore, suggest that pinacidil inhibits insulin release by a similar cellular mechanism as, but with a lower potency than diazoxide. Whether both drugs directly affect ATP-sensitive K^+ channels in the same way remains to be established.

It is well known that pinacidil has a direct arterial vasodilator effect (Kaergaard Nielsen & Arrigoni-Martelli, 1981), which was recently ascribed to its ability to open still unidentified K^+ channels in smooth muscle cells (Bray *et al.*, 1987; Cook *et al.*, 1988).

Effects of cromakalim

It has been reported in abstract form (Wilson *et al.*, 1988) that 10 μM cromakalim did not affect (basal?) insulin release by rat islets or interfere with the stimulatory effect of a very high concentration (50 μM) of glibenclamide. Our results show that high concentrations of cromakalim slightly inhibited glucose-induced insulin release. There is no evidence, however, to suggest that this effect is due to an increase in K^+ permeability of the β-cell membrane, since ^{86}Rb efflux was never increased. On the contrary, cromakalim decreased it at least in the absence of glucose and inhibited ATP-sensitive K^+ currents. These unexpected observations could be due to the fact that cromakalim is only a partial agonist of K^+ channels with antagonistic effects at high concentrations, or that the K^+ channels of β-cells are markedly different from K^+ channels of muscle cells. None of these explanations would satisfactorily explain the slight inhibition of insulin release. It is thus pertinent to point out that cromakalim can

inhibit contractile activity at concentrations lower than those required to increase ^{86}Rb efflux (Quast, 1987; Hollingsworth *et al.*, 1987). Whether this reflects an action of the drug at a site distinct from K^+ channels, is still unresolved.

Cromakalim activates ATP-sensitive K^+ channels in heart muscle cells (Escande *et al.*, 1988; Sanguinetti *et al.*, 1988), and indirect evidence suggests that similar K^+ channels could be activated in smooth muscle cells (Wilson *et al.*, 1988; Winquist *et al.*, 1989). In the heart, activation of ATP-sensitive K^+ channels by cromakalim could only be demonstrated at 37°C. It is unlikely that this phenomenon explains our inability to detect an increase in K^+ permeability by cromakalim. Thus ^{86}Rb efflux was measured at 37°C and, in the absence of glucose, an inhibitory effect of cromakalim occurred. This is consistent with the inhibition of ATP-sensitive K^+ currents measured at room temperature.

Effects of nicorandil

The slight acceleration of ^{86}Rb efflux produced by 500 μM nicorandil suggests that an increase in membrane K^+ permeability of β-cells could underlie the small inhibition of insulin release produced by the same concentration of the drug. Since no effect on ATP-sensitive K^+ currents was detected, it is possible that nicorandil affects another type of K^+ channel. However, we cannot exclude the possibility that the effect was too small to be detected by the method used. We can also not rule out an additional action of nicorandil which would contribute to the inhibition of insulin release. It has been suggested that the vasodilator action of nicorandil is not only due to K^+ channels opening but also to an unidentified mechanism mediated by the nitro group present in the nicorandil molecule (Weir & Weston, 1986). Inhibition of glucose-induced insulin release by sodium nitroprusside has been reported (Laychock, 1981).

Effects of minoxidil sulphate

The hypotensive effects of minoxidil (Freyburger *et al.*, 1981) are currently attributed to the opening of unidentified K^+ channels in vascular muscles by the active metabolite minoxidil sulphate (Meisheri *et al.*, 1988). We unexpectedly found that a high concentration of minoxidil sulphate decreased ^{86}Rb efflux and ATP-sensitive K^+ currents. This small effect was insufficient to trigger insulin release in the absence of glucose. However, since minoxidil sulphate decreased ^{86}Rb efflux also in the presence of 7 mM glucose (in contrast to cromakalim which decreased it only in the absence of glucose), it is possible, though not proven, that the drug potentiates glucose-induced insulin release by decreasing K^+ permeability of the β-cell membrane.

Conclusions

Although pinacidil, nicorandil, cromakalim and minoxidil sulphate affect insulin release *in vitro*, this 'side-effect' is unlikely to occur *in vivo* and to perturb glucose homeostasis of patients treated with these agents. These effects on β-cells were only observed at concentrations which are 1–3 orders of magnitude higher than those necessary to affect contractility of vascular muscles. Of the drugs tested here, which are known or thought to activate K^+ channels in muscle cells, only diazoxide and pinacidil decreased insulin release by opening K^+ channels in β-cells. Cromakalim and minoxidil sulphate were rather found to inhibit K^+ channels, whilst nicorandil did not affect them in β-cells. These observations add to the accumulating evidence that the effects of certain drugs on K^+ channels may be very tissue-specific. With the reservation that it has not yet been demonstrated that diazoxide relaxes muscle solely by opening K^+ channels, it is interesting to note that its effects on contraction (Winquist *et al.*, 1989) and on insulin secretion occur in the same concentration-range. In contrast, tolbutamide and glibenclamide must be used at much higher concentrations to affect K^+ permeability in heart muscle cells (Belles *et al.*, 1987; Escande *et al.*, 1988; Sanguinetti *et al.*, 1988) than in pancreatic β-cells (Henquin & Meissner, 1982; Garrino *et al.*, 1985; Zünkler *et al.*, 1988). This may reflect subtle differences in the structure of K^+ channels (even of the same type) between tissues or in their mechanisms of control.

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Effects of changes in extra- and intracellular K^+ on the endothelial production of prostacyclin

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1 Changes in the KCl concentration of the incubation medium, from 0 to 80 mM, had no effect on the basal or ATP-stimulated release of prostacyclin (PGI_2) from bovine aortic endothelial cells.

2 The monovalent cation ionophores, valinomycin and nigericin (5 μM), enhanced the release of PGI_2 from endothelial cells stimulated by ATP or bradykinin.

3 The action of nigericin, unlike that of valinomycin, was time-dependent, abolished in a high-KCl medium and associated with an increased efflux of ^{86}Rb and a time-dependent depletion of intracellular K^+.

4 Ouabain (1–100 μM) also enhanced the release of PGI_2 in response to ATP and induced a significant depletion of intracellular K^+ in bovine aortic endothelial cells.

5 In conclusion, modifications of the endothelial cell membrane potential, resulting from changes in the extracellular K^+ concentration, do not modulate the basal or ATP-stimulated production of PGI_2. An acute depletion of intracellular K^+ by nigericin or ouabain enhances the production of PGI_2 in aortic endothelial cells stimulated by ATP or bradykinin.

Introduction

Vascular endothelial cells contain several kinds of K^+ channels, whose opening in response to chemical or mechanical signals induces hyperpolarization. The earliest event induced by haemodynamic shear stress in bovine aortic endothelial cells is the activation of a K^+ current (Olesen *et al.*, 1988a) and the resulting hyperpolarization might play a crucial role in the process of mechanochemical transduction (Nakache & Gaub, 1988). In the same cells, a distinct K^+ current is activated by acetylcholine, via muscarinic receptors, but independently from a pertussis toxin-sensitive GTP-binding protein, as in the heart (Olesen *et al.*, 1988b). K^+ channels activated by Ca^{2+} have also been detected in these cells (Fichtner *et al.*, 1987; Sauve *et al.*, 1988). They are likely to mediate the increased efflux of ^{86}Rb (Gordon & Martin, 1983) and the K^+ current (Colden-Stanfield *et al.*, 1987; Sauve *et al.*, 1988) which are induced by agents known to increase cytosolic Ca^{2+}, such as bradykinin and adenosine 5'-triphosphate (ATP). The same agents stimulate the release of prostacyclin (PGI_2) and endothelium-dependent relaxing factor (EDRF) (Gordon & Martin, 1983; Pearson *et al.*, 1983; Van Coevorden & Boeynaems, 1984). ATP acts via P_{2y}-receptors (Needham *et al.*, 1987), which are coupled to the generation of inositol 1,4,5-trisphosphate (Pirotton *et al.*, 1987), resulting in a rise of cytosolic Ca^{2+} (Hallam & Pearson, 1986) and the activation of calmodulin-dependent kinases (Demolle *et al.*, 1988). On the other hand, the onset of flow triggers a burst of PGI_2 production in endothelial cells (Frangos *et al.*, 1985). It was speculated that the hyperpolarization induced by haemodynamic shear stress, acetylcholine, bradykinin or ATP might play a role in endothelium-dependent relaxation, either by direct junctional conductance to smooth muscle cells or by stimulating the endothelial cells themselves to release EDRF or PGI_2 (Olesen *et al.*, 1988b). Although the best known example of a voltage-dependent cell process is the opening of Na^+ and Ca^{2+} channels upon depolarization, there are indeed non-excitable cells in which Ca^{2+} influx is increased by hyperpolarization (Penner *et al.*, 1988). The membrane potential of endothelial cells is determined primarily by the K^+ permeability (Northover, 1980; Colden-Stanfield *et al.*, 1987). Therefore, in order to evaluate the role of hyperpolarization in the control of endothelial cell

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function, we have investigated the effects of monovalent cation ionophores and of changes in extracellular K^+ on the endothelial production of PGI_2.

Methods

Preparation, culture and incubation of endothelial cells

Bovine aortic endothelial cells were obtained by collagenase digestion of the aorta excised from a freshly slaughtered cow, as previously described (Booyse *et al.*, 1975; Van Coevorden & Boeynaems, 1984). The cells were seeded on 100 mm Petri dishes and incubated at 37°C under an atmosphere of 5% CO_2/95% air in the following medium: MEM D-valine (80%, v/v), foetal calf serum (20%, v/v), 2 mM glutamine, 100 u ml^{-1} penicillin, 100 $\mu g\,ml^{-1}$ streptomycin, 2.5 $\mu g\,ml^{-1}$ amphotericin B. The medium was changed after 24 h and then every 3 days. After 4 or 5 days, the primary cultures formed confluent monolayers and could be subcultured. The cells were detached by a 5 min incubation in a Ca^{2+}- and Mg^{2+}-free Hanks buffer containing trypsin (10 mg dl^{-1}) and EDTA (1 mM). Thereafter, they were seeded on 35 mm Petri dishes or 16 mm diameter wells of 24-well plastic trays and the culture was continued in the following medium: DMEM (60%, v/v), Ham's F_{12} (20%, v/v), foetal calf serum (20%, v/v) with the same concentrations of penicillin, streptomycin and amphotericin B.

Experiments were performed with confluent monolayers, between passage 2 and 5. The culture medium was removed and, after rinsing twice with DMEM, the cells incubated in 1 ml (dishes) or 0.5 ml (wells) medium containing the test agents, usually for 20 min. Incubation media of various compositions were used in this study: DMEM or Krebs-Ringer medium buffered with HEPES (25 mM HEPES buffer pH 7.4, 1.25 mM $MgSO_4$, 1.45 mM $CaCl_2$, 1.25 mM KH_2PO_4, 8 mM glucose) contained different concentrations of KCl (0, 5, 20, 80 mM) and NaCl (129, 124, 109, 49 mM), which were varied in a reciprocal way in order to maintain a constant osmolarity. Acute K^+ depletion by nigericin was performed in a Krebs-Ringer-HEPES medium containing no KCl and buffered at pH 7.1, as described by Delafontaine *et al.* (1987).

Prostaglandin radioimmunoassay

The production of PGI_2 was measured by radioimmunoassay (RIA) of its stable degradation product, prostaglandin 6-keto-$F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), performed directly in the incubation medium, without extraction or chromatography. A rabbit antiserum was raised against 6-keto-$PGF_{1\alpha}$ coupled to bovine serum albumin (BSA), as described by Maclouf (1981): the limit of detection was 16 pg and the cross-reactions were 1.2% with $PGF_{2\alpha}$, 0.3% with PGE_2 and <0.1% with thromboxane B_2. One hundred μl aliquots of incubation media, [^{3}H]-6-keto-$PGF_{1\alpha}$ (11,000 d.p.m.), anti-6-keto-$PGF_{1\alpha}$ antiserum (final dilution: 10^{-4}) and bovine gamma globulins (0.25 g dl^{-1}) in Tris buffer (50 mM, pH 7.4) were incubated in a total volume of 0.4 ml for 60 min at room temperature. Then 0.4 ml of a cold 25% (wt/wt) solution of polyethylene glycol was added to separate bound and free antigen.

Measurement of cell K^+

After removal of the incubation medium, the cells were rapidly washed 5 times with a cold solution of $MgCl_2$ (0.1 M), lysed with Triton (0.01% v/v), and K^+ was measured by flame photometry, using Li^+ as internal standard.

Measurement of ^{86}Rb efflux

Bovine aortic endothelial cells were seeded on plastic coverslips (Thermanox, Lux) at a density of 1.5×10^5 cells per coverslip. They were incubated for 3 h in culture medium containing ^{86}RbCl (10 μCi ml^{-1}). The coverslips were then washed once in a medium of the following composition (mM): NaCl 120, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, Na_2HPO_4 3, HEPES 20 (pH 7.4) and glucose 10. The time course of ^{86}Rb efflux was followed by transferring the coverslips every 4 min into test tubes containing 2 ml of the same medium, at 37°C. At the end of the experiment, the cells were lysed in trichloracetic acid (TCA) 10%. The radioactivity in each fraction was counted in liquid scintillation and expressed as % of the radioactivity remaining associated to the cells.

Materials

DMEM, Ham's F_{12}, MEM-D-valine, foetal calf serum, penicillin, streptomycin, amphotericin B and glutamine were obtained from Gibco. Collagenase type I was from Cooper (Worthington). Trypsin was purchased from Flow Laboratories. ATP, bradykinin, nigericin, valinomycin and ouabain were purchased from Sigma Chem Co.; ionophore A23187 was obtained from Boehringer-Mannheim and 6-keto- $PGF_{1\alpha}$ from Upjohn Diagnostics. [^{3}H]-6-keto- $PGF_{1\alpha}$ and ^{86}RbCl were purchased from Amersham.

Results

Changing the concentration of KCl added to the incubation medium from 0 to 80 mM had no effect

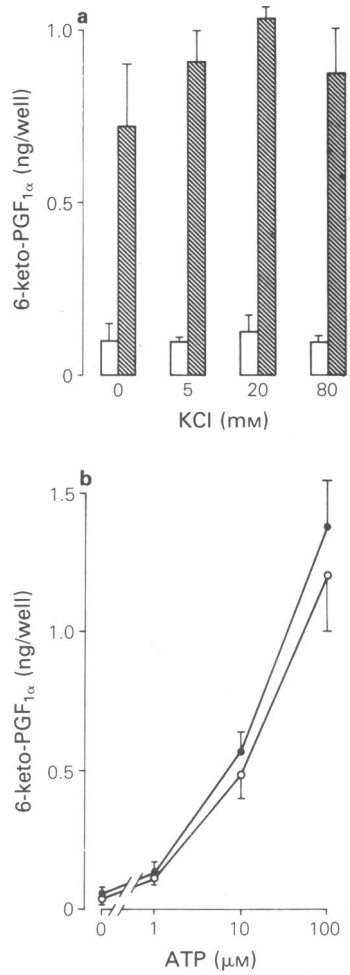

Figure 1 Effect of changes in the concentration of extracellular K^+ on the release of prostacyclin (PGI_2) and its stimulation by ATP. After removal of the culture medium, bovine aortic endothelial cells were rinsed and incubated in a Krebs-Ringer-HEPES buffer containing variable concentrations of KCl (0, 5, 20 and 80 mM) and NaCl (129, 124, 109 and 49 mM, respectively). Results represent the amount of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) accumulated in the medium at the end of a 20 min period (mean with vertical bars showing s.d. of triplicate determinations). Each column corresponds to 1 representative experiment out of 3. (a) Open columns show control and hatched columns effect of ATP (10 μM). (b) (○) Medium containing 5 mM KCl and (●) medium containing 80 mM KCl.

either on the basal or ATP-stimulated release of PGI_2 from bovine aortic endothelial cells (Figure 1). The monovalent cation ionophores valinomycin and nigericin were used to increase the permeability of endothelial cell membrane to K^+ (Pressman, 1976). Both agents amplified the response to ATP, with a minimal effect on PGI_2 release from unstimulated cells (Figure 2). The enhancement of the ATP-induced synthesis of PGI_2 was 3.8 fold in the presence of 5 μm valinomycin (range 2–8, 7 experiments) and 5.0 fold after exposure to 5 μM nigericin (range 3–8, 7 experiments). The response to bradykinin was amplified to the same degree as the effect of ATP by both valinomycin and nigericin (not shown). Exposure to valinomycin for 5 min before the addition of ATP was sufficient to obtain a maximal potentiation, whereas the effect of nigericin was time-dependent: it increased markedly when the preincubation with the ionophore was prolonged from 5 to 30 min (Figure 3a). Incubation in a medium containing 80 mM KCl abolished the enhancing action of nigericin, but had no effect on the potentiation induced by valinomycin (Figure 3b).

Nigericin produced a significant increase of the rate of ^{86}Rb efflux from prelabelled bovine aortic endothelial cells, whereas valinomycin had no effect (Figure 4). Cell K^+ did not change during an exposure to valinomycin (Figure 5), but fell progressively during incubation in presence of nigericin, reaching 31% of the control value after 5 min and 12% after 30 min (mean of 3 experiments, Figure 5). After a 40 min incubation with ouabain, the K^+ content of endothelial cells was decreased by 22% at 1 μM and by 35% at 100 μM (mean of 3 experiments: Figure 6a). In the same range of concentrations, ouabain amplified the stimulant effect of ATP on PGI_2 release by a factor of 2.2 (mean of 4 experiments: Figure 6b).

Discussion

The membrane potential of aortic endothelial cells is primarily determined by the permeability to K^+ (Colden-Stanfield *et al.*, 1987). Northover (1980) has shown that a 10 fold increase in K^+ concentration produces a (−) 20 mV reduction in membrane potential. Our data indicate therefore that depolarization of bovine aortic endothelial cells does not increase the basal output of PGI_2. This is consistent with the lack of functional voltage-dependent Ca^{2+} channels in these cells (Hallam & Pearson, 1986; Colden-Stanfield *et al.*, 1987). On the other hand the cellular response to ATP was not reduced in a high KCl medium, suggesting that the hyperpolarization induced by this agonist does not play an obligatory role in its stimulation of PGI_2 release.

Nigericin enhanced the release of PGI_2 from aortic endothelial cells stimulated by ATP or bradykinin. The abolition of this effect in a high KCl medium indicates that it is related to the endothelial

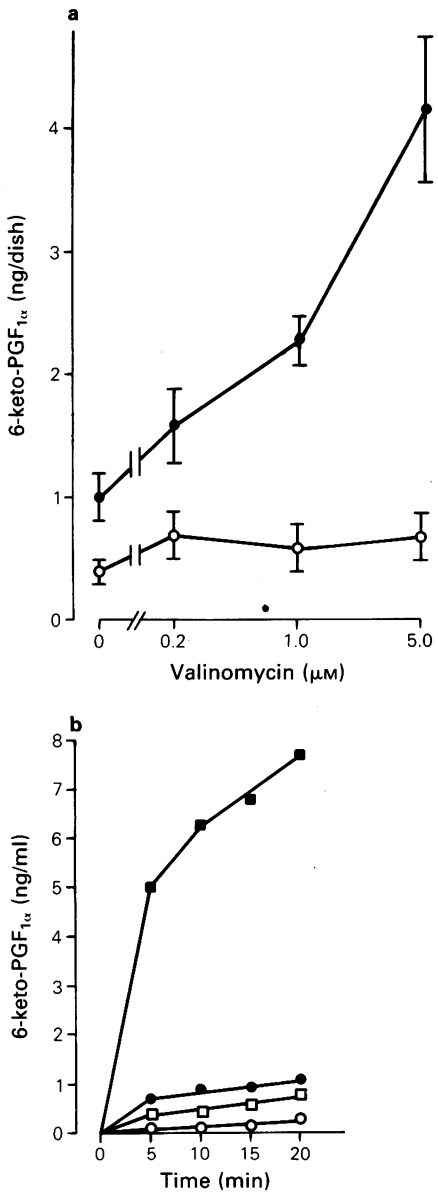

Figure 2 Effect of valinomycin and nigericin on the release of prostacyclin (PGI_2) from bovine aortic endothelial cells. (a) After removal of the culture medium, the cells were incubated in DMEM medium containing various concentrations of valinomycin. After 5 min, ATP (10 μM) was added and the incubation continued for 20 min. Results represent the mean amount 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) accumulated in the medium at the end of the incubation (mean of triplicate determinations in 1 representative experiment out of 3); vertical lines show s.d. (○) Control; (●) ATP (10 μM). (b) After removal of the culture medium, the cells were incubated for 30 min in Krebs-Ringer medium with no KCl and buffered at pH 7.1, with or without nigericin (5 μM). Then ATP (10 μM) was added and aliquots of the medium were collected every 5 min, for the measurement of 6-keto-$PGF_{1\alpha}$. Results represent the mean of triplicate determinations in 1 representative experiment out of 3. (○) Control, (●) ATP, (□) nigericin, (■) nigericin + ATP.

cell K^+ depletion produced by nigericin. Ouabain also decreased endothelial cell K^+ and enhanced the release of PGI_2 induced by ATP: both effects were smaller than those induced by nigericin. It is obviously impossible to determine if the enhancement of PGI_2 release is a direct consequence of the decreased cell K^+ concentration or results indirectly from a simultaneous increase in cell Na^+ content. Since the time course of ATP action was not modified by nigericin, it is unlikely that the amplification of PGI_2 release results from a decreased internalization of purinoceptors, as shown for angiotensin II receptors in K^+-depleted aortic smooth muscle cells (Delafontaine *et al.*, 1987). So far several biochemical consequences of K^+ depletion have been characterized: inhibition of protein and DNA synthesis (Ledbetter & Lubin, 1977; Lopez-Rivas *et al.*, 1982), arrest of coated pits formation resulting in a decreased receptor-mediated internalization of LDL, EGF and angiotensin II (Larkin *et al.*, 1983; 1986; Delafontaine *et al.*, 1987). According to Larkin *et al.* (1986), these cellular events induced by K^+ depletion constitute a pleiotropic response similar to the response to heat shock. The increased release of a prostaglandin in response to stimuli, observed in this study, might represent an additional component of this response. The significance of this observation is unclear, since it is not known whether cells in nature ever experience a severe depletion of intracellular K^+. Interestingly, Skaper & Varon (1981) have observed that freshly isolated root ganglia from the chick embryo lose more than 80% of their cell K^+ during a 6 h incubation without nerve growth factor.

Valinomycin amplified ATP-induced release of PGI_2 by a similar extent as nigericin. However, the mechanism of its action appears to be completely different: it was not associated with an increased efflux of ^{86}Rb or a decrease in cell K^+, and was maintained in a high KCl medium. This lack of effect on K^+ efflux from aortic endothelial cells is not surprising, as valinomycin exhibited a preference for the mitochondrial membrane over the plasma membrane in yeast cells (Kovac *et al.*, 1982), lymphocytes (Felber & Brand, 1982) and skeletal muscle (Kitazawa *et al.*, 1984). Despite these discrepancies, it is possible that the effects of ouabain, nigericin and valinomycin involve, as a common mechanism, an amplification of the Ca^{2+} transient induced by ATP.

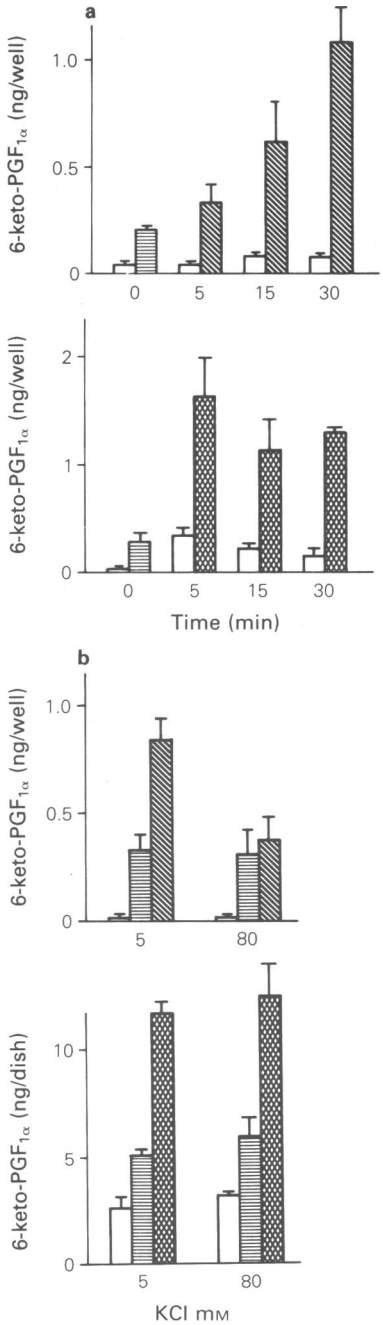

Figure 3 Enhancement by valinomycin and nigericin of the endothelial production of prostacyclin (PGI_2): (a) time-dependence and (b) sensitivity to K^+. (a) After removal of the culture medium, the cells were exposed for the indicated time to nigericin (5 μM) or valinomycin (5 μM). Thereafter ATP (10 μM) was added and the incubation was continued for 20 min. (b) After removal of the culture medium, the cells were incubated in Krebs-Ringer-HEPES medium containing either 5 or 80 mM KCl, and, as indicated, valinomycin (5 μM) or nigericin (5 μM). After 5 min (in the case of valinomycin) or 30 min (for the nigericin experiment), ATP (10 μM) was added and the incubation was continued for 20 min. Results represent the amount of 6-keto-prostaglandin $F_{1\alpha}$(6-keto-$PGF_{1\alpha}$) accumulated in the medium at the end of the experiment (mean, with vertical bars showing s.d., of triplicate determinations, in 1 representative experiment out of 3 for each column). Open columns, control; horizontally-hatched columns, ATP (10 μM); cross-hatched columns, valinomycin (5 μM) + ATP (10 μM); diagonally-hatched columns, nigericin (5 μM) + ATP (10 μM).

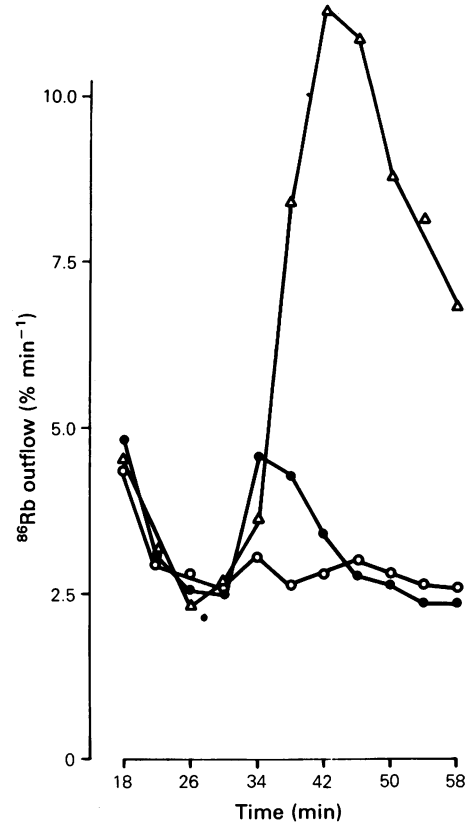

Figure 4 Comparison of the effects of 3 ionophores, A23187, valinomycin and nigericin, on the efflux of ^{86}Rb from prelabelled bovine aortic endothelial cells. Loading of the cells and measurement of ^{86}Rb efflux were performed as described in Methods. Valinomycin (5 μM, ○) and nigericin (5 μM, △) were present from 34 to 46 min; A23187 (5 μM, ●) from 30 to 38 min. Results represent the average efflux of ^{86}Rb per min, as % of the radioactivity remaining associated with the cells (mean of duplicate determinations in 1 representative experiment out of 3).

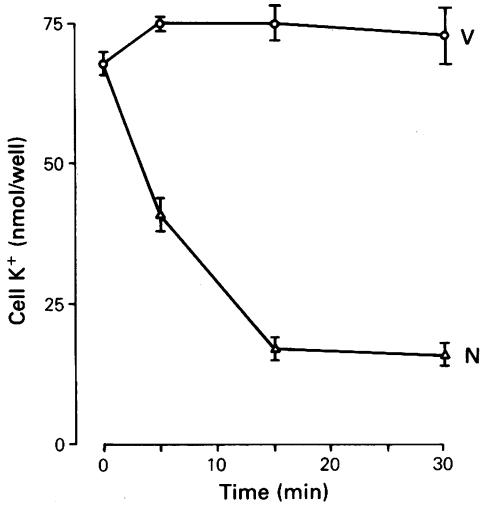

Figure 5 Effect of valinomycin (V) and nigericin (N) on the K^+ content of bovine aortic endothelial cells. After removal of the culture medium, the cells were incubated for 5, 15 or 30 min in DMEM containing valinomycin (5 μM, ○) or nigericin (5 μM, △). At the end of this incubation, they were rapidly washed with cold $MgCl_2$ and lysed with Triton, as described in Methods. K^+ was measured by flame photometry. Results represent the mean of triplicate determinations in 1 representative experiment out of 3. Vertical lines show s.d.

This amplification would result from an influx of Na^+, in the case of nigericin and ouabain, and of mitochondria uncoupling in the case of valinomycin. It has indeed been shown that ouabain and valinomycin increase the influx of ^{45}Ca, respectively into human umbilical vein endothelial cells (Nakagawa *et al.*, 1987) and rabbit coronary microvessel endothelial cells (Gerritsen *et al.*, 1987).

In conclusion, the endothelial production of PGI_2 and its stimulation by ATP are not modulated by modifications of the membrane potential, resulting from changes in the extracellular concentration of K^+. An acute depletion of intracellular K^+ by nigericin or ouabain enhanced the release of PGI_2 induced by ATP or bradykinin.

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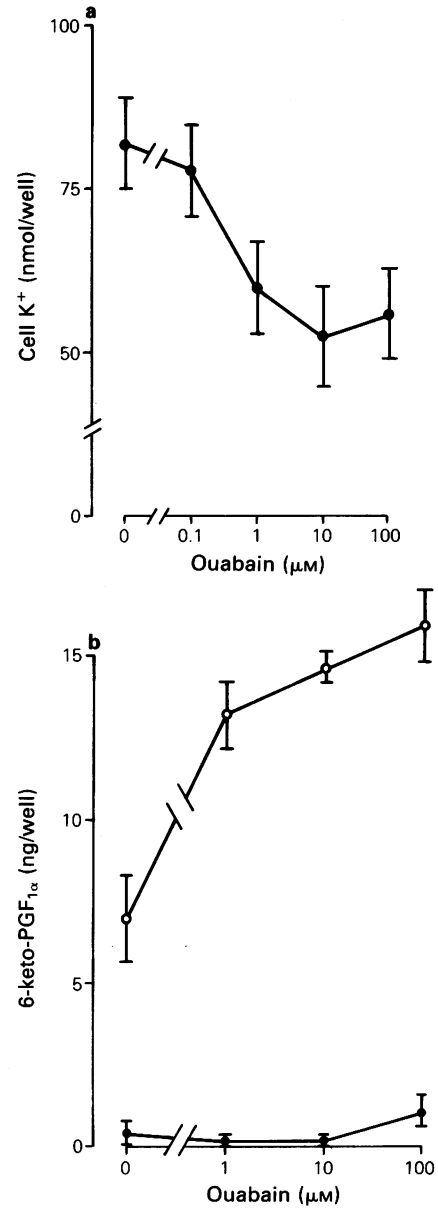

Figure 6 Effect of ouabain on the K^+ content (a) and production of prostacyclin (PGI_2) (b) of bovine aortic endothelial cells. After removal of the culture medium, the cells were incubated for 40 min in the presence of various concentrations of ouabain. They were then washed with cold $MgCl_2$, lysed with Triton and K^+ was measured by flame photometry (a). Alternatively, they were incubated for another 20 min, with (○) or without (●) ATP (10 μM) and 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) accumulated in the medium was measured (b). Results represent the mean of triplicate determinations in 1 representative experiment out of 3, for (a) and (b). Vertical lines show s.d.

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Effects of amiloride analogues on the production of prostacyclin by aortic endothelial cells

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1 The release of prostacyclin (PGI_2) from bovine aortic endothelial cells stimulated by adenosine 5'-triphosphate (ATP) was decreased by amiloride analogues bearing alkyl groups on the 5-amino nitrogen atom, like 5-(N-ethyl-N-isopropyl)amiloride (EIPA), which are inhibitors of the Na^+/H^+ exchanger. Analogues substituted on a terminal guanidino nitrogen atom were not inhibitory.

2 The release of PGI_2 induced by ATP was not significantly depressed in a Na^+-poor medium or in a medium acidified to pH 6.9, two conditions known to inhibit the Na^+/H^+ exchanger.

3 Cytoplasmic alkalinization by ammonium chloride did not suppress the inhibitory action of EIPA. By itself, ammonium chloride decreased the response of endothelial cells to ionophore A23187 and ATP, whereas sodium acetate had no effect.

4 EIPA did not decrease the mobilization of free arachidonic acid induced by ATP. It inhibited the conversion of exogenous arachidonate into PGI_2 and prostaglandin E_2 (PGE_2).

5 Although the intracellular pH was not measured in this study, it seems unlikely that cytoplasmic alkalinization via the activation of the Na^+/H^+ exchanger plays a significant role in the stimulatory action of ATP on the release of PGI_2 from endothelial cells. The inhibition of that release by EIPA and other amiloride analogues might involve a direct effect on cyclo-oxygenase, although an action on the reacylation of free arachidonic acid cannot be excluded.

Introduction

The cytoplasmic pH, which is important for many cellular processes, is tightly controlled (Madshus, 1988). In mammalian cells, this control involves two separate mechanisms, the relative importance of which differs from one cell type to the other: an amiloride-sensitive Na^+/H^+ exchanger and a Na^+-dependent Cl^-/HCO_3^- exchanger inhibited by stilbene disulphonic acids (L'Allemain *et al.*, 1985; Jeutsch *et al.*, 1985). Cell proliferation is the process whose dependency on cytoplasmic pH has been most studied in recent years. Optimal cell growth occurs at alkaline internal pH and several mitogens induce a cytoplasmic alkalinization, via the activation of the Na^+/H^+ exchanger (Moolenaar *et al.*, 1983; Pouyssegur *et al.*, 1984; Lucas *et al.*, 1988; Perona & Serrano, 1988). In contrast, it was recently suggested that cytosolic acidification might constitute an early transductory signal in neutrophil chemotaxis (Yuli & Oplatka, 1987) and in inhibitory synaptic transmission (Kaila & Voipio, 1987).

The role of cytoplasmic pH in the control of arachidonic acid metabolism has been thoroughly investigated in human platelets. It was shown that various procedures which inhibit the Na^+/H^+ exchanger decrease the mobilization of free arachidonic acid and the synthesis of thromboxane in response to adenosine 5'-diphosphate (ADP), adrenaline, low concentrations of thrombin and platelet-activating factor (Sweatt *et al.*, 1985; 1986; 1987). This suggests that changes in cytoplasmic pH and free calcium concentration might regulate phospholipase A_2 activity in a synergistic way.

Prostacyclin (PGI_2) is a potent inhibitor of platelet aggregation and *in vivo* its biosynthesis is increased in disease states involving the intravascular activation of platelets. This reflects a local compensatory response by the vascular endothelium (FitzGerald *et al.*, 1984; 1986). *In vitro*, the endothelial production of PGI_2 is indeed stimulated by factors released from activated platelets e.g. prostaglandin endoperoxides (Marcus *et al.*, 1980; Schafer *et al.*, 1984; Chesterman *et al.*, 1986) and adenine nucleotides, which act via P_{2y}-purinoceptors (Boeynaems & Galand, 1983; Pearson *et al.*, 1983; Van Coevorden & Boeynaems, 1984; Needham *et al.*, 1987). The biochemical events that mediate the

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action of adenine nucleotides on aortic endothelial cells have been recently characterized as involving generation of inositol trisphosphate, increase of cytosolic calcium and activation of calcium-dependent protein kinases (Pirotton *et al.*, 1987; Hallam & Pearson, 1986; Demolle *et al.*, 1988). In addition, Kitazono *et al.* (1988) have shown that adenosine 5'-triphosphate (ATP) and ADP induce a transient acidification followed by a sustained alkalinization, due to Na^+/H^+ exchanger activation, in bovine aortic endothelial cells.

The purpose of the present study was to investigate the possible role of cytoplasmic pH changes in the control by ATP of PGI_2 release from bovine aortic endothelial cells. Analogues of amiloride, some of which are specific inhibitors of the Na^+/N^+ exchanger (Vigne *et al.*, 1984; Simchowitz & Cragoe, 1986), were the main tools used in this study.

Methods

Preparation, culture and incubation of endothelial cells

Bovine aortic endothelial cells were obtained by collagenase digestion of the aorta excised from a freshly slaughtered cow, as previously described (Booyse *et al.*, 1975; Van Coevorden & Boeynaems, 1984). The cells were seeded on 100 mm Petri dishes and incubated at 37°C under an atmosphere of 5% CO_2/95% air in the following medium: MEM D-valine (80%, v/v), foetal calf serum (20%, v/v), 2 mM glutamine, 100 u ml^{-1} penicillin, 100 $\mu g\,ml^{-1}$ streptomycin, 2.5 $\mu g\,ml^{-1}$ amphotericin B. The medium was changed the following day and then every 3 days. After 4 or 5 days, the primary cultures formed confluent monolayers and could be sub-cultured. The cells were detached by a 5 min incubation in a Ca^{2+}- and Mg^{2+}-free Hanks buffer containing trypsin (0.1 $mg\,ml^{-1}$) and EDTA (1 mM). Thereafter, they were seeded on 35 mm Petri dishes and the culture was continued in the following medium: DMEM (60%, v/v), Ham's F-12 (20%, v/v), foetal calf serum (20%, v/v) with the same concentrations of penicillin, streptomycin, and amphotericin B as mentioned above.

Experiments were performed with confluent monolayers ($\pm 10^6$ cells per dish), between passage 2 and 5. The culture medium was removed and, after rinsing, the cells were incubated in 1 ml medium containing the tested agents, in general for 20 min. Amiloride analogues were dissolved in dimethyl sulphoxide (DMSO) and added to the cells 5 min before ATP and other stimuli. Control incubations contained the same amount of DMSO (0.5%, v/v). Incubation media of various compositions were used in this study: Dulbecco's MEM (DMEM); Krebs-Ringer medium buffered with HEPES (composition, mM, NaCl 124, KCl 5, $MgSO_4$ 1.25, $CaCl_2$ 1.45, KH_2PO_4 1.25, HEPES buffer 25, pH 7.4 and 8 mM glucose), Krebs-Ringer medium as above, except that sodium chloride was replaced by an equal concentration of choline chloride.

Prostaglandin radioimmunoassay (RIA)

The production of PGI_2 was measured by the RIA of its stable degradation product, prostaglandin 6-keto-$F_{1\alpha}$ (6-K-$PGF_{1\alpha}$), performed directly in the incubation medium, without extraction and chromatography. A rabbit antiserum was raised against 6-K-$PGF_{1\alpha}$ coupled to BSA, as described by Maclouf (1981). The limit of detection was 16 pg and the cross-reactivity was 1.2% with $PGF_{2\alpha}$, 0.3% with PGE_2 and $<0.1\%$ with thromboxane B_2. Aliquots (100 μl) of incubation media, [^{3}H]-6-K-$PGF_{1\alpha}$ (11,000 d.p.m.), anti-6-K-$PGF_{1\alpha}$ antiserum (final dilution 1:10,000) and bovine gamma globulins (2.5 $mg\,ml^{-1}$) in Tris buffer (50 mM, pH 7.4) were incubated in a total volume of 0.4 ml for 60 min at room temperature. Then 0.4 ml of a cold 25% (w/w) solution of polyethylene glycol was added to separate bound and free antigen. The various amiloride analogues tested did not interfere with the RIA of 6-K-$PGF_{1\alpha}$.

Assay of free arachidonic acid release

Arachidonic acid was measured by gas liquid chromatography (g.l.c.) with electron capture detection (ECD), as described previously (Boeynaems *et al.*, 1985). Incubations were performed in a medium containing BSA (1 $mg\,ml^{-1}$) and indomethacin (1 $\mu g\,ml^{-1}$). After the addition of 1 μg docosahexaenoic acid as an internal standard, the incubation medium was extracted with 1 volume of ethyl acetate. The free fatty acids were converted into pentafluorobenzyl esters by a modification of the method of Wickramasinghe *et al.* (1973). To the dry residue of the extract were added 290 μg pentafluorobenzylbromide (in 5 μl acetonitrile) and 44 μg diisopropylethylamine (in 5 μl acetonitrile). After 5 min at 40°C and evaporation of the solvent under nitrogen, the samples were redissolved in 50 μl hexane. The 20 fold reduction in the amounts of reagents used as compared with the original procedure made it possible to analyze the samples directly without prior purification. Analysis was performed in a Varian model 3700 chromatograph (Varian Associates, Palo Alto, CA, U.S.A.) equipped with a ^{63}Ni ECD and a 2 m column of 3% OV-1 on Gas chrom Q (Applied Science Laboratories) operated isothermally at 235°C.

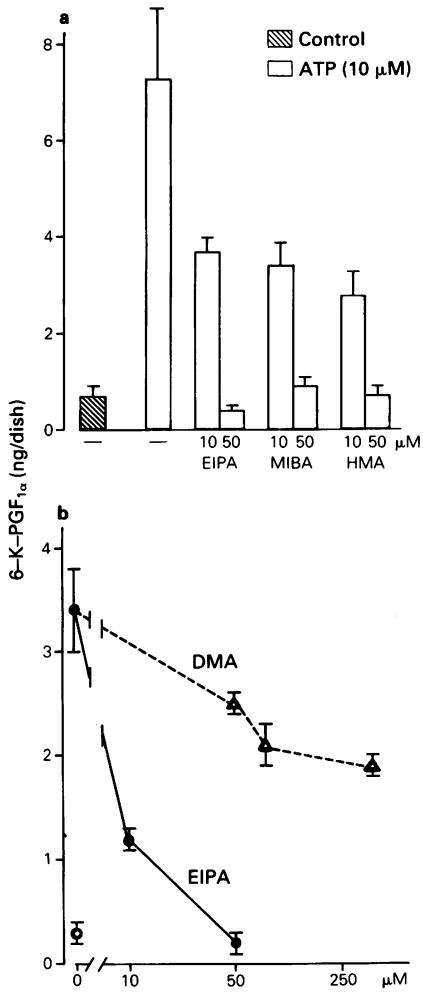

Figure 1 Effects of amiloride analogues, substituted by alkyl groups on the 5-amino nitrogen atom, on the release of prostacyclin (PGI_2) from bovine aortic endothelial cells stimulated by ATP. Incubation was performed in DMEM: the drugs were added to the cells 5 min prior to ATP (10 μM). Results represent the amount of 6-K-$PGF_{1\alpha}$ accumulated in the medium at the end of a 20 min incubation (mean ± s.d. (vertical bars) of triplicate determinations in 1 representative experiment out of 3 (a) or 2 (b)). (a) Hatched columns, control; open columns, ATP (10 μM). (b) (○) Control; (●) ATP (10 μM) + 5-(N-ethyl-N-isopropyl)amiloride (EIPA); (△) ATP (10 μM) + 5-(N,N-dimethyl)amiloride (DMA).

Metabolism of radiolabelled arachidonic acid

Confluent monolayers of bovine aortic endothelial cells were incubated in DMEM, with or without 5-(N-ethyl-N-isopropyl)-amiloride (50 μM). After 5 min, [^{3}H]-arachidonic acid (0.8 μCi ml^{-1}; 21 ng ml^{-1}) was added to all dishes and the incubation stopped after 60 min. The media were collected and 200 μl aliquots were directly analyzed by reversed-phase high performance liquid chromatography (r.p.-h.p.l.c.) on a C_{18} μBondapak column (3.9 × 300 mm, 10 μm particles; Waters Associates, Millipore Corp., Milford, MA, U.S.A.). The injector (model U6K) and the pump (model 600) were from Waters Associates. Elution was performed sequentially with acetonitrile-water-acetic acid (26:74:0.1, v/v/v; 40 min) and methanol (15 min) at a flow rate of 2 ml min^{-1}. Fractions of 2 ml volume were collected for liquid scintillation counting.

Table 1 Effect of 5-(N-ethyl-N-isopropyl)amiloride (EIPA) on the release of prostacyclin (PGI_2) from bovine aortic endothelial cells: comparison between ATP and ionophore A23187

	6-K-$PGF_{1\alpha}$ (ng per dish)	
—	0.5 ± 0.2	0.6 ± 0.3
ATP (10 μM)	3.3 ± 0.6	0.7 ± 0.2
A23187 (0.1 μM)	9.3 ± 2.1	3.1 ± 0.6
	—	EIPA (50 μM)

The cells were incubated for 20 min in DMEM containing the tested agents. EIPA was added 5 min before ATP or A23187. Results represent the amount of 6-K-$PGF_{1\alpha}$ accumulated in the medium at the end of the incubation (mean ± s.d. of triplicate determinations in 1 representative experiment out of 3).

Materials

All the amiloride analogues tested were prepared as previously published (Cragoe *et al.*, 1967; Cragoe & Woltersdorf, 1978).

DMEM, Ham's F-12, MEM-D-val, foetal calf serum, penicillin, streptomycin, amphotericin B and glutamine were from Gibco. Collagenase type I was from Cooper (Worthington). Trypsin was purchased from Flow Laboratories. Arachidonic acid and ATP were obtained from Sigma Chem. Co., ionophore A23187 from Boehringer-Mannheim, BSA (fatty acid-poor) from Calbiochem-Behring and 6-K-$PGF_{1\alpha}$ from Upjohn Diagnostics. [^{3}H]-arachidonic acid and [^{3}H]-6-K-$PGF_{1\alpha}$ were purchased from Amersham Corp.

Results

The presence of 50 μM 5-(N-ethyl-N-isopropyl)amiloride (EIPA) had no significant effect on the basal

Table 2 Effects of amiloride analogues, substituted by alkyl groups on the 5-amino nitrogen atom, on the release of prostacyclin (PGI_2) from bovine aortic endothelial cells stimulated by bradykinin

Drugs	*6-K-$PGF_{1\alpha}$* (ng per dish)	
—	0.2 ± 0.1	3.6 ± 0.4
EIPA (10 μM)		1.7 ± 0.6
EIPA (50 μM)		0.3 ± 0.1
MIBA (10 μM)		2.0 ± 0.2
MIBA (50 μM)		0.3 ± 0.1
HMA (10 μM)		1.0 ± 0.4
HMA (50 μM)		0.2 ± 0.1
	—	Bradykinin (0.1 μM)

Incubation was performed in DMEM, the drugs being added to the cells 5 min before bradykinin (0.1 μM). Results represent the amount of 6-K-$PGF_{1\alpha}$ accumulated in the medium at the end of a 20 min incubation (mean ± s.d. of triplicate determinations in 1 representative experiment out of 3).
EIPA = 5-(N-ethyl-N-isopropyl)amiloride;
MIBA = 5-(N-methyl-N-isobutyl)amiloride;
HMA = 5-(N,N-hexamethylene)amiloride.

release of PGI_2 from bovine aortic endothelial cells, with release of 103 ± 13% of the control (mean ± s.e.mean, $n = 13$). However, EIPA inhibited the stimulation of that release by ATP, in a concentration-dependent way (Figure 1). At 10 μM, EIPA produced a partial inhibition in some but not all experiments; at 50 μM, the release of PGI_2 in response to ATP (10 μM) was decreased to 19 ± 5% of the control value (mean ± s.e.mean, $n = 12$).

Likewise, the stimulatory effect of ionophore A23187 (0.1 μM) was decreased to a mean 39% of the control value ($n = 3$) by EIPA (50 μM) (Table 1). 5-(N-methyl-N-isobutyl)amiloride (MIBA) and 5-(N,N-hexamethylene)amiloride (HMA) produced the same degree of inhibition as EIPA (Figure 1a). These various compounds also inhibited the release of PGI_2 induced by bradykinin (Table 2). The ATP effect was only partially decreased by 5-(N,N-dimethyl)amiloride (DMA) at a concentration as high as 500 μM (Figure 1b), whereas amiloride itself did not produce a significant inhibition at 1 mM (116% of the control, mean of 3 experiments). 5-N-(4-chlorobenzyl)-2′,4′-dimethylbenzamil (CBDMB) slightly increased the basal release of PGI_2 and did not inhibit the response to ATP (Figure 2). 2′,4′-Dimethylbenzamil (DMB) itself increased the production of PGI_2, but this effect was not additive to that of ATP (Figure 2).

Replacement of sodium chloride by choline chloride in the incubation medium slightly decreased the release of PGI_2 induced by ATP (33% inhibition at

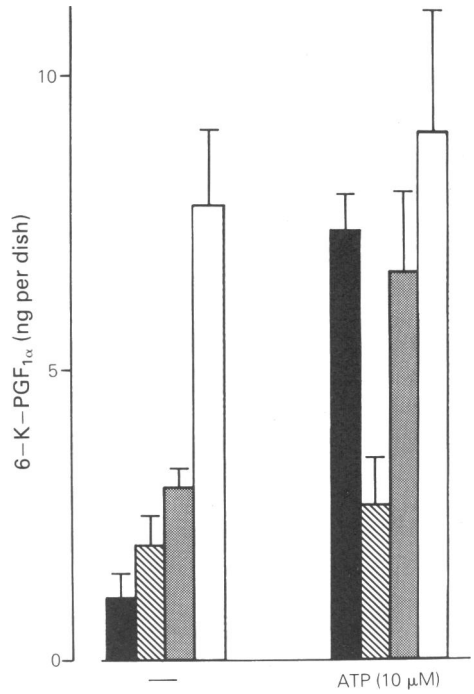

Figure 2 Effects of amiloride analogues on the release of prostacyclin (PGI_2) from bovine aortic endothelial cells and its stimulation by ATP: comparison between 5-(N-ethyl-N-isopropyl)amiloride (EIPA) and compounds bearing substituents on a terminal guanidino nitrogen. Incubation was performed in DMEM and the drugs were added to the cells 5 min before ATP (10 μM). Results represent the amount of 6-K-$PGF_{1\alpha}$ accumulated in the medium at the end of a 20 min incubation (mean ± s.d. (vertical bars) of triplicate determinations in 1 representative experiment out of 4): control; hatched columns, EIPA (50 μM); stippled columns, 5-N-(4-chlorobenzyl)-2′,4′-dimethylbenzamil (CBDMB) (50 μM); open columns, 2′4′-dimethylbenzamil (DMB) (50 μM).

