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Histamine H_3-receptors inhibit cholinergic neurotransmission in guinea-pig airways

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The histamine H_3-agonist (R)-α-methylhistamine (α-MeHA) caused a dose-dependent inhibition of vagally-mediated contraction of a guinea-pig tracheal tube preparation but did not alter tracheal contraction induced by exogenously-applied acetylcholine. Blockade of H_1- and H_2-histamine receptors, and α- and β-adrenoceptors failed to prevent the inhibitory effect of α-MeHA, whereas the specific H_3-antagonist thioperamide prevented the effect of α-MeHA on tracheal contraction. In the presence of H_1- and H_2-receptor antagonists, histamine also inhibited vagally-mediated tracheal contraction. The inhibitory effect of α-MeHA was greater with preganglionic (vagus nerve) stimulation than with postganglionic stimulation by electrical field stimulation, suggesting that H_3-receptors are localized both to cholinergic ganglia and to post-ganglionic nerve-endings. Our results suggest that H_3-receptors exist on the vagus nerve which modulate cholinergic neurotransmission in the airways.

Introduction Histamine causes bronchoconstriction through stimulation of H_1-receptors, and, in some species, bronchodilatation via H_2-receptors (Chand & DeRoth, 1979; Barnes, 1987). Recently, (R)-α-methylhistamine (α-MeHA), a histamine H_3-receptor agonist, has been developed (Arrang *et al.*, 1983; 1987). H_3-receptors have been localized within the central nervous system, where they appear to be involved in the feedback control of both histamine synthesis and release (Arrang *et al.*, 1987), and have been shown to inhibit sympathetic neurotransmission in perivascular nerves (Ishikawa & Sperelakis, 1987). Their functional significance in autonomic nerves of the airways, however, has not yet been elucidated.

In the airways, vagus nerve stimulation causes bronchoconstriction by releasing acetylcholine. Histamine may enhance vagally mediated bronchoconstriction via H_1-receptors (Kikuchi *et al.*, 1984). In this study, we have investigated whether H_3-receptors are present on airway cholinergic nerves in an innervated guinea-pig tracheal tube preparation (Blackman & McCaig, 1983).

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Methods Male Dunkin-Hartley guinea-pigs (400–600 g) were anaesthetized with an intraperitoneal injection of urethane (2 g kg^{-1}). The trachea was dissected out together with the right vagus nerve supply as previously described (Blackman & McCaig, 1983). The trachea was placed in a tissue bath containing Krebs-Henseleit solution, (mM): NaCl 118, KCl 5.9, $MgSO_4$ 1.2, $CaCl_2$ 2.5, NaH_2PO_4 1.2, $NaHCO_3$ 25.5, and glucose 5.05, maintained at 37°C and bubbled with 95% O_2, 5% CO_2, pH 7.4. Indomethacin (10^{-5} M) was present throughout. The trachea was filled with Krebs-Henseleit solution and mounted vertically at its *in vivo* length. The bottom of the trachea was closed and the top connected to a pressure transducer (Gould) for recording intraluminal pressure. The right vagus nerve was stimulated through a bipolar electrode, and trains of rectangular pulses (40 V, 0.2 ms, 8 Hz) were applied for 5 s every 90 s (Grass SD9). Postganglionic nerves were stimulated by electrical field stimulation with parallel platinum electrodes having the same stimulation parameters.

The following drugs were used: α-MeHA, thioperamide (Bioprojet, Paris); astemizole (Janssen, Wantage); cimetidine (Smith Kline & French); phentolamine (Ciba-Geigy); propranolol (ICI); indomethacin, histamine, acetylcholine chloride (Sigma).

Data were expressed as means ± s.e. and analyzed by Student's *t* test for paired and unpaired data. Probability values of $P < 0.05$ were considered significant.

Results Whilst having no effect on the resting intraluminal pressure, α-MeHA (10^{-12} to 10^{-6} M) gave a concentration-dependent inhibitory effect on vagal nerve-induced tracheal responses, which was maximal within 10 min. The EC_{50} value for this effect was 7.0×10^{-9} M. α-MeHA also reduced the contractile response to electrical field stimulation (10.8 ± 2.8% at 10^{-7} M compared with 27.8 ± 2.0% for vagus nerve stimulation). Matched contractile responses induced by exogenously-applied acetylcholine (10^{-7}–3×10^{-6} M) were unaffected by α-MeHA (Figure 1a).

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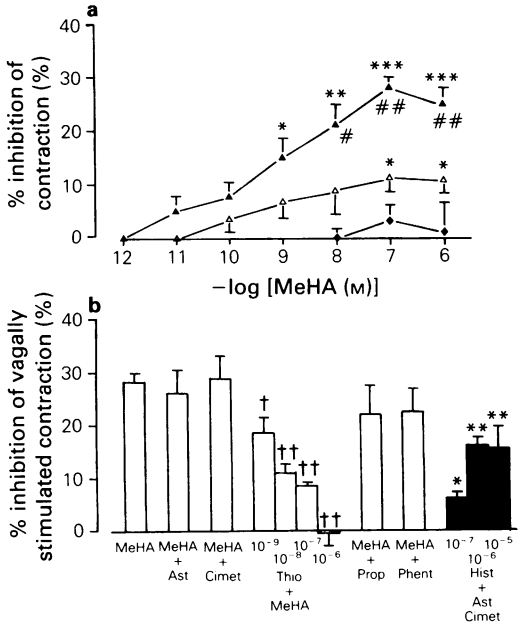

Figure 1 (a) Effect of α-methylhistamine (MeHA) on vagus nerve stimulation (VS; ▲, $n = 5$), electrical field stimulation (EFS; △, $n = 7$), and exogenously-applied acetylcholine (◆, $n = 6$). Values are means with s.e.means shown by vertical bars. (b) Open columns showing the effect of various blockers on the inhibition of VS by MeHA (10^{-7} M). The effects of astemizole (Ast, 10^{-6} M), cimetidine (Cimet, 10^{-4} M), thioperamide (Thio, 10^{-9}–10^{-6} M), propranolol (Prop, 10^{-6} M), and phentolamine (Phent, 10^{-6} M) are shown as mean values of 5 experiments; s.e.mean shown by vertical bars. Solid columns showing the effect of histamine (Hist, 10^{-7}–10^{-5} M, $n = 5$) on VS with Ast and Cimet; $^{*}P < 0.02$; $^{**}P < 0.002$; $^{***}P < 0.001$ as compared with control values; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ as compared with EFS; $\dagger P < 0.05$; $\dagger\dagger P < 0.01$ as compared with MeHA alone.

Neither H_1- nor H_2-receptor antagonism by astemizole (10^{-6} M) and cimetidine (10^{-4} M), respectively, altered the inhibitory effect of α-MeHA but its effect was prevented by the H_3-antagonist, thioperamide ($IC_{50} = 4.2 \times 10^{-9}$ M). Neither the α-blocker phentolamine (10^{-6} M), nor β-blocker propranolol (10^{-6} M) modified the α-MeHA-induced effect. Furthermore, with H_1- and H_2-receptor blockade, histamine (10^{-7}–10^{-5} M) caused significant inhibition of vagally-induced tracheal contraction (Figure 1b). The maximum effect of histamine (16.1 ± 1.0%) was significantly less than that of MeHA (27.8 ± 2.0%).

Discussion We have demonstrated that α-MeHA, a selective H_3-receptor agonist causes dose-dependent inhibition of airway smooth muscle contraction elicited by vagus nerve stimulation in a concentration-range similar to that observed for inhibition of histamine release in the CNS (Arrang *et al.*, 1987). The contractile response of the guinea-pig tracheal preparation is due to release of acetylcholine from cholinergic nerves (Blackman & McCaig, 1983). α-MeHA had no significant effect on matched acetylcholine-induced contraction in this preparation, suggesting that it may be inhibiting acetylcholine release. The inhibitory effect of α-MeHA was greater on vagus nerve stimulation than on electrical field stimulation of postganglionic nerves, indicating that it is acting predominantly on cholinergic ganglionic neurotransmission. The inhibitory effect of α-MeHA was blocked by the H_3-selective antagonist thioperamide with a potency similar to that seen in CNS (Arrang *et al.*, 1987) but not by an H_1-antagonist or an H_2-antagonist, confirming that this effect was mediated via H_3-receptors in the cholinergic pathways.

Cholinergic neurotransmission in airways may be modified by adrenergic mechanisms, such as α_2- (Grundstrom *et al.*, 1981) and β-adrenoceptor stimulation (Rhoden *et al.*, 1988). In this study, however, the inhibitory effect of α-MeHA was not influenced by α_2- or β-adrenoceptor blockade.

Histamine itself also inhibited cholinergic neurotransmission in the presence of H_1- and H_2-antagonists, although the inhibitory effect was less marked than with α-MeHA. The concentration of histamine inhibiting cholinergic neurotransmission is lower than that reported to cause direct contraction of this preparation via H_1-receptors (McCaig, 1986).

Our results suggest that H_3-receptors which inhibit acetylcholine release are present in the vagal pathway and could play a role in modulating neural bronchoconstriction in allergic disease when histamine is released from airway mast cells in the vicinity of airway ganglia and cholinergic nerves.

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Pertussis toxin prevents the inhibitory effect of adenosine and unmasks adenosine-induced excitation of mammalian motor nerve endings

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Pertussis toxin (PTX), which blocks certain classes of guanine nucleotide binding proteins (G proteins), consistently blocked the inhibitory effects of adenosine (100 μM–250 μM) on quantal acetylcholine (ACh) secretion in rat phrenic nerve hemidiaphragm preparations. PTX pretreatment also highlighted long-lasting increases in evoked ACh release elicited by adenosine. The results suggest that specific G proteins are involved in mediating the inhibitory effects of adenosine at motor nerve endings.

Introduction Studies on the mechanism by which adenosine receptor binding is transduced into the inhibition of neurotransmitter secretion are replete with controversy at present (see e.g. Ribeiro & Sebastiao, 1986; Silinsky, 1986; Fredholm & Dunwiddie, 1988). At motor nerve endings, it has been suggested that a guanine nucleotide binding protein (G-protein, see Graziano & Gilman, 1987) is an important transducer of inhibitory effects of adenosine (Silinsky, 1985, Figure 4). As a means of testing this hypothesis, it should be possible to inactivate several types of G proteins at motor nerve endings by ADP-ribosylating their alpha subunits with pertussis toxin (PTX; Graziano & Gilman, 1987). If PTX were observed to occlude the inhibitory action of adenosine, then such a result would be consistent with the suggestion that certain classes of G proteins are involved in coupling adenosine receptor binding to inhibition of ACh secretion.

Methods Conventional methods for intracellular recording and stimulation were employed in rat phrenic nerve hemi-diaphragm preparations (see Gage & Hubbard, 1966). For further details of the methods for nerve stimulation, electrophysiological recording and data analysis, see Silinsky (1987).

In these studies, Male-Sprague Dawley rats of 128–200 g were injected with 10 μg of Islet Activating Protein (pertussis toxin, List Biological Laboratories) in 500 μl of physiological saline + bovine serum albumin (1 mg ml^{-1}). Four–nine days after injection, the diaphragms were excised and studied *in vitro*.

Results Figure 1a illustrates the typical effect of 250 μM adenosine on preparations from control animals, namely a maximal inhibition of evoked ACh secretion (reflected as endplate potentials; e.p.ps) to approximately 50% of the control level. In contrast, when this concentration of adenosine was applied to preparations from rats injected with PTX, no inhibitory effect was observed during adenosine application (Figure 1b). The inability of adenosine (100–250 μM) to inhibit release after PTX-treatment was reproduced in all 8 experiments using the protocol described in the methods. In contrast to adenosine, the inhibitory effect of Mg, which exerts a direct blocking effect on the Ca channel, was unaffected by PTX treatment.

Whilst no inhibitory effect of adenosine was observed in PTX-treated preparations, rebound increases in evoked ACh release occurred after the cessation of superfusion with adenosine in such preparations (e.g. Figure 1b). Furthermore, in the experiment illustrated, subsequent addition of 250 μM adenosine failed to alter ACh release from its increased level in the PTX-treated preparation. The rebound increases in ACh release ranged from 1.4 to 2.7 times the control level ($n = 8$) with a mean increase of 1.9 fold over control ($n = 8$).

In two experiments in which accurate measurements of miniature endplate potentials (m.e.p.ps) were possible, the effects of adenosine on spontaneous ACh release, i.e., m.e.p.p. frequency, were also studied. Normally adenosine produces a parallel fall

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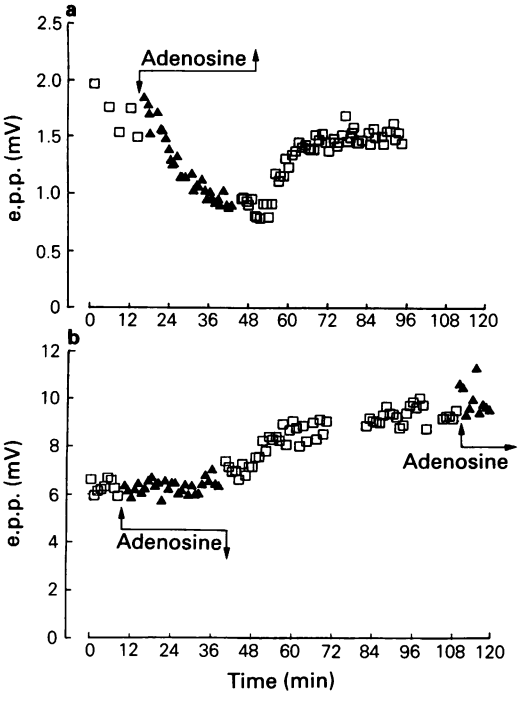

Figure 1 Comparison of the effects of adenosine (250 μM) in control (a) and in diaphragms from rats treated with pertussis toxin (PTX) (b). Each symbol represents the average of 30–32 e.p.ps (for statistical methods see Silinsky, 1987). As adenosine does not alter the post-junctional acetylcholine (ACh) sensitivity, changes in e.p.p amplitudes accurately reflect changes in ACh release. (□) Control; (▲) 250 μM adenosine. The physiological saline solution consisted of (mM): NaCl 137, KCl 5, $CaCl_2$ 1, $MgCl_2$ 20–25, NaH_2PO_4 1, $NaHCO_3$ 24, glucose 11, bubbled with 95% O_2: 5% CO_2 to pH 7.2 (see Gage & Hubbard, 1966). The Mg concentration was 25 mM in (a) and 20 mM in (b). The phrenic nerve was stimulated at a frequency of 0.1 Hz. Corrections for non-linear summation of e.p.ps were not required (see Silinsky, 1987). All experiments were done at room temperature. A large number of experiments were also done on frog cutaneous pectoris preparations using various protocols of injection and direct superfusion of isolated tissues with PTX. However, consistent results such as those found in the rat were not observed with frog preparations.

in evoked and spontaneous release (see references in Silinsky, 1986). After PTX treatment, however, no significant inhibitory effect of 250 μM adenosine on m.e.p.p. frequency was observed in these two experiments.

In the present studies, the level of Mg or curare needed for neuromuscular blockade was of a similar magnitude to that required by the untreated control preparations. Unfortunately, variability in the Mg composition of solutions and in the degree of curarization required to reduce the e.p.p. below threshold in control and in PTX-treated preparations prevented a detailed comparison of ACh release in control animals and in pertussis-treated rats.

Discussion The results demonstrate two features of note after PTX-treatment of rat motor nerve endings. The first is the elimination of the inhibitory effect of adenosine by PTX and the second is the unmasking of increases in ACh release after adenosine application.

With respect to the inhibition of the presynaptic effects of adenosine by PTX, the results are in accordance with those of Dolphin & Prestwich (1985) who studied glutamate overflow from cultured cerebellar neurones and of O'Regan & Phillis (1987) who studied the firing of cortical neurones (but see Fredholm & Dunwiddie, 1988). At motor nerve endings, these present studies of quantal ACh release argue in favour of coupling of adenosine receptors to GTP binding proteins in mediating the inhibitory effects of adenosine. Specifically, the results suggest that adenosine receptors are linked to either G_i (the inhibitory G protein negatively coupled to adenylate cyclase), G_o (a G protein that may be linked to Ca-dependent events), or an as yet unidentified G protein.

Since it has been proposed that adenosine reduces the intracellular apparent affinity for Ca of a strategic component of the secretory apparatus (Silinsky, 1986), it is of interest that a recent report has shown a direct coupling between G_i or G_o and the active site of calcium binding proteins (Katada *et al.*, 1987). Specifically, Katada *et al.* (1987) showed Ca-calmodulin binding to the beta/gamma subunits of PTX-sensitive G proteins. Adenosine, by increasing the free concentration of the beta/gamma subunits, might be expected to remove Ca binding proteins from participation in the secretory process.

The results also reduce the likelihood that G_s (and thus cyclic AMP elevation) is an important mediator of the inhibitory effects of adenosine in the rat. While some evidence is in support of the involvement of G_s in the action of adenosine has been provided in frog (Silinsky, 1986), the antagonism by PTX of the effect of adenosine would argue against an obligatory involvement of G_s, unless, as suggested by Cerione *et al.* (1985), active G_i is necessary for optimal activation of the G_s/adenylate cyclase system.

In summary the results implicate a PTX-sensitive G-protein in mediating the inhibitory effect of adenosine and a second, PTX-insensitive process for mediating the long-lasting increases in secretion provoked by adenosine. These effects occur without

occlusion by PTX of Ca-dependent depolarization-secretion coupling.

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Antagonism of calcium by zinc in guinea-pig isolated taenia caeci and trachealis muscle

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1 In guinea-pig isolated taenia caeci and trachealis bathed in a K^+-rich, Ca^{2+}-free medium, $CaCl_2$ (0.01–10 mM) produced a concentration-dependent contraction. Zn^{2+} (0.01–1 mM), Cd^{2+} (0.01–1 mM), verapamil (0.01–100 μM) and trifluoperazine (1–100 μM) were effective antagonists of $CaCl_2$-induced responses.

2 Zn^{2+} and Cd^{2+} in concentrations from 0.01 to 1 mM were without effect on the tone of taenia and trachea in normal Tris solution. Conversely, Zn^{2+} and Cd^{2+}, in concentrations of 1 mM, caused contraction of these tissues in a K^+-rich, Ca^{2+}-free medium. Zn^{2+} (1 mM)-induced contractions of taenia and trachea were completely inhibited by verapamil (10 μM).

3 In taenia and trachea skinned of their plasma membranes, tension development induced by Ca^{2+} (10 μM or 1 μM, respectively) was unaffected by verapamil (100 μM), whereas trifluoperazine (100 μM) depressed the maximal tension produced by Ca^{2+}. Segments of skinned preparations contracted in response to low concentrations of Zn^{2+} (10 μM) or Cd^{2+} (10 μM).

4 It is concluded that Zn^{2+} may suppress Ca^{2+}-induced spasm by a direct action on the binding sites of the Ca^{2+} channel.

Introduction

The term calcium-blocking agents refers to drugs that block the entry of Ca^{2+} through the Ca^{2+} channels (Spedding, 1985; Godfraind *et al.*, 1986). The first method of achieving Ca^{2+} channel blockade was the use of inorganic cations which interact with Ca^{2+} channels in a direct and reversible manner (Hurwitz, 1986).

Zinc, a divalent cation, is involved in many biological functions (Prasad, 1985). Thus, some of the observed physiological effects of Zn^{2+} may be related to competition between Zn^{2+} and several other cations such as Cd^{2+}, Ca^{2+} and Cu^{2+} but its exact mechanism of action has not yet been defined. Experimental evidence *in vitro* suggests the existence of a Zn^{2+}–Ca^{2+} antagonism because Zn^{2+} depresses the contractile responses to different Ca^{2+}-dependent agonists in rat aorta (Cortijo *et al.*, 1985). However, the characteristics of this antagonism are different from those reported for other calcium-blocking agents such as verapamil (Triggle, 1982). This suggests the possibility that the Zn^{2+}–Ca^{2+} antagonism could be the result of an intracellular modification in the regulation of the excitation–contraction coupling in the smooth muscle. This is in keeping with previous hypotheses proposed to explain some intracellular events promoted by Zn^{2+} in blood cells (Brewer *et al.*, 1979). Nevertheless, further studies are necessary to discover whether this divalent cation possesses calcium-blocking properties in other tissues and, if this is proved to be so, to study the nature of its calcium antagonistic action.

Therefore, the main objective of this study was to provide more direct evidence that this cation can produce calcium antagonism. To achieve this, we have assessed its ability to inhibit calcium-induced contractions in guinea-pig isolated taenia caeci and trachealis muscle, comparing its effects with those obtained with other calcium-blocking agents such as Cd^{2+}, verapamil and trifluoperazine. Furthermore, we have tested these four calcium-blocking agents on preparations in which the sarcolemma had been chemically disrupted. By comparing the effects of these agents on intact and skinned tissues we tried to

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determine whether they act on the external surface of, or within, the plasma membrane or conversely if they produce their calcium antagonistic action by acting intracellularly.

Methods

Guinea-pigs of either sex (350–450 g) were killed by stunning and bleeding. Strips of taenia were obtained from the caecum. Tracheae were excised, cleaned of adhering fat and connective tissue and opened by cutting longitudinally through the cartilage rings diametrically opposite the trachealis.

Antagonism of calcium

Strips of taenia or small segments of trachea were mounted, for the isometric recording of tension changes, as previously described by Cortijo *et al.* (1987a) and Foster *et al.* (1983). The tissues were subjected to a tension of 1 g in a Tris buffer solution containing normal amounts of Ca^{2+} and K^+ (composition mM: NaCl 134.8, KCl 4.75, $CaCl_2$ 2.55, $MgSO_4 7H_2O$ 1.2, Tris 5.0, HCl 5.0 and glucose 10.1). After equilibration, the bath fluid was changed to a Ca^{2+}-free (neither Ca^{2+} nor a Ca^{2+}-chelating agent was added) depolarizing solution containing 55 mM KCl in equimolar replacement for NaCl. This produced a spasm which was dissipated by regular (20 min) changes of the K^+-rich, Ca^{2+}-free bath fluid. These solutions were aerated with O_2 at 37°C and the pH adjusted to 7.4 with HCl 0.5 N. The Ca^{2+} concentration of the Ca^{2+}-free medium did not exceed 6 μM calculated from the impurity limits of the constituents. Changes in tone were recorded with a Hewlet-Packard FTA 100-1 (1 g) isometric force transducer connected to a Phillips PM-8222 pen recorder via a HP-8808B carrier amplifier. The spasmogenic response to $CaCl_2$ was studied by constructing cumulative concentration-effect curves. The contact time for each concentration of $CaCl_2$ was sufficient to allow the complete development of the tension attainable at that concentration (15 min). In these experiments the strips were divided into pairs. In test tissues, the effect of $CaCl_2$ was examined before and after tissue equilibration (20 min) with the calcium-blocking agents. Tissues were treated with the antagonist once the $CaCl_2$ induced spasm had been fully dissipated by washing with K^+-rich, Ca^{2+}-free medium. Only one concentration of the antagonist was tested in each strip. Paired tissues from the same taenia or trachea were not exposed to calcium-blocking agents but otherwise were treated identically, giving reproducible concentration-response curves to $CaCl_2$. The comparison of the effects produced by the calcium antagonist was made with their appropriate time-matched controls i.e. the concentration-response curve to $CaCl_2$ obtained in the presence of a certain concentration of the calcium antagonist was compared to the second concentration–response curve generated in the paired tissue in the absence of antagonist. No differences were found among these time-matched control concentration–response curves to $CaCl_2$ hence they were pooled for statistical purposes and presented as the control group. The inhibitory effect of each concentration of each calcium-blocking agent was quantified as a proportion (%) of the $CaCl_2$(10 mM)-induced contraction in the absence of antagonist. The concentration of calcium-blocking agent causing a 50% reduction in the spasm produced by $CaCl_2$ 10 mM (IC_{50}) was derived by interpolation and expressed as $-\log$ molar concentration.

Measurement of the effect of Zn^{2+} and Cd^{2+} on tone

In taenia and trachea batched in either normal Tris solution or K^+-rich, Ca^{2+}-free Tris solution, cumulative concentration-effect curves to Zn^{2+} (0.01–1.0 mM) or Cd^{2+} (0.01–1.0 mM) were constructed. In tissues equilibrated with K^+-rich, Ca^{2+}-free Tris solution the response to a single concentration (1 mM) of Ca^{2+}, Zn^{2+} and Cd^{2+} was generated to obtain the values of the latency and the initial velocity of contraction. The time elapsed between the time of drug addition to the bath and a just discernible increase in isometric tension was the latency which is expressed in s. The initial velocity of contraction was derived from the tangent to the initial part of the tension record and is expressed in mg s^{-1}. In another set of experiments, the contractile response to single concentration (1 mM) of Zn^{2+} (taenia and trachea bathed in K^+-rich, Ca^{2+}-free Tris solution) was generated in the absence (time-matched control) or presence (20 min of incubation time) of verapamil (10 μM). The inhibitory effect was quantified as a proportion (%) of the contraction obtained in the absence of antagonist.

Experiments with taenia and trachea skinned of their plasma membranes

Segments of taenia or trachea were prepared as described above and then skinned of their plasma membranes as previously reported (Cortijo *et al.*, 1987a, b). Tissue segments were incubated (4 h at 4°C) in a 1% (v/v) Triton X-100 solution which contained (mM): KCl 50, sucrose 150, EGTA 5, imidazole 20 and dithioerythritol 0.5 (pH 7.4). After rinsing for 15 min in a solution of the same composition but without Triton X-100, tissues were stored in a solution of (mM): EGTA 4, $MgCl_2$ 10, ATP 7.5,

Na_2N 1, imidazole 20) and dithioerythritol 0.5 (pH 6.7), with 50% glycerol at −20°C for up to 7 days.

With an imposed tension of 0.5 g, segments of skinned taenia or tracheae were set up at 20°C for isometric recording of tension changes in 5 ml of relaxing solution containing (mM): EGTA 4, $MgCl_2$ 10, ATP 7.5, KH_2PO_4 6, Na_3N 1 and imidazole 20, adjusted to pH 6.7 with KOH. The relaxing solution did not contain added calmodulin. Tension development was induced by addition of $CaCl_2$ in an amount calculated to yield a free Ca^{2+} concentration of 10 μM (taenia) or 1 μM (trachea). When the tension became maximal (25 min) it was subsequently dispelled by repeatedly washing with relaxing solution (90 min). The apparent stability constants used for Ca^{2+}-EGTA, Zn^{2+}-EGTA and Cd^{2+}-EGTA solutions were 1.2 μM, 0.0162 μM and 10.8 pM at 20°C and pH 6.7, which were calculated from the stability constants of EGTA–metal ion complexes (Martell, 1971), using the equations described by Portzhel *et al.* (1964).

The experimental design used was : in test tissues three successive Ca^{2+} challenges were performed. Verapamil (100 μM) or trifluoperazine (100 μM) was present for 20 min before and throughout the second Ca^{2+} challenge. Zn^{2+} (10 μM) or Cd^{2+} (10 μM) were also added 20 min before the second Ca^{2+} challenge but because they produce a contraction, the second and third Ca^{2+} challenge were not carried out. Acetylcholine (100 μM) was added to the tissue once full relaxation was achieved either after the third Ca^{2+} challenge (experiments with verapamil or trifluoperazine) or after the contraction elicited by Zn^{2+} or Cd^{2+}. Control tissues were treated similarly except that they were not exposed to calcium blocking agents. The effect in the second and third challenge was expressed as a proportion (%) of that in the first challenge.

Drugs and solutions/statistical analysis of results

The following substances were used: acetylcholine (Sigma), adenosine-5′-triphosphate (ATP disodium salt, Sigma), cadmium chloride (Merck), dithioerythritol (Sigma), ethyleneglycol-*bis*(β-aminoethylether)-*N*-*N*-tetraacetic acid (EGTA, Sigma), glycerol (Merck), imidazole (Sigma), trifluoperazine (Sigma), Tris (Merck), Triton X-100 (Merck), sucrose (Merck), verapamil hydochloride (Biosedra-Knoll, Paris, France), zinc sulphate (Merck). Concentration of drugs is expressed in terms of the molar concentration of the active species.

The significance of differences between means was assessed by use of two-tailed unpaired *t*-test. A difference between means was assumed to be significant when $P < 0.05$.

Results

Experiments in normal taenia preparations

When the bath fluid was changed from Tris solution to a K^+-rich, Ca^{2+}-free, Tris solution, the tissues generated tension which became maximal after approximately 3 min. Despite regular changes of the bath fluid, the tension developed by the tissue required approximately 20 min for dissipation. This was the period of incubation in K^+-rich, Ca^{2+}-free, Tris solution used in subsequent experiments. Addition of $CaCl_2$ (0.1 to 100 mM) at this time produced a concentration-dependent smoothly developing spasm, $E_{max} = 5.31 \pm 0.25$ g; $pD_2 = 3.30 \pm 0.05$; these values are the mean of 72 experiments corresponding to the time-matched controls. Verapamil, trifluoperazine, Zn^{2+} and Cd^{2+} caused a concentration-dependent right downward shift of the concentration–response curve to $CaCl_2$ (Figure 1, Table 1). The IC_{50} against $CaCl_2$ 10 mM and the corresponding relative potencies are shown in Table 2.

Experiments in normal trachea preparations

Spasm evoked by exposure to the depolarizing medium was dissipated by regular changes of the K^+-rich, Ca^{2+}-free Tris solution in approximately 70 min. This established the incubation time in this medium for subsequent experiments. A cumulative concentration–effect curve for $CaCl_2$ was then constructed as described above, $E_{max} = 905.7 \pm 127.2$ mg; $pD_2 = 3.93 \pm 0.10$; these values are the mean of 72 experiments corresponding to the time-matched controls. Verapamil, trifluoperazine, Zn^{2+} and Cd^{2+} antagonized the effect of $CaCl_2$ in a concentration-dependent manner producing a right downward shift of the concentration–effect curve for $CaCl_2$ (Figure 2, Table 1). The IC_{50} against $CaCl_2$ 10 mM and the corresponding relative potencies are shown in Table 2.

Effects of Zn^{2+} and Cd^{2+} on tone

Zn^{2+} (0.01 to 1 mM) and Cd^{2+} (0.01 to 1 mM) were without effect on the tone of taenia caeci and trachealis in normal Tris solution but the higher concentrations were able to contract tissues bathed in a K^+-rich, Ca^{2+}-free medium. This contraction was reversed by washing. Figure 3 shows the contraction elicited by a single concentration (1 mM) of Ca^{2+}, Zn^{2+} or Cd^{2+} in taenia caeci and trachea bathed in a K^+-rich, Ca^{2+}-free medium. The characteristics of the Zn^{2+} and Cd^{2+}-induced contractions are different (greater latency and lesser initial velocity) from those of the contraction elicited by Ca^{2+} (Table 4).

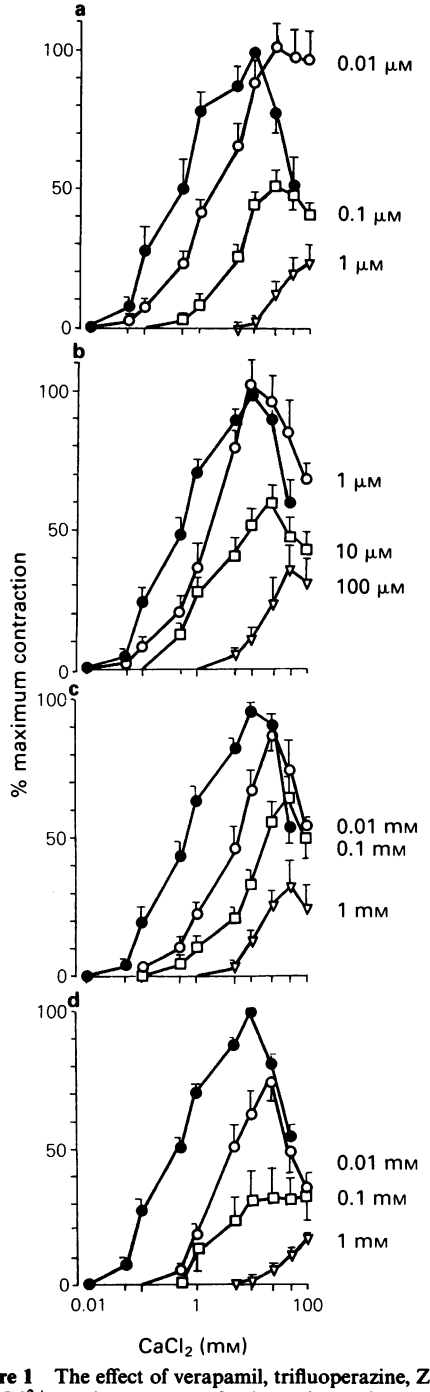

Figure 1 The effect of verapamil, trifluoperazine, Zn^{2+} and Cd^{2+} on the response of guinea-pig taenia caeci (in K^+-rich, Ca^{2+}-free, Tris solution) to $CaCl_2$. The abscissae indicate concentration of $CaCl_2$ (mM) on a log scale. The ordinates indicate response as a % of the maximal response to $CaCl_2$ in the absence of antagonists. Points represent the means and vertical lines the s.e.mean; (●), time-matched control concentration-effect curve for $CaCl_2$ in the absence of antagonists ($n = 18$); (○, □, ▽), curves obtained after verapamil (a) (0.01, 0.1, 1 μM), trifluoperazine (b) (1, 10, 100 μM), Zn^{2+} (c) (0.01, 0.1, 1 mM) and Cd^{2+} (d) (0.01, 0.1, 1 mM) respectively ($n = 6$ for each concentration of the antagonist).

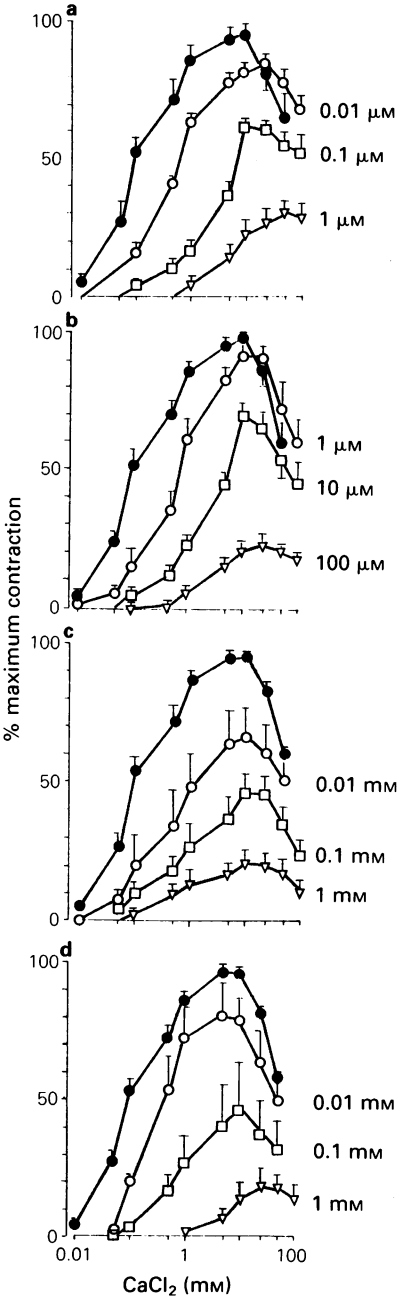

Figure 2 The effect of verapamil, trifluoperazine, Zn^{2+} and Cd^{2+} on the response of guinea-pig trachea (in K^+-rich, Ca^{2+}-free, Tris solution) to $CaCl_2$. The abscissae indicate concentration of $CaCl_2$ (mM) on a log scale. The ordinates indicate response as a % of the maximal response to $CaCl_2$ in the absence of antagonists. Points represent the means and vertical lines the s.e.mean; (●), time-matched control concentration-effect curve for $CaCl_2$ in the absence of antagonists ($n = 18$); (○, □, ▽), curves obtained after verapamil (a) (0.01, 0.1, 1 μM), trifluoperazine (b) (1, 10, 100 μM), (c) Zn^{2+} (0.01, 0.1, 1 mM) and Cd^{2+} (d) (0.01, 0.1, 1 mM), respectively ($n = 6$ for each concentration of the antagonist).

Table 1 Maximal effects (E_{max}) and EC_{50} values for $CaCl_2$ in the absence and presence of various concentrations of antagonists, in the guinea-pig taenia caeci and trachealis suspended in a Ca^{2+}-free, depolarizing solution

	Taenia caeci		*Trachealis*	
	E_{max} (g)	pD_2	E_{max} (g)	pD_2
Verapamil				
Control	5.01 ± 0.31	3.30 ± 0.05	0.937 ± 0.131	3.97 ± 0.08
0.01 μM	5.11 ± 0.29	2.70 ± 0.07*	0.834 ± 0.116	3.33 ± 0.10
0.1 μM	2.55 ± 0.51*	2.30 ± 0.06*	0.618 ± 0.098*	2.39 ± 0.12*
1 μM	1.18 ± 0.41*	1.60 ± 0.10*	0.328 ± 0.091*	2.25 ± 0.04*
Trifluoperazine				
Control	5.39 ± 0.36	3.25 ± 0.04	0.893 ± 0.099	4.09 ± 0.11
1 μM	5.55 ± 0.41	2.74 ± 0.11*	0.839 ± 0.092	3.18 ± 0.09*
10 μM	3.21 ± 0.47*	2.90 ± 0.13*	0.645 ± 0.086*	2.56 ± 0.14*
100 μM	1.95 ± 0.63*	1.77 ± 0.09*	0.218 ± 0.043*	2.57 ± 0.06*
Zn^{2+}				
Control	5.11 ± 0.39	3.24 ± 0.08	0.826 ± 0.123	3.80 ± 0.15
0.01 mM	5.23 ± 0.81	2.35 ± 0.09*	0.606 ± 0.197	3.31 ± 0.19
0.1 mM	3.40 ± 0.38*	2.01 ± 0.04*	0.422 ± 0.157*	3.19 ± 0.34*
1 mM	1.46 ± 0.34*	1.91 ± 0.11*	0.220 ± 0.080*	2.87 ± 0.32*
Cd^{2+}				
Control	5.52 ± 0.42	3.37 ± 0.07	1.006 ± 0.114	3.90 ± 0.90
0.01 mM	3.49 ± 0.59*	2.52 ± 0.06*	0.611 ± 0.202	3.35 ± 0.06
0.1 mM	2.16 ± 0.77*	2.38 ± 0.20*	0.308 ± 0.071*	3.08 ± 0.02*
1 mM	0.95 ± 0.28*	1.43 ± 0.11*	0.195 ± 0.041*	2.31 ± 0.21*

Data are mean ± s.e.mean of 18 experiments for time-matched controls and 6 experiments for each concentration of the antagonist in treated tissues. *$P < 0.05$ compared to control.

In addition, Zn^{2+} (1 mM)-induced contractions of taenia and trachea were completely inhibited (100% inhibition) by verapamil (10 μM).

Skinned taenia and trachea preparations

Control experiments showed that skinned taenia and trachea responded to an initial Ca^{2+} challenge (10 μM and 1 μM, respectively) by generating tension which reached a peak value (1.62–1.97 g, for taenia and 0.298–0.465 g for trachea) within 25 min. Responses to subsequent Ca^{2+} challenges became progressively smaller (about 15% for taenia and about 40% for trachea). Acetylcholine (100 μM) never produced contraction of the skinned preparations.

When test tissues were compared with their appropriate time-matched controls, it was evident that trifluoperazine (100 μM) depressed the maximal tension evoked by Ca^{2+}, whereas verapamil (100 μM) was ineffective (Figure 4). Segments of skinned taenia and trachea contracted in response to low concentrations of Zn^{2+} (10 μM) and Cd^{2+} (10 μM) (Figure 5). The magnitude of these contractions was similar to those elicited by Ca^{2+} in time-matched skinned tissues.

Discussion

The present experiments show that Zn^{2+}, which is without effect on the tone of taenia caeci and trachealis in normal Tris solution, causes a contraction of

Table 2 IC_{50} values of calcium blocking agents for responses to $CaCl_2$ (10 mM) of guinea-pig taenia and trachea maintained in K^+-rich, Ca^{2+}-free, Tris solution

	Verapamil	*Trifluoperazine*	Zn^{2+}	Cd^{2+}
IC_{50} (*−log molar concentration*)				
Taenia caeci	7.15 ± 0.12	4.93 ± 0.07	4.49 ± 0.48	4.60 ± 0.58
Trachealis	5.70 ± 0.32	4.37 ± 0.48	4.19 ± 0.82	4.10 ± 0.56
Potency relative to verapamil				
Taenia caeci	1	0.0060	0.0021	0.0028
Trachealis	1	0.0470	0.0300	0.0240

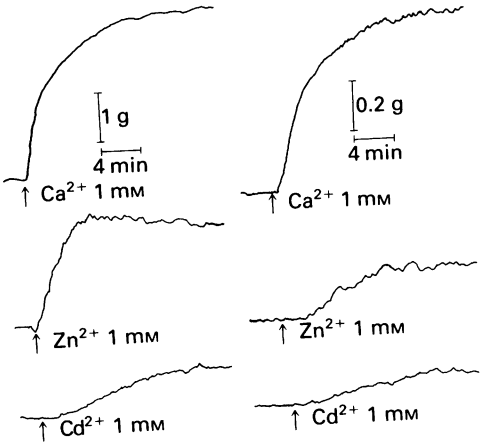

Figure 3 Original traces showing the contraction produced by a single concentration (1 mM) of Ca^{2+}, Zn^{2+} or Cd^{2+} when added to guinea-pig isolated taenia caeci (left panel) or trachealis (right panel) bathed in a K^+-rich, Ca^{2+}-free, Tris solution.

Table 3 Increase of resting tone produced by Zn^{2+} and Cd^{2+} in the guinea-pig taenia caeci and trachea bathed in K^+-rich, Ca^{2+}-free medium

	Taenia caeci	*Trachealis*
Zn^{2+}		
0.01 mM	NR	NR
0.1 mM	NR	0.045 ± 0.015
1 mM	1.8 ± 0.25	0.123 ± 0.024
Cd^{2+}		
0.01 mM	NR	NR
0.1 mM	NR	0.031 ± 0.019
1 mM	0.965 ± 0.17	0.101 ± 0.013

Numbers indicate the response in g. NR indicates the absence of response after drug addition. Data represents mean ± s.e.mean ($n = 9$)

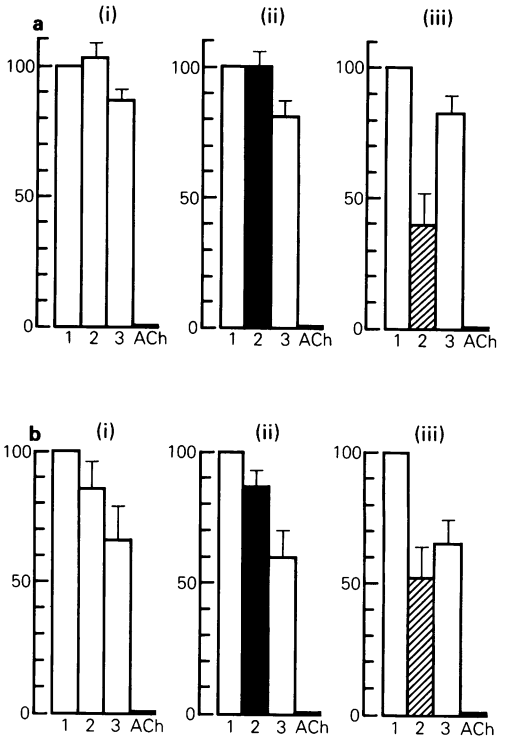

Figure 4 The effect of verapamil (ii) and trifluoperazine (iii), on responses of skinned taenia caeci (a) and trachea (b) to Ca^{2+} (i). The abscissae indicate the first, second and third (1, 2, 3) challenge with 10 μM Ca^{2+} (taenia) or with 1 μM Ca^{2+} (trachea) and with 100 μM acetylcholine (ACh). The ordinates indicate response as a % of the first Ca^{2+} challenge. Solid columns indicate the presence of verapamil 100 μM and hatched columns the presence of trifluoperazine 100 μM. Column heights represent the mean value of at least 6 experiments and vertical lines indicate the s.e.mean. There was a significant difference ($P < 0.05$) between time-matched control tissues and trifluoperazine treated tissues.

Table 4 Latency (L) and initial velocity (V) of the contraction produced by a single concentration (1 mM) of Ca^{2+}, Zn^{2+} or Cd^{2+} when added to guinea-pig isolated taenia caeci or trachealis bathed in a K^+-rich, Ca^{2+}-free, Tris solution

	Taenia caeci		*Trachealis*	
Agonist	*L* (s)	*V* (mg s^{-1})	*L* (s)	*V* (mg s^{-1})
Ca^{2+}	24.0 ± 1.8	30.00 ± 5.80	30.0 ± 2.3	2.89 ± 0.57
Zn^{2+}	72.0 ± 27.2	9.40 ± 1.69*	132.0 ± 49.1*	0.59 ± 0.12*
Cd^{2+}	180.0 ± 63.0*	1.44 ± 0.26*†	252.0 ± 86.3*	0.16 ± 0.03*†

* $P < 0.01$ compared to Ca^{2+}, † $P < 0.05$ compared to Zn^{2+}. $n = 6$ for each group.

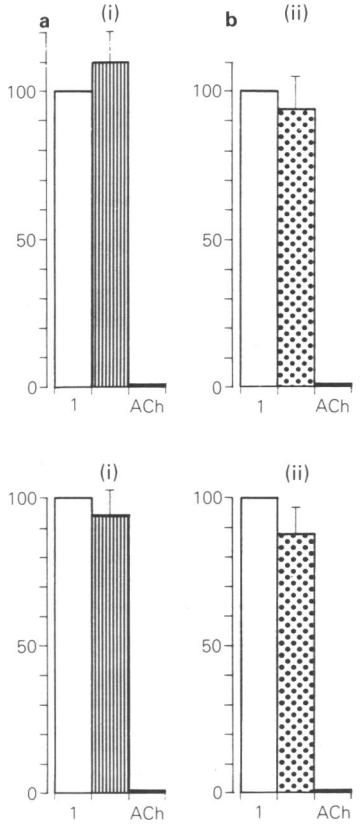

Figure 5 Responses to Zn^{2+} (i) and Cd^{2+} (ii) in skinned taenia caeci (a) and trachea (b). Open columns indicate responses to Ca^{2+} (1) (10 μM for taenia or 1 μM for trachea). Lined columns indicate the response to Zn^{2+} 10 μM and stippled columns the response to Cd^{2+} 10 μM. Acetylcholine (ACh) 100 μM produced no response. The ordinates indicate the magnitude of the response as a % of the Ca^{2+}-induced response. Column heights represent the mean values of at least 6 experiments and vertical lines indicate the s.e.mean.

these tissues when bathed in K^+-rich, Ca^{2+}-free medium or in skinned preparations. Zn^{2+} also produces an antagonism of the responses to $CaCl_2$ in normal (unskinned) but not in the skinned tissues. A similar profile of activity is found for Cd^{2+} in this study.

The effects of Zn^{2+} and Cd^{2+} on resting tone under the above experimental conditions may be explained by taking into account the structure and functional properties of Ca^{2+} channels (Hurwitz, 1986). Since Ca^{2+} has a higher affinity than Zn^{2+} or Cd^{2+} for the binding sites of the Ca^{2+} channel (Hagiwara & Byerly, 1981) it is understandable that this channel is readily permeable for these cations only in the absence of extracellular Ca^{2+}, thus permitting Zn^{2+} and Cd^{2+} to enter the cell and express their ability to activate the contractile machinery which is also unveiled when applied to skinned preparations. The conclusion that Zn^{2+} can enter the cell to cause contraction is reinforced by the finding that verapamil blocked such a contraction elicited in a K^+-rich, Ca^{2+}-free, Tris solution.

Zn^{2+}, as well as Cd^{2+}, verapamil and trifluoperazine, produced in this study a concentration-related, right downward displacement of the concentration–response curve to $CaCl_2$ in normal taenia and trachea bathed in a Ca^{2+}-free depolarizing solution as previously reported in other preparations under similar experimental conditions (Tand *et al.*, 1970; Hayashi & Toda, 1977; Spedding, 1983; Foster *et al.*, 1984; Cortijo *et al.*, 1987a; Sanz *et al.*, 1988).

The Zn^{2+}–Ca^{2+} antagonism may be due to an action on sarcolemma interfering with transmembrane fluxes of Ca^{2+} or to some type of intracellular action yielding to an interference with Ca^{2+}-induced contraction. As a matter of fact, Zn^{2+} inhibition of calmodulin has been proposed (Brewer *et al.*, 1979; Brewer, 1980) as the molecular mechanism of Zn^{2+} action. However we have demonstrated in skinned taenia and trachea that low concentrations (10 μM) of Zn^{2+} and Cd^{2+} are able to produce contractions *per se*. As expected, in the skinned preparations, only trifluoperazine depresses the Ca^{2+}-induced contraction whereas verapamil is ineffective, thus confirming the idea that verapamil works mainly in the cell membrane while trifluoperazine is capable of interfering with the contractile machinery (Cortijo *et al.*, 1987a).

Therefore the antagonism of Ca^{2+} produced by Zn^{2+} and other cations such as Cd^{2+} appears to be exerted at the level of the plasma membrane. These cations have reasonably high affinity for the binding sites of the Ca^{2+} channel but traverse it at low or even negligible speeds (Hagiwara & Byerly, 1981) so they behave as potent competitive inhibitors of Ca^{2+} influx through Ca^{2+} channels.

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The properties of 5-HT_3 receptors in clonal cell lines studied by patch-clamp techniques

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1 The characteristics of transmembrane currents evoked by 5-hydroxytryptamine (5-HT) in the neuroblastoma × Chinese hamster brain cell line NCB-20 and neuroblastoma clonal cell line N1E-115 have been studied under voltage-clamp conditions by the whole-cell recording and outside-out membrane patch modes of the patch-clamp technique.

2 In 73% of NCB-20 cells examined ($n = 221$), and all N1E-115 cells studied ($n = 80$), 5-HT (10 μM) elicited a transient inward current at negative holding potentials, this being associated with an increase in membrane conductance. In both cell lines responses to 5-HT reversed in sign at a potential of approximately −2 mV and demonstrated inward rectification.

3 The reversal potential of 5-HT-induced currents ($E_{5\text{-HT}}$) recorded from either NCB-20 or N1E-115 cells was unaffected by total replacement of internal K^+ by Cs^+. In N1E-115 cells, reducing internal K^+ concentration from 140 to 20 mM produced a positive shift in $E_{5\text{-HT}}$ of approximately 28 mV, whereas reducing external Na^+ from 143 to 20 mM was associated with a negative shift in $E_{5\text{-HT}}$ of about 37 mV. A large reduction in internal Cl^- concentration (from 144 to 6 mM) had little effect on $E_{5\text{-HT}}$.

4 5-HT-induced currents of NCB-20 cells were unaffected by methysergide (1 μM) or ketanserin (1 μM), but were reversibly antagonized by GR38032F (0.1–1.0 nM) with an IC_{50} of 0.25 nM. GR 38032F (0.3 nM) reduced 5-HT-induced currents in N1E-115 cells to approximately 26% of their control value.

5 On outside-out membrane patches excised from both NCB-20 and N1E-115 cells, 5-HT induced small inward currents which could not be clearly resolved into discrete single channel events. Such responses were: (i) reversibly antagonized by GR 38032F (1 nM) (ii) reversed in sign at 0 mV, and (iii) subject to desensitization.

6 Fluctuation analysis of inward currents evoked by 5-HT (1 μM) in N1E-115 cells suggests that 5-HT gates a channel with a conductance of approximately 310 fS. Such a relatively small conductance could readily explain why the response of outside-out membrane patches to 5-HT cannot at present be resolved into clear single channel events.

Introduction

It has been proposed that vertebrate 5-hydroxytryptamine (5-HT) receptors exist as three distinct subtypes termed '5-HT_1-like', 5-HT_2 and 5-HT_3 (Bradley *et al.*, 1986). 5-HT_3 receptors are present on various neurones of the peripheral nervous system, mediating membrane depolarization and neurotransmitter release (Wallis, 1981; Fozard, 1984a; Richardson & Engel, 1986). The action of 5-HT at these sites is potently and selectively antagonized by several recently synthesized compounds, including MDL72222 (1αH, 3α, 5αH-tropan-3-yl-3,5-dichlorobenzoate), ICS 205-930 [(3α-tropanyl)-1H-indole-3-carboxylic acid ester] and GR 38032F (1,2,3,9-tetrahydro-9-methyl-3-[(2-methyl-1H-imidazol-1-yl) methyl]-4H-carbazol-4-one) (Fozard, 1984b; Richardson *et al.*, 1985; Butler *et al.*, 1988). Such

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compounds are behaviourally active, possessing potentially anxiolytic (Tyers *et al.*, 1987; Jones *et al.*, 1988) and antipsychotic (Costall *et al.*, 1987) properties. Together with the recent demonstration of a binding site within brain tissue that displays a pharmacological profile consistent with that of a 5-HT_3 receptor (Kilpatrick *et al.*, 1987; Peroutka & Hamik, 1988; Barnes *et al.*, 1988), the behavioural data suggest that functional 5-HT_3 receptors may occur in the CNS. Consistent with this suggestion, 5-HT_3 receptor activation has recently been reported to elicit inward currents from a population of voltage-clamped rat hippocampal neurones in culture (Yakel & Jackson, 1988) and also to modulate the release of dopamine from rat striatal slices (Blandina *et al.*, 1988).

Current knowledge of the molecular events that occur at 5-HT_3 receptors is limited. Electrophysiological studies have shown that in sympathetic and visceral primary afferent neurones, 5-HT_3 receptor activation elicits a rapidly desensitizing membrane depolarization which is the result of a simultaneous increase in membrane permeability to sodium and potassium ions (Wallis & North, 1978; Nigashi & Nishi, 1982). Similar depolarizing responses to 5-HT have been observed in several neuronal clonal cell lines which are amenable to advanced electrophysiological techniques. In murine N1E-115 neuroblastoma cells, 5-HT increases membrane conductance to monovalent cations, generating a depolarizing response under current-clamp conditions (Guharay & Usherwood, 1981; Peters & Usherwood, 1983), and an inward current when the membrane is voltage-clamped at negative holding potentials ((Neijt *et al.*, 1986). Both the 5-HT-induced depolarization and inward current are antagonized by the selective 5-HT_3 receptor antagonists MDL 72222 and ICS 205-930, but are unaffected by antagonists acting at '5-HT_1-like' or 5-HT_2 sites (Neijt *et al.*, 1988). A binding site corresponding to the 5-HT_3 receptor has been identified in N1E-115 cells (Hoyer & Neijt, 1988) and the neuroblastoma × glioma clone NG 108-15 (Hoyer & Neijt, 1987). Collectively, these observations suggest that certain neuronal clonal cell lines may be appropriate model systems in which to study 5-HT_3 receptors and their associated ion channels.

In the present study, membrane currents elicited by 5-HT in N1E-115 cells and the clonal neuroblastoma × Chinese hamster brain cell line NCB-20 were examined under voltage-clamp conditions, by the patch-clamp technique (Hamill *et al.*, 1981). 5-HT has previously been shown to stimulate the formation of cyclic AMP in the latter system (Berry-Kravis & Dawson, 1983) and also to evoke a membrane depolarization, the two effects occurring via pharmacologically distinct subtypes of 5-HT receptor (MacDermot *et al.*, 1979). The present work demonstrates that the electrical response in NCB-20 cells is, like that of the N1E-115 clone, mediated by 5-HT_3 receptors. Additionally we have examined the ionic selectivity of 5-HT_3 receptor gated ion channels and present evidence from fluctuation analysis of 5-HT-induced currents, recorded from whole cells and excised outside-out membrane patches, which suggests that such channels are of unusually low conductance. Preliminary accounts of some of this work have appeared in abstract form (Hales *et al.*, 1988a,b).

Methods

Cell culture

Cells of the clonal lines NCB-20 (Minna *et al.*, 1975) and N1E-115 (Amano *et al.*, 1972) were grown essentially as described by MacDermot *et al.* (1979) and Khimi *et al.* (1976) respectively. Briefly, NCB-20 cells were cultured in 25 cm^2 'Nunclon' tissue culture flasks (Gibco) containing 10 ml of Dulbecco's modified Eagle medium (DMEM) supplemented with 5% (vol/vol) foetal calf serum (FCS), hypoxanthine (13.6 $mg\,l^{-1}$), aminopterin (0.178 $mg\,l^{-1}$), thymidine (3.88 $mg\,l^{-1}$), streptomycin (50 $mg\,l^{-1}$) and penicillin (5×10^4 $iu\,l^{-1}$). Cells were grown to confluency over a period of 7 days, from an initial innoculum of 2×10^5 cells per flask and collected by mechanical agitation and subsequent centrifugation (100 *g* for 5 min). N1E-115 cells were grown and harvested in a similar manner, but in a growth medium consisting only of DMEM supplemented with 10% (vol/vol) FCS and streptomycin and penicillin at the concentrations stated above. For electrophysiological experiments, approximately 10^4 NCB-20 or N1E-115 cells were plated into 35 mm diameter 'Nunclon' petri dishes (Gibco) containing 2 ml of their respective growth media. Prior to use in electrophysiological experiments, NCB-20 cells were exposed to 1 mM N^6, O^{12}-dibutyryl adenosine 3':5'-cyclic monophosphoric acid for 2 to 7 days to initiate cell differentiation. N1E-115 cells did not receive this treatment. The cell lines were incubated at 37°C in an atmosphere of 90% air/10% CO_2 at 100% relative humidity. A number of experiments utilized cultures of adult bovine adrenomedullary chromaffin cells. These were prepared following an established methodology (Cottrell *et al.*, 1987).

Electrical recordings

Agonist-activated currents from 'whole-cells' and outside-out membrane patches were recorded by

standard patch-clamp techniques (Hamill *et al.*, 1981) and a List Electronics L/M EPC7 converter headstage and amplifier. The extracellular recording medium used in the majority of experiments consisted of (in mM): NaCl 140, KCl 2.8, $MgCl_2$ 2.0, $CaCl_2$ 1.0 and HEPES 10 (pH 7.2). Cells were continually superfused with this solution at a rate of 3–5 ml min^{-1}. The pipette solution usually employed to dialyse the cell interior comprised (in mM): CsCl 140, $MgCl_2$ 2.0, HEPES 10, $CaCl_2$ 0.1, EGTA 1.1, (free $[Ca^{2+}] = 10^{-8}$ M at pH 7.2). Caesium was chosen as the predominant internal cation in order to suppress membrane potassium conductances. In experiments designed to evaluate the ionic-dependence of 5-HT-evoked currents, modified extracellular and intracellular media were employed. Pipette solutions containing potassium, rather than caesium, as the predominant internal cation were prepared by total replacement of CsCl by KCl or K gluconate. In experiments where the internal concentration of potassium was reduced, KCl was partially replaced by tetraethylammonium Cl. External sodium ion concentration was reduced to either 75 or 20 mM by partial substitution of L-glucosamine HCl for NaCl. All solutions were titrated to pH 7.0–7.2 with 1 M NaOH; Na ions so introduced were included in the calculation of external and internal sodium ion concentrations.

To minimize changes in reference electrode potential during the bath application of the modified recording media to the cells, the bath was held at virtual ground via a salt bridge containing 3 M KCl in 4% (w/v) agar. When the pipette and bath contained salines of differing ionic composition, a liquid junction potential developed at the tip of the patch pipette prior to 'giga-seal' formation. Such potentials, which were estimated by the method of Fenwick *et al.* (1982) to range between 2 and 10 mV (pipette negative), were taken into account when setting the holding potential in voltage-clamp recordings. Experiments started approximately 10 min after establishing the whole-cell recording and in all cases were conducted at room temperature (17–21°C).

In most experiments, 5-HT was locally applied to cells either by pressure ejection (1.4×10^5 Pa, 30–300 ms) from modified patch pipettes filled with 5-HT (10 μM) in recording medium or by ionophoresis. Ionophoretic pipettes contained 20 mM 5-HT in twice distilled deionized water (pH 3.5–4.0) and had resistances > 80 MΩ. A constant current pump based on the design of Dreyer & Peper (1974) was used to supply ejection and retaining currents to the pipette. Antagonist compounds were introduced to the bath via the superfusion system. In experiments where the unitary conductance of 5-HT-activated ion channels was to be estimated from fluctuation analysis of whole-cell currents, a relatively slowly rising response to 5-HT was desired. For this purpose, 5-HT (1 μM in recording medium) was applied to cells by diffusion from coarse-tipped (4–10 μm diameter) micropipettes. All quantitative data are reported as the arithmetic mean ± standard error of the mean (s.e.mean).

Fluctuation analysis of 5-HT-induced whole-cell currents

Estimates of single channel conductance were made by analysis of the random fluctuations in the 5-HT-induced whole cell membrane currents. Current fluctuations were a.c. coupled (high pass filter 2.0 Hz) and recorded on a separate tape channel after extra amplification.

Continuous records of mean d.c. current and current fluctuations, starting before the application of 5-HT (1 μM) and continuing throughout (1–3 min), were played back, digitised at a rate of 1 kHz using a Data Translation DT2801A or a Cambridge Electronic Design 502 laboratory interface and stored on an Amstrad PC1640 personal computer or a PDP 11 73 minicomputer respectively. Anti-aliasing filtration of the current fluctuation signal was performed using a Frequency Devices 901, 8 pole Butterworth low pass filter (500 Hz cut-off) and a Neurolog NL 125 high-pass filter (2 Hz cut-off).

The variance analysis method was used to estimate the single channel conductance (Marty *et al.*, 1984; Cull-Candy *et al.*, 1988; Ascher *et al.*, 1988). The record was split into a series of 0.5 s blocks (512 samples) with variance and mean current calculated for each block from the equation:

$$\sigma^2 = \sum_{J=1}^{n} \frac{(I_J - I_{av})^2}{(n-1)} \qquad (1)$$

where σ_2 is the variance, I_{av} the mean current, n the number of samples in the block, and I_J current sample J. σ^2 has a parabolic relationship to I_{av}, rising from zero to a maximum (at $P = 0.5$) and falling to zero again as the open channel probability, P, is varied from 0–1. For small values of P (≤ 0.2), the relation is approximately linear and the value of the unitary current can be calculated from the slope of the fitted line.

σ^2/I_{av} plots were obtained from blocks recorded during the onset of the 5-HT current, with P increasing as the 5-HT concentration increased. Unless specified otherwise, background variance contributed by sources other than the 5-HT-activated ion channels was removed by subtracting the mean value of 32–64 blocks recorded before 5-HT application. The σ^2/I_{av} plots were well fitted by straight lines using linear regression (correlation coefficient 0.71–

0.97), suggesting that P was indeed small. The slope was therefore used as an estimate of the unitary current.

Reagents

All constituents of cell culture media were obtained from Gibco Europe. The following compounds were used in experiments: γ-aminobutyric acid (GABA), 5-hydroxytryptamine creatinine sulphate complex (both from Sigma), citalopram hydrogen bromide (Lundbeck), GR 38032F (1,2,3,9-tetra-hydro-9-methyl-3-[(2-methyl-1H-imidazol-1-yl)methyl]-4H-carbazol-4-one hydrochloride dihydrate) (Glaxo), ICS 205-930 ([3α-tropanyl]1H-indole-3-carboxylate) (Research Biochemicals), ketanserin tartrate (Janssen) and methysergide hydrogen maleate (Sandoz). All drugs were freshly prepared as concentrates in twice distilled deionised water and diluted into recording medium as required.

Results

5-HT-induced responses

Local application of 5-HT (100 μM) to NCB-20 cells elicited a transient membrane depolarization under current-clamp conditions. Such responses were accompanied by an apparent increase in membrane conductance, this being reflected in a decrease in the amplitude of the anelectrotonic potential to current injection during a 5-HT-evoked depolarization (Figure 1a). The inward current which underlies the 5-HT-induced depolarization was recorded directly in cells voltage-clamped at −60 mV. Of 221 NCB-20 cells challenged with pressure applied 5-HT (10 μM), 161 responded with an inward current which ranged in amplitude between several pA and in excess of −1 nA. The remaining 27% of cells sampled had no discernible response to 5-HT. In comparison, all voltage-clamped N1E-115 cells tested responded to 5-HT (10 μM) with an inward current ($n = 80$). Figure 1b shows a typical response to 5-HT recorded under voltage-clamp from an NCB-20 cell. The large increase in clamp-current required to drive the membrane potential transiently between −60 and −80 mV during the action of 5-HT confirms that such responses result from an increase in membrane conductance. Temporally close applications of 5-HT resulted in a progressive decline of the amplitude of the 5-HT-induced current. Such desensitization could normally be avoided by applying 5-HT at intervals ≥90 s (data not illustrated).

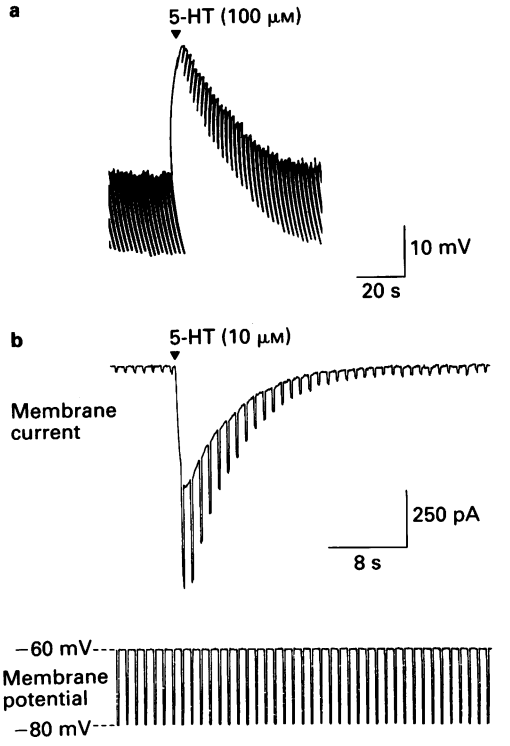

Figure 1 Responses of NCB-20 cells to 5-hydroxytryptamine (5-HT) in current- and voltage-clamp recording modes. (a) Current-clamp record illustrating a depolarizing response to microperfused 5-HT (100 μM). The downward deflections in the trace are anelectrotonic potentials to square pulses of current (−0.1 nA, 200 ms, 0.6 Hz) injected into the cell to monitor membrane conductance. The reduction in the size of the anelectrotonic potential during the 5-HT-evoked response indicates an apparent increase in conductance. (b) Voltage-clamp record showing an inward current (upper trace) to pressure applied 5-HT (10 μM). Membrane potential (lower trace) was held at −60 mV and transiently stepped to −80 mV (200 ms, 1.0 Hz) to assess membrane conductance. The increase in clamp current required to drive the membrane potential between −60 and −80 mV during the action of 5-HT indicates that the response is due to an increase in membrane conductance (of approximately 20 nS in this example). Voltage and current recordings were low-pass filtered at 1.0 kHz.

Current-voltage relationships and the ionic basis of the 5-HT-induced response

Figure 2 illustrates the results of an experiment in which the influence of holding potential upon the 5-HT-induced current was examined in an NCB-20 cell dialysed with a Cs^+-containing pipette solution.

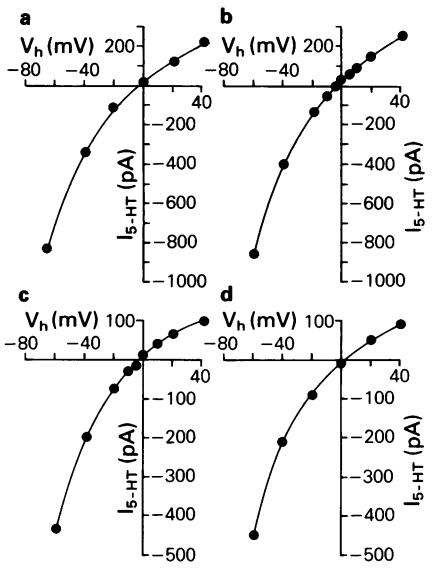

Figure 2 The relationship between the amplitude of the 5-hydroxytryptamine (5-HT)-induced current ($I_{5\text{-HT}}$) and holding potential (V_h) in NCB-20 and N1E-115 cells dialysed with various pipette solutions. The standard extracellular recording medium, with a composition detailed under Methods, was used in all experiments. (a and b) Current-voltage relationship in an NCB-20 cell (a) and N1E-115 cell (b) dialysed with a pipette solution containing Cs^+ as the predominant internal cation ($[Cs^+]_i = 140$ mM; $[Na^+]_i = 6$ mM). Note that in both cell types the 5-HT-induced response displays inward rectification and reverses in sign at a potential of approximately −2 mV. (c) Current-voltage relationship in an N1E-115 cell dialysed with a solution in which Cs^+ was completely replaced with K^+ ($[K^+]_i = 140$ mM; $[Na^+]_i = 6$ mM). Replacement of Cs^+ by K^+ has no effect upon the reversal potential of the response (−2 mV) and does not conspicuously alter the pattern of inward rectification. (d) Current-voltage relationship obtained from an N1E-115 cell dialysed with a Cl^--deficient solution ($[Cl^-]_i = 6$ mM; $[K^+]_i = 140$ mM; $[Na^+]_i = 6$ mM). The reduction in $[Cl^-]_i$ has little effect upon the reversal potential of the response (+1.0 mV) and inward rectification is still apparent. In (a) 5-HT (10 μM) was locally applied to the cell by pressure ejection from a modified patch pipette, whereas in (b), (c) and (d) 5-HT was ionophoretically applied with ejection charges of 1.24 to 1.6 nC.

The amplitude of the response to locally applied 5-HT (10 μM) decreased with membrane depolarization and reversed in sign, in this example, at a holding potential of −3.0 mV (Figure 2a). From similar experiments performed on 7 NCB-20 cells dialysed with Cs^+-based pipette solution, the reversal potential of the 5-HT-induced current ($E_{5\text{-HT}}$) was estimated by interpolation to be -1.4 ± 1.5 mV. Under identical recording conditions, $E_{5\text{-HT}}$ in N1E-115 cells was determined to be -2.2 ± 0.9 mV ($n = 5$). In both NCB-20 (Figure 2a) and N1E-115 cells (Figure 2b), the response to 5-HT demonstrated inward rectification. Such an effect is unlikely to result from the use of Cs^+ as the predominant internal cation because a qualitatively similar rectification was observed in recordings performed on NCB-20 and N1E-115 cells employing a KCl-based pipette solution (Figure 2c). The reversal potential of the 5-HT-induced current under the latter recording conditions was estimated to be -2.0 ± 2.6 mV ($n = 3$) in NCB-20 cells and -2.1 ± 1.0 mV ($n = 7$) in N1E-115 cells. However, these values may be subject to slight errors, as the activation of voltage-dependent K currents at depolarized potentials made the measurement of 5-HT-induced currents close to their reversal potential difficult.

In an attempt to determine the ionic basis of the 5-HT-evoked current, the influence of a number of cation and anion substitutions upon $E_{5\text{-HT}}$ was examined using N1E-115 cells and ionophoretic application of 5-HT. The effect of complete replacement of KCl by K-gluconate in the pipette solution, such that internal chloride ion concentration was reduced from 144 to 6 mM, was studied in 4 cells. With the Cl^--deficient solution, $E_{5\text{-HT}}$ was estimated to be $+1.2 \pm 1.3$ mV (Figure 2d), a value close to that of −2.1 mV found under control conditions (Figure 2c). Thus it is unlikely that 5-HT elicits an appreciable increase in the Cl^- permeability of the cell membrane. In contrast, partial replacement of extracellular Na^+ by glucosamine had marked effects upon the amplitude and reversal potential of 5-HT-induced currents recorded with pipettes containing a KCl-based intracellular solution. Reducing the external concentration of sodium ($[Na^+]_o$) from its control value of 143 mM to 75 mM depressed the amplitude of the 5-HT-induced current, recorded at a holding potential −60 mV, to $42.5 \pm 10.5\%$ ($n = 3$) of its control value and produced a negative shift in $E_{5\text{-HT}}$ to -18.0 ± 2.8 mV ($n = 3$; Figure 3a). When $[Na^+]_o$ was further reduced to 20 mM, the 5-HT-induced current was reduced to $6.9 \pm 1.2\%$ ($n = 3$) of control and its reversal potential shifted to -39.0 ± 2.6 mV ($n = 3$; Figure 3a). The effects of reduced $[Na^+]_o$ upon the amplitude and reversal potential of the 5-HT-induced current were reversed upon returning to control solution (not illustrated). Collectively, these observations suggest that the current evoked by 5-HT is at least partially mediated by an influx of Na^+. To test whether K^+ may also contribute to the response, the internal concentration of K^+ was reduced from its standard value of 140 mM to 20 mM by partial replacement of KCl with

tetraethylammonium Cl. Under such conditions, $E_{5\text{-HT}}$ was determined to be $+25.9 \pm 0.5$ mV ($n = 4$; Figure 3b), a value considerably more positive than that of -2.1 mV found in control, suggesting that an efflux of K^+ from the cell also contributes to the 5-HT-evoked response.

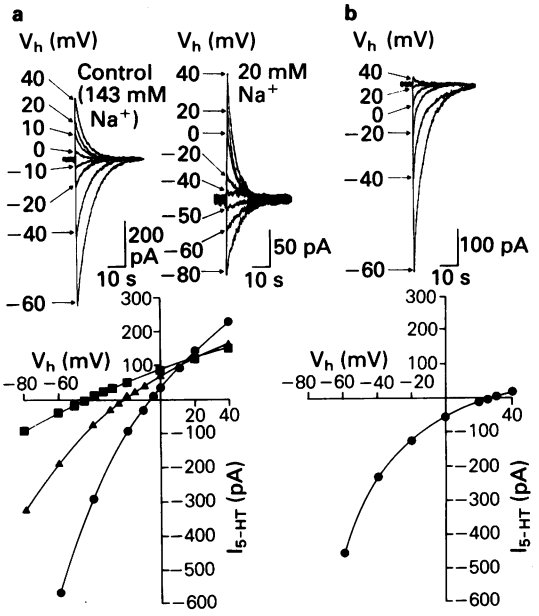

Figure 3 5-Hydroxytryptamine (5-HT)-induced currents are associated with an increase in membrane conductance to Na and K ions. (a) Influence of extracellular Na^+ concentration upon 5-HT-induced currents and their reversal potential in an N1E-115 cell. The traces illustrate transmembrane currents, recorded at various holding potentials (V_h) in response to ionophoretically applied 5-HT (3.0 nC, 0.01 Hz). The traces shown were obtained in standard extracellular medium containing 143 mM Na^+ (left) and in a solution in which $[Na^+]_o$ was reduced to 20 mM by partial replacement of NaCl with glucosamine HCl (right). Note that leakage currents have been subtracted. The graphical representation of the data shows the relationship between the amplitude of the 5-HT-induced current and holding potential with $[Na^+]_o$ equal to 143 mM (●), 75 mM (▲) and 20 mM (■). The reduction in $[Na^+]_o$ is accompanied by a hyperpolarizing shift in the reversal potential of the 5-HT-induced current. All data were obtained from the same cell which was dialysed with K^+-based pipette solution ($[K^+]_i = 140$ mM; $[Na^+]_i = 6$ mM). (b) 5-HT-induced currents recorded from an N1E-115 cell dialysed with a pipette solution which reduced $[K^+]_i$ to 20 mM. The experiment was performed with standard extracellular solution in the bath and ionophoretic applications of 5-HT (4.5 nC, 0.01 Hz). Note that in comparison to cells dialysed with a pipette solution containing 140 mM K^+ (e.g. Figures 2c and 3a), the reversal potential of the 5-HT-evoked response in this cell is displaced to a more positive potential (+25 mV).

Pharmacological categorization of the 5-HT receptor

Membrane currents evoked on NCB-20 cells by locally applied 5-HT (10 μM) were suppressed by bath applied GR 38032F (0.1–1.0 nM), a selective 5-HT_3 receptor antagonist. Such antagonism was concentration-dependent, as illustrated in the inset to Figure 4, and was readily reversed upon washout (Figure 5a). From pooled data obtained from several

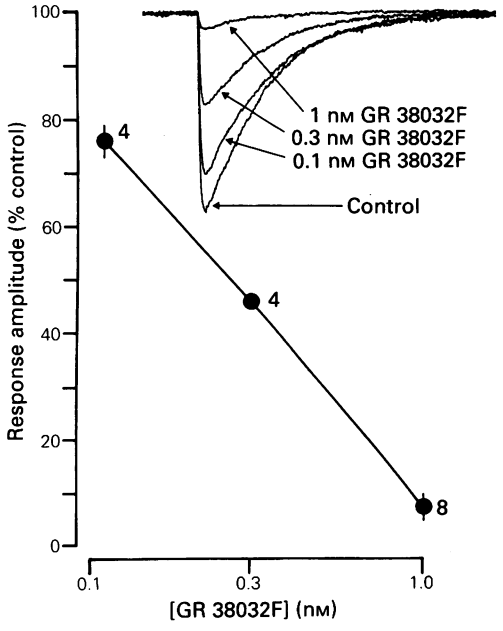

Figure 4 Concentration-dependent blockade of 5-hydroxytryptamine (5-HT)-induced inward currents in NCB-20 cells by GR 38032F. The inset depicts the results of an experiment on one cell where currents elicited by locally applied 5-HT (10 μM) were recorded in control and in the presence of a range of concentrations (0.1–1.0 nM) of bath-applied GR 38032F. Each current trace shown is the computer-generated average of 4 responses to 5-HT. Although not illustrated, antagonism by all concentrations of GR 38032F was completely reversible. In the graph, the amplitude of the 5-HT-evoked current, as a percentage of its control value, is plotted against the logarithm of the concentration of GR 38032F in the medium. From this plot, the IC_{50} for GR 38032F was estimated to be 0.25 nM. Each data point represents the mean value calculated from experiments performed on 4 to 8 cells and vertical lines indicate s.e.mean. All responses were recorded at a holding potential of -60 mV and low-pass filtered at 500 Hz.

cells, the IC_{50} for blockade by GR 38032F was estimated to be 0.25 nM (Figure 4). A similar antagonism of 5-HT-induced currents by GR 38032F (0.3 nM) was observed in N1E-115 cells, with responses being reduced to 26.0 ± 3.6% ($n = 4$) of their control value in the presence of the drug. In contrast to the potent blockade observed with GR 38032F, neither the mixed '5-HT$_1$-like'/5-HT$_2$ receptor antagonist methysergide (1 μM), nor the selective 5-HT$_2$ receptor antagonist ketanserin (1 μM), had any effect upon 5-HT-evoked currents in NCB-20 cells (Figure 5b,c). In preliminary experiments, the 5-HT$_3$ receptor antagonist ICS 205-930 had no effect upon the amplitude of 5-HT-evoked currents in NCB-20 cells when bath applied at a concentration of 10 pM. Virtually complete blockade could be demonstrated with 300 pM ICS 205-930, but recovery upon washout was incomplete (Figure 5d). Intermediate concentrations of ICS 205-930 (30–100 pM) produced a very slowly developing antagonism of the response, such that equilibrium was difficult to establish with certainty within the time that stable recordings could be maintained. In view of these practical difficulties, no attempt was made to determine the IC_{50} for the blocking action of ICS 205-930.

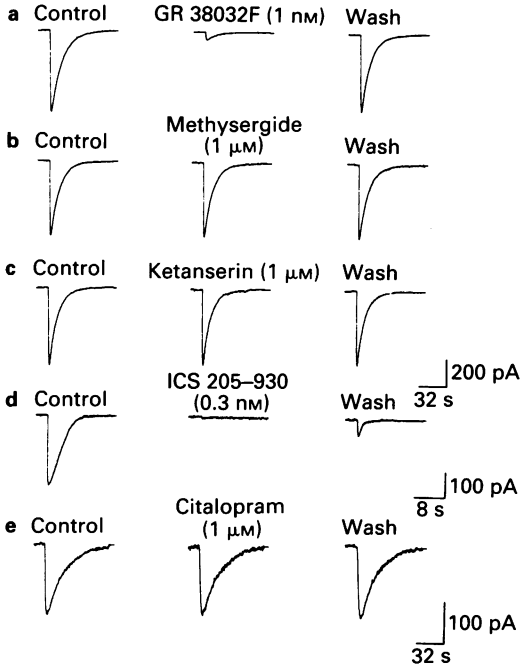

Figure 5 The pharmacology of 5-hydroxytryptamine (5-HT)-induced currents in NCB-20 cells. (a) Reversible antagonism of the 5-HT-evoked response by the 5-HT$_3$ receptor antagonist GR 38032F (1 nM). In contrast, methysergide (b) and ketanserin (c), each at a concentration of 1 μM, have no effect upon 5-HT-induced currents. (d) ICS 205-930 (0.3 nM) blocks the response to 5-HT, but reversal of the antagonism is incomplete. (e) The 5-HT uptake inhibitor citalopram (1 μM) does not modify responses to 5-HT. Traces depicted in (a), (b) and (c) were obtained from experiments performed on the same NCB-20 cell. Responses in (d) and (e) are the results from 2 other cells. All currents illustrated are the computer-generated average of 4 responses to pressure applied 5-HT (10 μM) recorded at a holding potential of −60 mV. Recordings were low-pass filtered at 500 Hz.

In the rat superior cervical ganglion, the presence of a 5-HT uptake system has been shown to influence estimates of antagonist potency when 5-HT is applied by superfusion (Ireland *et al.*, 1987). In the present investigation, the 5-HT uptake inhibitor citalopram (1 μM) had no effect on the amplitude of responses to 5-HT recorded from NCB-20 cells (Figure 5e). Similarly, citalopram does not potentiate the response of the rabbit superior cervical ganglion to bolus injections of 5-HT (Round & Wallis, 1986). The lack of effect of citalopram in the present work might suggest that uptake of 5-HT does not occur in NCB-20 cells, or that uptake does not exert a significant effect upon the rapid and transient increase in 5-HT concentration that is achieved with local pressure application of 5-HT.

5-HT-evoked membrane currents on outside-out membrane patches

In an effort to study the single channel events which underlie the 5-HT-induced current in NCB-20 and N1E-115 cells, 5-HT was locally applied to the extracellular face of outside-out membrane patches, either by pressure, or by ionophoresis. In a sample of 11 patches, all taken from 5-HT-sensitive NCB-20 cells, 5 had no detectable response to pressure applied 5-HT (10 μM), whereas the remainder responded with an inward current at negative holding potentials (−50 to −60 mV). Such currents, which appeared as relatively smoothly rising and decaying signals (Figure 6a), varied in amplitude between patches (range −1 to −6.5 pA). Despite the resolution of the recordings, discrete single channel events uniquely associated with the presence of 5-HT were not apparent during these inward currents. To illustrate this feature, Figure 6a compares a 5-HT-evoked current observed on an NCB-20 cell patch with single channel currents of similar amplitude, evoked by GABA (1 μM), on an outside-out patch excised from a bovine adrenomedullary chromaffin cell (Cottrell *et al.*, 1985). Responses qualitatively similar to those obtained on NCB-20 cell membranes were observed when 5-HT was ionophoretically applied to outside-out membrane patches taken from N1E-115 cells ($n = 4$; Figure 6c). This suggests that the unusual response to 5-HT is not a peculiarity of

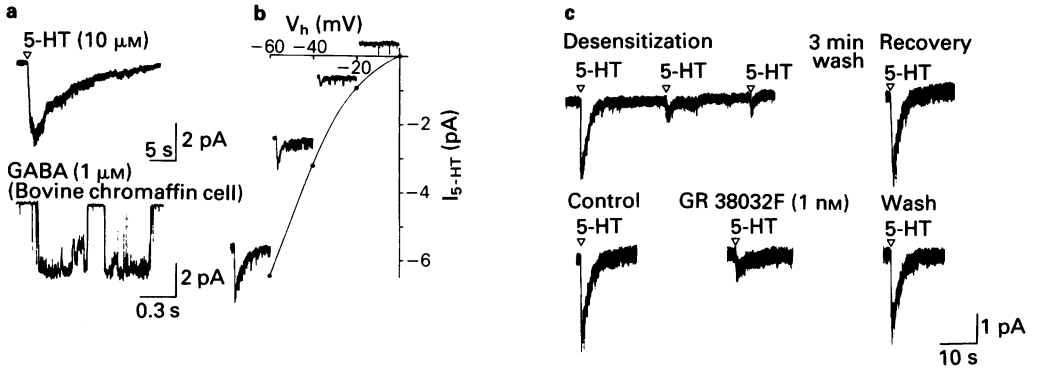

Figure 6 5-Hydroxytryptamine (5-HT)-evoked currents on outside-out membrane patches excised from NCB-20 and N1E-115 cells. (a) Upper trace: inward current evoked by pressure applied 5-HT (10 μM, 1s, 1.4×10^5 Pa) on an outside-out patch taken from an NCB-20 cell. The patch was voltage-clamped at a holding potential of −60 mV (inside negative) and the 5-HT response low-pass filtered at 500 Hz. Lower trace: single channel currents recorded on an outside-out patch excised from a bovine chromaffin cell in response to bath applied γ-aminobutyric acid (GABA, 1 μM). Currents were recorded at a holding potential of −100 mV and low-pass filtered at 1 kHz. The two traces have been deliberately selected to compare 5-HT- and GABA-evoked currents of similar amplitude. Note that unlike the response to GABA, the 5-HT-induced current is not resolved into discrete single channel events. (b) Influence of holding potential upon responses to pressure applied 5-HT (10 μM) recorded from an outside-out patch excised from an NCB-20 cell. Currents recorded at holding potentials of −60, −40, −20 and 0 mV are illustrated. Note that the single channel events superimposed upon the currents are unrelated to the action of 5-HT. Such single channel currents appeared as uncharacterized 'spontaneous' activity in some recordings. In common with the current-voltage relationship found in 'whole-cell' recordings (Figure 2), the relationship between holding potential and 5-HT-induced current amplitude on the outside-out patch shows inward rectification and a reversal potential close to 0 mV. (c) Inward currents elicited by ionophoretically applied 5-HT (40 nA, 200 mS) on an outside out patch excised from an N1E-115 cell. Currents were recorded at a holding potential of −50 mV. Repeated applications of 5-HT induce a reversible desensitization of the response. The introduction of GR 38032F (1 nM) to the bath reversibly suppressed the response to 5-HT. All currents illustrated in (b) and (c) were low-pass filtered at 500 Hz.

the NCB-20 cell line, or a consequence of the pressure application of 5-HT. It is conceivable that the inward current represents the summed activity of many small conductance 5-HT-gated ion channels (see below).

The response to 5-HT recorded on membrane patches from either NCB-20 or N1E-115 cells demonstrated properties similar to those found under 'whole-cell' recording conditions. Firstly, the amplitude of the response decreased with membrane depolarization and was nullified at a potential close to 0 mV (Figure 6b). Secondly, repeated applications of 5-HT produced a reversible desensitization of the response (Figure 6c). Finally, inward currents evoked by 5-HT on outside-out membrane patches were reversibly antagonized by 1 nM GR 38032F (Figure 6c), strongly suggesting that 5-HT_3 receptor activation underlies such responses.

Fluctuation analysis of 5-HT-induced currents

Figure 7 illustrates the results of a typical experiment in which 5-HT was applied by microperfusion to an N1E-115 cell voltage-clamped at a holding potential of −60 mV. Microperfusion was chosen as the method of agonist application since with careful positioning of the drug pipette it was possible to obtain relatively slowly rising inward currents in response to a low concentration (1 μM) of 5-HT, which were suited to the analysis of 5-HT-induced membrane noise. The onset of the response (Figure 7a) was in all cases accompanied by a modest, but clearly discernible, increase in membrane noise as revealed by high-gain a.c.-coupled recordings of the current elicited by 5-HT (e.g. Figure 7b). Fluctuation analysis of such records indicated that the noise variance (σ^2) was linearly related to the mean amplitude (I_{av}) of the 5-HT-evoked inward current during its rising phase. Assuming that the increase in σ^2 arises from the opening of an homogeneous class of channels (see Discussion), the slope of the relationship between σ^2 and I_{av} allows an estimate of the current (i) flowing through one 5-HT-activated channel to be made. In the exemplar cell, i was determined to be 21.6 fA at a holding potential (Vh) of −60 mV. Using this value, in conjunction with the reversal potential of the 5-HT-induced whole cell current determined under identical ionic conditions

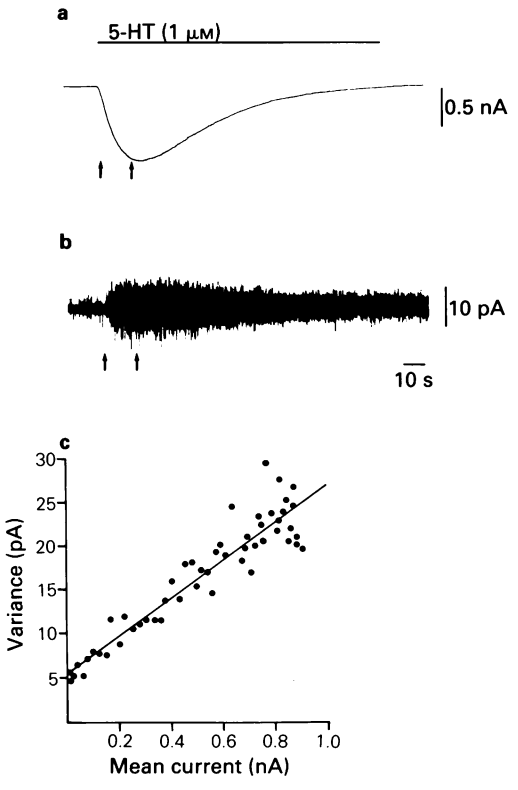

Figure 7 Fluctuation analysis of an inward current evoked by 5-hydroxytryptamine (5-HT) in an N1E-115 cell. (a) d.c.-coupled inward current, recorded at a holding potential of −60 mV, in response to 5-HT (1 μM) applied by microperfusion. Note the relatively slow rise of the current and its subsequent decline during the continued presence of the agonist, indicating the onset of some form of desensitization. (b) High-gain, a.c.-coupled, record of the response elicited by 5-HT. The record illustrates the clear increase in current fluctuation which parallels the development of the inward current evoked by 5-HT. High- and low-pass filtering was performed with cut-off frequencies of 2.0 and 500 Hz respectively. (c) Plot of membrane current variance against mean inward current during the rising phase of the 5-HT-induced response. The analysis was conducted over the bandwidth 2.0–500 Hz and restricted to the data segment contained within the arrows illustrated in (a). The slope of the line fitted to the data points by least squares regression analysis ($r = 0.97$), gives an estimate of the value of the elementary current (i) flowing through a single 5-HT-activated channel. In this example, i was estimated to be 21.6 fA, corresponding to an elementary conductance of 360 fS at a holding potential of −60 mV (see text for further details). In this instance, membrane current variance in the absence of agonist was not subtracted from the total variance observed during the response to 5-HT. The intercept of the fitted line with the ordinate thus yields the 'background' current variance in this recording.

Table 1 Estimates from fluctuation analysis of the current (i) flowing through a single 5-HT-activated ion channel and its conductance (γ) at a holding potential of −60 mV in N1E-115 cells

Cell	*i*(fA)	γ(fS)	*r*
1	19.2	320	0.92
2	9.6	160	0.77
3	11.4	190	0.80
4	15.6	260	0.71
5	27.0	450	0.75
6	19.8	330	0.87
7	22.2	370	0.93
8	21.0	350	0.79
9	21.6	360	0.97
mean ± s.e.	18.6 ± 1.9	310 ± 32	—

i was estimated from the slope of a straight line fitted to the relationship between mean agonist-induced current and current variance. *r* is the correlation coefficient associated with the fit. γ was calculated according to equation 2.

(i.e. −2.1 mV, Figure 2b), allows an estimation of the elementary conductance (γ) of the 5-HT-activated channel to be made since:

$$i = \gamma(V_h - E_{5\text{-HT}}) \quad (2)$$

For the example illustrated in Figure 7, γ was estimated to be 360 fS at a holding potential of −60 mV. Estimates of i and γ obtained from experiments performed on 8 other cells are listed in Table 1. As expected, on 3 N1E-115 cells voltage-clamped at $E_{5\text{-HT}}$, the application of 5-HT was not associated with any detectable increase in current variance.

Discussion

The results of the present study indicate that 5-HT-induced currents in NCB-20 cells are mediated exclusively by the 5-HT$_3$ subtype of receptor. This conclusion is based on the potent blockade of the response observed with GR 38032F and ICS 205-930, and the lack of effect of either ketanserin or methysergide. In particular, blockade of the 5-HT-induced current by GR 38032F occurred over a concentration-range (0.1 to 1.0 nM; $IC_{50} = 0.25$ nM) at which this compound exerts no antagonist action at either '5-HT$_1$-like' or 5-HT$_2$ receptors. Such concentrations are however similar to those at which GR 38032F acts as an antagonist of 5-HT$_3$ receptor-mediated depolarization in rabbit vagus nerve ($pA_2 = 9.4$; Butler *et al.*, 1988).

Adenylate cyclase activity is stimulated through the activation of '5-HT$_1$-like' receptors in NCB-20 cells (Berry-Kravis & Dawson, 1983), but several

lines of evidence suggest that this effect is distinct from the electrical response to 5-HT. Firstly, the enhancement of adenylate cyclase activity shows no decline in the continued presence of 5-HT, whereas the depolarization and inward current elicited by 5-HT desensitize rapidly (MacDermot *et al.*, 1979). Secondly, both D-lysergic acid diethylamide and methysergide act as partial agonists at '5-HT_1-like' receptors in NCB-20 cells, but neither compound affects the electrical response to 5-HT (MacDermot *et al.*, 1979 and present study). Cells of the phaeochromocytoma cell line PC-12, and neuroblastoma clone N-2a accumulate 5-HT from the external medium by diffusion and high affinity transport systems (Yoffe & Borchardt, 1982a,b). If the latter process also occurs in NCB-20 cells, it is unlikely to influence the present results because the selective 5-HT uptake blocker citalopram had no effect on 5-HT-induced currents.

No attempt was made to characterize in detail the 5-HT receptor mediating the inward current response to 5-HT in N1E-115 cells, since there is already substantial evidence that this effect is due to the activation of the 5-HT_3 receptor subtype (Neijt *et al.*, 1986; 1988). The blockade by GR 38032F of 5-HT-induced currents described here adds to this evidence.

5-HT_3 receptor activation is thought to result in the opening of an associated ion channel which conducts monovalent cations. Wallis & Woodward (1975) found that the extracellularly recorded depolarizing response to 5-HT in the rabbit superior cervical ganglion was diminished by the removal of Na^+ from the bathing solution, but was not influenced by replacing Cl^- with an impermeant anion. In a subsequent intracellular study (Wallis & North, 1978), a simultaneous increase in membrane conductance to Na^+ and K^+ was proposed to underlie the 5-HT-induced depolarization. Firm evidence for this suggestion was provided by Higashi & Nishi (1982) in a voltage-clamp study of 5-HT-induced currents in the nodose ganglion of the rabbit. Consistent with these reports, it has been proposed that a simultaneous increase in membrane conductance to Na^+ and K^+ mediates both the 5-HT-evoked depolarization and dopamine-induced inward current in N1E-115 cells (Peters & Usherwood, 1983; Kato & Narahashi, 1982). The latter response has recently been attributed to 5-HT_3 rather than dopamine receptor activation (Peters & Usherwood, 1984; Neijt *et al.*, 1986).

The present study provides evidence obtained under voltage-clamp, with known intra- and extracellular ion concentrations, that 5-HT induces a simultaneous increase in membrane conductance to both Na^+ and K^+. Chloride ions are unlikely to contribute to the response since a large reduction in internal Cl^- concentration had no significant effect upon $E_{5\text{-HT}}$. In a previous study (Peters *et al.*, 1988), varying the extracellular concentration of either Ca^{2+} or Mg^{2+} within the range 0.1 to 3.0 mM did not change $E_{5\text{-HT}}$ in N1E-115 cells, although these divalent cations were observed to modulate both the amplitude and duration of the current. The insensitivity of $E_{5\text{-HT}}$ to $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ might suggest that these ions do not permeate the channel to a significant extent. However, the range of concentrations of Ca^{2+} and Mg^{2+} examined was restricted, and the absence of an observable effect upon $E_{5\text{-HT}}$ does not necessarily exclude the possibility that these ions possess a finite permeability. Assuming that the 5-HT-induced current is carried exclusively by Na^+ and K^+ in physiological solutions, the permeability of $Na^+(P_{Na})$ relative to $K^+(P_K)$ may be calculated from the Goldman-Hodgkin-Katz voltage equation (e.g. Hille, 1984)

$$E_{5\text{-HT}} = \frac{RT}{zF} \quad \text{In} \quad \frac{P_{Na}[Na^+]_o + P_K[K^+]_o}{P_{Na}[Na^+]_i + P_K[K^+]_i} \quad (3)$$

where z, F, R and T have their usual meaning. Employing the known intra- and extra-cellular concentrations of the permeant ion species, and the value of $E_{5\text{-HT}}$ determined with the KCl based pipette solution (−2.1 mV), P_{Na}/P_K was estimated to be 0.92. However, it should be noted that such an estimate does not quantitatively account for the shifts in $E_{5\text{-HT}}$ observed with Na^+- and K^+-deficient media. Reducing $[K^+]_i$ from 140 to 20 mM would, on the basis of equation 3 and a P_{Na}/P_K ratio of 0.92, be expected to shift $E_{5\text{-HT}}$ to +41 mV rather than +25.9 mV as observed. Similarly, $E_{5\text{-HT}}$ found with $[Na^+]_o$ equal to 20 mM (i.e. −39.0 mV) is less negative than that expected (−48.7 mV), whereas the empirically determined and theoretically predicted values of $E_{5\text{-HT}}$ with $[Na^+]_o$ equal to 75 mM correspond perfectly, both being approximately −18.0 mV. The departure of the experimental data from ideal behaviour may be due to several factors (see Hille, 1984), one possibility being that the cations used to replace Na^+ and K^+ are themselves slightly permeant. Further experiments with a variety of replacement cations are required to clarify this issue.

The relationship between holding potential and the amplitude of 5-HT-induced currents in both NCB-20 and N1E-115 cells indicates that the response is inwardly rectifying (e.g. Figures 2 and 3). In NCB-20 and N1E-115 cells, such rectification was not conspicuously altered by replacing Cs^+ in the pipette solution with K^+. This suggests that voltage-dependent blockade of the 5-HT-gated ion channel by internal Cs^+ is unlikely to account for the non-linearity of the current-voltage relationship. Indeed, in both NCB-20 and N1E-115 cells, $E_{5\text{-HT}}$ was found

to be -1 to -2 mV with either Cs^+- or K^+-based pipette solutions, suggesting that these two ions permeate the 5-HT-activated channel equally well. Inward rectification has also been reported for dopamine-induced currents in N1E-115 cells (Kato & Narahashi, 1982) and 5-HT$_3$ receptor mediated membrane currents recorded from clonal NG108-15 hybrid cells and murine striatal and hippocampal neurones in cell culture (Yakel *et al.*, 1988; Yakel & Jackson, 1988). In contrast, the current-voltage relationship obtained with 5-HT in the nodose ganglion of the rabbit is essentially linear (Higashi & Nishi, 1982). We are at present performing voltage-jump experiments in an effort to elucidate the origin of the rectification in the clonal cell lines.

In an attempt to investigate the single channel correlate of 5-HT-induced whole-cell currents, 5-HT was applied locally to outside-out membrane patches excised from NCB-20 and N1E-115 cells. Although 5-HT clearly evoked an inward current in a proportion of the patches examined, such responses could not be resolved into discrete single channel events. Instead, the currents appeared as relatively smooth rising and decaying signals which varied in amplitude from patch to patch. Membrane currents induced by 5-HT on outside-out patches were reversibly antagonized by GR 38032F (1 nM), diminished in amplitude by membrane depolarization and nullified at 0 mV and reversibly desensitized by repeated agonist applications. Collectively, these features strongly suggest that the response is 5-HT$_3$ receptor-mediated.

One interpretation of the data obtained on outside-out patches is that 5-HT gates an ion channel with a conductance too small to allow the direct visualization of individual channel openings, despite the relatively high resolution afforded by this recording mode. The simultaneous activation of many such channels could conceivably produce responses with the characteristics illustrated in Figure 6. Such a mechanism is not unprecedented. Glutamate, or kainate can activate cation-conducting channels with a conductance as low as 140 fS in cerebellar granule cells and membrane patches excised from them (Cull-Candy & Ogden, 1985; Cull-Candy *et al.*, 1988). Additionally, in rat spinal neurones kainate can elicit currents which appear only as an increase in the membrane noise of outside-out patches (Ascher and Nowak, 1988). The results we have obtained using the technique of fluctuation analysis provide considerable support for the view that 5-HT activates a channel of small conductance. Analysis of high-gain a.c.-coupled records of the membrane noise of both NCB-20 and N1E-115 cells, in the absence and presence of 5-HT, indicates that 5-HT-evoked whole cell currents are accompanied by only a modest increase in current variance, which appears to correspond to the activation of single channels with a conductance of approximately 310 fS. However, the results obtained with fluctuation analysis require cautious interpretation. An underestimate of single channel conductance could occur if either the probability of an individual channel being in the open state was not low, or if the bandwidth over which the analysis was performed excluded significant frequency components (Marty *et al.*, 1984). The whole cell currents used in fluctuation analysis were evoked with agonist applied by microperfusion from pipettes containing 1 μM 5-HT and it is likely, that due to diffusion, the actual concentration of 5-HT at the receptor plane is somewhat lower than this value. Such concentrations constitute the foot of the dose-effect relationship for 5-HT in N1E-115 cells (Neijt *et al.*, 1988), suggesting that the probability of a single channel being open would indeed be low. The linear relationship between the variance of 5-HT-induced current fluctuations and mean current (e.g. Figure 7) is consistent with this suggestion. In a number of experiments the bandwidth over which current fluctuations were analysed was extended, either by reducing the high-pass filtering to 1.0 Hz, or by increasing the low-pass filtering to 1 kHz cut-off points. Neither of these changes had any significant effect upon the estimated single channel conductance.

It should be noted that our estimates of single channel conductance were derived under non-steady-state conditions during the period of time that agonist concentration was increasing from zero towards 1 μM. We have assumed that single channel conductance is independent of agonist concentration, and also that the desensitization which is likely to occur during the relatively slow rise of the 5-HT-induced current (e.g. Figure 7a) has not influenced our results. Recent studies have established that for several other agonist-activated cation selective channels, the concept of just two conductance states of the channel, open and closed, is an over-simplification. Instead it is now clear that in addition to a most frequently observed main conductance state, such channels may open to states of higher or lower conductance (e.g. Cull-Candy & Usowicz, 1987; Jahr & Stevens, 1987). Fluctuation analysis is likely to provide a weighted-mean estimate of channel conductance that is influenced by the relative frequency of all conducting states. Clearly, the present results may also suffer from this limitation, and although we have interpreted our data in terms of a 5-HT gated channel of unitary conductance, the possibility that 5-HT activates multiple conductance states certainly cannot be excluded. Indeed, on one outside-out membrane patch voltage-clamped at -60 mV and challenged with microperfused 5-HT (1 μM), distinct channel openings of small amplitude

(~0.5 pA) were clearly superimposed upon a relatively smooth inward current of the form described above. Such conductance states associated with the application of 5-HT have been observed on but one occasion, and a far more extensive study is required to establish the reproducibility of this effect. Despite these potential problems, the value of 310 fS (Table 1) thus far suggested for the elementary conductance of the 5-HT-evoked channel is entirely consistent with the lack of observable single channel activity during 5-HT-evoked currents on the majority of outside-out membrane patches. For example, at a holding potential of −60 mV and assuming a reversal potential of −2 mV, a single 5-HT-gated channel of 310 fS conductance would pass a current of only 19 fA, a value far too small to stand out from background noise as a discrete event.

A previous study (Guharay *et al.*, 1985) has shown that 5-HT activates a relatively large conductance (140 pS) ion channel on cell attached, inside-out, and outside-out mebrane patches from N1E-115 cells. On no occasion did we observe single channel currents with this characteristic, neither on whole cells, nor isolated membrane patches. At the present time, a satisfactory explanation for these disparate findings is not available. However, it should be noted that in contrast to the present study, the recordings of Guharay *et al.*, (1985) were conducted on N1E-115 cells differentiated by the inclusion of 2% (vol/vol) dimethylsulphoxide in the cell culture medium, a treatment known to interfere with nucleic acid metabolism (Friend & Freedman, 1978). Additionally, in the latter study membrane patches were bathed with a constant concentration of 5-HT (100 nM), rather than transiently exposed to the agonist as in the present work. We chose a transient method of agonist application in an attempt to minimize the desensitization which even low concentrations of 5-HT are known to induce in N1E-115 cells (Neijt *et al.*, 1988). Finally a degree of genetic instability is inherent to clonal cell systems, including N1E-115 cells, as even amongst cells of the same clone large variations in chromosome number may occur (Amano *et al.*, 1972). Although the results obtained from the two clones examined in the present study are consistent, it is nevertheless desirable to substantiate such observations with results obtained from untransformed tissue. We are presently investigating whether 5-HT induces currents similar to those reported here in rodent hippocampal neurones which have recently been reported to express 5-HT_3 receptors (Yakel & Jackson, 1988).

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The activation gate of the sodium channel controls blockade and deblockade by disopyramide in rabbit Purkinje fibres

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1 The effect of disopyramide on the maximum upstroke velocity ($\dot{V}_{max}$) and the sodium current of rabbit cardiac Purkinje fibres was studied with the two-microelectrode voltage-clamp technique.

2 In the absence of stimulation the drug did not cause block at membrane potentials ranging from -100 to -65 mV. Use-dependent block of $\dot{V}_{max}$ was most pronounced at -75 mV. At hyperpolarized membrane potentials development of use-dependent block was faster than at depolarized membrane potentials. The time course of development of use-dependent block was not significantly influenced by the duration of the depolarizing pulse. These results strongly suggest that disopyramide predominantly blocks activated sodium channels.

3 The relative decrease of the sodium current at the beginning of a 2 s depolarizing clamp to -45 mV was almost the same as at the end, implying a rapid blockade of activated sodium channels. The Hill plots were linear with slopes ranging from 0.98 to 1.08 indicating a first order reaction; the dissociation constant for activated channels was 70 μM.

4 Recovery of $\dot{V}_{max}$ from use-dependent block during rest was strongly voltage-dependent, the time constant of recovery increasing upon hyperpolarization. When the fraction of charged molecules was reduced by changing the pH of the external solution, the voltage-dependence of recovery was decreased. In contrast, recovery of $\dot{V}_{max}$ for a change in holding potential from -80 to -95 mV was very fast during repetitive stimulation.

5 It is concluded that disopyramide blocks the sodium channel during activation and is trapped in the channel when the activation gate closes.

Introduction

The modulated receptor hypothesis (Hille, 1977b; Hondeghem & Katzung, 1977) postulates a variable-affinity channel binding site and a modification of inactivation gate kinetics in drug complexed channels. Therapeutically useful antiarrhythmic drugs are assumed to have a low affinity for rested sodium channels but to bind preferentially when the membrane is depolarized. Therefore these agents show a pronounced use-dependent block of sodium channels whenever the diastolic interval during repetitive stimulation is too short for complete recovery from block. Since blocked channels have their inactivation kinetics shifted in the hyperpolarized direction and it has been shown for several drugs that dissociation of the drug from the binding site occurs more slowly in inactivated than in rested channels (Hondeghem & Katzung, 1984), recovery should be voltage-dependent, i.e. the rate of recovery should increase with hyperpolarization.