10 μM ATP, $n = 4$). In a medium acidified to pH 6.9, the stimulatory effect of ATP (10 or 100 μM) was not decreased (118% of the control, $n = 3$), whereas the PGI_2 response to a low concentration of ATP (1 μM) was partially inhibited (77% of the control, $n = 3$) (Figure 3).

The inhibitory action of EIPA was not suppressed by the addition to the medium of ammonium chloride, which induces a cytoplasmic alkalinization (Figure 4). The action of EIPA at low concentration was not potentiated by incubating the endothelial cells in a Krebs-Ringer medium buffered with HEPES and containing no bicarbonate, nor by the substitution of choline chloride for sodium chloride in such medium (data not shown).

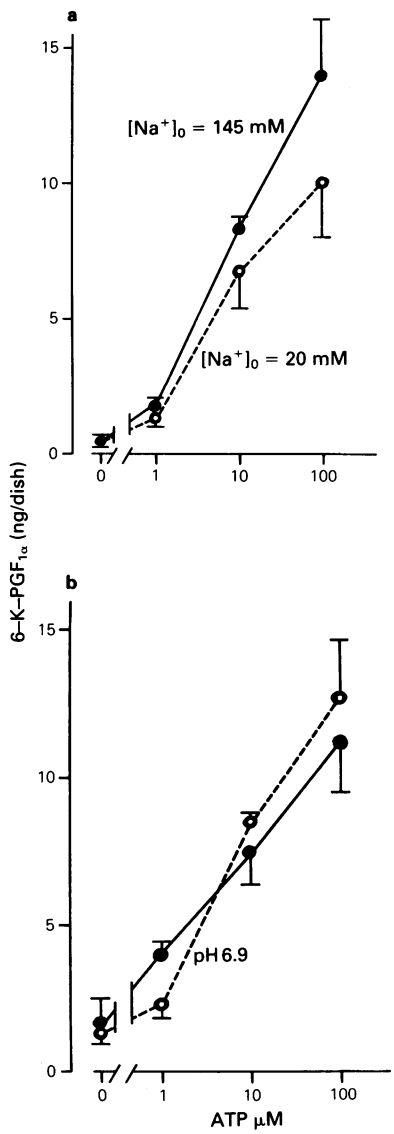

Figure 3 Effects of experimental conditions known to inhibit the activity of the Na^+/H^+ exchanger on the release of prostacyclin (PGI_2) from bovine aortic endothelial cells stimulated by ATP. The cells were incubated for 20 min in a medium containing various concentrations of ATP. This medium was either normal Krebs-Ringer-HEPES, or contained choline chloride instead of sodium chloride (a) or DMEM, normal or acidified to pH 6.9 by the addition of hydrochloric acid (b). Results represent the amount of 6-K-$PGF_{1\alpha}$ accumulated in the medium at the end of the incubation (mean ± s.d. (vertical bars) of triplicate determinations in 1 representative experiment out of 4 (a) or 3(b)). (a) (●) Normal medium; (○) Na^+-poor medium; (b) (●) normal medium; (○) medium acidified to pH 6.9.

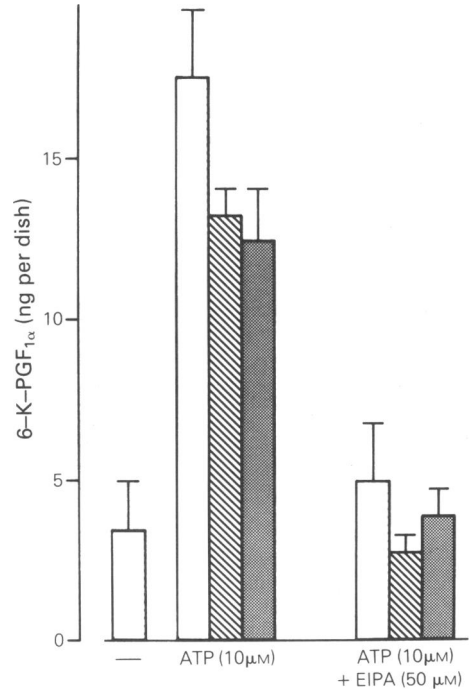

Figure 4 Inhibition by 5-(N-ethyl-N-isopropyl)amiloride (EIPA) of prostacylin (PGI_2) release from bovine aortic endothelial cells stimulated by ATP: insensitivity to cytoplasmic alkalinization by ammonium chloride. The cells were incubated for 20 min in DMEM containing various concentrations of ammonium chloride. EIPA (50 μM) was added 5 min before ATP (10 μM). Results represent the amount of 6-K-$PGF_{1\alpha}$ accumulated in the medium at the end of the incubation (mean ± s.d. (vertical bars) of triplicate determinations in one representative experiment out of 2). Open columns, control no NH_4Cl; hatched columns, NH_4Cl 5 mM; stippled columns, NH_4Cl 10 mM.

Cytoplasmic alkalinization by ammonium chloride partially inhibited the release of PGI_2 in response to ATP (63 ± 15% of the control, mean ± s.d. of 3 experiments: Figure 4) or ionophore A23187 (49 ± 12%, of the control, mean ± s.d. of 5 experiments: Figure 5). Acidification with sodium acetate had no significant effect on the response to either agonist (Figure 5).

In contrast to its inhibitory effect on the release of PGI_2, EIPA (50 μM) did not decrease the mobilization of free arachidonic acid induced by ATP, but had a slight potentiating effect (169% ± 27% of the control value, mean ± s.e.mean of 6 experiments: Figure 6). EIPA decreased the conversion of exogenous arachidonic acid into PGI_2 and PGE_2, evaluated either by RIA or by h.p.l.c. analysis of the

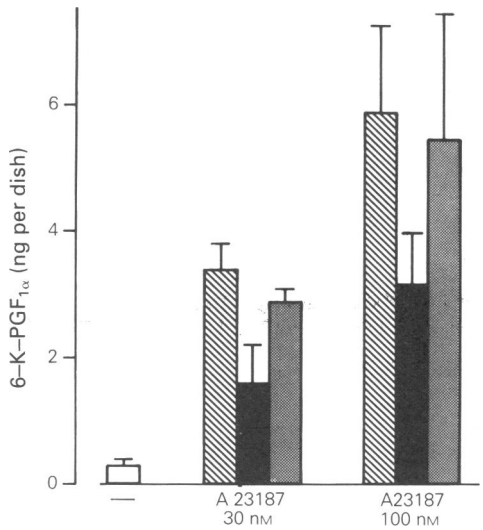

Figure 5 Modulation by cytoplasmic alkalinization or acidification of prostacylin (PGI_2) release from bovine aortic endothelial cells stimulated by ionophore A23187. The cells were incubated in DMEM, containing either ammonium chloride (solid columns), sodium acetate (stippled columns) or additional sodium chloride (hatched columns) as control (20 mM for each). Results represent the amount of 6-K-$PGF_{1\alpha}$ accumulated in the medium at the end of a 20 min incubation (mean ± s.d. (vertical bars) of triplicate determinations in one representative experiment out of 5).

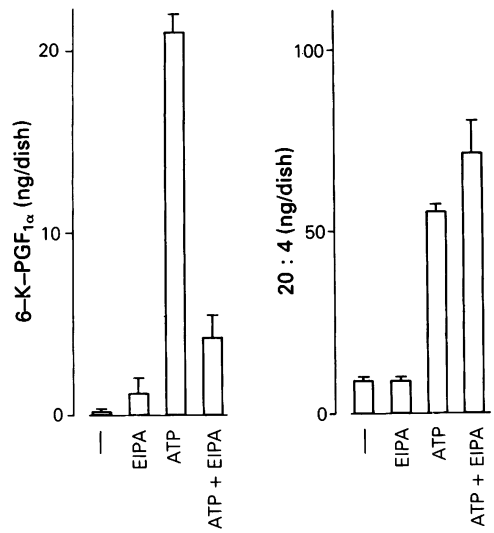

Figure 6 Comparison between the effects of 5-(N-ethyl-N-isopropyl)amiloride (EIPA) on prostacyclin (PGI_2) release and arachidonic acid mobilization in bovine aortic endothelial cells stimulated by ATP. EIPA (50 μM) was added 5 min before ATP (50 μM): the incubations were performed in Krebs-Ringer medium buffered with HEPES. Results represent the amounts of 6-K-$PGF_{1\alpha}$ or arachidonate (20:4) accumulated in the medium at the end of a 20 min incubation (mean ± s.d. (vertical bars) of triplicate determinations in one representative experiment out of 6).

incubation fluid, following the addition of radiolabelled substrate (Tables 3 and 4).

Discussion

The release of PGI_2 from bovine aortic endothelial cells stimulated by ATP was inhibited by amiloride analogues substituted by alkyl groups on the 5-amino nitrogen atom, which are well-known blockers of the Na^+/H^+ exchanger (Vigne *et al.*, 1984; Simchowitz & Cragoe, 1986). This inhibition was not mimicked by analogues bearing substitutents on a terminal guanidino nitrogen atom, which are inactive on the Na^+/H^+ exchanger, but inhibit the Na^+/Ca^{2+} exchanger (Kaczorowski *et al.*, 1985). DMB itself actually induced PGI_2 release.

Although the concentrations of amiloride analogues required to inhibit PGI_2 release were higher than those needed to inhibit the Na^+/H^+ exchanger in the bovine aortic endothelial cells (IC_{50} of HMA was less than 1 μM according to Kitazono *et al.*, 1988), this specificity would be consistent with a role of the Na^+/H^+ exchanger and of cytoplasmic alkalinization in the action of ATP on endothelial PGI_2 production. However several other arguments do not support this hypothesis. The ATP-induced release of PGI_2 was minimally affected by two other procedures classically used to inhibit the Na^+/H^+ exchanger: reduction of the Na^+ concentration or

Table 3 Effect of 5-(N-ethyl-N-isopropyl)amiloride (EIPA) on the conversion of exogenous arachidonic acid into prostacyclin (PGI_2) by bovine aortic endothelial cells

Arachidonic acid (μg ml^{-1})	6-*K-PGF*$_{1\alpha}$ (ng per dish)	
0	1.0 ± 0.2	0.5 ± 0.2
0.3	2.7 ± 0.6	1.3 ± 0.4
1.0	5.2 ± 1.1	2.9 ± 0.5
	—	EIPA (50 μM)

The cells were incubated for 20 min in DMEM containing different concentrations of arachidonic acid and EIPA; EIPA was added 5 min before arachidonic acid. Results represent the amount of 6-K-$PGF_{1\alpha}$ accumulated in the medium at the end of the incubation (mean ± s.d. of triplicate determinations in 1 representative experiment out of 4).

Table 4 Effect of 5-(N-ethyl-N-isopropyl)amiloride (EIPA) on the transformation of radiolabelled arachidonic acid by bovine aortic endothelial cells

	$^3H \times 10^{-3}$ (c.p.m.)	
6-K-$PGF_{1\alpha}$	32 ± 1	8 ± 1
PGE_2	33 ± 9	17 ± 1
Arachidonate	663 ± 103	790 ± 6
	—	EIPA (50 μM)

The cells were incubated in DMEM with or without EIPA, [^{3}H]-arachidonic acid (0.8 μCi ml^{-1}) was added 5 min later and the incubation was stopped after 60 min. The incubation media were analyzed by h.p.l.c. as described in Methods. Results represent the total radioactivity in the fractions where 6-K-$PGF_{1\alpha}$, PGE_2 and arachidonate were eluted (mean ± s.d. of triplicate determinations in 1 representative experiment out of 2).

the pH of the incubation medium. The inhibition by EIPA was not suppressed by ammonium chloride, which increases the cytoplasmic pH in bovine aortic endothelial cells (Kitazono *et al.*, 1988), independently from the Na^+/H^+ exchanger activity. The potency of EIPA was not increased in a medium with a reduced concentration of Na^+, which is known to compete with amiloride analogues for binding to the Na^+/H^+ exchanger. Kitazono *et al.* (1988) have shown that the action of ATP on the cytoplasmic pH is biphasic, with an initial acidification followed after 3 min by a lasting alkalinization. On the other hand, ATP induces a transient burst of PGI_2 release which lasts less than 5 min (Pearson *et al.*, 1983; Van Coevorden & Boeynaems, 1984); this release being coincident with the acidificiation phase and not with the alkalinization. Finally, we have observed that EIPA does not inhibit the mobilization of free arachidonic acid in ATP-stimulated endothelial cells and that it decreases the conversion of exogenous arachidonic acid into PGI_2 and PGE_2. These data suggest that EIPA might have a direct inhibitory effect on cyclo-oxygenase, although an effect on arachidonate reacylation into phospholipids cannot be excluded.

It is known that besides its three main targets, the epithelial Na^+ channel, the Na^+/H^+ exchanger and the Na^+/Ca^{2+} exchanger, amiloride inhibits protein synthesis (Lubin *et al.*, 1982), protein kinase C (Besterman *et al.*, 1985), the (Na^+, K^+) ATPase (Soltoff & Mandel, 1982) and the T calcium channel (Tang *et al.*, 1988). The inhibition of endothelial PGI_2 observed in this study might thus correspond to an additional side-effect of this class of drugs.

Sweatt *et al.* (1985, 1986, 1987) have shown that EIPA and related amiloride analogues, as well as the reduction of extracellular Na^+ concentration or pH, inhibit the activation of a phospholipase A_2 and the synthesis of thromboxane B_2, induced in human platelets by ADP, adrenaline, platelet-activating factor and low concentrations of thrombin. Dieter *et al.* (1987) made similar observations in rat Kuppfer cells stimulated by zymosan. Sweatt *et al.* (1986) proposed that changes in cytoplasmic pH and free calcium concentration might produce a synergistic activation of phospholipase A_2. Our results indicate that such a synergism does not operate in aortic endothelial cells.

Results described in this study are consistent with those of Ritter *et al.* (1987) who showed that amiloride analogues substituted on the 5-amino nitrogen inhibit the calcium-induced release of PGI_2 from rat aortic rings, while analogues bearing substitutents on the terminal nitrogen atom of the guanidino moiety were able to stimulate the basal production of PGI_2. Since the releases of PGI_2 and endothelial-dependent relaxing factor (EDRF) are often coupled and are both calcium-dependent, a stimulatory effect of this class of analogues on EDRF might be expected. Cocks *et al.* (1988) recently detected such a stimulation in canine coronary arteries. It is difficult to reconcile these last data with the observation that the same analogues inhibit the release of EDRF induced by acetylcholine or ionophore A23187 in the rat and rabbit aorta (Winquist *et al.*, 1985; Schoeffter & Miller, 1986).

In conclusion, cytoplasmic alkalinization via Na^+/H^+ exchanger activation does not appear to play a significant role in the stimulatory action of ATP on the release of PGI_2 from endothelial cells. The inhibition of that release by EIPA and other amiloride analogues might involve a direct effect on cyclo-oxygenase.

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Myenteric 5-HT-containing neurones activate the descending cholinergic excitatory pathway to the circular muscle of guinea-pig ileum

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1 Participation of myenteric 5-hydroxytryptamine (5-HT)-containing neurones in the ascending and descending pathways of the guinea-pig isolated ileum was investigated in a new preparation. Transmural electrical stimulation of the longitudinal muscle-myenteric plexus (LM-MP) portion of the preparation caused ascending and descending contractions of circular or longitudinal muscle in the attached, intact segments situated orally or anally to the point of stimulation

2 All contractions to LM-MP stimulation were abolished by tetrodotoxin (0.2 μM). The ascending and descending contractions of circular muscles were also abolished by atropine and inhibited to about 50% by hexamethonium. They were not affected by desensitization to substance P (SP) or by the SP antagonist, (D-Pro^2,D-Trp^{7,9})-substance P. The contractions of longitudinal muscles were inhibited by about 45% by hexamethonium and abolished by a combination of atropine with SP desensitization or the SP antagonist, (D-Pro^2,D-Trp^{7,9})-substance P.

3 Desensitization to 5-HT, ICS 205-930 (1 μM) or cocaine (1 μM) reduced the descending contraction of circular muscle by 80–90%, without significantly affecting the ascending contraction. Methysergide (0.2 μM) failed to alter either contraction.

4 5-HT desensitization, ICS 205–930 and cocaine only partially reduced the descending contraction of longitudinal muscle. A similar reduction of the ascending contraction (20–30%) was also observed. Methysergide had no effects on either contraction.

5 Contractions of either circular or longitudinal muscle produced by field stimulation of the intact segment were not significantly affected by any of the 5-HT receptor antagonists tested.

6 The results imply that 5-HT-containing neurones, as interneurones, are involved mainly in the descending cholinergic excitatory pathway to the circular muscles.

Introduction

5-Hydroxytryptamine (5-HT) has been proposed as a neurotransmitter in the enteric nervous system by Gershon *et al.* (1965). Some authors (Gaddum & Picarelli, 1957; Brownlee & Johnson, 1963; Drakontides & Gershon, 1968; Costa & Furness, 1979) have suggested that 5-HT acts on enteric ganglia, thereby activating neurones which release acetylcholine and cause contractions of the longitudinal and circular muscle layers. These suggestions imply that one role of 5-HT in the enteric nervous system is to serve as the transmitter of interneurones (Gershon, 1981).

The myenteric plexus of guinea-pig small intestine contains 5-HT-immunoreactive neurones that send their long processes in an anal direction to myenteric ganglia (Costa *et al.*, 1982; Furness & Costa, 1982). However, it is not clear whether such polar distributions of 5-HT-containing neurones participate in regulating motility of circular and/or longitudinal muscles of the small intestine and, if so, how they regulate the motility. The present experiments were therefore designed to study the involvement of myenteric 5-HT-containing neurones in excitatory pathway(s) of guinea-pig isolated ileum, by measuring the responses of the circular and longitudinal muscles situated orally and anally to a point of electrical stimulation of the myenteric plexus.

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Methods

Adult guinea-pigs of either sex weighing 250–400 g were used. They were stunned by a blow on the head and bled via the carotid arteries. Segments 3 cm long were taken from the ileum, excluding the most distal

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10 cm. The middle one-third of each segment was cut open longitudinally along its mesenteric border and pinned out flat in a dissection dish with the mucosa uppermost. The mucosa, submucosa and circular muscle layers of the mid-portion were successively stripped away, leaving the myenteric plexus with attached longitudinal muscle. In the resulting preparation, approximately one-third was intact at each of the oral and anal extremities, whereas the middle one-third constituted the exposed longitudinal muscle and myenteric plexus (LM-MP). As shown in Figure 1, the preparation was transversly or longitudinally suspended in an organ bath containing 15 ml Tyrode solution bubbled with a mixture of 95% O_2 and 5% CO_2 at 37 ± 1°C. The mechanical activity of the circular muscle of the intact segments was isotonically recorded under an imposed tension of 0.25 g from the preparation suspended transversely. Longitudinal muscle activity was recorded under 0.5 g of tension from the preparation suspended longitudinally. The Tyrode solution had the following composition (mM): NaCl 145, KCl 2.7, $CaCl_2$ 1.5, $MgCl_2$ 0.7, $NaHCO_3$ 4.8, Na_2HPO_4 0.3 and glucose 11.1.

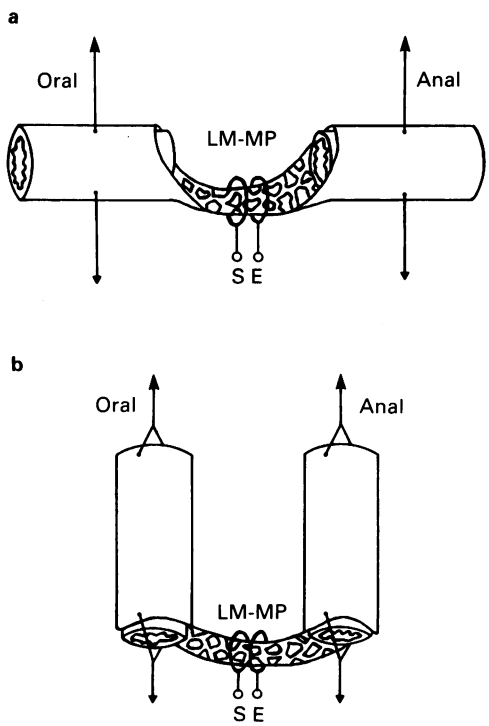

Figure 1 Schematic drawings of arrangements used for recording ascending and descending responses to electrical stimulation of myenteric plexus adhered on the longitudinal muscle (LM-MP), with stimulating electrodes (SE). (a) Recording from circular muscle of the intact ileal segment. (b) Recording from longitudinal muscle.

The exposed LM-MP was electrically stimulated via a pair of platinum ring electrodes (Figure 1). In some other experiments the intact segment of the ileum was directly stimulated by a pair of ring electrodes placed around the segment. Segments of ileum 1 cm long were used for recording from circular muscle and 3 cm long were used for recording from longitudinal muscle. The mechanical activity of the circular or longitudinal muscles was isotonically recorded as described above. Electrical stimulation of both LM-MP and the intact segments was carried out with rectangular pulses (1–50 Hz, 0.1–0.3 ms, 50–70 mA) for 15 s at 4.5 min intervals.

The ileum was desensitized to 5-HT by exposing it to 5 μM 5-HT. After a 4–8 min contact time, when the initial contraction had waned completely, the response to LM-MP stimulation was determined in the continuous presence of the desensitizing concentration of 5-HT. When the same dose of 5-HT was applied to the organ bath again, the contractile response was only 16.4 ± 4.6% of that on first administration ($n = 6$), while responses to histamine (0.3 μM) or nicotine (6 μM) were not reduced ($n = 6$), indicating specific desensitization to 5-HT. Desensitization to substance P was carried out with 0.2 μM of substance P and the same procedure described for 5-HT desensitization.

The following drugs were used: atropine sulphate (Tokyo-Kasei), hexamethonium bromide (Sigma), guanethidine sulphate (Tokyo Kasei Kogyo), tetrodotoxin (Sigma), histamine hydrochloride (Sigma), substance P (Peptide Institute), (D-Pro^2,D-$Trp^{7,9}$)-substance P (Peptide Institute), 5-hydroxytryptamine creatine sulphate (Sigma), ICS 205-930 (indole-3-carboxylic acid-tropine-ester hydrochloride, Sandoz), cocaine hydrochloride (Sigma) and methysergide hydrogen maleate (Sandoz). A stock solution of (D - Pro^2, D - $Trp^{7,9}$)-substance P (0.49 nM) was prepared with 0.01 M acetic acid and dilutions were made with fresh Tyrode solution; applied in volumes used for drug administration, the solvent had no effect on the responses to LM-MP stimulation. All other drugs were dissolved in 0.9% saline solution.

Quantitative data were presented as means ± s.e.mean. Statistical significance was determined by use of Student's t test for paired data. A probability of 0.05 or less was considered statistically significant.

Results

Transmural electrical field stimulation of the LM-MP at frequencies of 1, 2, 5, 10, 20 and 50 Hz

caused ascending and descending contractions of the circular or longitudinal muscle of the oral and anal intact ileal segments. Because the maximal contraction was obtained at a frequency of 20 Hz, electrical stimulation at this frequency was used in the following experiments. The contractions could be elicited repeatedly for 2 h or longer, without observing a significant diminution in amplitude provided the period of electrical stimulation was kept short (15 s), a 4.5 min rest period was allowed between contractions and the preparation was washed out after a series of stimulations. These responses were abolished by pretreatment with tetrodotoxin (0.2 μM) or by crushing the LM-MP between the intact segment and the stimulating electrodes.

Contractions of circular muscle evoked by LM-MP stimulation

As shown in Table 1, both the ascending and descending contractions of the circular muscles produced by LM-MP stimulation were abolished by atropine (1 μM) and reduced by hexamethonium (400 μM) to approximately half of the control ($n = 6$, $P < 0.001$ for each). Neither desensitization to substance P (SP, 0.2 μM) nor the addition of the SP antagonist, (D-Pro^2,D-Trp^{7,9})-substance P (20 μM) modified either contraction. On the other hand, after cholinergic transmission had been suppressed by atropine (1 μM) and the tone of the segment enhanced by histamine (1 μM), the LM-MP stimulation produced descending, but not ascending, relaxation which was not affected by guanethidine (5 μM) (results not shown).

High concentrations of 5-HT may desensitize its own receptors on the myenteric plexus and on the smooth muscle of the guinea-pig ileum (Brownlee & Johnson, 1963). In the presence of a desensitizing concentration of 5-HT (5 μM), the descending contraction was reduced to 19.8 ± 3.4% of the control ($n = 8$, $P < 0.001$), whereas 5-HT desensitization only slightly inhibited the ascending contraction to 90.3 ± 3.8% of the control ($n = 8$, not statistically significant) (Figure 2a).

ICS 205-930 (Table 1) decreased the descending contraction in a dose-related manner, without significantly affecting the ascending contraction. A typical inhibitory effect of ICS 205-930 (1 μM) is shown in Figure 2b. A similar effect was obtained with cocaine (1 μM) (Table 1 and Figure 2c).

It has been found that methysergide antagonizes only the 5-HT receptor on the smooth muscle of the guinea-pig ileum (Costa & Furness, 1979). Methysergide (0.2 μM) did not change the descending or the ascending contractions (Figure 2d), even when a concentration of 100 μM was used (results not shown).

Contraction of longitudinal muscle evoked by LM-MP stimulation

As shown in Table 1, the ascending and descending contractions of the longitudinal muscle to stimulation of the adjacent LM-MP were strongly diminished by atropine (1 μM). On the other hand, the ascending and descending contractions were partially reduced by SP desensitization or the SP antagonist, (D-Pro^2,D-Trp^{7,9})-substance P (20 μM) alone (Table 1). The combination of atropine (1 μM) and SP desensitization substantially reduced the ascending and descending contractions to 2.4 ± 0.9% ($n = 9$, $P < 0.001$) and 3.8 ± 1.4%

Table 1 The effect of drugs on the contractions evoked by LM-MP stimulation

		Circular muscle		*Longitudinal muscle*	
	Conc. (μM)	*Ascending*	*Descending*	*Ascending*	*Descending*
Control		100.0	100.0	100.0	100.0
Saline	0.15 ml	98.7 ± 1.3	102.3 ± 1.1 (5)	100.8 ± 0.9	99.1 ± 1.7 (4)
Atropine	1	2.5 ± 1.9***	1.1 ± 0.8*** (8)	30.5 ± 2.6***	11.4 ± 3.2*** (9)
Hexamethonium	10	55.4 ± 4.9***	49.9 ± 5.3*** (6)	47.4 ± 3.4***	45.4 ± 3.3*** (11)
	400	49.8 ± 5.1***	47.6 ± 5.6*** (5)	45.8 ± 4.5***	46.1 ± 6.1*** (5)
SP (desensitization)	0.2	105.9 ± 3.7	108.8 ± 4.1 (7)	61.4 ± 2.9***	54.7 ± 3.1*** (6)
(D-Pro^2,D-Trp^{7,9})-SP	20	92.7 ± 6.9	94.5 ± 5.7 (5)	79.9 ± 5.5*	81.2 ± 5.1* (5)
5-HT (desensitization)	5	90.3 ± 3.8	19.8 ± 3.4*** (8)	72.9 ± 5.0**	72.6 ± 6.2** (7)
ICS 205-930	0.01	105.4 ± 4.3	95.1 ± 2.2 (6)	106.9 ± 3.7	100.1 ± 3.3 (5)
	0.1	98.9 ± 3.7	66.4 ± 6.3** (6)	89.1 ± 5.8	90.1 ± 6.1 (6)
	1	91.1 ± 3.8	10.6 ± 2.5*** (7)	66.7 ± 5.9**	64.5 ± 6.3** (7)
Cocaine	1	89.8 ± 4.1	19.5 ± 4.1*** (6)	68.3 ± 6.1**	69.1 ± 5.5** (6)
Methysergide	0.2	103.4 ± 3.5	106.7 ± 4.9 (9)	107.3 ± 4.2	109.9 ± 7.8 (9)

Results shown are means ± s.e.mean. Significantly different from controls: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Number of preparations indicated in parentheses.

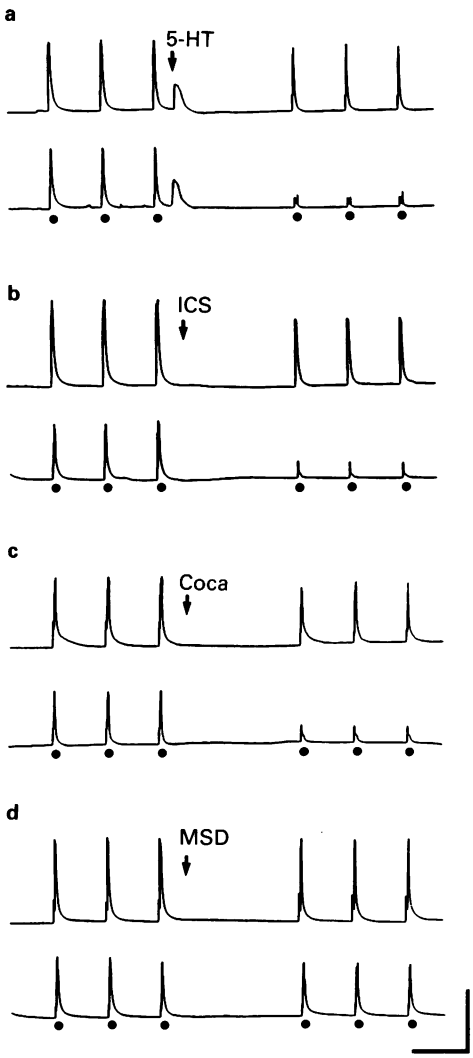

Figure 2 Effect of desensitization to 5-hydroxytryptamine (5-HT; 5 μM, a), ICS 205–930 (ICS; 1 μM, b), cocaine (Coca; 1 μM, c) and methysergide (MSD; 0.2 μM, d) on the ascending (upper traces) and descending responses (lower traces) to LM-MP stimulation (20 Hz, 0.1 ms, 50–70 mA, for 15 s). Vertical and horizontal lines represent 1 cm and 4 min, respectively.

($n = 9$, $P < 0.001$) of the control, respectively. Similar inhibitions were obtained with a combination of atropine (1 μM) and the SP antagonist, (D-Pro^2,D-Trp^{7,9})-substance P (20 μM), which reduced the ascending and descending contractions to $1.8 \pm 1.1\%$ ($n = 5$, $P < 0.001$) and $2.7 \pm 1.4\%$ ($n = 5$, $P < 0.001$) of the control. Hexamethonium (400 μM) reduced both the ascending and descending contractions to approximately half of the control (Table 1).

5-HT desensitization, ICS 205-930 and cocaine each produced only partial inhibitions of both the descending and ascending contractions. Methysergide failed to change either contraction (Table 1).

Effects of electrical stimulation of intact segments of the preparation

Transmural electrical stimulation of the intact ileal segment produced contractions of both circular and longitudinal muscles. The former were abolished by atropine (1 μM) and the latter by a combination of atropine and SP desensitization. Both contractions were unaffected by hexamethonium (400 μM).

As shown in Table 2, 5-HT desensitization, ICS 205-930 and cocaine each tended to reduce the contraction of both the circular and longitudinal muscles by about 10% of the control. Methysergide tended to increase both contractions. However, overall, these effects were not significant ($P > 0.05$, for each).

Discussion

Transmural electrical stimulation of the LM-MP caused both circular and longitudinal muscle contractions of adjacent intact ileal segments, situated orally and anally to the LM-MP. This preparation makes it possible to examine separately the neural pathways and neurotransmitters involved in the regulation of ascending and descending contractions of the circular and longitudinal muscles.

Since Bayliss & Starling (1899) formulated their law of the intestine, that mechanical stimuli applied to the wall of the intestine produce ascending excitation and descending inhibition, a great many experiments, using various methods, have confirmed this theory. However, no descending relaxation was obtained in the present study. These contrasting results may be attributable to differences in the methods of stimulation; in the present study, the entire mesh of the myenteric plexus was electrically stimulated. Therefore, the possibility that the descending inhibitory and descending excitatory pathways are activated simultaneously by electrical stimulation is increased. Yokoyama & Ozaki (1978) have suggested that, when both the descending pathways are electrically activated, the descending inhibition is normally masked by descending excitation. This suggestion may be supported by the findings of our previous study (Jin *et al.*, 1988) and those of the present experiments. Thus, the descending contraction, but not the ascending contraction, of either cir-

Table 2 The effect of drugs on contractions to electrical stimulation of the intact ileum

		Circular muscle	*Longitudinal muscle*
Control		100.0	100.0
Saline	0.15 ml	104.6 ± 3.9 ($n = 4$)	98.9 ± 3.4 ($n = 5$)
5-HT desensitization	5 μM	90.2 ± 4.3 ($n = 6$)	95.3 ± 3.7 ($n = 6$)
ICS 205-930	1 μM	87.9 ± 6.7 ($n = 6$)	91.7 ± 7.3 ($n = 5$)
Cocaine	1 μM	98.2 ± 2.2 ($n = 7$)	92.8 ± 4.7 ($n = 5$)
Methysergide	0.2 μM	109.3 ± 3.4 ($n = 5$)	112.2 ± 6.3 ($n = 6$)

Values are mean ± s.e.mean. n indicates the number of preparations used. $P > 0.05$, for each.

cular or longitudinal muscles, was converted to relaxation after suppression of cholinergic transmission by atropine and enhancement of the muscle tone by histamine.

Since tetrodotoxin prevents the generation of action potentials in the nerve but has no effect on the smooth muscle, it can be used to differentiate between non-neuronally-evoked responses of the smooth muscle and responses indirectly evoked by nerve stimulation (Kuriyama *et al.*, 1966; Bülbring & Tomita, 1967; Gershon, 1967). Thus, since all responses to LM-MP stimulation were abolished by tetrodotoxin, the responses must be mediated by excitation of neural elements. Both ascending and descending responses of the circular muscle to LM-MP stimulation were also abolished by atropine, so they appear to be cholinergically-mediated. On the other hand, both responses of the longitudinal muscle were reduced by atropine and then the residual portion was abolished by SP desensitization or by the SP antagonist, indicating that these responses are probably due to actions of cholinergic and SP-ergic neurones, as shown previously (Jin *et al.*, 1988).

5-HT receptors have been extensively studied by means of both radioligand binding techniques and physiological preparations. It is thought that there are three main types of 5-HT receptor, one of which, recently termed 5-HT_3-receptor, has clearly been identified in the peripheral nervous system by functional experiments (Fozard, 1984; Richardson *et al.*, 1985; Brittain *et al.*, 1987; Fake *et al.*, 1987; Ireland & Tyers, 1987; Sanger, 1987). Considerable data suggest that 5-HT_3-receptors are involved in the depolarization of peripheral neurones (Ireland & Tyers, 1987) and in increasing neurotransmitter release (Richardson *et al.*, 1985; Ireland & Tyers, 1987; Bradley *et al.*, 1986). ICS 205-930 has been shown to be a potent, selective 5-HT_3-receptor antagonist (Richardson & Engel, 1986). Cocaine is thought to be a non-selective 5-HT_3-receptor antagonist (Fozard *et al.*, 1979). The present findings with these compounds provide evidence that 5-HT_3-receptors are probably involved in the descending pathway to the circular muscle, but the possibility that other subtypes of receptor are involved (such as 5-HT_4: Dumuis *et al.*, 1988) cannot be ruled out. Indeed, ICS 205-930 (0.3–3.0 μM) has been shown to block receptors distinct from the 5-HT_3-subtype in the guinea-pig ileum (Craig & Clarke, 1989). Further studies are needed to clarify this point.

Immunohistochemical studies have revealed that 5-HT-like immunoreactive nerve cell bodies are present in the myenteric plexus of the guinea-pig ileum and send their long axons in an anal direction (Costa *et al.*, 1982; Furness & Costa, 1982). In accordance with this finding, the present results show that the descending contraction of the circular muscle was blocked by neuronal 5-HT receptor antagonists, which had no affect on the ascending one. Therefore, it is suggested that the long axons of the 5-HT-containing neurones are involved in the descending excitatory pathway which supply the circular muscle and that neuronal 5-HT receptors participate in this pathway.

However, the neuronal 5-HT receptor antagonists caused a small reduction of both the descending and ascending contractions of the longitudinal muscle, by approximately the same extent (about 20–30%). This finding implies that 5-HT-containing neurones with projecting anally long axons or orally short processes (Costa *et al.*, 1982; Furness & Costa, 1982) may be, at least partly, involved in both excitatory pathways. On the other hand, a non-specific inhibition, contributing to some extent, cannot be ruled out, since the neuronal 5-HT antagonists tended to reduce all contractile responses, except the descending one of circular muscle, by about 10% in the present study.

Physiological and immunohistochemical evidence strongly suggest that enteric 5-HT is contained in interneurones, i.e., 5-hydroxytryptaminergic neurones do not appear to form close contacts with the

smooth muscle but instead appear to act on other neurones (Bulbring & Gershon, 1976; Rothman *et al.*, 1976; Wood & Mayer, 1978; Costa & Furness, 1979; Erde *et al.*, 1985). Since the contractile responses to LM-MP stimulation were abolished by crushing the LM-MP between the intact segment and the electrode, the 5-HT cell bodies and/or the axons must be located in the stimulated LM-MP. Furthermore, all 5-HT receptor antagonists tested, as well as hexamethonium, failed to modify the contractile responses of the circular and longitudinal muscles to electrical stimulation of the intact segment; in this preparation the response may be produced mainly by activation of final neurones innervating smooth muscle. It is, therefore, reasonable to hypothesize that 5-HT is released from interneurones located in the myenteric ganglia and acts on cholinergic neurones and/or other neurones.

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Pharmacological characterization of 8-OH-DPAT-induced inhibition of rat hippocampal 5-HT release *in vivo* as measured by microdialysis

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1 We have previously found that the putative 5-HT_{1A} agonist 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) decreases hippocampal 5-hydroxytryptamine (5-HT) release in the anaesthetized rat, as measured by brain microdialysis. The present study attempted to characterize the receptor involved in this response using a range of monoamine receptor antagonists.

2 The classical 5-HT receptor antagonists, metergoline (5 mg kg^{-1} s.c.), methysergide (10 mg kg^{-1} s.c.) and methiothepin (10 mg kg^{-1} s.c.) each reduced dialysate levels of 5-HT which complicated their use as antagonists in these experiments. Nevertheless, pretreatment with metergoline but not methiothepin and methysergide partially reduced the 5-HT response to a maximally effective dose of 8-OH-DPAT (0.25 mg kg^{-1} s.c.).

3 The mixed 5-HT_1/β-adrenoceptor antagonist pindolol (8 mg kg^{-1} s.c.) was without effect on spontaneous 5-HT output but attenuated the effect of both maximally (0.25 mg kg^{-1} s.c.) and submaximally (0.05 mg kg^{-1} s.c.) effective dose of 8-OH-DPAT. In comparison, propranolol (10 mg kg^{-1} s.c.) did not affect 5-HT output when injected alone and did not alter the response to 8-OH-DPAT (0.25 mg kg^{-1} s.c.).

4 The 5-HT_2 receptor antagonist ritanserin (0.2 mg kg^{-1} s.c.) and the 5-HT_3 receptor antagonist BRL 43694 (0.5 mg kg^{-1} s.c.) neither altered 5-HT output alone nor significantly changed the response to 8-OH-DPAT (0.25 mg kg^{-1} s.c.).

5 The 8-OH-DPAT (0.25 mg kg^{-1} s.c.) response was not affected by pretreatment with either the dopamine D_2-receptor antagonist sulpiride (10 mg kg^{-1} s.c.) or the α_1/α_2-adrenoceptor antagonist phentolamine (10 mg kg^{-1} s.c.).

6 We conclude from these data that the decrease of hippocampal 5-HT output induced by 8-OH-DPAT does not involve 5-HT_2, 5-HT_3, adrenoceptors or dopamine D_2-receptors and that activation of a 5-HT_1 class of receptor seems probable. Full classification of the 8-OH-DPAT response awaits development of a suitably selective 5-HT_1 receptor antagonist with low intrinsic activity at the somatodendritic 5-HT autoreceptor.

Introduction

There is considerable evidence from neuropharmacological studies in animals that central 5-hydroxytryptamine (5-HT)-containing neurones are controlled by inhibitory 5-HT receptors localized both on nerve terminals in the forebrain and cell soma/dendrites in the mid-brain raphé nuclei. For example, it has been shown that exogenous 5-HT and direct acting 5-HT agonists both decrease the release of preloaded, radiolabelled 5-HT from rat brain tissue *in vitro* (Farnebo & Hamberger, 1974; Hamon *et al.*, 1974; Cerrito & Raiteri, 1979; Göthert & Weinheimer, 1979), and reduce the electrical activity of serotoninergic neurones when applied directly in the 5-HT somatodendritic area of the dorsal raphé nucleus *in vivo* (Haigler & Aghajanian, 1977; Vander Maelen *et al.*, 1986; Blier & De Montigny, 1987).

Current classification of the type of 5-HT receptor involved in 5-HT autoreceptor function relies to a large part on the association between the potencies of various 5-HT agonists in 5-HT autoreceptor

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models and their selectivity for various 5-HT receptor sub-types; 5-$HT_{1A, B, C, D,}$, 5-HT_2 and 5-HT_3. Thus, the rank-order of potency of 5-HT agonists at the nerve terminal 5-HT autoreceptor *in vitro* correlates best with the affinity of these drugs for the rat brain 5-HT_{1B} binding sites (Middlemiss, 1984a,b; Engel *et al.*, 1986). In contrast, inhibition of 5-HT cell-firing is observed following intra-raphé application of drugs with 5-HT_{1A} agonist activity, such as 8-OH-DPAT, but not those with 5-HT_{1B} agonist activity (Sprouse & Aghajanian, 1987; Sinton & Fallon, 1988). Accordingly, on the basis of such studies the somatodendritic and nerve terminal 5-HT autoreceptors are thought to be of the 5-HT_{1A} and 5-HT_{1B} subtypes, respectively.

Generally speaking, characterization of the 5-HT autoreceptor as being of the 5-HT_1 class is not well supported by studies with 5-HT antagonists. Despite having high affinity for the 5-HT_1 binding site in rat brain, the classical 5-HT antagonists metergoline and methysergide are weak or inactive as blockers of the nerve terminal 5-HT autoreceptor *in vitro*, although in this respect methiothepin is a potent antagonist (Martin & Sanders-Bush, 1982; Mounsey *et al.*, 1982; Moret, 1985). Certain β-adrenoceptor antagonists including cyanopindolol and propranolol, which bind with high affinity to brain 5-HT_1 sites, also act as nerve terminal 5-HT autoreceptor antagonists (Middlemiss, 1984c; Schlicker *et al.*, 1985). In comparison, whilst propranolol shows antagonistic activity towards the somatodendritic 5-HT autoreceptor (Sprouse & Aghajanian, 1986), metergoline, methysergide and methiothepin do not block the inhibition of serotoninergic cell firing rate by 5-HT receptor stimulation and indeed inhibit serotoninergic cell firing when administered alone (Haigler & Aghajanian, 1977).

Recently, we demonstrated that systemic administration of 8-OH-DPAT reduces 5-HT release in rat hippocampus *in vivo* as assessed by brain microdialysis (Sharp *et al.*, 1989a). Our proposal that this response was mediated via somatodendritic 5-HT_{1A} autoreceptors is directly supported by the finding that a similar effect is produced by injecting 8-OH-DPAT directly into the dorsal raphé nucleus (Sharp *et al.*, 1989b). It was therefore of interest to characterize the effect of 8-OH-DPAT on 5-HT release by the use of various monoamine receptor antagonists.

Methods

Brain microdialysis

The microdialysis methodology used in the present study is described in detail elsewhere (Sharp *et al.*, 1989a,b). Briefly, male Sprague Dawley rats (280–320 g) were anaesthetized with chloral hydrate (360 mg kg^{-1} i.p.) and a dialysis probe of the short-loop type was stereotaxically implanted into the ventral hippocampus (probe tip; rostral–caudal −4.8 mm, lateral −4.6 mm, ventral −8.5 mm, from bregma and dura surface according to Paxinos & Watson, 1982). Once secured in place with dental cement, the probe was continuously perfused (1 μl min^{-1}) with artificial CSF containing citalopram 1 μM. Every 20 min, perfusates were collected and injected immediately onto a high performance liquid chromatogram (h.p.l.c.) with electrochemical detection system (Sharp *et al.*, 1989b) for measurement of 5-HT content.

Experimental design

Dialysates were collected every 20 min until their 5-HT content was similar for 2–3 consecutive samples (typically 2–3 h post probe implantation), at which point either 8-OH-DPAT or a monoamine receptor antagonist was injected subcutaneously. For drug interaction experiments the control period was extended slightly and the antagonist was administered 30 min before injection of 8-OH-DPAT. Perfusates were collected for a further 2 h following 8-OH-DPAT or antagonist (when this was injected alone). Since several monoamine receptor antagonists were seen to decrease 5-HT output when injected alone, a maximally effective dose of 8-OH-DPAT (0.25 mg kg^{-1}; Sharp *et al.*, 1989a) was chosen for the interaction study.

The doses of methysergide, methiothepin, pindolol and propranolol were chosen on the basis of those previously shown to attenuate markedly 8-OH-DPAT-induced behaviour in rats (Tricklebank *et al.*, 1984). Similarly, on the evidence of earlier reports the doses of ritanserin (e.g. Goodwin & Green, 1985), BRL 43694 (Fake *et al.*, 1987) phentolamine (Doxey *et al.*, 1983) and sulpiride (Zetterström *et al.*, 1986) that were used would be expected to inhibit effectively *in vivo* 5-HT_2, 5-HT_3, α-adrenoceptors and dopamine D_2-receptors, respectively.

Data analysis

5-HT in dialysates is expressed as a percentage of the absolute amount of 5-HT contained in the dialysate collected immediately before administration of 8-OH-DPAT or, when injected alone, the monoamine antagonists. Areas under the curves (AUC) were calculated and statistical comparisons were made between groups on log transformed AUC data using Student's unpaired t test following one way ANOVA. A P value of less than 0.05 was considered to be statistically significant.

Drugs

Drugs from the following sources were used: 8-OH-DPAT (8-hydroxy-2-(di-n-propylamino) tetralin HBr., Research Biochemical Inc., SEMAT, St. Albans, U.K.), ritanserin (Janssen Pharmaceutical Ltd, Wantage, U.K.), BRL 43694 (endo-N-(9-methyl-9-azabicyclo [3,3,1]non-3-yl)-1-methyl-indazole-3-carboxamide HCl, Beecham Pharmaceuticals, Harlow, U.K.), metergoline (Farmitalia, Milan), methiothepin maleate (Roche, Welwyn Garden City, U.K.), methysergide maleate (Sandoz Pharmaceuticals, Feltham, U.K.), (±)-propranolol HCl (ICI Pharmaceuticals, Macclesfield, U.K.), (±)-pindolol HCl (Sandoz Pharmaceuticals, Feltham, U.K.), (±)-sulpiride (Sigma) and phentolamine mesylate (Ciba, Rogitine). Drugs were dissolved in saline (8-OH-DPAT, BRL 43694, propranolol) or alternatively a minimum amount of glacial acetic acid, then made up to volume with 5% glucose solution, and were injected subcutaneously in a volume of $1\,ml\,kg^{-1}$.

Results

Effect of 5-HT receptor antagonists on spontaneous 5-HT output in hippocampus of the anaesthetized rat

Administration of the classical 5-HT receptor antagonists methiothepin ($10\,mg\,kg^{-1}$), methysergide ($10\,mg\,kg^{-1}$) and metergoline ($5\,mg\,kg^{-1}$) reduced hippocampal dialysate levels of 5-HT over the 2 h postdrug period in comparison to saline injected controls ($P < 0.05$ in each case; Figure 1a). This effect was most pronounced for $10\,mg\,kg^{-1}$ methiothepin which decreased 5-HT levels by about 50% within 60 min of injection.

In comparison, the mixed 5-HT_1/β-adrenoceptor antagonist drugs pindolol ($8\,mg\,kg^{-1}$) and propranolol ($10\,mg\,kg^{-1}$) did not affect dialysate levels of 5-HT (Figure 1b, and 5a). Similarly, the 5-HT_2 antagonist ritanserin ($0.2\,mg\,kg^{-1}$) and the 5-HT_3 antagonist BRL 43694 ($0.5\,mg\,kg^{-1}$) had no consistent effect on basal 5-HT output (Figure 1b).

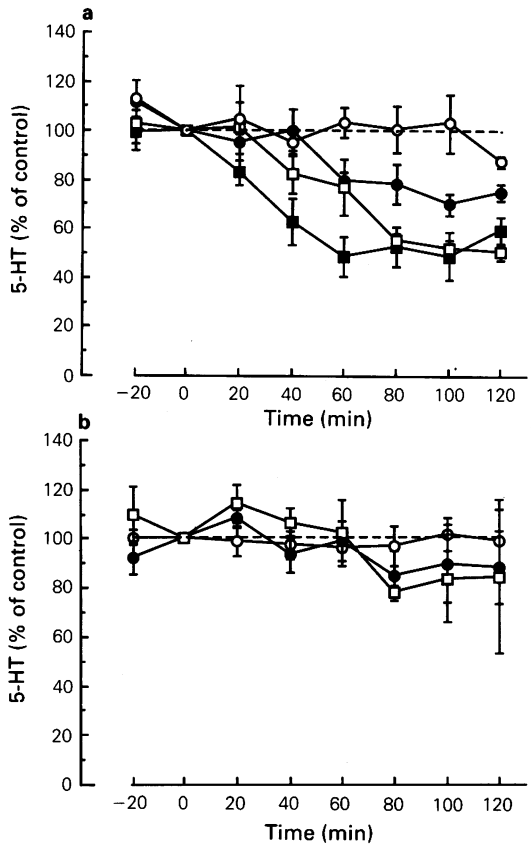

Figure 1 Effect of various 5-HT receptor antagonists on 5-hydroxytryptamine (5-HT) in ventral hippocampus dialysates of the anaesthetized rat. Drugs were injected subcutaneously at $t = 0$. (a) Saline (○, $n = 6$); metergoline $5\,mg\,kg^{-1}$ (●, $n = 4$); methysergide $10\,mg\,kg^{-1}$ (□, $n = 3$); methiothepin $10\,mg\,kg^{-1}$ (■, $n = 5$). (b) Pindolol $8\,mg\,kg^{-1}$ (○, $n = 3$), ritanserin $0.2\,mg\,kg^{-1}$ (□, $n = 3$), BRL 43694 $0.5\,mg\,kg^{-1}$ (●, $n = 3$). Mean values are shown with s.e.mean indicated by vertical bars. Each of metergoline, methysergide and methiothepin had a statistically significant effect on 5-HT compared to saline ($P < 0.05$).