In contrast, the guarded receptor hypothesis (Starmer *et al.*, 1984) assumes use-dependent block as the result of transient access to a binding site of constant affinity, controlled by the channel gate conformation. For lipid soluble agents, receptor access is postulated as being controlled by only the activation gate, whereas for hydrophilic drugs both activation and inactivation gates are required in the open configuration. Drug molecules bound to the binding site in the channel may become trapped by closure of the channel gates. The first evidence for gate-trapping was provided by Strichartz (1973) and by Yeh (1979). As regards the recovery time course, the gate trapping hypothesis predicts acceleration or slowing of recovery with hyperpolarization, depending on whether the drug is trapped by the inactivation or the activation gate. In the case of the activation gate trapping, recovery is slowed as the membrane is hyperpolarized because the probability of channel

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opening is decreased. Quaternary compounds and those with a low deprotonation rate, will only be able to leave the channel when both gates are in the open configuration.

According to those two hypotheses the determining factor for recovery is either the drug's dissociation from the binding site or the channel gating mechanism. In order to differentiate between those two alternatives the study of voltage sensitivity of recovery from block may be a discriminating test.

Recent results obtained in squid axons with QX-314 and QX-222 (Yeh & Tanguy, 1985) and with disopyramide and analogues (Yeh & Ten Eick, 1987) indicate a slowing of recovery as the membrane is hyperpolarized and give additional support for the activation gate trapping model. In cardiac muscle, disopyramide shows similar potential dependence of the time constant of recovery from use-dependent block, when the membrane potential is changed by using variable potassium concentrations in the perfusion solution (Gruber *et al.*, 1986).

Therefore, the present investigation was done to test this hypothesis under voltage-clamp conditions in rabbit Purkinje fibres, in an attempt to differentiate between the activation gate trapping and the modulated receptor hypothesis for the action of a sodium channel blocking drug in cardiac tissue.

Methods

The experiments were performed on rabbit Purkinje fibres, dissected from both ventricles. The fibres were shortened to less than 1 mm by crushing them with a wire grid on the bottom of the perfusion chamber.

The composition of the modified Tyrode solution was (mM): NaCl 137, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, $NaHCO_3$ 24, glucose 11. The fibres were superfused with this solution, which was gassed with 95% O_2–5% CO_2, at a rate of 3 ml min^{-1} at a constant temperature of 37°C. For experiments performed at a pH of 8.0, the concentration of HCO_3^- was increased and the CO_2 content of the gas mixture was reduced to 3%. Cs 20 mM was added to block the inward rectifying potassium channel; this allowed continuous clamping at very negative potentials without causing potassium depletion. Disopyramide (Mead Johnson) was applied in concentrations ranging from 10 to 300 μM.

The two-microelectrode technique was used to measure currents under voltage-clamp conditions, or to impose different degrees and durations of depolarizing pulses. Sodium channel activity was measured directly by recording the slowly inactivating sodium current (Carmeliet, 1987a) or indirectly by measuring the maximum rate of rise of an action potential ($\dot{V}_{max}$). The current-passing electrode penetrated the middle of the fibre, whereas the potential-measuring electrode was positioned between the first one and the end of the segment. Both electrodes were filled with 3 M KCl. This combination of voltage-clamp methods and measurement of $\dot{V}_{max}$ in unclamped fibres was chosen, because it is difficult to apply a well controlled voltage-clamp during periods of peak sodium current flow. Thus, the voltage-clamp allows the application of 'conditioning' protocols which permit testing of hypotheses concerning the importance of the state of voltage-dependent channels for drug binding, and $\dot{V}_{max}$ provides a good measure of sodium channel availability during periods of high sodium current density.

The membrane potential was amplified and led to the input of the voltage-clamp amplifier which also received rectangular command pulses. Transmembrane current was measured by an operational amplifier circuit that held the bath potential at virtual ground. Signals were displayed on an oscilloscope or a pen recorder, or were stored on magnetic tape.

For statistical analysis mean values ± s.e. are given.

Results

Figure 1a,b illustrates the existence of a pronounced use-dependent block of $\dot{V}_{max}$ by disopyramide (10 μM). The clamp protocol consisted of 50 ms clamp periods at −95 mV (holding potential) interrupted by 10 ms to −45 mV. Between the two clamp periods the preparation was stimulated to measure $\dot{V}_{max}$. $\dot{V}_{max}$, for the first pulse applied after a rest period of at least 3 min, was identical with the control value in the absence of the drug, indicating the absence of a tonic block. Repetition of the depolarizing clamp steps resulted in a gradual decline of $\dot{V}_{max}$, which could be described as a mono-exponential decay (Figure 1b). In the absence of drug such a decrease was not seen (Figure 1a). The existence of use-dependence implies extra-block occurring during activity and insufficient recovery during the rest period between stimuli. It also means that the extra block during one depolarization does not reach equilibrium concentration. The question to be answered is whether disopyramide binds preferentially to the activated or inactivated state.

Absence of inactivation block

In published studies (Kus & Sasyniuk, 1978; Kojima *et al.*, 1982) disopyramide was described as an inactivation blocker. In the present experiments the possibility of preferential binding to the inactivated state was tested in two ways, either by prolonging

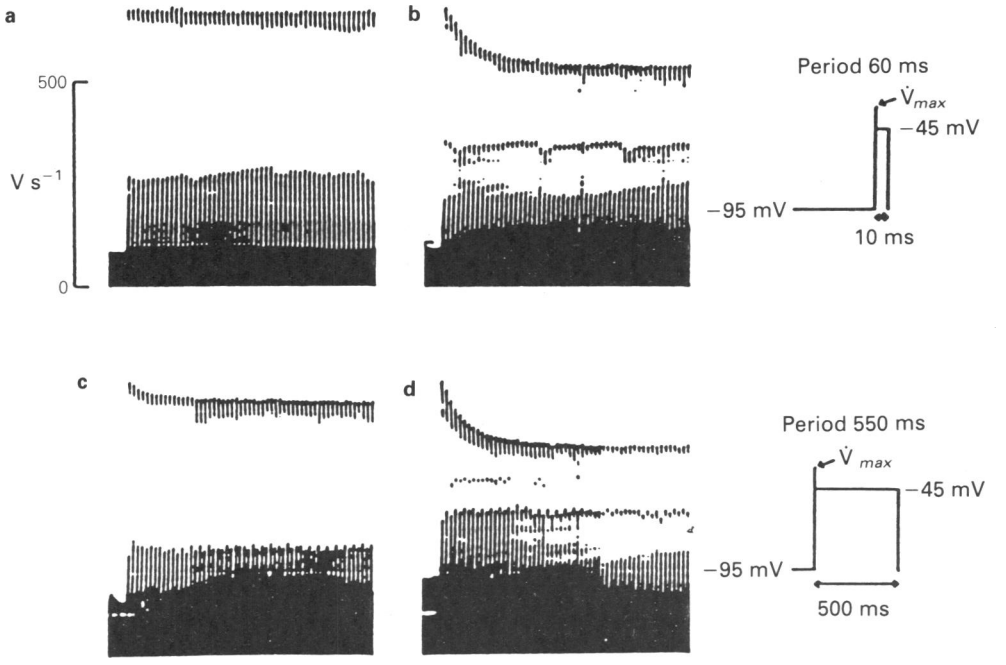

Figure 1 Development of use-dependent block for depolarizing pulses of different duration. After a depolarizing pulse and measuring $\dot{V}_{max}$ the fibres were held at a membrane potential of −45 mV for either 10 ms (a,b) or 500 ms (c,d) before clamping back to the holding potential of −95 mV for 50 ms (see inset: period is 60 ms for a and b and 550 ms for c and d). Original recordings of $\dot{V}_{max}$ are shown under control conditions (a,c) and during exposure to disopyramide (10 μM) (b,d). The time course of development of use-dependent block is largely independent of the duration of the clamp step to −45 mV.

the duration of the depolarizing step (Figure 1c,d) or by changing the holding potential preceding the test depolarization (Figure 2). As shown in Figure 1, prolongation of the depolarizing step from 10 ms to 500 ms had no effect on the amplitude of the block in steady-state. The time course of the block development was slightly faster with the long depolarizations, but a certain degree of $\dot{V}_{max}$ decay was also seen under control conditions and is due to the existence of a slow phase in the recovery from inactivation (Saikawa & Carmeliet, 1982). The fact that prolongation of the depolarization did not increase block means that inactivation beyond a duration of 10 ms had no effect on the block. A block of inactivated channels reaching steady-state in less than 10 ms can also be excluded because of the existence of use-dependency. The effect of different degrees of inactivation was further studied by varying the membrane holding potential between −100 mV and −65 mV and imposing a series of voltage-clamp depolarizations of the type shown in Figure 2 (inset) at a frequency of 1000 min^{-1}. Between each series and before changing the holding potential the preparation was rested for at least 3 min. Under control conditions $\dot{V}_{max}$ values at the start and the end of a series of depolarizations did not differ (Figure 2). The same values were also obtained in the presence of 10 μM disopyramide when only one single stimulus was applied. Therefore, it is evident that disopyramide did not cause any block in the absence of stimulation, although inactivation was clearly present under control conditions.

Use-dependent block and membrane potential

The results of Figure 2 further demonstrate that the steady-state use-dependent block obtained at the end of a series of depolarizing clamps was dependent on the holding membrane potential, although it is not a simple function of inactivation. In absolute terms the block was greatest at −75 mV, and gradually decreased at hyper- or depolarized levels (see also Figure 5a,b for an illustration of a greater block at a holding potential of −80 mV compared to −95 mV). In relative terms the depression of $\dot{V}_{max}$ was 9.7 ± 2.6% at −100 mV and this depression increased as the membrane potential became less negative, until a constant level of about 57% reduction was reached at −70 mV.

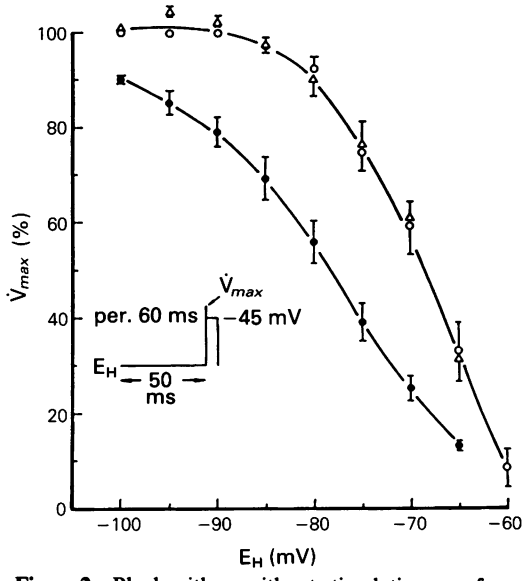

Figure 2 Block with or without stimulation as a function of membrane potential. From a distinct holding potential (E_H) between −100 to −60 mV a depolarizing pulse was applied and $\dot{V}_{max}$ measured. Following this pulse the fibre was kept at −45 mV for 10 ms before switching back to a certain holding potential for 50 ms. This protocol was repeated until steady-state block was obtained. Stimulation was then stopped and a rest period of at least 3 min was interpolated until complete disappearance of the block. Mean values of $\dot{V}_{max}$ for 5 experiments are given, vertical lines indicate s.e. Control is indicated by (○). Disopyramide (10 μM) did not cause tonic block (absence of stimulation) at any tested holding potential (△), whereas use-dependent block (●) was most pronounced at membrane potentials of −75 mV, declining when the membrane was hyper- or depolarized during diastolic intervals.

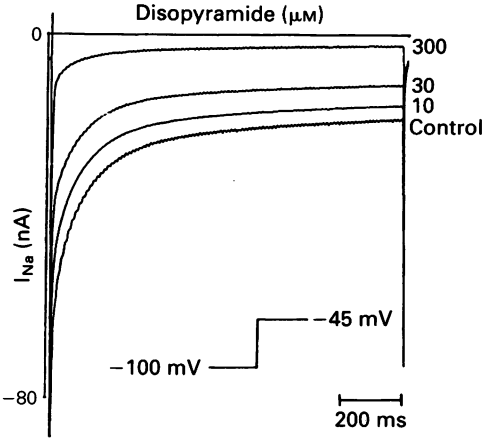

Figure 3 Slow sodium current in the absence and presence of disopyramide (10, 30 and 300 μM). From a holding potential of −100 mV a 2 s depolarizing clamp to −45 mV was applied. The control current is the total tetrodotoxin-sensitive current measured after exposure to 60 μM (see Carmeliet, 1987a,b for further details). Characteristic current traces recorded in the presence of 10, 30 and 300 μM disopyramide are shown. For these concentrations the current was 81%, 60% and 16% of control at the beginning and 83%, 59% and 13% at the end of the depolarizing pulse. The holding current in this experiment was −7 nA.

The time course of the development of use-dependent block can be described by one exponential and a constant. The time constant was shorter for hyperpolarizing and longer for depolarizing holding potentials. The mean values (ms) for three experiments are given in Table 1. Expressed in rates and taking the number of depolarizations (activations) into account these values changed from 0.239 pulse^{-1} at −100 mV to 0.025 pulse^{-1} at −65 mV.

Activation block

Since the preceding experiments have shown that disopyramide neither exerts a resting nor an inactivation block, we decided to measure the blocking of the sodium channels during activation directly. Since it is difficult to control voltage during the initial peak sodium current flow, the present analysis is limited to the slow component of the tetrodotoxin(TTX)-sensitive sodium current. This

Table 1 Time constants for development of use-dependent block (τ) at different membrane potentials

	Membrane potential (mV)							
	−100	−95	−90	−85	−80	−75	−70	−65
τ (ms)	251 ±10.2	310 ±26.1	376 ±81.5	450 ±47.7	691 ±91.6	817 ±24.6	1528 ±38.6	2379 ±99.3

Values are means ± s.e., $n = 3$.

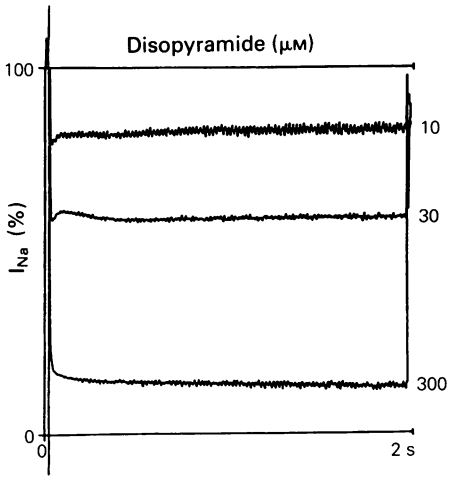

Figure 4 Time course of relative block during a depolarizing pulse. Data were taken from the same experiment as shown in Figure 3. The ratio of the current in the presence of 10, 30 and 300 μM disopyramide to control current is illustrated. The onset of block was so fast that no reliable time constants for the time course of development of block during the depolarizing pulse could be determined. The results indicate that disopyramide rapidly blocks activated sodium channels.

was studied by applying a voltage-clamp depolarization of 2 s from a holding potential of −100 mV to −45 mV ($n = 4$). The same method was used to measure the time course of TTX-binding (Carmeliet, 1987b) and of transcainide (Carmeliet, 1988a) to activated sodium channels. At any drug concentration tested between 10 and 300 μM, the initial slow sodium current was reduced to the same extent as the current at the end of the 2 s depolarizing pulse (Figures 3 and 4). The ratio of the current in the presence of disopyramide to the current in control Tyrode solution showed no obvious decay of relative block during a depolarizing pulse, indicating that no additional blockade of sodium channels could be detected during the depolarization. Since disopyramide did not cause resting block (Figures 1 and 2) this signifies that all or most of the blocking of sodium channels occurred during the initial ms of the depolarizing pulse (Figure 4). Taking those results into account, disopyramide can be classified as a fast activation blocker. The Hill plots ($n = 4$) of the data were linear with slopes ranging from 0.98 to 1.08, suggestive of a first order reaction between the drug and the binding site. The dissociation constant determined for activated sodium channels was 70 μM.

At first sight the finding of steady-state block which developed during the initial 10–15 ms of a clamp to −45 mV seems against the existence of a use-dependent block as illustrated in Figures 1 and 2. However one should take into account the fact that block is estimated in one case by measuring the slow Na current and in the other case by measuring $\dot{V}_{max}$, an estimate of the fast Na current. The time allowed for development of activation block of the fast Na current is very short, i.e. only part of the action potential upstroke. In the case of the slow Na current the time for development of block is much longer, and amounts to 10 to 15 ms, i.e. the time during which the clamp is out of control.

Recovery from use-dependent block

Since the block exerted by disopyramide at a given frequency was dependent on membrane potential (Figure 2) and disappeared in the absence of stimulation it is possible to distinguish between two types of recovery: (1) recovery can be studied by changing the membrane holding potential or (2) it can be estimated by lowering the frequency.

Fast recovery from block: activation deblockade Figure 5a and b first confirms the occurrence of a greater block at a holding potential of −80 mV compared to −95 mV (see also Figure 2). In the absence of drug (a) an abrupt decrease of $\dot{V}_{max}$ was seen when the membrane potential was changed from −95 mV to −80 mV. This fall in $\dot{V}_{max}$ is due to inactivation. In the presence of drug (b) and continuous stimulation, a certain degree of block had already developed at −95 mV (time course not shown). When the holding potential was changed to −80 mV, an instantaneous decrease, due to inactivation, was followed by a pronounced time-dependent decrease, due to extra-block.

Recovery from the extra block, when the reverse change in holding potential was applied, is seen in Figure 5d. (Figure 5c illustrates the change in $\dot{V}_{max}$ due to a difference in inactivation, when the same step in potential is applied in the absence of drug.) $\dot{V}_{max}$ reached the steady-state level of the use-dependent block at −95 mV within a few pulses. The time constants determined from two experiments were 194 and 185 ms. This result indicates a fast deblockade of sodium channels during activation.

Slow recovery from block at rest For estimation of the time constant of recovery during rest, repetitive stimulation was interrupted for variable periods at holding potentials in the range −95 to −70 mV. The time course of recovery of $\dot{V}_{max}$ can be described by one exponential and a constant and shows a strong voltage-dependence, in that recovery was markedly slowed at hyperpolarized levels. The time constant of recovery increased from 11.2 ± 1.0 s ($n = 5$) at a

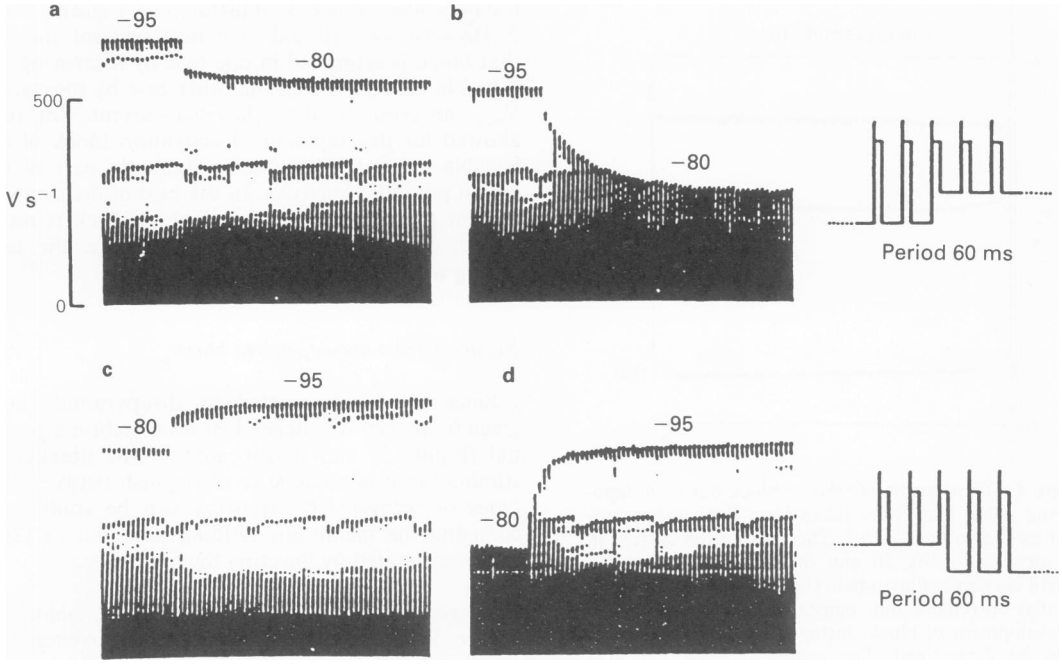

Figure 5 Fast recovery from block by changing the holding potential during continuous stimulation. From the holding potential of −95 (a and b) or −80 mV (c and d) a depolarizing pulse was applied every 60 ms and $\dot{V}_{max}$ measured. Before clamping back to one of these holding potentials the fibre was held at −45 mV up to 10 ms. Original recordings of $\dot{V}_{max}$ are shown under control conditions (a,c) and after exposure to 10 μM disopyramide (b,d). Under control conditions most of the change in $\dot{V}_{max}$ was abrupt; the small, slow changes are due to a process of slow inactivation. When, in the presence of disopyramide, the holding potential was switched from −95 to −80 mV, an abrupt change in $\dot{V}_{max}$ was followed by a gradual decrease to a new steady-state, demonstrating that use-dependent block is more pronounced at −80 than at −95 mV. On returning to the holding potential of −95 mV this extra block disappeared within a few pulses, indicating that sodium channels in their open state facilitate the escape of the drug from the binding site.

membrane potential of −75 mV to 15.5 ± 1.6 s ($n = 9$) at −80 mV, 21.9 ± 1.8 s ($n = 7$) at −85 mV, 45.7 ± 6.7 s ($n = 7$) at −90 mV and finally to 70.8 ± 8.0 s ($n = 4$) at −95 mV. When the membrane was depolarized from −75 to −70 mV ($n = 6$), the time constant of recovery did not decrease further (Figure 6). The semilogarithmic plot of the time constants of recovery as a function of the membrane potential gives a straight line between −75 and −95 mV ($r = 0.9885$, $P < 0.01$) (Figure 7). As demonstrated in one experiment the voltage-dependence of recovery was unaffected by prolongation of the depolarizing pulse from 10 to 500 ms. At a membrane potential of −80 mV the time constant was 20 s and 85 s at a holding potential of −95 mV. A more developed inactivation apparently has no effect on this slow recovery phenomenon.

Assuming that the charged disopyramide molecules are responsible for the slowed recovery of $\dot{V}_{max}$ at hyperpolarized membrane potentials, the pH of the external solution was increased to 8.0 in order to reduce the fraction of charged molecules. At all membrane potentials tested, the amplitude of use-dependent block at pH 8 was slightly more pronounced than at pH 7.4; its time course could be fitted as a mono-exponential. As expected, recovery from use-dependent block was accelerated, the relative change being dependent on the membrane potential. Between −80 and −70 mV time constants were practically constant (9.7 ± 0.7 s at −80 mV, $n = 6$; 9.6 ± 1.1 s at −75 mV, $n = 5$ and 9.04 ± 0.5 s at −70 mV, $n = 4$). Upon hyperpolarization to −85 mV the time constant increased to 12.2 ± 1.6 s ($n = 6$), to 18.1 ± 2.6 s ($n = 5$) at −90 mV and to 28.3 ± 3.0 s ($n = 3$) at −95 mV (Figure 6). The time constant of recovery, semilogarithmically plotted as a function of the membrane potential, followed a linear relation between −80 and −95 mV ($r = 1.007$, $P < 0.01$). On changing the pH of the external solution from 7.4 to 8.0 a shift of that correlation to the

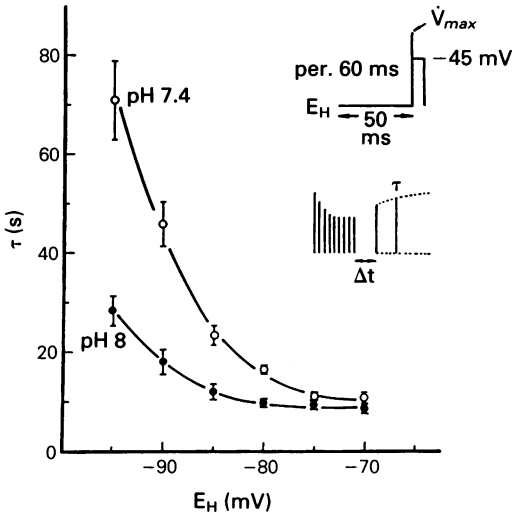

Figure 6 Time course of recovery as a function of the membrane potential and pH. From holding potentials (E_H) of between −70 and −95 mV depolarizing clamp pulses of 10 ms to −45 mV were applied and repeated every 60 ms until steady-state block was obtained. Disopyramide concentration was 10 μM. The time constants for recovery of $\dot{V}_{max}$ (τ) from use-dependent block were estimated by interruption of the repetitive stimulation for varying periods of time. (○) Indicate mean values of the time constants from 4–9 experiments performed at pH 7.4. The time constant is markedly increased when the membrane is hyperpolarized. Therefore, disopyramide can be considered an activation gate trapped agent. This strong voltage-dependence of the time constant of recovery is somewhat reduced and is shifted to the hyperpolarizing direction by 5 mV when the fraction of charged disopyramide molecules is decreased by raising the pH of the bathing solution to pH 8.0, as shown by (●) ($n = 4$–6). Mean values are shown with vertical lines indicating s.e.

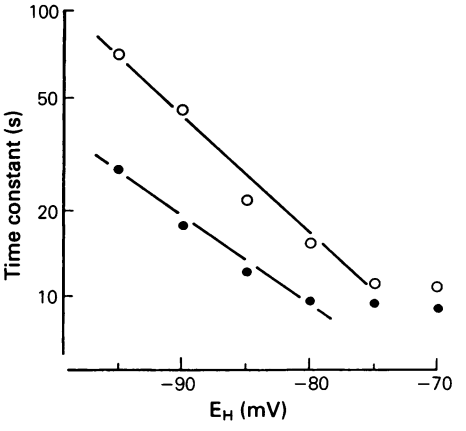

Figure 7 Correlation between time constant of recovery and membrane holding potential. The semi-logarithmic plot of the time constants as a function of the membrane potential gives a straight line in the range −95 to −75 mV at a pH of 7.4 (○, $r = 0.9885$, $P < 0.01$) and for membrane potentials ranging from −95 to −80 mV at a pH of 8.0 (●, $r = 1.007$, $P < 0.01$).

hyperpolarizing direction occurred, and additionally the slope of the straight line decreased somewhat (Figure 7).

Discussion

Blockade of open channels

In the present experiments evidence for blockade of open channels by disopyramide has been provided by demonstrating the existence of use-dependent block and the absence of inactivation block.

The conclusion that disopyramide does not bind preferentially to inactivated channels is based on two arguments: (1) disopyramide (10 μM) did not shift the instantaneous inactivation curve, obtained by applying a depolarizing pulse after a rest period of sufficient duration to ensure complete recovery of $\dot{V}_{max}$ from the effect of the drug. This indicates that disopyramide is no inactivation blocker at that concentration. Misinterpretation of the results due to the non-linear relationship between $\dot{V}_{max}$ and the sodium current (Strichartz & Cohen, 1978; Cohen *et al.*, 1984) is improbable. Fozzard *et al.* (1987) recently pointed out that, at least in single canine cardiac Purkinje cells, overestimation of sodium channel availability by $\dot{V}_{max}$ is less than found for rabbit Purkinje fibres (Cohen *et al.*, 1984). Therefore, we conclude from our results that disopyramide does not block closed sodium channels. (2) A similar conclusion can be reached from experiments in which the duration of a repetitive depolarization was prolonged from 10 ms to 500 ms without causing any important change of the use-dependent block. This conflicts with the data of Kojima *et al.* (1982), based on the increased effect of disopyramide in preparations bathed in low K concentrations when combined with low rates of stimulation (0.1 Hz). They interpreted their results to be due to a promoted inactivation block, caused by a lengthening of the action potential duration in low potassium solution. They did not take into account the marked hyperpolarization, which is accompanied by slow recovery.

Since disopyramide does not affect rested or inactivated sodium channels, the only possibility left is a block of activated sodium channels. A direct measurement of the time constant for binding to activated sodium channels, using the slowly inactivating

current, failed; the blockade of the sodium current was so fast that a steady-state level was already obtained during the period when the clamp was out of control, which is in the range of 10 ms. However, the existence of use-dependent blockade and its change with holding potential offers an indirect way to estimate τ_d, the binding constant for the change in bound drug at the depolarized level. Use-dependent blockade followed an exponential time course and can be characterized by λ, the rate constant for uptake of drug, expressed in pulse^{-1}, since the blockade associated with the n^{th} pulse can be described recursively in terms of blockade acquired during the previous pulse. The rate constant for uptake of the drug decreased for positive potentials (see Table 1). Using the formula

$$\lambda = \frac{t_d}{\tau_d} + \frac{t_r}{\tau_r}$$

(Starmer *et al.*, 1986; equation 6) τ_d can be calculated. The repolarizing interval t_r is defined by the clamp protocol, while recovery time constant τ_r and uptake rate λ were determined experimentally. The mean receptor access time t_d was taken as 1 ms. Then, for membrane potentials between -95 mV and -75 mV, a τ_d was calculated, which varied between 5 and 15 ms. These values should only be taken as an estimation of the order of magnitude.

Assuming this estimation to be valid, it is understandable why the $\dot{V}_{max}$ value of the first action potential following a long period of rest is not affected by the presence of disopyramide (Figure 2). The time between start of the upstroke and time at which $\dot{V}_{max}$ is measured does not allow sufficient channels to be blocked. On the other hand it also becomes understandable why most of the block was already present when the slow Na current was measured directly (Figures 3 and 4): a quantitative measurement, without interference of escape artefacts, was only possible after 10–15 ms.

Recovery from block

Block by antiarrhythmic drugs is generally assumed to be relieved by hyperpolarization (Hondeghem & Katzung, 1977; 1984), as has been demonstrated in the case of lidocaine (Bean *et al.*, 1983) and quinidine (Weld *et al.*, 1982). However our results show a completely different behaviour for disopyramide: (1) When recovery measured during rest and time constants were compared at different membrane potentials, recovery was found to be markedly slowed at hyperpolarized levels. (2) When recovery was measured during a pulse train by changing the holding potential from -80 to -95 mV, recovery was fast (in the order of 0.1–0.2 s instead of tens of seconds). Following the quasi-instantaneous recovery from inactivation of the non-blocked channels, $\dot{V}_{max}$ increased in an exponential way and reached steady-state within a few depolarizing pulses. Not only blockade, but also deblockade is apparently facilitated by opening of the sodium channels. Such results support the activation gate trapping hypothesis (Yeh & Tanguy, 1985; Starmer *et al.*, 1986). The activation gate trapping hypothesis suggests that hydrophilic charged agents bind to the channel during the depolarizing phase and are trapped within the channel by the activation gate. The trapped molecules can only escape from the channel when the channel is open. This can explain slower recovery at hyperpolarized membrane potentials, since the probability of channel random openings decreases with hyperpolarization. Voltage-dependent recovery kinetics of disopyramide (present paper) and its congener penticainide (Gruber & Carmeliet, 1987; Carmeliet, 1988b) are comparable to that of a quaternary compound or 9-aminoacridine, a molecule which deprotonates very slowly (Yeh & Tanguy, 1985). The slow time constant of recovery is not only a function of the membrane potential (Starmer *et al.*, 1986), but also of the pK_a of the drug and the pH of the external solution (Hille, 1977a; Schwarz *et al.*, 1977). Our results support these assumptions in that recovery of $\dot{V}_{max}$ is accelerated when the pH of the external solution is changed from 7.4 to 8.0, since the fraction of charged molecules will be reduced. However, the acceleration of the time course of recovery is notably greater at membrane potentials more negative than -80 mV. This indicates that, for disopyramide, unblocking predominantly occurs via the hydrophilic pathway as suggested for quaternary drugs.

According to the size-solubility hypothesis (Courtney, 1980) large tertiary compounds, thus having a small unblocking rate via the hydrophobic pathway, or tertiary compounds with high pK_as, thus providing larger protonation rates and/or smaller deprotonation rates, may behave like permanently charged molecules. Starmer & Courtney (1986) assume a higher pK_a value of the drug in the channel than in free solution. They argue that local potential fields near the drug binding site may influence the protonation-deprotonation reactions and that the relation of the protonation site to the proton access path may influence the rates, too. As regards the size of the molecule, disopyramide resembles quinidine, and its pK_a of 8.36 is even lower than that of quinidine which has a pK_a of 10.0 (besides one of 5.4). Quinidine, however, does not show voltage-dependence of recovery like disopyramide (unpublished observation). Weld *et al.* (1982) even found that the rate constant for dissociation of quinidine from quinidine-blocked channels becomes larger as the transmembrane voltage

becomes more negative; so quinidine behaves in the opposite way to disopyramide in respect to voltage-dependent recovery from use-dependent block of $\dot{V}_{max}$. These findings lead us to conclude that other determining factors may be important, such as stereochemical properties of a molecule.

If activation block and activation trapping were the only factors determining use-dependent block, this block should be most pronounced when applying depolarizations from very negative holding potentials: the number of available Na channels for activation is greater and recovery rate is slower. The use-dependent block as a function of holding potential (Figure 2), however, was quite different and showed a maximum block for intermediate potentials. We suggest that a voltage-shift to negative potentials of blocked inactivated channels may be responsible for this behaviour. Such a voltage shift results in more block at less negative potentials. The peculiar voltage relationship illustrated in Figure 2 could thus be explained as follows: (1) at very negative potentials the block is solely determined by activation trapping, (2) at intermediate potentials the block is greater because it consists of rested channels, in which the drug is trapped, and inactivated blocked channels. (3) At less negative potentials the block is again smaller because activation and therefore the possibility of the drug entering and blocking a channel is reduced.

From the therapeutical point of view slowed recovery from use-dependent block at membrane potentials more negative than -75 mV may not be advantageous. However, this is partly compensated for by the smaller amount of use-dependent block at these potentials. Furthermore it should be realized that changes in block (increase or decrease) induced by a change in membrane potential are rather fast phenomena when the preparation is stimulated.

In conclusion, disopyramide is thought to be an activation blocker and an activation gate trapped agent.

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Methylene blue but not changes in cyclic GMP inhibits resting and bradykinin-stimulated production of prostacyclin by pig aortic endothelial cells

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1 Primary cultures of pig aortic endothelial cells produced 6-keto-prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$), the stable breakdown product of prostacyclin, both in the resting state and in response to bradykinin. The rise in 6-keto-$PGF_{1\alpha}$ production induced by bradykinin (1–100 nM) was concentration-dependent.

2 Treating endothelial cells with the inhibitor of soluble guanylate cyclase, methylene blue (0.1–20 μM) produced an irreversible reduction in resting and bradykinin (0.1 μM)-stimulated production of 6-keto-$PGF_{1\alpha}$ with an IC_{50} of 0.5 ± 0.1 μM. Treating endothelial cells with haemoglobin (10 μM) had no effect on resting or bradykinin (0.1 μM)-stimulated production of 6-keto-$PGF_{1\alpha}$.

3 Two stimuli that elevate the level of guanosine 3':5'-cyclic monophosphate (cyclic GMP) in endothelial cells, 8-bromo cyclic GMP (30 μM) and atriopeptin II (0.1 μM), each had no effect on resting or bradykinin (0.1 μM)-stimulated production of 6-keto-$PGF_{1\alpha}$. Furthermore, treating endothelial cells with either 8-bromo cyclic GMP (30 μM) or atriopeptin II (0.1 μM) had no effect on the ability of methylene blue (20 μM) to inhibit resting or bradykinin (0.1 μM)-stimulated production of 6-keto-$PGF_{1\alpha}$.

4 Adding arachidonic acid (1 μM) to endothelial cells led to a marked stimulation of 6-keto-$PGF_{1\alpha}$ production. Treating cells with either methylene blue (20 μM) or the cyclo-oxygenase inhibitor, flurbiprofen (10 μM), inhibited both resting and arachidonic acid (1 μM)-induced production of 6-keto-$PGF_{1\alpha}$.

5 In pig aortic endothelial cells methylene blue appears to block prostacyclin production by a mechanism independent of inhibition of soluble guanylate cyclase. Care should be exercised when using methylene blue as a selective inhibitor of endothelium-derived relaxing factor due to its additional ability to block production of the other endothelium-derived vasodilator, prostacyclin.

Introduction

The vascular endothelium regulates vasomotor tone by producing two powerful vasodilators, namely prostacyclin (Moncada *et al.*, 1976), and endothelium-derived relaxing factor (EDRF; Furchgott & Zawadzki, 1980), which has recently been identified as nitric oxide (Palmer *et al.*, 1987; Furchgott, 1988; Ignarro *et al.*, 1988). These two vasodilators produce their effects by different mechanisms: prostacyclin by activating adenylate cyclase (Dembinska-Kiec *et al.*, 1979), and EDRF by activating soluble guanylate cyclase (Förstermann *et al.*, 1986). The actions of EDRF are blocked by methylene blue, which inhibits soluble guanylate cyclase, and haemoglobin, which binds and inactivates EDRF (Holzmann, 1982; Martin *et al.*, 1985). Methylene blue and haemoglobin show a high degree of selectivity in that the actions of vasodilators which activate soluble guanylate cyclase, e.g. EDRF and nitrovasodilators are blocked. Actions produced via different pathways, e.g. isoprenaline, are not (Martin *et al.*, 1985). Methylene blue and haemoglobin can therefore be used as analytical tools to investigate the role of EDRF in endothelium-mediated vasodilator responses. It is not known whether methylene blue or haemoglobin can interfere with the actions of the other major endothelium-derived vasodilator, prostacyclin. The purpose of this study was to determine whether

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methylene blue or haemoglobin influenced resting and bradykinin-stimulated production of prostacyclin by primary cultures of pig aortic endothelial cells. A preliminary account of these findings has already been published (Martin & Drazan, 1987).

Methods

Endothelial cell culture

Pig aortae were obtained from a local abattoir. Immediately after removal from the pig (within 10 min of death), the aorta was flushed with sterile saline containing benzyl penicillin (100 u ml^{-1}) and streptomycin (100 $\mu g\,ml^{-1}$), tied off at one end and cannulated at the other with a 60 ml syringe containing the same saline solution at ambient temperature. Saline was infused into the lumen before the vessel was transported to the laboratory within 30 min. The endothelial cells were then isolated as previously described (Gordon & Martin, 1983a). Briefly, after ligating the intercostal arteries, collagenase (0.2%, type II, Sigma) was introduced into the aortic lumen and the vessel was incubated at 37°C for 20 min. The endothelial cells from each aorta were then harvested in 60 ml of Dulbecco's Modification of Eagle's Medium supplemented with: foetal bovine serum (10%), newborn bovine serum (10%), glutamine (4 mM), benzyl penicillin (100 u ml^{-1}), streptomycin (100 $\mu g\,ml^{-1}$) and kanamycin (100 $\mu g\,ml^{-1}$) and were seeded into 3 Linbro 6 well (9.6 cm^2) plates and grown to confluence. The culture medium was replaced every 2 days and cells were used within 3–7 days. All tissue culture media and supplements were obtained from Flow Laboratories, Irvine, Scotland, except for benzyl penicillin (crystapen; Glaxo) and streptomycin sulphate (Evans Medical Ltd.).

The cells were characterized as endothelial cells by the following criteria: they grew as a strict monolayer and produced prostacyclin; in randomly selected cultures, no fewer than 98% of the cells were observed to fluoresce following incubation with acetylated low-density lipoprotein labelled with 1,1'dioctadecyl - 1 - 3,3',3' - tetramethyl - indocarbo - cyanine-perchlorate (CMD UK Ltd.; Voyta *et al.*, 1984).

Measurement of prostacyclin

Prostacyclin was measured as previously described (Martin *et al.*, 1988). Briefly, following removal of the tissue culture medium, the endothelial cells were washed with 2 × 2 ml of Krebs solution containing (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24, glucose 11 and Na_2 EDTA 0.03 and then incubated in 2 ml of Krebs solution at 37°C under an atmosphere of 5% CO_2 in air. After 60 min the bathing solution was removed and replaced with fresh Krebs solution. After a further 30 min, the Krebs solution was recovered and the cells immediately extracted with 2 × 0.5 ml volumes of 6% trichloroacetic acid (TCA). The prostacyclin content of the Krebs solution was determined by radioimmunoassay of the stable breakdown product, 6-keto-prostaglandin $F_{1\alpha}$(6-keto-$PGF_{1\alpha}$). The DNA content of the cell pellet was determined by the fluorescence technique of Kissane & Robins (1958). Prostacyclin production is expressed as ng 6-keto-$PGF_{1\alpha}\,\mu g^{-1}$ DNA.

Drugs

Arachidonic acid, bradykinin triacetate, 8-bromo guanosine 3':5'-cyclic monophosphate (8-bromo cyclic GMP), haemoglobin (bovine Type 1) and methylene blue were obtained from Sigma. Sodium flurbiprofen was a generous gift from Dr R.V. Holland, Boots Pure Drug Co. All drugs were dissolved in twice-distilled water except for arachidonic acid which was dissolved in ethanol.

Solutions of haemoglobin were reduced to the ferrous form with dithionite before use (Martin *et al.*, 1985).

Statistical analysis

Results are expressed as the mean ± s.e.mean and comparisons were made by means of Student's *t* test. A probability of 0.05 or less was considered significant.

Results

Resting and bradykinin-stimulated production of prostacyclin

Prostacyclin production (measured as 6-keto-$PGF_{1\alpha}$) was determined in the 30 min period immediately following replacement of the Krebs solution bathing primary cultures of pig aortic endothelial cells. Within 3 min the amount of 6-keto-$PGF_{1\alpha}$ produced was 245 ± 31 pg μg^{-1} DNA ($n = 6$) and by the end of the 30 min period this had risen significantly ($P < 0.05$) to 470 ± 88 pg μg^{-1} DNA ($n = 12$).

Adding bradykinin (1–100 nM) to the endothelial cells for the final 5 min of the 30 min incubation period led to a concentration-dependent increase in 6-keto-$PGF_{1\alpha}$ production (Figure 1). The stimulation of 6-keto-$PGF_{1\alpha}$ production induced by a standard concentration of bradykinin (0.1 μM) was complete within this 5 min period (data not shown). A maximum stimulation of 6-keto-$PGF_{1\alpha}$ pro-

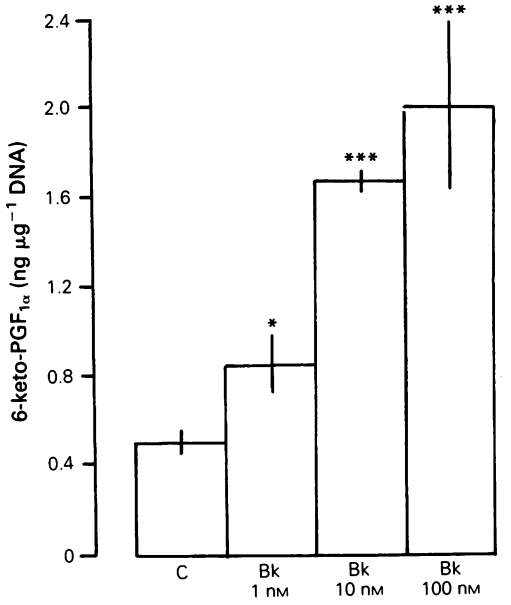

Figure 1 Resting and bradykinin (Bk)-stimulated production of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) by pig aortic endothelial cells. The resting production in a 30 min incubation period (C) is shown together with the production obtained following addition of bradykinin (1–100 nM) for the final 5 min of the 30 min incubation period. Results are presented as the mean obtained from 6–12 experiments, vertical lines show the s.e.mean. $*P < 0.05$; $***P < 0.001$, denotes a significant difference from control resting production.

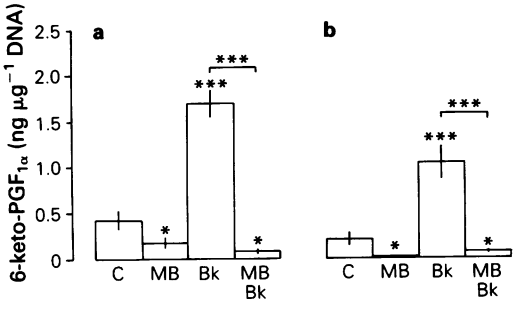

Figure 2 (a) The ability of methylene blue (MB, 20 μM) to inhibit resting and bradykinin (Bk, 0.1 μM)-stimulated production of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) by pig aortic endothelial cells. (b) Following washout of methylene blue, resting and bradykinin-stimulated production of 6-keto-$PGF_{1\alpha}$ were still inhibited. Results are presented as the mean of 5–17 experiments, vertical lines show the s.e.mean. $*P < 0.05$; $***P < 0.001$, indicates a significant difference from control resting production (C), or a significant difference between two groups joined by a bar.

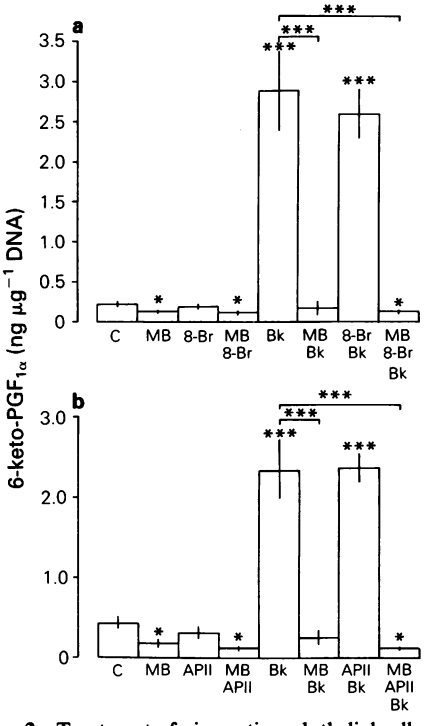

Figure 3 Treatment of pig aortic endothelial cells with (a) 8-bromo cyclic GMP (8-Br, 30 μM) or (b) atriopeptin II (APII, 0.1 μM) had no effect on resting or bradykinin (Bk, 0.1 μM)-stimulated production of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$). Furthermore, 8-bromo cyclic GMP or atriopeptin II had no effect on the ability of methylene blue (MB, 20 μM) to inhibit resting or bradykinin-stimulated production of 6-keto-$PGF_{1\alpha}$. Results are presented as the mean of 5–9 experiments; vertical lines show the s.e.mean. $*P < 0.05$; $***P < 0.001$, indicates a significant difference from control resting production (C), or a significant difference between two groups joined by a bar.

duction of 4.3 fold, over control ($n = 9$) was obtained.

Effects of methylene blue and haemoglobin

An inhibitor of soluble guanylate cyclase, methylene blue (20 μM), when present throughout the 30 min incubation period, inhibited both resting and bradykinin-stimulated production of 6-keto-$PGF_{1\alpha}$ (Figure 2). Removal of methylene blue from the cells by washing did not reverse this inhibition (Figure 2).

The blockade of bradykinin-stimulated production of 6-keto-$PGF_{1\alpha}$ by methyline blue was concentration-dependent with an IC_{50} value of 0.5 ± 0.1 μM ($n = 6$).

Haemoglobin (10 μM), when present throughout the 30 min incubation period had no effect on resting or bradykinin-stimulated production of 6-keto-$PGF_{1\alpha}$ by endothelial cells (data not shown).

Effects of 8-bromo cyclic GMP and atriopeptin II

The membrane permeant analogue of cyclic GMP, 8-bromo cyclic GMP (30 μM), when added to endothelial cells for the final 20 min of the 30 min incubation period or atriopeptin II (0.1 μM), a stimulant of particulate guanylate cyclase, when added for the final 10 min of the 30 min incubation period, had no effect on resting or bradykinin-stimulated production of 6-keto-$PGF_{1\alpha}$, and did not reverse the methylene blue-induced inhibition of 6-keto-$PGF_{1\alpha}$ production (Figure 3).

Effects of methylene blue and flurbiprofen on arachidonic acid-induced production of 6-keto-$PGF_{1\alpha}$

On two separate occasions arachidonic acid (1 μM), when added to endothelial cells for the final 5 min of the 30 min incubation period, increased production of 6-keto-$PGF_{1\alpha}$ 17.6 fold ($n = 6$) and 20.6 fold, ($n = 6$, Figure 4).

Endothelial cells were exposed to either methylene blue (20 μM), or the cylco-oxygenase inhibitor flurbiprofen (10 μM) for 60 min. When the Krebs solution and drugs were removed and replaced following a wash, both resting and arachidonic acid-stimulated production of 6-keto-$PGF_{1\alpha}$ were found to be inhibited (Figure 4).

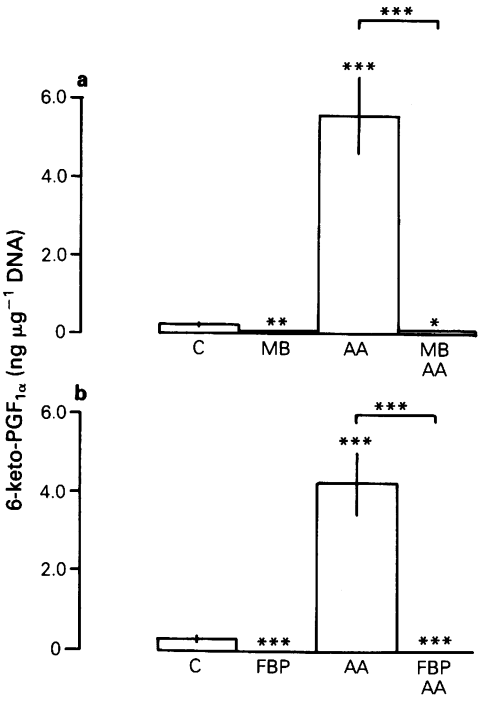

Figure 4 Arachidonic acid (AA, 1 μM) stimulated the production of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) by pig aortic endothelial cells. Treatment of cells with either (a) methylene blue (MB, 20 μM) or (b) the cyclo-oxygenase inhibitor, flurbiprofen (FBP, 10 μM), inhibited both resting and arachidonic acid-stimulated production of 6-keto-$PGF_{1\alpha}$. Results are presented as the mean of 5–8 experiments, vertical lines show the s.e.mean. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, indicates a significant difference from control resting production (C), or a significant difference between two groups joined by a bar.

Discussion

In this study 6-keto-$PGF_{1\alpha}$, the stable hydrolysis product of prostacyclin, was continuously released into the Krebs solution bathing primary cultures of pig aortic endothelial cells. The rate of production was highest immediately after replacing the Krebs and continued at a lower level thereafter. This initial high rate of production reflects mechanical stimulation of the cells induced by changing the Krebs solution (Pearson *et al.*, 1983), probably through activation of mechanoreceptors linked to calcium influx (Lansman *et al.*, 1987). In addition, bradykinin produced a concentration-dependent stimulation of prostacyclin production in accordance with previous findings (Hong, 1980; Gordon & Martin, 1983b).

Methylene blue, an inhibitor of soluble guanylate cyclase is known to block the actions of endothelium-derived relaxing factor (EDRF) and nitrovasodilators (Holzmann, 1982; Martin *et al.*, 1985). We have now found that methylene blue can, in addition, irreversibly inhibit both basal and bradykinin-stimulated production of prostacyclin. The concentration of methylene blue required to inhibit prostacyclin production is comparable or slightly less than that required to inhibit EDRF-induced vasodilatation.

Although methylene blue does lower the cyclic GMP content of pig aortic endothelial cells, it is unlikely that the blockade of prostacyclin production results from inhibition of soluble guanylate cyclase since haemoglobin, which also lowers endothelial cyclic GMP content (Martin *et al.*, 1988), has no effect on basal or bradykinin-stimulated production of prostacyclin. Furthermore, if inhibition of soluble guanylate cyclase were responsible for the blockade of prostacyclin production, raising endothelial cyclic GMP content should reverse the blockade. 8-bromo cyclic GMP, a membrane permeant analogue of cyclic GMP, or atriopeptin II, a stimu-

lant of the particulate form of guanylate cyclase, which markedly elevates endothelial cyclic GMP content in a methylene blue-insensitive manner (Adams-Brotherton, 1986; Martin *et al.*, 1988), did not reverse the methylene blue-induced inhibition of basal or stimulated prostacyclin production.

The actions of methylene blue on prostacyclin production could be due to interference with receptor coupling mechanisms that control release of arachidonic acid from membrane phospholipid, or inhibition of an enzyme involved in the synthesis of prostacyclin. Since in methylene blue treated endothelial cells the ability to form prostacyclin from exogenously added arachidonic acid is lost, its actions are similar to those of the cyclo-oxygenase inhibitor, flurbiprofen. Whether methylene blue inhibits production of prostacyclin at the level of cyclo-oxygenase or some other enzymatic step could not be determined in our experiments. Interestingly, it is believed that methylene blue inhibits soluble guanylate cyclase by oxidising the ferrous haem moiety of the enzyme (Gruetter *et al.*, 1979). The two components of prostaglandin endoperoxide synthetase, i.e. cyclo-oxygenase, which catalyzes the oxidation of arachidonic acid to PGG_2, and prostaglandin hydroperoxidase, which reduces PGG_2 to PGH_2, each require a haem moiety for activity (Ueno *et al.*, 1982). The possibility that methylene blue blocks prostacyclin production by interfering with the haem moiety of these enzymes warrants further investigation.

In a recent study (Doni *et al.*, 1988), it has been shown that nitric oxide, identified as EDRF (Palmer *et al.*, 1987; Furchgott, 1988), inhibits prostacyclin production by bovine aortic endothelial cells. The degree of inhibition was small (30%) and was seen only with concentrations of nitric oxide that were greatly in excess of those required to relax vascular smooth muscle. The additional finding that the selective cyclic GMP phosphodiesterase inhibitor, M & B 22,948, potentiated the inhibitory actions of nitric oxide was taken as evidence for the involvement of cyclic GMP in the inhibition of prostacyclin production. According to this scheme we would expect that lowering or elevating endothelial cyclic GMP content should increase or decrease, respectively, prostacyclin production. We found, however, that haemoglobin, which decreases endothelial cell cyclic GMP content, and atriopeptin II or 8 bromo cyclic GMP, which elevates cyclic GMP content (Martin *et al.*, 1988), had no effect on either basal or bradykinin-stimulated production of prostacyclin. Our data, in common with several other studies (Levin *et al.*, 1982; De Caterina *et al.*, 1985; Adams-Brotherton, 1986), therefore provide no evidence for a role of cyclic GMP in controlling endothelial prostacyclin production.