Effect of metergoline, methysergide and methiothepin on the decrease of hippocampal 5-HT output induced by 8-OH-DPAT

Pretreatment with metergoline ($5\,mg\,kg^{-1}$) weakly attenuated the marked decrease of 5-HT output induced by $0.25\,mg\,kg^{-1}$ 8-OH-DPAT when compared to the effect of 8-OH-DPAT alone ($P < 0.05$; Figure 2a). A higher dose of metergoline, $10\,mg\,kg^{-1}$, also partially prevented ($P < 0.05$) the inhibitory response of 8-OH-DPAT ($0.25\,mg\,kg^{-1}$) although the effect was not greater than that observed with $5\,mg\,kg^{-1}$ ($n = 4$ rats, data not shown). Similarly $20\,mg\,kg^{-1}$ metergoline was no more effective than 5 or $10\,mg\,kg^{-1}$ of the drug ($n = 2$ rats, data not shown).

The finding that both methiothepin and methysergide induced a marked decrease in 5-HT output complicated an examination of their 8-OH-DPAT antagonist activity. Furthermore, a pilot dose-response study on methiothepin suggested that doses of the drug which would be without effect on 5-HT

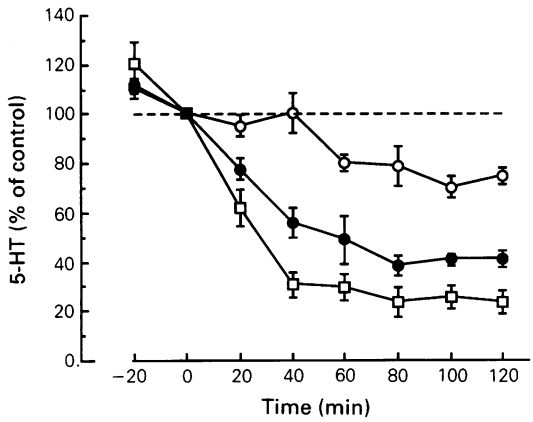

Figure 2 Effect of pretreatment with 5 mg kg^{-1} metergoline on the reduction of hippocampal 5-HT output induced by 0.25 mg kg^{-1} 8-OH-DPAT. Metergoline (○, $n = 4$) and 8-OH-DPAT (□, $n = 5$) were injected alone at $t = 0$. For combination (●, $n = 5$), metergoline was injected 30 min before 8-OH-DPAT ($t = 0$). Mean values are shown with s.e.mean indicated by vertical bars. The effect of metergoline plus 8-OH-DPAT is less than that of 8-OH-DPAT alone ($P < 0.05$).

output (<0.05 mg kg^{-1}) would have questionable 5-HT antagonist activity. Nevertheless it is clear from the data in Table 1 that a dose of methiothepin and methysergide which alone reduced 5-HT output by around 50% did not prevent an additional effect of 8-OH-DPAT.

Effect of pindolol and propranolol on the decrease of hippocampal 5-HT output induced by 8-OH-DPAT

Pretreatment with 8 mg kg^{-1} pindolol attenuated the decrease of 5-HT output induced by 0.25 mg kg^{-1} 8-OH-DPAT (Figure 3a, $P < 0.01$). In comparison, pindolol (8 mg kg^{-1}) also reduced ($P < 0.05$) the 5-HT response to a submaximally effective dose of 8-OH-DPAT (0.05 mg kg^{-1}) although the degree of inhibition was not clearly greater. A higher dose of pindolol (20 mg kg^{-1}) was no more effective in blocking the 5-HT response to 0.25 mg kg^{-1} 8-OH-DPAT than 8 mg kg^{-1} pindolol ($n = 2$, data not shown). Propranolol (10 mg kg^{-1}) did not alter the decrease in 5-HT induced by 0.25 mg kg^{-1} 8-OH-DPAT (Figure 5a).

Effect of ritanserin and BRL 43694 on the decrease of hippocampal 5-HT output induced by 8-OH-DPAT

As shown in Figure 4, pretreatment with ritanserin (0.2 mg kg^{-1}) or BRL 43694 (0.5 mg kg^{-1}) had no clear effect on the decrease of 5-HT output induced by 8-OH-DPAT (0.25 mg kg^{-1}).

Effect of phentolamine and sulpiride on the decrease of hippocampal 5-HT output induced by 8-OH-DPAT

Neither the α_1/α_2-adrenoceptor antagonist phentolamine (10 mg kg^{-1}), nor the dopamine D_2-antagonist sulpiride (10 mg kg^{-1}), altered basal 5-HT output.

Table 1 Effect of methiothepin and methysergide on 8-hydroxy-2(di-n-propylamino)tetralin (8-OH-DPAT)-induced decrease of 5-hydroxytryptamine (5-HT) in rat hippocampal dialysates

Treatment[a] (mg kg^{-1})	*Baseline level of 5-HT immediately before treatment* (pmol per 20 μl dialysate)	*Level of 5-HT*[b] *2 h after treatment* (pmol per 20 μl dialysate)
8-OH-DPAT (0.25)	0.063 ± 0.008 (5)[c]	0.017 ± 0.004 (5) [−73.8 ± 4.5%][d]
Methiothepin (1)	0.057 ± 0.002 (5)	0.025 ± 0.007 (5) [−56.8 ± 11.3%]
8-OH-DPAT (0.25) + methiothepin (1)	0.048 ± 0.010 (5)	0.014 ± 0.004 (5) [−70.8 ± 3.0%]
Methysergide (10)	0.081 ± 0.01 (3)	0.041 ± 0.005 (3) [−49.3 ± 3.5%]
8-OH-DPAT (0.25) + methysergide (10)	0.049 ± 0.007 (5)	0.013 ± 0.001 (5) [−73.4 ± 2.2%]

[a] 8-OH-DPAT injected 30 min after methiothepin or methysergide.
[b] In all cases this was close to maximum change during the 2 h post treatment period.
[c] Mean ± s.e.mean (*n*).
[d] % reduction from pretreatment value.

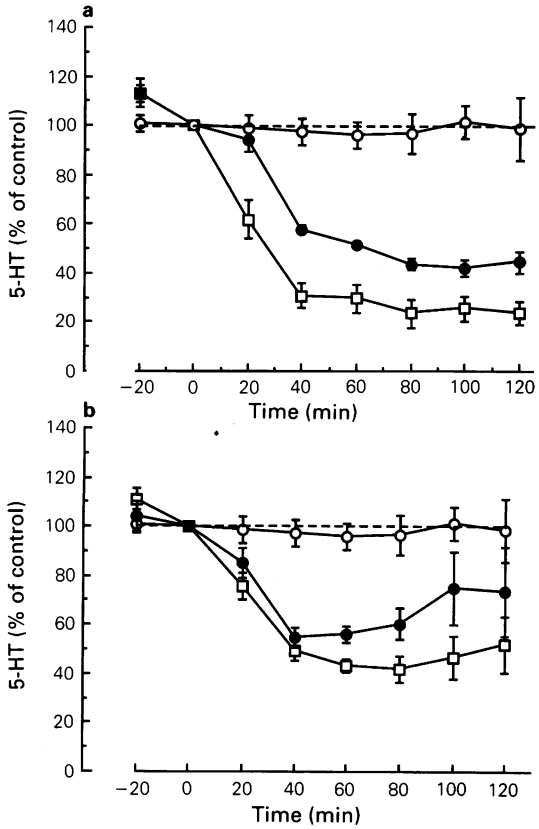

Figure 3 Effect of pretreatment with 8 mg kg^{-1} pindolol on the reduction of hippocampal 5-HT output induced by 8-OH-DPAT 0.25 mg kg^{-1} (a) and 0.05 mg kg^{-1} (b). Pindolol (○, $n = 3$) and 8-OH-DPAT (□, $n = 5$) were injected alone at $t = 0$. For combination (●, $n = 6$), pindolol was injected 30 min before 8-OH-DPAT ($t = 0$). Mean values are shown with s.e.mean indicated by vertical bars. The effect of pindolol plus 8-OH-DPAT is less than that of 8-OH-DPAT alone in both (a) ($P < 0.01$) and (b) ($P < 0.05$).

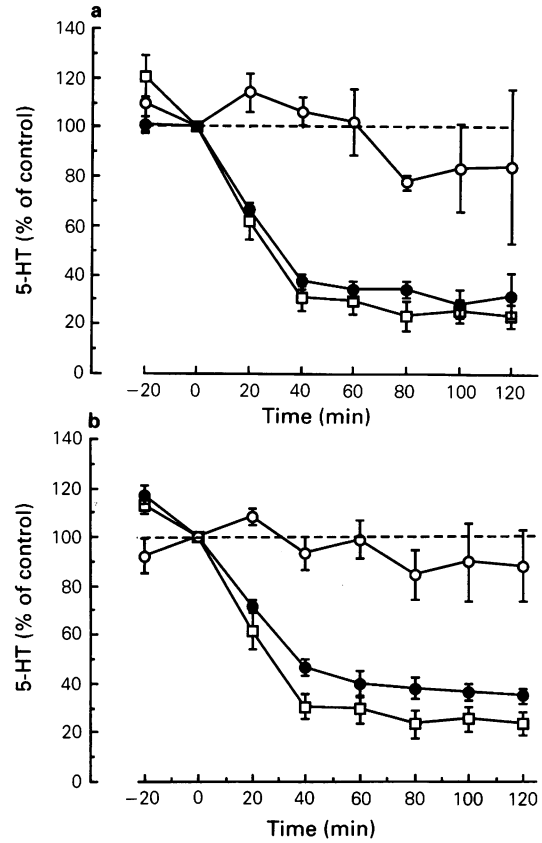

Figure 4 Effect of pretreatment with ritanserin 0.2 mg kg^{-1} (a) and BRL 43694 0.5 mg kg^{-1} (b) on reduction of 5-HT output induced by 8-OH-DPAT 0.25 mg kg^{-1}. Antagonist (○, $n = 3$) and 8-OH-DPAT (□, $n = 5$) were injected alone at $t = 0$. For combination (●, $n = 4$ ritanserin, $n = 5$ BRL 43694), antagonist was injected 30 min before 8-OH-DPAT ($t = 0$). Mean values are shown with s.e.mean indicated by vertical bars. Neither ritanserin nor BRL 43694 had a statistically significant effect on the 5-HT response to 8-OH-DPAT.

Pretreatment with either phentolamine (Figure 5) or sulpiride ($n = 4$ rats, data not shown) did not alter the 5-HT response to 8-OH-DPAT.

Discussion

We have previously shown that 8-OH-DPAT causes a marked reduction of 5-HT release in ventral hippocampus of the anaesthetized rat, as assessed by brain microdialysis (Sharp *et al.*, 1989a). For several reasons it seems likely that this effect is related to an inhibition of serotoninergic impulse flow following a direct action of the drug on somatodendritic 5-HT$_{1A}$ autoreceptors. Firstly, 8-OH-DPAT is a highly selective ligand for brain 5-HT$_{1A}$ binding sites (Middlemiss & Fozard, 1983; Hoyer 1988) which are in high concentrations on 5-HT neurones in the rat dorsal raphé nucleus (Verge *et al.*, 1985; Weissman-Nanopoulos *et al.*, 1985). Also, application of 8-OH-DPAT directly into the rat dorsal raphé nucleus causes inhibition of serotoninergic cell firing (Blier &

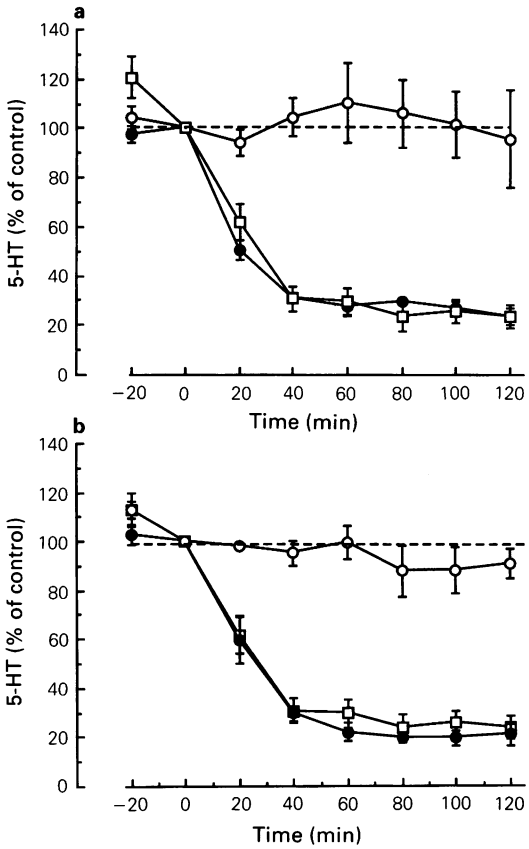

Figure 5 Effect of pretreatment with propranolol 10 mg kg^{-1} (a) and phentolamine 10 mg kg^{-1} (b) on reduction of 5-HT output induced by 8-OH-DPAT 0.25 mg kg^{-1}. Antagonist (○, $n = 3$) and 8-OH-DPAT (□, $n = 5$) were injected alone at $t = 0$. For combination (●, $n = 4$), antagonist was injected 30 min before 8-OH-DPAT ($t = 0$). Mean values are shown with s.e.mean indicated by vertical bars. Neither propranolol nor phentolamine had a statistically significant effect on the 5-HT response to 8-OH-DPAT.

De Montigny, 1987; Sprouse & Aghajanian, 1987), a reduction of regional brain 5-HT synthesis (Hjorth & Magnusson, 1989) and a fall of 5-HT output in ventral hippocampus *in vivo* (Sharp *et al.*, 1989b). Finally, 8-OH-DPAT does not decrease 5-HT release when locally applied to 5-HT nerve terminals *in vitro* (Middlemiss, 1984a; Engel *et al.*, 1986) or *in vivo* (10^{-5} M 8-OH-DPAT in microdialysis perfusion medium, unpublished observations).

The present study attempted to characterize pharmacologically the inhibitory effect of systemically administered 8-OH-DPAT on 5-HT output by use of various monoamine receptor antagonists. The main findings of the study (see Table 2) were; (i) of the three classical 5-HT receptor antagonists tested, metergoline, methysergide and methiothepin, each of which reduced 5-HT output when injected alone, only metergoline inhibited the response to 8-OH-DPAT, albeit weakly. (ii) Of the two β-adrenoceptor blockers pindolol and propranolol, both of which have high affinity for the 5-HT$_{1A}$ binding site (Hoyer, 1988), only the former drug attenuated the response to 8-OH-DPAT. This effect was weak and not clearly improved by reducing the dose of the agonist or increasing the dose of antagonist. (iii) The 5-HT response to administration of 8-OH-DPAT was not altered by pretreatment with ritanserin (5-HT$_2$ receptor antagonist), BRL 43694 (5-HT$_3$), phentolamine (α_1/α_2) or sulpiride (D_2).

The above findings, together with our previous observations, show that the receptor mediating the 8-OH-DPAT-induced reduction in hippocampal 5-HT output has some resemblance to the somatodendritic 5-HT autoreceptor characterized by electrophysiological studies. Not only is the autoreceptor modulating *in vivo* 5-HT release and serotoninergic cell-firing sensitive to 8-OH-DPAT and other putative 5-HT$_{1A}$ agonists such as gepirone, ipsapirone and buspirone (Vander Maelen *et al.*, 1986; Blier & De Montigny, 1987; Sprouse & Aghajanian 1987; Sharp *et al.*, 1989a) but methiothepin, methysergide and metergoline are poor antagonists (this study; Haigler & Aghajanian, 1977). Indeed the latter drugs reduce both 5-HT output and serotoninergic cell-firing in the anaesthetized rat (this study; Haigler & Aghajanian, 1977).

While propranolol is reported to block the inhibitory action of 8-OH-DPAT on the electrophysiologically identified somatodendritic 5-HT autoreceptor (Sprouse & Aghajanian, 1986), in this study pindolol but not propranolol attenuated the 5-HT release response to 8-OH-DPAT. Part of the reason that propranolol was inactive in this study may lie in the fact that we administered the drug systemically rather than directly onto the dorsal raphé nucleus (as done by Sprouse and Aghajanian). In this respect it is interesting that Adrien *et al.* (1989) recently found that propranolol attenuated the inhibitory effect of a putative 5-HT$_{1A}$ agonist on dorsal raphé neurones but only when locally applied *in vitro* and not when administered systemically *in vivo*. However, in the present study the inactivity of propranolol makes it unlikely that the effect of pindolol was mediated via β-adrenoceptors.

The observation that ritanserin, BRL 43694, phentolamine and sulpiride did not attenuate the 5-HT response to 8-OH-DPAT is certainly consistent with the idea that 8-OH-DPAT acts through a 5-HT$_1$ class of receptor. Further support for this idea comes

Table 2 Summary of effects of monoamine receptor antagonists on basal and 8-hydroxy-2(di-n-propylamino)tetralin (8-OH-DPAT)-reduced hippocampal 5-hydroxytryptamine (5-HT) output

Treatment	*Dose* (mg/kg s.c.[a])	*Monoamine receptor[b] selectivity*	*Effect on basal 5-HT output*	*Effect on 8-OH-DPAT reduced 5-HT output*
Methiothepin	1	5-HT/α/DA	Reduction	No effect
Methysergide	10	5-HT/DA	Reduction	No effect
Metergoline	5	5-HT/α/DA	Reduction	Partial attenuation
Pindolol	8	$5\text{-HT}_1/\beta$	No effect	Partial attenuation
Propranolol	10	$5\text{-HT}_1/\beta$	No effect	No effect
Phentolamine	10	α_1/α_2	No effect	No effect
Ritanserin	0.2	5-HT_2	No effect	No effect
BRL 43694	0.5	5-HT_3	No effect	No effect
Sulpiride	10	DA/D_2	No effect	No effect

[a] In certain cases a range of doses were used, see text for details.
[b] Information based on references in the text.

from our observation that the effect of 8-OH-DPAT is attenuated by pindolol, a drug with high affinity for the rat brain 5-HT_{1A} binding site (Hoyer, 1988) and which effectively inhibits various 8-OH-DPAT-induced responses in the rat including the 5-HT behavioural syndrome (Tricklebank *et al.*, 1984), hypothermia (Gudelsky *et al.*, 1986) hyperphagia (Hutson *et al.*, 1987) and adrenocorticotrophic hormone secretion (Gilbert *et al.*, 1988). However, in contrast to the findings of the latter studies, pindolol appeared not to be a truly competitive antagonist of 8-OH-DPAT in our experiments. We cannot rule out the possibility that a non-5-HT_{1A} mechanism is involved in the partial blockade of 8-OH-DPAT by pindolol, although this seems less likely given our recent findings that pindolol inhibits the decrease in 5-HT output induced by other putative 5-HT_{1A} agonists.

As well as our findings with pindolol, our experiments showing no inhibition of 8-OH-DPAT by methiothepin, methysergide or propranolol also contrast with the fact that these drugs bind with high affinity to the 5-HT_{1A} binding site (Hoyer, 1988) and reports that they effectively block 8-OH-DPAT-induced behavioural responses in rats at doses close to those used in this study (Goodwin & Green, 1985; Tricklebank *et al.*, 1984). Why certain drugs should effectively prevent some but not other 8-OH-DPAT-induced responses is a matter for speculation. However, one simple explanation is that at least some of these drugs are not full antagonists but rather partial agonists at the central 5-HT_{1A} receptor. In support of this idea is evidence that both metergoline and methysergide, like 8-OH-DPAT, reduce forskolin-stimulated adenylate cyclase activity in calf hippocampus (Schoeffter & Hoyer, 1988). Furthermore, the decrease of 5-HT output induced by metergoline, methysergide and methiothepin observed in the present study, together with the inhibitory effect of these drugs on serotoninergic cell firing (Haigler & Aghajanian, 1977) is consistent with activation of 5-HT_{1A} receptors. Also, metergoline and methiothepin, like 8-OH-DPAT, are reported to reduce brain 5-HT synthesis (Bourgoin *et al.*, 1978; Long *et al.*, 1983). Decreases in 5-HT synthesis/metabolism have also been described following administration of pindolol (Hjorth & Carlsson, 1986) and propranolol (Jones & Tackett, 1988; S. Hjorth unpublished findings) although in this study we were unable to detect an effect of these drugs on basal 5-HT output.

The intrinsic activity of partial agonists will vary relative to the amount of endogenous ligand (in this case 5-HT) available at the receptor site. Thus, the efficacy of such drugs will vary according to receptor tone which will be dependent on localization of the receptor both at the synapse (pre- or postsynaptic) and within the brain. Furthermore, we cannot rule out the possibility that while drugs such as methiothepin appear to behave as 5-HT agonists in the present experiments, altering our experimental conditions to say, exclude anaesthesia, might better reveal the 5-HT_1 antagonist profile of these drugs. However, one should be equally cautious in labelling drugs like methiothepin, methysergide and metergoline as partial 5-HT_{1A} agonists on the basis of the present dialysis data since their well documented activity at other neurotransmitter receptors (e.g. Leysen *et al.*, 1981) may contribute to the observed decrease in 5-HT output.

Other contributing factors for the lack of clear-cut antagonism of 8-OH-DPAT by methysergide, methiothepin and propranolol at the doses tested could be a very high efficacy of 8-OH-DPAT at the

somatodendritic 5-HT autoreceptor and the possibility of high autoreceptor reserve (compared to the postsynaptic 5-HT_{1A} receptor). If so, it is conceivable that these drugs might reveal some somatodendritic 5-HT autoreceptor antagonist activity if the antagonist/agonist ratios were increased even further than those used in the present study.

In summary, we present experiments which suggest that the 8-OH-DPAT-induced decrease of 5-HT in hippocampal dialysates of the anaesthetized rat is mediated via a 5-HT_1 class of receptor. Classification of this receptor to be of the 5-HT_{1A} subtype, which would be entirely consistent with the selective pharmacological profile of 8-OH-DPAT, awaits the development of a selective ligand with sufficiently low intrinsic activity at the brain 5-HT_{1A} binding site. Perhaps the most suitable such candidate published in the literature to date is BMY 7378 (Yocca *et al.*, 1987). This drug, however, appears to have a 5-HT agonist-like profile in the present model (Sharp *et al.*, 1989c).

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Difference in the potentiating effect of adenosine triphosphate and α,β-methylene ATP on the biphasic insulin response to glucose

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1 The effects of exogenous adenine nucleotides and structural analogues on the biphasic insulin response to an increase of glucose concentration in the physiological range (from 4.2 to 8.3 mM) were studied in the isolated perfused rat pancreas. Purinoceptor agonists were added either simultaneously or 15 min before increasing glucose.

2 ATP and ADP at 16.5 μM were ineffective *per se* in the presence of the non stimulatory glucose concentration (4.2 mM) but markedly potentiated the biphasic insulin response to glucose rise in both experimental protocols.

3 Two more stable analogues of ATP and ADP (adenylylimidodiphosphate and α,β-methylene ADP (α,β-MeADP)) at 16.5 μM behaved like the natural compounds: they were ineffective at a glucose concentration of 4.2 mM and potentiated both phases of insulin response to glucose rise.

4 α,β-MeATP added simultaneously with the high glucose concentration, markedly potentiated the first phase of insulin response to glucose rise but did not potentiate the second one. When α,β-MeATP infusion began 15 min before glucose rise, the biphasic response to glucose was not potentiated, in contrast to what occurred with ATP.

5 In the presence of α,β-MeATP, the ATP potentiating effect was unaffected.

6 It is concluded that ATP and ADP, via activation of β cell P_{2y} purinoceptors, potentiates the biphasic insulin response to an increase of glucose concentration. On the other hand, α,β-MeATP did not behave like natural and other structural analogues of ATP and ADP: this difference appears not to be the consequence of desensitization of β cell P_{2y} purinoceptors by α,β-MeATP.

Introduction

Previously we have demonstrated that exogenous adenosine tri and diphosphate (ATP and ADP) stimulated insulin secretion from rat pancreatic β cell, an effect mediated by P_2 purinoceptor activation (Loubatières-Mariani *et al.*, 1979; Chapal & Loubatières-Mariani, 1981).

P_2 purinoceptors have been subdivided into P_{2x} and P_{2y}. This subdivision is principally based on the rank order of potency of ATP and its structural analogues. Furthermore administration of α,β-MeATP is known to desensitize P_2 purinoceptors in various tissues; this effect would be specific for P_{2x} receptor according to Burnstock & Kennedy (1985). Recently on the basis of the agonist potency order of ATP and its structural analogues, we have provided evidence for a P_{2y} purinoceptor subtype on rat β cell (Bertrand *et al.*, 1987).

It has long been established that raising the concentration of glucose, the major physiological stimulator of the β cell, induced a biphasic pattern of insulin secretion (Cerasi & Luft, 1967; Grodsky *et al.*, 1968). The aim of the present work was to investigate, in the isolated rat pancreas, the effect of the P_2 purinoceptor activation on the biphasic insulin response to an increase in glucose concentration. For this purpose we tested and compared the effect of ATP, ADP and their more stable structural analogues: adenylylimidodiphosphate (AMP-PNP), α,β-methylene ADP (α,β-MeADP) and α,β-methylene ATP (α,β-MeATP), on the biphasic insulin response

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to an increase in glucose concentration in the range of that occurring in physiological conditions, namely during food intake in rat (Strubbe & Steffens, 1975).

Methods

Our experiments were performed on male Wistar rats fed *ad libitum* and weighing 320 to 350 g. The surgical procedure for the isolated perfused pancreas of the rat has been described (Loubatières *et al.*, 1969). After anaesthesia with sodium pentobarbitone (60 mg kg^{-1}), the pancreas was totally isolated from all neighbouring tissues; it was perfused through its own arterial system with a Krebs-Ringer bicarbonate buffer containing 2 g l^{-1} pure bovine serum albumin (Fraction V) and glucose 4.2 mM. The Krebs buffer had the following ionic composition (mM): NaCl 108, KH_2PO_4 1.19, KCl 4.74, $CaCl_2$ 2.54, $MgSO_4.7H_2O$ 1.19, $NaHCO_3$ 18. A mixture of O_2 (95%) and CO_2 (5%) was continuously bubbled through this medium; the pH was $\simeq$7.35. The preparation was maintained at 37.5°C. Each organ was perfused at a constant pressure, the flow rate was about 2.5 ml min^{-1}. In all the experiments, a 30 min adaptation period was allowed before taking the first sample for insulin assay. A second sample was taken 15 min later. At time 60 min, the glucose concentration was raised from 4.2 to 8.3 mM, the stimulation period lasting 30 min. In one experimental protocol ATP, ADP or α,β-MeATP were added simultaneously with glucose rise and remained for 30 min. In another protocol, adenine nucleotides were added to the physiological medium 15 min before glucose rise and remained for 45 min. For each group of experiments, controls (glucose stimulation) were performed. Samples were collected during a period of 1 min.

The kinetics of insulin output rate were studied for controls and for each adenine nucleotide. Samples were taken every min for 5 min, then at times 8, 10, 15, 20, 30 min just after increasing glucose concentration. The area under the curve was calculated for each experiment, both for the first phase, i.e. for the first 5 min, by adding the insulin output rates (ng min^{-1}) and for the second phase during the subsequent 25 min by applying the linear interpolation method.

Insulin in the pancreatic effluent was assayed by the radioimmunological method B of Hales & Randle (1963), using the SB-INSI-1 kit from CEA (France). Purified rat insulin (kindly supplied by NOVO, Copenhagen, Denmark) was used as the reference standard, the biological activity of which was 19 μu ng^{-1}. The intra and interassay coefficients of variations were respectively 9 and 13.5%.

Data are given as mean $\pm$ standard error of the mean (s.e.mean). For each group of experiments, areas under curves were submitted to analysis of variance by the multiple comparison test (Zar, 1974).

ATP and adenylylimidodiphosphate (AMP-PNP) were obtained from Boehringer Mannheim; ADP, α,β-MeATP and α,β-MeADP were from Sigma Chemical Company. ATP, ADP and α,β-MeADP were sodium salts, AMP-PNP and α,β-MeATP were lithium salts. Albumin (Fraction V) was from Sigma Chemical Company.

Results

Effect of exogenous ATP and ADP on the biphasic insulin response to a glucose rise

As shown in Figure 1, raising the glucose concentration in the perfusate from 4.2 to 8.3 mM (controls) elicited the classical biphasic pattern of insulin release.

To study the effects of ATP and ADP, two protocols were used: ATP or ADP was applied either simultaneously with (Figure 1a) or 15 min before (Figure 1b) the rise of glucose concentration from 4.2 to 8.3 mM. Since previous studies had shown that these nucleotides elicited half-maximum stimulating effect on insulin release at 16.5 μM, this concentration was used (Loubatières-Mariani *et al.*, 1979).

ATP or ADP 16.5 μM, introduced simultaneously with glucose 8.3 mM, markedly enhanced both phases of insulin response (Figure 1a). The areas under the curves were for the first phase respectively 62.9 $\pm$ 7.6 and 79.9 $\pm$ 18.9 ng ($P < 0.01$) for ATP and ADP versus 18.0 $\pm$ 2.7 ng in controls and for the second phase 573.6 $\pm$ 89.6 and 545.6 $\pm$ 114.4 ng ($P < 0.05$) versus 237.1 $\pm$ 44.8 ng in controls.

As shown in Figure 1b, ATP and ADP, introduced 15 min before the glucose rise, had no effect on basal insulin output rate recorded with a non stimulatory glucose concentration (4.2 mM). When glucose concentration was raised to 8.3 mM, ATP and ADP (16.5 μM) considerably enhanced the biphasic insulin response. The areas under the curves were for the first phase respectively 72.1 $\pm$ 9.9 and 71.2 $\pm$ 8.6 ng ($P < 0.001$) versus 22.4 $\pm$ 6.2 ng in controls, and for the second phase 554.6 $\pm$ 102.7 ($P < 0.01$) and 706.8 $\pm$ 59.6 ng ($P < 0.001$) versus 230.6 $\pm$ 59.6 ng in controls. These values are comparable to those recorded when the nucleotides were applied simultaneously with glucose rise.

Increasing the concentration of ATP (1.65, 16.5 and 165 μM) showed that the potentiation of the insulin response to glucose by ATP was concentration-dependent (results not shown). However, it should be noted that ATP at the lowest

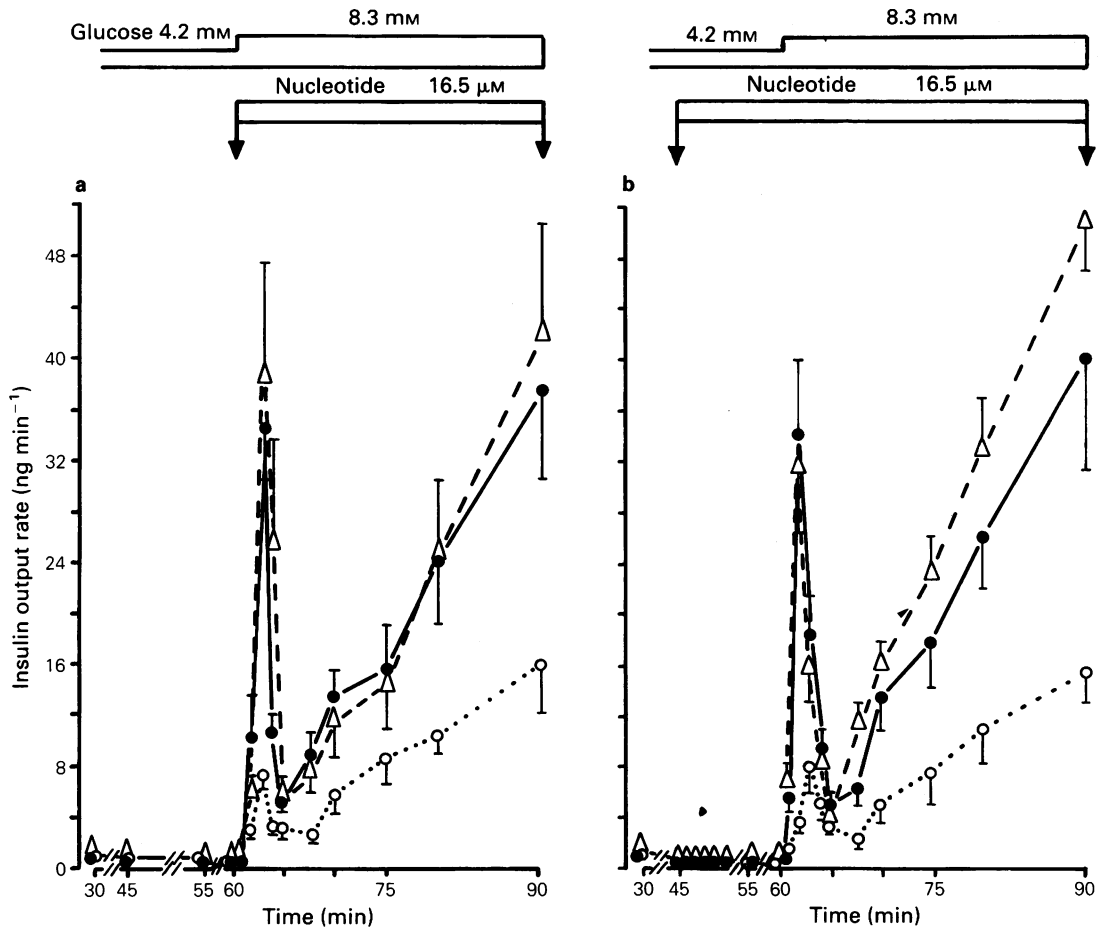

Figure 1 Effect of adenine nucleotides ATP (●) and ADP (△) (16.5 μM) on the biphasic insulin response to glucose rise; controls (○). (a) ATP and ADP were applied simultaneously with increased glucose. (b) ATP and ADP were applied 15 min before the glucose rise. Points represent the mean of 5–8 experiments and vertical lines the s.e.mean.

concentration (1.65 μM) significantly increased only the first phase.

Effect of two structural nucleotide analogues on the biphasic insulin response to a glucose rise (Figure 2)

In order to ascertain a potentiating effect of ATP and ADP *per se* and not after metabolic alteration, we studied the effects of AMP-PNP, a non phosphorylating ATP analogue and α,β-MeADP, a non hydrolysable methylene isostere of ADP (Yount, 1975). In these experiments we used the second protocol, the nucleotides were introduced 15 min before glucose increase.

While AMP-PNP and α,β-MeADP at 16.5 μM were ineffective in the presence of 4.2 mM glucose, they significantly increased the biphasic insulin response to glucose rise. The areas under the curves were respectively for the first phase 47.8 ± 10.1 and 59.1 ± 11.9 ($P < 0.05$) versus 17.9 ± 3.1 ng in controls, and for the second phase 460.1 ± 55.8 ($P < 0.05$) and 749.3 ± 97.4 ($P < 0.001$) versus 194.2 ± 27.2 ng in controls.

Effect of α,β-methylene ATP on the biphasic insulin response to a glucose rise (Figure 3)

As this substance is known to desensitize P_{2x} purinoceptors we used two experimental protocols.

α,β-MeATP (16.5 μM), when introduced simultaneously with the increase in glucose concentration from 4.2 to 8.3 mM (Figure 3a), induced a potentiation of the first phase of the insulin response to

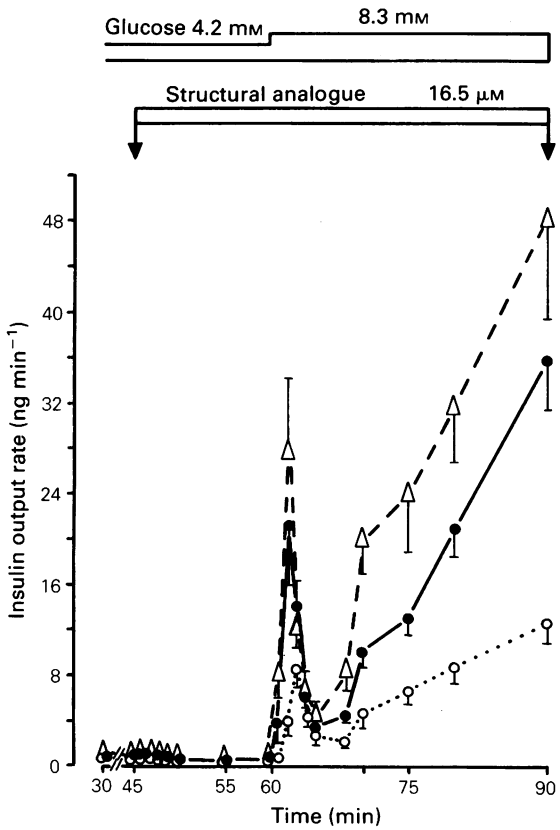

Figure 2 Effect of structural nucleotide analogues on biphasic insulin response to glucose rise. Adenylylimidodiphosphate (●); α,β-methylene ADP (△) and controls (○). Points represent the mean of 6–8 experiments and vertical lines the s.e.mean.

glucose, while the second phase was not significantly modified. It should be noted that in the presence of α,β-MeATP the response was delayed by about 3 min. This delay is explained by the interference of a vasoconstrictor effect of α,β-MeATP on the pancreatic vascular bed; this marked but transient effect has been described previously (Chapal & Loubatières-Mariani, 1983).

When α,β-MeATP was added 15 min before glucose rise (Figure 3b), the following results were obtained: (1) Unlike ATP, α,β-MeATP in the presence of a non stimulating glucose concentration induced a weak and transient but significant insulin secretion; the area under the curve was 15.0 ± 4.9 ng versus 3.2 ± 0.8 ng in controls ($P < 0.05$). (2) α,β-MeATP did not potentiate, as did ATP in the preceding experiments, the biphasic insulin response to glucose increase.

Effect of ATP on the biphasic insulin response to a glucose rise in presence of α,β-methylene ATP (Figure 4)

To determine whether the lack of effect of α,β-MeATP is due to a desensitization of P_2 purinoceptors of β cells, we have investigated whether the ATP effect was affected by the presence of this analogue.

To ensure an eventual desensitization we used a higher α,β-MeATP concentration (49.5 μM). As shown in Figure 4, ATP with and without α,β-MeATP elicited a comparable potentiating effect on the biphasic insulin response to glucose rise. The areas under the curves were respectively for the first phase 71.4 ± 5.3 and 69.5 ± 14.3 ng and for the second phase 438.7 ± 34.5 and 416.9 ± 84.7 ng in the absence or in the presence of α,β-MeATP. These values are comparable to those obtained in previous experiments with ATP (Figure 1a and 1b).

Discussion

The present results show that (1) ATP and ADP potentiate the biphasic insulin response to glucose; (2) α,β-MeATP behaves differently.

ATP and ADP are ineffective *per se* on insulin secretion in the presence of a substimulating glucose concentration and markedly potentiate the biphasic insulin response to a glucose increase in the physiological range (from 4.2 to 8.3 mM). Indeed, such an increase is close to that reported to occur in rat (Strubbe & Steffens, 1975) and man after a large carbohydrate meal (Müller & Weir, 1974). Similar results were obtained with two more stable structural analogues. Indeed, the non phosphorylating analogue of ATP, adenylylimidodiphosphate (AMP-PNP) potentiated as did ATP, both phases of insulin response to glucose rise, therefore excluding the involvement of ecto-ATPases for ATP action; on the other hand, the α,β-methylene isostere of ADP, resistant to hydrolysis to AMP and adenosine was as potent as ADP. So, the potentiating effect of ATP and ADP on the biphasic insulin response to a physiological glucose rise, appears to require neither hydrolysis of high energy phosphate bonds, nor the action of metabolic products such as AMP or adenosine. The potentiating action of ATP and ADP on the biphasic insulin response to glucose appears to be mediated through the P_2 purinoceptor previously characterized on the rat pancreatic β cell membrane (Loubatières-Mariani *et al.*, 1979; Chapal & Loubatières-Mariani, 1981).

α,β-MeATP potentiated the first but not the second phase, when it was introduced simultaneously with increased glucose. On the other hand,

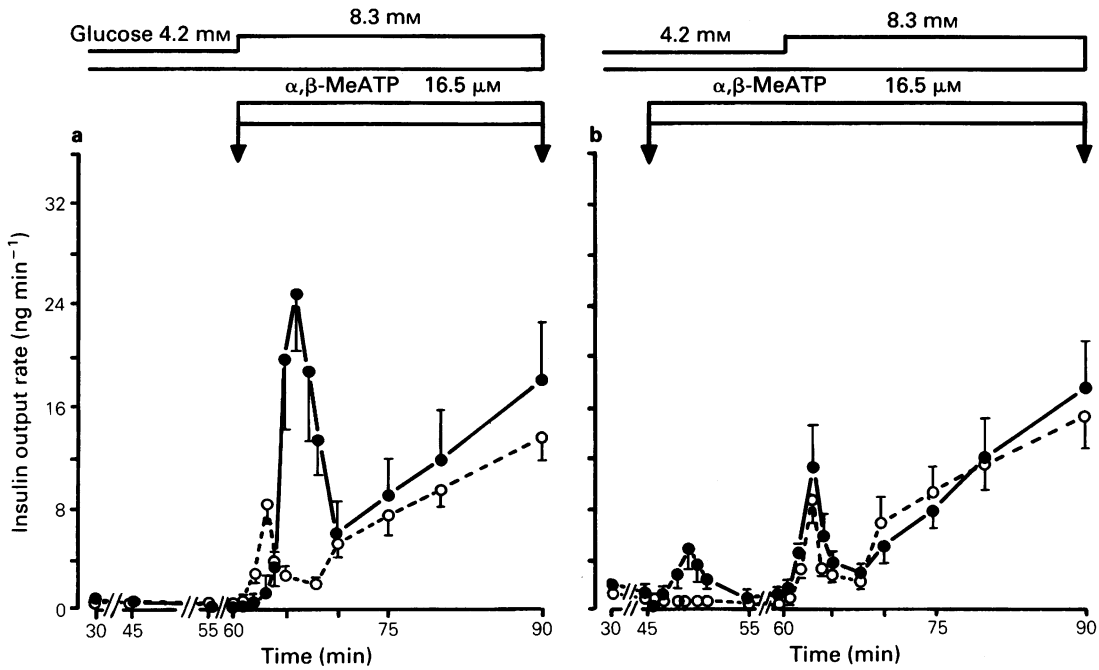

Figure 3 Effect of α,β-methylene ATP (α,β-MeATP) (16.5 μM) (●) on the biphasic insulin response to increased glucose. (a) α,β-MeATP was applied simultaneously with glucose rise. (b) α,β-MeATP was applied 15 min before glucose rise. Controls (○). Points represent the mean of 5–6 experiments and vertical lines the s.e.mean.

when α,β-MeATP was added 15 min before glucose rise, the biphasic response was not at all potentiated.

Thus there is a difference between the effects of α,β-MeATP and ATP on the biphasic insulin response to glucose increase. These results differ from those obtained when α,β-MeATP was applied 45 min after glucose stimulation (8.3 mM); in this case α,β-MeATP, like ATP, elicited a sustained insulin response (Chapal & Loubatières-Mariani, 1981). Since in both studies, α,β-MeATP induced a similar transient decrease in pancreatic outflow rate, the vasoconstrictor effect of this agent cannot explain the difference in the responses observed according to the experimental conditions concerning the glucose concentration. So, the previous glucose (substimulating or stimulating) concentration may play a role in the effects observed with α,β-MeATP. In this respect, the importance of the glucose concentration for the β-cell response to various stimulating agents must be recalled.

Tachyphylaxis has been shown to occur previously with α,β-MeATP on guinea-pig vas deferens (Meldrum & Burnstock, 1983) and cat colon (Hedlund *et al.*, 1983). This effect is generally the consequence of P_{2x} purinoceptor desensitization as it has been reported in such organs as mesenteric artery (Kügelgen & Starke, 1985; Muramatsu, 1986), vas deferens (Sneddon & Burnstock, 1984) and urinary bladder (Kasakov & Burnstock, 1983; Hoyle & Burnstock, 1985). However in our study since the ATP potentiating effect was unaffected in the presence of α,β-MeATP, the absence of the analogue potentiating effect is unlikely to be due to a desensitization of the P_2 purinoceptor which mediates ATP effect. We have previously characterized the P_2 purinoceptor of the rat β cell as a P_{2y} subtype on the basis of the agonist potency order of ATP and its structural analogues (Bertrand *et al.*, 1987). As it is known that α,β-MeATP selectively exerts a desensitization action at P_{2x} purinoceptors, our results provide further evidence for an ATP effect on rat β cell via the P_{2y} purinoceptor.

The discrepancy between ATP and α,β-MeATP remains to be explained. α,β-MeATP could act by an indirect action, e.g. via intramural nerves, as proposed by Moody & Burnstock (1982) in rat ileum. On the other hand, it might be suggested that the difference in the effect between α,β-MeATP and ATP could be due to the stimulation of the two different P_2 receptor subtypes. It is known that α,β-MeATP is more potent than ATP at P_{2x} receptors (Burnstock & Kennedy, 1985) and, in our experiments for the

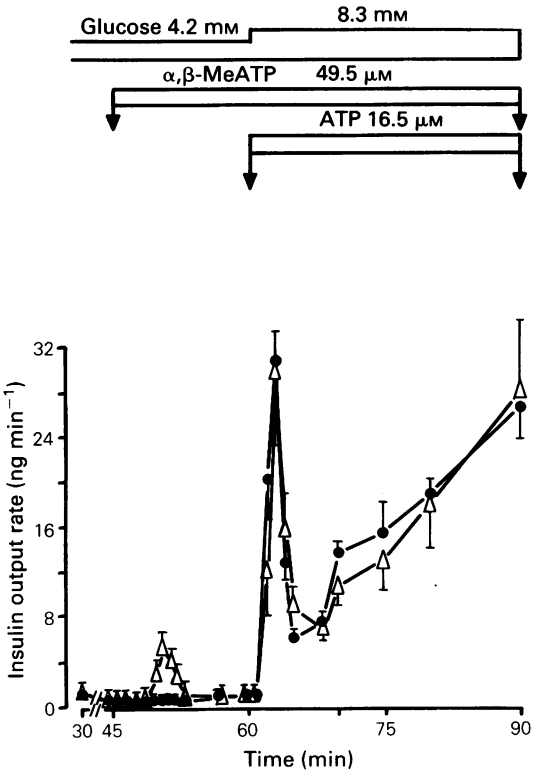

Figure 4 Effect of ATP (16.5 μM) on biphasic insulin response to glucose rise in absence (●) or in presence (△) of α,β-methylene ATP (49.5 μM). Points represent the mean of 4–5 experiments and vertical lines the s.e.mean.

same concentration (16.5 μM), α,β-MeATP, but not ATP, caused a small and transient increase of basal insulin release; at a ten fold higher concentration (165 μM), ATP elicited, only in some cases, a response in the same range (results not shown). However, further studies are needed to establish the existence of a P_{2x} receptor on β cells.

The potentiating effect of ATP and ADP on the biphasic insulin response to glucose rise might be of physiological relevance. Indeed, since ATP and ADP are present in the secretory granule and coreleased with insulin (Leitner *et al.*, 1975), they could exert a positive feedback control on the β cell during glucose stimulation. Furthermore as ATP strongly potentiates insulin release, particularly the first phase, it would be of interest to examine whether abnormalities in the P_2 purinoceptor of the β cell could be involved in the pathogenesis of non-insulin-dependent diabetes mellitus (NIDDM).

In conclusion, ATP and ADP potentiate the biphasic insulin response to a physiological glucose rise, an effect mediated by the P_{2y} purinoceptor of the β cell. On the other hand, α,β-MeATP has a different effect which cannot be explained by a desensitization of the P_{2y} purinoceptor of the β cell, but possibly implicates a P_{2x} purinoceptor.

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Cycloleucine blocks NMDA responses in cultured hippocampal neurones under voltage clamp: antagonism at the strychnine-insensitive glycine receptor

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1 Radioligand binding studies have demonstrated that the neutral amino acid cycloleucine may act as a competitive antagonist at the glycine modulatory site on the N-methyl-D-aspartate (NMDA) receptor complex. In the present study, we examined the effects of cycloleucine on NMDA-evoked inward current responses in dissociated hippocampal neuronal cultures using the whole cell voltage-clamp technique.

2 In the presence of 1 μM glycine, cycloleucine caused a reversible, dose-dependent inhibition of NMDA responses with an IC_{50} of 24 μM. An increase in glycine to 100 μM resulted in a shift to the right of the cycloleucine concentration-effect curve (IC_{50}, 1.4 mM). However, with cycloleucine concentrations $\leqslant 100\,\mu$M, a fraction of the block could not be overcome by glycine even at concentrations as high as 1 mM.

3 The cycloleucine block was unaffected by shifts in the holding potential (−60 to +60 mV), and there was no effect of cycloleucine on the reversal potential of the NMDA-evoked current.

4 Cycloleucine failed to effect kainic acid- and quisqualic acid-evoked currents at concentrations which inhibited NMDA responses.

5 We conclude that cycloleucine is a potent and selective antagonist of NMDA-receptor mediated responses. Although this effect occurs in part via competitive antagonism at the glycine modulatory site, the cycloleucine block cannot be completely reversed by glycine indicating an interaction with an additional site on the receptor-channel complex.

Introduction

Ionic current flow through the N-methyl-D-aspartate (NMDA) receptor-coupled channel is subject to modulation at a number of pharmacologically distinct sites on the receptor-ionophore complex (see e.g., Mayer *et al.*, 1988). These modulatory sites, which may either influence gating of the channel or serve as acceptors for steric blockers of the channel pore, provide a variety of targets for drugs directed at modifying NMDA receptor function. Of particular interest is the observation of Johnson & Ascher (1987) that glycine markedly enhances agonist-induced channel opening via its action at a strychnine-insensitive acceptor site (see also Verdoorn *et al.*, 1987; Bertolino *et al.*, 1988; Forsythe *et al.*, 1988). These electrophysiological observations have been corroborated in several recent studies using radioligand binding techniques (Reynolds *et al.*, 1987; Bonhaus *et al.*, 1987; Wong *et al.*, 1987; Johnson *et al.*, 1988), and it has been suggested that a glycine-like substance may be an endogenous modulator of NMDA-receptor mediated excitatory neurotransmission (Larson & Beitz, 1988). If this is the case, antagonists of the glycine modulatory site would be expected to produce an indirect suppression of synaptic responses mediated by NMDA receptors and could be of therapeutic benefit in pathological states associated with excess excitatory amino acid release.

Several allosteric blockers of NMDA-receptor-mediated responses may operate, at least in part, via

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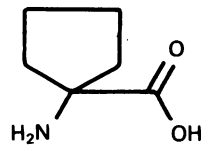
Glycine



Cycloleucine

Figure 1 Comparison of the structures of glycine and cycloleucine.

an action at the strychnine-insensitive glycine site including kynurenic acid, 7-chlorokynurenic acid, 1-hydroxy-3-amino-2-pyrrolidone (HA-966), quinoxalinedione analogues, 5-substituted indole-2-carboxylic acids, and 1-aminocyclobutane-1-carboxylic acid (Birch *et al.*, 1988a,b; Kemp *et al.*, 1988; Fletcher & Lodge, 1988; Grossman & Hayes, 1988; Mayer *et al.*, 1988; Ransom & Deschenes, 1988; 1989; Drejer *et al.*, 1989; Reynolds *et al.*, 1989; Verdoorn *et al.*, 1989; Huettner, 1989; Hood *et al.*, 1989). Recently, Snell & Johnson (1988) have identified the neutral amino acid cycloleucine (Figure 1) as a possible antagonist on the basis of studies demonstrating that the compound (i) displaces [^{3}H]-glycine from binding to strychnine-insensitive sites in rat brain homogenates and (ii) that it inhibits NMDA-induced [^{3}H]-thienylcyclohexylpiperidine binding, a measure of channel activation, in a glycine-dependent fashion.

We now provide electrophysiological evidence that cycloleucine is indeed an inhibitor of NMDA responses in brain neurones. While a major component of this effect is due to antagonism at the strychnine-insensitive glycine site, the compound appears to have a glycine-independent action as well.

Methods

Cell culture

Neurones from the hippocampi of 19-day-old Sprague-Dawley rat embryos were grown in primary cell culture according to methods described by Segal (1983). The hippocampal tissue was triturated by passage through a Pasteur pipette and the resulting cell suspension was plated onto polyornithine-coated 35 mm culture dishes at a density of $\sim 3 \times 10^5$ cells per dish in Eagle's Modified Minimal Essential medium supplemented with 10% foetal bovine serum, 10% horse serum and 1% glutamine. The cultures were incubated at 37°C in a humidified atmosphere containing 10% CO_2. One-half of the culture medium was replaced every four days with medium similar to that used for the initial plating except that it lacked foetal bovine serum. Culture media were obtained from Gibco laboratories (Grand Island, N.Y., U.S.A.); all other chemicals and drugs were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Whole-cell voltage-clamp recording

Electrophysiological recordings were performed at room temperature (23–25°C) on the stage of an inverted phase contrast microscope. Before each experiment, the culture medium was removed, the cells were rinsed twice, and the culture dish was partially filled with 1.5 ml of recording solution containing 140 mM NaCl, 5 mM KCl, 5 mM $CaCl_2$, and 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES). The bathing medium also contained 1 μM tetrodotoxin to block voltage-dependent Na^+ current, 1 μM strychnine to block glycine-evoked Cl^- current, and varying concentrations of glycine from 0.1 μM to 1 mM. Mg^{2+} was omitted from the solution to avoid depression of the NMDA-evoked current responses (Mayer *et al.*, 1984; Nowak *et al.*, 1984). The solution was adjusted to a pH of 7.4 with NaOH and an osmolality of 307 mOsm kg^{-1} H_2O with glucose.

Patch electrodes were prepared from filament fused, borosilicate glass capillaries (1.5 mm, o.d.) (TW150F-4, WPI instruments, New Haven, CT, U.S.A.) with a Flaming-Brown micropipette puller (Model P80, Sutter Instruments Co., San Rafael, CA, U.S.A.). The electrodes were filled with a solution containing 145 mM potassium gluconate, 2 mM $MgCl_2$, 1.1 mM EGTA, 0.1 mM $CaCl_2$ and 5 mM HEPES except in experiments where current-voltage curves were constructed in which case 145 mM CsCl was used instead of the potasssium gluconate. The electrode solutions were adjusted to an osmolality of 307 mOsm kg^{-1} H_2O with glucose and to a pH 7.4 with KOH (CsOH in the CsCl solution). The solutions were filtered through a 0.22 μm membrane filter (Millipore Corp., Bedford, MA) immediately prior to use. Pipet-to-bath resistances were typically 4–10 MΩ.

Whole-cell currents were recorded with an Axopatch 1B patch-clamp amplifier (Axon Instruments, Burlingame, CA). After seal formation and prior to establishing a whole-cell recording, electrode capac-

itance was neutralized. Series resistance compensation of 80–85% was obtained under voltage clamp. Voltages corresponding to the membrane currents were displayed on a high speed ink pen recorder with a direct current input stage (Gould Electronics, Cleveland, OH, U.S.A.).

Drug application

Drugs were dissolved in bathing medium and were applied to the cell surface with blunt micropipettes (tip diameter, 1 μm) using a pneumatic pressure ejection system (Medical Systems, Greenvale, N.Y., U.S.A.). The tip of the pressure pipette was situated $<4\,\mu$m from the cell membrane and the drug solution was expelled as needed during the experiment by applying a back pressure of 0.8–2.5 p.s.i. In most experiments, we used two pressure pipettes: one containing an excitatory amino acid and the second with cycloleucine. A third pressure pipette containing a second excitatory amino acid was used in some experiments. In a few experiments, the cycloleucine pressure pipette was replaced with a pipette containing bathing medium alone to verify that the hydrodynamic effects of fluid flow from a nearby pipette does not by itself interfere with the excitatory amino acid response ('blow-away' artifact). To assay for block, excitatory amino acids were applied with brief (300–400 ms) pressure pulses before, during and after the sustained ejection of cycloleucine. To avoid desensitization, excitatory amino acid ejections were not made more frequently than every 30 s. The pressure pipettes were tested for patency before and after each experiment by verifying that there was sufficient fluid flow from the tip to displace small particles of debris (1–2 μm in size) on the bottom of the culture dish.

Data analysis

We calculated the fractional block produced by cycloleucine according to the formula $B = 1 - I'/I$, where I is the maximum net current amplitude immediately prior to the onset of the drug superfusion and I' is current evoked 20 s after the onset of the superfusion. Group means were compared with the two-way analysis of variance and Newman-Keuls tests as performed by the Pharmacologic Calculation System computer programs accompanying Tallarida & Murray (1987); the criteria for statistical significance was $P < 0.05$. Concentration-effect data were fit with a nonlinear least squares programme (ALLFIT as described by De Lean *et al.*, 1978) according to the logistical equation

$$B = \frac{B_{min} - B_{max}}{1 + ([X]/IC_{50})^n} + B_{max}$$

where B_{min} and B_{max} are the asymptotic minimum and maximum block, $[X]$ is the drug concentration, IC_{50} is the concentration of X resulting in $(B_{min} + B_{max})/2$ block, and $|n|$ is an empirical parameter that describes the steepness of the curve and has the same meaning as the Hill coefficient. Final plotting was performed on a microcomputer with the Grapher software package (Golden Software, Golden, CO). All quantitative data are expressed as mean $\pm$ s.e.mean.

Results

The data presented represent results of whole-cell voltage-clamp recordings from 108 cultured hippocampal neurones.

Response to N-methyl-D-aspartate

In all experiments except those carried out explicitly to test the voltage sensitivity of the cycloleucine block, the holding potential was set at -60 mV. At this potential, 100 μM NMDA invariably elicited a robust inward current response that rose rapidly during the 300 ms superfusion and decayed in the 1–2 s period following the drug application. Peak NMDA responses ranged in amplitude from 200–3600 pA (mean $\pm$ s.e.mean, 1122 $\pm$ 99 pA; 73 cells; glycine concentration $\geqslant 1\,\mu$M).

Cycloleucine block

Figure 2 illustrates the depressant effect of cycloleucine on the NMDA-evoked inward current response. The cycloleucine achieves a maximum block within ~ 10 s after the onset of the superfusion and full recovery is observed 30–40 s following the termination of the superfusion. There was no change in the resting current level during the cycloleucine superfusion. Experiments like this in three other cells showed a similar time course for the onset and recovery of block. Consequently, in subsequent experiments, we quantitated the cycloleucine blocking effect 20 s after the onset of the superfusion, and we evaluated recovery 30 s after termination of cycloleucine superfusion.

Dose-dependence and reversal by glycine

The cycloleucine block of NMDA responses occurred in a dose-dependent fashion. Figures 3(a) and (b) compare the effect of 1 and 1000 μM cycloleucine. In the presence of 1 μM glycine, 1 μM cyclo-

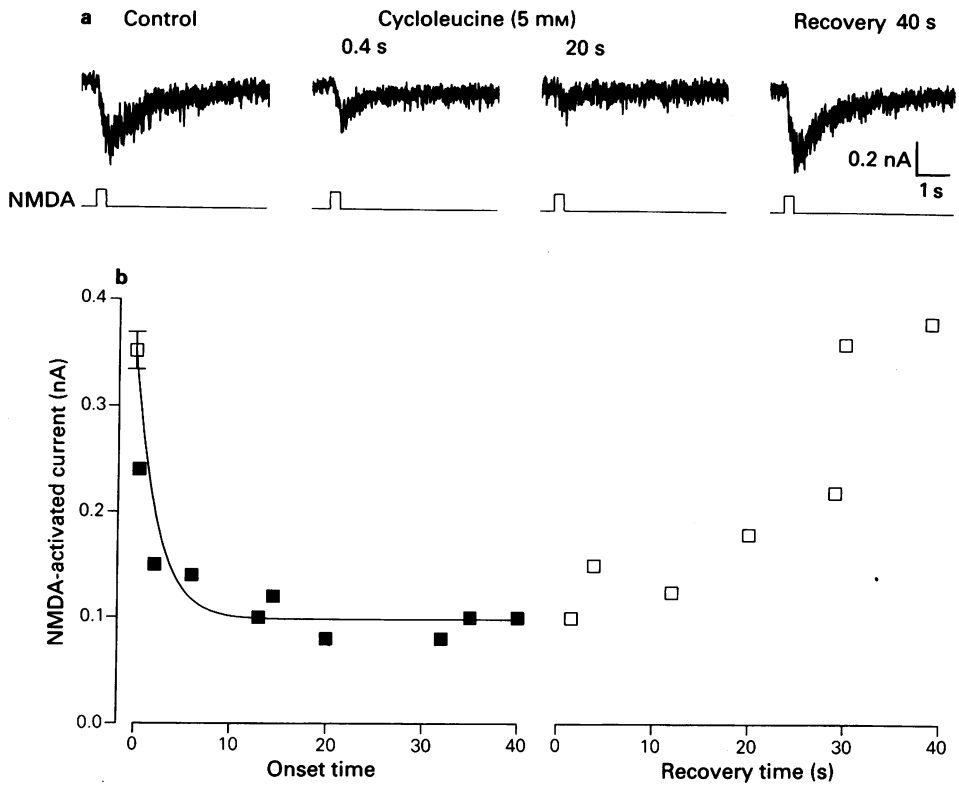


Figure 2 Time course of cycloleucine induced inhibition of NMDA-evoked inward current responses in the presence of 1 μM glycine. (a) Inward current responses produced by 300 ms pressure pulses of NMDA (100 μM) prior to (Control), at various times during (Cycloleucine), and after (Recovery) superfusion with 5 mM cycloleucine. Holding potential in this and all other figures is -60 mV unless otherwise noted. (b) Summary of several trials similar to those shown in the upper traces. The open square at 0 time represents the mean of the control NMDA responses with s.e.mean shown by vertical bar. Filled squares indicate amplitudes of single NMDA responses at various times after the onset of the cycloleucine superfusion. Open squares above the axis labelled "Recovery time" represent single NMDA responses at various times after the cycloleucine superfusion was terminated. Both (a) and (b) represent data from the same cell.

leucine produced a moderate degree of block and 1000 μM almost completely inhibited the response. However, increasing the glycine concentration to 100 μM reduced the effect of 1000 μM cycloleucine (Figure 3c).

Concentration-effect curves were constructed from experiments with 22 cells exposed to 1 μM glycine and 24 cells exposed to 100 μM glycine (Figure 4a). Increasing the glycine concentration resulted in a parallel shift to the right of the concentration-effect curve. The IC_{50} values in the presence of 1 and 100 μM glycine were 24 μM and 1.4 mM, respectively. The slopes of the best fit curves to the data are shallow, giving Hill coefficients at 1 and 100 μM glycine of 0.23 and 0.26, respectively. Control experiments in which the cycloleucine pipette was replaced by one containing bathing medium alone showed no significant reduction in the NMDA-induced current (fractional block, 0.04 ± 0.03; 4 cells).

In an additional series of experiments, we examined further the dependence of the cycloleucine block on the glycine concentration. These recordings were carried out with 10 μM (18 cells) and 100 μM (27 cells) cycloleucine and with bath concentrations of glycine from 0.1 μM to 1 mM (Figure 4b). Increasing the glycine concentration from 1 to 10 μM resulted in a sharp drop in the fractional block at both cycloleucine concentrations. No further reversal of the cycloleucine block was observed with glycine concentrations above 10 μM. The plateau in the curves at low glycine concentrations is probably due, at least in part, to the presence in the bathing medium of a

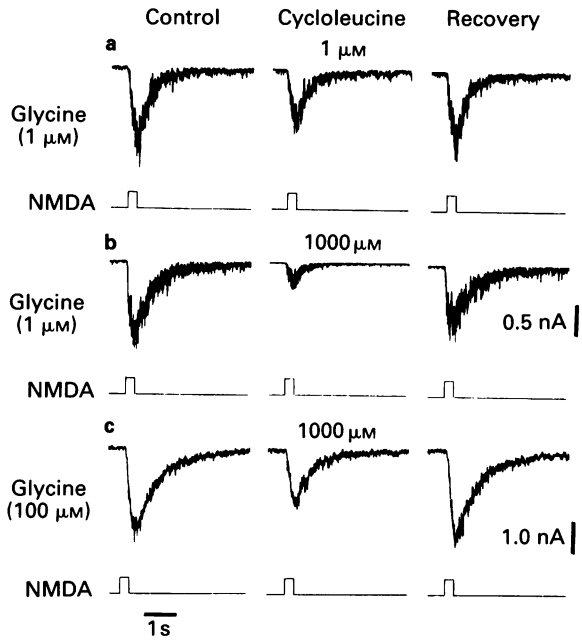


Figure 3 The effect of various concentrations of cycloleucine and glycine on NMDA-evoked inward current responses. In each panel, the upper traces are current recordings and the lower traces indicate the duration of the 100 μM NMDA pressure pulses. The NMDA response is shown prior to (Control), 20 s after the onset (Cycloleucine), and 30 s after the end (Recovery) of the cycloleucine superfusion. (a) In the presence of 1 μM glycine, 1 μM cycloleucine reduced the NMDA response by 40%. (b) In the presence of the same glycine concentration as (a) but with the 1000 μM cycloleucine, the block is 90%. (c) Increasing the glycine concentration to 100 μM results in a diminished block by 1000 μM cycloleucine (35%). (a), (b) and (c) represent different cells. Time scale for all records is the same; current scale to the right in (b), also applies to (a).

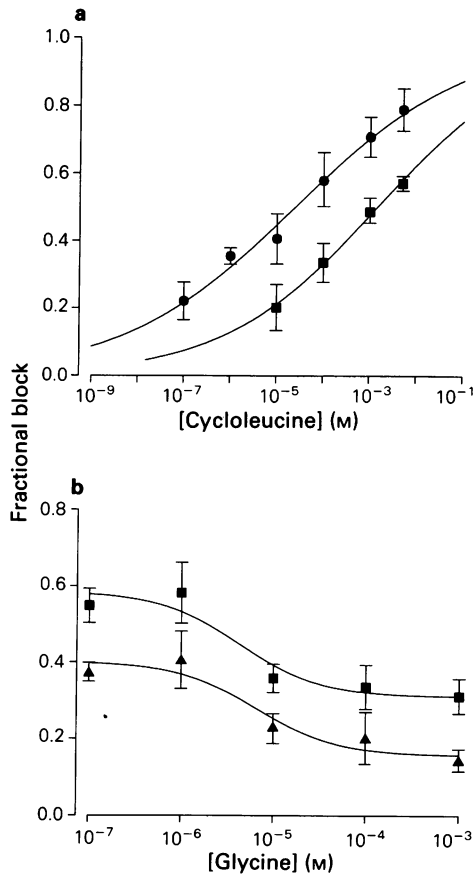


Figure 4 Concentration-dependence of cycloleucine block: effect of changes in glycine concentration. (a) Concentration-effect curves for cycloleucine inhibition of NMDA-evoked current in the presence of 1 μM (●, 22 cells) and 100 μM (■, 24 cells) glycine. (b) Concentration-effect curves for the effect of glycine on cycloleucine inhibition of the NMDA-evoked current at cycloleucine concentrations of 10 μM (▲, 18 cells) and 100 μM (■, 27 cells). Points represent the mean with s.e.mean shown by vertical bars.

glycine-like substance at concentrations equivalent to 0.1–0.5 μM of glycine (see Johnson & Ascher, 1987).

Voltage-dependence and reversal potential

We evaluated the voltage-dependence of the cycloleucine block in six cells (two with 100 μM and four with 1000 μM cycloleucine). The current recordings from one cell are shown in Figure 5a. There was no significant change in the fractional block as the holding potential was shifted from −60 to +60 mV. The reversal potential of the NMDA-evoked current for the cell shown was +20 mV and was unchanged by cycloleucine. The mean current amplitude data (normalized to the control current at −60 mV) for the six cells are plotted in Figure 5b. No rectification was apparent under control conditions or in the presence of cycloleucine and there was no significant change in reversal potential caused by the drug (range +5 to +20 mV in the six cells). We calculated the mean fractional block for the two cycloleucine concentrations at the various holding potentials and these values are plotted in Figure 4c. There were no statistically significant differences among the mean

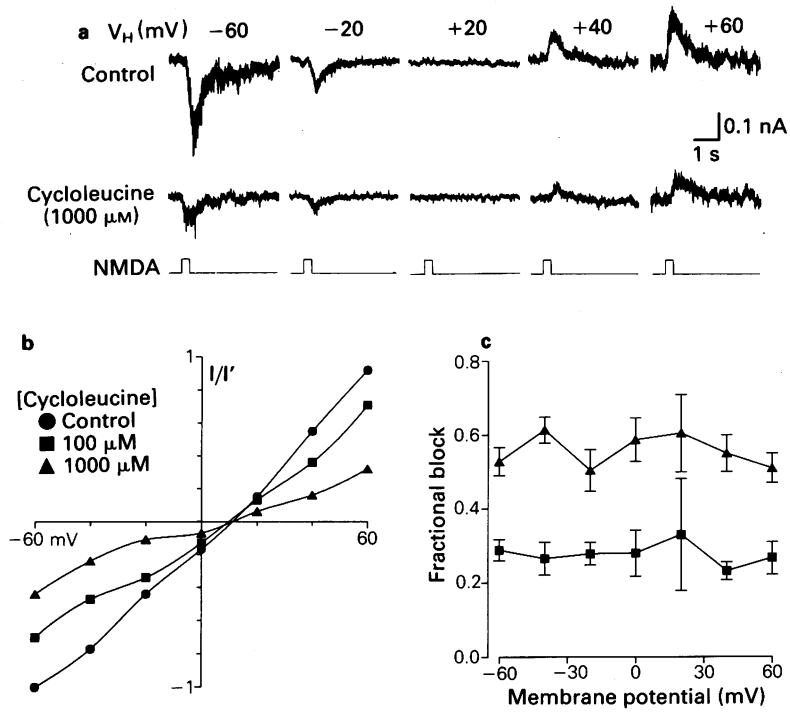


Figure 5 Lack of voltage-dependence of the cycloleucine block. (a) NMDA induced currents before (Control) and after (Cycloleucine) superfusion with 1000 μM cycloleucine at various holding potentials. (b) Current-voltage curves for NMDA-evoked current prior to (Control, ●) and during superfusion with 100 μM (■, 2 cells) and 1000 μM (▲, 4 cells) cycloleucine. Data were normalized to the control current at −60 mV. (c) Fractional block as a function of membrane holding potential derived from the data presented in (b); key to symbols is same as in (b). The glycine concentration was 1 μM.

fractional block values at the various holding potentials for either cycloleucine concentration, confirming the voltage independence of the block. The increase in the standard errors at a holding potential of +20 mV results from the increased variability in measuring the very small currents near the reversal potential.

Selectivity of block

To assess the specificity of cycloleucine, we compared the effects of the compound on inward current responses evoked by NMDA with its action on responses elicited by the selective excitatory amino acid agonists quisqualic acid (QA) or kainic acid (KA). As illustrated in Figure 6, cycloleucine caused a selective block of the NMDA response with little reduction in the QA-induced current. In a series of similar experiments, cycloleucine produced no statistically significant effects on QA (10 μM) or KA

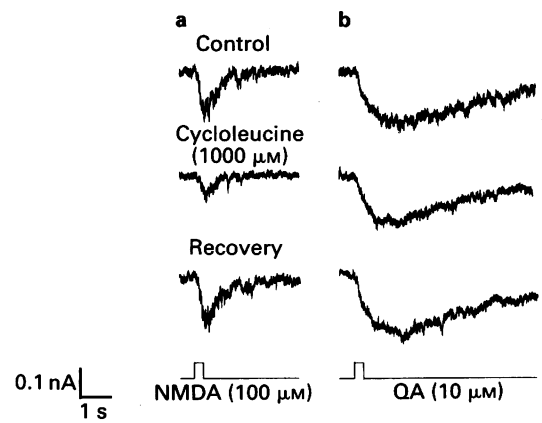


Figure 6 Selectivity of the cycloleucine block. Comparison of the effects of 1000 μM cycloleucine on responses evoked by 100 μM NMDA (a) and 10 μM quisqualic acid (QA) (b) in the same cell. The glycine concentration was 1 μM.

(100 μM) responses, although there were trends toward a small increase in the amplitude of the QA current (at the higher cycloleucine concentration) and toward a small depression of the KA current. The mean fractional block values for QA with 100 and 1000 μM cycloleucine were 0.02 ± 0.02 (8 cells) and -0.07 ± 0.04 (4 cells), respectively, and for KA with 100 μM cycloleucine was 0.08 ± 0.05 (9 cells).

Discussion

The main observation of this study is that cycloleucine causes a selective inhibition of NMDA responses, at least in part, as a result of competitive antagonism at the strychnine-insensitive glycine modulatory site on the NMDA receptor-channel complex. Thus, the cycloleucine block was partly reversible with glycine concentrations of 1–10 μM. However, increasing the glycine concentration above 10 μM produced no further reversal of the cycloleucine-induced inhibition, suggesting that cycloeucine can also inhibit NMDA responses by a mechanism distinct from its action at the glycine site. In fact, Johnson & Snell (1988) have demonstrated that cycloleucine binds to the NMDA receptor at concentrations 10 fold higher than those that antagonize glycine, indicating that cycloleucine may act as a competitive antagonist at both the NMDA receptor and the glycine modulatory site.

Our data are consistent with this conclusion. The concentration-effect curve of fractional block as a function of cycloleucine concentration exhibited a shallow slope with a Hill coefficient of less than one. Although numerous different mechanisms could theoretically account for this observation, one likely possibility is that cycloleucine has a dual action on both the glycine and NMDA receptor sites, and moreover that cycloleucine saturation of the glycine site does not result in complete suppression of the NMDA current. This latter condition would occur if cycloleucine were a partial agonist or if glycine were not absolutely required for the NMDA response as supported by the observations of Mayer *et al.* (1989) and Reynolds *et al.* (1989) (however, see Kleckner & Dingledine, 1988; Huettner, 1989). An additional requirement is that the affinity of cycloleucine for the NMDA receptor is less than that for the glycine receptor, as obtained by Johnson & Snell (1988). Under these conditions the concentration-effect curve would be an additive function of both the glycine and NMDA receptor-mediated actions of the drug, and should exhibit a shallow slope (Tallarida *et al.*, 1988). The lack of complete specificity toward the glycine site suggested by these results is similar to that observed for other glycine blockers, such as kynurenic acid and 7-chlorokynurenic acid, which also exhibit some (albeit weak in the case of the latter compound) activity at the NMDA recognition site (Kemp *et al.*, 1988; Ransom & Deschenes, 1989). In contrast, the recently described glycine-site antagonists which are analogues of indole-2-carboxylic acid have been reported to have little or no activity at the NMDA receptor, although these compounds are less potent than cycloleucine as NMDA antagonists (Huettner, 1989).

The observed shallow slope of the cycloleucine concentration-effect curve could also result solely from the type of reaction scheme involved in glycine modulation. For example, if glycine modulation involves a two-step reaction with the formation of a ternary complex (i.e., a glycine-NMDA-receptor complex), the concentration-effect curve would be shallow (De Hean, 1976; Jacobs & Cuatrecasas, 1976; however, see Huettner, 1989).

We found the potency of cycloleucine to be markedly greater than that reported in the radioligand binding studies of Snell & Johnson (1988). These workers obtained an IC_{50} for cycloleucine of 3.8 mM in the presence of 1 μM glycine whereas our data indicates an IC_{50} of 24 μM. The reason for this potency difference is not clear, but could relate to the use of intact cells as opposed to broken membrane preparations.

In contrast to its potent action on NMDA-mediated responses, we found no significant effect of cycloleucine on QA- and KA-evoked responses at concentrations that produced marked NMDA inhibition. We cannot, however, exclude the possibility that cycloleucine would affect these responses at higher concentrations than those tested.

The effect of cycloleucine on the NMDA response was not voltage-dependent, as expected of a molecule that is uncharged at physiological pH. In this regard, cycloleucine is similar to competitive NMDA receptor antagonists like 2-amino-5-phosphonovaleric acid (MacDonald *et al.*, 1987) and it differs from open channel blockers such as Mg^{2+}, ketamine, phencyclidine and MK-801. These latter agents exhibit a strong voltage-dependence to their blocking effect as a result of their intrinsic charge and because they bind to a site within the transmembrane electrostatic field (Nowak *et al.*, 1984; Mayer *et al.*, 1984; Honey *et al.*, 1985; Mayer & Westbrook, 1987; MacDonald *et al.*, 1987; Huettner & Bean, 1988).

The voltage-independent, allosteric block of NMDA responses produced by cycloleucine and other antagonists of the strychnine-insensitive glycine site may offer certain theoretical advantages over the block produced by either the competitive antagonists, whose effect can be overcome by an

increase in the agonist concentration, or the open channel blocking agents, which are expelled from the channel with depolarization (and also tend to have actions on many other channel types: ffrench-Mullen *et al.*, 1988; Rothman, 1989). While drugs that operate by this novel mechanism will certainly have value as tools to investigate the role of NMDA receptors in brain function, it remains to be determined if they will have practical usefulness as therapeutic agents in the treatment of neurological disorders.

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Evidence for two P_2-purinoceptor subtypes in human small pulmonary arteries

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1 P_2-purinoceptors have not been characterized in human pulmonary vessels and we therefore examined the effects of adenosine 5'-triphosphate (ATP) and its analogues on human isolated small pulmonary arteries (SPA) *in vitro*.

2 Contractile responses were induced by all of the analogues, with the rank order of potency α,β-methylene-ATP (α,β-meATP) = β,γ-methylene-ATP (β,γ-meATP) > ATP > 2-methylthio-ATP, indicating the presence of vasoconstrictor P_{2x} receptors.

3 In precontracted SPA, vasodilator responses were produced by all of the analogues. The rank order of potency for the analogues causing vasodilator responses was: 2-methylthio-ATP $\gg$ ATP $\gg$ β,γ-meATP = α,β-meATP, indicating a vasodilator P_{2y} receptor.

4 Removal of endothelial cells had no significant effect on either the contractile or relaxant responses to any of the analogues.

5 After pretreatment of the endothelium-denuded vessels with α,β-meATP (to desensitize P_{2x} receptors), the contractile response to β,γ-meATP (a potent P_{2x} receptor agonist) was abolished.

6 We conclude that both P_{2x}- and P_{2y}-purinoceptors are present in human SPA and that both receptors reside on the vascular smooth muscle.

Introduction

Since Burnstock & Kennedy (1985) first proposed the division of P_2-purinoceptors into two subtypes, P_{2x} and P_{2y}, these receptors have been identified on a variety of vessels and vascular beds of different species including rat pulmonary artery and vein (White *et al.*, 1985; Kennedy *et al.*, 1985; Burnstock & Warland, 1987; Bertrand *et al.*, 1987; Houston *et al.*, 1987; Liu *et al.*, 1989). Evidence from these studies suggests that the distribution of P_2-purinoceptor subtypes on blood vessels varies between species and different vascular beds. Thus, in rabbit mesenteric artery and portal vein both P_{2x} and P_{2y} receptors are located on the smooth muscle (Mathieson & Burnstock, 1985; Kennedy & Burnstock, 1985). By contrast, in rat mesenteric and dog coronary arteries, P_{2x} and P_{2y} receptors reside on the smooth muscle and endothelium respectively (Ralevic & Burnstock, 1988; Houston *et al.*, 1987). In rabbit, pig and rat aorta, endothelium-dependent relaxation by ATP has been reported (Furchgott, 1983; Martin *et al.*, 1985; White *et al.*, 1985). Additionally, it has been demonstrated that ATP, the P_2-purinoceptor agonist, relaxes precontracted human pulmonary arteries *in vitro* (Greenberg *et al.*, 1987) and causes pulmonary vasodilatation in subjects with chronic obstructive pulmonary disease (Gaba *et al.*, 1986). However, the function and distribution of P_2-purinoceptor subtypes in human pulmonary vessels have not been described.

P_{2x} and P_{2y}-purinoceptor subtypes are defined by the rank order of potency of ATP analogues in causing vasoconstrictor and vasodilator responses respectively. The potency order of ATP analogues at the P_{2x} receptor is α,β-methylene-ATP (α,β-meATP) β,γ - methylene - ATP = (β,γ - meATP) > ATP = 2 - methylthio-ATP, and at the P_{2y} receptor is 2-methylthio-ATP $\gg$ ATP > α,β-meATP, β,γ-meATP (Burnstock & Kennedy, 1985). The P_{2x}-receptor can be selectively desensitized by α,β-meATP (Kasakov & Burnstock, 1983), while reactive blue 2 (RB2) has been demonstrated to be an antagonist at the P_{2y} receptor (Manzini *et al.*, 1986; Burnstock & Warland, 1987; Reilly *et al.*, 1987).

Using this receptor classification and definition we have characterized the P_2-purinoceptor subtypes in human small pulmonary arteries (SPA). We also attempted to analyse the distribution of P_2-purinoceptors on human SPA by comparing the

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contractile and relaxant responses to ATP analogues in vessels with and without an intact endothelium.

Methods

Tissue preparation

Fresh human lung tissue was obtained from patients undergoing lobectomy or pneumonectomy for removal of bronchial carcinoma. No patient had clinical evidence of pulmonary vascular disease or asthma. The lung specimen was placed in oxygenated Krebs-Henseleit (KH) solution at 4°C and transported immediately to the laboratory. SPA (300–500 μm internal diameter) were dissected, carefully cleaned of surrounding connective tissue and cut into rings of 3–4 mm in length. Most of the vessels were used on the day of surgery and all tissues were used within 18 h. Rings were stored in KH solution of the following composition (mM): NaCl 118, KCl 5.9, $MgSO_4.7H_2O$ 1.2, $CaCl_2.6H_2O$ 2.5, $NaH_2PO_4.H_2O$ 1.2, glucose 5.6 and $NaHCO_3$ 25.5, maintained at 4°C and continuously aerated with a gas mixture of 95% O_2:5% CO_2. There were no differences in the responses of the vessels stored for 18 h and those used immediately. In some of the SPA rings, endothelium was removed mechanically by gently rotating a small steel probe in the lumen for 20–30 s. Removal of the endothelium was confirmed by loss of relaxant response to 0.1–10 μM acetylcholine in precontracted vessels (Greenberg *et al.*, 1987).

SPA rings were mounted over a pair of stainless steel wires and suspended in an organ bath containing 2 ml KH solution. One wire was fixed and the other attached to a force transducer (FT. 03 Grass, Quincy, Mass; U.S.A.). Changes in isometric force were recorded on a polygraph (Grass Model 7). The KH solution was maintained at 37°C and continuously bubbled with a gas mixture of 95% O_2:5% CO_2. The rings were gradually stretched to a resting tension of 600 mg, which was found to be optimal for determining changes in tension, and then allowed to equilibrate in the bath for at least 1 h. Tissues were washed with fresh KH solution every 20 min during the equilibration period.

Experimental protocol

To study contractile responses, α,β-meATP, β,γ-meATP, ATP and 2-methylthio-ATP were added to the organ bath in a cumulative manner starting with the vessels at resting tension, and dose-response curves to each analogue were obtained. In separate experiments SPA rings were pretreated with 100 μM α,β-meATP to desensitize the P_{2x} receptors (Kasakov & Burnstock, 1983). Treatment of the vessels with α,β-meATP caused a large transient contraction and after tension had returned to baseline, a cumulative dose-response curve to β,γ-meATP was obtained in the continued presence of α,β-meATP. At the end of each experiment vessels were maximally contracted with 60 mM KCl.

To study relaxation, rings of SPA were precontracted with 1 μM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and after a stable contraction was obtained, the ATP analogues (α,β-meATP, β,γ-meATP, 2-methylthio-ATP and ATP) were added to the organ bath in a cumulative manner. In some experiments, rings of SPA were incubated with 10 μM RB2 for 20 min before studying relaxation.

Since both contractile and relaxant responses to the ATP analogues were transient, the subsequent concentration of each analogue was added to the organ bath as soon as the previous concentration had attained a maximal effect.

Drugs

Prostaglandin $F_{2\alpha}$ sterile solution (Upjohn Ltd, Crawley, Sussex) was diluted with KH solution. Acetylcholine chloride, adenosine 5'-triphosphate, α,β-methylene-ATP, β,γ-methylene-ATP, reactive blue 2 (Sigma, Poole, UK), and 2-methylthio-ATP (a gift from Ciba-Geigy, Basle, Switzerland) were dissolved in distilled water and diluted with KH solution.

Analysis of results

Contraction was expressed as the percentage of the maximal contraction induced by KCl (60 mM). Relaxation was expressed as a percentage of 1 μM $PGF_{2\alpha}$-induced contraction. Agonists and antagonist potency were measured by $-\log EC_{15}$ and $-\log IC_{30}$ values, where $-\log EC_{15}$ is the negative logarithm of the concentration of agonist needed to produce 15% of the maximal KCl contraction. $-\log IC_{30}$ is the negative logarithm of the concentration of agonist required to cause a 30% reduction of the $PGF_{2\alpha}$ induced tone. Data were expressed as means $\pm$ s.e.mean. Statistical analysis of the results was performed by use of Student's t test for paired and unpaired data. A P value of <0.05 was considered significant.

Results

Contraction and P_{2x} receptor desensitization

In the endothelium-denuded SPA rings at resting tone, all of the ATP analogues produced concentration-dependent contraction, with a rank order of potency: α,β-meATP = β,γ-meATP > ATP >

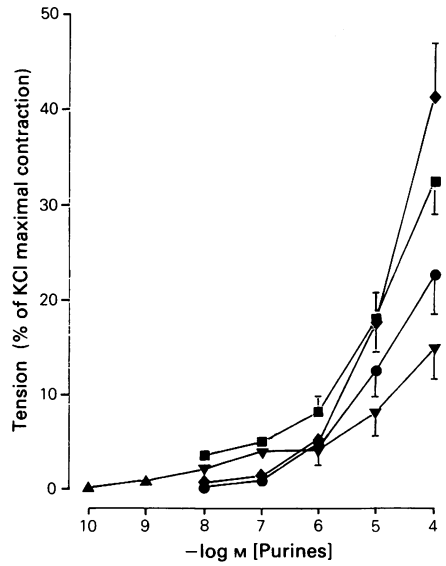

Figure 1 Cumulative dose-response curves for contractile responses to α,β-methylene-ATP (■),β,γ-methylene-ATP (◆), ATP (●) and 2-methythio-ATP (▲) in the endothelium-denuded rings of small pulmonary arteries at resting tone. Tension is expressed as the percentage of 60 mM KCl maximal contraction. Data represent the mean values of 13–15 rings from 6–9 patients. The vertical lines indicate s.e.mean.

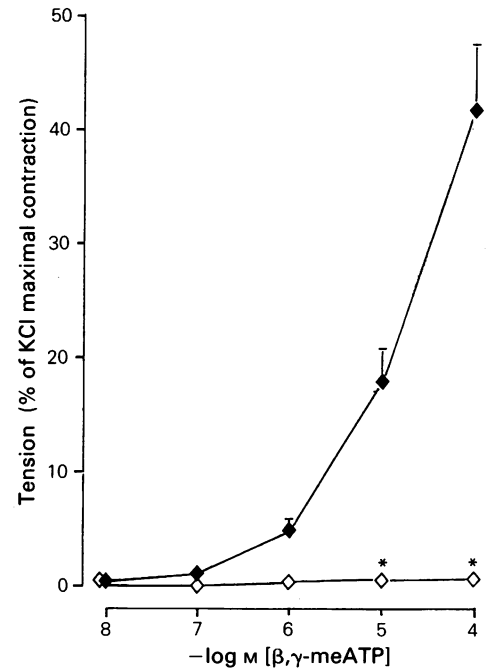

Figure 2 ' Effects of P_{2x} receptor desensitization by α,β-methylene-ATP (α,β-meATP) on the contractile response of the endothelium-denuded SPA rings to β,γ-methylene-ATP. Tension is expressed as the percentage of 60 mM KCl maximal contraction: (◆) control; (◇) after desensitization by α,β-meATP, * $P < 0.05$, compared to control. Data represent the mean values of 6–15 rings from 4–9 patients. Vertical lines indicate s.e.mean.

2-methylthio-ATP (Figure 1). Since the contractile responses to ATP analogues did not reach maximal amplitude at concentrations up to 100 μM, EC_{50} values for the analogues could not be obtained. However, comparison of the mean −log EC_{15} values showed that α,β-meATP was 4 and 21 fold more potent than ATP and 2-methylthio-ATP in inducing a vasoconstrictor response. These results indicate the presence of P_{2x} receptors on pulmonary vascular smooth muscle.

A single dose of α,β-meATP (100 μM) caused a large transient contraction of SPA. After tension had returned to baseline and in the continued presence of α,β-meATP, β,γ-meATP, a potent P_{2x}-agonist, failed to elicit contraction (Figure 2).

Relaxation

All the ATP analogues studied caused a dose-dependent relaxation of the precontracted SPA rings with intact endothelium. The most potent analogue was 2-methylthio-ATP, causing 97% relaxation at a concentration of 100 μM. The rank order of potency for the ATP analogues inducing vasodilator responses was: 2-methylthio-ATP ≫ ATP ≫ β,γ-meATP = α,β-meATP (Figure 3). The IC_{50} values could not be calculated therefore the mean −log IC_{30} values were compared. In inducing the relaxant response, 2-methylthio-ATP was 540 and 41687 fold more potent than ATP and β,γ-meATP. For α,β-meATP an IC_{30} value was not obtained as the maximal relaxation was 23% at a concentration of 100 μM.

RB2 was evaluated to determine whether it antagonized the P_{2y}-receptor-mediated vasodilator response to ATP and 2-methylthio-ATP. We found that RB2 reduced the relaxant responses to low concentrations of 2-methylthio-ATP (0.01 nM–0.1 μM) significantly (Figure 4), but had no effect on relaxant responses to higher concentrations of 2-methylthio-ATP (1–100 μM). Similar results was obtained with ATP ($n = 4$, data not shown).

Effect of endothelial removal

The contractile and relaxant responses to ATP analogues were compared between the SPA rings with

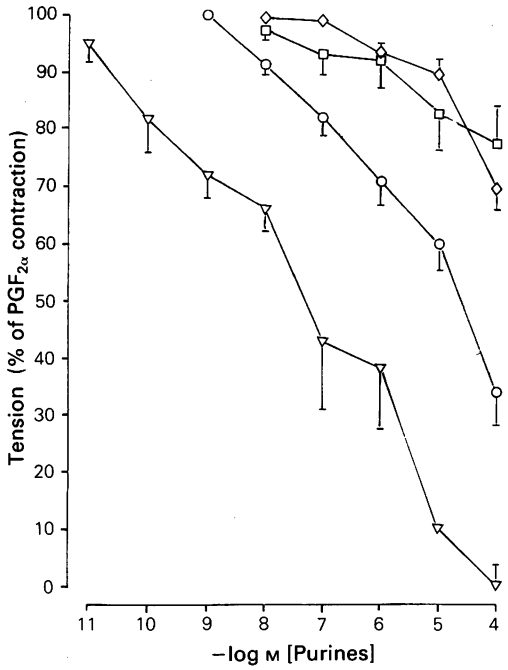

Figure 3 Cumulative dose-response curves for relaxant responses to 2-methylthio-ATP (▽), ATP (○), β,γ-methylene-ATP (◇) and α,β-methylene-ATP (□) in endothelium intact rings of small pulmonary arteries precontracted with 1 μM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Tension is expressed as a percentage of the $PGF_{2\alpha}$ contraction. Data represent the mean values of 6–10 rings from 4–6 patients. Vertical lines indicate s.e.mean.

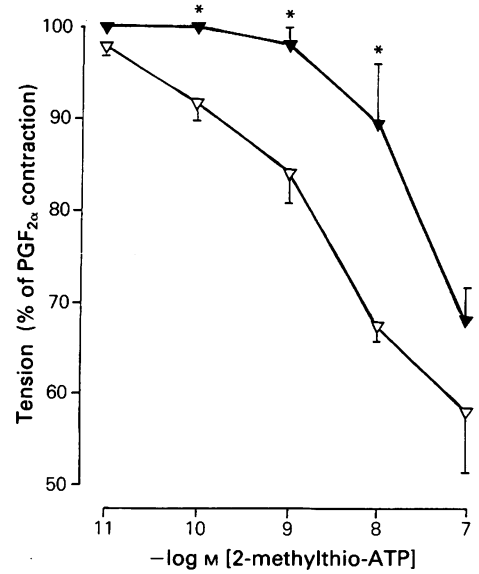

Figure 4 Effects of reactive blue 2 (RB2, 10 μM) on the relaxant response to 2-methylthio-ATP. Tension is expressed as a percentage of 1 μM $PGF_{2\alpha}$-induced contraction: (▽) control; (▼) RB2; * $P < 0.05$, compared to control. Data represent the mean value of 4 rings from 3 patients. Vertical line indicates s.e.mean.

and without an intact endothelium. Removal of the endothelium did not significantly affect either the contractile responses to any of the analogues (Figure 5) or the relaxant responses to 2-methylthio-ATP, ATP and β,γ-meATP (Figure 6), whereas the relaxation caused by α,β-meATP was variably altered by endothelial removal (Figure 6).

Discussion

The main findings of this study are that two types of P_2-purinoceptor are present in human isolated SPA and both P_{2x} and P_{2y} receptors are located on the vascular smooth muscle. P_{2x} receptors mediate a vasoconstrictor response and P_{2y} receptors mediate a vasodilator response.

P_{2x} and P_{2y}-purinoceptors are defined according to the potency rank order of ATP analogues in eliciting vasoconstrictor and vasodilator responses (Burnstock & Kennedy, 1985). In our study, the rank order of potency of ATP analogues producing vasoconstriction in endothelium-denuded rings of SPA was α,β-meATP = β,γ-meATP > ATP > 2-methylthio-ATP, indicating that the P_2 receptors involved in the vasoconstrictor response of human SPA to ATP is of the P_{2x}-purinoceptor subtype. This is consistent with the reported observations on both systemic vessels (Houston *et al.*, 1987; Burnstock & Warland, 1987; Hopwood & Burnstock, 1987) and rat pulmonary vessels (Liu *et al.*, 1989).

α,β-meATP can selectively desensitize P_{2x}-purinoceptors (Kasakov & Burnstock, 1983) and has been used to desensitize P_{2x} receptors both in blood vessels (Kugelgen & Starke, 1985; Muramatsu, 1986; Ralevic & Burnstock, 1988; McCormack *et al.*, 1989a) and other tissues (Stjarne & Astrand, 1985). Our results show that after pretreatment with 100 μM α,β-meATP, the vasoconstrictor response to β,γ-meATP, a potent P_{2x} agonist, was virtually abolished in human SPA, confirming that P_{2x} receptors are responsible for the vasoconstrictor response to ATP.

The rank order of agonist potency in causing vasodilatation of human SPA was: 2-methylthio-ATP $\gg$ ATP $\gg$ β,γ-meATP = α,β-meATP, establishing that the P_2-purinoceptor mediating vasodilatation is a P_{2y} receptor (Burnstock & Kennedy, 1985).

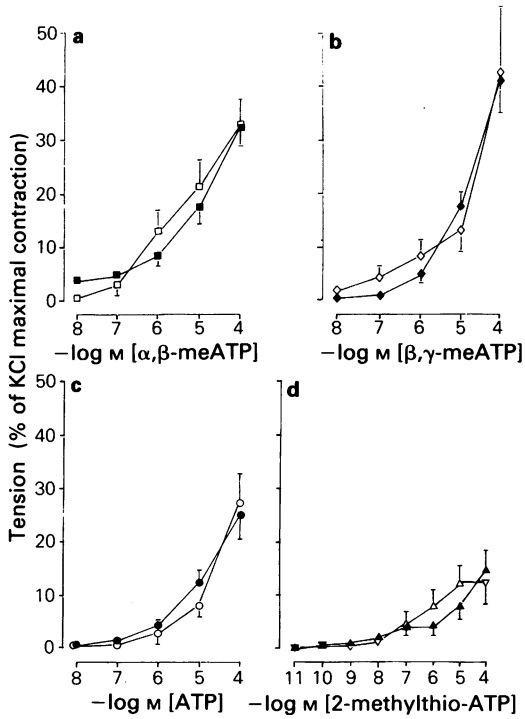

Figure 5 Effects of endothelial denudation on the contractile responses of small pulmonary artery rings to α,β-methylene-ATP (a), β,γ-methylene-ATP (b), ATP (c) and 2-methylthio-ATP (d). Tension is a percentage of maximal contraction to 60 mM KCl. Solid symbols represent rings without endothelium. Open symbols represent rings with endothelium. Data represent the mean values of 6–15 rings from 4–9 patients. Vertical lines indicate s.e.mean.

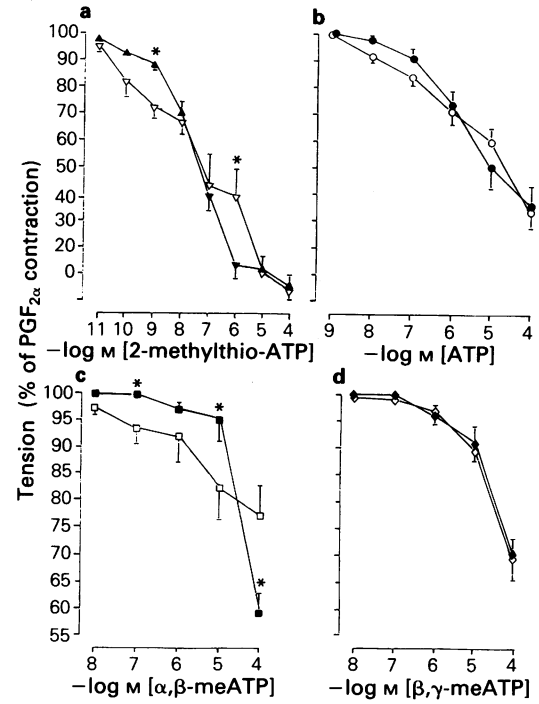

Figure 6 Effects of endothelial denudation on the relaxant responses of small pulmonary arteries to 2-methylthio-ATP (a), ATP (b), α,β-methylene-ATP (c) and β,γ-methylene-ATP (d). Tension is expressed as the percentage of 1 μM prostaglandin $F_{2\alpha}$-induced contraction. Solid symbols represent rings wihout endothelium. Open symbols represent rings with endothelium. * $P < 0.05$. Data represent the mean values of 5–14 rings from 4–9 patients. Vertical lines indicate s.e.mean.

We found in human SPA that RB2 antagonized the vasodilator responses to 2-methylthio-ATP and ATP, but this was only observed at low concentrations of 2-methylthio-ATP and ATP, presumably because RB2 is a weak competitive antagonist and higher concentrations of the ATP analogues are able to overcome the antagonist effect.