In conclusion, the commonly used EDRF inhibitor, methylene blue, has been shown to inhibit the production of the other major endothelium-derived vasodilator, prostacyclin. This inhibitory action of methylene blue appears to be unrelated to its ability to inhibit soluble guanylate cyclase, but is probably due to inhibition of an enzyme involved in the biosynthesis of prostacyclin. Thus, methylene blue can block two mechanisms by which the endothelium elicits vasodilatation, and we would therefore caution against its use as a selective inhibitor of EDRF due to this additional ability to block prostacyclin production.

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Inhibition by glibenclamide of the vasorelaxant action of cromakalim in the rat

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1 In rat isolated thoracic aortic rings pre-contracted with noradrenaline (10^{-6} M), cromakalim (3×10^{-7}–3×10^{-5} M) produced concentration-related relaxation. This effect was progressively inhibited by increasing concentrations of the anti-diabetic sulphonylurea drug, glibenclamide (10^{-6}–10^{-5} M).

2 In rat isolated portal veins, cromakalim (3×10^{-8}–10^{-6} M) produced concentration-related inhibition of the spontaneous contractive activity and glibenclamide (3×10^{-7}–3×10^{-6} M) prevented this inhibitory action in a concentration-dependent manner.

3 In both rat aortic rings and portal veins, cromakalim (10^{-5} M) stimulated ^{86}Rb efflux. Prior exposure to glibenclamide (10^{-7}–10^{-6} M) produced a concentration-related inhibition of this response.

4 In conscious rats, cromakalim, 0.075 mg kg^{-1} i.v., produced a rapid and sustained fall in arterial blood pressure which was not influenced by pretreatment (2 h) with a large oral dose of glibenclamide (100 mg kg^{-1}).

5 In conscious rats, the hypotensive action of cromakalim, 0.075 mg kg^{-1} i.v., was abolished by pretreatment (30 min) with glibenclamide, 20 mg kg^{-1}, given by the intravenous route.

6 The results suggest that the vasorelaxant and hypotensive actions of cromakalim involve a K^+ channel which can be inhibited by glibenclamide, but which may be distinct from the ATP-sensitive K^+ channel of the pancreatic β-cell.

Introduction

Cromakalim (BRL 34915) is a potent blood pressure lowering agent in laboratory animals (Buckingham *et al.*, 1986) and man (VandenBurg *et al.*, 1986; 1987; Eckl & Greb, 1987). It is thought that the ability of cromakalim to open smooth muscle cell membrane K^+ channels underlies its relaxant effect not only in rabbit, rat and guinea-pig isolated blood vessels (Coldwell & Howlett, 1986; Hamilton *et al.*, 1986; Weir & Weston, 1986; Quast, 1987), but also in rat isolated uterus (Hollingsworth *et al.*, 1987), guinea-pig trachealis (Allen *et al.*, 1986) and guinea-pig and pig urinary bladder (Foster & Brading, 1987).

In rat pancreatic islet cells the antidiabetic sulphonylurea, tolbutamide, reduces ^{86}Rb efflux (used as a tracer for K^+) (Henquin, 1980) and this action causes depolarization (Henquin & Meissner, 1982), a rise in the cytosolic free Ca^{2+} concentration (Abrahamsson *et al.*, 1985) and insulin secretion (see review by Gylfe *et al.*, 1984). Glucose is thought to stimulate insulin release by a similar mechanism (Atwater *et al.*, 1978; Ashcroft *et al.*, 1984). The glucose-sensitive pancreatic K^+ channel and the K^+ channel blocked by glibenclamide, a more potent sulphonylurea than tolbutamide, have both been shown to be regulated by intracellular ATP (Cook & Hales, 1984; Rorsman & Trube, 1985; Schmid-Antomarchi *et al.*, 1987). These results suggest that glucose and the sulphonylureas may act on the same membrane K^+ channel in pancreatic islet cells.

In the present paper we have tested the possibility that glibenclamide acts as an antagonist of the vasorelaxant action of cromakalim both *in vitro* and *in vivo*. A preliminary account of some of these findings has been communicated to the British Pharmacological Society (Wilson *et al.*, 1988).

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Methods

Male Sprague Dawley rats were used in all the experiments described below.

Isolated tissue experiments: tension studies

The thoracic aorta was removed rapidly from rats (200–300 g) and cut into 4 rings, each 3–4 mm in width. Two stainless steel wire hooks were passed through the lumen of each ring. One wire was attached to the base of a 10 ml tissue bath and the second to an isometric tension transducer (Dynamometer UFI). Tissues were bathed in Krebs-Henseleit solution of the following composition (mM): NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and D-glucose 10.1. The bathing solution was warmed to 37°C and gassed with 5% CO_2 in O_2(pH = 7.3).

For other experiments, portal veins were removed and suspended, by means of cotton ties, in 10 ml tissue baths containing Krebs-Henseleit solution as described above.

An initial tension of 2 g was applied to aortic rings and 1 g to portal veins; these tensions were then reset after 1 h and kept constant thereafter. The tissues were allowed to equilibrate for 2 h with exchange of bathing fluid by overflow every 20 min. Isometric tension was recorded on a Lectromed 4-channel system. The spontaneous contractile activity of the portal veins was quantified using integrators (Beecham Pharmaceuticals Research Division Instrument Services) working on a 1 min time cycle.

At the end of the equilibration period in rat aorta, a known maximally effective concentration of noradrenaline (10^{-6} M) was added to each bath and the tissue contractions allowed to reach a plateau. This was obtained within 10–15 min of the addition of noradrenaline. Cromakalim was then added, in volumes of 70–200 μl in a cumulative manner, in half log unit increments commencing at 10^{-7} M. The relaxation response to each concentration was allowed to reach a plateau before the next addition was made. Relaxation responses were usually complete within 10 min. When consecutive additions of cromakalim produced no further relaxation, the tissues were washed every 5 min over a 45 min period, during which the baseline was always restored. Subsequently, the rings were contracted once more with noradrenaline and, when a plateau was reached, glibenclamide (10^{-6}, 3×10^{-6} or 10^{-5} M) or an equivalent volume of vehicle (dimethylsulphoxide, DMSO) was added and allowed to equilibrate for 15 min before the cromakalim concentration-relaxation curve was repeated.

In rat portal vein the vasorelaxant action of cromakalim was studied in terms of its ability to inhibit spontaneous phasic activity. In these experiments, cumulative additions of cromakalim were made at 20 min intervals until a maximum inhibitory effect was produced. Washing the tissues every 5 min over a 45 min period then restored phasic contractile activity, and the cumulative concentration-inhibitory effect curve for cromakalim was repeated following 15 min incubation with glibenclamide (3×10^{-7}, 10^{-6} or 3×10^{-6} M) or vehicle, as described above.

The effects of cromakalim on aortic rings have been expressed as a percentage of the maximum possible relaxation i.e. the return of tension to the pre-noradrenaline level. Effects on portal vein have been expressed as a percentage reduction in the mean integrated response (the mean of the last 6 integrals in the 20 min period following each drug addition). Arithmetic mean peak responses (±s.e.mean) and geometric mean IC_{40} (aortic rings) or IC_{50} values (portal veins), together with 95% confidence limits, were calculated from individual experiments. The effects of glibenclamide have been quantified by calculating concentration-ratios at the IC_{40} or IC_{50} level.

Isolated tissue experiments: ^{86}Rb efflux studies

For ^{86}Rb efflux studies, hepatic portal veins and segments of thoracic aorta (each 10–20 mg wet weight) were removed rapidly from rats (200–350 g) and suspended in a tissue bath containing 200 ml of HEPES buffer which was gassed with 5% CO_2 in O_2 at 37°C (pH = 7.4). The composition of the HEPES medium was as follows (mM): NaCl 120, KCl 6.0, $CaCl_2$ 2.5, $MgCl_2$ 1.2, HEPES 5.0, and D-glucose 11.4. After 30 min, 100–200 μCi ^{86}Rb (1–6 mCi mg^{-1}) was added to the bath and the tissues allowed to equilibriate for 90 min. Each tissue was then transferred, at 3 min intervals, through a series of plastic vials containing 3 ml of aerated buffer but no radioactivity. Cromakalim (10^{-5} M) was present from the 30th to the 48th min of this efflux period. Experiments were conducted in the absence and in the continuous presence of three concentrations of glibenclamide (10^{-7}, 3×10^{-7} and 10^{-6} M). The radioactive content of the vials was determined by liquid scintillation counting. Results have been expressed as rate coefficients which were calculated as the ^{86}Rb released during each 3 min period as a percentage of the mean tissue ^{86}Rb remaining during that period. The average efflux rate between the 21st and 30th min of the efflux period was taken as the basal rate. The maximum stimulation of ^{86}Rb efflux in the presence of cromakalim was expressed as a percentage of the basal efflux rate.

Conscious rat studies

Rats (315–385 g) were anaesthetized with pentobarbitone sodium, 60 mg kg^{-1} i.p., and polyethylene catheters filled with 5% w/v dextrose solution, were implanted in the aorta and vena cava (Buckingham, 1976). Following recovery, the animals were housed singly in a quiet room and used for experiment on the second post-operative day. Food was removed on the evening prior to the experiments. On each study day, polyethylene tube connectors containing 5% w/v dextrose solution were used to link the arterial catheter to a Bell and Howell physiological pressure transducer (type 4-422-0001) and the venous catheter to a 3-way tap. The arterial connector and catheter were heparinsed (250 u ml^{-1}) to prevent blood clotting. Recordings of mean arterial blood pressure (MAP; 1 mmHg $\approx$ 133 Pa) and heart rate from 8 rats were displayed simultaneously and continuously on Ormed multi-channel recorders, heart rate being triggered by the arterial pulse wave.

Rats were assigned to two matching groups on the basis of their baseline mean arterial pressures, estimated after an acclimatisation period of 2–3 h. When recordings of blood pressure and heart rate were stable, animals were treated with either glibenclamide, 100 mg kg^{-1} orally, or its vehicle (water), 25 ml kg^{-1} orally (time 0). Venous catheters were then connected to an infusion pump (Harvard Apparatus, Compact Infusion Pump, Type 975A) and a 5% w/v dextrose infusion was started (0.55 mg min^{-1}; 0.65 ml h^{-1}). The infusion was interrupted briefly after 75 min when timolol, 1 mg kg^{-1} i.v., was injected, and again at 120 min when all rats also received cromakalim, 0.075 mg kg^{-1} i.v.

In a subsequent experiment, the effect of intravenously administered glibenclamide on the hypotensive response to cromakalim was studied. In this experiment timolol, 2 mg kg^{-1} i.v., was injected at time 0, immediately before starting the 5% w/v dextrose infusion. The intravenous infusion was stopped briefly at 30 min, to allow injection of glibenclamide, 20 mg kg^{-1}, or its vehicle, 5 ml kg^{-1} (see 'Drugs used' section), and again at 60 min when cromakalim, 0.075 mg kg^{-1} i.v., was given to all rats.

In both of these studies in conscious rats, blood pressure and heart rate were recorded for a further 2 h after the injection of cromakalim.

Statistical evaluation of results

For the tension experiments in isolated blood vessels the expression of results has been dealt with under an earlier heading in this section. Results in the ^{86}Rb efflux experiments and in the conscious rat studies have been expressed as group mean data ($\pm$s.e.mean). Statistical evaluation of the results was made using Student's t test for unpaired data. Probability values (P) of less than 0.05 were taken to indicate statistical significance.

Drugs used

Cromakalim was synthesized in these laboratories. Glibenclamide and timolol maleate were gifts from Roussel Laboratories and Merck, Sharp and Dohme, respectively. (−)-Noradrenaline bitartrate was purchased from Sigma.

For the tension experiments a cromakalim stock solution (10^{-2} M) was prepared in 50% v/v polyethylene glycol in water. (−)-Noradrenaline (10^{-2} M) was prepared in 0.1 M HCl to prevent oxidation. All dilutions were made in distilled water. Glibenclamide (10^{-3} M) was dissolved in and diluted with DMSO. All concentrations in the text refer to the concentration in the tissue bath.

For ^{86}Rb efflux studies, stock solutions of cromakalim and glibenclamide were prepared in ethanol (5×10^{-3} M). Subsequent dilutions of both solutions were in HEPES buffer.

For conscious rat studies, both timolol maleate and cromakalim were dissolved in sterile 0.9% w/v saline. The doses of timolol used in these experiments (1 or 2 mg kg^{-1}) are expressed as free base. Glibenclamide was suspended in water for oral administration. For intravenous administration, glibenclamide was sonicated in 1 M NaOH then diluted 1 in 40 with 5% dextrose.

Results

Isolated tissue experiments: tension studies

Aortic rings Cromakalim (3×10^{-7}–3×10^{-5} M) produced concentration-related relaxation of rat isolated aortic rings pre-contracted with noradrenaline (10^{-6} M) (Figure 1), with a mean ($\pm$s.e.mean) maximum response from the four experiments (i.e. $n = 20$) of 79.0 $\pm$ 2.3%. In the control experiment (Figure 1a), the tissues exhibited an increased sensitivity to cromakalim in the second concentration-response curve (IC_{40} = 4.4 [2.4–7.9] $\times 10^{-7}$ M compared with 7.5 [4.9–11.0] $\times 10^{-7}$ M) to yield a concentration ratio of 0.62. This value, therefore, was used in the calculation of concentration-ratios in the experiments with glibenclamide.

Addition of glibenclamide (10^{-6}–10^{-5} M) to noradrenaline-contracted aortic rings produced no change in tension. However, glibenclamide produced concentration-related displacements to the right of the cromakalim concentration-response curve (Figure 1b, c, d). The IC_{40} values for cromakalim were 2.7 [1.8–4.0] $\times 10^{-6}$ M, 1.1 [0.7–1.7] $\times 10^{-5}$ M

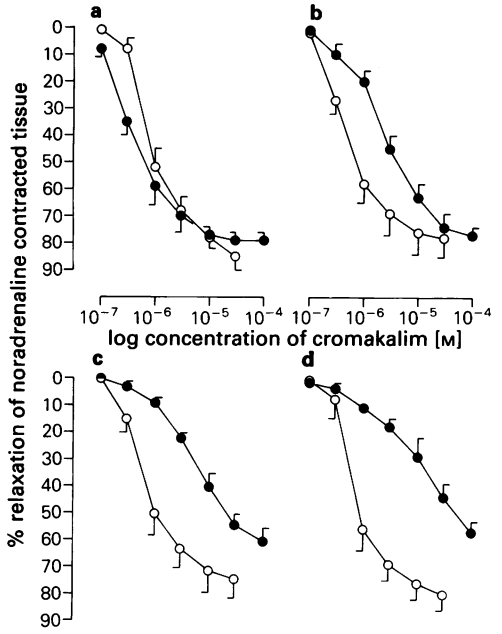

Figure 1 Demonstration of the antagonism of the vasorelaxant effect of cromakalim by glibenclamide in rat isolated aorta. In each of the four panels (○) depict the % relaxation of tissues pre-contracted with noradrenaline (10^{-6} M) produced by increasing concentrations of cromakalim; (●) depict a second cromakalim concentration-relaxation response in the presence of (a) glibenclamide vehicle, and in (b), (c) and (d), increasing concentrations of glibenclamide (10^{-6}, 3×10^{-6} and 10^{-5} M, respectively; $n = 5$).

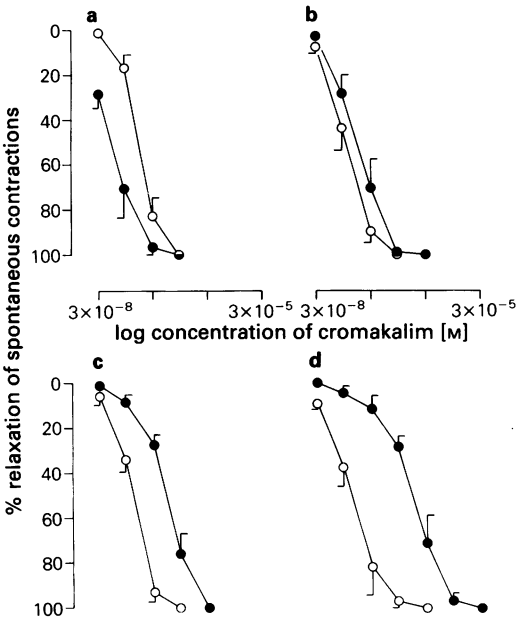

Figure 2 Demonstration of the antagonism of the vasorelaxant effect of cromakalim by glibenclamide in rat isolated portal vein. In each panel (○) depict the % relaxation of spontaneous contractions produced by increasing concentrations by cromakalim; (●) represent a second cromakalim concentration-relaxation response in the presence of (a) glibenclamide vehicle, and in (b), (c) and (d), increasing concentrations of glibenclamide (3×10^{-7}, 10^{-6} and 3×10^{-6} M, respectively; $n = 5$).

and 2.2 [1.0–4.7] $\times 10^{-5}$ M in the presence of 10^{-6}, 3×10^{-6} and 10^{-5} M glibenclamide, respectively. The calculated concentration ratios were 10.1, 26.1 and 103.2, respectively.

Portal veins Cromakalim (3×10^{-8}–10^{-6} M) produced concentration-related inhibition of the spontaneous contractile activity of rat isolated portal veins (Figure 2). Complete abolition of activity was always achieved. In the control experiment (Figure 2a), a considerable increase in sensitivity to cromakalim was observed in the second concentration-response curve (IC_{50} = 6.3 [4.8–8.3] $\times 10^{-8}$ M compared with 1.8 [1.6–2.0] $\times 10^{-7}$ M). This concentration-ratio (0.40), therefore, was used in the calculation of concentration-ratios in the experiments with glibenclamide.

When glibenclamide (3×10^{-7}–3×10^{-6} M) was added to portal veins there was no effect on the integrated activity of the preparations. However, glibenclamide produced concentration-related, parallel displacements to the right of the cromakalim concentration-response curve (Figure 2b, c, d). IC_{50} values for cromakalim were 2.0 [1.6–2.4] $\times 10^{-7}$ M, 5.6 [4.6–6.7] $\times 10^{-7}$ M and 1.9 [1.5–2.4] $\times 10^{-6}$ M in the presence of 3×10^{-7}, 10^{-6} and 3×10^{-6} M glibenclamide, respectively. The calculated concentration-ratios were 4.6, 10.4 and 36.1, respectively.

Isolated tissue experiments: ^{86}Rb efflux studies

Aortic rings At peak effect, cromakalim (10^{-5} M) caused a 107 ± 22% stimulation of ^{86}Rb efflux in control tissues and increasing concentrations of glibenclamide reduced progressively the degree of stimulation (Figure 3a). However, this inhibitory effect was only statistically significant at the highest concentration of the sulphonylurea (10^{-6} M), when ^{86}Rb efflux was raised only 16 ± 7% by cromakalim.

The basal ^{86}Rb efflux rate coefficient (1.9 ± 0.5% $3\,min^{-1}$) was not significantly changed by the presence of different concentrations of glibenclamide.

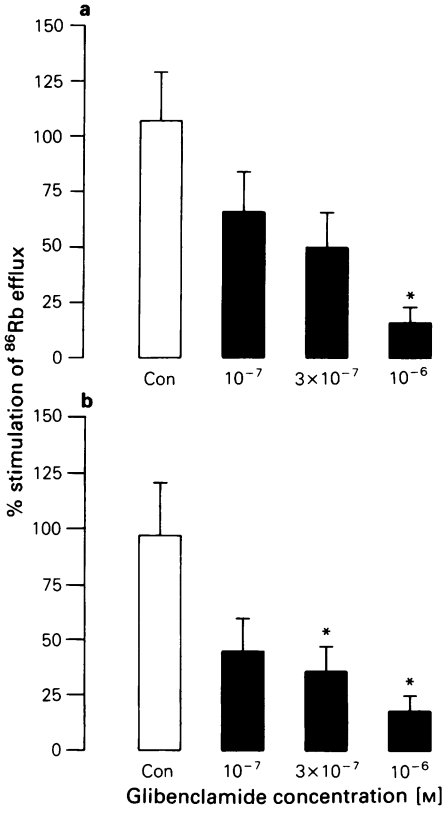

Figure 3 Demonstration of the antagonism by glibenclamide of the stimulation of ^{86}Rb efflux produced by cromakalim (10^{-5} M) in (a) rat isolated aorta, and (b) rat isolated portal vein. In each panel the open columns depict the group mean % stimulation of ^{86}Rb efflux evoked by cromakalim alone; s.e.mean shown by vertical bar. The consecutive solid columns depict responses to cromakalim in the presence of increasing concentrations of glibenclamide (10^{-7}, 3×10^{-7}, 10^{-6} M, respectively; $n = 7$ or 8). Statistically significant differences between the control and glibenclamide treatments: $^{*}P < 0.05$.

Portal veins In control tissues, ^{86}Rb efflux was increased by a maximum of 97 ± 24% in the presence of cromakalim, 10^{-5} M (Figure 3b). As in rat aorta, this stimulatory effect was reduced by increasing concentrations of glibenclamide. At a glibenclamide concentration of 10^{-6} M, ^{86}Rb efflux was elevated only 18 ± 7% by cromakalim.

Again the basal ^{86}Rb efflux rate coefficient (1.8 ± 0.5% 3 min^{-1}) was not significantly changed in the presence of the different concentrations of glibenclamide.

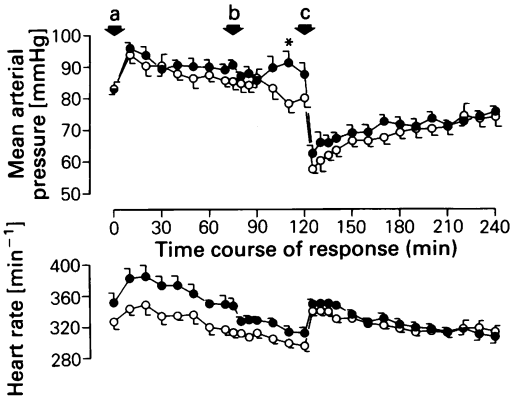

Figure 4 Demonstration of the lack of an interaction between orally administered glibenclamide and cromakalim on blood pressure and heart rate in conscious rats. In both panels (○) depict group mean responses (s.e.mean shown by vertical bars) in the control group ($n = 8$) which received a 25 ml kg^{-1} water load orally at time 0 (marked 'a' on the figure); (●) depict the group given glibenclamide, 100 mg kg^{-1} orally, at time 0 ($n = 7$). Subsequently, both groups received an intravenous injection of timolol, 1 mg kg^{-1}, at 75 min (marked 'b') and cromakalim, 0.075 mg kg^{-1} at 120 min (marked 'c'). Statistically significant differences between the groups: $^{*}P < 0.05$.

Conscious rat studies

Compared with vehicle-treated control rats, the oral administration of glibenclamide, 100 mg kg^{-1}, produced no significant change in either mean arterial blood pressure or heart rate in the 75 min before the intravenous administration of the β-adrenoceptor antagonist timolol, 1 mg kg^{-1}, to both experimental groups (Figure 4). In the period between 90 min and 120 min the group mean blood pressures did exhibit some divergence, though a significantly higher pressure in glibenclamide-treated rats occurred only at 110 min. However, group mean heart rates in the two groups were very similar following the administration of timolol. Intravenous injection of cromakalim, 0.075 mg kg^{-1}, at 120 min caused an immediate fall in blood pressure which was of a similar magnitude in the two groups. Despite the presence of timolol, cromakalim also increased heart rate slightly and to the same extent in both groups. For both parameters the peak responses were seen within 5 min, thereafter gradually returning towards their pre-cromakalim values. These results demonstrate a lack of effect of oral pretreatment with glibenclamide on the cardiovascular response to i.v. cromakalim.

In the second conscious rat experiment, both groups were treated with timolol, 2 mg kg^{-1} i.v., at

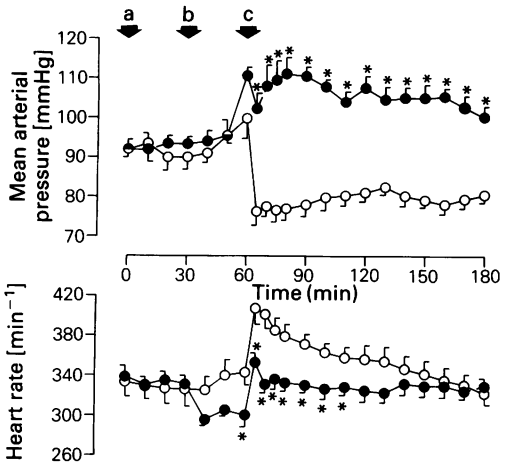

Figure 5 Interaction between intravenously administered glibenclamide and cromakalim on blood pressure and heart rate in conscious rats. In both panels, group mean ($n = 8$) responses (s.e.mean shown by vertical bars) of the control group are represented by (○) and the glibenclamide-treated group by (●). Both groups received an intravenous injection of timolol, 2 mg kg^{-1}, at time 0 (marked 'a' on figure). At 30 min (marked 'b'), the treated group received glibenclamide, 20 mg kg^{-1} i.v., and the control group received vehicle 5 ml kg^{-1}. At 60 min (marked 'c'), both groups received cromakalim, 0.075 mg kg^{-1} i.v. Statistically significant differences between the groups: $*P < 0.05$.

time 0 (Figure 5). Administration of glibenclamide, 20 mg kg^{-1} i.v., or its vehicle at time 30 min resulted in increases in blood pressure, though there was no statistically significant difference between the groups for the ensuing 30 min. Heart rate fell following glibenclamide injection though the difference between the groups was only significant at the 60 min time point. Administration of cromakalim, 0.075 mg kg^{-1} i.v., to control rats produced an immediate fall in blood pressure and, despite β-blockade with timolol, an increase in heart rate. In glibenclamide-treated rats, however, cromakalim caused only a small, transient fall in blood pressure showing that the sulphonylurea drug had prevented the hypotensive response. Despite β-blockade, and the absence of a hypotensive response in rats given the sulphonylurea, heart rate increased following cromakalim, suggesting a marginal fall in vascular resistance. However, following the peak response heart rate then fell to a plateau level similar to that seen just prior to glibenclamide injection.

Discussion

In the present paper we have demonstrated that the sulphonylurea, glibenclamide, is an inhibitor of the ability of cromakalim to (i) relax rat isolated aortic rings pre-contracted with noradrenaline, (ii) suppress spontaneous contractile activity of rat isolated portal veins and (iii) stimulate ^{86}Rb efflux from both of these tissues. Quast & Cook (1988) and Wilson (1989) have also recently demonstrated an interaction between glibenclamide and cromakalim in isolated blood vessels of the rat and rabbit. In view of the fact that vascular smooth muscle relaxant effects of cromakalim *in vitro* are thought to be mediated via an opening of cell membrane K^+ channels (see Introduction), it can be inferred from the present results that glibenclamide blocks these same channels. There are already several publications which associate sulphonylurea-mediated stimulation of insulin secretion from pancreatic islet cells with blockade of ATP-sensitive K^+ channels (see Introduction). Thus the present observations raise the question of the nature of the K^+ channel(s) opened by cromakalim in vascular smooth muscle. If pancreatic islet cells and vascular smooth muscle cells possess similar ATP-sensitive K^+ channels, two things might reasonably be expected: (i) the concentrations of glibenclamide required to block these channels would be of a similar order in the different tissues and (ii) cromakalim would stimulate insulin release from islet cells at concentrations similar to those causing vascular smooth muscle relaxation.

In the present study, 10^{-7} M glibenclamide appeared to be close to the threshold concentration required to reduce the ^{86}Rb efflux stimulated by cromakalim (10^{-5} M) in both aorta and portal vein. Similarly, Garrino *et al.* (1985) found that 10^{-7} M glibenclamide reduced ^{86}Rb efflux and increased insulin release from mouse islets perifused with a medium containing 3 mM glucose, although no attempt was made in that study to establish a threshold concentration for the sulphonylurea. Commonly, authors have tended to use higher concentrations of glibenclamide in their studies. For example, Matthews & Shotton (1984) employed 10^{-6}–10^{-4} M glibenclamide to inhibit ^{86}Rb efflux from rat isolated pancreatic islets. However, in a study by Schmid-Antomarchi *et al.* (1987), the ^{86}Rb efflux stimulated by lowering the intracellular ATP content of rat RIN M 5F insulinoma cells, was half-maximally inhibited by 6×10^{-11} M glibenclamide. Thus the issue of the glibenclamide concentrations required to block ATP-sensitive K^+ channels in pancreatic islet β-cells compared with the K^+ channels activated by cromakalim in isolated vascular smooth muscle is, at present, not completely resolved. Furthermore, although in the present studies cromakalim exerted

its maximum vascular relaxant effect at around 10^{-6} M (portal vein) or 10^{-5} M (aorta), Wilson *et al.* (1988) reported that in batch incubated rat pancreatic islets, cromakalim, 10^{-5} M, neither reduced insulin secretion nor inhibited the increase produced by glibenclamide, 5×10^{-5} M. In view of these observations we feel that at present there is insufficient evidence from *in vitro* studies to conclude that glibenclamide might be blocking a cromakalim-activated ATP-sensitive K^+ channel in vascular smooth muscle, though this possibility needs to be studied further.

Although we have been able to demonstrate an interaction between glibenclamide and cromakalim in rat isolated vascular smooth muscle, it was not possible to demonstrate a functional interaction *in vivo* when glibenclamide was administered by the oral route. Thus in conscious rats, a large oral dose of glibenclamide ($100\,mg\,kg^{-1}$) did not antagonize the blood pressure lowering effect of what we would consider to be a modest intravenous dose of cromakalim ($0.075\,mg\,kg^{-1}$) (Buckingham, 1988). Taking into account the necessity to minimize the dose of cromakalim, animals received the β-adrenoceptor antagonist, timolol, to prevent an anticipated sympathetically driven reflex increase in heart rate in order to amplify the hypotensive response. The fact that heart rate did increase slightly following cromakalim injection suggests that either the chosen dose of timolol ($1\,mg\,kg^{-1}$) was insufficient for complete cardiac β-adrenoceptor blockade or that a pre-existing vagal tonic component was withdrawn as blood pressure fell. Grosset & Hicks (1986) detected no effect of cromakalim on the rate of beating of guinea-pig right atria *in vitro*, suggesting that this compound has no direct stimulatory effect on the cardiac pacemaker.

In a further (unpublished) experiment in conscious normotensive rats we found that a lower oral dose of glibenclamide ($10\,mg\,kg^{-1}$) also did not antagonize the hypotensive action of cromakalim. Interestingly, Lorch *et al.* (1972) demonstrated that glibenclamide $1–30\,\mu mol\,kg^{-1}$ orally (approximately 0.5–$15\,mg\,kg^{-1}$) caused a dose-dependent hypoglycaemia in rats. In those experiments doses of 3 and $30\,\mu mol\,kg^{-1}$ orally were maximally effective within 1–3 h, suggesting that in our own studies glibenclamide would already have been maximally effective in stimulating insulin secretion at the time cromakalim was injected (2 h). Again in our studies, a continuous infusion of 5% w/v dextrose solution ($0.55\,mg$ dextrose min^{-1}) was given since in a preliminary experiment this protocol reduced, but did not prevent, the hypoglycaemia caused by glibenclamide, $100\,mg\,kg^{-1}$ orally (results not shown).

Following the disclosure by Cavero *et al.* (1988) that, in anaesthetized rats, glibenclamide, $20\,mg\,kg^{-1}$ i.v., prevented the hypotensive response to i.v. infusion of cromakalim, $5\,\mu g\,kg^{-1}\,min^{-1}$ for 20 min, we used this dose of the sulphonylurea for a study in conscious rats. Indeed, glibenclamide, $20\,mg\,kg^{-1}$ i.v., almost completely blocked the hypotensive response to cromakalim, $0.075\,mg\,kg^{-1}$ i.v. In a recent study, Cavero *et al.* (1989) found that the threshold i.v. dose of glibenclamide for inhibition of cromakalim responses in the rat was between 5 and $10\,mg\,kg^{-1}$. Such doses, however, are far in excess of those reported by Lorch *et al.* (1972) to cause hypoglycaemia in rats ($0.1–3.0\,\mu mol\,kg^{-1}$, 0.05–$1.5\,mg\,kg^{-1}$ i.v.).

Nonetheless, the present evidence supports the view that cromakalim causes vascular relaxation both *in vitro* and *in vivo* by virtue of its ability to activate a glibenclamide-sensitive K^+ channel in the smooth muscle cell membrane. However, the fact that there is such a large difference between the dose of glibenclamide required to block the hypotensive effect of cromakalim and that required to cause hypoglycaemia in the rat, suggests that the vascular K^+ channel is much less sensitive to the sulphonylurea than the ATP-sensitive K^+ channel in the pancreatic islet cell. Although this differential sensitivity of the two tissues to glibenclamide does not rule out the possibility that the cromakalim-activated K^+ channel in vascular smooth muscle is also regulated by ATP, it is difficult to use the present evidence in favour of such a proposition.

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Prejunctional modulation of acetylcholine release from the skeletal neuromuscular junction: link between positive (nicotinic)- and negative (muscarinic)-feedback modulation

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1 Presynaptic receptor-mediated modulation of stimulation-evoked [^{3}H]-acetylcholine([^{3}H]-ACh) release from the neuromuscular junction was 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 $[Ca^{2+}]_o$-dependent,quantal release of [^{3}H]-ACh in response to axonal stimulation.

2 Atropine enhanced, and non-depolarizing muscle relaxants ((+)-tubocurarine, pancuronium and pipecuronium) reduced, the release of [^{3}H]-ACh evoked by high frequency trains of stimulation (50 Hz, 40 shocks) of the phrenic nerve. The effect of (+)-tubocurarine was frequency-dependent as at 5 Hz (40 shocks) it was less effective than at 50 Hz. The resting release of [^{3}H]-ACh was not affected by these compounds. These findings indicate that ACh released into the synaptic gap by axonal firing reaches a concentration sufficient to influence its own release by a prejunctional effect.

3 The anticholinesterase, physostigmine sulphate, enhanced the release of [^{3}H]-ACh in a concentration-dependent manner. This effect was mediated via prejunctional nicotinic receptor stimulation: (+)-tubocurarine, pancuronium and pipecuronium completely prevented the effect of physostigmine.

4 When the prejunctional nicotinic and muscarinic receptors were stimulated by a high concentration of extracellular ACh which had accumulated in the junctional gap in the presence of physostigmine, atropine did not influence the evoked release of [^{3}H]-ACh. However, when the effect of endogenous ACh on nicotinic receptors was prevented by (+)-tubocurarine, atropine enhanced the release.

5 It is concluded that quantally-released ACh from motor endplates is subject to prejunctional automodulation: (a) ACh facilitates its own release via an effect on prejunctional nicotinic receptors (positive feedback), (b) ACh release is reduced by an action on muscarinic receptors. When the nicotinic receptor-mediated facilitation is fully operative, the muscarinic receptor-mediated negative feedback is much less effective. It is supposed that there is a link between the two feedback mechanisms possibly at the level of the second messenger system(s).

Introduction

Electrophysiological (Hubbard *et al.*, 1969; Blaber, 1973; Gibb & Marschall, 1983; Matzner *et al.*, 1988) and pharmacological (Bowman & Webb, 1976; Bowman, 1980a, b; cf. Bowman *et al.*, 1988) studies have provided evidence to suggest that (+)-tubocurarine might, in addition to its post-junctional actions, exert a prejunctional effect, and inhibit the evoked release of acetylcholine (ACh). Bowman (1980b) suggested that ACh facilitates its own release through a positive feedback mechanism at high frequency nerve stimulation. The radioisotope method (Foldes *et al.*, 1984) has made it possible to measure the Ca^{2+}-dependent quantal release of ACh from motor nerves at the neuromuscular junction independently of non-quantal resting release (Somogyi *et al.*, 1987; Somogyi & Vizi, 1987) which is Ca-independent.

In recent years, neurochemical evidence has been obtained (Halank *et al.*, 1985; Vizi *et al.*, 1985; 1987;

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Wessler *et al.*, 1986; Somogyi & Vizi, 1987; Somogyi *et al.*, 1987) showing that non-depolarizing muscle relaxants, in addition to post-junctional blockade, exert prejunctional actions. (+)-Tubocurarine (Wessler *et al.*, 1986; 1987a; Vizi *et al.*, 1987), pancuronium and hexamethonium (Vizi *et al.*, 1987) reduced the stimulation-evoked release of ACh from motor nerve endings of diaphragm. In agreement with these findings, 1,1-dimethyl-4-phenyl piperazinium (DMPP), a nicotinic receptor agonist, was shown to enhance the release of ACh from the rat hemidiaphragm preparation (Wessler *et al.*, 1986). It was shown that nicotinic agonists as well as anticholinesterase agents generated antidromic axonal firing which was inhibited by nicotinic antagonists (cf. Bowman, 1980a). The effect of anticholinesterase may have been caused by an accumulation of spontaneously released ACh (Katz & Miledi, 1977) which activated the prejunctional nicotinic receptors. No electrophysiological evidence is available to suggest that prejunctional muscarinic stimulation reduces chemical transmission (Beranek & Vyskocil, 1967). However, it has been shown that the muscarinic receptor agonist oxotremorine reduced (Somogyi *et al.*, 1987), and the antagonist atropine enhanced (Somogyi *et al.*, 1987; Wessler *et al.*, 1987b) the release of ACh evoked by electrical stimulation. This suggests that negative feedback modulation mediated via muscarinic receptors occurs at the neuromuscular junction (Somogyi *et al.*, 1987; Wessler *et al.*, 1987b).

In this study an attempt was made to investigate the possible interaction between muscarinic autoinhibition and nicotinic receptor-mediated positive feedback modulation of ACh release at prejunctional axon terminals of the mouse hemidiaphragm preparation. In view of the multiple effects of physostigmine (cf. Bowman, 1980a) on neuromuscular transmission in some experiments the cholinesterase activity was inhibited with physostigmine sulphate to amplify the effect of released ACh on prejunctional axon terminals. In this way the role of prejunctional cholinoceptors was more easily studied.

Methods

Male Swiss-Webster mice, weighing 25–30 g, were decapitated. Both hemidiaphragms were prepared (Somogyi *et al.*, 1987) and the phrenic nerves were cut about 1 mm from the muscle. Strips with the innervated region containing the phrenic nerve terminals about 2 mm to the right and left of the insertions of the phrenic nerves were mounted in separate 2 ml baths in Krebs solution gassed with a mixture of 95% O_2 and 5% CO_2 at 37°C. The composition of the Krebs solution was (mmol l^{-1}): NaCl 113, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11.5.

The preparations were incubated for 60 min in a solution containing 185 kBq ml^{-1} methyl [^{3}H]-choline (Somogyi *et al.*, 1987). To facilitate the uptake of [^{3}H]-choline and the synthesis of [^{3}H]-ACh the preparation was stimulated (square wave pulses, supramaximal, 0.2 ms duration, 1 Hz) during incubation. After incubation the preparations were transferred to a 1 ml bath and superfused continuously with solution at a rate of 2 ml min^{-1} for 90 min to wash out excess radioactivity. Subsequently, the perfusion rate was reduced to 0.5 ml min^{-1}, and 10 μmol l^{-1} hemicholinium-3 was added to the perfusing solution to prevent reabsorption of [^{3}H]-choline originating from the hydrolysis of [^{3}H]-acetylcholine released by electrical stimulation. After the washout period, 3 min aliquots of the effluent were collected by an automated fraction collector. The preparations were stimulated for 3 min on two occasions, the times depending on the experimental schedule. This endplate-rich preparation of the phrenic nerve-hemidiaphragm synthesizes [^{3}H]-ACh from [^{3}H]-choline (Wessler & Steinlein, 1987) and the newly synthesized [^{3}H]-ACh (Somogyi *et al.*, 1987) is released by electrical stimulation.

Measurement of radioactivity

The tritium content of the tissue was determined after perfusion by placing the preparation into 1 ml Soluene (Packard) and an aliquot of 0.1 ml was used for scintillation counting.

Ten ml of scintillation fluid containing toluene + Triton X-100 (3:1, v/v) were added to each 3 min aliquots of perfusate. The activity of the samples was measured in a LKB scintillation beta spectrometer. The radioactivity was expressed as Bq $3\,min^{-1}\,g^{-1}$ wet weight of the tissue determined at the end of the experiment. The effect of drugs was expressed by changes in the S2/S1 ratio. The calculation of S2/S1 ratio was based on fractional release of radioactivity.

Stimulation

The preparations were stimulated by square wave pulses of 0.2 ms duration at a voltage five times higher than that which produced maximal contractions (20 V, 50 mA). Unless otherwise stated, trains of 40 impulses (50 Hz) were administered every 10 s for 3 min through a pair of platinium electrodes placed at the top and the bottom of the organ bath (field stimulation). The preparations were stimulated twice for 3 min each starting at the 6th (S1) and 36th (S2) min.

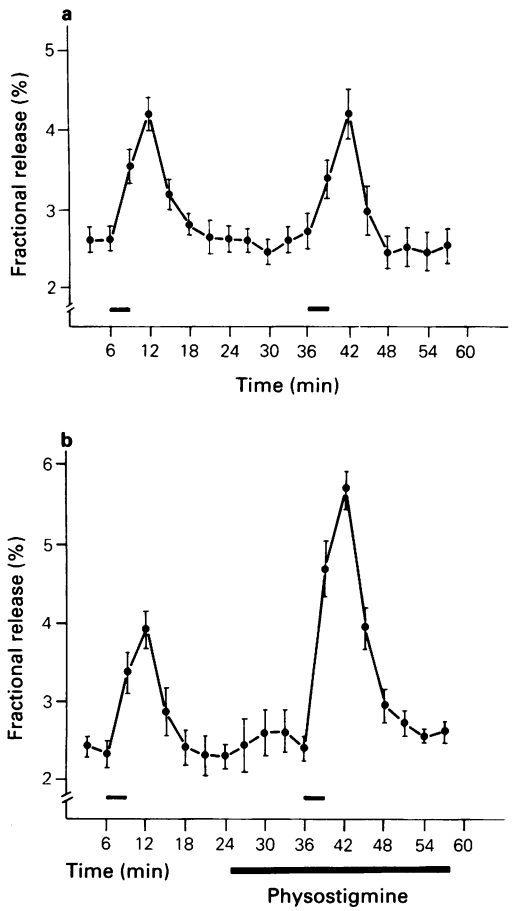

Figure 1 The effect of physostigmine (5×10^{-5} M) on the release of radioactivity from the endplate rich region of neuromuscular junction (mouse hemidiaphragm) in response to electrical stimulation. (a) Control experiments. Periods of stimulation (50 Hz, trains of 40 shocks) were applied every 10 s as indicated by the horizontal bars. (b) Effect of physostigmine sulphate (5×10^{-5} M). Each point represents the mean (n = 3(a) or 4(b)) and vertical lines indicate s.e.mean.

Statistics

Paired t test, Student's t test, or one way analysis of variance was used, and $P < 0.05$ was accepted as significant.

Drugs

Methyl-[^{3}H]-choline (2.89 TBq mmol^{-1}, Amersham, Arlington Heights, IL, U.S.A.), atropine sulphate (Burroughs Wellcome, Burlingame, CA, U.S.A.), hemicholinium-3 (Aldrich, Steinheim, F.R.G), pancuronium (Pavulon, Organon) and pipecuronium (Gedeon Richter, Budapest, Hungary) were used. The chemicals used for the Krebs solution were of analytical grade from Fischer (New York, U.S.A.).

Table 1 Effect of (+)-tubocurarine (Tc) and atropine on [^{3}H]-acetylcholine ([^{3}H]-ACh) release from the endplate-rich portion of mouse hemidiaphragm

Drugs	n	*Effect on [^{3}H]-ACh release (S2/S1)* 50 Hz	5 Hz
Control	(8)	0.86 ± 0.04	0.91 ± 0.07
Tc, 50 μM	(4)	0.41 ± 0.07*	0.65 ± 0.02*
Atropine, 1 μM	(4)	2.52 ± 0.30*	1.46 ± 0.16*
Tc, 50 μM + atropine, 1 μM	(4)	2.81 ± 0.19*	1.51 ± 0.11*

The drugs were administered 12 min before S2. Between the two stimulation periods (50 Hz, 40 shocks every 10 s for 3 min or 5 Hz 40 shocks every 10 s for 3 min) 30 min elapsed. Significance: ANOVA followed by Dunn's test. *Significant difference ($P < 0.05$) between the control values and the value obtained in the presence of drug. Note that the differences between the effect of drugs on 50 Hz and 5 Hz are significant. Data show means ± s.e.mean (n = number of experiments).

Results

Resting and stimulation-evoked release of [^{3}H]-ACh

After loading, the preparation contained 225,600 ± 20,450 Bq g^{-1} ^{3}H and the resting release was 3450 ± 230 Bq g^{-1} in 3 min (16 experiments). Electrical field stimulation (50 Hz, 40 shocks every 10 s for 3 min) released 3680 ± 170 Bq g^{-1} [^{3}H]-ACh. The stimulation was repeated within 30 min (S2) and the release was 3015 ± 195 Bq g^{-1} (n = 8). The S2/S1 ratio was constant (Figure 1a). Therefore, the effect of different drugs was evaluated from changes in S2/S1 ratios. (+)-Tubocurarine (50 μM) reduced the ratio, (i.e. the release of [^{3}H]-ACh), while atropine (1 μM) enhanced the release of [^{3}H]-ACh (Table 1). When both receptors were inhibited the release of [^{3}H]-ACh was higher than in control conditions which suggests that the release of ACh is modulated by both nicotinic and muscarinic receptors. In order to enhance the biophase concentration of ACh in the junctional cleft and thereby amplify the possible prejunctional effect of ACh on nicotinic and muscarinic receptors an anticholinesterase, physostigmine sulphate, was applied. Figure 1b shows the effect of physostigmine on evoked release of [^{3}H]-ACh. In the presence of physostigmine the

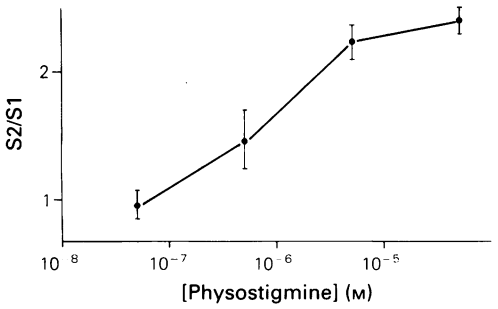

Figure 2 Effect of physostigmine sulphate on the stimulation-evoked release of [^{3}H]-acetylcholine from the endplate-rich region of mouse-hemidiaphragm preparation. Physostigmine was added in different concentrations between S1 and S2 as indicated in Figure 1b. Stimulation: trains of 40 stimuli (50 Hz), with an interval of 10 s between trains, were applied. Stimulation period: 3 min. Each point represents the mean and vertical lines indicate s.e.mean.

release was significantly higher (S2/S1 = 1.46 ± 0.22, ($P < 0.01$) than in its absence (S2/S1 = 0.89 ± 0.08, $n = 4$). The effect of physostigmine on the release of [^{3}H]-ACh was concentration-dependent (Figure 2).

Interaction between muscarinic and nicotinic presynaptic modulation

Figure 3 shows that (+)-tubocurarine (2×10^{-5} M) significantly reduced the release of [^{3}H]-ACh enhanced by physostigmine (S2/S1 = 0.37 ± 0.12). That this reduction in [^{3}H]-ACh release is mediated not only via removal of nicotinic receptor stimulation but by concomitant stimulation of muscarinic

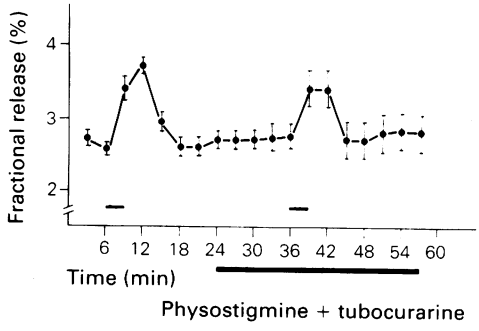

Figure 3 Inhibitory effect of (+)-tubocurarine (2×10^{-5} M) on [^{3}H]-acetylcholine release enhanced by physostigmine (5×10^{-5} M). Two periods of stimulation (50 Hz, trains of 40 shocks) were applied as indicated by the horizontal bars. Each point represents the mean ($n = 4$) and vertical lines indicate s.e.mean.

receptors, is indicated by the finding (Figure 3) that atropine (10^{-6} M) completely reversed this effect of (+)-tubocurarine (S2/S1 = 2.70 ± 0.28). Pancuronium (10^{-5} M) and pipecuronium (10^{-5} M) also prevented the effect of physostigmine. The S2/S1 ratio in the presence of pancuronium was 0.64 ± 0.08 ($n = 3$) and with pipecuronium 0.51 ± 0.11 ($n = 4$). These values are significantly ($P < 0.01$ in both cases) smaller than that obtained in the absence of muscle relaxants, 1.61 ± 0.12 ($n = 4$).

In the presence of physostigmine, atropine (10^{-6} M) failed to affect the release of [^{3}H]-ACh. However, under control conditions atropine (10^{-6} M) enhanced the release from 0.83 ± 0.09 to 2.52 ± 0.39 (Table 1).

Discussion

Dale *et al.* (1936) found that the amount of ACh released into the solution perfusing striated muscle was not diminished when the muscles were completely paralysed by curare. They concluded that curare had principally a postjunctional action. Since then several attempts have been made to study the prejunctional effect of (+)-tubocurarine (Chang *et al.*, 1967; Fletcher & Forrester, 1975), but until now attempts to demonstrate the inhibitory effect of (+)-tubocurarine on the evoked release of ACh by neurochemical methods (i.e. assay of the released ACh) have been unsuccessful. This was always considered a strong argument in favour of a purely postjunctional action of curare. In all of these studies the total (quantal + non-quantal) release of ACh was measured in the presence of an anticholinesterase. The failure to demonstrate any changes in the evoked, quantal release of ACh by (+)-tubocurarine or muscarinic stimulation may have been due to the fact that most of the endogenous ACh released from mammalian muscle is released in a non-quantal, Ca-independent way (Mitchell & Silver, 1963; Katz & Miledi, 1977; Vizi & Vyskocil, 1979). Very recently a sensitive radioactive method that measures selectively the evoked (Ca-dependent) release of ACh from the mouse (Somogyi *et al.*, 1987) or rat (Wessler & Kilbinger, 1986) phrenic nerve-hemidiaphragm preparation *in vitro* has made it possible to measure the effect of drugs on the quantal release of labelled ACh from motor nerve endings. The mouse hemidiaphragm preparation (Somogyi *et al.*, 1987) seems to be a suitable tissue for studying quantally released ACh, because it is both rich in endplates (Waser *et al.*, 1978) and thin which allows easy access to drugs as well as diffusion of ACh into the perfusing fluid. The signal:noise (evoked:resting release) ratio in this preparation is much higher than that obtained in the rat hemidiaphragm (Wessler & Kilbinger, 1986). Based on electrophysiological (cf. Gibb &

Marshall, 1983) and pharmacological (Bowman & Webb, 1976; Bowman, 1980a; Bowman *et al.*, 1988) data, it was suggested that there is a prejunctional nicotinic receptor-mediated facilitation (mobilization) of ACh release by a positive feedback mechanism.

In this study neurochemical evidence was obtained that non-depolarizing muscle relaxants (+)-tubocurarine, pancuronium, pipecuronium) reduced the stimulation-evoked release of [^{3}H]-ACh, and that atropine, a muscarinic receptor antagonist, enhanced it. These compounds did not affect the resting release in the absence of nerve stimulation. In a different set of experiments physostigmine was used to enhance the biophase concentration of ACh in the junctional gap, thereby amplifying the possible effect of ACh on prejunctional receptors. Physostigmine, by itself, enhanced the release of [^{3}H]-ACh. When cholinesterase is inhibited, ACh is allowed to make multiple interactions with the cholinoceptors before escaping by diffusion from the receptor sites. Enhancement of ACh release by physostigmine was completely inhibited by (+)-tubocurarine and other non-depolarizing muscle relaxants (pancuronium, pipecuronium) which indicates that the effect of physostigmine was mediated via prejunctional nicotinic receptors. The possibility that physostigmine *per se* might increase the amount of transmitter released by each nerve impulse cannot be ignored.

As far as the muscarinic negative feedback modulation is concerned, the literature offers very controversial data. While Abbs & Joseph (1981) have shown that oxotremorine reduces and atropine enhances the release of ACh from the neuromuscular junction, others (Gundersen & Jenden, 1980; Haggblad & Heilbronn, 1983) failed to find such an effect. In addition, electrophysiological (Beranek & Vyskocil, 1967) and histochemical data (Gillberg *et al.*, 1984) oppose the presence of muscarinic receptors at the neuromuscular junction. Wessler *et al.* (1987b) found that the stimulation by oxotremorine of muscarinic receptors resulted in a biphasic response: in low concentrations (10 nM) oxotremorine enhanced whereas in higher concentrations (1 μM) it inhibited the release of ACh. These data and our earlier observations that oxotremorine reduces the release of ACh (Vizi *et al.*, 1985; Somogyi *et al.*, 1987) suggest that the release of ACh from the neuromuscular junction is modulated by muscarinic receptors. However, in our study atropine enhanced the release evoked by electrical stimulation and attenuated the reduction of release following (+)-tubocurarine administration. Atropine did not change the resting release of ACh. It is generally accepted that inhibition of presynaptic muscarinic autoreceptors enhances (cf. Kilbinger, 1977; Vizi, 1984) ACh release in both central (Szerb & Somogyi, 1973) and peripheral cholinergic synapses (Szerb & Somogyi, 1973; Vizi *et al.*, 1984; Baux & Tauc, 1987), whereas muscarinic receptor agonists (ACh, oxotremorine) inhibit the release. Therefore, the concept of a muscarinic feedback modulation of ACh release may be extended to the neuromuscular junction.

It is interesting to consider how our results obtained with different frequencies compare with already published data. It is known that (+)-tubocurarine is more effective at high frequencies of stimulation (cf. Bowman, 1980a) and in our experiments it was more effective in reducing ACh release at 50 Hz than at 5 Hz.