Since the crucial role of endothelium in acetylcholine-mediated vasodilatation was first demonstrated (Furchgott & Zawadski, 1980), many vasodilators have been found to exert their vasodilator action via receptors on the endothelium. ATP has been reported to cause endothelium-dependent relaxation of both pulmonary (De Mey & Vanhoutte, 1982; Frank & Bevan, 1983; Greenberg *et al.*, 1987; Liu *et al.*, 1989) and systemic (De Mey & Vanhoutte, 1982; Martin *et al.*, 1985; Houston *et al.*, 1987) vessels of several species. In these studies removal of the endothelium abolished the vasodilator effects of ATP and/or 2-methylthio-ATP, leading to the conclusion that P_{2y} receptors are located on the endothelium (Burnstock & Kennedy, 1986; Houston *et al.*, 1987; Ralevic & Burnstock, 1988). It is possible that ATP may be metabolised to adenosine, which relaxes human SPA via A_2-receptors (McCormack *et al.*, 1989b), but this would not account for the relaxant effect of ATP analogues (such as 2-methylthio-ATP) which are not metabolised to adenosine. Our results demonstrating that removal of the endothelium (which was confirmed by lack of vasodilator response to acetylcholine) did not have a significant effect on the vasodilator responses to any of the ATP analogues indicates that P_{2y} receptors are located on the smooth muscle of SPA. Similar findings have been obtained for *in vitro* rabbit portal vein and mesenteric artery preparations (Kennedy & Burnstock, 1985; Mathieson & Burnstock, 1985). However, in a previous report

from our laboratory we have shown that endothelium removal significantly diminishes (but did not abolish) the ATP-induced relaxation of large (2–4 mm i.d.) human pulmonary arteries (Greenberg *et al.*, 1987). One explanation for this difference may be that P_{2y} receptor localisation differs between large and small pulmonary arteries such that as vessels become smaller a greater proportion of the P_{2y} receptors reside on smooth muscle.

In conclusion, P_2 purinoceptors mediate both contraction and relaxation of human small pulmonary arteries. The vasoconstrictor effect of ATP is mediated via P_{2x}-purinoceptors located on the smooth muscle, and the vasodilator effect of ATP is mediated via P_{2y}-purinoceptors which reside on the smooth muscle.

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Multiple sources of calcium for contraction of the human urinary bladder muscle

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1 KCl, carbachol, neurokinin A and endothelin produced concentration-dependent contractions of mucosa-free muscle strips from the dome of the human urinary bladder. The maximal response to carbachol or neurokinin A exceeded that to KCl, while the maximal response to endothelin approached that to KCl.

2 Nifedipine (1 μM) abolished the response to KCl, reduced the response to carbachol or neurokinin A but had no effect on the response to endothelin. Bay K 8644 (1 μM) markedly potentiated the response to KCl but had little or no effect on the response produced by the other stimulants.

3 Superfusion of the strips with a nominally calcium (Ca)-free medium containing EDTA (1 mM) for 30 min markedly reduced the response to carbachol, neurokinin A and endothelin, although a small response was still evident at high concentrations. Likewise, after a prolonged (60 min) superfusion of the strips with a high K (80 mM) Ca-free medium plus EDTA (1 mM) these three agonists still produced a small contractile response.

4 The nifedipine (1 μM) resistant response to carbachol, neurokinin A or endothelin was markedly depressed by $LaCl_3$ (1 mM). In contrast, the nifedipine-(1 μM) resistant response to carbachol was not modified by $NiCl_2$ (0.1 mM) or ω-conotoxin (0.1 μM).

5 Caffeine produced divergent effects depending upon the temperature of incubation: a relaxation at 37°C and a concentration-dependent (2.5–20 mM) contraction at 25°C. The latter was markedly inhibited by procaine (3 mM) but unaffected by nifedipine (1 μM).

6 After a prolonged (60 min) superfusion with a high K, Ca-free medium containing EDTA the response to carbachol (100 μM) was abolished by previous exposure to procaine (3 mM). Conversely, the response to endothelin (1 μM) was unaffected by procaine. The response to endothelin in these experimental conditions was also resistant to $LaCl_3$ (1 mM).

7 These findings indicate that multiple sources of Ca are mobilized for contraction of the human bladder muscle by different stimulants. Dihydropyridine- and voltage-sensitive Ca channels provide the major if not the sole source of Ca for the response to KCl, play some role in the response to muscarinic (carbachol) or NK-2 tachykinin receptor stimulation but are not involved in the response to endothelin. Carbachol, neurokinin A and endothelin all mobilize a Ca pool (either extracellular or located at membrane level) which is $LaCl_3$-sensitive but nifedipine-resistant. Neither T- nor N-type channels appear to be involved in the response to carbachol. In addition, these agents mobilize a tightly bound Ca pool independently from membrane depolarization. This latter pool is probably a procaine-sensitive intracellular source of activator Ca mobilized by caffeine and carbachol. The failure of procaine to prevent the response to endothelin in high K, Ca-free medium raises the possibility that this peptide mobilizes an intracellular source of activator Ca, distinct from the caffeine- and carbachol-sensitive pool.

Introduction

Much evidence indicates that smooth muscles have multiple sources of calcium ions (Ca) which are utilized for contraction (Somlyo & Somlyo, 1968; Bolton, 1979; Brading & Sneddon, 1980; Casteels & Droogmans, 1982; Van Breemen *et al.*, 1982; Bolton & Kitamura, 1983). Extracellular Ca enters the smooth muscle cells via two pathways, that is

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voltage-operated Ca channels (VOCs) and receptor-operated channels (see Bolton 1979, for review). In addition, release of intracellular Ca can be activated by several agonists either via receptor-mediated pathways or via a direct release from the storage site(s) such as in the case of caffeine (Bolton 1979; Brading & Sneddon, 1980; Itoh *et al.*, 1982; 1983; Leijten & Van Breemen, 1984).

Sources of activator Ca for contraction have been widely investigated for vascular and intestinal smooth muscle. Less information is available with regard to the detrusor muscle of the urinary bladder. Information in this field appears of particular interest because of the possible use of drugs with Ca entry blocking properties in the management of bladder hypermotility disorders such as detrusor instability or detrusor hyperreflexia.

Studies in animals have suggested that multiple sources of activator Ca are present in the bladder muscle. Ca entry via dihydropyridine-sensitive Ca channels has been proposed as a likely mechanism for bladder contraction in the detrusor muscle from rabbits, guinea-pigs or rats (Huddart & Butler, 1986; Batra *et al.*, 1987; Maggi *et al.*, 1988a). In the guinea-pig and rat isolated bladder muscle mobilization of intracellular Ca seems also a relevant process for agonist-induced contraction (Mostwin, 1985; Maggi *et al.*, 1988a). *In vivo* studies in anaesthetized rats suggested that Ca mobilization via nifedipine-resistant pathways might be a relevant mode of contraction during a physiological-like event, that is, volume-evoked bladder voiding (micturition reflex) (Maggi *et al.*, 1988a). Fovaeus *et al.* (1987) investigated the effect of nifedipine and Bay K 8644 on carbachol-induced contractions of human isolated bladder muscle and concluded that both voltage-dependent and receptor-operated Ca channels mediate this response.

Recently we studied the effect of endothelin, an endothelium-derived vasconstrictor peptide proposed to act as an endogenous agonist at dihydropyridine-sensitive Ca channels (Yanagisawa *et al.*, 1988) in the human isolated bladder (Maggi *et al.*, 1989a) and found that the contractile response to this peptide was almost totally nifedipine-resistant while the KCl-induced contraction was abolished by nifedipine-pretreatment. These observations prompted us to re-address (Fovaeus *et al.*, 1987) the question of the sources of activator Ca for contraction of the human detrusor muscle. With this aim, four agonists were selected, that is KCl, carbachol, neurokinin A and endothelin, in order to assess whether different agonists might mobilize different Ca sources for contraction, and the effect of nifedipine, Bay K 8644, $LaCl_3$ and Ca-free media on the responses produced by these agents was investigated.

Methods

Specimens from the dome of human urinary bladder were obtained from 25 (18 males and 7 females) patients (age range 51–79 years) undergoing total cystectomy for carcinoma of the bladder base. Specimens were collected from patients showing no signs of urinary retention or detrusor instability. No patient received radio- or chemotherapy before intervention. Pre-anaesthetic medication was intramuscular atropine (1.0 mg) and diazepam (10 mg), 2 h before surgery. Anaesthesia was induced by thiopentone sodium (500 mg, i.v.) and maintained with NO_2/O_2 (1/2) and halothane (0.6–1%). During induction of anaesthesia, the patients received pancuronium bromide (6 mg, i.v.). All specimens appeared macroscopically normal, with no signs of tumour or inflammation. Tissues were placed in ice-cold physiological salt solution within 2–3 min from surgical removal. Most experiments were performed on strips which were stored overnight in ice-cold physiological salt solution and continuously gassed with 96% O_2 and 4% CO_2 as described previously (Maggi *et al.*, 1988b; 1989a). Some experiments were performed within 3 h of surgery ('fresh' strips).

From each sample, 4 to 6 small strips of mucosa-free muscular tissue were excised and mounted in 5 ml baths for isolated organs, maintained at 37°C, containing physiological salt solution continuously gassed with 95% O_2 and 5% CO_2. In some experiments the bath temperature was kept at 25°C. Changes in isometric tension were recorded by means of a force-displacement transducer connected to a Basile 7050 pen recorder. Resting load was 10 mN.

After a 60–90 min equilibration period the strips were repeatedly exposed to KCl (80 mM, added to the bath) until responses were reproducible. This response served as an internal standard for all experiments.

Cumulative concentration-response curves to the various agonists were constructed as described by Van Rossum (1963), the next concentration being added when the effect of the preceding one had reached a steady state.

The composition (mM) of the physiological salt solution was as follows: NaCl 119, $NaHCO_3$ 25, glucose 11, KCl 4.7, $MgSO_4$ 1.5, KH_2PO_4 1.2 and $CaCl_2$ 2.5.

In some experiments a nominally Ca-free solution was prepared by omission of $CaCl_2$, replaced by NaCl. In other experiments a high K, Ca-free solution was prepared by adding 80 mM KCl to the Ca-free solution. In these 'Ca-free' solutions the contaminating Ca due to impurities present in the other salts was 6.7 μM. EDTA (1 mM) was added to both the Ca-free and the high K, Ca-free solutions. In

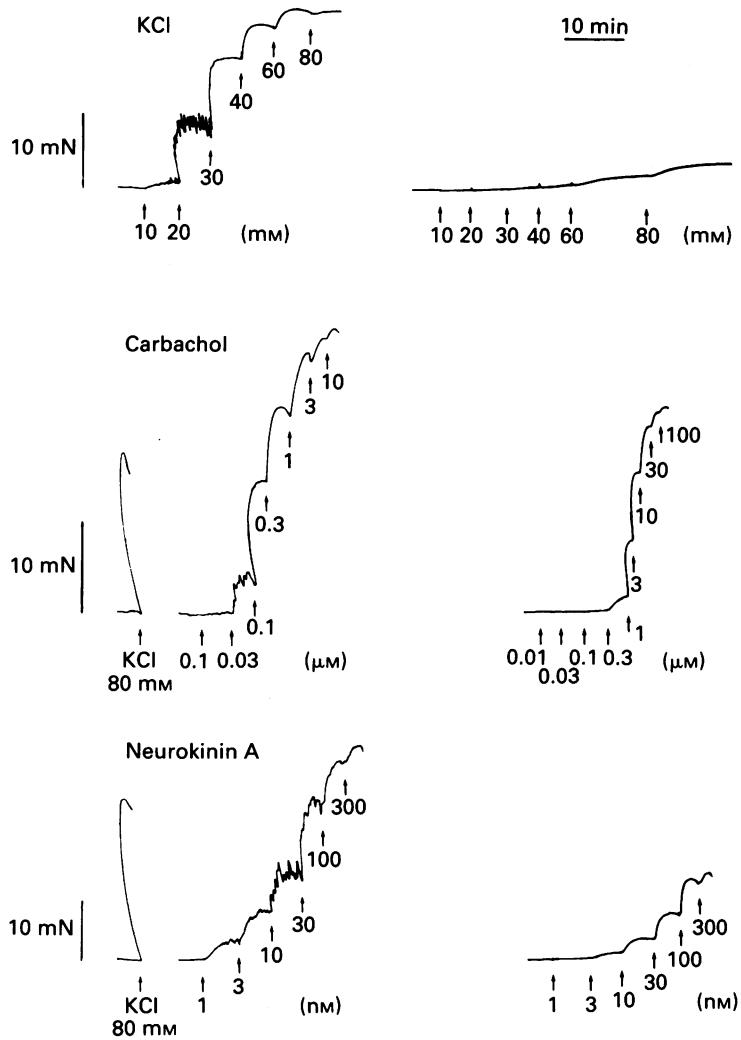

Figure 1 Typical tracings showing the contractile response of the human isolated bladder muscle strips to cumulative addition of KCl, carbachol or neurokinin A and inhibition of the response by 1 μM nifedipine (30 min beforehand, right section in each panel).

experiments involving the effect of $LaCl_3$, the physiological salt solution had the following composition (mM): NaCl 144, glucose 11, Trizma 7.7 (Sigma) 5, KCl 4.7, $MgSO_4$ 1.5 and $CaCl_2$ 2.5 which was gassed with 100% oxygen. The high K, Ca-free solution used for experiments with $LaCl_3$ had the following composition (mM): NaCl 147.8, KCl 80, glucose 11, Trizma 5, EDTA 1, $MgSO_4$ 1.5.

In experiments involving the Ca-free media, the strips were superfused with the modified solution for 30 or 60 min at a rate of 10 ml min^{-1} by means of a peristaltic pump. Superfusion of the strips was stopped 5 min before application of the agonists which were added directly to the bath.

Statistical analysis

Each value given in Results section is mean $\pm$ s.e.mean. Statistical analysis of the data was made by Student's t test or analysis of variance, when applicable.

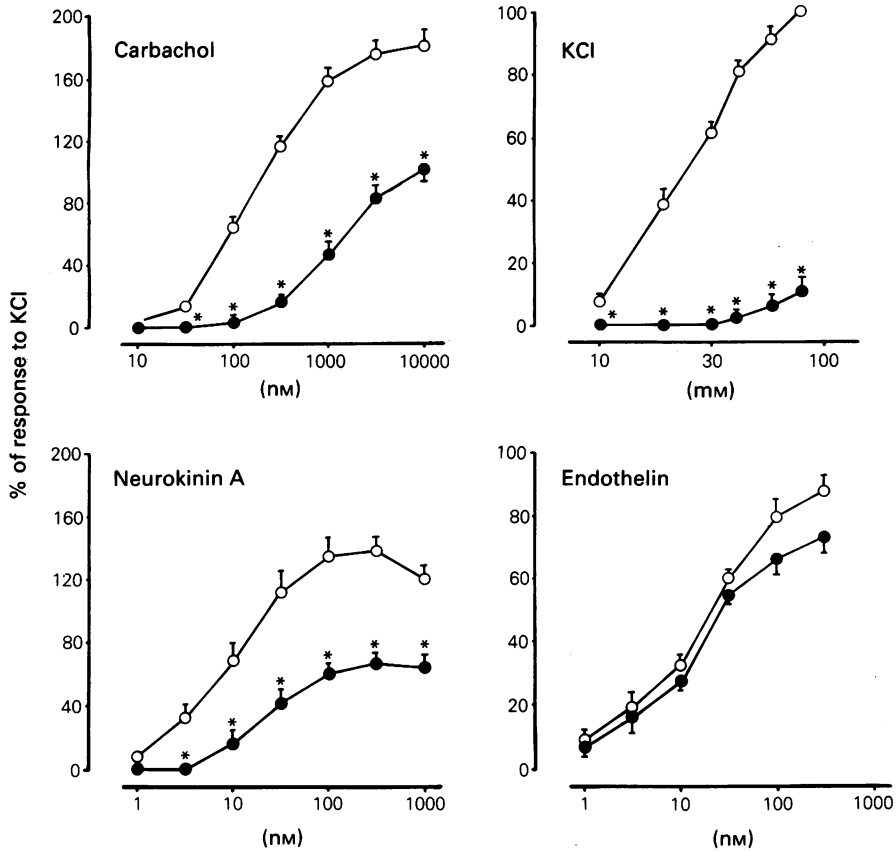

Figure 2 Effect of nifedipine (1 μM, 30 min beforehand, ●) on the concentration-response curve to carbachol, KCl, neurokinin A or endothelin in the human isolated bladder muscle strips; (○) control response. Each value is mean of at least 5 experiments; s.e. indicated by vertical bars. *Significantly different from controls, $P < 0.05$.

Drugs

Drugs used were: carbachol (Merck), atropine HCl (Serva), caffeine, nifedipine and $NiCl_2$ (Sigma), Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate, Bayer), ω-conotoxin fraction GVIA and porcine endothelin (Peninsula), $LaCl_3$ (Fluka) and procaine HCl (Gianni). Neurokinin A was synthesized by conventional solid-phase methods by Dr P. Rovero, Chemistry Department, Menarini Pharmaceuticals.

Results

General

The study was performed on 123 strips from 25 patients; 32 strips were studied within 3 h of surgery ('fresh' strips) while the remainders were stored overnight. In some experiments, when reproducible contractile responses to KCl (80 mM, added to the bath) had been obtained, the strips were allowed to equilibrate for 60 min to determine whether spontaneous activity was present. This was observed in 8 out of 15 'fresh' strips (from 5 patients) and in 10 out of 50 'stored' strips (from 15 patients) ($P < 0.01$).

All strips responded to carbachol, neurokinin A, endothelin or KCl with concentration-related contractions. No major differences in sensitivity or maximal response to these agonists were observed in fresh vs. stored strips and therefore data obtained in these two different conditions were pooled.

The contractile response produced by carbachol, neurokinin A or KCl was readily reversed on washing. A second concentration-response curve to these agonists, made 45 min after the first one gave reproducible results ($n = 3$ each, data not shown). By contrast, the effect of endothelin was difficult to remove by washing (Maggi *et al.*, 1989a).

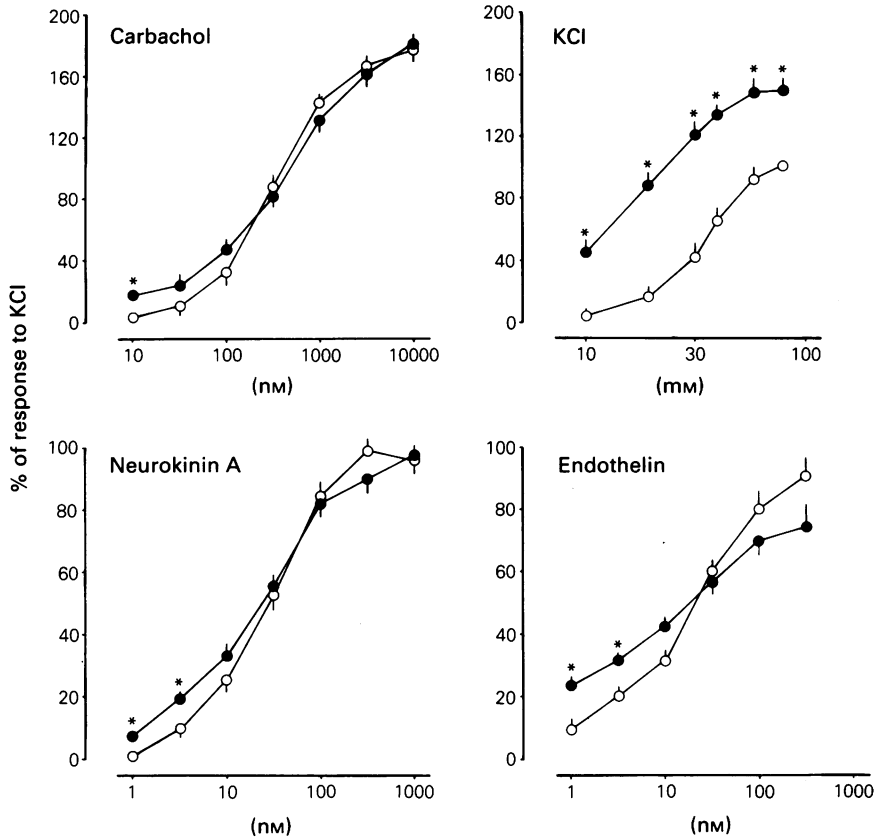

Figure 3 Effect of Bay K 8644 (1 μM, 30 min beforehand ●) on the concentration-response curve to carbachol, KCl, neurokinin A or endothelin in the human isolated bladder muscle strips; (○) control response. Each value is mean of at least 5 experiments; s.e. indicated by vertical lines. *Significantly different from controls, $P < 0.05$.

Effect of nifedipine

In accordance with our previous findings (Maggi *et al.*, 1989a), nifedipine (1 μM for 30 min) abolished the contractile response to KCl but had no significant effect on the response to endothelin (Figures 1 and 2). Carbachol- or neurokinin A- induced contractions were inhibited by nifedipine, the effect being more evident at low concentrations of the agonists. A response to carbachol or neurokinin A was always observed at the highest concentrations of the agonists (Figures 1 and 2).

Effect of Bay K 8644

Human bladder strips responded to Bay K 8644 (1 μM) with a contraction in 14 out of 18 cases. In responders, the magnitude of this contraction ranged between 8 and 37% of that to KCl (80 mM); it peaked in 5–10 min and then declined slowly to baseline. At the time of administration of the various agonists (30 min after Bay K 8644), tension had fully recovered or was below 15% of the maximal response to KCl (80 mM). In the presence of Bay K 8644, the concentration-response curve to KCl was markedly enhanced as compared to controls and a higher maximal contraction was attained (Figure 3).

By contrast, the concentration-response curves to carbachol, neurokinin A or endothelin were barely influenced by Bay K 8644 (Figure 3) with the exception of low concentrations of the agonists (10 nM for carbachol, 1 and 3 nM for neurokinin A, 1 and 3 nM for endothelin) for which slightly higher contractions than controls were noted (Figure 3).

Effect of a Ca-free medium plus EDTA

The responses to carbachol, neurokinin A and endothelin were markedly reduced after superfusion (30 min) with a Ca-free medium containing 1 mM EDTA (Figure 4) although a response was still

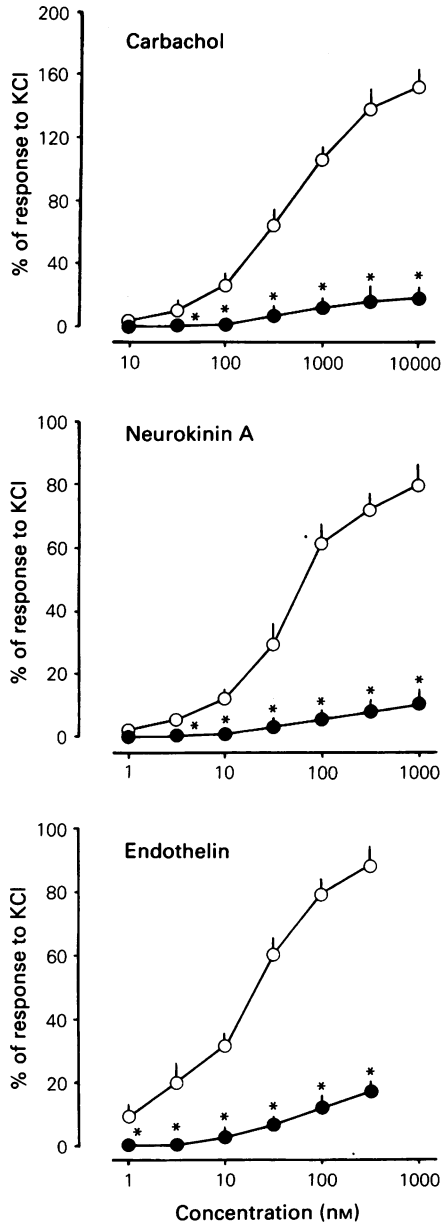

Figure 4 Effect of 30 min superfusion with a Ca-free plus EDTA (1 mM, ●) solution on the concentration-response curve to carbachol, neurokinin A or endothelin in the human isolated bladder muscle strips; (○) control response. Each value is mean of at least 5 experiments; s.e. indicated by vertical lines. *Significantly different from controls, $P < 0.05$.

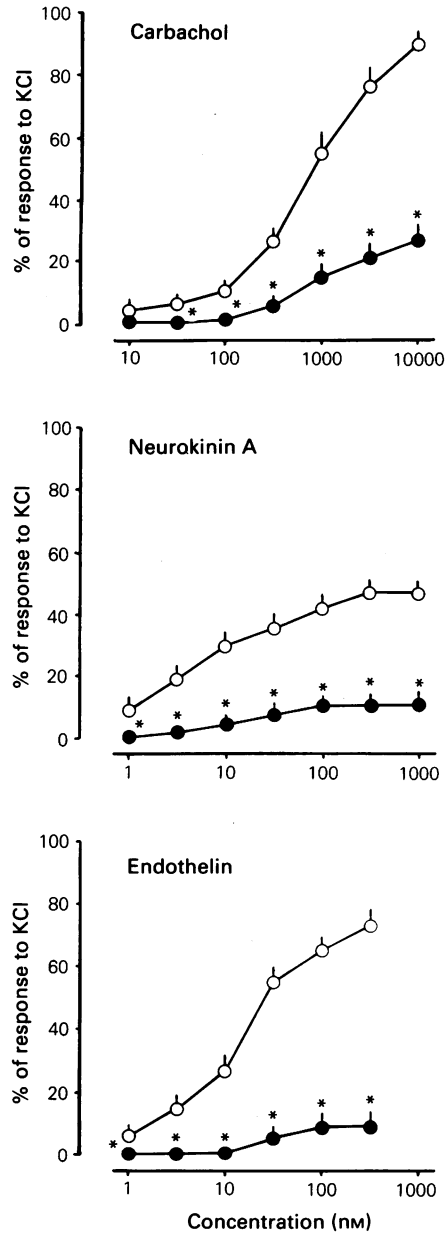

Figure 5 Effect of $LaCl_3$ (1 mM, 30 min beforehand, ●) on the concentration-response curve to carbachol, neurokinin A or endothelin in the human isolated bladder muscle strips; (○) control response. All experiments performed in presence of 1 μM nifedipine. Each value is mean of at least 5 experiments; s.e. indicated by vertical lines. *Significantly different from controls, $P < 0.05$.

Table 1 Contractile response to carbachol, neurokinin A or endothelin of detrusor strips from the human urinary bladder after prolonged (60 min) superfusion with a high K Ca-free medium plus EDTA

		A	B	C
	No. of experiments	*Response to KCl* (mN)	*Response to high K, Ca-free medium* (% of A)	*Response to the agonist* (% of A)
Carbachol	7	26 ± 4	85 ± 4	25 ± 7
Neurokinin A	3	21 ± 3	92 ± 3	5 ± 2*
Endothelin	6	20 ± 4	96 ± 4	18 ± 4

Each value is mean ± s.e.mean. The strips were first exposed to KCl (80 mM, added to the bath for 10 min) until reproducible responses were obtained (A). Then the strips were superfused with a high K (80 mM), Ca-free solution containing EDTA (1 mM). This produced at first a transient phasic contraction (B) followed by a slow return to baseline. At 60 min from the start of superfusion, carbachol (100 μM) neurokinin A (1 μM) or endothelin (1 μM) was added to the bath (superfusion was stopped at 55 min) and its effect recorded (C).
*Significantly different from the response to carbachol, $P < 0.05$.

observed consistently at high concentrations (Figure 4).

Effects of $LaCl_3$ on the response to carbachol, neurokinin A or endothelin

To determine further the role of extracellular and/or membrane-bound Ca, the effect of $LaCl_3$ (1 mM for 30 min) was investigated. In these experiments, after having established reproducible responses to KCl, nifedipine (1 μM) was added to the Krebs solution in order to block Ca influx via dihydropyridine-sensitive Ca channels. Figure 5 shows that $LaCl_3$ further depressed the nifedipine-resistant response to carbachol, neurokinin A and endothelin but failed to abolish completely the response produced by high concentrations of these agonists.

Effect of ω-conotoxin or $NiCl_2$ on the response to carbachol

These experiments were designed to assess whether the nifedipine-resistant but $LaCl_3$-sensitive component of the response to an agonist such as carbachol might involve influx of extracellular Ca via T- or N-type channels. Therefore, concentration-response curves to carbachol were constructed in the presence of nifedipine (1 μM, 30 min before), nifedipine plus $NiCl_2$ (0.1 mM, 30 min before) or nifedipine plus ω-conotoxin fraction GVIA (CTX, 0.1 μM, 30 min before). As shown in Figure 6 neither $NiCl_2$ nor CTX, known blockers of T- and N-type channels, respectively, was capable of exceeding the inhibitory effect produced by nifedipine alone.

Effect of a high K, Ca-free medium plus EDTA

In these experiments, after having recorded reproducible responses to KCl (80 mM added to the bath), the strips were superfused with a high K (80 mM), Ca-free medium plus EDTA (1 mM) at a rate of 10 ml min^{-1} for 60 min. Exposure to this medium produced an immediate large contraction which peaked in 6–10 min and then declined slowly toward baseline. Full recovery of initial tension was achieved in 30–45 min. At 55 min from start of superfusion, the flow was stopped and the preparations allowed to equilibrate for 5 min. At this time carbachol (100 μM, $n = 7$), neurokinin A (1 μM, $n = 3$) or endothelin 1 μM, $n = 6$) was added to the bath. As shown in Table 1, all the agonists produced a contraction,

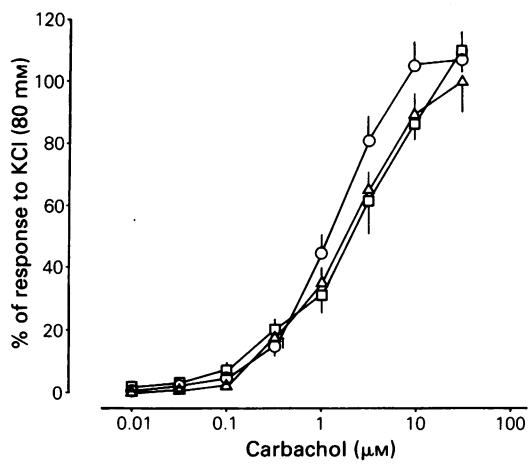

Figure 6 Effect of $NiCl_2$ (0.1 mM, 30 min beforehand) or ω-conotoxin (0.1 μM, 30 min beforehand) on the concentration-response curve to carbachol in the human isolated bladder muscle strips. All experiments performed in presence of 1 μM nifedipine: (○) nifedipine alone; (□) nifedipine plus $NiCl_2$; (△) nifedipine plus ω-conotoxin. Each value is mean of 5 experiments; s.e. indicated by vertical lines.

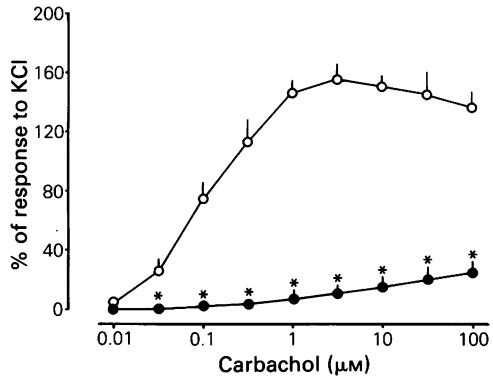

Figure 7 Effect of a prolonged (60 min) superfusion with a high K, Ca-free medium, plus EDTA (1 mM) (●) on the concentration-response curve to carbachol in the human isolated bladder muscle strips; (○) control response. Each value is mean of 5 experiments; s.e. indicated by vertical lines. *Significantly different from controls, $P < 0.05$.

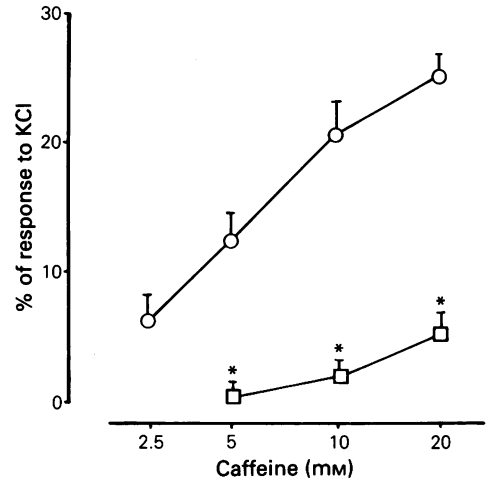

Figure 8 Non-cumulative concentration-dependent contractile response to caffeine (○) on the human isolated bladder muscle strips and its blockade by procaine (3 mM, □). Bath temperature was 25°C. Increasing concentrations of caffeine were added to the bath at 45 min intervals. Each value is mean of 4 experiments; s.e. indicated by vertical bars. *Significantly different from controls, $P < 0.05$.

the response to neurokinin A being significantly smaller than that produced by carbachol or endothelin. The complete concentration-response curve to carbachol obtained under similar experimental conditions and compared to that obtained in controls is shown in Figure 7.

Effect of caffeine

At 37°C, caffeine (5–20 mM) had either no effect or produced a relaxation ($n = 4$ strips from 3 patients). In both cases, the contractile response to KCl (80 mM, added to the bath) was markedly reduced in the presence of caffeine (40–60% inhibition). In other experiments, the bath temperature was kept at 25°C. In these conditions caffeine (2.5–20 mM) produced a concentration-related contraction (Figure 8) which peaked 1–3 min after its addition and then faded to baseline. In two strips from different patients the effects of caffeine were investigated first at 37°C and then at 25°C. In both instances, caffeine produced a contraction only at the lower bath temperature. The caffeine- (5 mM) induced contractions were fully reproducible at 30 min intervals with changes of the bath solution every 5 min. Procaine (3 mM, 15 min beforehand) inhibited the caffeine-induced contractions of the human bladder while nifedipine (1 μM, 30 min beforehand) did not ($n = 4$ from 3 bladders). In fact the caffeine-(5 mM) induced contraction averaged 13 ± 3 and 16 ± 4% of the response to KCl before and after addition of nifedipine, respectively.

Effect of procaine or $LaCl_3$ on the response to carbachol or endothelin in high K, Ca-free medium

In presence of procaine (3 mM, 15 min beforehand) the contractile response produced by 100 μM carbachol in a high K, Ca-free medium (plus EDTA) (25 ± 7% of the response to 80 mM KCl in normal Krebs) was abolished ($n = 3$). By contrast, endothelin (1 μM) induced a response (14 ± 4% of the response to KCl in normal Krebs) not significantly different from that (18 ± 4%, $n = 4$) observed in controls. The response to endothelin in high K, Ca-free medium was also unaffected by previous exposure to 1 mM $LaCl_3$, being 15 ± 3% ($n = 4$) of the response to KCl in normal Krebs solution.

Discussion

In agreement with previous observations (Fovaeus *et al.*, 1987), we found evidence for the presence of dihydropyridine- and voltage-sensitive (L-type) Ca channels in the smooth muscle of the human urinary bladder. This pathway sustains entirely the contractile response to KCl and probably plays some role in the response to carbachol and neurokinin A, as indicated by the partial inhibitory effect of nifedipine. These latter agents exert their effect directly on

smooth muscle cells by activating muscarinic (Zappia *et al.*, 1986) and NK-2 tachykinin receptors (Maggi *et al.*, 1988b), respectively. It appears therefore conceivable that L-type Ca channels might be activated indirectly through membrane depolarization initiated by a receptor-mediated mechanism.

A large fraction of the response to carbachol or neurokinin A was insensitive to nifedipine, although largely dependent upon extracellular Ca as indicated by experiments using the Ca-free medium plus EDTA and antagonism by $LaCl_3$.

We therefore interpret these findings as an indication that carbachol, neurokinin A or endothelin activate influx of extracellular Ca and/or mobilization of Ca pools associated with the plasma membrane, which can refill from the extracellular Ca via a nifedipine-resistant pathway (cf. Fovaeus *et al.*, 1987). This could occur through activation of receptor-operated mechanisms as proposed for many other smooth muscles (Somlyo & Somlyo, 1968; Bolton, 1979). The resulting contractile response could be operationally defined as nifedipine-resistant but $LaCl_3$-sensitive. At least for carbachol, we can exclude T- or N-type voltage-sensitive channels as playing a role in the nifedipine-resistant component of the response, as it was unaffected by either $NiCl_2$ or ω-conotoxin (see Fox *et al.*, 1987; Miller, 1987).

In addition, since high concentrations of the three agonists were still able to induce a contraction in the absence of extracellular Ca, these findings suggest that they may mobilize Ca from an intracellular store (see below).

Caffeine induced a procaine-sensitive but nifedipine-resistant contraction of the human bladder. This occurred only at a low (25°C) bath temperature, while at 37°C a relaxant effect was observed. Caffeine has been shown to contract various smooth muscles by mobilizing an intracellular Ca pool from the sarcoplasmic reticulum (Weber & Hertz, 1968; Casteels & Raeymaekers, 1979; Haeusler *et al.*, 1981; Itoh *et al.*, 1982; 1983; Leijten & Van Breemen, 1984) but smooth muscle relaxation via inhibition of phosphodiesterases and other mechanisms has also been noted (Leijten & Van Breemen, 1984; Sato *et al.*, 1988). We could speculate that at 37°C the relaxant effect of caffeine prevails while at 25°C the relaxant action is somehow slowed or inhibited thus unmasking a contractile effect. However, a relaxant effect of caffeine was evident after waning of the contractile response at low temperature. These findings are in keeping with data of Sato *et al.* (1988) who showed that the transient elevation of intracellular Ca produced by caffeine on rat aorta is enhanced at low temperature (see also Karaki *et al.*, 1987).

In view of the above, we interpret the procaine-sensitive caffeine-induced contraction as evidence for the existence of an intracellular Ca pool in the human bladder muscle. This pool is probably activated also by neurotransmitters via receptor-mediated stimulation. In fact, stimulation of either muscarinic or NK-2 tachykinin receptors produced a small but consistent contractile response after a prolonged incubation in a Ca-free medium plus EDTA and this effect was also observed in K-depolarized preparations.

The fact that procaine was able to block the response to carbachol or endothelin produced after a prolonged superfusion with a Ca-free depolarizing medium suggests that the contraction might involve Ca release from the sarcoplasmic reticulum (Endo, 1977; Itoh *et al.*, 1981). The ability of procaine to block Ca release from the internal store in the human urinary bladder muscle is further indicated by its action toward the caffeine response.

A final point deserving discussion is related to the nature of Ca pools mobilized by endothelin. After the original discovery (Yanagisawa *et al.*, 1988) the suggestion was made that endothelin may act as an endogenous direct activator of dihydropyridine-(L-type) voltage-sensitive Ca channels in both vascular, tracheobronchial and cardiac mucle (Ishikawa *et al.*, 1988a,b; Uchida *et al.*, 1988; Tomobe *et al.*, 1988). However, in some smooth muscles, the contractile response to endothelin is largely or totally resistant to dihydropyridine type Ca channel blockers (Auguet *et al.*, 1988; Maggi *et al.*, 1989a,b). Here we confirm that the response to this peptide in the human bladder muscle is nifedipine-resistant and also substantially unaffected by Bay K 8644. Van Renterghem *et al.* (1988) showed that endothelin acts in a complex manner on the cell membrane: it stimulates metabolism of inositol phosphates (see also Sugiura *et al.*, 1989; Marsden *et al.*, 1989), inducing mobilization of Ca from intracellular stores (Miasiro *et al.*, 1988; Kai *et al.*, 1989), it opens both a Ca-sensitive K channel (transient hyperpolarization) and a non-specific cation channel (prolonged depolarization) which in turn may activate L-type voltage-dependent Ca channels (Van Renterghem *et al.*, 1988). Thus, the contribution of L-type Ca channels to tension generation by endothelin might vary from one smooth muscle to another depending upon the relative role of the various mechanisms involved.

In this study, we extended our observations (Maggi *et al.*, 1989a) about the Ca sources utilized by endothelin in producing a contraction of the human bladder muscle. Present data confirm the view that L-type Ca channels play a very minor if any role in the response to endothelin in this tissue. The response to endothelin can be divided into a large (about 80% of total tension generated), $LaCl_3$-sensitive component (this fraction of the response conceivably overlaps with that prevented

by superfusion with Ca-free media) and a small (about 20% of total response) $LaCl_3$-resistant response, probably reflecting Ca mobilization from internal store(s). In rat aortic smooth muscle cells in culture, Kai *et al.* (1989) showed that the intracellular Ca pool activated by endothelin overlaps the caffeine-sensitive one. This does not seem to occur in the human urinary bladder muscle, because the caffeine response was procaine-sensitive while that to endothelin (in high K, Ca-free medium) was procaine-resistant. These findings give rise to the hypothesis, which needs further experimental evaluation, that the Ca store mobilized by endothelin in the human bladder muscle might be distinct from that mobilized by caffeine (or carbachol).

In conclusion, the present findings indicate that multiple sources of activator Ca can be mobilized, in a differential manner, by several agonists which induce contraction of the human urinary bladder muscle. Ca influx via L-type channels plays some role in the response to carbachol and neurokinin A but not in the response to endothelin. Receptor-operated and lanthanum ion-sensitive mechanisms promoting Ca influx could play a role in the response to carbachol, neurokinin A or endothelin. A caffeine- and procaine-sensitive intracellular Ca pool is mobilized by carbachol. Further, endothelin mobilizes a tightly bound (intracellular ?) Ca pool which is procaine-resistant. The relevance of these findings to the possible use of drugs interfering with Ca mobilization in the therapy of bladder motility disorders awaits elucidation of the nature of the Ca pools which are utilized *in vivo* for physiological (micturition contraction) and 'pathological' (detrusor instability) human bladder contractions.

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α_2-Adrenoceptor agonists enhance vasoconstrictor responses to α_1-adrenoceptor agonists in the rat tail artery by increasing the influx of Ca^{2+}

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1 The α_2-adrenoceptor agonists TL99 (2-(*NN*-dimethyl)amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene) and UK14304 (5-bromo-6-[2-imidazoline-2-yl-aminol]-quinoxaline), in concentrations that are less than 1% of those producing vasoconstriction, enhance vasoconstrictor responses to noradrenaline and phenylephrine in isolated perfused preparations of the rat tail artery.

2 The enhancing effect was abolished when Ca^{2+} was absent and by the calcium channel blocking drug diltiazem.

3 α_2-Adrenoceptor agonists had no effect on the component of the responses to noradrenaline and phenylephrine that is attributable to mobilization of intracellular Ca^{2+}, but enhanced the component attributable to influx of extracellular Ca^{2+}.

4 These results suggest that the enhancing effect of α_2-adrenoceptor agonists on responses of the rat tail artery to α_1-adrenoceptor agonists involves an increase in Ca^{2+}-influx into smooth muscle cells through Ca^{2+} channels that are opened when α_2-adrenoceptors are activated.

Introduction

There is clear evidence for the presence of postjunctional α_2-adrenoceptors as well as α_1-adrenoceptors on the smooth muscle of many vascular preparations (McGrath, 1982). The presence of postjunctional α_2-adrenoceptors on the smooth muscle of the rat tail artery has been demonstrated in binding studies with selective radioligands (Weiss *et al.*, 1983; Dashwood & Jacobs, 1985; Nasseri *et al.*, 1985; Abel & Minneman, 1986), and activation of these α_2-adrenoceptors produces contractile responses (Medgett & Langer, 1984; Rajanayagam & Medgett, 1987; Abel & Minneman, 1986; Abe *et al.*, 1987).

Pressor responses to α_2-adrenoceptor agonists *in vivo* can be blocked by calcium channel blocking drugs such as verapamil, nifedipine and diltiazem (Van Meel *et al.*, 1981a,b; Cavero *et al.*, 1983; Timmermans *et al.*, 1984). Furthermore, contractions of isolated vascular smooth muscle elicited by α_2-adrenoceptor activation are reduced by lowering the Ca^{2+} concentration and by calcium channel blocking drugs in the rat tail artery (Medgett & Rajanayagam, 1984; Hicks *et al.*, 1985; Su *et al.*, 1986) and the saphenous vein of the rabbit (Schümann & Lues, 1983) and dog (Jim & Matthews, 1985). Hence, influx of Ca^{2+} appears to be involved in the vasoconstrictor action of α_2-adrenoceptor agonists.

In concentrations well below those producing vasoconstriction, α_2-adrenoceptor agonists enhance responses of the isolated perfused rat tail artery to sympathetic nerve stimulation and to a number of vasoconstrictor agents including α_1-adrenoceptor agonists, adenosine 5′-triphosphate (ATP), vasopressin and high K^+ solution (Xiao *et al.*, 1987a; Xiao & Rand, 1989a). The aim of the present study was to examine the possible involvement of Ca^{2+} entry in the enhancing effect of the α_2-adrenoceptor agonists TL99 (Hicks & Cannon, 1980) and UK14304 (Grant & Scrutton, 1980; Cambridge, 1981) on vasoconstrictor responses to noradrenaline and phenylephrine in the rat isolated perfused tail artery. A preliminary account of part of this work was presented at the 6th Symposium on Neurovascular Mechanisms (Xiao *et al.*, 1987b).

Methods

Male Sprague-Dawley rats weighing 250 to 350 g were anaesthetized with pentobarbitone (30 mg kg^{-1},

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i.p.) and approximately 1 cm segments of the proximal portion of the tail artery were dissected out and set up as isolated preparations perfused at 4 ml min^{-1} as described by Xiao *et al.* (1987a) and Xiao & Rand (1989a). The perfusion pressure was measured with a Statham P23Db pressure transducer and recorded on a Rikadenki chart recorder. Vasoconstriction was measured as an increase in perfusion pressure in mmHg. A 30 min stabilization period was allowed before any experimental procedures were carried out.

Perfusion solutions

The physiological salt solution (PSS) was of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 0.45, $NaHCO_3$ 25, KH_2PO_4 1.03, D-(+)-glucose 11.1 and disodium edetate 0.067. For some experiments, the $CaCl_2$ concentration was reduced to 0.61 mM. For others, $CaCl_2$ was omitted and 2 mM EGTA (ethylene-*bis*-(β-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid) was added to the solution. The reservoirs of solutions were gassed with 5% CO_2 in O_2 and maintained at 37°C.

Vasoconstrictor responses

Responses to noradrenaline and phenylephrine were obtained by replacing the drug-free PSS with one containing the required concentration of an agonist, which was subsequently perfused until the response reached its peak value (usually less than 30 s) before returning to drug-free PSS. Two non-cumulative concentration-response curves were constructed; the second was begun 30 min after completing the first. When the effects of drugs or altered Ca^{2+} concentrations on concentration-response curves were to be determined, the PSS was modified after the first curve had been constructed and was used throughout the construction of the second curve. Only two curves were obtained from any one artery preparation.

Mobilization of intracellular Ca^{2+} and Ca^{2+} influx

The method of Manzini *et al.* (1982) was used to examine the effect of α_2-adrenoceptor agonists on the components of the responses to noradrenaline and phenylephrine attributable to mobilization of intracellular Ca^{2+} and to influx of extracellular Ca^{2+}. The procedure was to replace normal PSS with a Ca^{2+}-free solution containing EGTA 5 min before determining the response to noradrenaline or phenylephrine. When the response (due to the mobilization of intracellular Ca^{2+}) had been obtained, the solution was replaced with normal PSS and the secondary response (due to Ca^{2+} influx) then occurred. Perfusion with normal PSS was continued for a further 30 min before repeating the procedure since Casteels & Droogmans (1981) showed that at least 10 min period of perfusion with normal Ca^{2+}-containing solution was required for refilling the intracellular Ca^{2+} store in the rabbit ear artery.

Drugs

The following drugs were used: diltiazem hydrochloride (Synthelabo); idazoxan hydrochloride (Reckitt & Colman); (−)-noradrenaline hydrochloride (Sigma); phenylephrine hydrochloride (Sigma); TL99 (2-(*NN*-dimethyl)amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene, synthesized by Synthelabo, L.E.R.S.); UK14304 (5-bromo-6-[2-imidazoline-2-Yl-aminol]-quinoxaline, Pfizer). Stock solutions of noradrenaline and TL99 were dissolved in distilled water which contained ascorbic acid (0.05 mg ml^{-1}). Other drugs were dissolved in distilled water.

Statistical analysis

Differences between means were analysed by Student's paired or unpaired *t* test, as appropriate. Differences between lines (slopes or displacements) were analysed by comparing the parameters of regression equations. Probability levels of less than 0.05 were taken as indicating significant differences.

Results

Vasoconstrictor responses

Noradrenaline (3–300 nM) and phenylephrine (0.01–3 μM) produced concentration-dependent increases in perfusion pressure (Figure 1). The concentration-response curves were linear between perfusion pressures of 25 mmHg to about 200 mmHg. No attempt was made to obtain maximal responses because rises in perfusion pressure greater than about 250 mmHg resulted in loss of reproducibility of responses, presumably because of damage to the preparation. When log concentration-response relationships were analysed by determining the regression equations, only responses with a mean value of 25 mmHg or greater were used. In control experiments, there were no significant differences between two successive concentration-response curves to either noradrenaline or phenylephrine.

Vasoconstrictor responses to the α_2-adrenoceptor agonists were produced only when the concentrations reached 10 μM for UK14304 and 3 μM for TL99, as found previously by Medgett & Langer (1984).

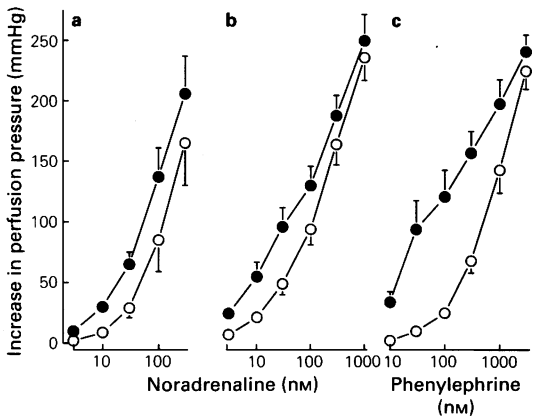

Figure 1 Concentration-response curves for noradrenaline (a and b) and phenylephrine (c): (○) first curves for the vasoconstrictor agonist alone; (●) second curves in the presence of 10 nM TL99 (a) or 30 nM UK14304 (b and c). Symbols indicate mean values and vertical bars show standard errors (a: $n = 7$; b, c: $n = 6$).

Effects of α_2-adrenoceptor agonists on vasoconstrictor responses

During infusions of concentrations of TL99 (10 nM) or UK14304 (30 nM) that were well below the thresholds for direct vasoconstrictor effects, the vasoconstrictor responses to noradrenaline and phenylephrine were enhanced. The log concentration-response line

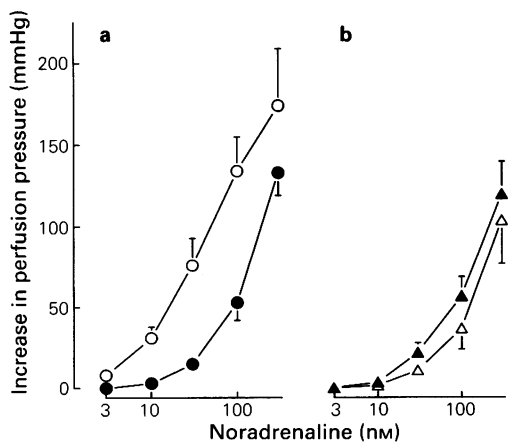

Figure 2 Concentration-response curves for noradrenaline: (a) (○) first curve for noradrenaline alone; (●) second curve in the presence of idazoxan (300 nM). (b) (△) First curve in the presence of idazoxan (300 nM); (▲) second curve in the presence of idazoxan (300 nM) plus TL99 (10 nM). Symbols are mean values and vertical bars show standard errors ($n = 6$).

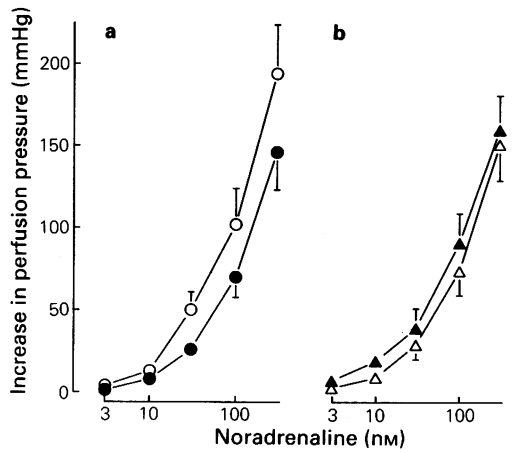

Figure 3 Concentration-response curves for noradrenaline: (a) (○) first curve for noradrenaline alone; (●) second curve in the presence of 1 μM diltiazem. (b) (△) First curve in the presence of 1 μM diltiazem; (▲) second curve in the presence of 1 μM diltiazem plus 10 nM TL99. Symbols are mean values and vertical bars show standard errors ($n = 6$).

for noradrenaline was significantly shifted to the left in the presence of TL99 (Figure 1a) and UK14304 (Figure 1b) by amounts corresponding to increases in potency of 2.4 fold and 2.1 fold, respectively. In the presence of UK14304, responses to the lower concentrations of phenylephrine were enhanced to a greater extent than were those to the higher concentrations, and the log concentration-response lines in the presence and absence of UK14304 departed significantly from parallelism (Figure 1c).

The α_2-adrenoceptor antagonist idazoxan (300 nM) had no effect on the resting perfusion pressure; however, it reduced vasoconstrictor responses to noradrenaline and significantly displaced the log concentration-response line to the right, to an extent amounting to a 5.1 fold decrease in potency (Figure 2a). When two successive curves for noradrenaline were obtained in the presence of idazoxan, they did not differ significantly. In the presence of idazoxan, TL99 had no significant effect on responses to noradrenaline and the log concentration-response lines did not depart significantly from coincidence (Figure 2b).

Effect of diltiazem

The calcium channel blocking drug diltiazem (1 μM) had no effect on the resting perfusion pressure, but significantly reduced vasoconstrictor responses to noradrenaline and shifted the log concentration-response line to the right by an amount corresponding to a 2.1 fold decrease in potency (Figure 3a). When two successive curves for noradrenaline were

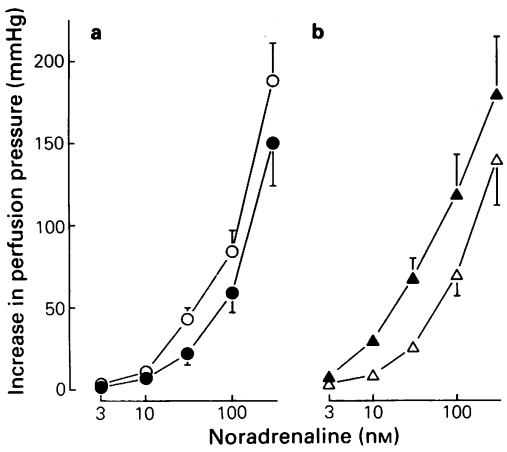

Figure 4 Concentration-response curves for noradrenaline: (a) (○) first curve for noradrenaline alone; (●) second curve after reducing the Ca^{2+} concentration from 2.5 to 0.61 mM. (b) (△) First curve in 0.61 mM Ca^{2+}; (▲) second curve in 0.61 mM Ca^{2+} with 10 nM TL99 present. Symbols are mean values and vertical bars show standard errors ($n = 6$).

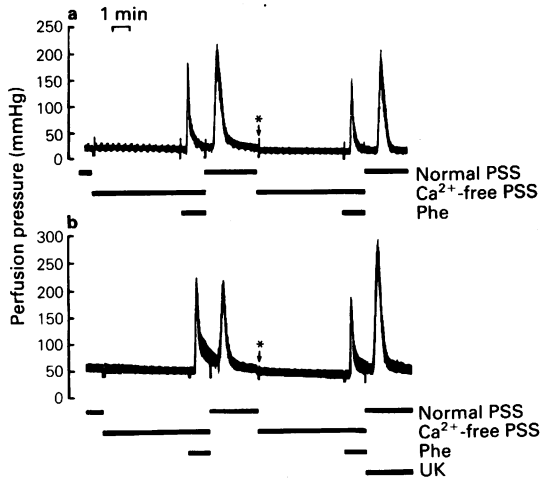

Figure 5 Records illustrating the transient vasoconstrictor responses to 10 μM phenylephrine (Phe) in Ca^{2+}-free PSS and after returning to normal PSS. The initial response in the Ca^{2+}-free PSS is due to mobilization of intracellular Ca^{2+}; the secondary response occurring when the Ca^{2+}-free solution was replaced by normal PSS is due to Ca^{2+} influx. (a): Two consecutive sets of responses (control); (b): when the second set of responses was elicited, 30 nM UK14304 (UK) was present in the normal PSS. There was a 30 min period of perfusion with normal PSS (indicated by *) between the first and second set of responses.

obtained in the presence of diltiazem, they did not differ significantly. In the presence of diltiazem, responses to noradrenaline were not significantly affected by TL99 (Figure 3b).

Effects of lowering the Ca^{2+} concentration

Reducing the concentration of Ca^{2+} in the PSS from 2.5 to 0.61 mM resulted in significant decreases in responses to noradrenaline, the log concentration-response line being shifted to the right by an amount corresponding to a 1.8 fold decrease in potency (Figure 4a). When first and second curves were obtained in a 0.61 mM Ca^{2+} solution, they did not differ significantly. When the second curve was obtained with TL99 (10 nM) present, responses to noradrenaline in a 0.61 mM Ca^{2+} solution were enhanced and the shift in the log concentration-response line corresponded to a 2.8 fold increase in potency (Figure 4b). The shifts caused by TL99 in the log concentration-response lines for noradrenaline to the left did not differ significantly between 0.61 and 2.5 mM Ca^{2+} solutions (*cf.* Figures 4b and 1a).

Effects of α_2-adrenoceptor agonists on the components of responses to noradrenaline and phenylephrine dependent on mobilizing intracellular Ca^{2+} and on Ca^{2+}-influx

When a Ca^{2+}-free, EGTA-containing, modified PSS was perfused, vasoconstrictor responses to 1 μM noradrenaline or 10 μM phenylephrine rose sharply to peak values that were approximately equivalent in magnitude to those produced by one-tenth the concentrations in normal PSS. Then, in the continued presence of the agonist, the perfusion pressure declined towards the base-line value, as shown for phenylephrine in Figure 5. This initial transient vasoconstrictor response in Ca^{2+}-free medium was attributed by Manzini *et al.* (1982) to mobilization of intracellular Ca^{2+}. On changing to perfusion with drug-free PSS containing the normal (2.5 mM) concentration of Ca^{2+}, there was a second transient vasoconstrictor response: this secondary response was attributed by Manzini *et al.* (1982) to the influx of extracellular Ca^{2+} into the vascular smooth muscle cells.

Neither TL99 (10 nM) nor UK14304 (30 nM) had any significant effect on the initial responses to noradrenaline or phenylephrine in the Ca^{2+}-free modified PSS. However, the secondary transient vasoconstrictor responses that appeared on changing to normal PSS were increased by UK14304 (Figure 5) and TL99. The secondary response to phenylephrine decreased by 3.5 ± 5.6% ($n = 8$) from the first to

the second occasion in control experiments (as in Figure 5a), but increased by $23.7 \pm 5.6\%$ ($n = 6$) when UK14304 was added before the second occasion (as in Figure 5b). With noradrenaline there was a decrease by $12 \pm 4.4\%$ ($n = 4$) in control experiments, but an increase of $14.7 \pm 5.4\%$ ($n = 6$) when UK14304 was added before the second occasion.

Discussion

Vasoconstrictor responses elicited by noradrenaline or phenylephrine in the rat isolated perfused tail artery were enhanced by concentrations of the α_2-adrenoceptor agonists TL99 and UK14304 that were subthreshold for producing vasoconstriction. This is consistent with the findings of Xiao *et al.* (1987a) and Xiao & Rand (1989a), using the same preparation, that vasoconstrictor responses to α_1-adrenoceptor agonists are markedly enhanced by α_2-adrenoceptor agonists in concentrations that are one-hundredth to one-thousandth of the concentrations producing other prejunctional or postjunctional effects. Similar observations were made by Shepperson (1984), who found that UK14304 in doses producing only small contractions of the cat nictitating membrane markedly enhanced contractile responses to phenylephrine.

The enhancing effect of TL99 on vasoconstrictor responses to noradrenaline was abolished by the relatively selective α_2-adrenoceptor antagonist idazoxan (Doxey *et al.*, 1983), confirming our previous findings that idazoxan blocked the enhancement by α_2-adrenoceptor agonists of responses resulting from activation of α_1-adrenoceptors by sympathetic nerve stimulation (Xiao *et al.*, 1987a; Xiao & Rand, 1989a) and responses elicited by certain other vasoconstrictor agents (unpublished observations).

When the Ca^{2+} concentration was zero and the chelating agent EGTA was present, UK14304 had no effect on the responses to noradrenaline or phenylephrine. Furthermore, the enhancing effect of TL99 on vasoconstrictor responses to noradrenaline was abolished in the presence of the calcium channel blocking drug diltiazem. When the component of the responses to noradrenaline and phenylephrine attributable to the influx of Ca^{2+} was studied by the method of Manzini *et al.* (1982), this component was enhanced by UK14304, but the component attributable to mobilization of intracellular Ca^{2+} was not affected. From these results it would appear that an influx of extracellular Ca^{2+} is required for producing the enhancing effect of α_2-adrenoceptor agonists on responses to α_1-adrenoceptor agonists.

Abe *et al.* (1987) found that the relatively selective α_1-adrenoceptor agonists methoxamine and phenylephrine produced an influx of Ca^{2+} into smooth muscle cells of the rat tail artery. More recently, Abe *et al.* (1988) reported that activation of α_1-adrenoceptors results in mobilization of intracellular Ca^{2+} and influx of extracellular Ca^{2+}, whereas activation of α_2-adrenoceptors results only in the influx of extracellular Ca^{2+}. Since idazoxan in a concentration that selectively blocks α_2-adrenoceptors reduces vasoconstrictor responses to methoxamine and phenylephrine in the rat tail artery (unpublished observations), it is possible that the Ca^{2+} influx produced by them could have been due to the activation of α_2-adrenoceptors. If this were the case, then the primary effects of methoxamine and phenylephrine on α_1-adrenoceptors resulting in mobilization of intracellular Ca^{2+} would be augmented by their secondary effect on α_2-adrenoceptors resulting in influx of extracellular Ca^{2+}.

TL99 enhanced vasoconstrictor responses to the relatively selective α_1-adrenoceptor agonist phenylephrine to a greater extent than those to noradrenaline, which acts on α_2- as well as α_1-adrenoceptors. We have previously found that another α_2-adrenoceptor agonist, rilmenidine, also enhanced responses to phenylephrine to a greater extent than those to noradrenaline (Xiao & Rand, 1989a). Furthermore, UK14304 enhanced the component of the response attributable to Ca^{2+} influx evoked by phenylephrine to a greater extent than that evoked by noradrenaline. The greater enhancement of responses to phenylephrine than to noradrenaline by α_2-adrenoceptor agonists may be because the noradrenaline had already activated a greater proportion of the α_2-adrenoceptor population than had phenylephrine. Responses to low concentrations of both phenylephrine and noradrenaline were enhanced to a greater extent than were those to high concentrations (Figure 1b,c). The decreasing enhancement with increasing concentrations, which was particularly marked with phenylephrine, could be due to increasing α_2-adrenoceptor activation.

Vasoconstrictor responses of the rat tail artery were reduced by idazoxan (Figure 2), diltiazem (Figure 3) and lowering the Ca^{2+} concentration of the perfusing solution from 2.5 to 0.61 mM (Figure 4). It is possible that these reductions may be partly due to elimination of an enhancement resulting from activation of α_2-adrenoceptors of the primary vasoconstrictor response produced by activating α_1-adrenoceptors. The reductions of responses to noradrenaline produced by idazoxan and diltiazem were accompanied by loss of the enhancing effect of TL99, but the enhancing effect of TL99 was still present when the Ca^{2+} concentration was decreased to 0.61 mM, suggesting that the increased Ca^{2+} influx produced by TL99 more than compensated for the decrease in the extracellular concentration.

We have previously suggested that the function of postjunctional α_2-adrenoceptors may be to augment the stimulus produced by activation of α_1-adrenoceptors (Xiao & Rand, 1989a). A similar suggestion has been made by Shepperson (1984) on the basis of experiments with the cat nictitating membrane. Such an ancillary role for postjunctional α_2-adrenoceptors might account for the functional interaction between α-adrenoceptor subtypes observed by Daly *et al.* (1988) in isolated vascular preparations, and for the suggestion by Sulpizio & Hieble (1987) that a circulating factor was necessary for the expression of pressor responses to α_2-adrenoceptor agonists *in vivo*.

5-Hydroxytryptamine in concentrations below those causing vasoconstriction produces a similar enhancement of vasoconstrictor responses to that produced by α_2-adrenoceptor agonists, and this effect also appears to be due to an increased Ca^{2+} influx in the rat tail artery (Xiao & Rand, 1989b) and rabbit ear artery (Meehan *et al.*, 1988). These observations lead us to suggest that the vasodilator and antihypertensive actions of calcium channel blocking drugs could be largely or even entirely due to inhibition of Ca^{2+} influx through channels that are opened by endogenous agonists that enhance vasoconstriction, rather than to blockade of calcium channels that are opened by the primary vasoconstrictor stimulus.

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δ-Opioid mediated inhibitions of acute and prolonged noxious-evoked responses in rat dorsal horn neurones

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1 The effects of a selective δ-opioid agonist Tyr-D-Ser(Otbu)-Gly-Phe-Leu-Thr (DSTBULET) were examined on the C- and Aβ-evoked responses of convergent dorsal horn neurones in the halothane anaesthetized, intact rat.

2 Intrathecal DSTBULET produced selective dose-dependent inhibitions of electrically-evoked C fibre responses of both superficial and deep neurones. A near-complete inhibition of 83 ± 5% followed 100 μg of DSTBULET and the ED_{50} was 9 μg (13.5 nmol). Inhibitions were antagonised by intrathecal naloxone and ICI 174,864 but were not antagonised by pretreatment with intrathecal β-funaltrexamine at a dose that blocked μ-opioid effects. By contrast, DSTBULET produced excitations of electrically-evoked responses of cells recorded in a zone intermediate between the superficial and deep neurones.

3 DSTBULET (50 μg) was also tested on the more prolonged noxious neuronal response produced by subcutaneous formalin (5%, 50 μl) into the receptive field. DSTBULET profoundly inhibited the response to formalin. Pretreatment with ICI 174, 864 before DSTBULET antagonised the effects of the δ-agonist on the formalin response.

4 The full peptidase inhibitor kelatorphan, known to protect endogenous enkephalins, was also tested on the formalin response. The intrathecal administration of 50 μg kelatorphan has previously been shown to inhibit electrically-evoked C fibre resonses of dorsal horn neurones and to be antagonised by ICI 174,864. The same dose of kelatorphan inhibited the formalin response in the present study.

5 From this study it appears that the δ-opioid agonist DSTBULET can produce profound inhibitions of the responses of convergent neurones to nociceptive afferent inputs. Furthermore, activation of δ-opioid receptors either by DSTBULET, or by protection of endogenous enkephalins with kelatorphan, can inhibit a more prolonged chemically-evoked nociceptive input onto these dorsal horn neurones.

Introduction

The existence of the three main opiate receptor subtypes, μ, δ and κ is well established and all three appear to be present in the spinal cord of many species including man (Maurer *et al.*, 1983; Quirion *et al.*, 1983; Gourderes *et al.*, 1985; Morris & Herz, 1987; Zajac *et al.*, 1989 and refs within). However, although agonists at the μ-opioid receptor subtype clearly mediate spinal analgesia (for review see Yaksh, 1984), it has proved more difficult to establish the role of the δ-opioid subtype in spinal antinociception. δ-Opioid binding sites are located throughout the dorsal horn, particularly in the superficial laminae where they appear to be less dense than the μ subtype (Quirion *et al.*, 1983; Gouarderes *et al.*, 1985; Waksman *et al.*, 1986; Morris & Herz, 1987; Zajac *et al.*, 1989). Despite evidence for the existence of a μ/δ-receptor complex in supraspinal sites (see Rothman & Westfall, 1982; Schoffelmeer *et al.*, 1988), in the spinal cord the δ-receptor appears distinct from the μ-receptor subtype and only a very low percentage of these receptor subtypes are colocalised on dorsal root ganglion cells (Werz *et al.*, 1987).

The peptides [Met] and [Leu]enkephalin are considered as selective but not specific endogenous

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ligands for the δ-opioid subtype and are located in neurones intrinsic to the spinal cord and in descending systems (Hokfelt *et al.*, 1977; Hunt *et al.*, 1980; Gibson *et al.*, 1981; Lamotte *et al.*, 1983). Spontaneous release of enkephalins *in vivo* (from local and descending fibres) is increased after noxious stimulation and is altered in chronic pain states (Cesselin *et al.*, 1985; Bourgoin *et al.*, 1986). The intrathecal application of exogenous [Met] and [Leu]enkephalin and other more or less selective synthetic δ-receptor agonists produces antinociception in behavioural tests of analgesia (Tung & Yaksh, 1982; Hylden & Wilcox, 1983; Yaksh, 1983; Porreca *et al.*, 1984; Schmauss & Yaksh, 1984; Kuraishi *et al.*, 1985; Rodriguez *et al.*, 1986; Heyman *et al.*, 1987) and in electrophysiological studies on dorsal horn neurones (Duggan *et al.*, 1976; 1977; Randic & Miletic, 1978; Satoh *et al.*, 1979; Zieglgansberger & Tulloch, 1979; Sastry & Goh, 1983; Willcockson *et al.*, 1984; Fleetwood-Walker *et al.*, 1985; 1986; Dickenson *et al.*, 1987b). Antinociceptive effects also occur following the protection from degradation of endogenous enkephalins by intrathecal administration of the peptidase inhibitors bestatin, thiorphan and kelatorphan (Bourgoin *et al.*, 1986; Dickenson *et al.*, 1986; 1987a). However, in an *in vitro* study with the isolated spinal cord it has been shown that the enkephalins modulate excitatory synaptic transmission, at least in the superficial dorsal horn, by acting through the μ-opioid receptor (Jeftinija, 1989).

When examining the consequences of δ-receptor activation in the spinal cord as discussed above, an important factor to consider is the specificity of the ligands for the δ-receptor. Although the μ- and δ-receptor subtypes in the cord are thought to be separate binding sites, many δ-ligands show a lack of selectivity between the two, and similarly with μ-ligands. In addition selective δ-opioid antagonists have not been available to confirm the opioid subtype involved until relatively recently. The endogenous agonists [Leu] and [Met]enkephalin are only 10 fold more selective for δ- than for μ-binding sites (Kosterlitz & Paterson, 1985). Tyr-D-Thr-Gly- Phe-Leu-Thr (DTLET), commonly used in binding studies, has good affinity for the δ-receptor but does show residual μ-receptor cross-reactivity (Zajac *et al.*, 1983), and elicits a biphasic dose-response in electrophysiological studies (Dickenson *et al.*, 1987b). Conformational restriction of the basic peptide structure has improved receptor selectivity, as exemplified by the two cyclic enkephalin analogues Tyr-D-Pen-Gly-Phe-D-Pen (DPDPE) and Tyr-D-Pen-Gly-Phe-L-Pen (DPLPE) (Mosberg *et al.*, 1983) but this involves a significant loss of affinity and potency (Delay-Goyet *et al.*, 1985). The linear hexapeptide Tyr-D-Ser(Otbu)-Gly-Phe-Leu-Thr (DSTBULET) has good selectivity for the δ-opioid receptor ($K_i(\delta)/K_i(\mu) = 0.012$) and better affinity than either DPDPE or DPLPE and thus is a suitable agonist for determining the role of δ-opiates in the spinal cord (Delay-Goyet *et al.*, 1987). We have already investigated the actions of DPDPE and the enkephalinase, aminopeptidase and dipeptidylaminopeptidase inhibitor, kelatorphan (Fournie-Zaluski *et al.*, 1984) on noxiously-evoked responses of dorsal horn neurones in the anaesthetized rat. Both compounds produced submaximal inhibitions which were antagonised by the selective δ-antagonist ICI 174,864 (Dickenson *et al.*, 1986; 1987b). In the present study DSTBULET was used to demonstrate the complete inhibition of C fibre-evoked responses of dorsal horn neurones in the spinal cord by a selective δ-agonist with a potency similar to the classical μ-opiate agonist morphine. The selective μ- and δ-antagonists β-funaltrexamine (Portuguese *et al.*, 1980) and ICI 174,864 (Cotton *et al.*, 1984) respectively further confirm the involvement of the δ-receptor subtype in the spinal actions of DSTBULET.

In addition to the effects of DSTBULET on electrically-evoked neuronal activation we also studied the action of this δ-agonist on more prolonged noxiously-evoked neuronal responses produced by subcutaneous formalin. The lower analgesic potency of δ-agonists against mechanical stimuli and in the acetic acid writhing test, in behavioural studies of antinociception, has led to the suggestion that spinal opiate receptor subtypes differentiate between thermal, mechanical and chemical noxious stimuli (Schmauss & Yaksh, 1984). The potency of μ-opioid agonists in inhibiting the formalin-evoked response of dorsal horn neurones has been shown previously (Dickenson & Sullivan, 1987). In the present study DSTBULET and the peptidase inhibitor kelatorphan were also found to be potent against the formalin response and do not therefore appear to differentiate between electrically-evoked and chemically-evoked cutaneous afferent input.

Methods

Male Sprague-Dawley rats (200–250 g) were initially anaesthetized with 3% halothane in a 66% N_2O, 33% O_2 gaseous mix and then maintained on 1.5–2% halothane for subsequent surgery. Following cannulation of the trachea the rat was mounted in a stereotaxic frame and a laminectomy performed, exposing the L1–L3 region of the spinal cord. The vertebrae caudal and rostral to the exposed cord were supported by clamps to hold the cord rigid and a glass-coated tungsten electrode lowered into the dorsal horn. Throughout the experiment the halothane concentration was held at between 0.8 and

1.5% depending on the individual animal. This level of anaesthesia was sufficient to maintain complete areflexia whilst maintaining the animal in good physiological condition with no apparent depression of respiration or neuronal activity. Recording of a single neurone could be continued for several hours.

Single unit extracellular recordings were made of dorsal horn neurones responding to both innocuous (brush) and noxious (pinch, heat) stimuli applied to the ipsilateral receptive fields in the plantar region of the hind paw. The depth from the surface of the cord of the neuronal recording site was established using a SCAT microdrive (Digitimer). Once the neurone had been characterized by means of natural stimuli, 16 electrical pulses (0.5 Hz, 2 ms wide) were applied via needles inserted into the centre of the receptive field. This provided a constant reproducible test stimulus for the experiment. Stimulation was applied at 3 times the threshold current for C fibre activation and a post-stimulus time histogram (PSTH) was built and displayed by means of a C.E.D. interface and MRATE software from which the C fibre-evoked neuronal response could be separated by latency and threshold from the Aβ fibre-evoked response and quantified. Aβ fibre-evoked responses were measured separately again at 3 times the threshold for Aβ fibre activation. For the acute studies, after at least 3 control tests at 5 min intervals which did not differ by more than 10%, drugs were applied directly onto the cord in a volume of 50 μl. Tests were carried out 5 and 10 min after each application and subsequently every 10 min.

In the experiments where formalin was used as the noxious stimulus, neurones were initially characterized as already described. C and Aβ fibre-evoked responses to electrical stimulation were measured by PSTH recordings. Then the spontaneous activity of the neurone was recorded for 10 min. Typically the convergent neurones studied in this preparation had no or very low levels of spontaneous activity and any cell showing a rate higher than 20 spikes min^{-1} was not used for these experiments. Formalin (5%, 50 μl) was then injected into the receptive field and the neuronal response monitored for 60 min. Intrathecal drugs were applied as described above 20 min before the formalin injection.

All results are expressed as a percentage of the mean control response and analysed by Student's t test.

Drugs

Tyr-D-Ser(Otbu)-Gly-Phe-Leu-Thr (DSTBULET), kelatorphan and Tyr-D-Ala-Gly-MePhe-Gly-ol (DAGOL), were synthesised by B.P. Roques (Delay-Goyet *et al.*, 1985). Allyl$_2$-Tyr-Aib-Aib-Phe-Leu-OH (ICI 174,864) was obtained from Cambridge Research Biochemicals and β-funaltrexamine was a gift from Prof. Portuguese.

Results

A total of 72 convergent cells was recorded from 34 rats. All cells had very little or no spontaneous activity and were separated into 3 distinct populations in terms of depth. Superficial cells (recorded between 0 and 250 μm from the surface of the dorsal horn, mean depth 125·8 $\pm$ 24 μm, $n = 25$) and deep cells (greater than 500 μm from the surface, mean depth 762.1 $\pm$ 48 μm, $n = 38$), were separated by an intermediate group recorded between 250 μm and 500 μm (mean depth 381.1 $\pm$ 27, $n = 9$). Superficial and deep cells responded similarly to opiates and could not be distinguished from each other by their neuronal responses. The threshold for activation of C fibre evoked responses in superficial and deep cells was 0.82 $\pm$ 0.14 and 0.64 $\pm$ 0.1 mA respectively. These intensities evoked a mean C fibre resonse of 201 $\pm$ 32 and 252 $\pm$ 36 spikes per 16 stimuli. The threshold for Aβ fibre-evoked responses were approximately 10 times less in most cells, a mean 0.058 $\pm$ 0.017 mA in superficial cells and 0.043 $\pm$ 0.001 mA in deep cells. Since cells occurring in either the superficial or deep layers of the dorsal horn responded similarly to opiates, dose-response curves were constructed from the results of all these cells. Neurones recorded between 250 and 500 μm in an intermediate zone showed similar thresholds to the other cells for Aβ and C fibre activation (0.041 $\pm$ 0.11, 0.68 $\pm$ 0.11 mA respectively) and a similar C fibre-evoked response (230 $\pm$ 19 spikes). However, the response of these neurones to intrathecal opiates were generally opposite to that of the superficial and deep cells and they have therefore been considered separately in the results.

Acute neuronal responses to electrically-evoked C and Aβ fibre stimulation

DSTBULET produced dose-dependent selective inhibitions of C fibre-evoked responses in all superficial and deep cells (Figure 1a). An almost complete mean inhibition of 83 $\pm$ 5% ($n = 14$) was produced by 100 μg of the peptide and the ED_{50} was estimated from the dose-response curve to be 9 μg (13.5 nmol). The peak effect occurred 31 $\pm$ 3 min (mean $\pm$ s.e.mean) after drug administration and was then maintained for over 30 min. There was no difference in response of the superficial and the deep cells to the effects of the peptide, either in terms of degree of inhibition or time-course. Aβ fibre-evoked activity was only significantly inhibited by the higher doses of the peptide and to a much lesser degree than the

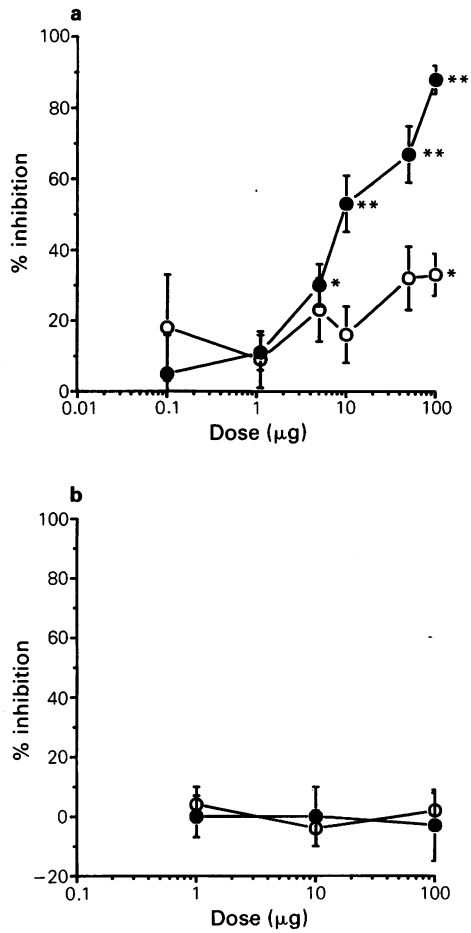


Figure 1 Dose-response curve of the effects of (a) DSTBULET and (b) L-DSTBULET on dorsal horn neuronal responses evoked by electrical stimulation at C fibre (●) and A fibre (○) intensities. Each point represents the mean and vertical lines show s.e.mean; $n = 4$ to 14. * $P < 0.05$; ** $P < 0.005$ (tested for significant difference against controls).

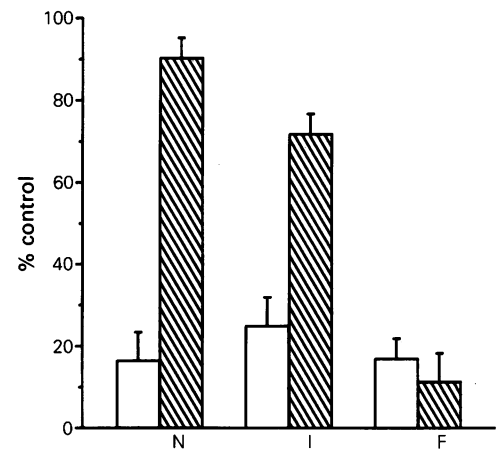
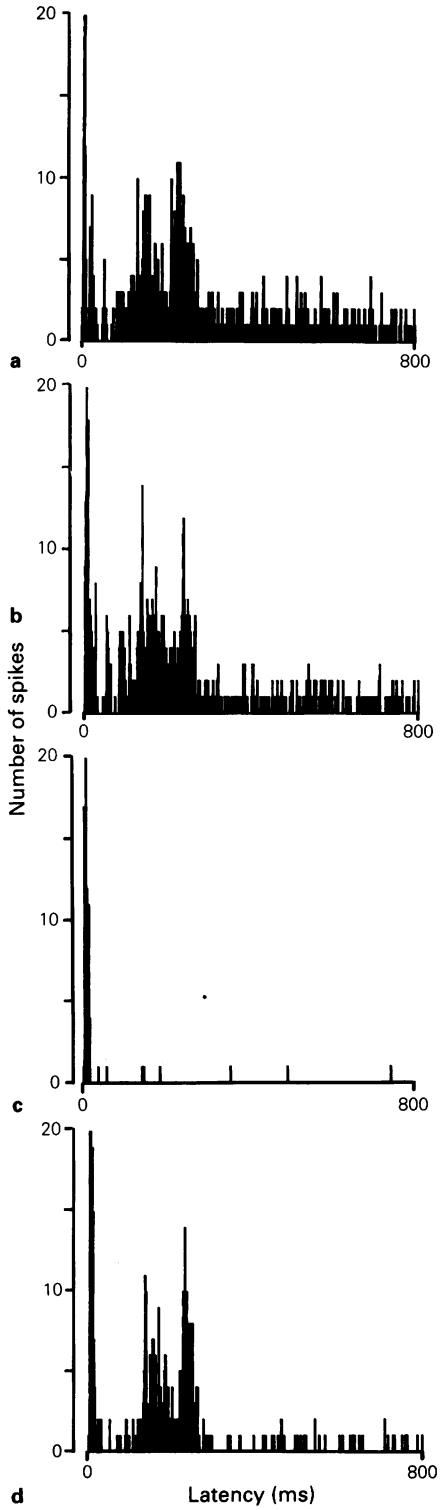


Figure 2 The antagonism of the inhibition by DSTBULET (open columns) on C fibre evoked responses of deep and superficial cells by 25 μg naloxone (N), 125 μg ICI 174,864 (I) but not by pretreatment with 0.05 μg β-funaltrexamine (F) (hatched columns).

corresponding C fibre response (Figure 1a). A maximal inhibition of only 33 ± 6% of Aβ fibre-evoked responses occurred with 100 μg DSTBULET compared to the almost complete inhibition of C fibre responses by this dose.

In a group of 9 cells recorded in the intermediate zone only 2 cells were inhibited by DSTBULET. For the other 7 cells enhancement of the C fibre-evoked responses above control levels occurred after intrathecal application of the peptide. The sample was too small to determine any dose-response relationship overall and individual cells varied considerably in the degree of excitation. However, a mean excitation of 48 ± 12% was produced by 5 μg DSTBULET in 4 cells compared with a mean inhibition of 30 ± 6% produced by the same dose of DSTBULET in the superficial/deep cells. The subsequent application of a higher dose of DSTBULET reversed the excitation produced by the agonist in 2 cells and an inhibition of C fibre-evoked responses occurred.

Several antagonists were used to determine whether the inhibitions produced by the higher doses of DSTBULET in the superficial and deep cells were mediated via the δ-receptor subtype. Intrathecal naloxone (25 μg) antagonised the effects of DSTBULET back to a mean of 83 ± 4% of control (Figure 2). However, this dose would not be expected to be selective for the μ-opioid receptor type. Intrathecal ICI 174,864 (125 μg), a selective δ-opioid antagonist was tested against both μ- and δ-agonist mediated effects. In 3 cells the inhibition (15 ± 4% of control) of C fibre-evoked responses by 5 μg DAGOL was not antagonised by application of ICI 174,864, the inhibition remaining unchanged over 1 h (14 ± 5% of control). Subsequent application of 5 μg naloxone rapidly antagonised the DAGOL-mediated inhibitions back to 73 ± 18% of control. In contrast to the lack of antagonism of μ-mediated effects, 125 μg ICI 174,864 completely antagonised the inhibitory effects of DSTBULET in 3 out of 11 cells and partially antagonised the agonist in the remaining 8 cells. Overall the responses of all 11 cells were antagonised to 72 ± 5% of control (Figure 2). The action of the selective μ-antagonist β-funaltrexamine (βFNA) was



also investigated. An intrathecal dose of 0.05 μg β-FNA was given 2 h before the administration of 5 μg DAGOL and subsequently 100 μg DSTBULET. An example of the antagonism of DAGOL by βFNA and subsequent inhibition of a single neurone by DSTBULET is shown in Figure 3. In 6 cells in which the action of DAGOL was completely blocked by βFNA the highest dose of DSTBULET still produced maximal inhibitions (mean 89 ± 7%), which were not significantly different from the effects of that dose of DSTBULET alone (Figure 2). Thus the effects of DSTBULET are likely to be mediated solely through the δ-receptor, although the incomplete antagonism of DSTBULET by ICI 174,864 must also be considered.

The inactive isomer of DSTBULET was tested on Aβ and C fibre-evoked responses in 6 cells. No inhibition or excitation of electrically evoked neuronal activity was seen to occur after intrathecal application of 1 to 100 μg Tyr-L-Ser(Otbu)-Gly-Phe-Leu-Thr (Figure 1b).

Prolonged neuronal responses to subcutaneous formalin

DSTBULET was also tested on the response of convergent dorsal horn neurones to a more prolonged chemical noxious stimulus. In the control experiments a subcutaneous injection of 5% formalin produced a biphasic pattern of activity in these neurones over a period of 1 h, as shown in Figure 4a. The activity within the first 10 min following the injection of formalin was denoted as the 1st peak and the more prolonged response over the next 50 min the 2nd peak. A dose of 50 μg DSTBULET 20 min before the injection of formalin completely abolished the 2nd peak of the response and profoundly inhibited the first peak (Figure 4b). The co-administration of ICI 174,864 with DSTBULET completely antagonised the effect of the agonist on the 1st peak (Figure 4c). ICI 174,864 also partially but significantly ($P < 0.005$) antagonised the action of DSTBULET on the 2nd peak. The peptidase inhibitor kelatorphan was also tested on the formalin

Figure 3 An example of the selective antagonism of DAGOL but not DSTBULET by pretreatment with β-funaltrexamine (βFNA). (a) The post-stimulus-time histogram of the responses of a single deep dorsal horn neurone to electrical stimulation after 2 h of pretreatment with 0.05 μg βFNA. There was no change in the response 30 min after the administration of 5 μg DAGOL (b). This dose would normally produce a complete inhibition of the C fibre-evoked response. Subsequently 100 μg of DSTBULET abolished the C fibre-evoked response within 30 min (c) and the inhibition was reversed by 25 μg naloxone (d).

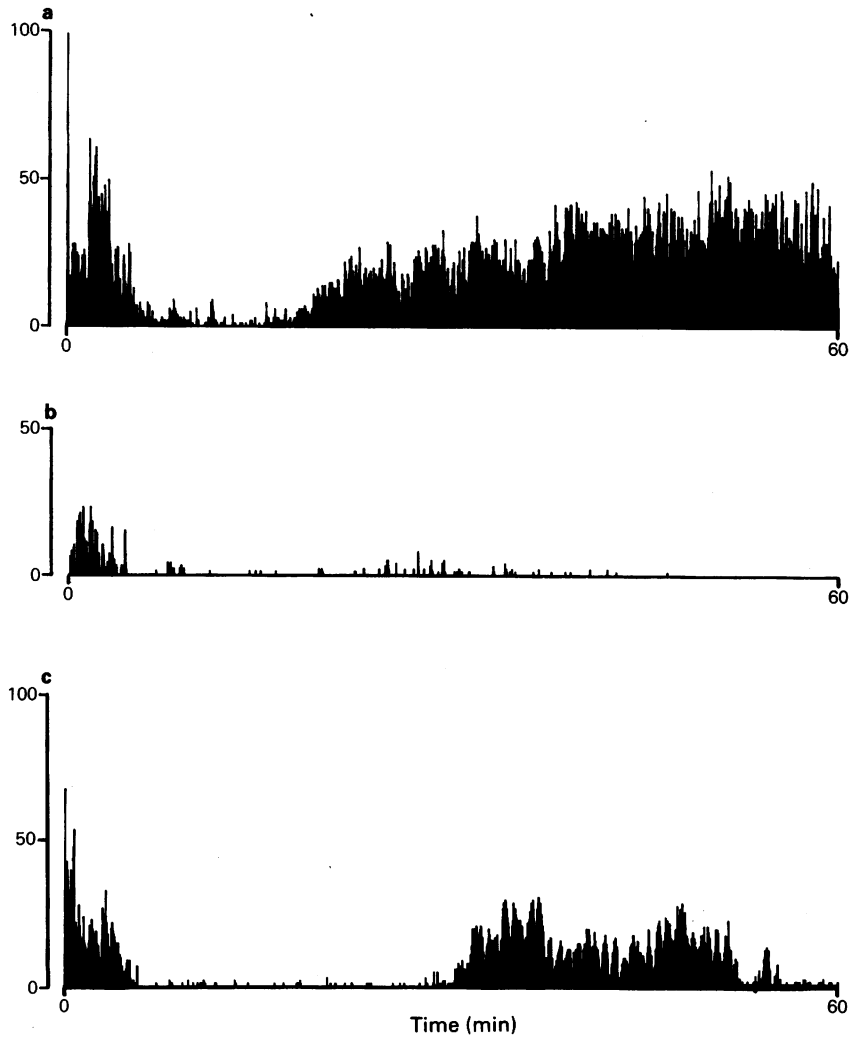


Figure 4 Rate recordings (1 s bins) of the neuronal response evoked in individual dorsal horn neurones by subcutaneous formalin. In a neurone with saline pretreatment 2 peaks of activity occurred over the 1 h of recording (a). Pretreatment with 50 μg DSTBULET 20 min before the injection of formalin inhibited both phases of the response in another neurone (b). When 125 g ICI 174,864 was coadministered with DSTBULET there was a partial recovery of the formalin response (c).

response. In previous studies we have found that the intrathecal application of 50 μg kelatorphan inhibited the C fibre-evoked response by 51% and this effect was antagonised by ICI 174,864 (Dickenson *et al.*, 1986). In the present study a similar dose of the peptidase inhibitor completely abolished the 2nd peak of the neuronal response in 4 cells. Figure 5 shows the overall results for the effects of DSTBULET and kelatorphan on both phases of the formalin-evoked response.

Discussion

In previous studies on the same preparation the intrathecal application of the selective δ-opioid agonist DPDPE or the enkephalinase inhibitor kelatorphan produced a maximal 50–60% inhibition of C fibre-evoked responses (Dickenson *et al.*, 1986; 1987a,b). DTLET, a mixed δ/μ-agonist could produce 100% inhibitions of C fibre-evoked activity but the biphasic dose-response curve suggested that

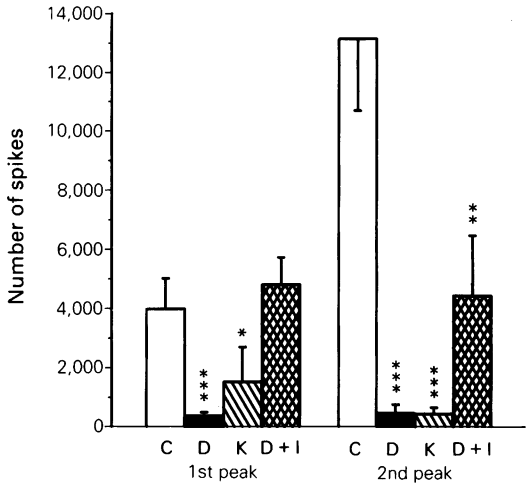


Figure 5 The overall inhibitory actions of 50 μg DSTBULET (D) and 50 μg kelatorphan (K) on the 1st acute peak (0–10 min) and the 2nd more prolonged phase (11–60 min) of the formalin response (C, control). Note the complete antagonism of DSTBULET effects on the 1st peak and partial antagonism in the 2nd peak by ICI 174,864 (I) coadministered with DSTBULET. $n = 3$ to 5. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.001$ (tested for significant difference against the control response.

μ-receptor activation was necessary to inhibit completely C fibre-evoked responses (Dickenson *et al.*, 1987b). It therefore appeared that δ-receptor activation either via an exogenous agonist, or through the protection of endogenous enkephalins, was unable to inhibit completely noxiously-evoked responses of convergent dorsal horn neurones in the spinal cord. This was in contrast to the profound maximal inhibitions of these responses of convergent dorsal horn neurones by a large range of μ-agonists, including DAGOL, morphine and methadone (Dickenson & Sullivan, 1986; Dickenson *et al.*, 1987b; 1988; McQuay *et al.*, 1989). However, the present study clearly demonstrates that a selective δ-opioid agonist DSTBULET can almost completely abolish C fibre responses in superficial and deep dorsal horn neurones with a potency similar to morphine. Although antagonism of the peptide by ICI 174,864 was often incomplete, pretreatment with βFNA, an irreversible selective μ-antagonist, at doses which blocked μ-mediated effects on the same neurone, did not reduce the inhibitory action of DSTBULET. Thus, it can be concluded that even at the highest doses tested DSTBULET is likely to be acting solely on the δ-receptor opioid subtype. The marked but incomplete antagonism of DSTBULET by ICI 174,864 may result from the relatively lower affinity of this antagonist for the δ-opioid receptor (Kosterlitz & Paterson, 1985).

The dose-response curve for the inhibition of C fibre-evoked responses by DSTBULET is similar to that of μ-opioid agonists with respect to the maximum drug effect attained. However, no excitations of neuronal activity were seen after the lowest doses of DSTBULET, unlike the pronounced facilitations which occur after low doses of the μ-agonists morphine and DAGOL in this preparation (Dickenson & Sullivan, 1986; 1987b). In addition other δ-opioid agonists studied in this preparation did not produce facilitations of responses at low doses (Dickenson *et al.*, 1987b). μ-Opioid mediated biphasic actions have also been observed on the nociceptive flexion reflex in the rat (Wiesenfeld-Hallin & Duranti, 1987), on glutamate-evoked responses of primate spinothalamic tract neurones (Willcockson *et al.*, 1986) and on action potentials in primary afferent neurones *in vitro* (Higashi *et al.*, 1982; Crain & Shen, 1988). The enhanced potassium- or capsaicin-evoked release of substance P-like immunoreactivity in the rat spinal cord slice by μ-opioid receptor activation (Mauborgne *et al.*, 1987; Pohl *et al.*, 1989) could be one mechanism by which the facilitations occur. Reduction rather than enhancement of substance P-like immunoreactivity occurs with DPDPE, DTLET, DADLE, [Met]- and δ-enkephalin (Hirota *et al.*, 1985; Go & Yaksh, 1987; Mauborgne *et al.*, 1987). Another possible explanation for the facilitations is presynaptic autoinhibition of [Met]enkephalin release (Ueda *et al.*, 1986). The lack of low-dose excitation by DSTBULET implies that this putative autoreceptor might be the μ-opioid receptor subtype. However, in *in vitro* studies low doses of both μ- and δ-opioid agonists prolonged the duration of the calcium component of the action potential (APD) in dorsal root ganglion cells in culture, before higher doses of the agonists shortened the APD leading to depression of neurotransmitter release (Crain & Shen, 1988).

In the convergent neurones examined in this study C fibre-evoked responses were selectively inhibited with little effect on Aβ fibre responses, except at the highest doses. The loss of only 50% of δ-binding sites in laminae 1 and 2 after dorsal rhizotomy (Zajac *et al.*, 1989) indicates the presence of presynaptic receptors on the primary afferent terminals but also postsynaptic receptors, presumably located on intrinsic neurones and on the dendrites and soma of convergent output cells (see Besson & Chaouch, 1987). Both pre- and postsynaptic δ-receptor activation could result in the inhibition of C fibre-evoked responses in convergent neurones (Sastry, 1979; Zieglansberger & Tulloch, 1979; Miletic & Randic, 1982; Hentall & Fields, 1983). The selectivity of opioid action on C fibre-evoked responses has also been demonstrated in iontophoretic studies where opiates, ejected into the substantia gelatinosa, inhibit

the noxiously evoked responses of deep dorsal horn neurones without affecting the responses to non-noxious stimuli (Duggan *et al.*, 1976; 1977; 1981; Davies & Dray, 1978; Fleetwood-Walker *et al.*, 1988). However, when ejected close to the cell bodies opiates generally either had no effect or mediated a non-selective inhibition of both noxious and non-noxious evoked responses (Duggan *et al.*, 1976; 1977; Zieglgansberger *et al.*, 1970; Fleetwood-Walker *et al.*, 1988). Although in several studies enkephalins inhibit noxiously-evoked responses of deep cells whilst sparing non-noxious responses when iontophoretically applied into the SG (Duggan *et al.*, 1976; 1977; 1981; Davies & Dray, 1978), it has also been found that specific δ-opioid agonists were only selective in inhibiting the response of multireceptive neurones located in lamina I and not those of laminae III-V spinocervical tract neurones (Fleetwood-Walker *et al.*, 1986). We have not characterised the cells in the present study in terms of output but, nevertheless, we could not distinguish between the effects of DSTBULET on superficial compared with deep convergent neurones.

We have not investigated the extent to which intrathecal opiates penetrate into the dorsal horn. However, from the electrophysiological studies mentioned above, and the evidence from binding studies indicating a superficial location of δ-opiate receptors, it is likely that a selective C fibre inhibition results from anatomical differences in Aβ and C fibre termination in the dorsal horn. It is interesting to note that inhibition of Aβ fibre-evoked responses did occur at higher doses of DSTBULET and the maximum degree of inhibition (40%) was similar to that observed previously for other opiates, including DAGOL and morphine (Dickenson & Sullivan, 1986; Dickenson *et al.*, 1987b). Possibly this represents the penetration of the drug to deeper opiate receptors postsynaptic to both Aβ and C fibre terminals and therefore results in the inhibition of all types of response of the cell. The thick myelinated Aβ afferent fibres terminate much deeper in the dorsal horn in laminae III-VI, whereas the smaller unmyelinated C fibres terminate in the superficial laminae I and II (Brown, 1982). It is also possible that the inhibition of Aδ fibre-evoked activity contributes to effects of higher doses of DSTBULET on the short latency response. It is often difficult to separate Aβ- and Aδ-evoked responses in terms of latency. Certainly it appears that a certain degree of inhibition of the short latency response, whether evoked by Aβ or Aδ fibres, always occurs whenever C fibre-evoked responses are completely inhibited.

Unlike the profound inhibitions of superficial and deep cells DSTBULET produced excitations of neuronal responses recorded in the intermediate zone. An excitatory effect on substantia gelatinosa (SG) neurones by systemic morphine and iontophoretic [Met] enkephamide has been previously demonstrated in electrophysiological studies *in vivo* (Woolf & Fitzgerald, 1981; Sastry & Goh, 1983). We have also observed a selective excitation of neurones recorded in an intermediate zone by other intrathecal opiates including morphine in this *in vivo* preparation (unpublished). We have not demonstrated that cells recorded in this intermediate zone are in fact located in the SG. However, excitations of neuronal responses by opiates including DSTBULET have also been recorded in the isolated spinal cord slice (Dickenson & Magnuson, 1989). In this preparation the cells are clearly located in the translucent zone of the dorsal horn which is generally accepted to be the SG (Brown, 1982). It is likely that the neurones recorded *in vivo* are also located in the same zone i.e. the SG, since they exhibit a similar excitatory response to opiates. The SG has been implicated in segmental control of nociceptive transmission via inhibitory interneurones releasing enkephalins and γ-aminobutyric acid (GABA) (see Besson & Chaouch, 1987). It seems probable that the mechanism of excitation of the intermediate cells by DSTBULET in this study is indirect and is in fact the consequence of the blockade by DSTBULET of a tonic interneuronal inhibition of these cells. A similar mechanism occurs in the hippocampus where opioid-induced excitation is mediated by inhibition of inhibitory interneurones (Zieglgansberger *et al.*, 1970; Neumaier *et al.*, 1988).