The data presented here also show that the modulation of ACh release at the neuromuscular junction is balanced through nicotinic and muscarinic autoreceptors. If it is assumed that nicotinic autoreceptors influence ACh release, then while positive feedback modulation is operative, inhibition of negative feedback modulation should not have any significant action. This was the case in our experiments. Atropine had no effect on the release evoked by physostigmine, i.e. when the nicotinic positive feedback modulation was fully operative. Therefore, we may conclude that positive feedback is dominant in the modulation of ACh release.

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6-Cyano-7-nitroquinoxaline-2,3-dione as an excitatory amino acid antagonist in area CA1 of rat hippocampus

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1 A quantitative pharmacological investigation of the excitatory amino acid antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) has been made in area CA1 of rat hippocampal slices bathed in 1 mM Mg^{2+} containing medium.

2 At a concentration of 10 μM, CNQX reversibly antagonized responses to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), quisqualate and kainate; it produced a parallel shift in their log dose-response curves. Responses to N-methyl-D-aspartate (NMDA) were not antagonized by 10 μM CNQX (dose-ratio: 1.04 ± 0.06, $n = 3$).

3 Schild plots (constructed over the range 1–100 μM) yielded the following estimated pA_2 values, AMPA 5.8, quisqualate 5.9, and kainate 5.9. NMDA was antagonized by 100 μM CNQX, giving an apparent log K of 4.44 ± 0.06.

4 The slopes (±s.e.mean) of the Schild plots were for AMPA 0.84 ± 0.06, quisqualate 0.79 ± 0.04 and kainate 0.68 ± 0.07. These were all significantly less than unity.

5 Synaptic responses elicited by low frequency activation of the Schaffer collateral-commissural pathway were blocked completely by CNQX (10 μM) providing that a low stimulus intensity was used. With high intensity stimulation a small component remained that was blocked by the selective NMDA antagonist D-2-amino-5-phosphonovalerate (APV).

6 These results suggest that CNQX does not differentially affect the responses of CA1 neurones to AMPA, quisqualate and kainate. It does, however, depress responses to these agonists to a greater degree than it does responses to NMDA and it is a highly effective synaptic antagonist.

Introduction

Excitatory amino acids, such as L-glutamate, act on several types of receptor, three of which are commonly named after the agonists N-methyl-D-aspartate (NMDA), kainate and quisqualate (Watkins & Evans, 1981). The NMDA receptor has been well characterized pharmacologically due to availability of various classes of antagonists: selective competitive antagonists, such as D-2-amino-5-phosphonovalerate (APV) (Davies *et al.*, 1981), 'non-competitive' antagonists, such as phencyclidine (Anis *et al.*, 1983) and divalent cations, in particular Mg^{2+} (Ault *et al.*, 1980).

Recently, two quinoxalinediones have been reported to be the first potent and selective antagonists active at kainate and quisqualate receptors (Honoré *et al.*, 1987). These compounds displace binding of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), a quisqualate receptor ligand, and to a lesser extent kainate; they have little or no effect on binding to NMDA or other neurotransmitter receptor sites (Honoré *et al.*, 1988). They also antagonize kainate- and quisqualate-induced [^{3}H]-γ-aminobutyric acid ([^{3}H]-GABA) release from mouse cultured cortical neurones, with a lesser effect on release induced by NMDA (Drejer & Honoré, 1988). In electrophysiological experiments these substances depress responses to quisqualate and kainate to a greater extent than responses to NMDA (Honoré *et al.*, 1988; Blake *et al.*, 1988a; Neuman *et al.*, 1988; Fletcher *et al.*, 1988; Andreasen *et al.*, 1988a). They are also effective antagonists of synaptic responses (Blake *et al.*, 1988a; Fletcher *et al.*, 1988; Andreasen *et al.*, 1988b; Collingridge *et al.*, 1988a; Neuman *et al.*, 1988) and certain types of epileptiform activity (Fletcher *et al.*, 1988; McBain *et al.*, 1988) in the hippocampus and other regions of the brain (Fletcher *et al.*, 1988).

Despite the considerable recent interest in these compounds, there have been few quantitative pharmacological assessments of quinoxalinediones as

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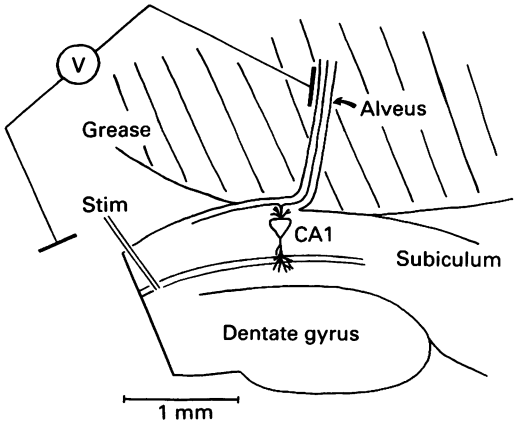

Figure 1 Schematic diagram of a rat hippocampal slice set up for grease gap recording. The potential difference was recorded (d.c.) between the alveus and the bathing medium. Stim = stimulating electrode.

excitatory amino acid antagonists. The purpose of the present investigation, therefore, has been to evaluate CNQX as an excitatory amino acid antagonist in the CA1 region of rat hippocampal slices; the region where most synaptic studies using CNQX have been performed.

Methods

Experiments were performed on 400 μm slices obtained from adult female rats, prepared and set up for grease gap recording (at 27°C) as described previously (Blake *et al.*, 1988b). In all experiments, the CA3 region was removed to prevent effects recorded from CA1 being contaminated by agonist-induced effects in CA3. A stimulating electrode (Collingridge *et al.*, 1988b) was placed in stratum radiatum to elicit synaptic responses (Figure 1); stimuli comprised square wave pulses of 0.1 ms duration and were

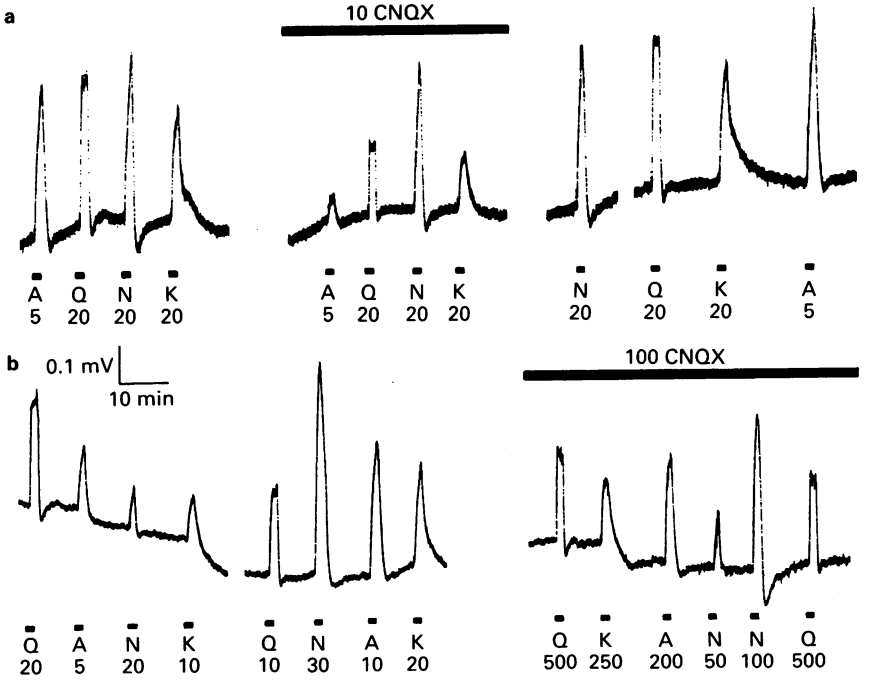

Figure 2 Antagonism of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA, A), quisqualate (Q), N-methyl-D-aspartate (NMDA, N) and kainate (K) by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). (a) Responses to the agonists in control medium, in the presence of 10 μM CNQX and after washout of CNQX. The gaps in the record represent (from left to right) 20 min, 85 min and 10 min. CNQX 10 μM was perfused for 20 min and was washed out for 70 min before agonists were tested. (b) Responses to the agonists in control medium and in the presence of 100 μM CNQX. The gaps in the record both represent 30 min. CNQX 100 μM was perfused for 20 min before the agonists were tested.

Table 1 Apparent log affinity constants for CNQX against four different agonists at CA1 neurones of rats

CNQX (μM)	*AMPA*	*Quisqualate*	*Kainate*	*NMDA*
1	5.71 ± 0.03	5.91 ± 0.03	5.89 ± 0.07	—
10	5.81 ± 0.04	5.71 ± 0.06	5.72 ± 0.09	—
100	5.39 ± 0.06	5.50 ± 0.08	5.26 ± 0.03	4.44 ± 0.06*
10^+	5.83 ± 0.03	5.74 ± 0.06	5.51 ± 0.15	—

CNQX = 6-cyano-7-nitroquinoxaline-2,3-dione; AMPA = α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid. NMDA = N-methyl-D-aspartate.
Data are presented as means ± s.e.mean for 3–6 slices. $^+$Experiments performed in Mg^{2+}-free medium (data calculated from dose-ratios presented in Blake *et al.*, 1988).
Apparent log *K* values for the different agonists were compared for each concentration of CNQX. Analyses of variance showed no significant differences between AMPA, kainate and quisqualate (1 μM: $F(2, 8) = 5.97$, $P > 0.05$; 10 μM: $F(2, 12) = 0.57$, $P > 0.05$; 100 μM: $F(2, 8) = 4.19$, $P > 0.05$; 10^+ μM: $F(2, 17) = 3.00$, $P > 0.05$.
Inclusion of NMDA in the statistical analysis (at 100 μM CNQX) yielded a highly significant effect ($F(3, 11) = 65.23$, $P < 0.0002$). * NMDA significantly different from the other agonists ($P < 0.001$; unpaired *t* tests).
– = no antagonism.

delivered at 0.033 Hz. The bathing medium comprised (mM): NaCl 124, $NaHCO_3$ 26, KCl 3, $CaCl_2$ 2, $MgSO_4$ 1, D-glucose 10 (bubbled with 95% O_2, 5% CO_2) and was perfused at 1.5 ml min^{-1}. Antagonists were added to this perfusate; agonists were perfused as 2 ml aliquots. Only slices where electrical stimulation elicited a synaptic response of at least 1 mV were selected for study.

Drugs

Drugs were stored frozen in stock solutions of 3 mM KCl. N-methyl-D-aspartate, quisqualate and kainate were obtained from Sigma. α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid and D-2-amino-5-phosphonovalerate were provided by Dr J.C. Watkins (Dept. Pharmacology, University of Bristol) and 6-cyano-7-nitroquinoxaline-2,3-dione was provided by Dr T. Honoré (Ferrosan Group).

Results

The four excitatory amino acids AMPA, kainate, quisqualate and NMDA produced dose-dependent depolarizations of CA1 neurones as described previously (Blake *et al.*, 1988*b*). CNQX depressed responses to all four agonists (Figure 2) in a reversible manner (Figure 2a). Maximal effects of CNQX were seen within 20 min (Figure 2b) but full recovery varied between 70 and 180 min. Of the four agonists tested NMDA was affected by far the least.

In the example illustrated in Figure 2a, doses were chosen to give roughly equal submaximal depolarizations. CNQX (10 μM) depressed responses to AMPA to a greater extent than responses to either kainate or quisqualate. However, this greater depression need not represent real differential antagonism since the dose-response curves of the various agonists do not have the same slopes (Blake *et al.*, 1988b).

To quantify the antagonism by CNQX two methods were adopted to generate dose-ratios. In the first method, full dose-response curves for each agonist were constructed using one agonist per slice. CNQX (10 μM) produced a parallel shift in the log dose-response curve to AMPA ($n = 2$; Figure 3), kainate ($n = 2$) or quisqualate ($n = 1$). In the second method (Figure 2b) four agonists were tested on the same slice; responses in the presence of CNQX (1, 10 or 100 μM) were bracketed between two control

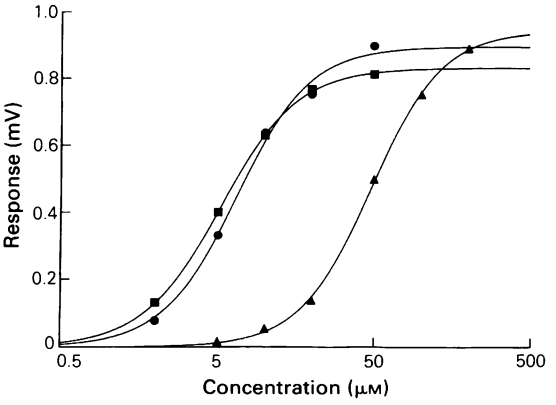

Figure 3 Log dose-response curve for α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) in control medium (●), in 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX)-containing medium (▲) and following washout of CNQX (■). Agonists were applied 30–100 min after the start of the perfusion of CNQX and 70–140 min after the start of the washout of CNQX. The EC_{50} values of each plot are 6.6, 47.0 and 5.2 μM.

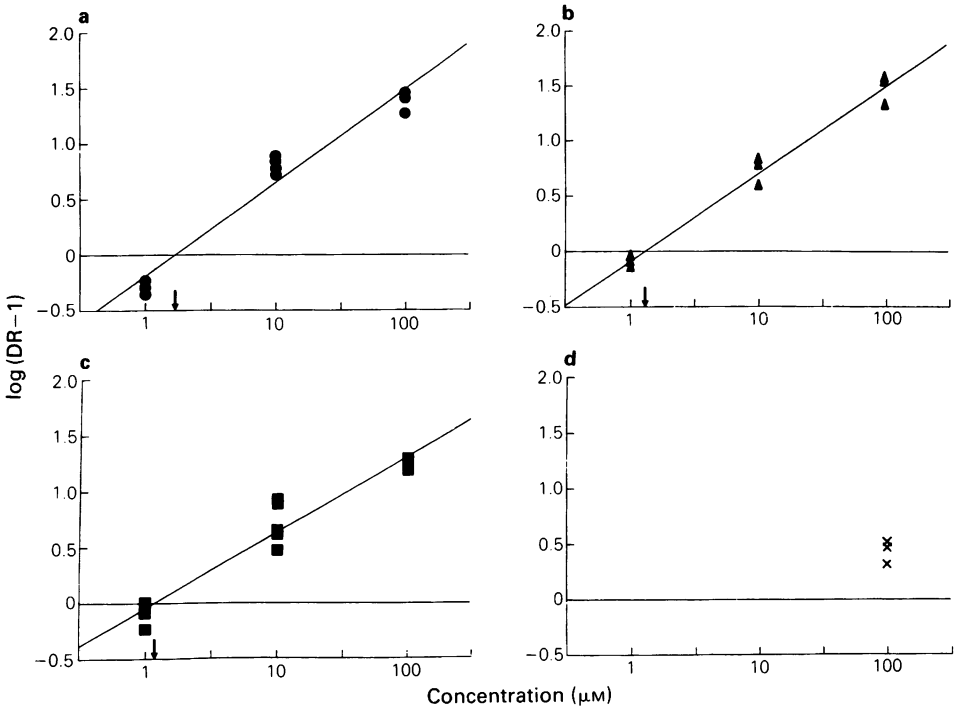

Figure 4 Schild plots for antagonism of (a) α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (●), (b) quisqualate (▲), (c) kainate (■) and (d) N-methyl-D-aspartate (NMDA) (×) by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). The estimated pA_2 values (indicated by arrows) for AMPA, quisqualate and kainate are 5.8, 5.9 and 5.9, respectively. For each agonist, every point was obtained from a separate slice and the lines were fitted by linear regression. The regressions for AMPA, kainate and quisqualate are highly significant ($P < 0.001$) and their slopes do not differ significantly from one another ($P > 0.05$) but they are significantly less than unity ($P < 0.05$).

responses (on the linear region of the log dose-response plots (Blake *et al.*, 1988b)) (Figure 2b). Both methods produced similar dose-ratios for 10 μM CNQX (see Figure 4). From the dose-ratios, apparent log K values were calculated for each antagonist concentration (Table 1) and Schild plots were also constructed (Figure 4). The respective pA_2 values were for AMPA 5.8, quisqualate 5.9 and kainate 5.9. Responses to NMDA were only affected by 100 μM CNQX (Figure 4); at 10 μM the mean ± s.e.mean dose-ratio was 1.04 ± 0.06 ($n = 3$). When all data are considered together, there was no difference in the antagonism of responses to AMPA, quisqualate and kainate, whereas NMDA was antagonized to a much lesser extent.

The slopes (±s.e.mean) of the Schild plots were for AMPA 0.84 ± 0.06, quisqualate 0.79 ± 0.04 and kainate 0.68 ± 0.07; these were all significantly ($P < 0.05$) less than unity.

In 5 slices, the effect of 10 μM CNQX upon synaptic responses evoked by two or more stimulus intensities was examined. When low intensities (approximately twice threshold for evoking a response) were used CNQX completely abolished the response (Figure 5a). However, when higher stimulus strengths were used (4–8 times threshold) a small component remained in CNQX; this was reversibly blocked by 10–50 μM APV (Figure 5b).

Discussion

The present data are consistent with the suggestion that CNQX is a moderately potent antagonist at excitatory amino acid receptors of the non-NMDA type but is only a weak NMDA antagonist (Honoré *et al.*, 1988). The estimated pA_2 values (and slopes of the Schild equations) are similar to those derived from the study of Fletcher *et al.* (1988) using rat neocortical wedges; their values were for quisqualate 6.2 (0.8), for kainate 5.5 (0.8) and for NMDA 4.5. The reason for the shallow Schild slopes in both of these studies is not known. It is interesting that kynurenate also produces a shallow Schild slope for

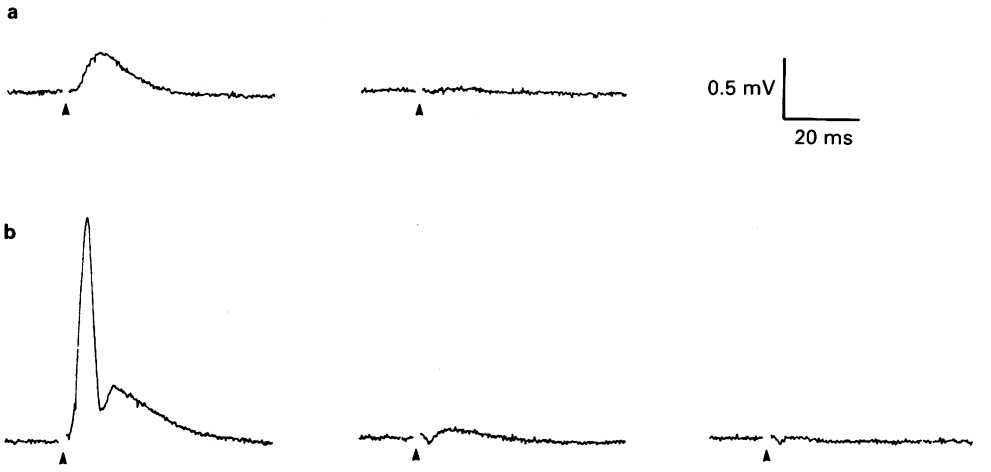

Figure 5 The effect of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) on synaptic responses evoked by stimulation of the Schaffer collateral-commissural pathway. Records in (a) were obtained with 20 V (twice threshold) and in (b) with 60 V stimulus intensities. They show (from left to right) control records, the effects of 10 μM CNQX (applied for 20–25 min) and the effect of the addition of 10 μM APV for 7 min (at 60 V). All traces are averages of 5 successive records; stimulus artefacts have been blanked and times of stimulation are indicated by arrowheads.

antagonism of responses to kainate and quisqualate whereas 1-(*p*-bromobenzoyl)-piperazine-2,3-dicarboxylate gives a Schild slope of unity (Evans *et al.*, 1987). One explanation for these data is that the antagonism of non-NMDA receptor ligands by CNQX and kynurenate is not (entirely) competitive.

One difference compared with the study of neocortical wedges was that we were unable to obtain any consistent differentiation in the antagonism of responses to AMPA, quisqualate and kainate. This also contrasts with binding studies where CNQX was 5 times more potent at displacing AMPA than kainate from rat neocortical membranes (Honoré *et al.*, 1988). It is possible, however, that the kainate receptor recorded in the present electrophysiological experiments is different from that determined in binding assays. The simplest interpretation of our results is that AMPA, kainate and quisqualate are all acting (predominantly at least) on the same receptor (see also Kemp *et al.*, 1987; Fletcher *et al.*, 1988). This receptor may be the one responsible for the generation of the fast e.p.s.p. in the CA1 region of the hippocampus as suggested by both the present and previous studies (Neuman *et al.*, 1988; Collingridge *et al.*, 1988a; Blake *et al.*, 1988a; Andreasen *et al.*, 1988b).

In a number of investigations, CNQX has been evaluated as an antagonist on the basis of the percentage depression of responses induced by single doses of agonist. In spinal cord *in vivo* kainate and quisqualate responses were depressed about equally (Honoré *et al.*, 1988), while in hippocampal slices kainate has been found to be either the more sensitive (Andreasen *et al.*, 1988a) or the less sensitive (Neuman *et al.*, 1988) of the two agonists to the action of CNQX. In neocortical wedges, agonists were depressed in the order AMPA > kainate > quisqualate (Fletcher *et al.*, 1988). However, as exemplified by Figure 2a, it is not possible to draw accurate conclusions about the selectivity of CNQX when percentage depressions (rather than dose-ratios) are measured, since the dose-response curves of the agonists have very different slopes (and additionally for quisqualate, a smaller maximum response) (Blake *et al.*, 1988b).

The effect of 10 μM CNQX as an excitatory amino acid antagonist in the CA1 region was essentially the same in the present study (in 1 mM Mg^{2+}) as that reported previously in Mg^{2+}-free medium (Blake *et al.*, 1988a). This shows that the antagonism of non-NMDA receptor ligands by CNQX is not influenced by Mg^{2+} (up to 1 mM). At 10 μM, CNQX has no significant effect on responses to NMDA, in either the presence or absence of Mg^{2+}. In the hippocampus this concentration of CNQX totally blocks fast e.p.s.ps evoked by low stimulus strengths, as reported previously (Blake *et al.*, 1988a). However, in agreement with intracellular studies (Collingridge *et al.*, 1988a; Andreasen *et al.*, 1988a), at high stimulus strengths a CNQX-resistant component is seen; this can be blocked by APV.

Very recently it has been reported that CNQX blocks responses to NMDA via an action at the allosteric glycine site (Birch *et al.*, 1988). As a consequence the ability of CNQX to block responses to NMDA will depend on the glycine concentration. It

is generally believed that in brain slices, glycine is present at supersaturating levels for this site; this will increase the effective selectivity of the antagonist.

In summary, CNQX is sufficiently selective in area CA1 of hippocampal slices to block non-NMDA receptor-mediated synaptic components completely whilst having no detectable effect on synaptic or agonist-induced responses mediated via NMDA receptors.

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Interactions between the tachykinins and calcitonin gene-related peptide lead to the modulation of oedema formation and blood flow in rat skin

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1 The mechanisms involved in tachykinin-induced oedema were investigated in rat skin and interactions between the tachykinins and calcitonin gene-related peptide (CGRP) were studied.

2 Intradermal injections of the tachykinins, substance P, neurokinin A and neurokinin B, stimulated local oedema formation which was in each case potentiated by co-injection of the vasodilator CGRP. Oedema induced by substance P, in the presence and absence of CGRP, was significantly inhibited by pretreatment of rats with a combination of the histamine H_1 antagonist, mepyramine, and the 5-hydroxytryptamine antagonist, methysergide. Oedema induced by neurokinin A or B was not inhibited by this pretreatment.

3 Intradermally-injected CGRP induced a long lasting increase in local blood flow, which was measured with a laser Doppler blood flow meter. The simultaneous injection of substance P, but not of the structurally-related neurokinins, caused a loss of the prolonged vasodilator activity of CGRP.

4 These results show that oedema induced by substance P is partially dependent on mast cell amines and that only substance P causes a loss of the prolonged vasodilator activity of CGRP.

5 We suggest that the ability of substance P to prevent the persistent vasodilator activity of CGRP may be a direct consequence of substance P-induced activation of mast cells.

Introduction

Neuropeptides, released from sensory nerves, exert potent pro-inflammatory effects on the microvasculature. These substances have been implicated in inflammatory reactions. Most early studies focused on substance P, an 11 amino acid peptide (Chang *et al.*, 1971; see Table 1) which is localised in sensory C-fibres (Hokfelt *et al.*, 1975). Substance P is a potent mediator of increased microvascular permeability (Chahl, 1977; Lembeck & Holzer, 1979) which acts through more than one mechanism (Foreman *et al.*, 1983). Substance P releases mast cell histamine which increases microvascular permeability. The activation of mast cells by substance P is dependent on its basic N-terminus (Foreman *et al.*, 1983), possibly through the insertion of a hydrophobic region into the mast cell membrane lipid to stimulate mast cell degranulation (Repke *et al.*, 1987). Substance P can also increase microvascular permeability directly via an interaction of the carboxy terminus with specific vascular receptors (Foreman *et al.*, 1983).

A family of peptides related to substance P, the tachykinins, have now been characterized. The tachykinins have a common carboxy-terminal amino acid sequence (see Table 1) and include two neurokinins (A and B). Both neurokinin A (substance K) and neurokinin B (neuromedin K) were extracted from mammalian spinal cord (Kimura *et al.*, 1983; Kangawa *et al.*, 1983; Minamino *et al.*, 1984). Like substance P, neurokinin A and neurokinin B increase microvascular permeability causing local oedema formation when injected into rat skin (Gamse & Saria, 1985) and human skin (Devillier *et al.*, 1986; Fuller *et al.*, 1987). The tachykinins do not possess a common N-terminus (see Table 1) and the neurokinins are >50 times less potent than substance P in stimulating histamine release from rat peritoneal mast cells (Devillier *et al.*, 1986). This suggests that oedema formation induced by the neurokinins is not mediated through histamine release from mast cells.

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Table 1 Structure of the tachykinins

Tachykinin	*Amino acid sequence*
Substance P	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2
Neurokinin A	His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2
Neurokinin B	Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH_2

The 37 amino acid peptide, calcitonin gene-related peptide (CGRP), is now known to be localised with substance P in many sensory neurones in organs that include skin (Gibbins *et al.*, 1985; 1987). CGRP is a potent vasodilator in several species including man (Brain *et al.*, 1985). In human and animal skin the intradermal injection of CGRP causes a prolonged increase in local blood flow (Brain & Williams, 1985; Brain *et al.*, 1986). As a consequence of its vasodilator activity CGRP potentiates oedema induced by mediators of increased microvascular permeability which include substance P and the neurokinins (Brain & Williams, 1985; Gamse & Saria, 1985).

We are interested in interactions between co-localised neuropeptides that can lead to modified microvascular responses. In this study we have, firstly, investigated mechanisms involved in tachykinin-induced oedema formation, the contribution by mast cell amines and potentiation by CGRP. Secondly, we have investigated the effect of tachykinins on blood flow changes induced by CGRP.

Methods

Measurement of oedema

Local oedema formation was measured in male Sprague-Dawley rats (160–600 g), in response to intradermal injections of the neuropeptides, by the extravascular accumulation of intravenously-injected [^{125}I]-human serum albumin (0.18 MBq kg^{-1}), as described previously (Brain & Williams, 1985). Rats were anaesthetized with pentobarbitone (30 mg kg^{-1}) and intradermal injections (0.1 ml in Tyrode bicarbonate solution) were given according to a balanced site pattern into the shaved dorsal skin of the rat with 2 replicates for each agent. The rats were killed after a 30 min accumulation period by pentobarbitone overdose, the dorsal skin was removed and injection sites (17 mm diameter) were counted in a gamma-counter. Oedema formation at each site was expressed as a plasma volume, calculated from the counts in 1 ml plasma. In experiments where mepyramine (6 mg kg^{-1}) and methysergide (6 mg kg^{-1}) were used, the antagonists were injected intraperitoneally 15 min before the intradermal injections were given. When the time course of the activity of substance P in mediating increased microvascular permeability was determined, substance P (100 pmol) was injected into untreated rats at different times, 2–16 min before and then 2 min after, the intravenous injection of [^{125}I]-albumin and oedema formation was measured over the following 30 min period.

Measurement of blood flow

Local blood flow was measured in the dorsal skin of male Sprague-Dawley rats (160–400 g) with a Perimed II laser Doppler blood flow meter (Holloway, 1977; Brain *et al.*, 1986). The rats were anaesthetized by intraperitoneal injection of pentobarbitone (Sagatal 60 mg kg^{-1}). The dorsal skin was shaved and depilated with a commercial depilatory cream (Immac). All rats were pretreated with mepyramine (6 mg kg^{-1}) and methysergide (6 mg kg^{-1}), given intraperitoneally 15 min before the start of the intradermal injections. Skin sites were marked out on the dorsal skin according to a balanced site pattern with two sites for each test agent. The agents under test in Tyrode (0.1 ml) were injected intradermally at 1 min intervals. Three blood flow readings, each for 12 s were taken sequentially at each site 30 min after the intradermal injection. Measurements were taken by placing the laser probe at a site 2–3 mm from the mark made by the injection needle. Care was taken to ensure that both the rat and laser probe remained still whilst each measurement was taken. The mean of the six readings (from the two replicate injections) was calculated and results were expressed as % change in blood flow from that observed at a Tyrode-injected site. Results were recorded as % flux value in approximately 1–2 mm^3 of tissue (number of moving red blood cells detected in the path of the laser beam × mean cell velocity). The laser Doppler flow meter was set at 4 Hz on either gain 3 or 10. Readings from a Tyrode-injected site, measured 30 min after injection, were 5–17% flux (gain 3) and 14–29% flux (gain 10). In experiments where the time course of vasodilator activity of CGRP was determined, blood flow was measured at 5, 15, 30, 45, 60, 75 and 90 min after intradermal injections of CGRP and Tyrode. Oedema formation was also assessed at injected sites, over the experimental period of the blood flow experiments, by the extravascular accumulation of intravenously-injected iodinated albumin (see above).

Materials

CGRP, substance P, neurokinin A and neurokinin B were purchased from Bachem U.K., Saffron Walden, Essex. Neuropeptides were stored in aliquots at −30°C until just before use. Pentobarbitone sodium (Sagatal) and mepyramine maleate (Anthisan) were purchased from May & Baker, Dagenham, Essex, U.K. Methysergide maleate was a gift from Dr W.L. Shaw, Sandoz Products Ltd, Eltham, Middx. [^{125}I]-human serum albumin was purchased from Amersham International, Bucks, U.K. Immac was purchased from Anne French.

All experiments were carried out using Tyrode solution of the following composition (mM): NaCl 137, KCl 2.7, $MgCl_2$ 0.5, NaH_2PO_4 0.4, $NaHCO_3$ 1.2 and glucose 5.6.

Results

Substance P, neurokinin A and neurokinin B induced local oedema formation in a dose-dependent (1–100 pmol) manner as shown in Figure 1. In each case oedema was potentiated when the tachykinin was injected with the vasodilator CGRP (20 pmol). CGRP did not induce oedema when injected alone at this dose. Pretreatment of the animals with the antagonists mepyramine and methysergide abolished oedema resulting from mast cell amine release induced by compound 48/80 and significantly inhibited oedema induced by susbtance P. The amine antagonists had no effect on oedema induced by neurokinin A or B. Inhibition of substance P-induced oedema by the amine antagonists was observed in both the presence and absence of CGRP.

Experiments were designed to determine the duration of action of substance P in increasing microvascular permeability, by injecting substance P at selected times before giving intravenous [^{125}I]-albumin. The time taken for substance P to lose 50% of its permeability-increasing activity was 2.7 ± 0.1 min (mean ± s.e.mean, $n = 4$).

Local blood flow was measured at injected sites with a laser Doppler blood flow meter. Three readings were taken for each injection site as shown in Figure 2. Blood flow was significantly increased in response to a 20 pmol dose of CGRP, for up to 60 min after intradermal injection (60 min: $P < 0.01$,

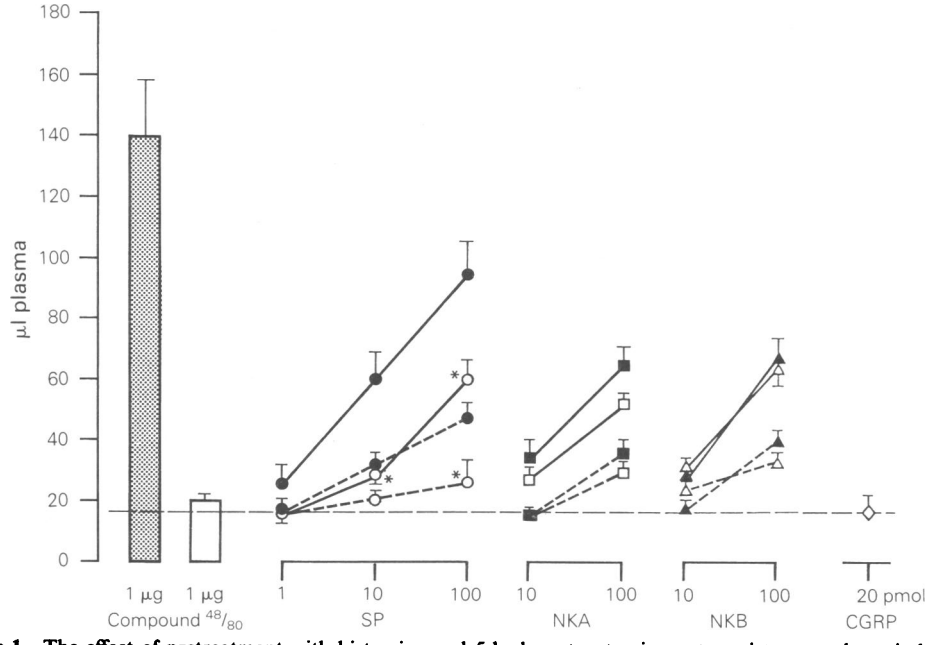

Figure 1 The effect of pretreatment with histamine and 5-hydroxytryptamine antagonists on oedema induced by the tachykinins in rat skin. Oedema induced by the intradermal injection of compound 48/80 in control rats (stippled column) and rats pretreated with mepyramine and methysergide (open column) is shown. Oedema induced by intradermal injection of the tachykinins; substance P (SP, ●), neurokinin A (NKA, ■) and neurokinin B (NKB, ▲) is shown alone (dashed lines) and in the presence of CGRP, 20 pmol per site (solid lines). Results from similar experiments carried out in mepyramine and methysergide pretreated rats are shown by the open symbols. The dotted line represents sites injected with Tyrode alone. The results are expressed as mean values from 9–13 rats and the bars represent s.e.mean. * $P < 0.01$, paired t test.

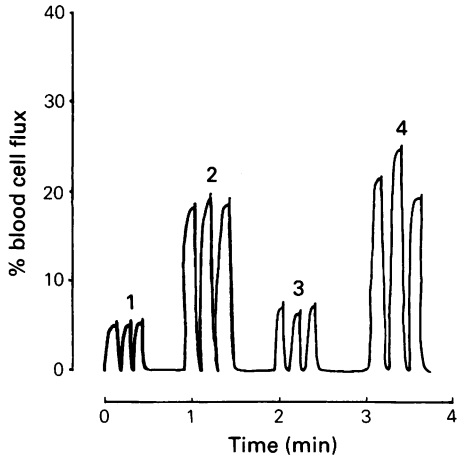

Figure 2 Measurement of blood flow using the laser Doppler blood flow meter. Blood flow was measured 30 min after the injection of either 0.1 ml Tyrode bicarbonate solution at two sites (1 and 3) or CGRP (20 pmol) at two sites (2 and 4) into the rat dorsal skin. Three sequential readings were taken at each site. Changes in blood flow were measured as % blood cell flux (see Methods) with the laser Doppler blood flow meter set at 4 Hz, gain 3. The mean blood flow was calculated from the six readings obtained from the two replicates for each sample.

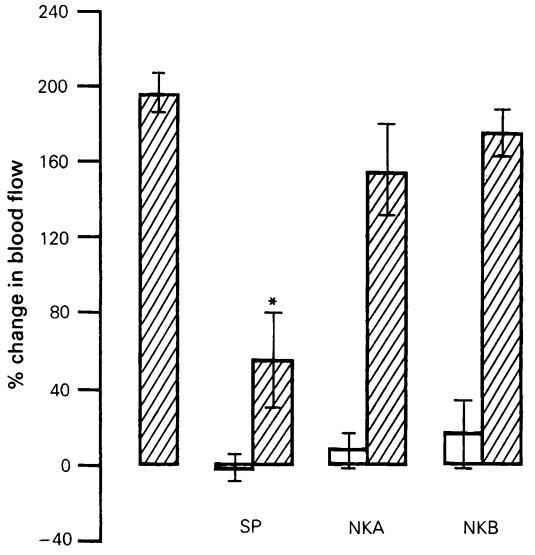

Figure 3 Effect of tachykinins on blood flow induced by calcitonin gene-related peptide (CGRP) in rat skin. The percentage increase in blood flow at sites injected with CGRP (20 pmol) when compared with blood flow at Tyrode-injected sites is shown by the hatched columns. The effect of tachykinins alone is shown by the open columns. The hatched columns show the effect of CGRP alone and in the presence of 100 pmol of each substance P (SP), neurokinin A (NKA) and neurokinin B (NKB). Results are expressed as the mean with bars representing s.e.mean, $n = 6$ rats. $^*P < 0.01$, paired t test.

paired t test, $n = 6$) when compared with blood flow at a site which received 0.1 ml Tyrode solution. In contrast neither substance P nor the neurokinins, at doses up to 100 pmol, caused any significant increase in blood flow. In experiments where the effect of the tachykinins on blood flow induced by CGRP was investigated, readings were taken 30 min after intradermal injections. As shown in Figure 3, blood flow induced by 20 pmol of CGRP was increased by a mean value of 200% above blood flow at Tyrode-injected sites. The injection of substance P (100 pmol) with CGRP caused a loss of the vasodilator activity of CGRP, as shown in Figure 3. In contrast, similar doses of either neurokinin A or neurokinin B did not inhibit the vasodilator activity of CGRP.

It is possible that the measurement of blood flow in skin with a laser Doppler blood flow meter could be distorted at oedematous sites because of the increased extravascular space between capillaries. However, the oedema induced by the tachykinins (100 pmol), when injected in the presence of CGRP (20 pmol), was similar in these experiments (substance P $81.7 \pm 10.1\,\mu l$, neurokinin A $71.0 \pm 14.6\,\mu l$ and neurokinin B $82.8 \pm 7.0\,\mu l$, mean $\pm$ s.e.mean of 6 experiments). For this reason it is unlikely that the loss of the vasodilator activity of CGRP, as measured by the laser Doppler blood flow meter, is due to an inability of the laser Doppler blood flow meter to measure blood flow at oedematous sites.

Discussion

The results show that the effects of substance P on microvascular tone and permeability *in vivo* are different from those induced by neurokinin A and B. Antagonists of histamine and 5-hydroxytryptamine partially inhibited oedema induced by substance P but not that induced by the neurokinins. This indicates that substance P-induced oedema in rat skin is partially dependent on the stimulation of mast cell amine release, which is mediated via the N-terminal amino acids of substance P (Foreman *et al.*, 1983).

Our previous experiments have shown that CGRP acts in a synergistic manner to potentiate oedema induced by mediators of increased microvascular permeability, such as substance P (Brain & Williams, 1985). This potentiating activity of CGRP is a dose-dependent effect which correlates with its potent vasodilator activity as does the potentiating activity of other vasodilators, such as the prostaglandins

(Williams & Morley, 1973; Williams & Peck, 1977). CGRP, at the doses used in these experiments did not increase microvascular permeability when injected alone. At very high doses, CGRP has been shown to cause a wheal and flare in human skin, due to histamine release (Brain *et al.*, 1985; Piotrowski & Foreman, 1986). However, CGRP is far more potent as a vasodilator than in stimulating histamine release from mast cells (Brain *et al.*, 1985). In this study, CGRP potentiated oedema induced by the neurokinins in both the presence and absence of the mast cell amine antagonists. Therefore it is unlikely that the CGRP-induced release of mast cell amines is involved in the potentiation of tachykinin-induced oedema by CGRP.

The loss of the prolonged vasodilator activity of CGRP at substance P-injected sites is striking and is dependent on the concentration of substance P injected (Brain & Williams, 1988). A similar loss of the vasodilator activity of CGRP was observed in human skin at sites injected with substance P (Brain & Williams, 1988). As neurokinins A and B did not affect the vasodilator activity of CGRP, it is tempting to speculate that the effect of substance P is a consequence of mast cell activation. However, the experiments were performed in rats pretreated with mepyramine and methysergide, suggesting that the mechanism involved in the loss of CGRP-induced vasodilator activity is independent of mast cell amine release.

Degranulation of mast cells leads to the activation of mast cell-derived proteases. The major mast cell proteases are tryptase and chymotryptase. Experiments with trypsin and chymotrypsin, both from bovine pancreas, have revealed that both proteases cleave CGRP to fragments which are inactive as vasodilators (Brain & Williams, 1988). Mast cell proteases are capable of inactivating a variety of peptides *in vitro* which apart from CGRP, include substance P, vasoactive intestinal peptide (Caughey *et al.*, 1988) and the complement fragment C3a (Gervasoni *et al.*, 1986). An effect of mast cell-derived proteases *in vivo* has been established by the finding that compound 48/80, which activates mast cells, caused a loss of the vasodilator activity of CGRP (Brain & Williams, 1988). This was inhibited when the experiments were carried out in the presence of soya bean trypsin inhibitor. Therefore we suggest that substance P-stimulated rat skin mast cells release proteases which degrade CGRP and thus terminate its vasodilator activity. The present experiments show that CGRP potentiated oedema induced by substance P and the neurokinins in a similar dose-dependent manner, i.e. irrespective of whether the CGRP prematurely lost its activity due to proteolysis. As substance P is active for only a short period after injection into rat skin the results suggest that CGRP is active as a vasodilator over the short period that substance P is increasing microvascular permeability.

In conclusion, by studying interactions between the tachykinins and CGRP, peptides which are often co-localized in sensory nerve terminals, we have observed two distinct mechanisms by which substance P and CGRP can interact to cause a modified effector response. Firstly, CGRP, as a consequence of its vasodilator activity, potentiated oedema induced by all the tachykinins. Secondly, substance P caused a loss of the prolonged vasodilator activity of CGRP, as discussed above. Further, Le Greves and co-workers (1985) have shown that CGRP *in vitro* inhibits the breakdown of substance P by an endopeptidase isolated from human cerebrospinal fluid. Thus if CGRP and substance P are released together from sensory nerve endings a series of interactions between the two peptides can occur leading to modified microvascular responses. These findings highlight the complex nature of the sensory nervous system. However, it is only when interactions between neuropeptides are fully understood that the precise role of neuropeptides in inflammatory conditions can be established.

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The effects of calcium antagonists on calcium overload contractures in embryonic chick myocytes induced by ouabain and veratrine

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1 The protective effects of some calcium antagonists against different forms of calcium overload contracture were investigated in embryonic chick cardiac myocytes.

2 Tetrodotoxin-sensitive sodium currents were recorded from the myocytes by the whole-cell voltage-clamp technique. Although the peak current was attenuated by veratrine, the inactivation process was markedly inhibited, resulting in a large increase in the total inward current. Action potentials were prolonged by veratrine, automaticity was inhibited and the membrane potential depolarized from −79 to around −45 mV.

3 Measurements of contraction were made from aggregates of myocytes using a video edge detection technique which quantified edge movement. Veratrine caused an initial positive inotropism then inhibited automaticity of aggregates with subsequent development of a tonic contracture to around 300% of the twitch contraction.

4 Veratrine-induced contractures were not significantly affected by 10 μM diltiazem or verapamil. Nifedipine (5 μM), nimodipine (5 μM) and ryanodine (5 μM) also had little effect whilst nicardipine and flunarizine caused a concentration-dependent inhibition of veratrine-induced contractures with IC_{50}s of 3 μM and 2 μM respectively.

5 Veratrine-induced contractures were found to be very sensitive to extracellular calcium concentration with an EC_{50} of 32 μM. Edge movement associated with beating of the myocytes was much less sensitive to calcium (EC_{50} = 1 mM). Submaximal veratrine contractures in 20–50 μM extracellular calcium were not potentiated by 1 μM Bay K 8644.

6 Tetrodotoxin also inhibited veratrine-induced contractures but did not affect contractions induced by ouabain in the presence of 10 μM diltiazem.

7 Ouabain-induced contractures were also inhibited by nicardipine and flunarizine indicating that these drugs can protect against calcium overload in embryonic chick heart by a mechanism independent of the normal form of voltage-sensitive sodium or calcium channels.

Introduction

The level of intracellular calcium in quiescent 13 day-old embryonic chick myocytes has been shown to be around 105 nM (Kim & Smith, 1988). These low levels are maintained by sequestration into intracellular stores such as mitochondria and the sarcoplasmic reticulum and extrusion from the cell, predominantly by the sodium-calcium exchanger (Barry *et al.*, 1985). Since the activity of this exchange mechanism is dependent on the gradient of sodium across the plasma membrane, its ability to extrude calcium is dependent on maintaining a low level of intracellular sodium. Intracellular sodium in myocytes is normally 10 mM at rest (Chapman & Rodrigo, 1987) and this can increase with contractile activity to 20 mM (Mullins, 1981). Under conditions of ischaemia where the ATP supply is compromised and the activity of the Na-K-ATPase reduced it has been shown that intracellular sodium levels rise (Turnstall *et al.*, 1986). This will result in a reduction of the sodium gradient across the cell membrane and thus a reduction of extrusion of calcium by the calcium-sodium exchanger. Such a process may lead to calcium overload which is known to result in cell death (Allen *et al.*, 1985). Thus the basic process preceding calcium overload may be sodium overload of the cell. We were interested in the effect of calcium antagonists on calcium overload caused by sodium overload and have followed up the work of Ver

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Donk *et al.* (1986) who caused rounding up of rat myocytes with veratrine, a mixture of the ceveratrum alkaloids which are known to inhibit sodium channel inactivation and thus increase membrane sodium permeability in nerve (Ulbricht, 1969) and heart muscle (Horakova & Vassort, 1973; see Honerjäger, 1982 for a review). Sperelakis & Pappano (1969) and later Romey *et al.* (1980) showed that veratrine and veratridine (the active component of veratrine) caused a depolarization and cessation of beating in chick myocytes. Ver Donk *et al.* (1986) demonstrated selective protection against veratrine-induced rounding-up of myocytes by calcium antagonists. Embryonic chick myocytes have been used in the study of calcium transport and homeostatic mechanisms. In common with other heart muscle these cells have two types of calcium current (Anderson *et al.*, 1988; Anderson & Patmore, 1988) and have been extensively used in the study of cardiac ionic transport processes. Aggregates of embryonic chick myocytes display automaticity and develop a 'tone' which increases when the rate of beating is increased or in the presence of positive inotropic agents and is decreased if automaticity is abolished, if extracellular calcium is reduced or in the presence of calcium antagonists (Patmore & Whiting, 1985; Patmore, 1987). The diastolic tone of these cells thus appears to reflect the level of intracellular calcium which cannot be extruded from the cell or sequestered before the next beat. This preparation is thus a useful model for investigating calcium homeostasis in cardiac cells and we have assumed that the contractile state is a reflection of free intracellular calcium.

In the present work we have looked at the effect of veratrine on contractions of embryonic chick myocytes and give an account of the underlying electrophysiological changes which occur. Sodium overload and subsequent calcium overload of embryonic chick myocytes has also been induced by the cardiac glycoside, ouabain. The effects of several different calcium antagonists on veratrine- and ouabain-induced contractures are described.

Methods

Cell cultures

Hearts were obtained from 11 day old Leghorn K chicken embryos by sterile dissection. Cell dispersion and culture techniques were as described by Clusin (1981). Briefly, ventricular fragments were washed in Hanks calcium- and magnesium-free balanced salt solution (GIBCO) and thereafter in 0.05% trypsin solution. This digestion process was repeated 3–4 times and trypsinisation was terminated after 5 min by dilution of the cells in an equivalent volume of Hanks balanced salt solution containing 20% foetal calf serum. The resulting cell suspension was pooled, centrifuged and the pellet resuspended in culture medium to give a final cell density of 3–4 $\times 10^5$ cells ml^{-1}. This cell suspension was then dispensed into 96 well trays (Costar), the bottom of each well having been previously coated with a layer of Sylgard 184 (Dow Corning) silicon elastomer resin. This treatment prevents the formation of monolayers and promotes the aggregation of cells into spherical clusters (80–160 μm diameter) over 3 days in culture. The culture medium contained 20% M-199 (GIBCO), 6% heat-inactivated foetal calf serum (Northumbria Biologicals), 1% penicillin-streptomycin solution and 73% potassium-free salt solution which was composed of (mM): NaCl 116, $NaHCO_3$ 26.2, $MgSO_4$ 0.8, NaH_2PO_4 0.9, $CaCl_2$ 1.8 and dextrose 5.5, adjusted to pH 7.3.

Solutions

The perfusion solution was designed to resemble the ionic composition of the culture media and contained (mM): NaCl 110, KCl 1, $MgSO_4$ 0.8, HEPES 19.3, $NaHCO_3$ 0.83 and dextrose 5.5, pH was adjusted to 7.4. The calcium concentration was routinely 2 mM $CaCl_2$. In some experiments the calcium concentrations were varied by either adding more $CaCl_2$ or buffering the calcium with EGTA. Free calcium concentrations were calculated taking into account the ionic effects of the salt solution, temperature and pH on the dissociation constant for EGTA according to Miller & Smith, 1984 and Miller, D.J. (personal communication) ($-\log K_{app} = 6.44$). In some experiments the superfusate Na level was also raised to 150 mM by adding more NaCl to increase the extra/intracellular gradient for sodium ions. When 40 mM TMA was added in place of sodium no osmotic contracture was observed.

Electrical measurements

Whole-cell voltage-clamp recordings were made on single myocytes which were typically around 5 μm wide and 20 μm in length. Pipettes were made from Garner glass (Corning No. 7052) and were fire polished and filled with a solution containing (mM): KCl 140, NaCl 10, $MgCl_2$ 1, EGTA 11, $CaCl_2$ 2.43, HEPES 10, glucose 5 at pH 7.2. Experiments were carried out at room temperature and under these conditions the free calcium ion concentration was estimated to be 10^{-7} M. Electrodes had resistances of 2–3 MΩ. Recordings were made according to the methods of Hamill *et al.* (1981) using an EPC-7 amplifier (List Electronics, FRG). Both current clamp and voltage clamp recordings were made.

Intracellular recordings were made from aggregates of embryonic chick myocytes which were prepared as described above. Micropipettes were pulled from 1.5 mm o.d. borosilicate glass and filled with 3 M KCl. Differential recordings were made with reference to a bath micropipette using a high impedence differential amplifier (Firbank Electronics, Norwich). Electrical signals were recorded on a galvanometric chart recorder.

Assessment of contractility

Contractility was estimated by measuring edge movement of aggregates of embryonic chick heart cells. Aggregates were initially grown on silicon elastomer and then transferred to normal tissue culture treated Lux select-a-well plates. After a period of 10–15 min the aggregates developed contacts with the dish such that they could be superfused quite rapidly. The wells contained approximately 200 μl of fluid and were superfused at a rate of 2.5 ml min^{-1} with salt solution prewarmed to 37°C. The well was mounted on the stage of an inverted microscope with a video camera (National Panasonic WV1500) attached to the camera port. A video image of an aggregate of cells magnified to 200 × was displayed on a monitor screen. An area of the aggregate which contained an edge which displayed significant movement was selected for analysis using a video motion detection system (Tephcotronics Ltd, Edinburgh). A window was superimposed on the image (see Patmore & Whiting, 1985), this was typically one-third the width of the screen and 20–30 video lines high. Each of these lines was scanned to determine the point where the image changed from light (background) to dark (image) and the area of the window occupied by the aggregate calculated by integration and updated every 20 ms. On contraction the area of window occupied by the aggregate changes and an analogue output of this area describes the waveform of edge movement of the aggregate which was typically 2–5 μm. Responses were recorded on a potentiometric chart recorder and in some cases recorded on magnetic tape for reproduction. Contractures or relaxations of aggregates are expressed as % of the twitch edge movement of an individual aggregate.

Drugs

Tetrodotoxin, veratrine, veratridine, ouabain (strophanthin G), diltiazem, verapamil and flunarizine were all obtained from Sigma and dissolved in distilled water. Nicardipine (Syntex), nifedipine and nimodipine (Bayer) were dissolved in ethanol. Concentrated stock solutions of all drugs were prepared and aliquots were added to the salt solution to achieve the concentrations required. Final ethanol levels were always less than 0.1%.

Statistical analyses

The effects of drugs on veratrine- or ouabain-induced contractures were analysed for significance by an unpaired Student's t test. The control group were responses obtained in the absence of drug on the same day. Data shown are mean values ± s.e. of mean for n experiments as shown.

Results

Effects of veratrine on sodium currents and action potentials

When single myocytes were held under voltage clamp at potentials more negative than −60 mV, applying step depolarizations evoked inward currents. Peak inward currents were observed with steps to around −35 mV. The action of veratrine on the inward current is shown in Figure 1A. In the presence of 100 μg ml^{-1} veratrine the peak inward current is attenuated and the inactivation process is inhibited. Under control conditions the sodium currents inactivated rapidly with a $t_{1/2} = 1.0 \pm 0.1$ ms ($n = 3$) and this process could be fitted by two exponentials with time constants of 0.85 ms and 5 ms. In the presence of veratrine there was an initial rapid inactivation but the second slower (5 ms) inactivation process was inhibited and this resulted in a very slow decay of the current which was not complete after 300 ms and produced a large increase in the total inward current. This effect is shown quite clearly on a longer time-scale in Figure 1B. Subsequent application of tetrodotoxin abolished the inward currents indicating that they were carried by sodium ions (Figure 1A). The effects of veratrine on the action potential are shown in Figure 1C. This cell was held under both voltage-clamp and current-clamp. In the current clamp mode the membrane potential, initially −64 mV, was held at −85 mV and small depolarizing current pulses of 1 ms duration were applied which evoked action potentials. Under control conditions the action potentials were 100 mV magnitude and around 70 ms long (duration to 90% repolarization). In the presence of veratrine the peak potential was reduced and the duration greatly increased such that complete repolarization took almost 1000 ms.