Differential inhibition of thermal, pressure and chemical noxious inputs have been observed for agonists acting at different opiate receptor subtypes in the spinal cord (Schmauss & Yaksh, 1984). It has been suggested that whereas μ-opioid agonists inhibit all three noxious stimuli, δ-agonists are selective for thermal stimuli and are ineffective against chemically-evoked noxious input. In behavioural studies DPDPE and [Met]enkephalin appear more potent against thermal compared with mechanically-evoked noxious stimuli (Kuraishi *et al.*, 1985; Rodriguez *et al.*, 1986) and both DADLE and [Met]enkephalin are ineffective in the acetic acid writhing test (Schmauss & Yaksh, 1984). However, in the present study DSTBULET was equally effective in inhibiting neuronal responses evoked by both electrical and chemical noxious stimulation. Furthermore, the enhancement of endogenous enkephalins in the spinal cord by kelatorphan was also sufficient to abolish the neuronal excitation by formalin. It is possible that the δ-agonists distinguish between cutaneous and visceral inputs rather than the modality of input. Both the noxious activity evoked by electrical stimulation and by the chemical irritant formalin were cutaneous in origin, whereas the acetic writhing test involves visceral inputs. An alternative explana-

tion arises from the differences in analgesic potency of μ- and δ-agonists in behavioural tests. Generally, δ-agonists are less effective than μ against noxious stimuli (Hylden & Wilcox, 1983; Porreca *et al.*, 1984; Heyman *et al.*, 1987; Russell *et al.*, 1987). If the intensity of the noxious stimuli is much greater in the writhing test, the lower potency of DPDPE or [Met]enkephalin might be insufficient to produce any visible analgesia. However, in a comparison of the writhing test and the hot-plate test, Shaw *et al.* (1988) concluded that the former is a less intense stimulus than the latter. It would be interesting to study DSTBULET against a visceral stimulus to see if the ligand, test or receptor type determines these differences.

To summarise, the selective δ-opioid DSTBULET is as potent as morphine in inhibiting noxiously-evoked input to the rat spinal cord. C fibre-evoked responses of dorsal horn neurones due to either electrical stimulation of the receptive field or subcutaneous injection of formalin could be abolished by this agonist. Kelatorphan was equally effective in inhibiting the formalin response, confirming the involvement of endogenous enkephalins in spinal antinociception. We conclude that the activation of δ-opioid receptors can play an important role in the mediation of antinociception in the spinal cord.

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Convulsions induced by centrally administered NMDA in mice: effects of NMDA antagonists, benzodiazepines, minor tranquilizers and anticonvulsants

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1 Convulsions were induced reproducibly by intracerebroventricular injection of N-methyl-D-aspartic acid (NMDA) to conscious mice.

2 Competitive (carboxypiperazine-propylphosphonic acid, CPP; 2-amino-7-phosphonoheptanoic acid, AP7) and non-competitive (MK801; phencyclidine, PCP; thienylcyclohexylpiperidine, TCP; dextrorphan; dextromethorphan) NMDA antagonists prevented NMDA-induced convulsions.

3 Benzodiazepine receptor agonists and partial agonists (triazolam, diazepam, clonazepam, Ro 16-6028), classical anticonvulsants (diphenylhydantoin, phenobarbitone, sodium valproate) and meprobamate were also found to prevent NMDA-induced convulsions.

4 Flumazenil (a benzodiazepine receptor antagonist) and the GABA agonists THIP and muscimol (up to subtoxic doses) were without effect.

5 Flumazenil reversed the anticonvulsant action of diazepam, but not that of MK801.

6 Results obtained in this model differ somewhat from those described in a seizure model with systemic administration of NMDA. An explanation for this discrepancy is offered.

7 This model is a simple test for assessing the *in vivo* activity of NMDA antagonists and also expands the battery of chemically-induced seizure models for characterizing anticonvulsants not acting at NMDA receptors.

Introduction

N-methyl-D-aspartate (NMDA) receptors have been implicated in a number of physiological and pathological phenomena such as learning, regulation of synaptic growth (see Cotman & Iversen, 1987), certain forms of epilepsy (Meldrum, 1985), excitotoxicity (see Rothman & Olney, 1987) and hypoxic/ischaemic brain insults (see Choi, 1988).

In order to study compounds potentially active at the NMDA receptor level, tests based on neurological and behavioural changes have been developed by use of systemic (i.p.) administration of high doses of NMDA (Czuczwar & Meldrum, 1982; Leander *et al.*, 1988). However, the effects induced by such injections were not very reproducible. Lower doses (e.g. 125 mg kg^{-1}) produced only scratching in some mice but tonic seizures and death in others. At higher doses (150, 175 mg kg^{-1}), more animals convulsed and died, but some only exhibited tail biting. As no i.p. dose of NMDA was found consistently to produce clonic convulsions, lethality was selected as the most reliable end-point following an injection of a high dose of NMDA (200 mg kg^{-1}) (Leander *et al.*, 1988). However, even the development of these symptoms was highly variable and the time of occurrence varied from 1 to 15 min. In view of these shortcomings (high dose of NMDA required, inconsistent behavioural effects, variable time course), we were interested in evaluating another *in vivo* method for testing compounds for antagonism of NMDA-induced convulsions. In the present study results were obtained by freehand injection of NMDA into the lateral brain ventricle of conscious mice. A very low dose of NMDA (1 nmol in 1 μl) yielded very reproducible effects which developed in less than 1 min. Such a methodology has already been used for the detection of anticonvulsant properties of two

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κ-opioid agonists and two anticonvulsants, but other known or putative anticonvulsant drugs have not been studied (Vonvoigtlander *et al.*, 1987).

Methods

Animals

Female SPF-Fü albino mice (18–22 g) were used. The animals were housed in groups and had unlimited access to food and water. They were transferred from the colony room to the testing room 2 h before the experiment, which was routinely performed between 09 h 00 min and 15 h 00 min. Groups of eight mice were used per drug dose.

Experimental protocol

Seizures were induced by injection of NMDA (1 nmol in 1 μl) into a lateral ventricle of conscious mice by use of a procedure similar to that described by Clark *et al.* (1968). This dose of NMDA was selected as the minimal dose that reliably induced the appearance of tonic convulsions in at least 90% of treated animals. Immediately after injection, animals were placed in plexiglas boxes (12 × 12 × 15 cm) and were observed for a period of 5 min. Typically, seizure activity consisted of wild running, clonic convulsions, tonic seizures and, in about 20–40% of the animals, death. The episode typically began a few seconds after injection and evolved to its maximal intensity in less than 1 min. The number of mice per group exhibiting tonic convulsions was recorded.

Test drugs were dissolved in phosphate buffer and administered in a volume of 1% body weight. Poorly water soluble drugs were administered as a suspension. For each drug, the route (i.p., p.o. or s.c.) and time-point of injection before NMDA administration were selected as corresponding to its peak activity as previously determined in other behavioural tests, such as prevention of convulsions induced by pentylenetetrazol, maximal electroshock or auditory stimulation.

Data analysis

Data were analysed by means of the Fisher exact probability test (Siegel, 1956). All results are expressed as percentages of control values and the significance level was predetermined at 0.01.

Drugs

The drugs used were: N-methyl-D-aspartate (NMDA, Sigma), MK801 (5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine, Merck, Sharp & Dohme), phencyclidine (Duphar), phenobarbitone (Siegfried), diphenylhydantoin (Fluka), meprobamate (Lederle), sodium valproate (Aldrich), THIP (tetrahydroisoxazolopyridin, Sandoz). All other chemicals were synthesized at F. Hoffmann-La Roche (Basle, Switzerland, or Nutley, U.S.A.).

Results

In all experiments, NMDA-induced tonic seizures appeared to be more sensitive than clonic convulsions to inhibition by the test compounds. This confirms our experience with other convulsants (3-mercaptopropionic acid, pentylenetetrazol) and their prevention by benzodiazepines (Pieri, unpublished observation). Consequently, and for more clarity, only results based on tonic convulsions are presented (with the exception of the experiment shown in Figure 3). For each drug tested vs NMDA, we determined time to peak activity and optimal route of administration in preliminary experiments. Therefore, we felt confident that we were in fact comparing like with like and thus justified in presenting an estimate of the order of potency.

Effects of competitive and non-competitive NMDA antagonists

Prevention of NMDA-induced tonic convulsions by selected competitive and non-competitive NMDA antagonists is represented in Figure 1. The order of potency of these compounds was MK801 > phencyclidine > thienylcyclohexylpiperidine (TCP) = carboxypiperazine-propylphosphonic acid (CPP) > dextrorphan = dextromethorphan > 2-amino-7-phosphonoheptanoic acid (AP7).

Phencyclidine and TCP at the highest doses tested (10 mg kg^{-1}) induced tremors in all animals. MK801 (1–10 mg kg^{-1}) also induced tremors and ataxia, and dextromethorphan (100 mg kg^{-1}) tremors and death.

Effects of benzodiazepine receptor ligands and GABA agonists

Figure 2 shows the inhibition of NMDA-induced tonic convulsions by selective benzodiazepine receptor (BZR) ligands and γ-aminobutyric acid (GABA) agonists, as compared to MK801.

Triazolam and clonazepam were considerably more potent than MK801. Diazepam and Ro 16-6028 (a BZR partial agonist, Martin *et al.*, 1988) were about equipotent and slighly less effective than MK801. Flumazenil (a BZR antagonist, Hunkeler *et*

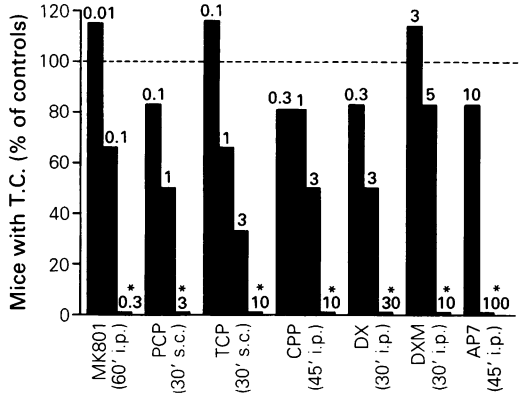

Figure 1 Prevention by competitive and non-competitive N-methyl-D-aspartate (NMDA) antagonists of tonic convulsions (T.C.) induced by i.c.v. NMDA (1 nmol in 1 μl). Results are expressed as % of control values and significant effects ($P < 0.01$) are marked by asterisks. The different routes and times of administration before NMDA injection are shown below the name of each compound and the different doses (in $mg\,kg^{-1}$) used for each drug are shown above the corresponding columns. PCP = phencyclidine; TCP = thienylcyclohexylpiperidine; CPP = carboxypiperazine-propyl-phosphonic acid; DX = dextrorphan; DXM = dextromethorphan; AP7 = 2-amino-7-phosphonoheptanoic acid.

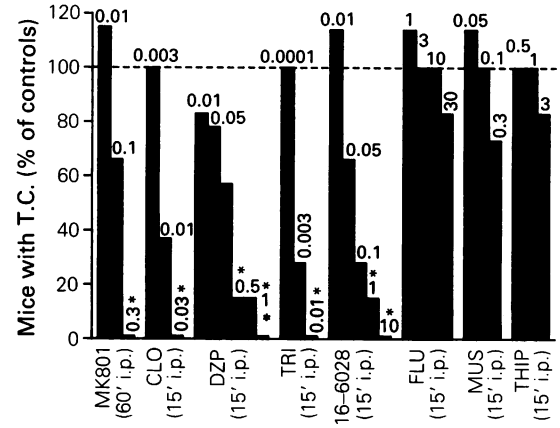

Figure 2 The effects of benzodiazepine receptor ligands and γ-aminobutyric acid (GABA) agonists on tonic convulsions (T.C.) induced by i.c.v. N-methyl-D-aspartate (NMDA, 1 nmol in 1 μl). Results are expressed as % of control values and significant differences ($P < 0.01$) are marked by asterisks. The different times of administration are shown below the name of each compound and the different doses (in $mg\,kg^{-1}$) used for each drug are shown above the corresponding columns. CLO = clonazepam; DZP = diazepam; TRI = triazolam; FLU = flumazenil; MUS = muscimol; THIP = tetrahydroisoxazolopyridin.

al., 1981), and the GABA agonists, muscimol and THIP, were devoid of anticonvulsant activity. The two latter compounds produced pronounced hypomotility and reduced reactivity to the environment at the highest doses tested (1 and 10 $mg\,kg^{-1}$). Flumazenil totally reversed the effects of diazepam but did not reduce those of MK801 (Figure 3).

Effects of non BZR ligands: classical anticonvulsants and a minor tranquilizer

Effects of these compounds are shown in Figure 4. Diphenylhydantoin was the most potent, followed by phenobarbitone, meprobamate and sodium valproate.

Discussion

In the present study we have used intracerebroventricular injection of NMDA in conscious mice as an *in vivo* test for detection of anticonvulsant drugs. This technique offers the following advantages: it is a simple and very rapid method, which requires a minute amount of NMDA (1 nmol in 1 μl) to be injected and produces reliable neurological and behavioural effects which develop in a very short

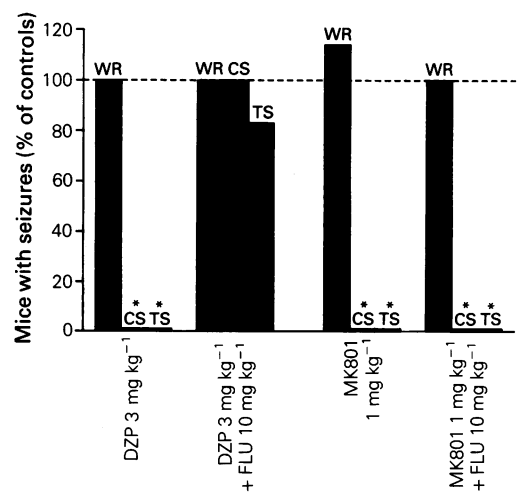

Figure 3 Effects of flumazenil (FLU) on prevention by diazepam (DZP) and MK801 of wild running (WR), clonic (CS) and tonic (TS) seizures induced by i.c.v. N-methyl-D-aspartate (NMDA) (1 nmol in 1 μl). Results are expressed as % of control values and significant differences ($P < 0.01$) are marked by asterisks.

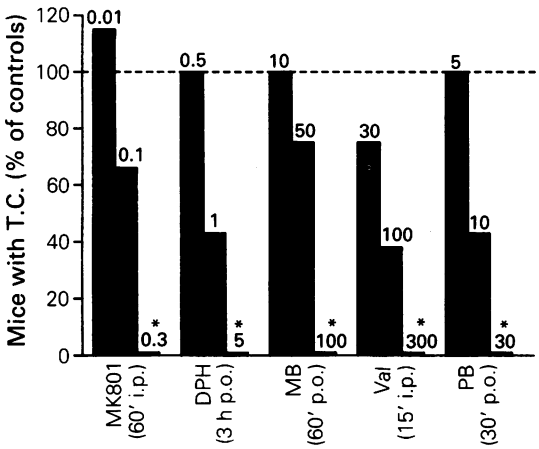

Figure 4 The effect of classical anticonvulsants and minor tranquilizers on tonic convulsions (T.C.) induced by i.c.v. N-methyl-D-aspartate (NMDA, 1 nmol in 1 μl). Results are expressed as % of control values and significant differences ($P < 0.01$) are marked by asterisks. The different routes and times of administration before NMDA injection are shown below the name of each compound and the different doses (in mg kg^{-1}) used for each drug are shown above the corresponding columns. DPH = diphenylhydantoin; MB = meprobamate; Val = sodium valproate ; PB = phenobarbitone.

period of time (wild running, clonic and tonic convulsions usually developing within 10 to 15 s, followed by death in about 20% of animals).

All competitive and non-competitive NMDA antagonists tested were active in this test, together with BZR full or partial agonists (triazolam, diazepam, clonazepam, Ro 16-6028) as well as another minor tranquilizer (meprobamate) and various classical anticonvulsants (diphenylhydantoin, sodium valproate, phenobarbitone). Therefore, this approach seems to be particularly suitable for the early detection of compounds with anticonvulsant activity.

In another study in which the same technique was used, phenytoin and phenobarbitone were found to be ineffective antagonists of the convulsions induced by i.c.v. NMDA (VonVoigtlander *et al.*, 1987). However, it should be noted that in this instance the two drugs were administered by routes and at times before NMDA injection differing from those described in our protocol. We estimated the optimal route and time to peak activity for all drugs tested, and this careful methodology is probably one of the grounds for our positive results with phenytoin and phenobarbitone. Furthermore, this discrepancy could also be explained by the fact that different endpoints were chosen for assessing convulsions (tonic versus clonic convulsions). Indeed, it is well known that diphenylhydantoin, whilst blocking tonic convulsions in experimental animals, is able to unmask the clonic component of convulsions (as in maximal electroconvulsive shocks experiments). It is therefore not surprising that in our experiment (with tonic seizures as end-point), tonic seizures were effectively blocked by phenytoin, whereas in the study of Von-Voigtlander *et al.* (1987) (with clonic convulsions as end-point), the clonic convulsions were not affected.

Previous studies have used intraperitoneal administration of high doses of NMDA to induce convulsions (Czuczwar *et al.*, 1985; Ferkany *et al.*, 1988; Leander *et al.*, 1988). In these studies, benzodiazepine receptor ligands, such as diazepam, and classical anticonvulsants, such as phenobarbitone or diphenylhydantoin, were either totally ineffective or only weakly active. In the course of our study, we repeated these experiments and found results similar to those formed in the literature (data not shown). Thus, it appears that the route of administration (i.p. versus i.c.v.) of NMDA is an important factor in determining the activity of various compounds in this test. In other words, with i.p. NMDA injection, only competitive and non-competitive NMDA antagonists will be detected, whereas i.c.v. NMDA administration allows the detection of a wider range of anticonvulsant compounds. A possible explanation for this unexpected finding might be that the minute amount of i.c.v. NMDA does not diffuse far away from the site of injection, whereas NMDA reaches numerous sites within the CNS after the high systemic dose. Therefore, i.c.v. injection of NMDA is likely to induce primary epileptic activity in a restricted area, which may be more sensitive to NMDA antagonists as well as to compounds acting in a different manner. The prevention of NMDA-induced seizures by benzodiazepine receptor agonists was mediated by the benzodiazepine receptor and not by the NMDA receptor, as shown by the specific blockade with flumazenil (a specific benzodiazepine receptor antagonist).

Our results with dextrorphan are in good agreement with those of Ferkany *et al.* (1988), who found a similar anticonvulsant activity in i.p. NMDA-induced convulsions. Several morphinans have been shown to act as non-competitive NMDA antagonists at the same site as phencyclidine and MK801 (see Kemp *et al.*, 1987).

The observed prevention of tonic convulsions induced by centrally administered NMDA by a variety of compounds with greatly differing mechanisms of anticonvulsant activity was not surprising. Seizure activity, in general, is reduced by agents that antagonise the effect of chemical convulsants at their receptors (in the present model competitive and non-competitive NMDA antagonists), as well as by agents that reduce paroxysmal neuronal activity by inhibiting various cation channels (diphenyl-

hydantoin, phenobarbitone, sodium valproate) or enhancing the synaptic inhibitory action of GABA (benzodiazepine receptor agonists, phenobarbitone, sodium valproate). The mechanism of action of meprobamate is still unknown. No significant protective effect was obtained with $GABA_A$-receptor agonists in a tolerated dose-range, probably because they indiscriminately stimulate $GABA_A$-receptors on excitatory as well as inhibitory neurones.

In summary, the present model of tonic convulsions induced by i.c.v. NMDA is very sensitive to competitive and non-competitive NMDA antagonists and also to various classes of classical anticonvulsants. The model is a convenient test for assessing the *in vivo* activity of agents that interact with the NMDA receptor *in vitro* and it expands the battery of chemically-induced seizure models for the characterization of potential anticonvulsants.

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Effects of moderate hypoxia, hypercapnia and acidosis on haemodynamic changes induced by endothelin-1 in the pithed rat

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1 Pithed rats were respired at a fixed rate of 54 cycles min^{-1} and with a ventilation volume of either 20 (control) or 10 ml kg^{-1}. In these two preparations, the dose-response relationships for the systemic blood pressure responses to endothelin-1, administered i.v., were examined. Also, cardiac output, its distribution, tissue blood flows and vascular resistances were determined at both respiratory volumes in pithed rats given saline or during pressor responses to endothelin-1 (750 ng, i.v.). Finally, a comparison was made of the pressor responses to endothelin-1 in the blood perfused superior mesenteric arterial bed of pithed rats respired at 10 or 20 ml kg^{-1}.

2 In control rats the systemic blood pressure responses to i.v. endothelin-1 were biphasic with an initial, transient (30 s) decrease in blood pressure followed by a well sustained pressor response. These responses were dose-dependent (the ED_{50} for the pressor response being 0.27 ± 0.04 μg). The pressor effect of endothelin-1 was due to an increase in total peripheral resistance with no change in heart rate or cardiac output. This increased total peripheral resistance was due to vasoconstriction of the spleen, stomach, large intestine, small intestine and the pancreas/mesentery (in which it was most severe). Endothelin-1 also increased blood flow through the heart, lungs, liver, epididimides, fat and skin through redistribution of cardiac output to these vascular beds.

3 At the lower ventilation volume there was moderate acidosis, hypoxia and hypercapnia relative to those rats respired at 20 ml kg^{-1}. With respiration at 10 ml kg^{-1}, the pressor response to endothelin-1 was not sustained and, after oscillations in both blood pressure and heart rate, death occurred 15–20 min after administration. The pressor effect resulted from increases in cardiac output (due to increased stroke volume) and total peripheral resistance: the latter was caused by vasoconstriction in the stomach, small intestine, large intestine and pancreas/mesentery. Endothelin-1 increased blood flow through the heart, lungs, liver, kidneys, testes, fat and skin due to either an increase in cardiac output, redistribution of cardiac output or both.

4 Endothelin-1 induced dose-dependent pressor responses in the mesenteric bed *in situ*. At the lower ventilation volume the potency of endothelin-1 in this vascular bed was increased approximately two fold with the ED_{50} being 68 ± 7 pmol compared to 113 ± 15 pmol in the rats respired at 20 ml kg^{-1}.

5 This study indicates that, in normoxic control pithed rats, the pressor response to endothelin-1 was due largely to vasoconstriction of the splanchnic vascular bed. In rats with moderate hypoxia, hypercapnia and acidosis, the pressor response was due to vasoconstriction of the gastrointestinal tract as well as an increase in cardiac output. Endothelin-1 induced profound vasoconstriction in the mesenteric bed of the pithed rat both *in vivo* and *in situ*. The potency of endothelin-1 on this bed *in situ* was doubled by lowering the ventilation volume. An increase in cardiac contractility and severe gastrointestinal vasoconstriction may be the initial events leading to the eventual toxic effect of endothelin-1 in the hypoxic pithed rat.

Introduction

It has been known for some time that hypoxic conditions potentiate vasoconstrictor responses to noradrenaline (Detar & Gellai, 1971). When it was found that pig pulmonary artery strips *in vitro* responded to hypoxia with a contraction that was reduced upon removal of the endothelium (Holden & McCall, 1984), it was suggested that the endothe-

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lium may release a contractile factor. Previously, it had been demonstrated that the anoxic facilitation of the canine arterial responsiveness to noradrenaline was endothelium-dependent (De Mey & Vanhoutte, 1982; 1983). Rubanyi & Vanhoutte (1984, 1985) demonstrated that hypoxia stimulates the release of a diffusible contractile factor from the canine coronary artery whilst Iqbal & Vanhoutte (1988) showed its activity was dependent upon extracellular calcium.

Other evidence that endothelial cells might mediate vasoconstriction has come from studies of cultured cells. Medium conditioned by endothelial cells was shown to contain a polypeptide which was a potent vasoconstrictor of coronary arteries and the contractions produced by this factor were found to be sensitive to calcium channel blocking agents (Hickey *et al.*, 1985; Gillespie *et al.*, 1986). Very recently, the sequence of endothelin-1, a vasoactive peptide released from endothelial cells in culture, has been determined and the vasoconstrictor activity of this peptide also shown to be dependent on the presence of extracellular calcium (Yanagisawa *et al.*, 1988). This has led to speculation that endothelin-1 may be the endothelium-derived vasoconstrictor which is wholly, or in part, responsible for the vasoconstriction and the potentiation of vascular responses to noradrenergic stimulation that occur in anoxia or hypoxia.

In the present study we have investigated the effect of a lower ventilation volume, which induces a mild hypoxia, hypercapnia and acidosis, on the systemic pressor response to endothelin-1 in the pithed rat. We have also determined the effects of endothelin-1, in both normoxic and hypoxic conditions, on cardiac output, cardiac output distribution, regional blood flows and regional vascular resistances in the pithed rat. Preliminary results from this study were presented to the Spring 1989 Meeting of the British Pharmacological Society (MacLean & Hiley, 1989).

Methods

Male Wistar rats (250–330 g; Bantin & Kingman Ltd, Hull) were pithed under halothane anaesthesia with a 16 gauge steel needle. Immediately after being pithed, the rats were respired with air through a tracheal cannula by means of a respiratory pump (BioScience, Sheerness, Kent) operating at 54 cycles min^{-1}. Body temperature was monitored by means of a rectal probe and maintained at 37°C by means of a homeothermic blanket system (BioScience, Sheerness, Kent).

Determination of dose-response curves

After pithing, the right carotid artery, left jugular vein and the right femoral artery of the rats were cannulated respectively for recording arterial blood pressure (in all animals blood pressure was recorded by means of a Bell & Howell type 4-422-0001 pressure transducer connected to a Grass Model 7D polygraph), for drug administration and for removal of the arterial blood samples used for determination of arterial blood gases and pH (see below). The rats were ventilated at a volume of 20 ml kg^{-1} and a dose-response curve to endothelin-1 constructed by giving bolus injections of the peptide (0.01–1 μg in 0.1–0.5 ml saline). The blood pressure was allowed to return to baseline between each dose and the doses of endothelin-1 were given in random order. The ED_{50} for endothelin-1 was calculated for each rat and the results are given as the mean ± s.e.mean. It was not possible to construct dose-response curves to endothelin-1 in rats ventilated at 10 ml kg^{-1}.

Determination of cardiac output, its distribution, regional blood flow and regional vascular resistance

Rats were pithed under halothane anaesthesia and were artificially respired with a volume of either 20 ml kg^{-1} (control) or 10 ml kg^{-1}. The right femoral artery was cannulated to measure systemic arterial blood pressure and heart rate was determined from the systemic blood pressure trace by a Grass 7P44 cardiotachograph. The left femoral artery was also cannulated and connected to a Perfusor IV pump (Braun, Melsungen, F.R.G.) for the withdrawal of blood. With the aid of pressure monitoring, a cannula was passed down the right common carotid artery into the left ventricle. Either endothelin-1 (750 ng; Novabiochem, Läufelfingen, Switzerland) in 0.3 ml saline, or 0.3 ml saline was administered through a cannula previously placed in the left external jugular vein. When a sustained blood pressure response had been obtained, 60,000–80,000 ^{113}Sn labelled microspheres (15 ± 3 mm; NEN, Boston, MA, U.S.A.), suspended by ultrasonication in saline containing 0.01% Tween 80, were injected into the ventricle over 20 s. Blood was withdrawn at 0.5 ml min^{-1} from the left femoral artery during, and for 70 s after, the microsphere injection. The circulation was stopped with an air embolism and the organs dissected out, weighed and placed in scintillation vials for counting in a Packard Autogamma 500 γ-scintillation counter.

The number of counts in the blood sample was also determined in order that cardiac output, tissue blood flow and organ vascular resistances could be measured as described by McDevitt & Nies (1976) and by us previously (MacLean & Hiley, 1988a,b). All results are given as the mean ± s.e.mean. Statistically significant differences between groups were assessed by analysis of variance followed by the least

significant difference procedure (Snedecor & Cochran, 1980).

Determination of dose-response curves in the blood perfused superior mesenteric arterial bed preparation of the pithed rat

Rats were anaesthetized with sodium thiopentone (Trapanal; BYK Gulden, Konstanz, F.R.G.), 120 mg kg^{-1} i.p., pithed and respired with a volume of either 20 ml kg^{-1} (control) or 10 ml kg^{-1} as described above. The left jugular vein was cannulated for the administration of drugs and 0.9% saline at 6 ml h^{-1} (to prevent volume depletion). The right common carotid artery was cannulated to record systemic blood pressure and heart rate as before. The preparation used for *in situ* blood perfusion of the superior mesenteric bed was a modification of the method of Jackson & Campbell (1980), which we have previously described in detail (Hiley *et al.*, 1985; Randall & Hiley, 1988; Randall *et al.*, 1989).

Endothelin-1 was administered into the extracorporeal circuit in 0.9% NaCl and in volumes of 100 μl or less. Dose-response relationships were determined by administration of doses in random order and the perfusion pressure allowed to return to baseline levels (5–20 min) before administration of the subsequent dose. EC_{50} values were calculated for each animal and final values are given as the mean ± s.e.mean.

Blood gases and pH were analysed in all animals by removing 125 μl blood samples from the extracorporeal circuit immediately before the first administration of endothelin-1 or saline. These samples were subsequently analysed with a Corning 166 micro blood gas analyser.

Results

Effect of lowering ventilation volume on blood Po_2, Pco_2 *and pH*

In 12 animals, lowering the ventilation volume from 20 to 10 ml kg^{-1} at 54 cycles min^{-1} induced moderate blood acidosis, hypoxia and hypercapnia. Blood pH was reduced from 7.44 ± 0.26 to 7.22 ± 0.12, blood Po_2 (mmHg) was reduced from 83.1 ± 2.4 to 59.8 ± 2.8 and blood Pco_2 (mmHg) was increased from 38.7 ± 1.9 to 61.4 ± 3.5. ($P < 0.001$ for each change, Student's paired t test).

Dose-response curves for the changes in mean arterial pressure induced by endothelin-1 in pithed rats ventilated at 20 ml kg^{-1} and 10 ml kg^{-1}

In rats respired at 20 ml kg^{-1}, endothelin-1 (25 ng–1 μg) induced biphasic changes in arterial blood pressure as shown in Figure 1; there was an initial, transient depressor response (lasting approximately 30 s) which was followed by a well-sustained pressor response (lasting 30 min–1 h). It can be seen from Figure 2 that the pressor responses to endothelin-1 were dose-dependent with an ED_{50} of 0.27 ± 0.04 μg. The initial depressor response was not clearly dose-dependent and the ED_{50} was therefore not calculated.

We have previously shown that even very low doses of endothelin-1 (10–30 ng) become toxic in rats in which the ventilation volume is 10 ml kg^{-1} (MacLean & Hiley, 1989) and for this reason a dose-response curve for endothelin-1 could not be constructed using this artificial respiratory volume. Figure 3 shows the response obtained to 100 ng endothelin-1 administered to a pithed rat respired at 10 ml kg^{-1}. Whilst all doses up to 1 μg were well tolerated in the normoxic state, it may be seen that, at the lower ventilation volume, a single dose of endothelin-1 induced a pressor response which was not maintained and which was followed by fluctuations in blood pressure and heart rate; death invariably occurred 15–20 min after endothelin-1 administration. Figure 3 also shows that the transient depressor response to endothelin-1 is absent in the hypoxic rat.

Effect of endothelin-1 on systemic haemodynamics

Table 1 shows the effect of endothelin-1 on haemodynamics at ventilation volumes of both 20 and 10 ml kg^{-1}. The lower ventilation volume in itself produced a basal mean arterial blood pressure which

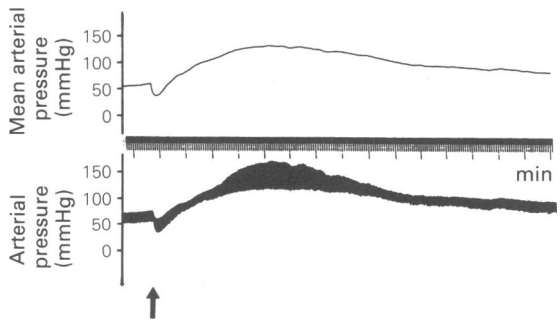

Figure 1 Experimental trace of blood pressure changes occurring after a single i.v. bolus injection of endothelin (750 ng) in a pithed rat respired with air at a ventilation volume of 20 ml kg^{-1}. The uppermost trace is the mean arterial pressure and the lowest trace shows instantaneous blood pressure; the middle trace shows timing marks with the distance between the largest marks indicating 1 min. The biphasic blood pressure response to endothelin may be seen clearly. The arrow indicates the point of endothelin administration.

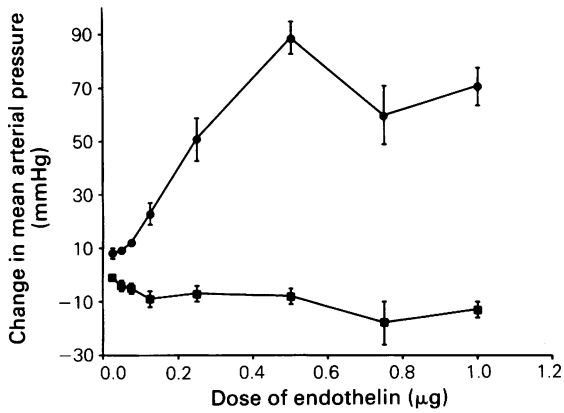

Figure 2 Blood pressure responses to endothelin in the pithed rat respired with air at a ventilation volume of $20\,ml\,kg^{-1}$ ($n = 5$). (■) The dose-response relationship for the initial, transient decrease in blood pressure to endothelin and (●) the dose-response relationship for the prolonged secondary pressor response to endothelin. The values are given as means with the vertical lines representing s.e.mean.

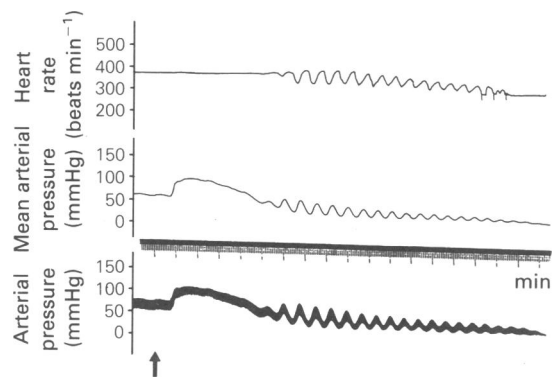

Figure 3 Experimental trace showing the blood pressure and heart rate responses to a single i.v. bolus injection of endothelin (100 ng) in a pithed rat respired with air at a ventilation volume of $10\,ml\,kg^{-1}$. From top to bottom the traces are: heart rate, mean blood pressure, timing scale with the largest marks indicating 1 min, and instantaneous blood pressure. The arrow indicates the point of endothelin administration.

was lower by 27% than in the rats respired at $20\,ml\,kg^{-1}$ (which will subsequently be referred to as normoxic rats). This difference was largely due to a lower pulse pressure since the basal diastolic pressures were not significantly different at the two respiratory volumes. In the normoxic rats, 750 ng endothelin-1 produced 51% and 61% increases in mean arterial and diastolic blood pressures, respectively, relative to the basal pressures observed before endothelin-1 administration. In the rats ventilated at $10\,ml\,kg^{-1}$ (subsequently referred to as hypoxic rats), the increases in mean arterial and diastolic blood pressures induced by 750 ng endothelin-1 were very similar in absolute terms to those occurring in the normoxic animals but, because of the lower basal mean arterial pressure, the increase in this in percentage terms, at 63%, was somewhat larger than in the controls. The diastolic blood pressure in the hypoxic animals was increased by 58% over the basal value.

In the normoxic rats there were no significant dif-

Table 1 The effects of endothelin-1 on haemodynamics in pithed rats ventilated with air at a rate of $54\,min^{-1}$ and a volume of either 20 or $10\,ml\,kg^{-1}$

	Saline ($20\,ml\,kg^{-1}$)	*Endothelin-1* ($20\,ml\,kg^{-1}$)	*Saline* ($10\,ml\,kg^{-1}$)	*Endothelin-1* ($10\,ml\,kg^{-1}$)
Basal mean arterial pressure (mmHg)	45 ± 2	41 ± 2	33 ± 3†	35 ± 3
Change in mean arterial pressure	−2 ± 1	21 ± 3***	−1 ± 1	22 ± 3***
Basal diastolic pressure (mmHg)	35 ± 2	33 ± 2	31 ± 3	31 ± 2
Change in diastolic pressure (mmHg)	−2 ± 1	20 ± 2***	−1 ± 1	18 ± 3***
Basal heart rate (beats min^{-1})	295 ± 15	325 ± 16	281 ± 8	284 ± 12
Change in heart rate (beats min^{-1})	1 ± 3	0 ± 7	−3 ± 1	−1 ± 3
Cardiac index (ml min^{-1} 100 g b.wt)	13.1 ± 0.9	13.8 ± 0.8	11.2 ± 1.0	14.8 ± 0.7*
Stroke volume (μl)	120 ± 7	111 ± 7	107 ± 13	151 ± 11*
TPR (mmHg ml^{-1} min 100 g b.wt)	3.3 ± 0.3	4.6 ± 0.3***	2.8 ± 0.3	4.0 ± 0.3**

For all groups, $n = 8$. Basal heart rate, diastolic and mean blood pressure values are those before administration of saline or endothelin-1. TPR = total peripheral resistance. The changes in blood pressure and heart rate are given as the differences between the values immediately before saline or endothelin-1 injection and those at the mid-point of the microsphere injection. Statistical differences were assessed by analysis of variance: between the two endothelin groups and their respective saline controls; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$: between the saline ($20\,ml\,kg^{-1}$) group and the saline ($10\,ml\,kg^{-1}$) group; † $P < 0.05$.

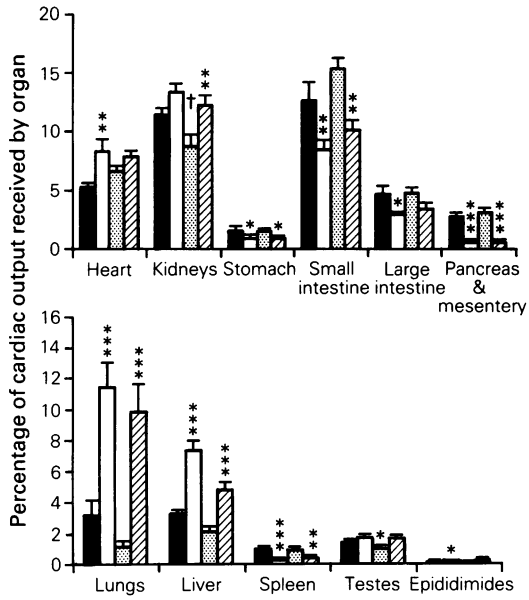

Figure 4 Effect of endothelin on the fractional distribution of cardiac output in pithed rats respired with air at either $20\,ml\,kg^{-1}$ or $10\,ml\,kg^{-1}$. The solid columns represent the results from rats given saline and ventilated at $20\,ml\,kg^{-1}$; the open columns are data from rats given 750 ng endothelin i.v. and ventilated at $20\,ml\,kg^{-1}$; the stippled columns are the data from rats given saline and ventilated at $10\,ml\,kg^{-1}$; and the hatched columns are the results from rats given 750 ng endothelin i.v. and ventilated at $10\,ml\,kg^{-1}$. $n = 8$ for all groups. Statistical differences were assessed by analysis of variance followed by the least significant difference procedure: between the endothelin groups and their respective saline controls; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$: between the saline group respired at $20\,ml\,kg^{-1}$ and the same group respired at $10\,ml\,kg^{-1}$; † $P < 0.05$.

ferences between the saline and endothelin-1 groups with respect to cardiac output, stroke volume or heart rate; the greater blood pressure after endothelin-1 was due to a 39% increase in total peripheral resistance. On the other hand, the hypoxic rats showed a 43% greater total peripheral resistance and a 32% greater cardiac index in the endothelin-1 group than in the saline group. The greater cardiac index was due to a 41% greater stroke volume since endothelin-1 had no effect on heart rate.

Effect of endothelin-1 on cardiac output distribution

Figure 4 shows that, in the normoxic animals, the endothelin-1 group showed fractions of cardiac output passing to the heart, lungs, liver and epididimides that were greater by 58%, 262%, 128% and 57% respectively relative to those in the saline group. There were, however, lesser proportions of the cardiac output passing to the spleen, stomach, small intestine, large intestine and pancreas/mesentery (by 73%, 43%, 34%, 39% and 79%, respectively) in the endothelin-1 relative to the saline group. Figure 4 also shows that, in the hypoxic saline group, the proportion of the cardiac output passing to the renal vascular bed was lower by 23% than in the equivalent normoxic group. Also, it may be seen that, in the hypoxic state, the endothelin-1 group showed, relative to the saline group, greater proportions of the cardiac output passing to the lungs (greater by 728%), liver (125%), kidneys (40%) and testes (57%) but lower proportions reached the spleen (lower by 52% than the saline group), stomach (45%), small intestine (34%) and pancreas/mesentery (81%).

Effects of endothelin-1 on regional blood flow

Table 2 shows that, in the normoxic animals, the endothelin-1 group had greater blood flows, relative to the saline group, through the heart, lungs, liver, fat, epididimides and skin (of 83%, 258%, 135%, 138%, 61% and 58% respectively) whilst there were lower flows through the spleen (less by 71%), small intestine (34%) and pancreas/mesentery (74%). Table 2 also shows that, in the hypoxic groups, the animals receiving endothelin-1 had, relative to the saline group, greater blood flows through the heart (45%), lungs (516%), liver (130%), kidneys (65%), testes (76%), fat (186%) and skin (107%) whilst blood flow through the pancreas/mesentery was decreased by 71% after endothelin.

Effects of endothelin-1 on regional vascular resistances

In the normoxic animals, vascular resistances in the splenic, stomach, small intestinal, large intestinal and mesenteric beds were 552%, 159%, 93%, 97% and 569% respectively greater in the endothelin-1 than in the saline group (Table 3). Coronary vascular resistance was 40% lower, and pulmonary vascular resistance greater by 149%, in the saline hypoxic group relative to the saline normoxic group. In the hypoxic group of rats given endothelin-1, coronary vascular resistance was still 26% lower than in the normoxic saline animals ($P < 0.05$). Table 3 also shows that, in the hypoxic animals, pulmonary and hepatic vascular resistances were lower by 88% and 43%, respectively, in the endothelin-1 group relative to the saline group but vascular resistances in the gastrointestinal tract were all significantly greater in the hypoxic, endothelin-1 group than in the hypoxic, saline group; the change for the stomach was 191% and those for the small intestine, large intestine and

Table 2 The effects of endothelin or saline on organ blood flow (ml min^{-1} g^{-1} organ wt) in pithed rats ventilated with air at a rate of 54 min^{-1} and a volume of either 20 ml kg^{-1} or 10 ml kg^{-1}

Organ	*Saline* (20 ml kg^{-1})	*Endothelin-1* (20 ml kg^{-1})	*Saline* (10 ml kg^{-1})	*Endothelin-1* (10 ml kg^{-1})
Heart	1.72 ± 0.11	3.14 ± 0.43**	2.19 ± 0.19	3.17 ± 0.25*
Lungs	0.92 ± 0.28	3.29 ± 0.51***	0.53 ± 0.21	3.26 ± 0.58***
Liver	0.091 ± 0.009	0.214 ± 0.022***	0.07 ± 0.017	0.161 ± 0.018***
Spleen	0.454 ± 0.063	0.132 ± 0.023**	0.385 ± 0.099	0.285 ± 0.046
Kidneys	1.85 ± 0.09	2.26 ± 0.08	1.48 ± 0.28	2.44 ± 0.23**
Testes	0.19 ± 0.013	0.231 ± 0.021	0.154 ± 0.024	0.271 ± 0.017**
Fat	0.026 ± 0.007	0.062 ± 0.013**	0.014 ± 0.004	0.040 ± 0.007*
Epididimides	0.082 ± 0.012	0.132 ± 0.012*	0.112 ± 0.011	0.13 ± 0.017
Skeletal muscle	0.094 ± 0.012	0.107 ± 0.024	0.081 ± 0.014	0.101 ± 0.018
Skin	0.073 ± 0.008	0.115 ± 0.017*	0.046 ± 0.011	0.095 ± 0.01**
Stomach	0.38 ± 0.055	0.226 ± 0.044	0.322 ± 0.045	0.251 ± 0.051
Small intestine	0.983 ± 0.149	0.646 ± 0.04*	0.966 ± 0.146	0.881 ± 0.064
Large intestine	0.691 ± 0.094	0.453 ± 0.051	0.674 ± 0.123	0.59 ± 0.074
Pancreas and mesentery	0.329 ± 0.031	0.084 ± 0.018***	0.31 ± 0.066	0.091 ± 0.016***

For all groups, $n = 8$. Statistical differences between the two endothelin groups and their respective saline controls were assessed by analysis of variance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

pancreas/mesentery were respectively 94%, 108% and 389%.

Effect of lowering ventilation volume on the pressor responses to endothelin-1 in the blood perfused superior mesenteric bed of the pithed rat

In the rats used in this part of the study, there were no significant differences in mean arterial blood pressures or heart rates between the rats artificially respired at the two volumes; mean arterial blood pressure for the 20 ml kg^{-1} animals was 41 ± 4 mmHg and heart rate was 361 ± 21 beats min^{-1}, whilst for the hypoxic animals the respective values were 49 ± 4 mmHg and 353 ± 16 beats min^{-1}. Basal mesenteric perfusion pressure was, however, significantly lower ($P < 0.05$) in the hypoxic animals (49.1 ± 3.5 mmHg) when compared to the controls (65.0 ± 5.8). Figure 5 illustrates the log dose-response relationships for the pressor effects of endothelin-1 in the mesenteric bed of pithed rats ventilated at either 20 or 10 ml kg^{-1}. The curve was

Table 3 The effects of endothelin-1 or saline on organ vascular resistances (mmHg ml^{-1} min, g) in pithed rats ventilated at a rate of 54 min^{-1} and with a volume of 20 ml kg^{-1} or 10 ml kg^{-1}

Organ	*Saline* (20 ml kg^{-1})	*Endothelin-1* (20 ml kg^{-1})	*Saline* (10 ml kg^{-1})	*Endothelin-1* (10 ml kg^{-1})
Heart	25.8 ± 2.6	22.2 ± 2.5	15.5 ± 1.6†	19.2 ± 2.1
Lungs	69.9 ± 13.0	23.4 ± 4.4	174 ± 68†	21.0 ± 3.1*
Liver	490 ± 39	318 ± 37	654 ± 146	373 ± 18*
Spleen	109 ± 20	711 ± 228**	149 ± 52	251 ± 48
Kidneys	23.7 ± 2.29	27.8 ± 0.98	24.8 ± 2.7	24.8 ± 2.2
Testes	238 ± 34	286 ± 26	232 ± 25	216 ± 15
Fat	4165 ± 2073	1452 ± 340	3523 ± 737	1872 ± 388
Epididimides	603 ± 105	498 ± 42	301 ± 25	505 ± 81
Skeletal muscle	535 ± 99	1033 ± 341	454 ± 66	976 ± 371
Skin	619 ± 61	653 ± 110	941 ± 171	674 ± 106
Stomach	133 ± 25	344 ± 55**	110 ± 13	320 ± 75**
Small intestine	51.4 ± 9.4	99.2 ± 6.3***	35.7 ± 2.6	69.3 ± 7.9**
Large intestine	76.2 ± 17.6	150 ± 16**	54.7 ± 6.8	114 ± 19*
Pancreas and mesentery	143 ± 22	956 ± 147***	157 ± 51	767 ± 122***

For all groups, $n = 8$. Statistical differences were assessed by analysis of variance: between the two endothelin groups and their respective saline controls; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$: between the saline (20 ml kg^{-1}) group and the saline (10 ml kg^{-1}) group; † $P < 0.05$.

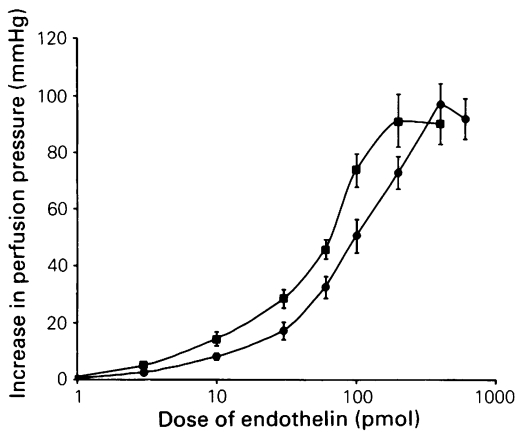

Figure 5 Pressor effects of endothelin in the *in situ* blood perfused, mesenteric arterial bed of rats respired at a ventilation volume of either $20\,ml\,kg^{-1}$ (●) or $10\,ml\,kg^{-1}$ (■) and at a rate of $54\,min^{-1}$. The values are given as means with the vertical lines representing s.e.mean ($n = 6$ for both groups).

shifted significantly ($P < 0.05$) to the left in the hypoxic animals with the ED_{50} decreased from 113 ± 15 pmol (normoxic) to 68 ± 7 pmol (hypoxic). The maximum pressor responses were not significantly different at the two ventilation volumes (Figure 5).

Discussion

Changes in mean arterial pressure induced by endothelin-1 in pithed rat

The results presented in Figures 1 and 2 show that endothelin-1 induces dose-dependent changes in blood pressure which are biphasic in nature in pithed rats artificially respired with room air at $20\,ml\,kg^{-1}$. It has been shown that endothelin-1 releases endothelium-derived relaxing factor (EDRF) in the rat isolated perfused mesenteric arterial bed (Warner *et al.*, 1989) although not, apparently, from cultured bovine aortic endothelial cells (De Nucci *et al.*, 1988). Thus, the initial, transient decrease in blood pressure observed before the onset of the sustained pressor response may be due to regional vasodilatation resulting from release of endothelium-derived relaxant factor (EDRF). However, this transient depressor response is absent in pithed rats ventilated at $10\,ml\,kg^{-1}$ and, in these animals, endothelin-1 becomes toxic at very low doses (MacLean & Hiley, 1989). It has been suggested that anoxia-induced inhibition of endothelium-dependent relaxation may contribute to anoxic facilitation of arterial contractions (De May & Vanhoutte, 1983) and, if so, this may explain why, in hypoxic conditions, the initial transient response to endothelin-1 was absent.