Effects of veratrine on aggregates of myocytes

Aggregates of embryonic chick myocytes display automaticity with a beating rate of 60–115

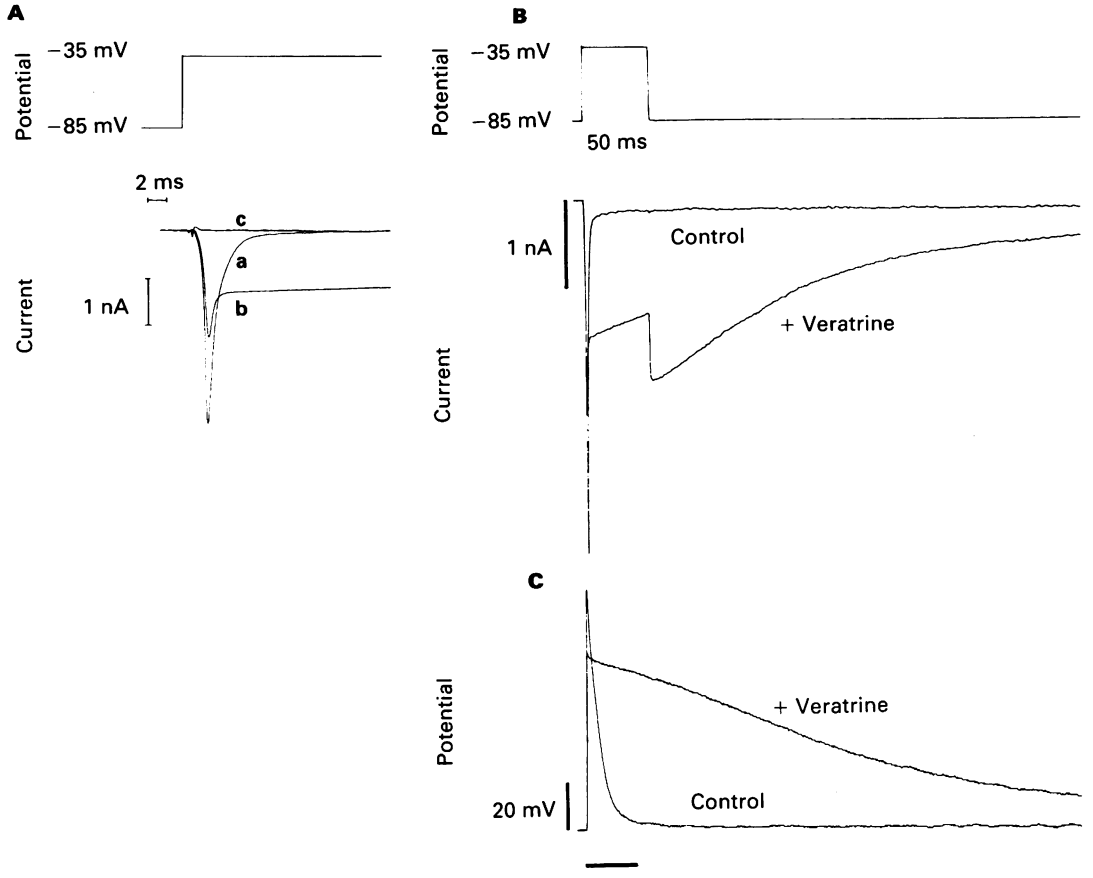

Figure 1 The effects of veratrine on sodium currents and action potentials in embryonic chick heart myocytes. (A) Averaged records of 4 current responses evoked from depolarizations from −85 mV to −35 mV, (a) control, (b) in the presence of 100 $\mu g\,ml^{-1}$ veratrine, (c) with subsequent application of 1 μM tetrodotoxin (TTX). (B) Inward sodium currents on a slower timebase showing the very slow inactivation of the sodium current during the clamp pulse and then on repolarization a slowly decaying tail current. (C) In the same cell switched into current clamp with the membrane potential held at −85 mV, small depolarizing current pulses of 1 ms duration evoked action potentials. Action potential duration is prolonged in the presence of veratrine.

(mean = 107 ± 4, $n = 20$) beats min^{-1}. Intracellular recordings from these aggregates showed maximum diastolic potentials of −79 ± 4 mV and peak potentials of around 20 mV. Veratrine caused a progressive prolongation of the action potential and a concomitant depolarization until automaticity was abolished and the membrane potential stabilised at −45 ± 10 mV ($n = 3$). Simultaneous recordings of edge movement were made from a few cells and these changes in electrical behaviour were paralleled by an initial positive inotropism, a transient relaxation as beating ceased and then a slowly developing tonic contracture as shown in Figure 2(a). The magnitude of these contractures was 293 ± 11% ($n = 118$) of the initial twitch edge movement.

Effects of calcium antagonists on veratrine-induced contractures

The pharmacological sensitivity of these tonic contractures is of interest. Ver Donk *et al.* (1986) have shown that only certain calcium antagonists protected against veratrine-induced rounding-up of rat myocytes. We found similar differences between calcium antagonists. The effects of the dihydropyridine calcium antagonists nifedipine and nicardipine are shown in Figure 2. Pretreatment with nicardipine, but not nifedipine prevented veratrine-induced contraction. The effects of a range of calcium antagonists from the different subgroups (diltiazem, verapamil, nifedipine, nimodipine, nicardipine and

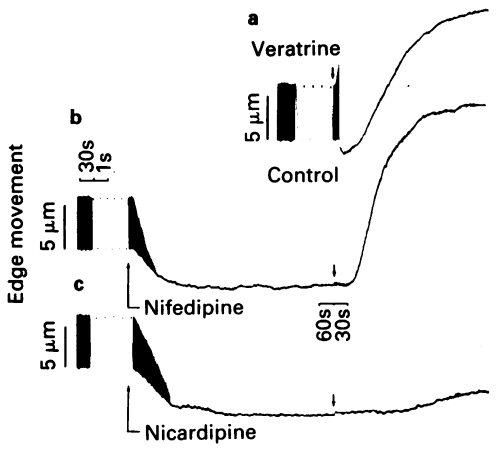

Figure 2 Effects of calcium antagonists on veratrine-induced contractures of embryonic chick myocytes. (a) Effect of veratrine ($100\,\mu g\,ml^{-1}$) on edge movement recorded from an aggregate of embryonic chick myocytes. Effect of pretreatment for 10 min with (b) $5\,\mu M$ nifedipine and (c) $5\,\mu M$ nicardipine on veratrine-induced tonic contractures. Expanded sections are all calibrated by the 1s bar.

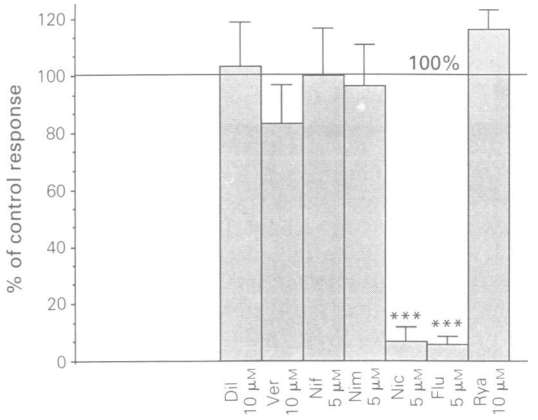

Figure 3 Effects of calcium antagonists on veratrine-induced contractions of embryonic chick myocytes. Responses to veratrine in the presence of calcium antagonists have been expressed as a percentage of the control response to veratrine obtained in parallel experiments on the same day. Aggregates were exposed to drugs for 10 min before application of veratrine. Data shown are mean values, with vertical lines indicating s.e.mean, for n experiments. Diltiazem (Dil) ($n = 6$), verapamil (Ver) ($n = 6$), nifedipine (Nif) ($n = 11$), nimodipine (Nim) ($n = 4$), nicardipine (Nic) ($n = 5$), ryanodine (Rya) ($n = 5$). Statistical significance is denoted as *** for $P \leqslant 0.001$ where drug-treated and control groups were compared using an unpaired Student's t test.

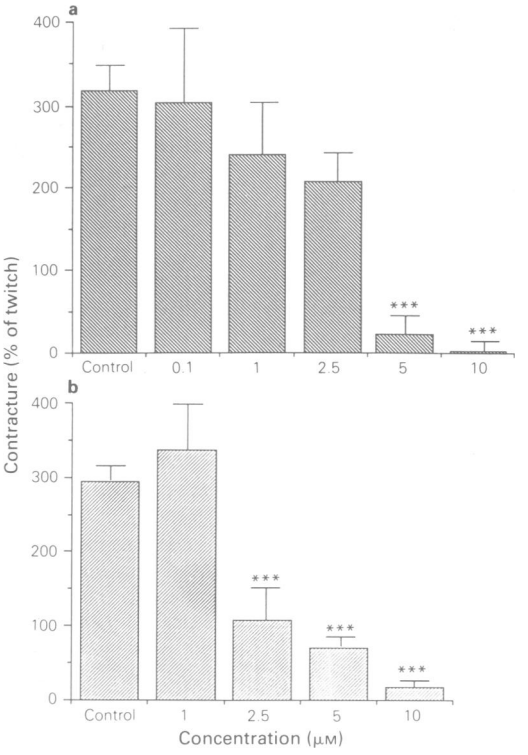

Figure 4 Concentration-response relationships for the effects of (a) nicardipine and (b) flunarizine on veratrine-induced contractions of embryonic chick myocytes. Responses are calculated as a % of the twitch response and values are expressed as mean, with vertical lines indicating s.e.mean; (a) $n = 4$–5, (b) $n = 4$. Contractures in the presence of the drugs were compared with control groups by use of an unpaired Student's t test. Significant differences from control groups are indicated by *** where $P \leqslant 0.001$.

flunarizine) are shown in Figure 3. Relatively high concentrations of these drugs in relation to their calcium channel blocking activity were chosen. Diltiazem ($10\,\mu M$), verapamil ($10\,\mu M$), nifedipine ($5\,\mu M$) and nimodipine ($5\,\mu M$) had no significant effect on veratrine-induced contractures. The inhibitory effects of nicardipine were concentration-dependent (Figure 4a) and were statistically significant at 5 and $10\,\mu M$ with an IC_{50} of $3\,\mu M$. Similar protection against veratrine-induced contracture was observed with the diphenylalkylamine calcium antagonist flunarizine. This compound significantly reduced contractures at concentrations between 2.5 and $10\,\mu M$ and had an IC_{50} of $2\,\mu M$ (Figure 4b). Calcium antagonists of different subgroups (Spedding, 1985) have different interactions with the calcium channel facilitator Bay K 8644 in smooth muscle (Spedding & Berg, 1984),

in embryonic chick heart (Patmore & Duncan, 1988) and rabbit heart (Bodekke *et al.*, 1987). These authors have found that the inhibitory effects of the class I (dihydropyridines) and class II (verapamil and diltiazem) calcium antagonists can be reversed by Bay K 8644. Bay K 8644 did not reverse the effects of the class III diphenylalkylamine calcium antagonists. This site of action of the class III drugs may be of importance in the protection against veratrine-induced contractions.

Calcium sensitivity of veratrine-induced contractures

The twitch edge movement of embryonic chick myocyte aggregates is near maximal in 2 mM calcium and several authors have reduced extracellular calcium in order to observe positive inotropic responses to drugs (Laurent *et al.*, 1985; Duncan *et al.*, 1987). The relationship between extracellular calcium levels and twitch edge movement is shown in Figure 5. An EC_{50} value of 1 mM was calculated. The sensitivity of veratrine-induced contractures to external calcium is somewhat different. These contractures are very sensitive to extracellular calcium with an EC_{50} of 32 μM. In the presence of submaximal concentrations of calcium (20–50 μM) the dihydropyridine calcium facilitator Bay K 8644 at

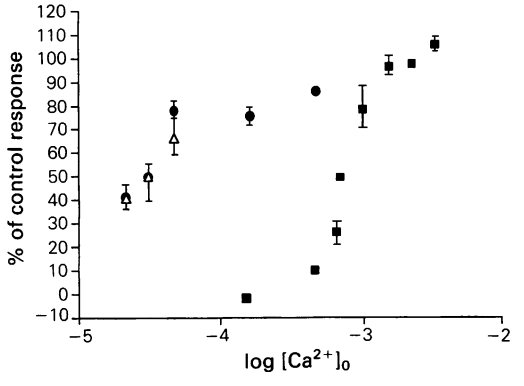

Figure 5 Calcium dependence of twitch and veratrine-induced contractions. The magnitude of the twitch response (■) has been expressed as a % of the control response in normal (2 mM) extracellular calcium. An EC_{50} of 1 mM was calculated from these data. Veratrine-induced contractures (●) are much more sensitive to extracellular calcium with an EC_{50} of 32 μM. At submaximal concentrations of calcium (20–50 μM), application of 1 μM Bay K 8644 (△) did not significantly increase contractures. Data shown are means, with vertical lines indicating s.e.mean, for $n = 5$–8 determinations for the twitch response and $n = 3$–7 determinations for the veratrine (and Bay K 8644) responses.

10^{-6} M, a concentration which is positively inotropic in these cells (Patmore & Whiting, 1985), had no effect on the veratrine-induced contracture (Figure 5). The possibility that calcium from intracellular stores such as the sarcoplasmic reticulum (SR) is involved in veratrine-induced contractures was explored by treatment of aggregates with the intracellular calcium antagonist ryanodine which has been shown to block release of SR calcium (Sutko & Willerson, 1980; Mitchell *et al.*, 1984). Ryanodine (10^{-5} M) inhibited twitch edge movement by 65 ± 3% ($n = 4$). Similar effects were observed by Rasmussen *et al.* (1987). Subsequent application of veratrine caused contractures to 355% of the twitch magnitude which was not significantly different from those obtained under control conditions (Figure 3). A direct interaction at the sodium channel could explain the effects of these drugs. We therefore investigated the actions of the sodium channel blocker tetrodotoxin (TTX) on veratrine-induced contractures. The effects of adding 1 μM TTX to the superfusate 2 min before exposure to veratrine and up to 90 s after are shown in Figure 6. TTX applied before veratrine inhibited beating in these aggregates and blocked the contracture. Interestingly, even when applied up to 90 s after veratrine, TTX relaxed the veratrine-induced contracture.

Application of 5 μM nicardipine 60 s after exposure to veratrine failed to relax the already well established contracture (not shown). However, these data do not unequivocally show that the action of nicardipine was not at the sodium channel since there may be differences in the access time of nicardipine and TTX to the sodium channel. In order to differentiate between protection against veratrine-induced contraction and sodium channel blockade by these drugs we investigated the effects of nicardipine and flunarizine against ouabain-induced contractures.

Effects of calcium antagonists on ouabain-induced contractures

In embryonic chick myocytes ouabain causes a depolarization, arrest of automaticity and a small tonic contracture (Koidl & Tritthart, 1980; Hasin & Barry, 1984; Nathan & Bhattacharya, 1984). We found similar responses in our preparations. Inhibition of twitch contractions of aggregates of myocytes with diltiazem and subsequent exposure to ouabain caused a larger contracture of around 150% of the original twitch magnitude. These responses were not affected by 1 μM TTX and thus appear to be independent of TTX-sensitive, voltage-dependent sodium channels. The contracture evoked by ouabain was only about half of that induced by veratrine and somewhat variable. To enhance the rise of intracellular sodium caused by ouabain-induced inhibition of

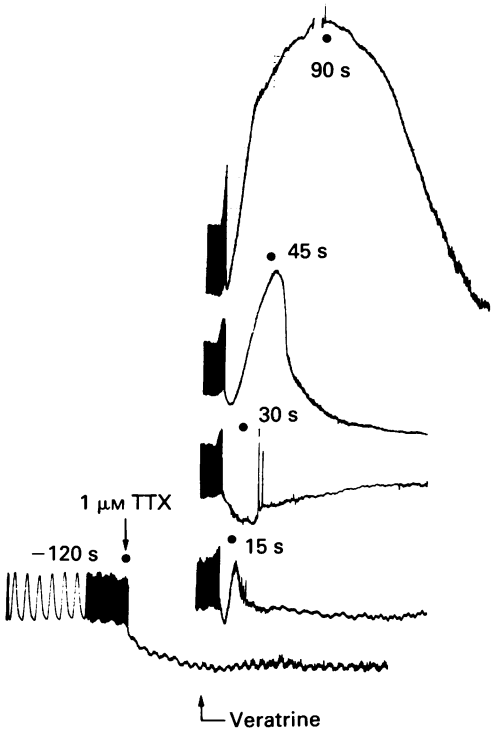

Figure 6 Effects of tetrodotoxin (TTX) on veratrine-induced contractures of embryonic chick myocytes. TTX (1 μM) was applied to the superfusate at times shown by the closed circles before or after application of 100 μg ml^{-1} veratrine. Note the break in the top trace.

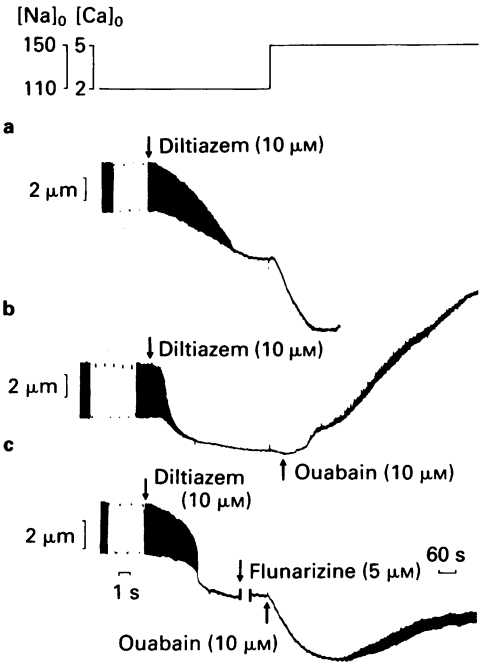

Figure 7 Effects of calcium antagonists on ouabain-induced contractures of embryonic chick myocytes. In all the records the sodium and calcium concentrations of the superfusate were increased as shown in the top panel. The twitch contracture was inhibited by application of 10 μM diltiazem. In (a) increasing extracellular sodium and calcium resulted in a relaxation of the aggregate. (b) Simultaneous application of ouabain (10 μM strophanthin G) caused a slowly developing tonic contracture. (c) Incubation with 5 μM flunarizine for 10 min before ouabain treatment inhibited the tonic contracture. Similar responses were obtained with 5 μM nicardipine. Note the break in the record in (c) for 8.5 min.

the Na-K-ATPase we increased the level of extracellular sodium. The contracture caused by ouabain can be explained by a reduction in the sodium gradient (due to an increased level of intracellular sodium) which drives the sodium-calcium exchange mechanism which normally extrudes calcium from the cell. Thus to maximize the contracture to ouabain we also increased extracellular calcium. In all experiments the twitch contractures were inhibited by 10 μM diltiazem which caused a relaxation of the tone of the aggregate. Raising calcium from 2 to 5 mM had little effect on tone. Interestingly when extracellular sodium was also increased from 110 to 150 mM the aggregate relaxed by 87 $\pm$ 36% ($n = 8$) of the twitch contracture (Figure 7a). Application of 10 μM ouabain in the presence of raised calcium and sodium caused a slowly developing tonic contracture of 266 $\pm$ 38% ($n = 37$) of the twitch magnitude (Figure 7b) and is comparable to that attained with veratrine. Diltiazem (10 μM) or verapamil (10 μM) had no effect on these contractures and responses to ouabain obtained in the presence of 1 μM TTX to inhibit the twitch were not significantly different from those obtained in the presence of diltiazem. Nicardipine and flunarizine both inhibited ouabain contractures (Figure 7c and Figure 8). Responses were not significantly different from those obtained to 150 mM Na and 5 mM Ca in the absence of ouabain, but were significantly different at the $P < 0.01$ level when compared with the control ouabain contracture.

Time-dependence of drug application

It is interesting to examine whether nicardipine and flunarizine can inhibit ouabain-induced contractures if applied after ouabain. Whilst established veratrine-induced contractures were relaxed by TTX, we found

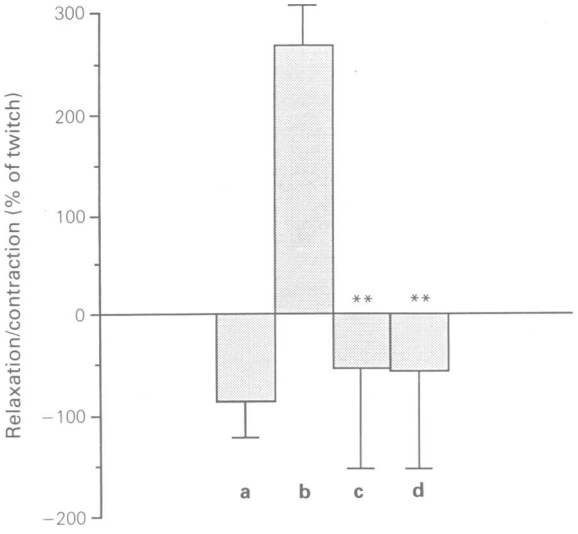

Figure 8 Effects of nicardipine and flunarizine on ouabain-induced contractures of myocyte aggregates. Responses are expressed as a % of the twitch contraction and mean values are shown, with vertical lines indicating s.e.mean. Aggregates were treated with 10 μM diltiazem to suppress beating. Increasing extracellular sodium from 110 to 150 mM and extracellular calcium from 2 to 5 mM (150 Na/5 Ca) caused a relaxation ($n = 8$). (a) Simultaneous application of ouabain (10 μM strophanthin G) caused a contraction to around 266 $\pm$ 38% of the twitch ($n = 37$). (b) Pretreatment for 10 min with 5 μM nicardipine (Nic) ($n = 11$) (c) or 5 μM flunarizine (Flu) ($n = 10$) (d) inhibited contraction to ouabain. Statistical significance is denoted by ** where $P \leqslant 0.01$ when compared with control (ouabain alone) responses.

that nicardipine did not relax established veratrine-induced contractures. To investigate the time-dependence of drug application, we applied ouabain to aggregates in low calcium solutions ($[Ca^{2+}]_0 = 10^{-9}$ M). Aggregates were pretreated with 10 μM diltiazem to inhibit the twitch contracture. Under these circumstances, the aggregates remained relaxed. After 10 min nicardipine was added to the superfusate and a further 10 min later calcium was readmitted. Rapidly developing contractures to 293 $\pm$ 41% ($n = 6$) of the twitch magnitude were observed (Figure 9). These responses were not significantly different from those obtained after 20 min superfusion with ouabain alone (248 $\pm$ 26%) ($n = 6$)).

Discussion

The action of the veratrine on contractility and ionic currents of heart muscle has been well documented and has been reported to inhibit the inactivation of the inward sodium current (see Honerjäger, 1982 for a review). In embryonic chick myocytes we have shown that veratrine also markedly slows the inactivation of the TTX-sensitive sodium current and inhibits one of the two processes which contribute to the bi-exponential decay of the sodium current. This results in an increase in the total inward sodium current with a concomitant increase in action potential duration and a depolarization of the membrane potential. Fosset *et al.* (1977) have shown that veratridine enhanced ^{22}Na uptake in embryonic chick heart cells, leading to a rise in intracellular sodium and enhanced calcium uptake, similarly Pang & Sperelakis (1982) demonstrated stimulation of calcium uptake by veratridine in chick myocytes. Calcium entry may occur through a number of different mechanisms.

The depolarization caused by sodium entry may increase the membrane permeability to calcium by activating voltage-sensitive calcium channels and these effects will result in a rise in the level of intracellular calcium and a tonic contracture. We find that the role of voltage-sensitive calcium channels in this process is questionable: firstly veratrine-induced contractures are insensitive to the calcium antagonists diltiazem, verapamil, nifedipine and nimodipine at concentrations which greatly exceed their calcium channel blocking activities in this and other tissues (Clarke *et al.*, 1984). Secondly, the sensitivity of veratrine-induced contractures to extracellular calcium and the sensitivity of beating is very different (see Figure 5) with EC_{50} values of 32 μM and 1 mM respectively. Whilst twitch edge movement is dependent on voltage-sensitive calcium channels these differences suggest that veratrine-induced contractures are not dependent on calcium entry through these channels. This idea is further supported by the lack of potentiation of submaximal veratrine-induced contractures in 20–50 μM calcium by the calcium channel facilitator Bay K 8644.

The possibility that the calcium which causes these contractures was released from intracellular stores such as the sarcoplasmic reticulum (SR) has been examined in experiments with ryanodine. Ryanodine has been shown by several authors to block the release of calcium from the SR (Sutko & Willerson, 1980; Clarke & Patmore, 1984). Marban & Wier (1985) have also used ryanodine to determine the contributions from calcium entry and calcium release to contractile calcium. In embryonic chick myocyte aggregates ryanodine (10^{-5} M) reduced contractions by 62% which suggests that almost two thirds of contractile calcium comes from the SR in this tissue. However, ryanodine had no significant effect on subsequent veratrine-induced contractures, indicating that calcium from the SR is not involved.

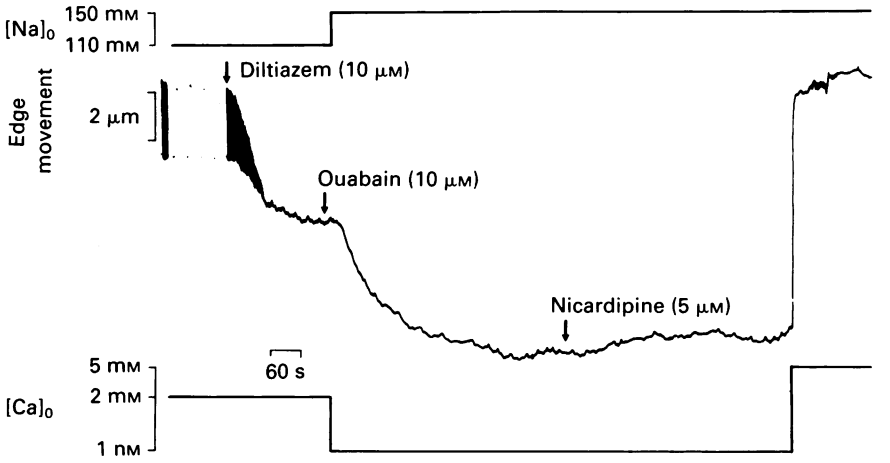

Figure 9 Time dependence of the protection against ouabain-induced contractures by nicardipine. Aggregates were treated with 10 μM diltiazem throughout to suppress twitch activity. Lowering extracellular calcium to approximately 10^{-9} M caused a relaxation. At the same time the aggregate was superfused with 10 μM ouabain and extracellular sodium was raised from 110 to 150 mM. After 10 min 5 μM nicardipine was added to the superfusate and after a further 10 min the calcium concentration was increased to 5 mM which evoked a rapidly developing tonic contracture.

Raised levels of intracellular sodium will cause a reduction in the extrusion of calcium via the calcium-sodium exchanger. The sensitivity of veratrine-induced contractures to extracellular calcium also suggests that the calcium-sodium exchanger may itself contribute to calcium entry by working in the reverse direction extruding sodium ions and allowing calcium to enter. Whilst this would be down the concentration gradient for calcium ions this direction of the exchanger would be against the concentration gradient for sodium and since the exchange process is electrogenic $-3Na^+ : 1Ca^{2+}$ (in the normal calcium extrusion mode positive charge enters the cell), this reverse process would result in the exit of positive charge from the cell which is against the electrochemical gradient. However, whilst the normally highly negative membrane potential of these cells favours the calcium extrusion mode of the exchanger, the depolarization caused by veratrine and the reduction of the sodium gradient may shift the equilibrium between calcium extrusion and calcium entry modes and significantly more time will be spent transporting calcium into the cell. The sodium calcium exchange mechanism has been extensively studied in mammalian ventricular muscle (Miller & Moiescu, 1976) and has been shown to exist in embryonic chick heart (Kim *et al.*, 1987). Of the two drugs which have been shown to inhibit veratrine-induced contractures nicardipine has been found to have no effect on low sodium contractures of embryonic chick myocyte aggregates (Patmore, 1987). It thus seems that calcium must come from another source independent of the calcium sodium-exchanger, the SR or the normal form of voltage-sensitive calcium channels.

The possibility that the protective action of nicardipine and flunarizine against veratrine-induced contractions is a direct action on sodium channels cannot be entirely discounted. We have shown that veratrine-induced contractures are blocked by TTX and can be relaxed after they have already developed by subsequent application of TTX, although nicardipine did not relax established contractures. The protection of these drugs against ouabain-induced contractures which are insensitive to TTX does, however, indicate that nicardipine and flunarizine can protect against calcium overload by a mechanism independent of TTX-sensitive, voltage-dependent sodium channels.

The dependence on time of application of these drugs throws an interesting light on their mechanism of action. If nicardipine was applied before ouabain in the presence of external calcium it prevented contracture. When applied after ouabain in the absence of external calcium nicardipine did not prevent the contracture following calcium re-admission.

This result has several implications: (i) nicardipine does not affect the calcium sensitivity of the contractile proteins or act on the contraction pathway at some point subsequent to a rise in calcium since contractures were of similar magnitude in the presence and absence of nicardipine. Thus it must act on processes which occur before calcium

entry. (ii) These data also provide further clear evidence against a direct effect on the calcium-sodium exchange process operating in the intrusion or calcium entry mode. We therefore suggest that nicardipine and flunarizine block sodium entry or sodium overload which precedes calcium overload.

In guinea-pig myocytes, Chapman & Rodrigo (1987) observed a rise in intracellular sodium from 10.2 to 49.5 mM when exposed to low calcium solutions and on reapplication of calcium the cells irreversibly rounded-up. This rise in intracellular sodium was associated with a prolongation of action potentials and depolarization to −20 mV indicative of increased sodium permeability (Chapman *et al.*, 1984; 1986). When these cells were superfused with low calcium solutions under voltage-clamp, Chapman & Rodrigo (1988) observed the development of a large sustained inward current and they suggested that sodium enters the cells in this process through the voltage-sensitive calcium channels. These phenomena may only occur in very low extracellular calcium and have not been observed in normal calcium solutions (Almers & McCleskey, 1984a, b). However, contractures of myocytes and ventricular fibres have been shown to occur following metabolic blockade (Lederer *et al.*, 1987; Allen *et al.*, 1987) and it is difficult to determine what conditions occur during ischaemia and, thus, whether these may modify the ionic selectivity of the calcium channel. In neuronal tissue modifications of calcium channels to conduct sodium ions have also been described (Carbone & Lux, 1987) in low calcium conditions and by Konnerth *et al.* (1987) on transient acidification of dorsal root ganglion cells. This effect is in response to a step change in pH which is unlikely to occur during ischaemia, although prolonged acidosis may play a role in modifying calcium channels during ischaemia in both heart and cerebral tissue. In both these tissues similar changes in the ionic selectivity of the calcium channel may give rise to sodium entry which may be blocked by nicardipine and this could explain why nicardipine has been shown to be more effective in protecting ischaemic heart and cerebral tissue than other dihydropyridines (Alps *et al.*, 1983; 1988).

Sodium overload associated with ouabain-induced contractures of embryonic chick myocytes will occur due to inhibition of sodium efflux and sodium entry through a transport process independent of the TTX-sensitive sodium channel. One possibility may be sodium entry through voltage-sensitive calcium channels. These may be stimulated to translocate sodium by raised intracellular sodium directly or raised intracellular sodium may reduce the rate of the sodium-hydrogen ion exchanger in these myocytes (Kim & Smith, 1988) which would lead to intracellular acidosis and thus acidosis may modify the selectivity of the calcium channel. Such channels (or states of channels) would be resistant to diltiazem and nifedipine but sensitive to nicardipine and flunarizine. The same process may also contribute in the contractures induced by veratrine even though it is clear that the majority of sodium entry occurs via TTX-sensitive sodium channels. Another possible route for sodium entry may be through non-specific cation channels and it may be at these or at modified voltage-sensitive calcium channels that nicardipine and flunarizine act to prevent sodium overload and thus calcium overload in embryonic chick myocytes. Should non-specific cation channels be involved in calcium entry as well as sodium entry, then one would have to envisage some selectivity in the action of these drugs to block sodium but not calcium entry through these channels.

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Sympathetic denervation caused by long-term noradrenaline infusions; prevention by desipramine and superoxide dismutase

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1 The effects of continuous intravenous infusion of noradrenaline (0.01 and 0.1 $\mu g\,kg^{-1}\,h^{-1}$) were studied in both the infused lateral saphenous vein and the contralateral saphenous vein of normal dogs. Noradrenaline, saline, noradrenaline + desipramine or noradrenaline + superoxide dismutase were infused using Alzet osmotic minipumps.

2 After a 5 day infusion period, the noradrenaline content in plasma and in both saphenous veins was determined, and the venous tissues submitted to light microscope morphometry and ultrastructural study and used for the determination of their *O*-methylation capacity (with [^{3}H]-isoprenaline as a substrate).

3 Noradrenaline caused dose-dependent damage to the sympathetic nerve endings of the lateral saphenous veins. Concomitant changes in extraneuronal structure and function were observed (hypertrophy of smooth muscle cells, nuclear dismorphy, thickening of the vessel wall, impairment in *O*-methylation capacity).

4 Desipramine and superoxide dismutase prevented or reduced the effects of noradrenaline on both the morphological and the biochemical parameters; the protection afforded by superoxide dismutase was more marked than that by desipramine.

5 It is concluded that moderately high doses of noradrenaline exert a 6-hydroxydopamine-like effect and that this chemical sympathectomy is partially or totally prevented by desipramine or superoxide dismutase. The data suggest that a substance derived from noradrenaline, in the formation of which free oxygen radicals are involved and which is subject to neuronal uptake, is the chemical entity responsible for the neurotoxic effect observed.

Introduction

We have found that denervation of the lateral saphenous vein of the dog induces marked morphological changes at the extraneuronal level, as well as an impairment of extraneuronal uptake and metabolism of noradrenaline and isoprenaline (Branco *et al.*, 1984). The same work provided evidence for the inability of infused noradrenaline to prevent the described denervation-induced changes; it was observed that noradrenaline infusion appeared instead to aggravate the effects of denervation.

Therefore, we decided to investigate the effects of noradrenaline infusion on the veins of normal dogs, i.e. on vessels with an intact adrenergic innervation.

Some of the results were presented to the 6th Meeting on Adrenergic Mechanisms (Azevedo *et al.*, 1987).

Methods

Male mongrel dogs (9–15 kg body weight) were anaesthetized with sodium pentobarbitone, 30 $mg\,kg^{-1}$ i.v., injected in the forelimb. After a stabilizing period of 15 min, a blood sample was obtained by venipunction. An incision in the right hindlimb was made and an Alzet 2 ML 1 osmotic

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minipump was implanted in the subcutaneous tissue above the knee and connected through a fine polyethylene catheter to the plantar branch of the saphenous vein and the surgical wound closed. The pump was loaded with sterile saline containing heparin (5000 units) and EDTA (1 mg) for infusion in the control series; in another experimental series, the saline contained heparin and EDTA plus noradrenaline alone or with superoxide dismutase (SOD) or desipramine (DMI). The amounts of noradrenaline, SOD or desipramine were calculated so as to ensure infusion rates of 0.01 or 0.1 $\mu g kg^{-1} h^{-1}$ for noradrenaline, 5 $\mu g kg^{-1} h^{-1}$ for SOD and 25 $\mu g kg^{-1} h^{-1}$ for DMI. This dose of DMI was chosen after preliminary experiments (not described in this paper) with different doses and schedules of administration of DMI. In 3 dogs treated with the above-mentioned dose of DMI the neuronal uptake in the venous tissue (as measured by the amount of noradrenaline accumulating in the tissue after a 60 min incubation with [^{3}H]-noradrenaline 0.2 $\mu mol l^{-1}$) was only 5, 10 and 12% of that found in control animals (no DMI injected). The animals receiving noradrenaline plus DMI infusions additionally received an i.v. injection of DMI 0.25 $mg kg^{-1}$ one hour before the infusion was started. Before implantation, the loaded minipump was immersed in sterile saline at 37°C for 4–6 h to ensure constant flow of the fluid.

Five days later the dogs were anaesthetized with sodium pentobarbitone, as before. A blood sample was obtained from the left lateral saphenous vein 15 min after induction of anaesthesia and the osmotic minipump as well as both saphenous veins removed. The function of the minipump was checked by visual inspection of the catheter (absence of kinks, correct implantation in the plantar branch, absence of formation of clots) and visual inspection of the pump after longitudinal sectioning: lumen collapsed. The remaining fluid was recovered and its volume measured.

The blood samples and fragments of the veins were used for determination of noradrenaline by high performance liquid chromatography with electrochemical detection (h.p.l.c.-e.d.), as described earlier (Branco *et al.*, 1984). The remaining venous tissue was used for morphological studies, using the methods previously described (Azevedo *et al.*, 1981; Branco *et al.*, 1984; Sarmento *et al.*, 1987) for light and ultrastructural microscopy. Briefly, tissue was fixed in glutaraldehyde-osmium and embedded in Epon. Semi-thin sections of 1 μm were prepared and stained with toluidine blue. Camera lucida drawings were used for determination of individual smooth muscle cell diameter. Thin sections of the outer third of the media were cut in a LKB Ultratome, stained with uranyl acetate and lead citrate and observed in a Siemens Elmiskope. For the study of the *O*-methylating capacity of the venous tissue, fragments weighing about 60 mg were incubated in 3 ml of Krebs-Henseleit solution containing [^{3}H]-isoprenaline 0.2 $\mu mol l^{-1}$, at 37°C, with constant shaking, for 30 min. The solution was gassed with 95% O_2 + 5% CO_2 and had the following composition (in $mmol l^{-1}$): NaCl 118, KCl 4.7, $CaCl_2$ 2.6, $MgCl_2$ 1.2, $NaHPO_4$ 1, $NaHCO_3$ 25, glucose 11.1, sodium EDTA 0.04, ascorbic acid 0.11. At the end of the incubation period the tissue was rapidly washed in ice-cold Krebs-Henseleit solution, blotted and weighed. [^{3}H]-isoprenaline and its metabolite [^{3}H]-*O*-methylisoprenaline were separated by column chromatography, as described earlier in detail (Azevedo & Osswald, 1976).

In order to exclude any chemical alteration of noradrenaline in the pump during the five days long infusion, samples of the fluid remaining in the pump (which empties itself in 7 days) were subjected to determination of noradrenaline content by h.p.l.c.-e.d.; no such alteration was found in any of the pumps used.

Statistics

Results are indicated as arithmetic means ± s.d.mean and differences between means were assessed by use of Student's *t* test (unpaired) and considered significant when $P < 0.05$.

Drugs used

(−)-Noradrenaline bitartrate (NA; Koch-Light, Colnbrook, U.K.); desmethylimipramine hydrochloride (Ciba-Geigy, Basle, CH); superoxide dismutase (Peroxinorm, Chemie Grunenthal GmbH, Stolberg, BRD); (±)-7-[^{3}H]-isoprenaline hydrochloride, 9.3 Ci $mmol^{-1}$ (New England Nuclear Chemical, Dreieich, BRD); sodium pentobarbitone (Abbott, Lisboa, P); sodium heparin, USP 5000 u ml^{-1} (Medac, Hamburg, BRD); L-(+)-ascorbic acid (May & Baker, Dagenham, U.K.); ethylenediaminetetraacetic acid disodium salt (EDTA; Sigma, St. Louis MO, U.S.A.).

Results

Morphological results

When observed with the light microscope, the veins in which the infusion of NA was effected showed pronounced differences from control sections. The vein wall was thicker, smooth muscle cells were larger and more irregular, presenting a larger nucleus. Determination of smooth muscle cell dimensions confirmed that NA infusion induced an increase in the diameter of smooth muscle cells,

which attained statistical significance only in the animals infused with the higher dose of noradrenaline (Table 1). Therefore, all further experiments were done with this noradrenaline dose. At the electron microscope level, smooth muscle cells were enlarged, and showed indented nuclei rich in euchromatin and nucleoli (Figure 1); fibroblasts exhibited exuberant nuclei, nucleoli and enlarged rough endoplasmic reticulum (Figure 2); and sympathetic nerves were badly damaged or fully degenerated (Figure 3). The smooth muscle cells and the fibroblasts were very rich in pinocytotic vesicles and exhibited abundant microbodies, most probably peroxisomes. Several mast cells (a cell type absent in control saphenous vein) were observed.

In these dogs, contralateral saphenous vein smooth muscle cells were enlarged in comparison

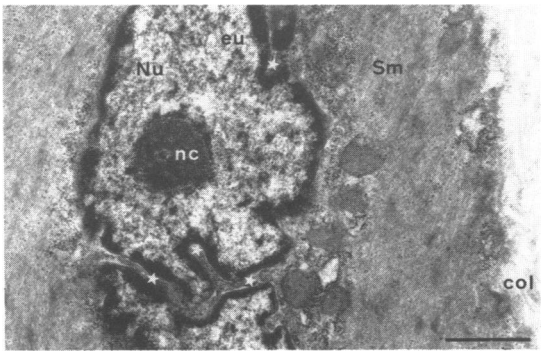

Figure 1 Smooth muscle cell (Sm) in a noradrenaline infused saphenous vein. The nucleus (Nu), rich in euchromatin (eu), exhibits indentations (*) and a prominent nucleolus (nc). col = collagen. Bar = 1 μm.

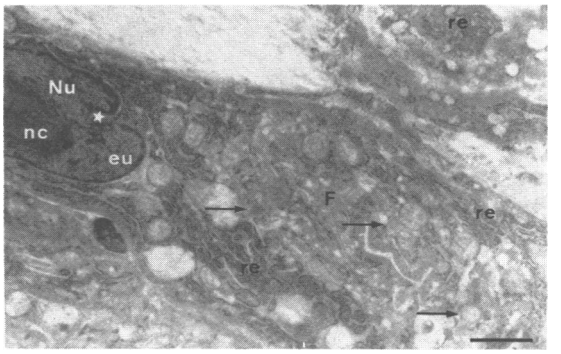

Figure 2 Fibroblast (F) in a noradrenaline-infused saphenous vein. The nucleus (Nu) rich in euchromatin (eu) shows an indentation (*) and a voluminous nucleolus (nc). The cytoplasm exhibits a prominent rough endoplasmic reticulum (re) and several microbodies (arrows). Bar = 1 μm.

with control veins, but significantly less than in the vein where the infusion took place ($P < 0.001$; Table 1). At the electron microscope level, sympathetic nerves showed a marked decrease in the proportion of large dense core vesicles; thus 9.7 $\pm$ 16.1% of all vesicles were large dense core vesicles, against 52.5 $\pm$ 25.0% in the control (75 nerve profiles observed in 5 dogs infused with NA, 110 nerve profiles observed in 7 control dogs).

When desipramine was infused simultaneously with NA, smooth muscle cells of the infused saphenous vein showed a diameter which was not different from that of controls, and significantly smaller than in the veins infused with NA alone (Table 1). At the electron microscope level, desipramine + NA infused

Table 1 Diameter of smooth muscle cells and of the nuclei of fibroblasts in dog saphenous veins under control conditions or after 5 days infusion of noradrenaline (NA), NA + desipramine (DMI) or NA + superoxide dismutase (SOD)

Drug infused		*Smooth muscle cell diameter* (μm)	*Fibroblast nuclei diameter* (μm)	n
Control (vehicle)	Inf. vein	6.2 $\pm$ 0.8	3.9 $\pm$ 1.0	4
	Contr. vein	6.1 $\pm$ 0.7	3.8 $\pm$ 0.9	
NA (0.01 $\mu g kg^{-1} h^{-1}$)	Inf. vein	7.8 $\pm$ 1.2	4.5 $\pm$ 1.2	2
	Contr. vein	7.3 $\pm$ 0.7	4.2 $\pm$ 1.5	
NA (0.1 $\mu g kg^{-1} h^{-1}$)	Inf. vein	9.8 $\pm$ 1.3*	6.2 $\pm$ 1.6*	3
	Contr. vein	8.7 $\pm$ 1.3*	4.0 $\pm$ 0.9	
NA (0.1 $\mu g kg^{-1} h^{-1}$) + DMI (25 $\mu g kg^{-1} h^{-1}$)	Inf. vein	6.1 $\pm$ 0.4	4.8 $\pm$ 1.2	4
	Contr. vein	6.0 $\pm$ 0.8	3.9 $\pm$ 1.1	
NA (0.1 $\mu g kg^{-1} h^{-1}$) + SOD (5 $\mu g kg^{-1} h^{-1}$)	Inf. vein	6.4 $\pm$ 0.8	4.4 $\pm$ 1.0	4
	Contr. vein	6.4 $\pm$ 0.8	3.9 $\pm$ 0.7	

Shown are means $\pm$ s.d.mean, n indicates the number of animals used; 200–350 cells or nuclei were measured in each animal. Inf. vein = vein in which the infusion was effected; Contr. vein = contralateral vein. The asterisk indicates significant difference from controls ($P < 0.05$).

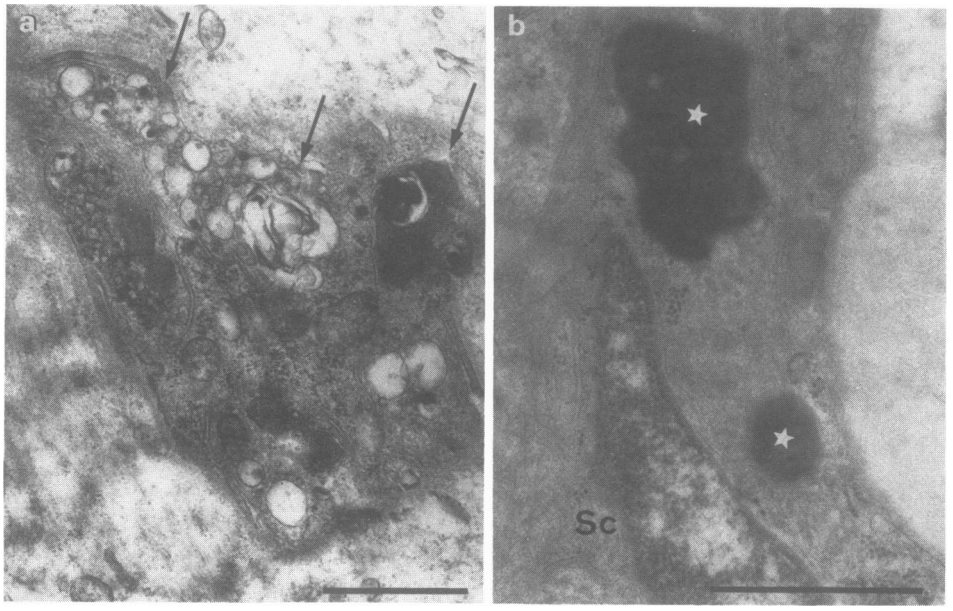

Figure 3 Sympathetic nerves in a noradrenaline-infused saphenous vein. (a) Noradrenergic dense core vesicles are still identifiable in badly damaged varicosities (arrows). (b) Dense degeneration of two nervous profiles (*). Sc = Schwann cell. Bars = 1 μm.

veins only occasionally showed slightly damaged nerves, although the density of vesicles was reduced (5.7 ± 3.8 vesicles per nerve profile; $n = 59$), when compared with control tissues (12.5 ± 11.2 vesicles per nerve profile; $n = 110$). This is shown in Figure 4. Both smooth muscle cells and fibroblasts showed nuclei with normal aspect; a special ultrastructural feature of these cells, under these conditions, was marked vacuolation of the cytoplasm and an abundance of microbodies (Figure 5).

When superoxide dismutase was infused simultaneously with NA ($0.1\,\mu g\,kg^{-1}\,h^{-1}$), the alterations induced by NA alone, in the same concentration, were no longer observed. Smooth muscle cell diameter was not different from the control (Table 1). At the ultrastructural level, sympathetic nerve terminals appeared well preserved (70 nerve profiles observed in 5 dogs; Figure 6); smooth muscle cells and fibroblasts showed no signs of increased nuclear activity, exhibiting, instead, some vacuolation of the cytoplasm (Figure 7). In these NA + SOD infused preparations only a few peroxisomes were observed.

Noradrenaline content and O-methylating capacity

The infusion of noradrenaline ($0.1\,\mu g\,kg^{-1}\,h^{-1}$) caused an elevation of the plasma noradrenaline level, which was roughly doubled (Table 2). Significant reductions in the content of noradrenaline and in the formation of *O*-methylisoprenaline in the veins occurred as shown in Table 3. In good agreement with the morphological data (see above), the reduction of noradrenaline content caused by noradrenaline on the contralateral vein was prevented by DMI or SOD. The protective effect of the latter drug was more complete, since it prevented all the effects of noradrenaline, whereas DMI was not able to impede the reduction in *O*-methylation capacity caused by noradrenaline. Again, this is in good agreement with the morphological data, which

Table 2 Plasma noradrenaline levels in control and noradrenaline-infused dogs

Experimental situation	n	*Plasma noradrenaline level* ($ng\,ml^{-1}$)
Controls (vehicle infusion)	5	0.57 ± 0.06
Noradrenaline infusion	5	0.96 ± 0.04*
Noradrenaline + desipramine infusion	4	0.80 ± 0.05*
Noradrenaline + SOD infusion	4	0.98 ± 0.15*

Blood samples were obtained from the lateral saphenous vein contralateral to that in which the infusion of noradrenaline $0.1\,\mu g\,kg^{-1}\,h^{-1}$ took place.
* Significant difference from control values ($P < 0.05$).

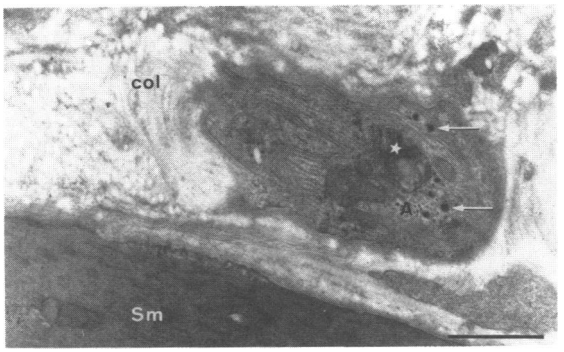

Figure 4 Noradrenaline + desipramine-infused saphenous vein. Lammellate dense body (*) in a nervous profile of an otherwise apparently normal sympathetic nerve. A = adrenergic varicosity with a few large dense core vesicles (arrows), Sm = smooth muscle cell, col = collagen. Bar = 1 μm.

showed that the structure was more preserved in veins of animals treated with SOD than in those which received DMI.

However, it is evident that there is some discrepancy between the morphological and the biochemical data; in fact total protection against the neurotoxic action of noradrenaline by SOD or DMI, as ascertained by morphological results, was accompanied only by partial protection at the biochemical level (for example, the nerve terminals in veins infused with NA plus DMI were not degenerated but the noradrenaline content was not significantly different from that found in veins infused with NA only). This may be attributed to the lower vesicular density observed to occur in the terminals of veins infused with NA plus DMI, when compared with controls (see morphological results section).

It is noteworthy that DMI or SOD, when added to noradrenaline, resulted in a noradrenaline content of the contralateral vein which was significantly higher than that found in control animals; this appears to indicate that some uptake of the infused noradrenaline occurred into the preserved nerve endings of the animals thus protected.

Discussion

It has been shown that the lateral saphenous vein of the dog is endowed with noradrenergic innervation and that fluorescence, fine structural and autoradiographic methods reveal no other type of nerve terminals in this tissue (Coimbra *et al.*, 1974). In good agreement, recent (unpublished) results from this laboratory show that this vascular tissue contains no neuronal elements which are resistant to chemical sympathectomy by 6-hydroxydopamine (6-OHDA).

The present results show that prolonged intravenous infusion of moderate doses of noradrenaline induces marked morphological changes of the venous vessels studied. These are characterized by degeneration of sympathetic nerve varicosities and extraneuronal changes of the same type as those previously described to occur after surgical denervation of these vessels (Branco *et al.*, 1984). Thus, noradrenaline is shown to exert 6-hydroxydopamine-like effects.

The neurotoxic effect of noradrenaline is apparently difficult to reconcile with the notion that, being a sympathetic transmitter, the amine should not induce toxic alterations of the varicosities where it is synthesized and stored. One could argue that under physiological circumstances the concentration of free noradrenaline in the axoplasm is always low, thus not reaching the levels that may exert a neurotoxic

Table 3 Effects of noradrenaline (NA) infusion on NA content and *O*-methylation capacity of saphenous vein tissue; influence of desipramine (DMI) and superoxide dismutase (SOD) on the effects of NA

Experimental situation		*Noradrenaline content* ($\mu g\,g^{-1}$)	[^3H]-*OMI* ($pmol\,g^{-1}$)	n
Controls (vehicle infusion)	Inf. vein	1.80 ± 0.22	544 ± 49	4
	Contr. vein	2.47 ± 0.24	766 ± 85	
NA infusion	Inf. vein	0.66 ± 0.11*	184 ± 28*	5
	Contr. vein	1.51 ± 0.11*	579 ± 54*	
NA + DMI infusion	Inf. vein	0.73 ± 0.37*	158 ± 16	5
	Contr. vein	4.13 ± 0.31**	606 ± 62	
NA + SOD infusion	Inf. vein	1.18 ± 0.21**	324 ± 35**	5
	Contr. vein	3.59 ± 0.28**	755 ± 72**	

NA was infused at a rate of 0.1 $\mu g\,kg^{-1}\,h^{-1}$; DMI, 25 $\mu g\,kg^{-1}\,h^{-1}$ and SOD, 5 $\mu g\,kg^{-1}\,h^{-1}$.
[^{3}H]-OMI – amount of *O*-methylisoprenaline formed by the tissue after 30 min incubation with 0.2 $\mu mol\,l^{-1}$ [^{3}H]-isoprenaline.
Differences between the means for infused (Inf.) and contralateral (Contr.) vein were all significant ($P < 0.01$); *significant differences from controls ($P < 0.01$); **significant differences from the NA infusion group ($P < 0.01$).

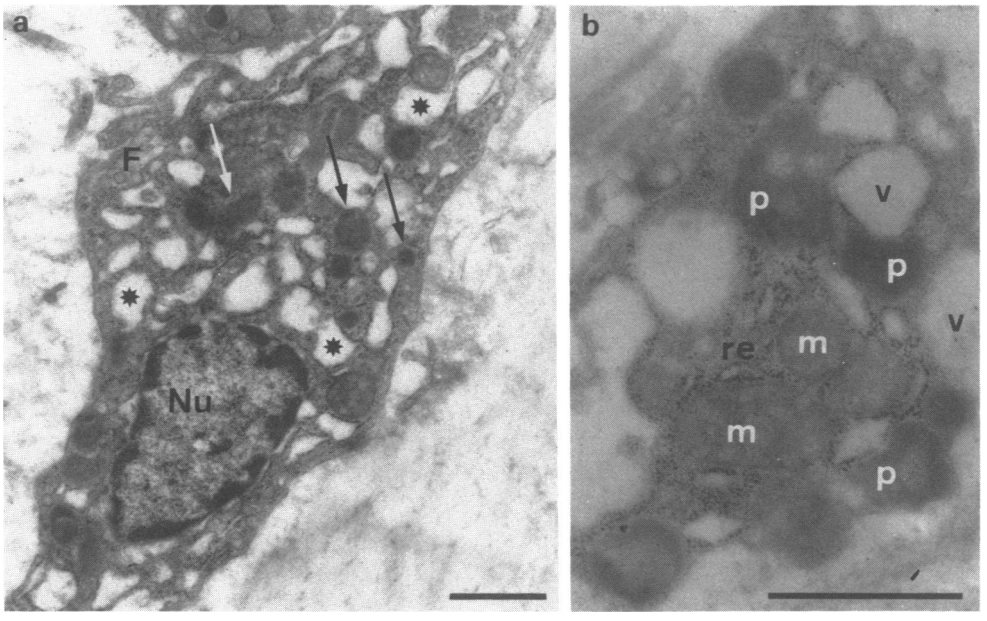

Figure 5 Noradrenaline + desipramine-infused saphenous vein. (a) Fibroblast (F) with a discrete nucleus (Nu); the cytoplasm shows many vacuoles (*) and microbodies reminiscent of peroxisomes (arrows). (b) A higher magnification of fibroblast cytoplasm. v = vacuoles; m = mitochondria; p = peroxisomes; re = rough endoplasmic reticulum. Bars = 1 μm.

effect. It is well known that after inhibition of monoamine oxidase (MAO) relatively high axoplasmic concentrations of noradrenaline are reached. However, to our knowledge, no chemical sympathectomy has been described to occur even after prolonged periods of inhibition of MAO, in animals chronically treated with irreversible enzyme inhibitors.

On the other hand, the blood levels of noradrenaline achieved during the infusion were relatively low, although significantly higher than those observed under control conditions. It appears therefore difficult to speculate that the plasma levels induced by intravenous infusion of noradrenaline (which were roughly twice as high as control plasma levels) could swamp efficient disposal mechanism, causing a very marked increase in axoplasmic concentration.

Our findings that blockade of neuronal uptake, as

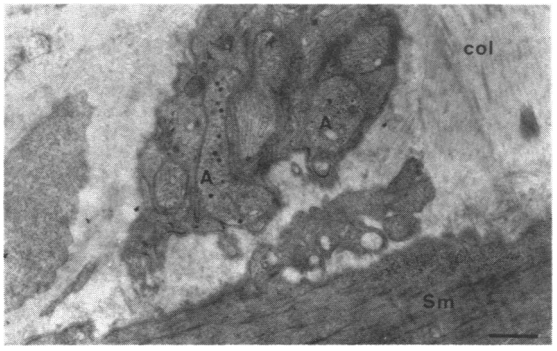

Figure 6 Well preserved sympathetic nerve in a noradrenaline + superoxide dismutase infused saphenous vein. A = noradrenergic varicosities with a few dense core vesicles; Sm = smooth muscle cell; col = collagen. Bar = 1 μm.

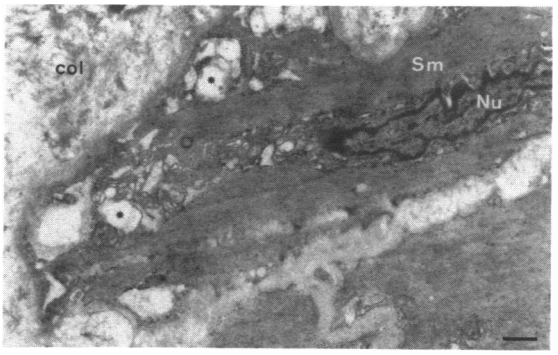

Figure 7 Smooth muscle cell (Sm) with a discrete nucleus (Nu) and a vacuolated cytoplasm (*) in a noradrenaline + superoxide dismutase-infused saphenous vein. col = collagen. Bar = 1 μm.

a result of DMI pretreatment (see Methods), or trapping of free radicals induced by SOD infusion partially or totally prevented the chemical sympathectomy caused by noradrenaline, allow us to suggest a mechanism of action for the neurotoxic effect of noradrenaline. It appears evident that it is not the noradrenaline molecule itself that is responsible for the neurotoxic action but rather a metabolite formed through a reaction with free radicals. The involvement of free radicals in the neurotoxic effect of NA is also suggested by the appearance of increased numbers of microbodies, most probably peroxisomes, in smooth muscle cells and fibroblasts of NA-infused veins. Unfortunately we had not anticipated the increase in microbodies and thus did not prepare the tissue for their histochemical characterization, but morphologically they were reminiscent of peroxisomes which are organelles rich in H_2O_2-forming oxidases and catalase (Bock *et al.*, 1980) and are known to be inducible (Lazarow & Fujiki, 1985). The increase of peroxisomes in NA-infused veins, as compared to control, was not observed in SOD-treated animals.

Quinones and semiquinones originating from catecholamines may cross-link with proteins through a thioether compound, resulting in denaturation (Graham, 1978; Saner & Thoenen, 1980), and this could be the basis for the neurotoxic effect. Since SOD is a protein of high molecular weight, it distributes, after exogenous administration, strictly to the extracellular space (Marklund, 1980) and therefore it is highly improbable that the protection afforded by SOD can be attributed to an intraneuronal trapping of free radicals. It is probable that the reaction occurs extraneuronally and that the toxic metabolite of noradrenaline is formed outside the neurone and then taken up into the neurone by the classical uptake process inhibited by DMI; and this would account for the protective effect shown to be exerted by the latter drug. As is well known, neuronal uptake inhibitors also prevent chemical sympathectomy induced by 6-hydroxydopamine (Stone *et al.*, 1964).

In this context, it is relevant that tetrahydroisoquinoline compounds formed by condensation of adrenaline with acetaldehyde cause chemical sympathectomy (Azevedo & Osswald, 1977; Osswald & Azevedo, 1980; Collins *et al.*, 1982). Formation of a similar compound originating from noradrenaline could be responsible for the neurotoxic effects observed.

We are aware that the interpretation of our results is speculative, since we had no means to detect and characterize the putative substance derived from noradrenaline that appeared to be involved in the neurotoxic effects of this amine. However, the fact remains that relatively low doses of noradrenaline have the potential to cause chemical sympathectomy and alterations at the extraneuronal level identical to those previously described to be induced by surgical or chemical denervation (Branco *et al.*, 1984; Azevedo & Osswald, 1986; Dimitriadou *et al.*, 1988). Furthermore, a clear-cut correlation between the amount of noradrenaline offered to the tissue and the degree of impairment of neuronal and extraneuronal structures was evident: first, because the chemical sympathectomy was evident only in the animals infused with the higher dose of noradrenaline; second, because for each dose the effects were consistently more marked on the vein where the infusion was effected (and therefore the plasma levels were substantially higher) than in the contralateral vein (where the plasma levels were lower, due to dilution and disposition of noradrenaline in the rest of the body).

There was also a fair correlation between the two markers of damage to sympathetic nerves used in this study, namely electron microscopic evidence of degeneration and tissue noradrenaline content. Furthermore, the increase in smooth muscle cell dimensions and nuclear activity of both these cells and fibroblasts, as well as the reduction in *O*-methylation capacity, were parallel to the degree of damage to sympathetic nerves. Moreover, the prevention of the neurotoxic effect of noradrenaline by SOD or DMI was accompanied by partial or total prevention of the changes occurring at the extraneuronal level. Thus, the present results confirm, for the denervation induced by noradrenaline, what we described for the denervation caused by surgery or 6-hydroxydopamine (Branco *et al.*, 1984; Azevedo & Osswald, 1986; Sarmento *et al.*, 1987).

Our observations also suggest that a decrease in the number of large dense core vesicles in varicosities may represent a first sign of damage to adrenergic nerves by neurotoxic agents; this was observed to occur in the vein contralateral to that where the infusion was effected and was accompanied by discrete, although significant, morphological changes at the extraneuronal level. We are not aware of similar changes being described after other neurotoxic agents, especially 6-hydroxydopamine.

It is tempting to correlate the morphological changes described in this study with those observed in humans with high levels of circulating catecholamines. Pheochromocytoma patients have been observed to present such changes, namely focal myocardial lesions, most often around small blood vessels, arterial thickening of the small and medium sized arteries, partial fibrous replacement of the smooth muscle of the media (Van Vliet *et al.*, 1966). Unfortunately, the authors do not refer to the state of the adrenergic innervation which may well be damaged. If this is so, the neurotoxic action of nor-

adrenaline described in this work would also be observed in human tissues exposed for long periods of time to high circulating levels of endogenous catecholamines, and free radical scavengers, as for example SOD, might find a place in the therapy of such patients.

In conclusion, we found that chemical sympathectomy is caused by long-lasting, continuous intravenous infusion of moderate doses of noradrenaline and that this 6-hydroxydopamine-like effect of noradrenaline is accompanied by the extraneuronal changes which have been described after surgical denervation of vascular tissues of the dog and rabbit (Branco *et al.*, 1984; Dimitriadou *et al.*, 1988). Furthermore, these actions of noradrenaline were partially or totally prevented by DMI or SOD, which suggests that a substance formed from noradrenaline by interaction with free oxygen radicals and subject to neuronal uptake is the chemical entity responsible for the neurotoxic effect.

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$GABA_A$ receptor-mediated positive inotropism in guinea-pig isolated left atria: evidence for the involvement of capsaicin-sensitive nerves

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1 Isolated left atria from reserpine-pretreated guinea-pigs, electrically driven (3 Hz) in the presence of atropine (1 μM), phentolamine (0.3 μM) and propranolol (1 μM), responded to a train of stimuli (10 Hz for 2.5 s) with a delayed neurogenic positive inotropic response which was insensitive to hexamethonium (10 μM) but abolished by either tetrodotoxin (1 μM), ω-conotoxin (0.1 μM), *in vitro* capsaicin desensitization or desensitization to calcitonin gene-related peptide (CGRP).

2 In these experimental conditions, γ-aminobutyric acid (GABA) produced a concentration-related (10 μM–1 mM) positive inotropic response similar to that produced by electrical field stimulation. The effect of GABA was competitively antagonized by bicuculline methiodide (10 μM), a $GABA_A$ receptor antagonist.

3 The selective $GABA_A$ receptor agonists, muscimol and homotaurine mimicked the positive inotropic effect of GABA while baclofen, the selective $GABA_B$ receptor agonist, did not.

4 The action of GABA (1 mM) was abolished by either tetrodotoxin (1 μM), ω-conotoxin (0.1 μM), *in vitro* capsaicin desensitization or desensitization to CGRP, while it was unaffected by hexamethonium. In contrast, the inotropic response to CGRP was unaffected by tetrodotoxin, ω-conotoxin, bicuculline methiodide, hexamethonium or *in vitro* capsaicin desensitization, but was abolished by CGRP desensitization.

5 In the spontaneously beating guinea-pig right atrium, GABA (1 μM) produced a small and transient positive chronotropic effect that was no longer observed after *in vitro* desensitization with capsaicin (1 μM).

6 In the guinea-pig isolated perfused heart from reserpine-pretreated animals (with atropine, phentolamine and propranolol in the perfusion medium), GABA (1 μM) produced a transient tachycardia and a small increase in coronary flow. Both capsaicin (1 μM) and CGRP (1 μM) produced marked tachycardias and increases in coronary flow. After exposure to capsaicin (1 μM), no effect of GABA could be detected.

7 We conclude that, in the guinea-pig heart, $GABA_A$ receptors, presumably located on the preterminal region of capsaicin-sensitive sensory nerves, initiate a conducted impulse (since it is tetrodotoxin-sensitive) which leads to transmitter release (endogenous CGRP-like material) by activation of ω-conotoxin-sensitive, voltage-sensitive calcium channels and a functional response.

Introduction

In recent years, much interest has been directed towards the study of the pharmacological and physiological effects of γ-aminobutyric acid (GABA) in the mammalian peripheral nervous system (see Jessen *et al.*, 1979; Giotti *et al.*, 1983; Erdö & Bowery, 1986). From these experiments evidence has accumulated which suggests that GABA has a widespread neuromodulatory action on autonomic nerves (both parasympathetic and sympathetic) in the cardiovascular system and in the gastrointestinal and genitourinary tracts. The actions of GABA are mediated, depending on either the species or the preparations, or both, used, by at least two distinct types of GABA receptors termed $GABA_A$ and $GABA_B$ according to the classification proposed by Bowery *et al.* (1981).

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It is well known that GABA modulates the excitability of primary afferents in the spinal cord (Levy, 1977). Indeed, both $GABA_A$ and $GABA_B$ receptors have been shown to be involved in the modulatory action of GABA on sensory neurones (Gallagher *et al.*, 1978; Desarmenien *et al.*, 1984a, b) and these effects are thought to be of physiological relevance for the processing of nociceptive information in the spinal cord. At this level both $GABA_A$ and $GABA_B$ receptors seem to be present on the central terminals of primary sensory neurones as the number of binding sites labelled by muscimol and baclofen (selective agonists at $GABA_A$ and $GABA_B$ sites, respectively) were significantly reduced after systemic capsaicin desensitization (Singer & Placheta, 1980; Price *et al.*, 1984) which has a selective neurotoxic effect on certain primary sensory neurones (Jancsó *et al.*, 1977; Szolcsányi, 1984).

GABA receptors have also been described on unmyelinated and myelinated sensory peripheral nerve fibres (Brown & Marsh, 1978; Allan *et al.*, 1980; Morris *et al.*, 1983; Bhisitkul *et al.*, 1987). The electrophysiological action of GABA at non-synaptic sites on peripheral axons is membrane depolarization brought about by an increase in chloride conductance (Morris *et al.*, 1983), an effect which apparently involves stimulation of the $GABA_A$ receptor (Bhisitkul *et al.*, 1987). In this study we present functional evidence indicating that stimulation of bicuculline-sensitive $GABA_A$ receptors activates the peripheral terminals of capsaicin-sensitive nerves in the guinea-pig atrium and produces functional responses mimicking those observed following electrical field stimulation of these sensory nerve terminals.

Methods

Isolated left atria

Inotropic responses of electrically driven guinea-pig left atria due to antidromic activation of capsaicin-sensitive nerves were obtained by using a preparation similar to that described by Goto *et al.* (1987) and Saito *et al.* (1987). Male albino guinea-pigs weighing 280–350 g received intraperitoneal reserpine (5 mg kg^{-1}) 24–48 h before being killed by stunning and bleeding. The whole heart was rapidly removed and placed in Tyrode solution containing atropine (1 μM), phentolamine (0.3 μM) and propranolol (1 μM) to block α- and β-adrenoceptors, respectively. The left atrium was cleaned of adhering tissues and placed in a 5 ml organ bath at 37°C containing oxygenated (96% O_2 and 4% CO_2) Tyrode solution of the following composition (mM): NaCl 137, KCl 2.68, $CaCl_2$ 1.8, $MgCl_2$ 1.05, NaH_2PO_4 0.42, $NaHCO_3$ 11.9, glucose 5.5.

The atrium was connected, under a resting tension of 5 mN, to an isometric force transducer (Basile 7003) connected to a Basile 7050 Unirecord. The atrium was stimulated by means of two platinum wire electrodes placed at the top and the bottom of the organ bath and connected to a Grass S11 stimulator. The atria were driven at a frequency of 3 Hz (0.5 ms pulse width, maximal voltage). At 10–15 min intervals, a train of stimuli was delivered at a frequency of 10 Hz for 2.5 s (1 ms pulse width, 60 V) by means of a second stimulator, which superimposed on the stimuli delivered by the first stimulator. During the train, the atria exhibited a flutter-like response, followed, at the end of the train, by a short (4–5 s) period of increased contractility (post-stimulus potentiation or post-extrasystolic potentiation) while the neurogenic inotropic response to the train of stimuli appeared somewhat later (Figure 1). As described by Goto *et al.* (1987) and Saito *et al.* (1987), this stimulation produces, in left atria from reserpine-treated guinea-pigs and in the presence of atropine, a delayed positive inotropic response mediated by antidromic invasion of capsaicin-sensitive sensory nerve terminals.

A non-cumulative concentration-response curve to GABA was constructed at 20–30 min intervals between doses, either in the absence or in the presence of bicuculline methiodide (10 μM) which was added to the Tyrode solution. Responses were expressed as percentage variation of resting force as compared to pre-drug values.

The effects of tetrodotoxin, hexamethonium or ω-conotoxin on the inotropic responses to field stimulation or GABA were studied after a 20 min contact period. In order to inactivate the capsaicin-sensitive sensory fibres a protocol of *in vitro* capsaicin desensitization was used (see Barthó *et al.*, 1987; Maggi *et al.*, 1987b). After the equilibration period, a maximally effective concentration of capsaicin (1 μM) was added to the bath and maintained for 15 min. Thereafter the drug was removed by repeated washings and the preparation allowed to equilibrate for a further 30 min. For calcitonin gene-related peptide (CGRP) desensitization we used a protocol essentially similar to that described previously for the guinea-pig heart (Franco-Cereceda & Lundberg, 1985) or rat ureter (Maggi *et al.*, 1987a): a maximally effective concentration of CGRP (0.1 μM) was added to the bath and left in contact with the tissue (no washing) until its positive inotropic effect had ceased. At this time a second application of CGRP had no further inotropic effect while the response to isoprenaline was unaffected. In some experiments the effects of GABA on the spontaneous chronotropic and inotropic activity of the right atrium were

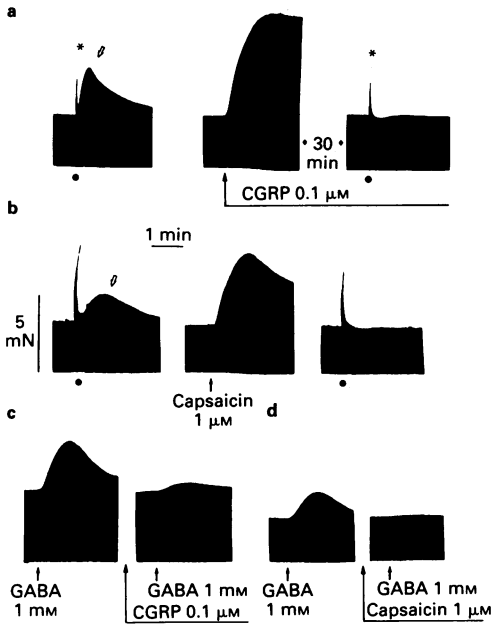

Figure 1 Typical tracings showing the effects of calcitonin gene-related peptide (CGRP) desensitization (a and c) or *in vitro* capsaicin desensitization (b and d) on inotropic responses, produced by electrical field stimulation (applied at the dots, a and b) or γ-aminobutyric acid (GABA; c and d), of guinea-pig isolated left atria from reserpine-pretreated animals (atropine, phentolamine and propranolol were present in the bath). The response to electrical field stimulation was characterized by an early, myogenic (tetrodotoxin-resistant) response (indicated by asterisks in a and b) and a late, neurogenic (tetrodotoxin-sensitive) response (indicated by open arrows in panels a and b). Note that both the late neurogenic response to electrical field stimulation or the inotropic response to GABA were almost abolished by CGRP or capsaicin desensitization.

studied in experimental conditions similar to those described above.

Isolated perfused heart

In other experiments we studied the effects of GABA (1 mM) in the guinea-pig isolated perfused heart. The hearts were excised from reserpine-pretreated animals, cannulated through the aorta and perfused under constant pressure (coronary flow 5.5 ± 0.3 ml min^{-1}) with oxygenated Tyrode solution containing atropine, phentolamine and propranolol as described above. Coronary flow was measured with an electronic optical flowmeter (MARB Gx-84, Chiesina Uzzanese, Lucca, Italy). Contractile force and heart rate were assessed by means of a hook inserted in the cardiac apex and connected to an isometric transducer under a resting tension of 2 g. Preparations were allowed to equilibrate for 60 min. Drugs were injected in the coronary flow through a side arm of the perfusion apparatus, just above the cannula. The injection vehicle (0.05 ml of Tyrode) on its own did not alter the cardiac performance. *In vitro* capsaicin desensitization was brought about by the injection of 1 μM capsaicin, 30 min before GABA administration.

Statistical analysis

Each value is presented as the mean ± s.e.mean. Statistical analysis was performed by means of Student's *t* test for paired or unpaired data, or by means of analysis of variance followed by the two-tailed Dunnett test, when applicable. Regression analysis was performed by means of the least squares method. EC_{50} and 95% confidence limits were calculated using a statistical package for the Apple IIe computer (Tallarida & Murray, 1981). The pA_2 for bicuculline methiodide antagonism of GABA responses was calculated as described by Van Rossum (1963).

Drugs

Drugs used were: γ-aminobutyric acid (GABA) hexamethonium bromide, capsaicin, homotaurine, propranolol HCl (Sigma, St Louis, MO, U.S.A.), ω-conotoxin fraction GVIA and rat calcitonin gene-related peptide (CGRP) (Peninsula, San Carlos, CA, U.S.A.), tetrodotoxin (Sankyo), atropine HCl, muscimol and reserpine (Serva, Heidelberg, FRG), (±)-baclofen and phentolamine mesylate (Ciba Geigy, Basel, Switzerland).

Results

We confirmed (Goto *et al.*, 1987; Saito *et al.*, 1987) that in the electrically-driven left atria from reserpine-pretreated guinea-pigs, in the presence of atropine (1 μM), phentolamine (0.3 μM) and propranolol (1 μM), application of a train of stimuli (10 Hz, 0.5 ms, 60 V for 2.5 s) produced two distinct responses. These were, firstly, an immediate short-lasting post-stimulus potentiation (indicated by asterisks in Figure 1a,b) and, secondly, a delayed positive inotropic response which occurred 20–30 s after the end of the train and reached its maximum within 60–90 s (indicated by open arrows in Figure 1a,b). Both responses were fairly reproducible at 5–10 min intervals for several hours without any sign of fading. The delayed response was suppressed by tetrodotoxin (1 μM, $n = 4$) thus indicating its neural origin, while the immediate post-stimulus potentiation was totally resistant to tetrodotoxin (cf. Goto

et al., 1987). ω-Conotoxin (0.1 μM) produced a marked (83 ± 3%, $n = 7$) inhibition of the neurogenic positive inotropic response to field stimulation while leaving the tetrodotoxin-resistant, immediate inotropic response unaffected. Capsaicin (1 μM) produced a marked positive inotropic response which reached a maximum within 2–5 min from application and then declined slowly. The delayed neurogenic inotropic response to train field stimulation averaged 20 ± 2%, $n = 16$ (range 15–42%) of the response to capsaicin. After *in vitro* desensitization with 1 μM capsaicin, the tetrodotoxin-sensitive delayed inotropic response was completely abolished (Figure 1b), indicating that it is attributable to activation of sensory nerve terminals and consequent transmitter release (cf. Goto *et al.*, 1987; Saito *et al.*, 1987). CGRP desensitization abolished the delayed neurogenic inotropic response to field stimulation while leaving the immediate myogenic response unaffected (Figure 1a, $n = 4$).

Effect of GABA and antagonism by bicuculline methiodide

GABA (10 μM–1 mM, $n = 5$) produced a concentration-related positive inotropic response in the electrically-driven isolated left atria of the guinea-pig (Figure 2). The response occurred within 10 s and reached a maximum within 50–60 s (Figure 1c,d). The ED_{50} for this stimulant action of GABA was 76 μM (95% confidence limits were 62–94 μM). The maximal response to GABA ranged between 50 and 170% of the neurogenic delayed inotropic response produced by a train of stimuli. Bicuculline methiodide (10 μM) did not significantly affect the inotropism. In the presence of bicuculline methiodide, the concentration-response curve to GABA was shifted to the right, but a maximal response similar to that observed before addition of the antagonist was observed at high GABA concentrations (3–10 mM), suggestive of competitive antagonism (Figure 2). From the curves presented in Figure 2, an apparent pA_2 value of 6.13 was calculated.

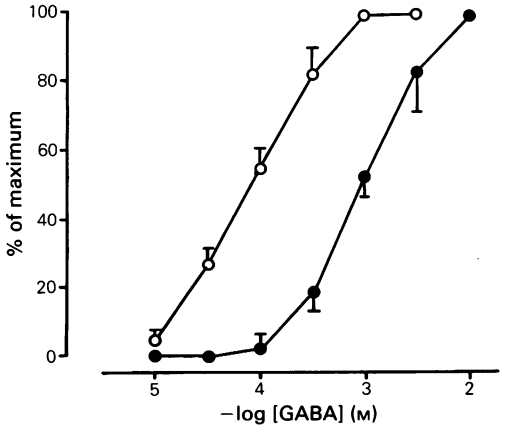

Figure 2 Effect of bicuculline methiodide on the positive inotropic effect induced by γ-aminobutyric acid (GABA) in the guinea-pig isolated left atria. Each point is the mean of 5 experiments; vertical lines show s.e.mean. (○) Control responses, (●) responses in the presence of bicuculline 10 μM.

Effects of muscimol, homotaurine and baclofen

Both muscimol (1 mM) and homotaurine (1 mM), two $GABA_A$ receptor agonists, produced a positive inotropic response (38 ± 9 and 34 ± 7% increases for muscimol and homotaurine, respectively, $n = 4$ for each) similar to that produced by GABA in the same preparations (32 ± 7% increase, $n = 4$). However, baclofen (0.3 mM, $n = 4$), a selective $GABA_B$ receptor agonist, had no significant effects on atrial inotropism.

Further characterization of the response to GABA

The positive inotropic response to GABA (1 mM) was no longer observed after *in vitro* capsaicin desensitization (Figures 1d and 3) suggesting involvement of these sensory nerves. Likewise, the response to GABA was almost abolished after desensitization to CGRP (Figures 1c and 3), the putative endogenous mediator responsible for the effect of capsaicin in this tissue (Franco-Cereceda & Lundberg, 1985; Saito *et al.*, 1986; 1987). The response to GABA was unaffected by hexamethonium (10 μM, $n = 4$) but was abolished by ω-conotoxin (0.1 μM, $n = 4$) and greatly reduced by tetrodotoxin (1 μM, $n = 6$) (Figure 3).

The response to exogenous CGRP (10 nM, 30–50% of the maximal response) was unaffected by either hexamethonium (10 μM, $n = 4$, Figure 3) or bicuculline methiodide (10 μM, $n = 4$), but was abolished by CGRP desensitization ($n = 4$, Figure 3). Neither tetrodotoxin, ω-conotoxin nor *in vitro* capsaicin desensitization had any effect ($n = 4$ each, Figure 3) on the inotropic response produced by exogenous CGRP (10 nM).

Effect of GABA on the spontaneously beating right atrium

In the right atrium, GABA (1 mM) produced transient increases in frequency (from 150 ± 6 to 169 ± 8 beats min^{-1}, $P < 0.05$) and contractile force (from 3.4 ± 0.3 to 4.9 ± 0.3 mN, $P < 0.05$, $n = 4$). The time course of this effect was similar to that described for the positive inotropic effect in left atria. Likewise capsaicin (1 μM) produced a marked positive chrono-

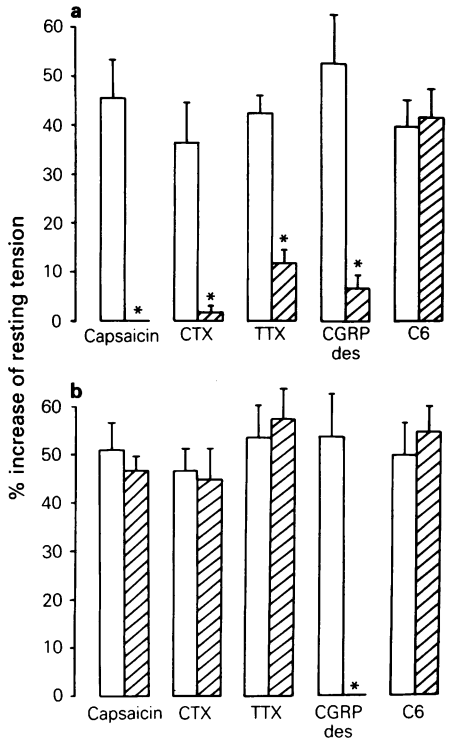

Figure 3 Effect of *in vitro* capsaicin desensitization, ω-conotoxin (CTX, 0.1 μM), tetrodotoxin (TTX, 1 μM), calcitonin gene-related peptide desensitization (CGRP des) or hexamethonium (C6, 10 μM) on the positive inotropic effect of γ-aminobutyric acid (GABA, 1 mM, a) or CGRP (10 nM, b) in electrically driven isolated left atria of the guinea-pig. Open columns, control responses to GABA or CGRP. Hatched columns, effect of GABA or CGRP after treatment. * Significantly different from controls, $P < 0.05$.

tropic and inotropic effects. A second application of GABA (1 mM), 30 min after *in vitro* capsaicin desensitization, had no effect on the rate or force of contraction of the guinea-pig right atrium ($n = 4$).

Effect of GABA on the guinea-pig isolated heart

GABA (1 mM, $n = 6$) consistently produced a small and transient increase in heart rate (from 250 ± 15 to 278 ± 15 beats min^{-1}, $P < 0.05$) and coronary flow (from 5.3 ± 0.3 to 5.8 ± 0.3 ml min^{-1}, $P < 0.05$) which peaked at 2 min from GABA administration, but did not affect the contractile force of the guinea-pig isolated perfused heart. Also both capsaicin (1 μM, $n = 6$) and CGRP (1 μM, $n = 6$) had positive chronotropic effects (heart rate increased by 58.8 and 81 ± 10 beats min^{-1} for capsaicin and CGRP, respectively) and produced transient increases in coronary flow (of 1.8 ± 0.3 and 1.6 ± 0.3 ml min^{-1} with capsaicin and CGRP, respectively). A second application of capsaicin 30 min later had no effect on heart rate and also the action on coronary flow was markedly reduced, although not abolished ($n = 4$, 0.8 ± 0.3 ml min^{-1}, $P < 0,05$ as compared to the first administration).

On the other hand a second application of GABA or CGRP, 30 min after the first challenge provided reproducible effects on heart rate and coronary flow ($n = 4$ each). After *in vitro* capsaicin desensitization, GABA (1 mM) had no effect while CGRP still retained its action on heart rate and coronary flow. In fact, heart rate and coronary flow were increased by 77 ± 8 beats min^{-1} and 1.7 ± 0.3 ml min^{-1}, respectively ($n = 4$, NS as compared to values obtained before application of capsaicin).

Discussion

Anatomical data indicate that a dense plexus of nerve fibres containing CGRP- and tachykinin-like immunoreactivities innervates the guinea-pig right and left atria. These nerve fibres cannot be observed in the hearts of capsaicin-pretreated animals, indicating that they represent peripheral terminals of primary sensory neurones (Saito *et al.*, 1986; Franco-Cereceda *et al.*, 1987a). Likewise, both CGRP- and tachykinin-like immunoreactivities were detected in extracts of guinea-pig atria and were depleted following systemic capsaicin desensitization (Wharton *et al.*, 1986; Geppetti *et al.*, 1988). Recently, the release of both CGRP- and tachykinin-like immunoreactivities from the guinea-pig heart or isolated atria was demonstrated in response to capsaicin, ischaemia or bradykinin (Franco-Cereceda *et al.*, 1987b; 1988; Geppetti *et al.*, 1988; Geppetti, Manzini, Santicioli & Maggi, unpublished data). Endogenous CGRP-like material seems the main mediator of the positive chrono- and inotropic responses to capsaicin in guinea-pig atria (Franco-Cereceda & Lundberg, 1985; Saito *et al.*, 1986; Franco-Cereceda *et al.*, 1988).

The capsaicin-sensitive sensory nerve terminals in guinea-pig atria can be activated by capsaicin itself and electrical field stimulation. Thus, in experimental conditions effective in preventing the inotropic effects due to activation of cholinergic and noradrenergic nerves, electrical field stimulation produces a positive inotropic response attributable to the release of endogenous CGRP-like immunoreactive material (Saito *et al.*, 1986; 1987; Miyauchi *et al.*, 1987; Goto *et al.*, 1987).

The depolarizing action of capsaicin on sensory nerve endings is tetrodotoxin-resistant but produces a calcium-dependent secretion of stored transmitters

e.g. the sensory receptor potential-coupled 'efferent' response which is produced by the orthodromic (from the environment) mode of activation of sensory nerve terminals (Maggi & Meli, 1988). On the other hand, the response produced by electrical field stimulation is tetrodotoxin-sensitive, e.g. requires a propagated action potential corresponding to the antidromic mode of activation of sensory nerve terminals. As these responses are hexamethonium-resistant, we can assume that nerve impulses generated by electrical field stimulation in the preterminal region of the sensory nerves are conducted to the nerve terminals where transmitters are secreted (see Maggi & Meli, 1988; Holzer, 1988).

A further element of differentiation between the orthodromic (by capsaicin) and antidromic (by electrical field stimulation) mode of activation of the 'efferent' function of these sensory nerves is their differential sensitivity to ω-conotoxin (Maggi *et al.*, 1988a), a peptide modulator of neuronal calcium channels (Fox *et al.*, 1987) which inhibits transmitter release from various peripheral nerve terminals (Kerr & Yoshikami, 1984; Miller, 1987; Maggi *et al.*, 1988b). Responses produced by capsaicin are completely resistant to ω-conotoxin, while activation of the fibres by electrical field stimulation is sensitive to ω-conotoxin (Maggi *et al.*, 1988a).

GABA has been shown previously to affect indirectly cardiac function at the peripheral level by inhibiting, through prejunctional $GABA_B$ receptors, noradrenaline release from sympathetic nerves (Bowery *et al.*, 1981). Neely *et al.* (1983) showed that, following selective perfusion of the sinus node artery, GABA had no direct effects on heart rate but attenuated, in a picrotoxin-sensitive manner, the bradycardia resulting from vagal stimulation. The present findings provide functional evidence that the capsaicin-sensitive primary afferents in the guinea-pig left atrium are endowed with $GABA_A$ receptors, the stimulation of which by GABA, muscimol or homotaurine induces secretion of stored transmitters (presumably an endogenous CGRP-like peptide) and generates a positive inotropic response. As the effects of GABA were totally resistant to hexamethonium, it appears unlikely that a local reflex takes place in these conditions. Rather the action of GABA involves a mechanism similar to that produced by electrical field stimulation, that is induction of a propagated action potential (sensitive to tetrodotoxin) and transmitter secretion through activation of ω-conotoxin- and voltage-sensitive calcium channels. These findings suggest that $GABA_A$ receptors are probably located at the preterminal (axonal) level on sensory nerves and their stimulation leads to antidromic invasion of sensory nerve terminals. Evidence for the presence of functional $GABA_A$ receptors on unmyelinated sensory axons has been obtained recently (Bhisitkul *et al.*, 1987). At this level (sensory axons in the rat sciatic nerve) GABA and muscimol produce a depolarization.

This is not the first time an interaction has been described between GABA and capsaicin-sensitive nerves in the periphery. Tonini *et al.* (1986) showed that capsaicin (0.2 μM given 30 min before) attenuated the contractile response to $GABA_A$ receptor agonists in the guinea-pig isolated ileum (see also Tonini *et al.*, 1987). From these experiments the authors concluded that capsaicin-sensitive nerves might be involved in the action of GABA in this tissue. However, since capsaicin was apparently not washed out from the bath before administration of GABA, the possibility cannot be ruled out that this depressant effect might have been due to some non-specific effect of the drug (e.g. not exhibiting desensitization) or to depression of contractility due to release of CGRP-like endogenous peptides (see Maggi *et al.*, 1988c).

On the other hand, we used a protocol of *in vitro* capsaicin desensitization (prolonged exposure to a maximally effective concentration of capsaicin; cf. Maggi *et al.*, 1987b; Bartho *et al.*, 1987) which could be assumed to produce inactivation of sensory nerves (as assessed by the disappearance of the response to field stimulation) without the continuing influence of capsaicin itself, which was washed out from the bath, during the experiment. The experimental design should also avoid problems due to desensitization of peptide receptors, or actions of endogenous peptides released during the application of capsaicin.

The physiological significance of these findings is, at this stage, a matter of speculation. GABAergic neurones have been described at various levels in the periphery (see Erdö & Bowery, 1986). Both GABA and the enzymes responsible for GABA synthesis (L-glutamate decarboxylase) and catabolism (GABA transaminase) have been detected in the rodent heart (Erdö & Kiss, 1986) but no other information is available at this stage about a possible transmitter role of GABA at this level. In the whole heart, GABA exerted a mild effect on heart rate and coronary flow which could no longer be observed after *in vitro* capsaicin desensitization. The chronotropic action of GABA is probably due to an action on capsaicin-sensitive fibres in the right atrium, as suggested by *in vitro* experiments. It appears therefore conceivable that even these effects are due to transmitter release from the peripheral endings of capsaicin-sensitive nerves (cf. Franco-Cereceda & Lundberg, 1985). In conclusion, the present findings indicate that peripheral terminals of primary sensory neurones could be a target for the action of GABA and, in principle, also for drugs which act by modifying GABAergic transmission.

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Sex-related differences in central adrenergic function and responsiveness to repeated administration of desipramine or electroconvulsive shock

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1 Clonidine induces hypoactivity in rodents. Male rats were found to be markedly more susceptible to the sedative effects of this α_2-adrenoceptor agonist than females. Thus to obtain identical hypoactivity responses for subsequent experiments, clonidine was administered to male and female rats at doses of 0.2 and 0.5 mg kg^{-1}, respectively.

2 The clonidine-induced hypoactivity response of female rats was not affected by the oestrous cycle.

3 Repeated injection of desipramine (DMI; 5 mg kg^{-1} b.d.) for up to 14 days progressively attenuated clonidine-induced hypoactivity in both male and female rats. However, in males the attenuation was more rapid in onset and a greater overall reduction was obtained. This α_2-adrenoceptor-mediated response was also progressively reversed by repeated daily administration of an electroconvulsive shock (ECS; 110 V, 1 s). In this case, although the maximum decrease was greater in males, the time of onset was identical in both sexes.

4 There were no sex-related differences in either the number or affinity of α_2- and β-adrenoceptors in rat cortex. Cortical α_2-adrenoceptors were decreased by 14 days of DMI injection or 10 days of ECS treatment (ECS × 10) and these effects were identical in both sexes. These receptors were not altered by 2 days administration of DMI or ECS. Cortical β-adrenoceptors were reduced in male and female rats by 2 and 14 days of DMI injection and by ECS × 10, but not ECS × 2.

5 Viewed overall, the data show differences in α_2-adrenoceptor function between the sexes, as determined by clonidine-induced hypoactivity and the responsiveness of this paradigm to repeated administration of DMI and ECS. In contrast, no differences were observed in complementary α_2- and β-adrenoceptor binding experiments using rat cortical tissue.

Introduction

The effects of administration of various antidepressant treatments, including electroconvulsive shock (ECS), a model of electroconvulsive thereapy, on central noradrenergic function have been extensively investigated (see Green & Nutt, 1987). The most widely studied and consistent effect of antidepressant treatment in animals is a decrease in the number of β-adrenoceptors in the brain (Bergstrom & Kellar, 1979a,b; Sellinger-Barnette *et al.*, 1980; Asakura *et al.*, 1982; Sugrue, 1982; Stanford & Nutt, 1982). However, presynaptic α_2-adrenoceptor function is also decreased by repeated administration of desipramine (DMI; Tang *et al.*, 1979; McMillen *et al.*, 1980; Sugrue, 1983; Heal *et al.*, 1983; 1987a) and ECS (Heal *et al.*, 1981; 1987a; Sugrue, 1983). Several groups have also shown that postsynaptic α_2-adrenoceptors are decreased by repeated ECS (Stanford & Nutt, 1982; Pilc & Vetulani, 1982; Heal *et al.*, 1987a), but the effect of chronic DMI treatment is much more controversial (Johnson *et al.*, 1980; Asakura *et al.*, 1982; Stanford *et al.*, 1983; Sugrue, 1983; Heal *et al.*, 1987a).

Recently, studies by this group have shown that some of these changes in α_2- and β-adrenoceptor

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function which are produced by DMI and ECS treatment are markedly influenced by the circulating levels of oestradiol (Bristow *et al.*, 1986; Heal *et al.*, 1988) and the thyroid hormone, triiodothyronine (T_3; Heal *et al.*, 1987a). In view of this, an investigation has been carried out to compare α_2- and β-adrenoceptor function in male and female rat brain. In addition, the effects of repeated administration of DMI and ECS have also been investigated to determine whether there are any sex-related differences in the responsiveness of central α_2- and β-adrenoceptors.

Methods

Animals

Sexually mature male and female rats weighing 75–100 g and 100–125 g respectively, at the commencement of experiments were used. These were housed 6–8 in a cage at 21 ± 1°C on a 12 h light–dark cycle (starting 08 h 00 min). The rats were allowed free access to 41-B diet and tap water and all behavioural experiments were performed between 09 h 00 min–18 h 00 min.

Protocol for administration of desipramine and ECS

DMI (5 mg kg^{-1}) was injected twice daily at 10 h 00 min and 17 h 00 min commencing on day 1 and continuing to day 14. Controls were injected with saline (0.5 ml 100 g^{-1}).

ECS (110 V, 1 s) was administered via earclip electrodes while rats were lightly anaesthetized with halothane and this always resulted in a full tonic-clonic seizure. ECS was given once daily commencing on day 1 and was continued up to day 10.

Behavioural and biochemical measurements were made at various points on the testing schedule and these were performed 18 h and 24 h after the last administration of DMI and ECS, respectively. Rats were used once only.

Measurement of clonidine-induced hypoactivity responses

The hypoactivity responses of rats to clonidine were used as an index of presynaptic α_2-adrenoceptor function in the brain. This behaviour was rated 0–3 on 4 separate parameters (passivity, tactile responsiveness, posture and gait) by an observer who was unaware of the treatment received by the animals. This protocol is based on the method described by Drew *et al.* (1979) and is fully described by Heal *et al.* (1981).

Initial experiments to establish dose-response relationships for hypoactivity induced by clonidine (0.05–1.0 mg kg^{-1}) showed that male rats were markedly more susceptible to the sedative effects of this α_2-adrenoceptor agonist than females (Figure 1). In order to obtain equivalent hypoactivity scores in male and female rats when investigating the actions of DMI and ECS, clonidine was administered at a dose of 0.2 mg kg^{-1} to male rats and 0.5 mg kg^{-1} to females.

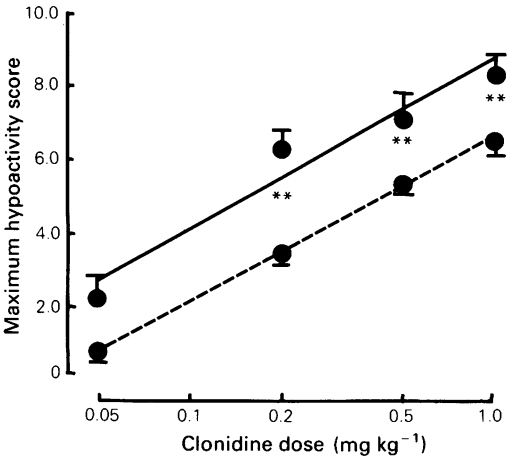

Figure 1 The dose-response relationship for clonidine-induced hypoactivity in male and female rats. Groups of rats, males (●——●) and females (●– – –●), were injected with clonidine (0.05–1.0 mg kg^{-1}). Hypoactivity was rated 0–3 on four behavioural parameters at 10 min intervals for the 60 min period following clonidine administration. Results show the maximum hypoactivity score which was obtained in the 60 min period plotted against the dose of clonidine injected shown on a log scale. The number of rats tested was 10–20 for each point. Vertical lines indicate s.e.mean. ** Significantly different from appropriate female response, $P < 0.01$.

Identification of stages of the oestrous cycle of the rat

Female rats were lightly restrained and a small nickel-chromium wire loop was inserted and rotated against the vaginal wall to collect cell samples. The cells were then mixed with a drop of sterile water and transferred to a microscope slide to make a smear. This was allowed to dry and the stage of the oestrous cycle was determined by examination of the smear under a light microscope.

Measurement of α_2- and β-adrenoceptor binding in rat cerebral cortex

The effects of antidepressant treatments on α_2- and β-adrenoceptor binding in male and female rat brain were assessed using [^{3}H]-idazoxan and [^{3}H]-dihydroalprenolol, respectively. Rats were killed and cerebral cortices from individual rats were frozen on

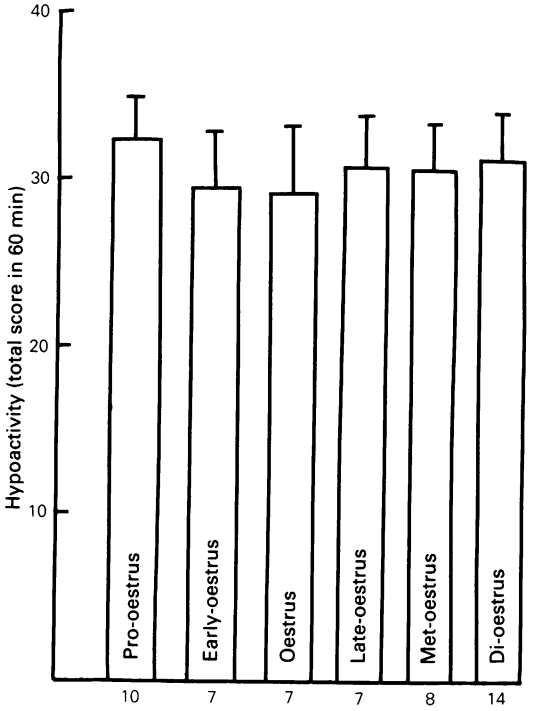

Figure 2 The effects of the oestrous cycle on the hypoactivity responses of rats to clonidine. The hypoactivity responses of rats to injection of clonidine (0.5 mg kg^{-1}) and the stages of the oestrous cycle were determined as described in Methods. Results shown are means of the number of rats tested given under each column; vertical lines indicate s.e.mean.

solid carbon dioxide and stored at −40°C. Individual cortices were initially homogenised in hypotonic buffer (5 mM Tris-HCl, 5 mM EDTA, pH 7.5) and nuclei and other large particles were sedimented by slow centrifugation (1000 × *g*, 15 min, 4°C). The remaining membrane fraction was washed twice by centrifugation (30,000 × *g*, 10 min, 4°C) and resuspension in the above buffer.

For measurement of α_2-adrenoceptor binding the membrane pellet was resuspended in 50 mM Tris-HCl buffer pH 7.5 containing 0.5 mM EDTA and 0.1% ascorbic acid at a tissue concentration of approximately 1 mg ml^{-1}. Saturation binding analysis was carried out using [^{3}H]-idazoxan (range 0.5–5.0 nM) and specific binding was defined by 5 μM phentolamine. Incubations were performed in triplicate at 2°C for 30 min and were terminated by rapid filtration through GF-B equivalent filters (Skatron), using a Skatron cell harvester, followed by washing with a large excess of incubation medium.

Cortical membranes were suspended in 50 mM Tris-HCl, 8 mM $MgCl_2$ pH 7.4 (1 mg tissue ml^{-1}) for measurement of β-adrenoceptor binding. Saturation binding analysis was carried out using [^{3}H]-dihydroalprenolol (0.5–5 nM) with specific binding being defined by the presence of 100 μM isoprenaline and 300 μM guanosine 5'-triposphate (GTP). Incubations were performed at 37°C for 30 min and were terminated by filtration through GF-B filters (Whatman) using a Skatron cell harvester.

Bound radioligand was measured by liquid scintillation counting in either Instagel or 299 scintillation fluid (Packard) at an efficiency of approximately 40%.

Binding characteristics of affinity (K_D, dissociation equilibrium constant) and capacity (B_{max}) were determined by non-linear regression analysis of specific binding data according to the method of Duggleby (1981).

Protein determinations for [^{3}H]-idazoxan binding were carried out by the method of Lowry *et al.* (1951). Protein content of membranes for [^{3}H]-dihydroalprenolol binding was determined by the method of Bradford (1976).

Statistical evaluation of the data

Biochemical and behavioural data have been analysed by ANOVA 2-way analysis of variance combined with Tukey's test, where appropriate. All other behavioural results have been analysed non-parametrically by use of Wilcoxon's 2-tailed rank order test. Data from receptor binding experiments have also been analysed by use of Student's unpaired *t* test. Although some figures show combined data for control experiments, all statistical analyses have been carried out between control and experimental groups of rats which have been tested at the same time.

Drugs

Drugs and reagents were obtained from the following sources: clonidine HCl (Boehringer-Ingelheim), desipramine HCl, phentolamine HCl (Geigy Pharmaceuticals), isoprenaline HCl (Sigma), [^{3}H]-idazoxan 40–60 Ci mmol^{-1}, [^{3}H]-dihydroalprenolol 70–110 Ci mmol^{-1} (Amersham International). Drugs were dissolved in 0.9% w/v NaCl solution (saline) and were injected by the intraperitoneal route.

Results

The effects of the oestrous cycle on clonidine-induced hypoactivity in female rats

The hypoactivity responses of female rats to clonidine (0.5 mg kg^{-1}) were measured at all stages of the oestrous cycle and this was found to have no effect

on the degree of sedation induced by the α_2-adrenoceptor agonist (Figure 2).

The effects of repeated desipramine administration on clonidine-induced hypoactivity in male and female rats

The effects of repeated administration of DMI (5 mg kg^{-1} b.d.) on clonidine-induced hypoactivity in male and female rats were examined after 4, 6, 9, 12 and 14 days treatment. The hypoactivity responses induced by clonidine at a dose of 0.2 mg kg^{-1} in male rats and 0.5 mg kg^{-1} in females were identical for both groups of saline-treated controls (Figure 3). Repeated administration of DMI progressively attenuated this α_2-adrenoceptor-mediated behavioural response in both male and female animals and this reduction was maximal after 9 to 12 days of DMI treatment (Figure 3). However, there were differences between the sexes, both in the time-course and the extent of this DMI-induced effect. In the males, attenuated responses to clonidine were observed after 4 days of DMI administration, but this antidepressant had to be given for 9 days before producing a significant attenuation in the female rats (Figure 3). In addition, the maximum reduction produced by repeated DMI treatment was markedly greater in the males than in the females (Figure 3).

The effects of repeated ECS administration on clonidine-induced hypoactivity in male and female rats

The effects of repeated ECS administration on clonidine-induced hypoactivity was investigated 24 h after 2, 3, 5, 7, and 10 days of ECS administration. The hypoactivity responses induced by clonidine administered at doses of 0.2 mg kg^{-1} and 0.5 mg kg^{-1} to male and female rats, respectively, were identical for both halothane-treated control groups of rats (Figure 4). Repeated ECS administration induced a progressive decrease in clonidine-induced hypoactivity in rats of either sex and the reduction was maximal after 7 to 10 days of ECS treatment (Figure 4). This attenuation was observed in both males and females after 2 days of ECS, but the maximum reduction was again greater in the males than in the females (Figure 4).

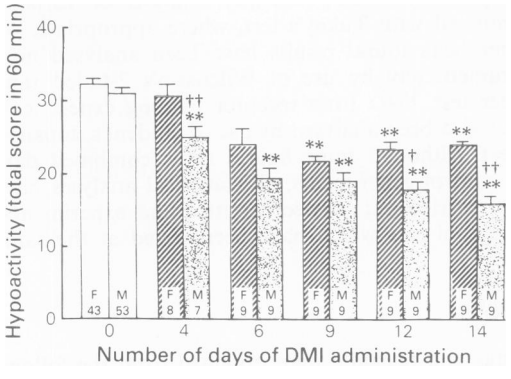

Figure 3 The time-courses in male (stippled columns) and female (hatched columns) rats for the attenuation of clonidine-induced hypoactivity by repeated injection of desipramine (DMI). Groups of rats were given twice daily injections of DMI (5 mg kg^{-1}; hatched and stippled columns) or saline (0.5 ml; open columns). The hypoactivity responses to clonidine (0.2 mg kg^{-1}, males; 0.5 mg kg^{-1}, females) were assessed 18 h after the final injection. The results are the mean total hypoactivity scores obtained in 60 min of the numbers of rats tested shown in each column; vertical lines indicate s.e.mean. Although saline control data are plotted as the combined results for all testing points, statistical comparisons were always made against controls tested at the same time. Rats were used once only. $^{**}P < 0.01$, significantly different from appropriate saline control. $^{\dagger}P < 0.05$; $^{\dagger\dagger}P < 0.01$, significantly different from appropriate DMI-treated female response.