Haemodynamic changes induced by endothelin-1 in pithed rats artificially respired with air at $20\,ml\,kg^{-1}$

The results of this study show that, in the normoxic rats, the sustained increases in mean arterial pressure and diastolic blood pressure induced by endothelin-1 were due to an increase in total peripheral resistance as the peptide, at the dose used here, had no effects on cardiac output, stroke volume or heart rate. The increase in diastolic blood pressure was 10% greater than that observed from mean arterial pressure, an observation also made in anaesthetized, chemically denervated rats by Yanagisawa *et al.* (1988).

In comparison to saline-treated animals, endothelin-1 caused an increase in the fractional distribution of cardiac output to the heart, inducing an increase in coronary blood flow. Despite previous findings that endothelin-1 is a potent coronary vasoconstrictor and inotrope (Yanagisawa *et al.*, 1988; Hu *et al.*, 1988), neither of these effects of endothelin-1 were observed in this study. The increase in the fraction of cardiac output passing to the heart was not due to coronary vasodilatation but rather to redistribution of blood away from other vascular beds in which there was vasoconstriction, that is primarily the splenic and gastrointestinal circulations. Similarly, endothelin-1 also caused a redistribution of cardiac output towards the lungs, liver and epididimides, inducing increases in blood flow in these organs, without producing decreases in their vascular resistances. Endothelin-1 had no effect on blood flow through the testes or the renal vascular bed in the normoxic animals, but caused increases in blood flow through the fat and skin vascular beds. As there was no significant change in vascular resistance of these beds, the increase in flow must be due to redistribution of cardiac output.

Endothelin-1 caused decreases in splenic, small intestinal and mesenteric blood flows by causing vasoconstriction in these vascular beds; this resulted in corresponding decreases in the proportions of cardiac output they received. Although blood flows in the gastric and large intestinal vascular beds did not decrease significantly, there were increases in vascular resistance in these regions and these produced reductions in the fractional distribution of cardiac output to these vascular beds. In this study, endothelin-1-induced vasoconstriction was most profound in the mesenteric bed. Endothelin-1-induced mesenteric vasoconstriction has previously been observed in the anaesthetized spontaneously hypertensive rat (Wright & Fozard, 1988) and in conscious freely moving rats (Han *et al.*, 1989). From the

present study, it is evident that, in the pithed rat, the increase in total peripheral resistance observed following administration of endothelin-1 was largely due to vasoconstriction in the splanchnic region. Previous studies by other workers have also demonstrated that endothelin-1, at a dose which induced a higher increase in systemic blood pressure than observed in this study, increased total peripheral resistance by increasing vascular resistance in the kidneys and skeletal muscle as well as in the gastrointestinal tract (Thomas *et al.*, 1989; Walder *et al.*, 1989).

Effects of endothelin-1 on haemodynamics in pithed rats ventilated with air at 10 ml kg^{-1}

At the lower ventilation volume there was a reduced basal mean arterial blood pressure relative to the normoxic rats. In the absence of a functional sympathetic nervous system, as is the case in the pithed rat, humoral mechanisms, such as increased circulating angiotensin II (de Jonge *et al.*, 1982), must be involved in the maintenance of blood pressure. It is evident from the present study that these compensatory mechanisms are compromised in the hypoxic pithed rat and this confirms our previous results (MacLean & Hiley, 1988b). Since there were no significant differences between the hypoxic animals and the normoxic ones with respect to cardiac index or total peripheral resistance, although both means were numerically lower in the hypoxic animals, it is not possible to determine which of these was responsible for this slightly lower basal diastolic blood pressure. However, previous studies have shown that the direct effect of hypercapnia and hypoxia on the myocardium is to depress cardiac contractility (Price & Helrich, 1955; Sonnenblick & Kirk, 1972) and thus it is possible that decreased cardiac efficiency may be contributing to this effect. It is interesting to note that, in these pithed rats, the hypoxia combined with the hypercapnia and acidosis also induced coronary vasodilatation.

In the hypoxic rats, the increases in mean arterial and diastolic blood pressures induced by endothelin-1 were the result of an increase in both total peripheral resistance and cardiac output, unlike the situation in the control rats where the increase in blood pressure was solely due to an increase in total peripheral resistance. The increased total peripheral resistance in the hypoxic animals given endothelin-1 was due to profound vasoconstriction of the gastrointestinal tract, whilst the increased cardiac index was the result of an increase in stroke volume as, at this dose, endothelin-1 had no effect on heart rate. In the pithed rat, an increase in cardiac stroke volume could be caused by an increase in cardiac contractility or an increase in venous return. Endothelin-1 has been shown to exert potent inotropic effects in the rat atria (Hu *et al.*, 1988) and also to be a more potent vasoconstrictor of veins than arteries (D'Orleans-Juste *et al.*, 1988). With the knowledge that hypoxia-induced vasoconstriction can be endothelium-dependent and that the Po_2 of venous blood is lower than that of arterial, there is evidence to suggest a role for endothelin-1 in venous constriction, and hence, in maintenance of venous return. Thus, the increased stroke volume observed after endothelin-1 administration in the hypoxic state could be due to the combined effects of both increased venous return and cardiac contractility.

When comparing the saline groups, the lower ventilation volume produced a lower calculated coronary vascular resistance than in the normoxic animals. However, for the hypoxic rats, the increased coronary blood flow observed after endothelin-1 administration would appear to be the result of the increase in cardiac output since there was no significant difference between the two groups of rats given endothelin-1.

Hypoxia, hypercapnia and acidosis have previously been shown to induce increases in pulmonary vascular resistance (Bergofsky *et al.*, 1962; Sykes *et al.*, 1972) and this is observed here, the lowering of ventilation volume inducing an increase in the vascular resistance of the lungs. There was an increase in blood flow through the lungs following endothelin-1 administration in the hypoxic rats and this was due to the combined effects of the increased cardiac index and the apparent decrease in vascular resistance observed in this vascular bed. It should be noted that the microspheres detected in the lungs represent not only those passing through the bronchial arteries, but also those passing through arteriovenous anastomoses and which are subsequently trapped in the lungs after passage along the great veins. Thus these-apparent effects of endothelin-1 on lung blood flow could be due to actions on either the anastomoses or the bronchial arteries.

In the hepatic artery also there was a greater blood flow in the hypoxic animals given endothelin-1 relative to the hypoxic control group given saline. Here, as in the lungs, there was a lower calculated vascular resistance after the peptide and, thus, the enhanced rate of perfusion would appear to be the result of this reduced resistance as well as the increased cardiac output. It is known that the two vascular inputs into the liver interact by, apparently, purely mechanical processes, such that increases or decreases in hepatic portal venous supply are at least in part compensated by changes in hepatic arterial inflow resistance (Sato *et al.*, 1977). Thus an increase in resistance to portal inflow results in a decrease in hepatic arterial resistance and the changes in hepatic arterial resistance to flow seen in this study may

reflect opposing effects in gastrointestinal vascular resistance.

The decrease in the distribution of cardiac output to the spleen following endothelin-1 administration in these hypoxic rats was presumably due to redistribution of the cardiac output to other vascular beds (e.g. the lungs and liver), as there was no change in splenic vascular resistance. In view of the increased cardiac index, this decrease in distribution to the spleen resulted in there being no net effect on splenic blood flow. The endothelin-induced increases in renal and testicular blood flows observed in these animals were due to the combined effects of the increase in cardiac index and the proportions passing to these vascular beds; since there was no change in either testicular or renal vascular resistances, then this redistribution in their favour must be the result of them being relatively less resistant to flow after endothelin-1 than the gastrointestinal tract.

Despite the greater vascular resistances in the stomach, large intestinal and small intestinal beds relative to the hypoxic saline controls, the increased cardiac index resulted in there being no net alteration in the blood flow through these organs. The most profound vasoconstriction observed with endothelin-1 in the hypoxic rats was, as for the normoxic rats, observed in the mesenteric bed. In this region there was a much lower blood flow than in the hypoxic saline controls since the redistribution of cardiac output away from this bed, resulting from the vasoconstriction induced by endothelin-1, was sufficient to overcome the effects of the increase in cardiac output.

Effect of a lower ventilation volume on the pressor responses to endothelin-1 in the blood perfused superior mesenteric bed of the pithed rat

Dose-dependent vasoconstriction of the superior mesenteric arterial bed by endothelin-1 has previously been formed both in isolated (De Nucci *et al.*, 1988; Hiley *et al.*, 1989) preparations and in similarly anaethetized intact rats in which the bed has been perfused *in situ* with blood (Hiley *et al.*, 1989). The present study confirms that dose-dependent increases in perfusion pressure also occur in the blood perfused mesenteric arterial bed of pithed rats. Hence, both in the intact circulation and in the *in situ* isolated blood perfused bed, endothelin-1 causes profound constriction in the mesenteric vasculature of the pithed rat.

The results also show that it is the potency of endothelin-1 on this vascular bed which changes when the rats are respired with the lower respiratory volume. Thus, in hypoxic, hypercapnic and acidotic conditions the peptide is twice as potent as in the normoxic group of animals and the maximum response is unchanged. This may be due to the inhibition by hypoxia of the release of EDRF by endothelin, but we have been unable to confirm this because we have found that, in similarly anaesthetized rats, removal of the endothelium with detergent in this preparation severely reduces the maximum response to endothelin-1, something that we have not observed for other vasoconstrictor agonists (Randall *et al.*, 1989). Other possibilities for this apparent increase in potency include changes in receptor number, affinity or efficiency of coupling to effector mechanisms.

Conclusions

In conclusion, the results of this study indicate that, in the normoxic pithed rat, the systemic pressor effect of endothelin-1 is mainly due to profound vasoconstriction of the splanchnic vascular beds. In the hypoxic rats, the pressor effect is due to a combination of an increase in gastrointestinal vasoconstriction and an increase in cardiac output. Endothelin-1 is very toxic in rats with moderate hypoxia, hypercapnia and acidosis with death occurring within 20 min of a low dose and being associated with disturbances of heart rate. The results presented here are consistent with this being due to an effect of endothelin-1 on cardiac contractility which eventually leads to deterioration of cardiac performance. The severe endothelin-induced gastrointestinal vasoconstriction, particularly that of the mesenteric bed, may contribute to the eventual cardiovascular collapse. It may be that any physiological role for endothelin release and activity are compromised in vascular disease states such as hypotension, hypertension and hypoxia. If so, it may play a role in phenomena like the acute renal failure observed in hypotension and hypoxia (Firth *et al.*, 1988), in myocardial ischaemic conditions and in splanchnic vasoconstriction associated with shock states.

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Amelioration of glycerol-induced acute renal failure in the rat with 8-cyclopentyl-1,3-dipropylxanthine

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1 Previous studies have shown that 8-phenyltheophylline (8-PT), a non-selective antagonist at adenosine A_1- and A_2-receptors, can ameliorate the severity of glycerol-induced acute renal failure (ARF) in the rat. In the present study we have examined the effects of an antagonist with selectivity for adenosine A_1-receptors (8-cyclopentyl-1,3-dipropylxanthine, CPX) on the development of ARF.

2 In the anaesthetised rat 8-PT ($4\,mg\,kg^{-1}$, i.v.) and CPX ($0.1\,mg\,kg^{-1}$, i.v.) antagonised adenosine-evoked responses which are thought to be mediated via A_1-receptors (bradycardia and decrease in renal blood flow). The agonist dose-ratio produced by CPX was equal to or greater than that found with 8-PT (heart rate and renal blood flow respectively). The hypotensive response to adenosine which is predominantly due to A_2-receptor activation was also antagonised by 8-PT, whereas CPX was a much less effective antagonist of this response.

3 Administration of CPX ($0.1\,mg\,kg^{-1}$, i.v.; twice daily for two days) significantly attenuated the increase in plasma levels of urea and creatinine, the increased kidney weight and the renal tubule damage observed in rats 2 days following induction of ARF with intramuscular glycerol injection. In addition treatment with CPX significantly enhanced the clearances of inulin and *p*-aminohippurate.

4 After glycerol injection, the mortality rate over 7 days in untreated and vehicle-treated rats was 43% and 21% respectively. In contrast, all animals treated with CPX survived over the 7 day observation period.

5 These results support the suggestion that adenosine is an important factor in the development of ARF and indicate that this effect of the purine is likely to be mediated via an adenosine A_1-receptor.

Introduction

Recent studies in the rat have demonstrated that administration of the alkylxanthine 8-phenyltheophylline (8-PT) can reduce the severity of acute renal failure (ARF) produced by intramuscular glycerol injection (Bowmer *et al.*, 1986; Yates *et al.*, 1987; Bowmer *et al.*, 1988). We have suggested that the beneficial effects of 8-PT are due to its ability to block adenosine receptors, particularly those which mediate vasoconstriction of the afferent renal arterioles. This proposal is supported by the observation that in the initial stage of glycerol-induced ARF renal blood flow is reduced (Stein *et al.*, 1978) and the resultant ischaemia is likely to evoke release of adenosine (Miller *et al.*, 1978). In addition, ARF is associated with a fall in glomerular filtration rate, an effect which is also produced by an infusion of adenosine (Churchill, 1982). Furthermore, blockade of adenosine uptake with dipyridamole augments the impairment in renal function associated with various models of ARF (Arend *et al.*, 1987; Lin *et al.*, 1987).

The renal vasculature possesses both A_1- and A_2-adenosine receptors and activation of A_1-receptors mediates arteriolar constriction, whereas activation of A_2-receptors mediates arteriolar dilatation (Murray & Churchill, 1984). Thus, an A_1-selective antagonist may prove as beneficial in the treatment of ARF as 8-PT which has been found to block both sub-classes of adenosine receptor (Collis *et al.*, 1985). We have pursued this idea by studying 8-cyclopentyl-1,3-dipropylxanthine (CPX) which has been shown, *in vitro*, to be a selective A_1-antagonist (Haleen *et al.*, 1987; Collis *et al.*, 1989). Initial studies were concerned with evaluating the ability of CPX to antagonise adenosine-induced

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responses *in vivo* mediated predominantly by A_1-receptors (heart rate and renal blood flow) or A_2-receptors (blood pressure). In further experiments we investigated the effect of CPX on biochemical, functional and morphological indices of glycerol-induced ARF.

Methods

Evaluation of adenosine antagonism

Measurement of blood pressure and heart rate Male Wistar rats (300–350 g) were anaesthetised with thiobutabarbitone (120 mg kg^{-1}, i.p.). Polyethylene cannulae were inserted into the left jugular vein for drug administration, into the left carotid artery for measurement of blood pressure and into the trachea. The rats were artificially respired (90 strokes min^{-1}, 3.5–4.0 ml tidal volume) with air supplemented with oxygen. Body temperature was monitored via a rectal probe and maintained at 37–38°C by heat lamps. Systemic blood pressure was recorded directly via a pressure transducer (Bell and Howell L221) and heart rate was derived electronically from the blood pressure signal.

After a 15–30 min stabilisation period, adenosine dissolved in saline (0.9% w/v NaCl) was administered via the venous catheter (20–2000 μg kg^{-1}, in 4–400 μl) with a micrometer controlled syringe. Blood pressure and heart rate were allowed to return to control values after each administration of adenosine.

Preliminary studies demonstrated that repeatable dose-response curves for adenosine in terms of its hypotensive and bradycardic responses could be obtained in this preparation. Dose-response curves to adenosine were then repeated in rats before and 15 min after administration of CPX (0.1 mg kg^{-1}, i.v.) or 8-PT (4 mg kg^{-1}, i.v.). CPX was prepared at 0.1 mg ml^{-1} in 1% v/v DMSO, 0.75% v/v 1 M NaOH in saline, and 8-phenyltheophylline (8-PT) at 10 mg ml^{-1} in 50% v/v 0.1 M NaOH and polyethylene glycol 400. These vehicles had no significant effects on adenosine-evoked vasodilatation and bradycardic responses in the anaesthetised rat.

Measurement of renal blood flow Male Wistar rats (200–250 g) were anaesthetised with thiobutabarbitone (120 mg kg^{-1}, i.p.) and the trachea cannulated. A polyethylene cannula was inserted into the left jugular vein to allow a continuous infusion of 6% w/v dextrose (2.4 ml h^{-1}) delivered by a Sage Infusion pump. The right carotid artery was exposed and a cannula was passed down the artery and into the thoracic aorta to a position just above the branch of the left renal artery. The correct position of the cannula was verified at the end of the experiment on post mortem examination. A flank incision was made and an electromagnetic flow probe (internal circumference 2.0 mm; Carolina Medical Electronics) was placed around the left renal artery. This was connected to an electromagnetic flow meter (Model FM 501, Carolina Medical Electronics) for a direct reading of renal blood flow (RBF). A polyethylene cannula was inserted into the left femoral artery and attached to a transducer for the measurement of blood pressure and heart rate.

Forty-five min after the completion of surgery, increasing bolus doses of adenosine (0.165 to 500 μg dissolved in saline) were given via the carotid cannula in a constant volume of 50 μl and the resultant fall in RBF was recorded. After initial doses of adenosine, rats received CPX (0.1 mg kg^{-1}, i.v.) or 8-PT (4 mg kg^{-1}, i.v.) and ten minutes later, responses to adenosine were recorded. In order to assess the shift of the dose-response curve produced by CPX or 8-PT, the dose-ratio of adenosine required to reduce RBF by 1.0 ml min^{-1} was estimated.

Evaluation of 8-cyclopentyl-1,3-dipropylxanthine in glycerol-induced acute renal failure

Induction of acute renal failure The method for inducing ARF has been described previously (Bowmer *et al.*, 1982). Briefly, male Wistar rats (200–300 g) were deprived of drinking water for 24 h and ARF was produced by an intramuscular injection of 50% v/v glycerol in sterile saline, 10 ml kg^{-1}. Control rats were similarly dehydrated but injected with saline (10 ml kg^{-1}) instead of glycerol. Immediately after injection of either saline or glycerol, the drinking water was returned for the remainder of the experiment.

Experimental protocol Before the administration of glycerol or saline, a 0.75 ml control blood sample (0 h) was taken from the tail vein. Further blood samples were taken at 24 h from the tail vein and at 48 h from a carotid cannula following clearance determinations. Immediately after glycerol or saline injection, rats were treated with either CPX (0.1 mg kg^{-1}) or vehicle (1.0 ml kg^{-1}; 1.0% v/v DMSO, 0.75% v/v 1 M NaOH in saline), administered via the tail vein. Additional doses of CPX or vehicle were also given i.v. at 12, 24 and 36 h after the initial dose. A group of saline-injected and a group of glycerol-injected rats received no treatment. At 48 h after injection of saline or glycerol, rats were anaesthetised and the clearances of [^{14}C]-*p*-aminohippuric acid (C_{PAH}) and [^{3}H]-inulin (C_{IN}) determined. At the end of the experiment rats were killed by an overdose of anaesthetic, the kidneys removed, weighed and prepared for histology.

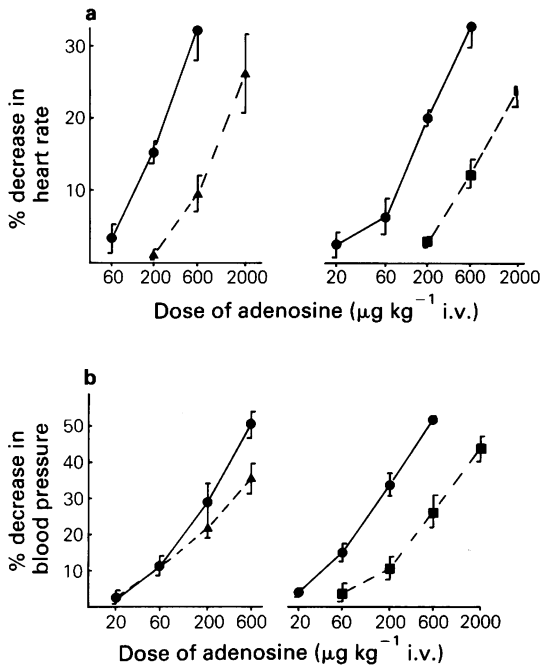

Figure 1 The effect of 8-phenyltheophylline (8-PT) and 8-cyclopentyl-1,3-dipropylxanthine (CPX) on the heart rate (a) and mean blood pressure (b) response evoked by adenosine (i.v.) in the anaesthetised rat. (●) Control ($n = 6$), (▲) CPX 0.1 mg kg^{-1} ($n = 6$), (■) 8-PT 4 mg kg^{-1} ($n = 6$). Results are given as mean and vertical lines indicate s.e.mean.

In a separate series of experiments, a group of glycerol-injected rats and a group of rats treated with either vehicle or CPX as above were studied over a 7 day period. Blood samples were taken from the tail vein prior to and on the 2nd and 5th days after glycerol injection. A further blood sample was taken from a carotid cannula following determinations of C_{PAH} and C_{IN} on the 7th day.

Measurement of plasma urea and creatinine concentrations Plasma urea and creatinine concentrations were determined by standard spectrophotometric assays: urea by reaction with diacetyl monoxime and creatinine by reaction with picrate in alkaline solution.

Determination of [^{3}H]-inulin and [^{14}C]-p-aminohippuric acid clearances Rats were anaesthetised with sodium pentobarbitone (60 mg kg^{-1}, i.p.). The trachea was cannulated to maintain a clear airway and rats allowed to breath spontaneously throughout the experiment. Cannulae were inserted into the left jugular vein and right carotid artery and the animals were heparinised (500 i.u. kg^{-1}, i.v.). The single injection method of Hall *et al.* (1977) was then used to measure simultaneously the clearances of [^{3}H]-inulin (100 mg kg^{-1}; 20 μCi kg^{-1}, i.v.) and [^{14}C]-*p*-aminohippurate (40 mg kg^{-1}; 4 μCi kg^{-1}, i.v.).

Kidney histology At the end of the experiment, kidneys were removed, cleared of adherent tissue and their weights recorded. The kidneys were bisected longitudinally, washed and placed in formal saline (BDH). A longitudinal section was cut from one kidney of each rat and stained with haematoxylin and eosin. Sections were examined by a pathologist, who was unaware of the treatment the donor animal had received, and the extent of renal damage was assessed according to the scoring system previously described (Bowmer *et al.*, 1986). The degree of necrosis and presence of casts were each scored out of 5. The two scores for each kidney were added to give the total damage score (maximum 10).

Statistical methods Results are expressed as mean ± s.e.mean. Statistical comparison of creatinine, urea, C_{IN}, C_{PAH} and total kidney weight data were made by either an unpaired Student's *t* test or one way analysis of variance (ANOVA) with means being compared by the method of Least Significant Difference (Snedecor & Cochran, 1967). Statistical analysis of the histological damage score was made by a two-sided Mann-Whitney test. Other comparisons utilised an unpaired Student's *t* test.

Materials

Adenosine and 8-PT were purchased from Sigma Chemical Co. [^{3}H(G)]-inulin (180–215 mCi g^{-1}) of stated radioactive purity 98–99% and *p*-[glycyl-1-^{14}C]-aminohippuric acid (46.4–57.6 mCi mmol^{-1}; purity 97.5–98%) were both obtained from New England Nuclear Ltd and were used without further purification. CPX was synthesised by Dr R. James of ICI. Kits for the assay of creatinine and urea were obtained from Pierce and Warriner and BDH Ltd, respectively.

Results

Evaluation of adenosine antagonism

Blood pressure and heart rate studies Adenosine evoked dose-related decreases in both mean blood pressure and heart rate of anaesthetised rats, such that at a dose of 600 μg kg^{-1} there was a reduction of about 30 and 50%, respectively, in these variables (Figure 1). CPX alone had no effect on resting heart rate (control = 371 ± 9, CPX = 357 ± 9 beats

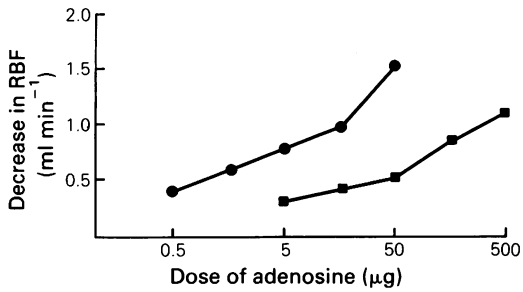

Figure 2 Decreases in renal blood flow (RBF) in response to bolus injections of adenosine given into the thoracic aorta of an anaesthetised rat before (●) and 10 min (■) following 8-cyclopentyl-1,3-dipropylxanthine (0.1 mg kg^{-1} i.v.).

min^{-1}; $n = 6$), although mean arterial blood pressure was slightly, but significantly, decreased (control = 145 ± 7, CPX = 129 ± 7 mmHg; $n = 6$, $P < 0.05$). Similarly, 8-PT did not significantly alter resting heart rate (control = 348 ± 9, 8-PT = 337 ± 8 beats min^{-1}; $n = 6$), but it did produce a significant reduction in mean arterial blood pressure (control = 124 ± 7, 8-PT = 111 ± 8 mmHg; $n = 6$, $P < 0.05$).

Both CPX (0.1 mg kg^{-1}) and 8-PT (4 mg kg^{-1}) significantly antagonised bradycardic responses evoked by all doses of adenosine (Figure 1). These effects of CPX and 8-PT were similar in magnitude as the log dose-ratios measured at a 15% decrease in heart rate were not significantly different (8-PT = 0.84 ± 0.10, CPX = 0.75 ± 0.11). 8-PT also antagonised the hypotensive response evoked by all doses of adenosine. However, CPX was less potent in this respect and only caused a significant ($P < 0.05$) reduction in the response evoked by 600 μg kg^{-1} of the purine (Figure 1).

Renal blood flow Adenosine given via the thoracic aorta elicited dose-dependent falls in blood flow to the left kidney. The results of a typical experiment are shown in Figure 2. Administration of the vehicle (1.0 ml kg^{-1}) for either alkylxanthine did not affect the response of renal blood flow to adenosine. Injection of CPX (0.1 mg kg^{-1}) did not alter basal renal blood flow (control = 5.13 ± 0.33, CPX = 5.15 ± 0.36 ml min^{-1}; $n = 6$). Similarly administration of 8-PT (4 mg kg^{-1}) had no effect on basal renal blood flow (control = 5.60 ± 0.28, 8-PT = 5.21 ± 0.47 ml min^{-1}; $n = 7$). Both alkylxanthines produced shifts to the right of the adenosine dose-response curve. The mean log-dose-ratio for the shifts produced by CPX was 1.47 ± 0.18 ($n = 6$) which was significantly greater ($P < 0.001$) than the shift produced by 8-PT (1.06 ± 0.07; $n = 7$). When larger doses of adenosine (50–500 μg) were given after CPX, falls in mean arterial blood pressure of up to 50 mmHg were noted. These changes in blood pressure occurred after the peak decreases in renal blood flow. By contrast, when the same doses of adenosine were injected after 8-PT, only small falls in mean arterial pressure were observed (5–10 mmHg). Administration of adenosine following either CPX or 8-PT resulted in only minor changes in heart rate (increases of 5–10 beats min^{-1}) which occurred in the dose range 165–500 μg.

Treatment with 8-cyclopentyl-1,3-dipropylxanthine

Two day study

Saline-injected rats The plasma urea levels of the CPX-treated group were significantly lower before any injections (0 h) and 24 h later compared to the untreated group (Table 1). The biological significance of this is questionable since the differences are relatively small and in addition, there was no difference in plasma creatinine concentrations between the two groups (Table 2). Kidney weight and morphology were unaffected by CPX treatment (Figure 4, Table 3). In addition, there was no change in C_{IN}, but by contrast, C_{PAH} was significantly increased by almost 50% (Figure 3).

Table 1 Plasma urea concentrations (mg 100 ml^{-1}) in saline and glycerol-injected rats treated by i.v. injection of either vehicle (1.0 ml kg^{-1}) or 8-cyclopentyl-1,3-dipropylxanthine (CPX, 0.1 mg kg^{-1}) twice daily for 2 days

	Saline-injected		*Glycerol-injected*		
	No treatment ($n = 8$)	*CPX* ($n = 8$)	*No treatment* ($n = 15$)	*Vehicle* ($n = 15$)	*CPX* ($n = 15$)
0 h	41 ± 1	33 ± 1[b]	42 ± 1	45 ± 3	40 ± 1
24 h	38 ± 1	35 ± 1[a]	213 ± 15[c]	217 ± 15	95 ± 10*†
48 h	43 ± 3	39 ± 1	262 ± 19[c]	273 ± 22	94 ± 8*†

Mean ± s.e.mean and number of rats in parentheses.
[a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$, relative to saline-injected no treatment group (t test).
* $P < 0.001$ relative to glycerol-injected no treatment group (ANOVA).
† $P < 0.001$ relative to glycerol-injected vehicle-treated group (ANOVA).

Table 2 Plasma creatinine concentrations (mg 100 ml^{-1}) in saline and glycerol-injected rats treated by i.v. injection of either vehicle (1.0 ml kg^{-1}) or 8-cyclopentyl-1,3-dipropylxanthine (CPX, 0.1 mg kg^{-1}) twice daily for 2 days

	Saline-injected		*Glycerol-injected*		
	No treatment ($n = 8$)	*CPX* ($n = 8$)	*No treatment* ($n = 15$)	*Vehicle* ($n = 15$)	*CPX* ($n = 15$)
0 h	0.58 ± 0.04	0.57 ± 0.03	0.49 ± 0.01	0.52 ± 0.03	0.49 ± 0.02
24 h	0.56 ± 0.02	0.56 ± 0.01	3.07 ± 0.25[c]	3.69 ± 0.29	1.08 ± 0.09*†
48 h	0.62 ± 0.06	0.55 ± 0.03	3.23 ± 0.41[c]	4.15 ± 0.46	0.85 ± 0.08*†

Mean ± s.e.mean and number of rats in parentheses.
[c] $P < 0.001$ relative to saline-injected no treatment group (t test).
* $P < 0.001$ relative to glycerol-injected no treatment group (ANOVA).
† $P < 0.001$ relative to glycerol-injected vehicle treated group (ANOVA).

Glycerol-injected rats Following an injection of glycerol, plasma urea and creatinine concentrations increased five to six fold within 24 h and increased even further by 48 h (Tables 1 and 2). At the end of this 48 h period, C_{IN} and C_{PAH} were both reduced by about 75% compared to untreated, saline-injected rats (Figure 3). Moreover, kidney weight and histological damage were significantly increased after glycerol administration (Figure 4; Table 3). In glycerol-injected rats treated with vehicle, no changes above those produced by glycerol-injection alone were seen in any indices of renal function measured.

Plasma urea and creatinine concentrations in glycerol-injected rats treated with CPX were significantly lower at 24 and 48 h than those seen in vehicle and untreated rats. In contrast to these two groups, the elevated urea and creatinine levels in the CPX treated group did not increase further from 24 to

Table 3 Effect of i.v. treatment with either vehicle (1.0 ml kg^{-1}) or 8-cyclopentyl-1,3-dipropylxanthine (CPX, 0.1 mg kg^{-1} i.v.) administered twice daily for 2 days on kidneys from rats injected with saline or glycerol

Group	n	*Damage score*
Saline-injected no treatment	8	0.1 ± 0.3
Saline-injected CPX-treated	8	0.0 ± 0
Glycerol-injected no treatment	15	6.2 ± 0.2[c]
Glycerol-injected vehicle-treated	15	5.5 ± 0.2
Glycerol-injected CPX-treated	15	4.7 ± 0.2*†

[c] $P < 0.001$ relative to saline-injected no treatment.
* $P < 0.001$ relative to glycerol-injected no treatment.
† $P < 0.05$ relative to glycerol-injected vehicle-treated (two tailed Mann-Whitney test).
Kidneys were fixed and sectioned 48 h after injection of either glycerol or saline. Maximum possible damage score = 10.

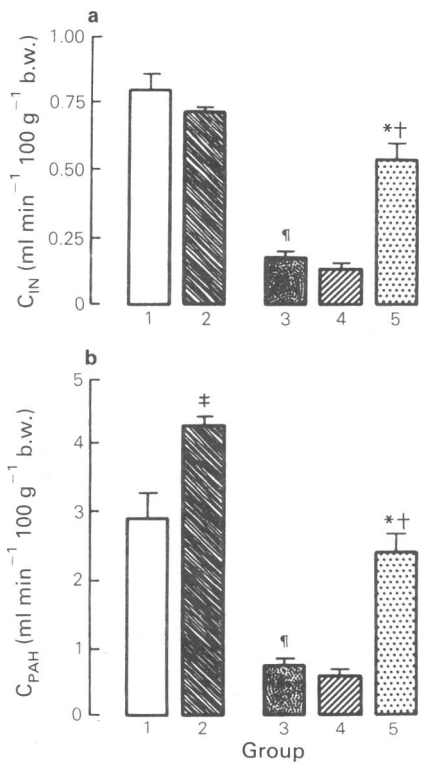

Figure 3 The clearances of (a) [^{3}H]-inulin (C_{IN}) and (b) [^{14}C]-p-aminohippurate (C_{PAH}) 48 h after injection of either saline or glycerol. Rats were given vehicle (1.0 ml kg^{-1}) or 8-cyclopentyl-1,3-dipropylxanthine (CPX) (0.1 mg kg^{-1}) twice daily i.v. for 2 days. Key to groups: (1) saline-injected, no treatment ($n = 8$); (2) saline-injected, CPX treated ($n = 8$); (3) glycerol-injected, no treatment ($n = 15$); (4) glycerol-injected, vehicle-treated ($n = 15$); (5) glycerol-injected, CPX-treated. Columns represent means and vertical bars s.e.mean. ‡ $P < 0.01$; ¶ $P < 0.001$ relative to group 1 (t test). * $P < 0.001$ relative to group 3; † $P < 0.001$ relative to group 4 (ANOVA).

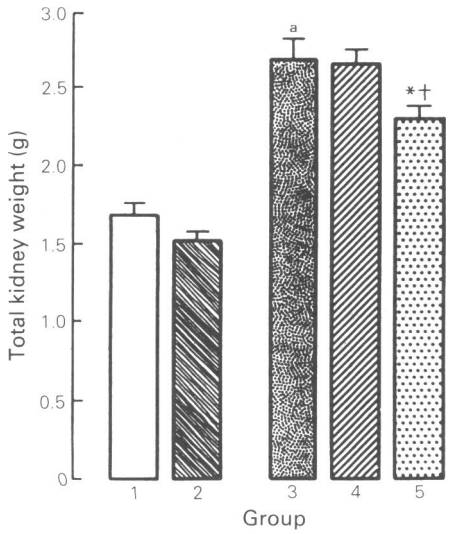

Figure 4 Total kidney weight of rats 48 h following an injection of either saline or glycerol. Rats were given vehicle (1.0 ml kg^{-1}) or 8-cyclopentyl-1,3-dipropylxanthine (CPX) (0.1 mg kg^{-1}) twice daily i.v. for 2 days. Key to groups: (1) saline-injected, no treatment ($n = 8$); (2) saline-injected, CPX-treated ($n = 8$); (3) glycerol-injected, no treatment ($n = 15$); (4) glycerol-injected, vehicle-treated ($n = 15$); (5) glycerol-injected, CPX-treated ($n = 15$). Columns represent means and vertical bars s.e.mean. [a] $P < 0.001$ relative to group 1 (t test). * $P < 0.001$ relative to group 3; † $P < 0.001$ relative to group 4 (ANOVA).

48 h (Tables 1 and 2). In rats injected with glycerol, C_{IN} was significantly greater in CPX-treated rats compared to vehicle and untreated animals and was 70% of the mean control value from saline-injected rats (Figure 3a). C_{PAH} was similarly larger in CPX-treated rats (Figure 3b) compared to untreated or vehicle-treated rats. The kidney weights of the CPX-treated group were significantly less than in the vehicle and untreated groups (Figure 4). Moreover, the degree of morphological damage associated with glycerol injection was also reduced ($P < 0.001$) following treatment with CPX (Table 3).

Seven day study Two days following glycerol injection, plasma urea and creatinine concentrations were much lower in the CPX-treated rats compared to untreated and vehicle treated animals (Tables 4 and 5). These findings are similar to those noted in the 48 h study (Tables 1 and 2). Between 2 and 5 days following the glycerol injection, mortality in untreated rats was 43% (8 out of 14 survived) and in vehicle-treated rats the mortality rate was 21% (11 out of 14 survived). In these two groups of rats there were no further fatalities between 5 and 7 days. In contrast, all 14 rats in the CPX group survived to 7 days. On day 5, plasma urea and creatinine concentrations were significantly reduced in the CPX-treated rats compared to the surviving animals in the untreated and vehicle-treated groups (Tables 4 and 5). Urea and creatinine levels on day 7 in the CPX-treated rats were also lower than the survivors in the other two groups, but the only statistically significant difference was that plasma creatinine levels in the CPX group were less than in the vehicle-treated rats.

C_{IN} and C_{PAH} in rats which received CPX were significantly greater than the clearance values recorded in either the untreated or vehicle-treated animals (Figure 5). The mean total kidney weight of rats injected with CPX (3.87 ± 0.18 g, $n = 14$) was significantly smaller ($P < 0.01$, ANOVA) than the mean

Table 4 Plasma urea concentrations (mg 100 ml^{-1}) in glycerol-injected rats treated with either 8-cyclopentyl-1,3-dipropylxanthine (CPX, 0.1 mg kg^{-1}) or vehicle (1.0 ml kg^{-1}) i.v. twice daily for 2 days and studied over 7 days

	No treatment ($n = 14$)	*Vehicle* ($n = 14$)	*CPX* ($n = 14$)
Day 0	43 ± 3	49 ± 2	47 ± 2
Day 2	215 ± 18 ($n = 8$)	210 ± 23 ($n = 11$)	130 ± 10**† ($n = 14$)
Day 5	163 ± 27	168 ± 29	81 ± 9*†
Day 7	77 ± 11	100 ± 23	54 ± 5

Results are given as mean ± s.e.mean.
* $P < 0.05$; ** $P < 0.01$ relative to no treatment group (ANOVA).
† $P < 0.01$ relative to vehicle-treated group (ANOVA).

Table 5 Plasma creatinine concentrations (mg 100 ml^{-1}) in glycerol-injected rats treated with either 8-cyclopentyl-1,3-dipropylxanthine (CPX, 0.1 mg kg^{-1}) or vehicle (1.0 ml kg^{-1}) i.v. twice daily for 2 days and studied over 7 days

	No treatment ($n = 14$)	*Vehicle* ($n = 14$)	*CPX* ($n = 14$)
Day 0	0.50 ± 0.04	0.59 ± 0.03	0.44 ± 0.03§
Day 2	3.34 ± 0.60 ($n = 8$)	3.58 ± 0.50 ($n = 11$)	1.36 ± 0.13**§ ($n = 14$)
Day 5	2.11 ± 0.41	2.30 ± 0.58	0.77 ± 0.09*†
Day 7	1.17 ± 0.20	1.54 ± 0.38	0.53 ± 0.04†

Results are given as mean ± s.e.mean.
* $P < 0.05$; ** $P < 0.01$ relative to no treatment group (ANOVA).
† $P < 0.01$; § $P < 0.001$ relative to vehicle-treated group (ANOVA).

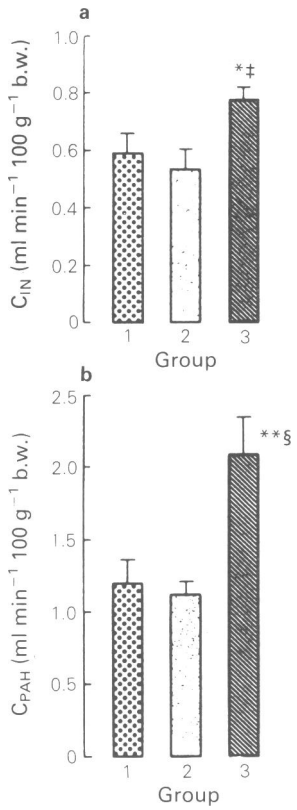

Figure 5 The clearances of (a) [^{3}H]-inulin (C_{IN}) and (b) [^{14}C]-*p*-aminohippurate (C_{PAH}) 7 days after glycerol injection. Rats were treated with either 8-cyclopentyl-1, 3-dipropylxanthine (CPX, 0.1 mg kg^{-1} i.v.) or vehicle (1.0 ml kg^{-1}, i.v.) twice daily for 2 days following glycerol injection. Columns represent mean values and vertical bars s.e.mean. Group 1 received no treatment ($n = 8$), group 2 vehicle ($n = 11$) and group 3 CPX ($n = 14$). * $P < 0.05$, ** $P < 0.01$ relative to group 1; ‡ $P < 0.01$; § $P < 0.001$ relative to group 2 (ANOVA).

weight noted in untreated (5.29 ± 0.35 g, $n = 8$) or vehicle-treated rats (4.98 ± 0.29 g, $n = 11$).

Discussion

The results of this study demonstrate that the A_1-selective adenosine receptor antagonist CPX is effective in ameliorating some of the biochemical, functional and morphological correlates of ARF in the rat. These observations support the results of our previous investigation with 8-PT (Bowmer *et al.*, 1986) and together with those of Bidani & Churchill (1983), with theophylline, they provide further evidence that adenosine antagonism is beneficial in ARF. The present findings also imply that it is activation of the A_1 sub-type of adenosine receptor which is involved in the pathogenesis of this disorder.

In vitro studies have shown that CPX is equieffective with 8-PT as an A_2-receptor antagonist (Collis *et al.*, 1989), but that it has a 30–100 fold higher affinity for the A_1-receptor. In the present study we provide *in vivo* evidence that CPX is A_1-selective at 0.1 mg kg^{-1}. Thus CPX antagonised the bradycardic and the renal vasoconstrictor response evoked by adenosine in the anaesthetised animal. The bradycardic response is mediated via A_1-receptors in the sino-atrial node and perhaps on adrenergic nerve terminals innervating the node (Hedquist & Fredholm, 1979; Brown & Collis, 1983; Collis & Saville, 1984). The renal vasoconstrictor response is also elicited via A_1-receptors located on the afferent renal arterioles (Murray & Churchill, 1984). In contrast, CPX had little effect on hypotensive responses evoked by adenosine. This response is due mainly to intraluminal adenosine acting to relax vascular smooth muscle via an A_2-receptor (Collis & Brown, 1983). The small degree of antagonism of the hypotensive response to larger doses of the purine by CPX may have been due to blockade of a pre-junctional inhibitory effect of adenosine on adrenergic vascular neurotransmission or to an A_1-mediated decrease in cardiac output. By contrast to CPX, a dose of the non-selective antagonist 8-PT, which was chosen to match the effect of CPX on heart rate responses to adenosine, also antagonised the hypotensive effects of the purine at all doses administered.

CPX treatment had little effect on renal function in normal rats except for an increase in C_{PAH}. We have no immediate explanation for this change in C_{PAH}, but C_{PAH} is a measure of effective renal plasma flow and an increase would indicate a rise in renal blood flow. However, in the studies designed to assess adenosine antagonism, CPX did not affect basal renal blood flow when recorded directly with an electromagnetic flow probe. Recent studies with a non-xanthine adenosine antagonist, CGS 15943A, have also demonstrated antagonism of adenosine-evoked renal vasoconstriction without an effect on basal renal blood flow (Callis *et al.*, 1989). Moreover, the duration of action of CPX (0.1 mg kg^{-1}) in the conscious rat is 3–5 h (Collis *et al.*, unpublished results) whilst the increase in C_{PAH} was noted 12 h following the last dose of CPX. Thus the increase in C_{PAH} may not be directly related to adenosine antagonism. It is noteworthy that CPX administration resulted in no morphological damage in normal rats (Table 3). This is in contrast to 8-PT, since we found that this alkylxanthine produced a significant degree of kidney damage in normal rats

compared to treatment with its vehicle (Bowmer *et al.*, 1986).

In glycerol-treated rats, the progression of ARF over a 48 h and a 7 day period was similar to that previously described in this model of renal failure (Bowmer *et al.*, 1986). Thus, plasma urea and creatinine were elevated with decreases in C_{IN} and C_{PAH}. There was morphological evidence of renal damage, with tubular necrosis and cast formation and an increase in kidney weight, probably due to oedema, and deposition of haem pigments in the renal tubules. CPX treatment caused a marked and significant reduction in the severity of ARF over the 48 h period following glycerol injection. This amelioration of ARF was seen for all the variables measured. The beneficial effects of treatment with CPX for the first 2 days following glycerol administration persisted over the following 5 days. There was an appreciable mortality rate over this period in untreated (43%) and vehicle-treated (21%) rats but no deaths were noted in CPX-treated animals. The lower mortality rate in the vehicle-treated rats is surprising since vehicle treatment did not produce significant improvements in any of the indices of renal function when compared to untreated rats. Furtermore, at 7 days there was no significant difference in plasma urea, plasma creatinine, C_{IN}, C_{PAH} and kidney weight between the surviving animals in the untreated and vehicle groups. Those animals from the untreated and vehicle-treated groups which survived to 7 days were clearly recovering from ARF as plasma urea and creatinine levels and C_{IN} together with C_{PAH} were returning towards normal levels. However, rats which had been treated with CPX showed a more complete recovery.

The results of this study support the hypothesis put forward by Churchill & Bidani (1982) that adenosine is an important factor in the pathogenesis of glycerol-induced ARF. It is postulated that adenosine has a deleterious effect by increasing afferent arteriolar resistance, probably via an action at the A_1-adenosine receptor. However, adenosine can also have vasodilator effects in the kidney via A_2- receptor activation (Murray & Churchill, 1984). This prompts the question, is an A_1-selective adenosine antagonist more effective at ameliorating ARF than a non-selective antagonist? Some indication of the answer to this question can be gained from a comparison of the results obtained in this study with those we observed in a previous study with the non-selective antagonist 8-PT (Bowmer *et al.*, 1986).

To make a comparison between the effects of CPX and 8-PT in rats with ARF, it is necessary to show that the A_1-antagonistic potency of the dose of CPX ($0.1\,mg\,kg^{-1}$) used in this study was similar to that of the dose of 8-PT ($10\,mg\,kg^{-1}$) used previously. Data in the present paper show that $4\,mg\,kg^{-1}$ 8-PT is equi-effective with $0.1\,mg\,kg^{-1}$ of CPX in terms of blocking A_1-mediated bradycardic responses to adenosine and is slightly but significantly less potent as an antagonist of A_1-mediated renal vasoconstriction. However, extrapolation of the dose-ratios obtained in the renal blood flow studies indicates that $10\,mg\,kg^{-1}$ 8-PT and $0.1\,mg\,kg^{-1}$ CPX would produce similar degrees of antagonism of the renal vasoconstrictor effects of adenosine. Thus, a comparison of the effects of $0.1\,mg\,kg^{-1}$ CPX with those previously found for $10\,mg\,kg^{-1}$ 8-PT (Bowmer *et al.*, 1986) appears valid. When compared to data from vehicle-treated rats with ARF, CPX caused a 66% decrease in plasma urea, an 80% decrease in plasma creatinine and a 300% increase in inulin clearance at the 48 h time point. The effect of 8-PT on these parameters was consistently less at this time after the induction of ARF (−50%, −40% and +70%, respectively). This comparison, therefore, suggests that selective A_1-adenosine receptor blockade may be more effective in ameliorating ARF than non-selective antagonism of both A_1- and A_2-receptors. However, confirmation of this would require a more detailed study of the relationship between dose, blockade of adenosine responses and beneficial effect of A_1-selective and non-selective adenosine antagonists in ARF.

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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 Street, New York, NY 10021, U.S.A.)

4–5 December 1989

Sensory Nerves and Neuropeptides in Gastroenterology, Florence, Italy. (Fondazione Internazionale Menarini, Piazza del Carmino, 4-20121 Milan, Italy).

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.)

10–14 December 1989

Sino-Brazilian Symposium on Chemistry and Pharmacology of Natural Products, Rio de Janeiro, Brazil. (Secretariat, Fundacao Oswaldo Cruz, Departmento de Farmacodinamica, Aremida, Brasil, 6365, Rio de Janeiro, Brazil).

3–5 January 1990

British Pharmacological Society Winter Meeting. London, U.K. (Closed meeting for members and guests)

7 February 1990

Royal Society of Chemistry, Free Radicals in Biotechnology and Medicine, London, U.K. (Miss P.E. Hutchinson, The Analytical Division, Royal Society of Chemistry, Burlington House, London W1V 0BN).

26–28 March 1990

Brain Research Association National Meeting, Bristol, U.K. (Dr. I.C. Kilpatrick, Dept. of Pharmacology, The Medical School, University Walk, Bristol BS8 1TD).

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.)

2–4 April 1990

Central and Peripheral Significance of Neuropeptide Y and its Related Peptides, Baltimore, U.S.A. (Conference Department, New York Academy of Sciences, 2 East 63rd Street, New York, NY 10021, U.S.A.).

4–6 April 1990

New Concepts in Anxiety, Castres, France. (Martin Dehaye, Centre de Recherche Pierre Fabre, 17 avenue Jean Moulin, 81100 Castres, France).

18–20 April 1990

British Pharmacological Society Spring Meeting. Sheffield, U.K. (Closed meeting for members and guests)

27–30 May 1990

Heterocycles in Bio-organic Chemistry, Solbacka, Sweden (Secretariat, PO Box 1136, S-11181 Stockholm, Sweden).

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).

28–30 June 1990

IUPHAR Satellite — Mediators in Airway Hyperreactivity, The Hague, The Netherlands. (F.P. Nimkamp, Department of Pharmacology, PO Box 80176, 3508 TD Utrecht, Netherlands).

30 June–4 July 1990

6th Southeast Asian/W. Pacific Meeting of Pharmacologists, Hong Kong. (Secretary, Department of Pharmacology, Faculty of Medicine, University of Hong Kong, 5 Sassoon Road, Hong Kong).

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).

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IUPHAR 11th International Congress of Pharmacology, Amsterdam, Netherlands (Contact to be advised).

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