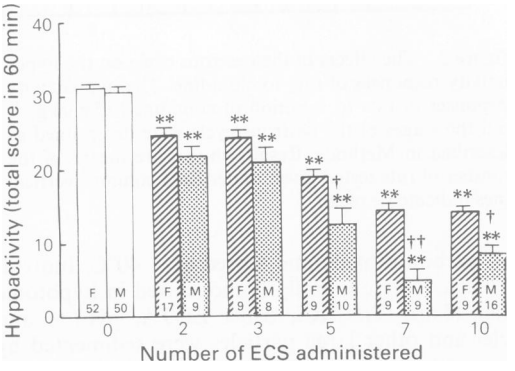

Figure 4 The time-courses in male (stippled columns) and female (hatched columns) rats for the attenuation of clonidine-induced hypoactivity by repeated administration of electroconvulsive shock (ECS). Groups of rats were given either an ECS (110 V, 1 s) under halothane anaesthesia (hatched and stippled columns) or halothane alone (open columns). The hypoactivity responses to clonidine (0.2 mg kg^{-1}, males; 0.5 mg kg^{-1} females) were assessed 24 h after the final treatment. The results are the mean total hypoactivity scores obtained in 60 min of the number of rats tested shown in each column; vertical lines indicate s.e.mean. Although halothane control data are plotted as the combined results for all testing points, statistical comparisons were always made against controls tested at the same time. Rats were used once only. $^{**}P < 0.01$, significantly different from appropriate halothane-treated control. $^{\dagger}P < 0.05$; $^{\dagger\dagger}P < 0.01$, significantly different from ECS-treated female response.

Table 1 A comparison of the effects of repeated desipramine (DMI) administration on the number of cortical α_2- and β-adrenoceptors (B_{max}) in male and female rats

	Saline × 2d	*DMI × 2d*	*Saline × 14d*	*DMI × 14d*
α_2-Adrenoceptors				
Male rats	104 ± 7	100 ± 5	82 ± 6	71 ± 2
Female rats	85 ± 8	90 ± 4	81 ± 3	73 ± 3*
β-Adrenoceptors				
Male rats	222 ± 6	191 ± 6**	221 ± 7	160 ± 7***
Female rats	226 ± 6	186 ± 8**	233 ± 6	156 ± 6***

Rats were injected with DMI (5 mg kg^{-1}) or saline twice daily for 2 or 14 days. Animals were killed 18 h after the final injection and binding to cortical α_2- or β-adrenoceptors was measured as described in Methods. Results are presented ± s.e.mean for data from 5–12 separate experiments on individual rats. B_{max} = fmol mg^{-1} protein. K_D values (nM) for combined saline-treated controls were: [^{3}H]-idazoxan, males = 0.48 ± 0.04, n = 16; females = 0.50 ± 0.03, n = 14; [^{3}H]-dihydroalprenolol, males = 2.55 ± 0.09, n = 12; females = 2.43 ± 0.23, n = 12. DMI treatment did not significantly alter these values.
Significantly different from appropriate saline control *P < 0.05; **P < 0.01; ***P < 0.001 (Student's unpaired t test).

The effects of repeated administration of desipramine and ECS on cortical α_2-adrenoceptors in male and female rats

The effects of antidepressant treatments on cortical α_2-adrenoceptors were assessed using [^{3}H]-idazoxan and were determined after 2 and 14 days of DMI administration and 2 and 10 days of ECS treatment.

There was no difference between either the number of cortical α_2-adrenoceptors (B_{max}) or the affinity (K_D) of the ligand for the α_2-adrenoceptor between the male and female saline-treated controls (Table 1). Two days of DMI treatment had no effect on cortical α_2-adrenoceptors (Table 1). However, after 14 days of DMI administration there was a small decrease in the number of cortical α_2-adrenoceptors in both male and female rats (Table 1). This reduction was identical for both sexes, but in the case of the males failed to reach statistical significance (Table 1).

Administration of an ECS once daily for 2 days produced no changes in cortical α_2-adrenoceptors in either male or female rats (Table 2). After 10 days of ECS treatment (ECS × 10) there was a significant reduction in cortical α_2-adrenoceptor numbers in both male and female rats with no change in K_D and the extent of this decrease in B_{max} was identical in both sexes (Table 2).

The effects of repeated administration of desipramine and ECS on cortical β-adrenoceptors in male and female rats

The actions of the two antidepressant treatments on cortical β-adrenoceptors were assessed by [^{3}H]-dihydroalprenolol binding. These investigations were performed after 2 and 14 days of DMI injection and also ECS × 2 and ECS × 10. A comparison of the β-adrenoceptor binding characteristics of the saline-treated control groups showed no differences

Table 2 A comparison of the effects of repeated electroconvulsive shock (ECS) administration on the number of cortical α_2- and β-adrenoceptors (B_{max}) in male and female rats

	Halothane × 2	*ECS × 2*	*Halothane × 10*	*ECS × 10*
α_2-Adrenoceptors				
Male rats	113 ± 4	108 ± 6	120 ± 6	102 ± 4*
Female rats	104 ± 5	114 ± 6	121 ± 6	103 ± 5*
β-Adrenoceptors				
Male rats	214 ± 7	217 ± 10	226 ± 7	188 ± 6**
Female rats	227 ± 7	221 ± 4	226 ± 6	185 ± 10**

Rats were given either a single ECS (110 V, 1 s) or halothane anaesthesia alone, once daily for 2 or 10 days. Animals were killed 24 h after the final treatment and binding to cortical α_2- and β-adrenoceptors was measured as described in Methods. Results are presented ± s.e.mean for data on 6–12 separate experiments on individual rats. B_{max} = fmol mg^{-1} protein. K_D values (nM) for combined halothane-treated controls were: [^{3}H]-idazoxan, males = 0.72 ± 0.06, n = 22; females = 0.72 ± 0.04, n = 21; [^{3}H]-dihydroalprenolol, males = 2.51 ± 0.09, n = 12; females = 2.44 ± 0.12, n = 12. ECS treatment did not significantly alter these values.
Significantly different from appropriate halothane control *P < 0.05, **P < 0.01 (Student's unpaired t test).

between B_{max} and K_D values of cortical β-adrenoceptors of male and female rats (Table 1). Two days of DMI injection produced a rapid decrease in the number of cortical β-adrenoceptors in both male and female rats (Table 1). This reduction was more pronounced in both sexes after 10 days of DMI treatment (Table 1).

Repeated ECS treatment did not produce a rapid decrease in the number of cortical β-adrenoceptors, with no change being observed in either male or female rats after 2 days of administration (Table 2). However, after ECS had been administered for 10 days, there was a marked reduction in the cortical β-adrenoceptor number in both sexes with no change of ligand-receptor affinity (Table 2).

Discussion

In this study, central α_2-adrenoceptor function has been assessed by measuring the hypoactivity responses to the α_2-adrenoceptor agonist, clonidine, and by ligand-receptor binding studies with [^{3}H]-idazoxan. In rat brain, the overwhelming majority of α_2-adrenoceptors are located on postsynaptic sites and are not presynaptic terminal autoreceptors (U'Prichard *et al.*, 1980; Dausse *et al.*, 1982; Dooley *et al.*, 1983). The majority of studies favour the hypothesis that sedative responses to clonidine are mediated via presynaptic α_2-adrenoceptors (Zebrowska-Lupina *et al.*, 1977; Drew *et al.*, 1979; Strömbom & Svensson, 1980; Nomura *et al.*, 1980; Heal *et al.*, 1987b), although two studies have suggested that the α_2-adrenoceptors involved are postsynaptic (Spyraki & Fibiger, 1982; Nassif *et al.*, 1983). By contrast, the cortical α_2-adrenoceptors monitored by receptor binding techniques are almost exclusively postsynaptic (U'Prichard *et al.*, 1980; Dausse *et al.*, 1982; Dooley *et al.*, 1983).

When the hypoactivity responses of male and female rats were tested using clonidine, the former were markedly more susceptible to the sedative effects of this α_2-adrenoceptor agonist. Since the behavioural parameters assessed by the method of Drew *et al.* (1979) are specifically α_2-adrenoceptor-mediated (Drew *et al.*, 1979; Heal *et al.*, 1981) and are not induced by other sedative agents, such as pentobarbitone or diazepam (Heal *et al.*, 1981), this strongly suggests a difference in central α_2-adrenoceptor function between male and female rats. This is unlikely to result from a generalised increase in activity in females because Heal *et al.* (1987b) have shown that the parameters scored by the method of Drew *et al.* (1979) were not influenced by increased locomotor activity after apomorphine injection (Heal *et al.*, 1987b). Furthermore, this difference is probably not related to levels of circulating oestrogen and progesterone, because hypoactivity responses to clonidine were not influenced by different phases of the oestrous cycle (this study), or by repeated administration of high doses of oestradiol (Heal *et al.*, 1988). Although the female rats were less responsive than the males to the sedative effects of clonidine, the resulting hypoactivity response was more resistant to attenuation by repeated DMI or ECS administration. In males, DMI significantly reduced hypoactivity after four days of treatment, but it required nine days of DMI injection to attenuate this response in female rats. Moreover, the maximum reduction induced by DMI administration was markedly greater in male rats than females. Similar effects were also observed with repeated ECS administration, but in this case, the onset of the reduced clonidine responses was identical in both sexes and it was only the maximum reduction which was greater in the male rats. Hence, while hypoactivity with clonidine is more pronounced in male rats, it is easier to reverse by administration of these two antidepressant treatments. Since the difference was observed for ECS, as well as for DMI, then this was unlikely to be due to pharmacokinetic effects. Furthermore, Heal *et al.* (1983) have previously shown that there is no correlation between the attenuation of clonidine-induced hypoactivity and plasma DMI concentrations. Another possible explanation for this dissimilarity between the sexes is that clonidine had to be given at a higher dose to the females to induce the same degree of hypoactivity. However, this is unlikely for two reasons. Firstly, this did not prevent the rapid reduction of the response by ECS × 2 and, secondly, we have found, albeit in male mice, that the attenuation of this α_2-adrenoceptor-mediated behaviour by ECS × 10 was independent of the dose of clonidine administered (percentage inhibition by ECS × 10: 0.1 mg kg^{-1}, 39%; 0.5 mg kg^{-1}, 43%; 1.0 mg kg^{-1}, 42%; Heal & Akagi, unpublished observations).

In the case of postsynaptic cortical α_2-adrenoceptors, no differences were observed between male and female rats. The receptor number (B_{max}) and ligand-receptor affinity (K_D) were identical in both sexes. No rapid decrease in the number of cortical α_2-adrenoceptors was observed after 2 days of DMI injection, but there was a small reduction in both sexes after 14 days of treatment. This effect was identical for both males and females, but only reached statistical significance in the latter. However, Heal *et al.* (1987a) have previously demonstrated a significant reduction in cortical α_2-adrenoceptors in male rats using an identical 14 day DMI treatment protocol. The reduction in cortical α_2-adrenoceptors induced by ECS administration was also relatively slow in onset being observed after 10 days, but not 2 days of ECS treatment. Once again, the effects were identical in both males and females.

There was no difference in either the number or ligand-receptor affinity of cortical β-adrenoceptors between the controls of either sex. Moreover, the effects of repeated administration of DMI and ECS were also identical in male and female rats. Administration of DMI reduced cortical β-adrenoceptors and this effect was progressive and rapid in onset, being observed after only 2 days of treatment. Repeated ECS also decreased β-adrenoceptors, but the effect was slower, was not induced by 2 days of ECS administration and was only significant after ECS $\times$ 10.

The present findings are in general agreement with previous results, in so far as repeated administration of DMI or ECS has been shown to decrease presynaptic α_2-adrenoceptor function (Tang *et al.*, 1979; McMillen *et al.*, 1980; Heal *et al.*, 1981; 1987a; Sugrue, 1983) and cortical β-adrenoceptor number (Bergstrom & Kellar, 1979a, b; Sellinger-Barnette *et al.*, 1980; Asakura *et al.*, 1982; Sugrue, 1982; 1983; Stanford & Nutt, 1982; Stanford *et al.*, 1983). Moreover, chronic ECS has previously been demonstrated to reduce cortical α_2-adrenoceptors (Pilc & Vetulani, 1982; Stanford & Nutt, 1982; Heal *et al.*, 1987a). However, the literature concerning the effects of repeated DMI on α_2-adrenoceptor binding is conflicting with findings of a decrease (Heal *et al.*, 1987a), an increase (Johnson *et al.*, 1980; Asakura *et al.*, 1982) and no change (Stanford *et al.*, 1983; Sugrue, 1983). The reason for this discrepancy may be that the effect of DMI on cortical α_2-adrenoceptors is relatively small. In this study, the reduction of cortical α_2-adrenoceptor number in the DMI-treated males failed to reach significance, because of the variability of the saline-treated controls. Other explanations for these discrepancies may be the different DMI treatment regimes employed or the ligand selected to quantify the α_2-adrenoceptors.

One point of note is that these results show a clear separation between the effects of the two antidepressant treatments on clonidine-induced hypoactivity and postsynaptic α_2-adrenoceptors, determined by [^{3}H]-idazoxan binding. This, therefore, provides additional indirect evidence to show that this behavioural response is not mediated by cortical postsynaptic α_2-adrenoceptors.

Overall, the data show clear sex-related differences in presynaptic α_2-adrenoceptor function with no corresponding alterations in postsynaptic α_2- or β-adrenoceptors. This focus of hormonal action on presynaptic α_2-adrenoceptor function is in agreement with our previous studies showing that T_3 and oestradiol both accelerate the ECS- or DMI-induced attenuation of clonidine-induced hypoactivity, despite having no effect alone. Now we have found that in comparison with male rats, female rats have an increased resistance to the sedative effects of clonidine and an attenuated reversal of this hypoactivity response by two antidepressant treatments. The most probable explanation for these effects is the difference in hormonal profiles. One way of confirming a role for sex hormones in presynaptic α_2-adrenoceptor function would be to conduct studies in ovarectomised females and castrated males.

In conclusion, therefore, the data show that differences in central presynaptic α_2-adrenoceptor function exist between male and female rats and this may be related to divergences in hormonal profiles. By contrast, no differences were observed in postsynaptic α_2- or β-adrenoceptors in complementary binding experiments on rat cortical tissue.

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Contraction of the rat isolated aorta caused by *Clostridium perfringens* alpha toxin (phospholipase C): evidence for the involvement of arachidonic acid metabolism

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1 Alpha toxin produced by *Clostridium perfringens* contracted the rat isolated aorta and stimulated release of arachidonic acid in the tissue.

2 Quinacrine did not inhibit contraction caused by the toxin.

3 Indomethacin blocked contraction caused by the toxin in a dose-dependent manner and markedly increased levels of arachidonic acid released by the toxin.

4 The toxin-induced contraction was blocked by the thromboxane synthetase inhibitor OKY-046 and the thromboxane A_2 (TXA_2) antagonist ONO-3708.

5 The toxin stimulated production of TXB_2 and this was blocked by pretreatment with either indomethacin or OKY-046.

6 Toxin-induced contraction was diminished by pretreating aorta with collagenase or by rubbing the intimal surface to remove the endothelium.

7 These data suggest that the contractile response to the toxin is associated with stimulation of TXA_2 production from arachidonic acid released by the toxin in the endothelial cells of the aorta.

Introduction

Clostridium perfringens alpha toxin, which is lethal, dermonecrotic and haemolytic, has been thought to be an important toxin in gas gangrene caused by the organism (Aikat & Dible, 1957; MacLennan, 1962). The toxin is known to possess phospholipase C activity (MacFarlane & Knight, 1941) and has been shown to hydrolyze various phospholipids such as phosphatidylcholine and sphingomyelin in biological membranes (Tobias *et al.*, 1962; Lenard & Singer, 1968; McIlwain & Rapport, 1971; Knickelbein & Rosenberg, 1980). Various effects have been observed with crude preparations of alpha toxin, including increases in vascular permeability in guinea-pig skin (Elder & Miles, 1957; Stark & Duncan, 1972), increases in the ionic permeability of skeletal muscle membranes (Albuquerque & Thesleff, 1967) and a significant reduction in the acetylcholine response of smooth muscle that was partially restored by the addition of Ca^{2+} (Lodge & Leach, 1973). However, *C. perfringens* produces toxins (enterotoxin, haemolysin) and enzymes (collagenase, hyaluronidase) which may be responsible for some of these effects (Willis, 1969) and interfere with characterization of the specific biological activities of alpha toxin.

We have recently investigated the effects of purified alpha toxin on tissues of rat and found that it potentiates the contraction of the rat isolated vas deferens elicited by noradrenaline (NA) (Sakurai *et al.*, 1985a), and causes a rise in blood pressure with a decrease in blood flow (Sakurai *et al.*, 1985b). Using alpha toxin purified on a large scale from culture supernatant fluid of *C. perfringens* type A (which produces alpha toxin, but not beta and epsilon toxins), we found that purified alpha toxin caused contraction of the rat isolated aorta (Fujii *et al.*, 1986). The present study was undertaken to define the mode of action of this contractile effect.

Methods

Rat isolated aorta

Male rats, weighing 280 to 300 g, were killed by cervical dislocation and exsanguination. The isolated

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aorta was cut helically into strips (20 mm long and 2 mm wide) that were suspended in organ baths (10 ml) containing Tyrode solution of the following composition (mM): NaCl 137, $CaCl_2$ 1.8, KCl 2.7, $MgCl_2$ 1.8, NaH_2PO_4 0.42, $NaHCO_3$ 11.9 and glucose 5.6. The tissue bath solution was maintained at 37°C and bubbled with a mixture of 95% O_2 and 5% CO_2. Contractions were determined isotonically as described previously (Fujii *et al.*, 1986).

Procedures for removing endothelial cells

Two separate procedures described by Furchgott & Zawadzki (1980) were used: (a) intact aorta were cannulated and the intima exposed to 0.2% collagenase (CLS-type 1A, Worthington) for 15 min at 37°C; (b) the intimal surface was gently abraded by rubbing.

Purification of C. perfringens *alpha toxin*

Procedures for culture of *Clostridium perfringens* type A (strain NCTC 8237) and purification of alpha toxin have been described previously (Sakurai *et al.*, 1985a; Fujii *et al.*, 1986). This toxin preparation was free from detectable collagenase, hyaluronidase, neuraminidase and theta toxin activities.

Thromboxane B_2 assay

Rings of aorta (2 to 3 mm, weighing 20–30 mg) were equilibrated in 5 ml of Tyrode solution for 1 h at 37°C. During the equilibration period, the solutions were replaced every 15 min with fresh Tyrode solution. They were then incubated in 1 ml of Tyrode solution containing the toxin for 20 min at 37°C and thromboxane B_2 (TXB_2) in the medium was measured by radioimmunoassay (Amersham, U.K.).

Arachidonic acid assay

Rings of aorta (2 to 3 mm, weighing 15–20 mg) were equilibrated in 5 ml of Tyrode solution for 1 h at 37°C. During the equilibration period, the solutions were replaced every 15 min with fresh Tyrode solution. Next, the tissues were incubated in 2 ml of Tyrode solution containing the toxin for 20 min at 37°C, and homogenized in a fluid volume of 4 ml of Tyrode solution. The homogenates (2 ml) were vigorously mixed with a mixture containing 4 ml of isopropyl alcohol-hexane (7:3), 1 ml of 2 N H_2SO_4, and nonadecanoic acid ($C_{19:0}$) as the internal standard for 30 s and allowed to separate on standing. The hexane phase was pipetted into a vial containing 60 mg of silicic acid. The vial was rotated, allowed to stand for 10 min at room temperature and dried in a rotary evaporator. The residue was esterified with excess ethereal diazomethane, and excess reagent was evaporated under a nitrogen stream. Esterified fatty acids were analysed by gas chromatography using a flame ionization detector (Shimazu GC-7AG, Kyoto, Japan). The silanized, coiled glass column (2.1 m × 3 mm i.d.) used was packed with either 2% (w/w) OV-17 on Chromosorb W (AW-DMCS), 80–100 mesh (Shimazu) or 3% (w/w) FFAP on Chromosorb W (AW-DMCS), 80–100 mesh (Shimazu).

Materials

The following drugs were used: indomethacin (Sigma Chemical Company, St. Louis, MO, U.S.A.), aspirin (Sigma Chemical Company), arachidonic acid (Sigma Chemical Company), quinacrine (Sigma Chemical Company), noradrenaline (NA) (Sankyo Company, Ltd., Tokyo, Japan), nonadecanoic acid (Serdary Research Lab., London, Ontario, Canada), collagenase type 1A (Sigma Chemical Company). OKY-46 ((E)-3-[4-(imidazolylmethyl)phenyl]-2-propenoic-acid hydrochloride-monohydrate) and ONO-3708 ((9,11)(11,12)-dideoxa-9α,11α-dimethylmethano-11,12-methano-13,14-dihydro-13-aza-14-oxo-15-cyclopentyl-16,17,18,19,20-pentanor-15-epithromboxane-A_2) were kindly provided by Ono Pharmaceutical Company (Osaka, Japan).

Results

Effect of alpha toxin on arachidonic acid release in the rat isolated aorta

The relationships between doses of the toxin and release of arachidonic acid were investigated (Figure 1). After treatment with 0.54 $\mu g\,ml^{-1}$ of the toxin, release of arachidonic acid was increased from 22.0 ± 3.8 to 42.1 ± 5.6 $ng\,mg^{-1}$ wet weight, suggesting that the toxin stimulates release of arachidonic acid. On the other hand, when the tissue was treated with the toxin in the presence of the cyclo-oxygenase inhibitor indomethacin, arachidonic acid release was 61.0 ± 3.0 $ng\,mg^{-1}$ wet weight (Figure 1). The data shows that in the presence of indomethacin arachidonic acid released by the toxin accumulates at a significantly higher level.

To test if the action of the toxin was dependent on phospholipase A_2, the effect of the phospholipase A_2 inhibitor quinacrine (Frye & Holz, 1983) was evaluated. Treatment with quinacrine (5×10^{-5} M) for 15 min, which was sufficient to prevent a submaximal response to NA (1×10^{-7} M), did not affect either

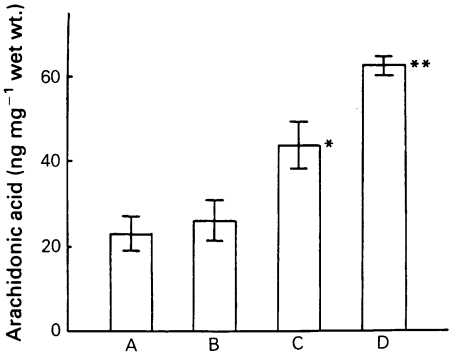

Figure 1 Effect of indomethacin on arachidonic acid release induced by alpha toxin in the rat isolated aorta. Tissues were incubated for 15 min in the absence (A) or the presence (B) of indomethacin (1×10^{-5} M). The toxin ($0.54\,\mu g\,ml^{-1}$) was added after 15 min of incubation in the absence (C) or the presence (D) of indomethacin (1×10^{-5} M). These treated tissues were incubated at 37°C for 1 h in Tyrode solution, and the arachidonic acid assay was carried out as described in the text. * Significantly different from (A) or (B) ($P < 0.01$). ** Significantly different from (C) ($P < 0.02$). Vertical lines indicate s.e.mean ($n = 4$).

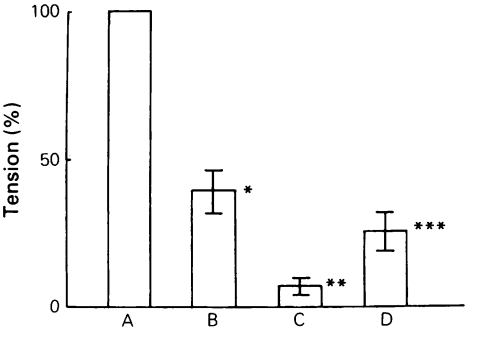

Figure 2 Effect of indomethacin or aspirin on alpha toxin-induced contraction. The tension (%) was expressed as a percentage of control contractions induced by alpha toxin alone (A). The toxin ($0.54\,\mu g\,ml^{-1}$) was applied 15 min after administration of indomethacin 1×10^{-6} M (B) or 3×10^{-6} M (C) or aspirin 5×10^{-5} M (D). * Significantly different from (A) ($P < 0.002$). ** Significantly different from (A) ($P < 0.001$) and (B) ($P < 0.01$). *** Significantly different from (A) ($P < 0.001$). Vertical lines indicate s.e.mean ($n = 5$).

the contraction or the release of arachidonic acid caused by the toxin (data not shown).

The relationship between arachidonic acid metabolism and contraction induced by the toxin

Figure 2 shows that the cyclo-oxygenase inhibitors indomethacin and aspirin both decreased contraction caused by the toxin.

In order to investigate further the role of cyclo-oxygenase products in toxin-evoked contractions, the effects of the TXA_2 synthetase inhibitor OKY-46 (Naito *et al.*, 1983; Cerletti *et al.*, 1986) and TXA_2 antagonist ONO-3708 (Fujioka *et al.*, 1986; Toda *et al.*, 1986) were investigated and compared with their actions on NA-induced contractions (Table 1). When the tissue pretreated with 10^{-5} M and 10^{-4} M OKY-046, the toxin-induced contraction was decreased to approximately 36% and 14% of control, respectively. ONO-3708 also inhibited the toxin-induced contraction in a dose-dependent manner. The NA-induced contraction was not inhibited by either OKY-046 or ONO-3708 at 10^{-4} M. These data suggest that TXA_2 is an important factor in the contraction induced by the toxin, but not by NA. Consistent with this, the stable metabolite of TXA_2, TXB_2, was significantly increased from 18.0 (basal release) to $40.2\,pg\,mg^{-1}$ wet weight by the treatment with the toxin (Table 2). This toxin-induced increase in TXB_2 production was blocked by indomethacin or OKY-046.

Effect of the toxin on the isolated aorta without endothelium

The effect of the toxin or NA on the untreated and the collagenase-treated tissue was investigated. Collagenase treatment resulted in no loss of the contractile response to NA (data not shown), as observed by

Table 1 Effect of OKY-046 and ONO-3708 on alpha toxin- and noradrenaline (NA)-induced contractions

Inducer	*Agent*	*Tension* (%)
Toxin	None	100
Toxin	OKY-046, 1×10^{-5} M	36.0 ± 4.6*
Toxin	OKY-046, 1×10^{-4} M	14.5 ± 4.8**
Toxin	ONO-3708, 1×10^{-5} M	18.0 ± 10.3***
Toxin	ONO-3708, 1×10^{-4} M	11.3 ± 5.6***
NA	None	100
NA	OKY-046, 1×10^{-4} M	98.2 ± 10.3
NA	ONO-3078, 1×10^{-4} M	94.0 ± 8.0

The toxin ($0.54\,\mu g\,ml^{-1}$) or NA (1×10^{-5} M) was applied 15 min after administration of OKY-046 or ONO-3708. The tensions (%) were expressed as a percentage of contraction induced by alpha toxin ($0.54\,\mu g\,ml^{-1}$) or NA (1×10^{-5} M). The results are the mean ± s.e.mean of 4 experiments.

* Significantly different from control ($P < 0.001$).

** Significantly different from treatment with 1×10^{-5} M OKY-046 ($P < 0.05$).

*** Significantly different from control ($P < 0.005$).

Table 2 Effect of indomethacin and OKY-046 on thromboxane B_2 generated by alpha toxin in rings of rat isolated aorta

Toxin	*Agent*	*Thromboxane B_2* (pg mg^{-1} wet wt.)
−	None	18.0 ± 2.3
+	None	40.2 ± 3.9*
−	Indomethacin	20.0 ± 2.0
+	Indomethacin	21.0 ± 1.4**
+	OKY-046	24.0 ± 3.4***
−	OKY-046	25.5 ± 2.1

Rings of isolated aorta were incubated for 15 min in the absence or presence of indomethacin (1×10^{-6} M) or OKY-046 (1×10^{-5} M), where indicated. (+) Toxin (0.54 μg ml^{-1}) was added after this incubation. The tissues were incubated in Tyrode solution at 37°C for 1 h, and the thromboxane B_2 assay was carried out as described in the text. The results are the mean ± s.e.mean of 6 experiments.

* Significantly different from the untreated tissues ($P < 0.005$).

** Significantly different from the toxin-treated tissues ($P < 0.01$).

*** Significantly different from the toxin-treated tissues ($P < 0.05$).

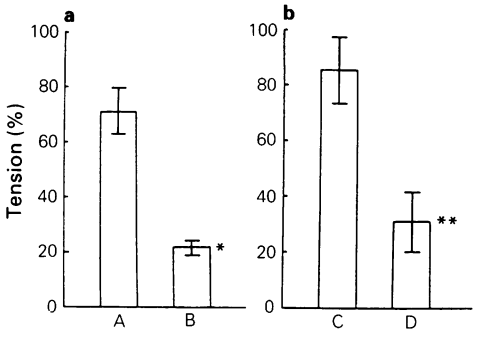

Figure 3 Effect of alpha toxin on the isolated aorta without endothelium. (a) Treatment of the tissue with collagenase. (A) The untreated tissue (control), (B) the treated tissue. (b) Rubbing of the intimal surface of the tissue. (C) The untreated tissue (control), (D) the treated tissue. The toxin (0.54 μg ml^{-1}) was added 15 min after incubation of the treated tissue in Tyrode solution. The tension (%) was expressed as a percentage of the reference contraction induced by noradrenaline (1×10^{-5} M). * Significantly different from control (A) ($P < 0.01$). ** Significantly different from control (C) ($P < 0.01$). Vertical lines indicate s.e.mean ($n = 5$).

Furchgott & Zawadzki (1980), suggesting that aortic smooth muscle was not damaged by collagenase. However, when endothelial cells had been removed by preincubation with collagenase (Furchgott & Zawadzki, 1980), the toxin-induced contraction was reduced by about 75%, compared to the toxin-induced contraction of the untreated tissue (Figure 3a). To obtain further evidence for the relationship between the presence of endothelium and contraction induced by the toxin, endothelial cells were removed by rubbing the intimal surface (Furchgott & Zawadzki, 1980). Contractions evoked by the toxin were decreased to approximately 35% of control (Figure 3b).

Discussion

Alpha toxin purified from culture supernatant fluid of *Clostridium perfringens* alpha toxin has been shown to possess phospholipase C activity (Krug & Kent, 1984), and we have recently demonstrated that toxin-induced contraction of rat isolated aorta was associated with *de novo* production of phosphatidylinositol and phosphatidic acid (Fujii *et al.*, 1986). In the present study, we found that the toxin-induced contraction was significantly inhibited by cyclo-oxygenase inhibitors such as indomethacin and aspirin. Furthermore, the toxin was found to stimulate release of arachidonic acid in the tissue. Therefore, stimulation of arachidonic acid release may be related to activation of phospholipid metabolism by the toxin. Arachidonic acid has generally been thought to be metabolized to cyclo-oxygenase products (Hamberg & Samuelsson, 1973) and lipoxygenase products (Murphy *et al.*, 1979). The release of arachidonic acid caused by the toxin in the presence of the cyclo-oxygenase inhibitor indomethacin was approximately 1.5 times higher than that in the absence of the inhibitor, suggesting that arachidonic acid is mainly metabolized to cyclo-oxygenase products under our experimental conditions. These data support the view that toxin-induced contractions are associated with cyclo-oxygenase product(s) metabolized from arachidonic acid.

The toxin-evoked contractions were unaffected by quinacrine at concentrations which completely inhibited NA-induced contractions. Furthermore, the release of arachidonic acid caused by the toxin was not inhibited by quinacrine. In these experiments, quinacrine was used to inhibit phospholipase A_2 (Lapetina, 1982; Frye & Holz, 1983). However, arachidonic acid can be released not only from phosphatidic acid by phospholipase A_2 (Lapetina, 1982) but also from monoacylglycerol by monoacylglyceride lipase (Chau & Tai, 1981). Therefore, since quinacrine was inactive, the release of arachidonic acid seems to be due to hydrolysis of monoacylglycerol or diacylglycerol by monoacylglyceride or diacylglyceride lipase. This is consistent with the

toxin causing hydrolysis of membrane phospholipids resulting in a rise in the level of diacylglycerol.

To investigate further the role of cyclo-oxygenase products, OKY-046 was used as an inhibitor of TXA_2 synthetase (Naito *et al.*, 1983; Cerletti *et al.*, 1986), and ONO-3708 as an antagonist of TXA_2 (Fujioka *et al.*, 1986; Toda *et al.*, 1986). OKY-046 and ONO-3708 were found to inhibit contractions caused by the toxin in a dose-dependent manner. Furthermore, the production of TXB_2, the stable metabolite of TXA_2, was stimulated by the toxin and this effect was also blocked by indomethacin and OKY-046. Although these agents completely inhibited the production of TXA_2 by the toxin, they did not completely inhibit toxin-induced contractions. In contrast, indomethacin 3×10^{-6} M almost abolished toxin-induced contractions. Thus, it is possible that other cyclo-oxygenase products apart from TXA_2 contribute to the contraction.

Several workers have shown that TXA_2 is produced enzymatically by endothelial cells of blood vessels from rabbit (Ingerman-Wojenski *et al.*, 1981b), bovine (Ingerman-Wojenski *et al.*, 1981a), and human umbilical blood vessels (Tuvemo *et al.*, 1976). Thus, it was possible that toxin-induced contractions of rat isolated aorta associated with the production of TXA_2 required intact endothelium. Consistent with this, the toxin did not induce contraction after treatment of the isolated aorta with collagenase or rubbing of the tissue to remove endothelial cells from the intimal surface of the tissue (Furchgott & Zawadzki, 1980). Since collagenase treatment and rubbing resulted in no effect on the sensitivity of the tissues to NA, we conclude that aortic smooth muscle was not damaged by these treatments.

From these observations it appears that in endothelial cells of rat aorta, alpha toxin stimulates arachidonic acid release, leading to the production of TXA_2 which then elicits contraction of adjacent aortic smooth muscle.

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The effect of medetomidine, an α_2-adrenoceptor agonist, on plasma atrial natriuretic peptide levels, haemodynamics and renal excretory function in spontaneously hypertensive and Wistar-Kyoto rats

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1 The effects of the selective α_2-adrenoceptor agonist, medetomidine, were assessed on plasma levels of immunoreactive atrial natriuretic peptide (IR-ANP), haemodynamics and on urine water and solute excretion in conscious, chronically cannulated, 7 month-old spontaneously hypertensive (SHR) and age-matched Wistar-Kyoto (WKY) rats, in order to examine the role of α_2-adrenoceptors in the control of ANP secretion.

2 A 60 min i.v. infusion of medetomidine (0.2 or 0.6 $\mu g\,kg^{-1}\,min^{-1}$) decreased heart rate dose-dependently in both strains. Medetomidine infusion (0.6 $\mu g\,kg^{-1}\,min^{-1}$) resulted in an increase in mean arterial pressure in WKY, whereas both doses decreased blood pressure in SHR. There was a slight increase in the right atrial pressure in both strains (WKY: $+1.18 \pm 0.26$ mmHg; SHR: $+1.64 \pm 0.64$ mmHg, NS) in response to infusion of 0.6 $\mu g\,kg^{-1}\,min^{-1}$ of medetomidine.

3 No differences were found in resting plasma IR-ANP levels between WKY (114 ± 8 pg ml^{-1}, $n = 19$) and SHR (117 ± 10 pg ml^{-1}, $n = 21$). Infusion of equibradycardic doses of medetomidine increased dose-dependently plasma IR-ANP levels in WKY, but did not affect the plasma IR-ANP concentration in SHR rats.

4 Despite the different effect of medetomidine on ANP release in WKY and SHR rats, i.v. administration of medetomidine affected renal excretory functions similarly in both strains; urine flow and sodium excretion increased and urine osmolality decreased significantly, while there was no consistent change in urinary potassium excretion. Urine osmolality decreased to hypo-osmotic levels during the infusion of 0.6 $\mu g\,kg^{-1}\,min^{-1}$ of medetomidine, suggesting a possible interaction between α_2-adrenoceptor stimulation and the vasopressin system.

5 These results show that the α_2-adrenoceptor agonist medetomidine increased plasma levels of ANP in WKY rats, probably through an increase in mean arterial and right atrial pressures, whereas the SHR had attenuated ANP release to α_2-adrenoceptor stimulation. Our findings, that medetomidine caused marked natriuretic and diuretic effects in both strains and that these effects on the excretory functions of the kidneys were not related to changes in plasma levels of IR-ANP, demonstrate that changes in plasma ANP levels alone do not account for the diuretic and natriuretic effect of α_2-agonists.

Introduction

Atrial natriuretic peptide (ANP) is a hormone released from cardiac atria which acts upon the kidneys, vasculature, adrenal glands and brain to regulate volume and blood pressure (Laragh, 1985; Brenner & Ballerman, 1986; Needleman & Greenwald, 1986). The exact mechanism by which ANP is released is not known, although an increase in atrial pressure, presumably leading to augmented mechanical distension of the atrial wall, may be the primary physiological regulator (Lang *et al.*, 1985; Ruskoaho

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et al., 1987). Little is known about other factors that may influence ANP release. Adrenaline, noradrenaline, vasopressin and other agents have been shown to increase the release of ANP into the circulation both in man and experimental animals, probably via their pressor effects (Manning *et al.*, 1985; Uehlinger *et al.*, 1986; Ruskoaho *et al.*, 1987; Sanfield *et al.*, 1987). In addition, clonidine, an α_2-agonist, has been shown to increase plasma levels of ANP in conscious rats (Baranowska *et al.*, 1987a,b,c). However, because atrial pressure or other haemodynamic variables were not measured in these studies, the role of α_2-adrenoceptors in the control of ANP release remains unclear.

The present study was thus undertaken to examine the effects of an α_2-agonist, medetomidine, on plasma levels of immunoreactive ANP (IR-ANP), haemodynamics (mean arterial pressure, heart rate, right atrial pressure) and renal excretory function simultaneously in genetically hypertensive and age-matched normotensive control rats. Medetomidine has been shown to be a potent, selective and specific α_2-adrenoceptor agonist both in experimental animals and in man (Savola *et al.*, 1986; Scheinin *et al.*, 1986; 1987; Virtanen *et al.*, 1988). Binding studies carried out in rat brain membranes showed a relative α_2/α_1 selectivity ratio of 1620 for medetomidine (compared to 220 for clonidine, 300 for UK 14304) and negligible binding to, or effects at, β-adrenoceptors and opiate, adenosine, dopamine, histamine or 5-hydroxytryptamine receptors (Virtanen *et al.*, 1988). Biochemical experiments in rats have indicated that the compound reduces the release and metabolism of noradrenaline in the central nervous system (Scheinin *et al.*, 1986). In anaesthetized rats, medetomidine reduced blood pressure and heart rate, and in the pithed rat it caused peripheral vasoconstriction and inhibition of tachycardia induced by preganglionic sympathetic nerve stimulation (Savola *et al.*, 1986). Idazoxan and other α_2-adrenoceptor antagonists, but not prazosin, antagonized these effects (Savola *et al.*, 1986; Scheinin *et al.*, 1986).

Methods

Animals

Male 7 month-old spontaneously hypertensive rats (SHR) of the Okamoto-Aoki strain (F61) and age-matched Wistar-Kyoto (WKY) rats from the Department of Pharmacology colony at the University of Oulu, Oulu, Finland, were used. Both strains were originally obtained from Mollegaards Avslaboratorium, Skensved, Denmark. The rats were housed in plastic cages in a room with controlled 40% humidity and a temperature of 22°C. A 12 h light/dark cycle (lights on 06 h 00 min) was maintained. The experimental protocols were approved by the Committee for Animal Experimentation of the University of Oulu.

Experimental procedures

The surgical preparation has been described previously (Ruskoaho & Leppäluoto, 1988). Briefly, rats were anaesthetized (for 30–40 min) with chloral hydrate (300 mg kg^{-1} i.p.), the left femoral artery was cannulated for recording of blood pressure and heart rate and the left femoral vein for drug infusions. A cannula was inserted into the right atrium via the jugular vein for measurement of right atrial pressure. After surgery, rats were housed individually and had continuous access to food and water until the day of experiment.

The day after the operation, a rat was placed in the metabolic cage, and the arterial and right atrial catheters were attached to pressure transducers (model MP-15, Micron Instruments Inc., Los Angeles, CA, U.S.A.) and a Grass polygraph (model 7D, Grass Instruments, Quincy, Mass., U.S.A.) for mean arterial pressure, heart rate and right atrial pressure recording. The venous catheter was connected to an infusion pump (B. Braun PerfusorED, Braun Melsungen AG, FRG) for infusions of drug and replacement fluid.

After an equilibration period (30 min), haemodynamic variables were recorded in the conscious animals for 60 min (control period). Then, either vehicle (0.9% saline) or the α_2-adrenoceptor agonist, medetomidine was administered i.v. as a continuous infusion at a rate of 66 $\mu l\ min^{-1}$ for 60 min, followed by a recovery period of 60 min. The doses of medetomidine infused (0.2 or 0.6 $\mu g\ kg^{-1}\ min^{-1}$) were chosen based on preliminary experiments in conscious rats. These experiments revealed that infusion of medetomidine at these doses resulted in increases in plasma IR-ANP comparable to those observed earlier after acute volume expansion under identical experimental conditions (Ruskoaho & Leppäluoto, 1988). Arterial blood samples (0.8 ml) for plasma IR-ANP were taken 30 and 5 min before and at 10, 30, 60, and 90 min after the start of infusion. Each blood sample taken was replaced by an equal volume of blood from a donor rat. Urine was collected during control, infusion and recovery periods. To establish correlations between haemodynamic variables and renal excretory functions and plasma levels of IR-ANP, mean arterial pressure, heart rate and right atrial pressure were measured continuously throughout the experiments.

Urinary osmolality was measured with an Advanced wide-range osmometer 3W2 (Advanced Instruments, Inc., Needham Heights, Massachusetts, U.S.A.), and electrolytes were determined with FLM3 Flame Photometer (Radiometer, Copenhagen, Denmark). The volume of urine was measured gravimetrically.

Assay of immunoreactive atrial natriuretic peptide in plasma

All blood samples (0.8 ml) were taken into pre-cooled EDTA tubes on ice, immediately centrifuged (2000 *g*, 10 min, at +4°C) and the plasma stored at −20°C until assayed by radioimmunoassay (RIA). ANP was extracted by Sep-Pak C_{18} cartridges from plasma as described previously (Ruskoaho & Leppäluoto, 1988). The recovery of added rat ANP_{1-28} (50 pg) was 81.7 ± 3.3% (mean ± s.e.mean, $n = 6$). The ANP values were not corrected for recovery.

The plasma extracts were diluted with the radioimmunoassay buffer and incubated in duplicates of 100 µl with the same volume of the specific rabbit ANP antiserum at the final dilution of 1/25,000 (Vuolteenaho *et al.*, 1985; Ruskoaho & Leppäluoto, 1988). Synthetic rat ANP_{1-28} (Sigma) ranging from 0 to 1250 pg per tube was used as standard. The ANP tracer was rat [^{125}I]-ANP_{1-28} from Amersham, England. After incubation for 20–44 h at +4°C the bound and free fractions were separated by double antibody in the presence of 0.5 ml 1.2 M $(NH_4)SO_4$, pH 7. The sensitivity of the assay was 1 pg per tube. The intra-assay and inter-assay coefficients were 5% and 14%, respectively. The rat plasma extracts diluted serially displaced [^{125}I]-ANP in parallel with the synthetic ANP_{1-28} standard and eluted (over 90%) in the reverse phase high performance liquid chromatography as rat ANP_{1-28}.

Materials

Medetomidine hydrochloride was a gift from Dr A. Karjalainen, Farmos Group Ltd, Farmos Research Center, Turku, Finland. Synthetic rat ANP_{1-28} was obtained from Sigma Chemicals and heparin from Medica, Helsinki, Finland. Other chemicals were from Sigma Chemicals.

Data analysis

The results are expressed as mean ± s.e.mean. The data were analysed with two- or one-way analysis of variance (ANOVA). For the comparison of statistical significance between two independent groups, Student's *t* test for unpaired data was used. For the multiple comparison, one-way analysis of variance followed by the Bonferroni *t* test was used. Differences at the 95% level were considered significant.

Table 1 Body weight, baseline cardiovascular and urinary excretory variables and plasma levels of immunoreactive atrial natriuretic peptide (IR-ANP) in conscious 7 month-old spontaneously hypertensive (SHR) and Wistar-Kyoto (WKY) rats

	WKY ($n = 19$)	*SHR* ($n = 21$)
Body weight (g)	420 ± 6	388 ± 6***
Mean arterial pressure (mmHg)	125 ± 2	165 ± 3***
Heart rate (beats min^{-1})	336 ± 7	363 ± 8*
Right atrial pressure (mmHg)	3.0 ± 0.3	3.7 ± 0.5
Plasma IR-ANP (pg ml^{-1})	114 ± 8	117 ± 10
Urinary volume (ml h^{-1} 100 g^{-1})	0.47 ± 0.04	0.42 ± 0.05
$U_{Na}V$ (mEq h^{-1} 100 g^{-1})	11.5 ± 2.4	19.7 ± 4.1
U_KV (mEq h^{-1} 100 g^{-1})	34.3 ± 3.0	25.3 ± 3.4
U_{osm} (mosm kg^{-1} H_2O)	521 ± 70	515 ± 45

Results are expressed as mean ± s.e.mean. * $P < 0.05$ and *** $P < 0.001$ WKY vs SHR (Student's *t* test). $U_{Na}V$ = urinary sodium excretion; U_KV = urinary potassium excretion; and U_{osm} = urine osmolality.

Results

Effect of medetomidine infusion on haemodynamics in conscious SHR and WKY rats

The mean basal arterial pressure, as measured directly in chronically cannulated conscious rats, and heart rate were significantly higher in SHR than in WKY at the age of 7 months but their right atrial pressures did not differ significantly (Table 1). The body weight of SHR was lower than that of WKY rats (Table 1). Table 2 shows the effect of medetomidine infusions on haemodynamics in WKY and SHR. A sixty min infusion of 0.2 µg kg^{-1} min^{-1} of medetomidine decreased heart rate maximally by 23% ($F = 3.85$ for drug and time interaction, $P < 0.01$) in WKY and by 18% in SHR and an infusion of 0.6 µg kg^{-1} min^{-1} by 34% and by 35%, respectively.

The changes in mean arterial pressure evoked by medetomidine infusions were clearly different in WKY and SHR rats at both doses tested and showed an opposite pattern. Infusion of 0.6 µg kg^{-1} min^{-1} of medetomidine in WKY rats resulted in an increase in mean arterial pressure, whereas the lower dose of medetomidine had no significant effect (Table 2). In contrast, both doses of medetomidine decreased blood pressure in SHR

Table 2 Effects of a 60 min infusion of the α_2-agonist medetomidine (Med) on cardiovascular variables in spontaneously hypertensive (SHR) and Wistar-Kyoto (WKY) rats

	Control	*Infusion At 10 min*	*At 30 min*	*At 60 min*	*Recovery*	*F*
			Heart rate (beats min^{-1})			
WKY						
Vehicle	346 ± 12	328 ± 13	313 ± 12	318 ± 14	325 ± 18	—
Med 0.2 $\mu g kg^{-1} min^{-1}$	318 ± 8	277 ± 6	263 ± 14	245 ± 15	300 ± 9	F = 3.83; $P < 0.01$
Med 0.6 $\mu g kg^{-1} min^{-1}$	343 ± 14	275 ± 23	226 ± 22	228 ± 22	318 ± 34	F = 12.51; $P < 0.001$
SHR						
Vehicle	361 ± 13	346 ± 7	334 ± 12	346 ± 12	337 ± 13	—
Med 0.2 $\mu g kg^{-1} min^{-1}$	367 ± 12	337 ± 10	300 ± 13	300 ± 7	336 ± 12	F = 5.68; $P < 0.001$
Med 0.6 $\mu g kg^{-1} min^{-1}$	361 ± 18	296 ± 13	243 ± 10	233 ± 11	321 ± 23	F = 15.06; $P < 0.001$
			Mean arterial pressure (mmHg)			
WKY						
Vehicle	122 ± 3	120 ± 4	120 ± 3	116 ± 3	116 ± 2	—
Med 0.2 $\mu g kg^{-1} min^{-1}$	123 ± 5	123 ± 5	130 ± 8	129 ± 9	118 ± 7	F < 2; NS
Med 0.6 $\mu g kg^{-1} min^{-1}$	128 ± 3	138 ± 5	137 ± 6	138 ± 8	121 ± 4	F = 4.76; $P < 0.003$
SHR						
Vehicle	157 ± 6	155 ± 6	156 ± 7	156 ± 7	154 ± 9	—
Med 0.2 $\mu g kg^{-1} min^{-1}$	171 ± 5	157 ± 6	141 ± 9	131 ± 7	134 ± 4	F = 9.11; $P < 0.001$
Med 0.6 $\mu g kg^{-1} min^{-1}$	169 ± 4	148 ± 5	149 ± 6	152 ± 6	142 ± 4	F = 3.66; $P < 0.01$
			Right atrial pressure (mmHg)			
WKY						
Vehicle	2.6 ± 0.5	2.5 ± 0.4	2.4 ± 0.4	2.3 ± 0.5	2.7 ± 0.5	—
Med 0.2 $\mu g kg^{-1} min^{-1}$	3.4 ± 0.7	3.2 ± 0.6	3.3 ± 0.5	3.0 ± 0.5	2.9 ± 0.7	F = 2.46; NS
Med 0.6 $\mu g kg^{-1} min^{-1}$	3.1 ± 0.6	3.6 ± 0.8	3.9 ± 0.8	3.3 ± 0.7	2.5 ± 0.6	F = 6.26; $P < 0.001$
SHR						
Vehicle	3.3 ± 0.7	3.0 ± 0.5	3.2 ± 0.6	3.4 ± 0.7	3.7 ± 0.6	—
Med 0.2 $\mu g kg^{-1} min^{-1}$	3.3 ± 0.7	3.1 ± 0.7	2.9 ± 0.6	2.9 ± 0.6	2.4 ± 0.7	F = 2.00; NS
Med 0.6 $\mu g kg^{-1} min^{-1}$	3.4 ± 0.5	4.0 ± 0.6	3.8 ± 0.4	3.2 ± 0.6	3.0 ± 0.7	F = 3.45; $P < 0.02$

Results are expressed as mean ± s.e.mean from 5–8 animals. Recovery = 30 min after the end of infusion. The data were analysed with two-way analysis of variance (ANOVA). F = drug and time interaction, medetomidine vs vehicle.

(0.2 $\mu g kg^{-1} min^{-1}$: maximal decrease of 23%; and 0.6 $\mu g kg^{-1} min^{-1}$: −10%).

The right atrial pressure increased in both strains in response to an infusion of 0.6 $\mu g kg^{-1} min^{-1}$ of medetomidine (Table 2). The maximal increases in right atrial pressure were noted between 5–30 min after the start of medetomidine infusion and were 1.18 ± 0.26 mmHg in WKY and 1.64 ± 0.64 mmHg

Table 3 Effects of a 60 min infusion of the α_2-agonist medetomidine (Med) on plasma levels of immunoreactive atrial natriuretic peptide (IR-ANP in pg ml^{-1}) in spontaneously hypertensive (SHR) and Wistar-Kyoto (WKY) rats

	Control	*Infusion At 10 min*	*At 30 min*	*At 60 min*	*Recovery*	*F*
WKY						
Vehicle	93 ± 7	126 ± 20	133 ± 14	140 ± 21	131 ± 27	—
Med 0.2 $\mu g kg^{-1} min^{-1}$	136 ± 18	179 ± 29	260 ± 68	216 ± 70	174 ± 35	F = 2.89; $P < 0.09$
Med 0.6 $\mu g kg^{-1} min^{-1}$	112 ± 11	194 ± 31	261 ± 45	239 ± 35	148 ± 18	F = 10.57; $P < 0.003$
SHR						
Vehicle	123 ± 19	130 ± 20	128 ± 15	123 ± 17	125 ± 21	—
Med 0.2 $\mu g kg^{-1} min^{-1}$	105 ± 18	114 ± 12	120 ± 18	108 ± 21	121 ± 12	F < 2; NS
Med 0.6 $\mu g kg^{-1} min^{-1}$	120 ± 24	119 ± 22	149 ± 15	139 ± 19	120 ± 24	F < 2; NS

Results are expressed as mean ± s.e.mean from 6–8 animals. Recovery = 30 min after the end of infusion. The data were analysed with two-way analysis of variance (ANOVA). F = drug and time interaction, medetomidine vs vehicle.

in SHR ($t = 0.63$, NS, paired t test). The lower dose of medetomidine had no signficant effect on right atrial pressure (Table 2).

Effect of medetomidine infusion on plasma ANP in conscious SHR and WKY rats

The resting values of plasma IR-ANP were similar in conscious 7 month-old WKY and SHR rats with indwelling catheters (Table 1). Basal IR-ANP levels in these rats were similar to those obtained earlier at this age in our strains (Ruskoaho & Leppäluoto, 1988). A marked difference was observed in the plasma IR-ANP response to medetomidine infusion between the two strains. In WKY rats, medetomidine increased dose-dependently the plasma concentration of IR-ANP: a maximal increase of $105 \pm 48\%$ after the lower dose and $196 \pm 62\%$ after the higher dose of medetomidine infusion was noted (Table 3). In contrast, the i.v. infusions of medetomidine did not affect plasma levels of IR-ANP in SHR rats (Table 3).

Urine volume and solute excretion in SHR and WKY rats

Table 1 shows the mean values of the renal excretory functions in conscious WKY and SHR rats measured before the infusion of medetomidine. No significant differences in urine flow, urinary sodium ($U_{Na}V$) and urinary potassium (U_KV) excretion or, in urine osmolality (U_{osm}) were found between these strains.

The mean values of the urinary excretory function in WKY and SHR during control (C), infusion (E), and recovery (R) periods are shown in Figure 1. Infusion of vehicle (0.9% saline) caused a slight increase in urine osmolality in WKY rats but had no signficant effect on urine volume or $U_{Na}V$ and U_KV. A 60 min constant infusion of medetomidine dose-dependently increased urine flow and urinary sodium excretion in WKY and SHR rats, while urine osmolality decreased in both strains. Excretion of potassium was unaffected, except that it increased during the infusion of the lower dose of medetomidine in the WKY rats (Figure 1).

Discussion

The present results show that in the conscious normotensive and genetically hypertensive rats medetomidine caused marked bradycardia, an effect typical for clonidine and other α_2-adrenoceptor agonists (Kobinger, 1978). Although decreasing similarly heart rate in both strains, the effect of medetomidine on blood pressure differed in WKY and SHR rats, causing a decrease in mean arterial pressure in SHR, while in normotensive WKY it increased blood pressure. In anesthetized rats, medetomidine had a blood pressure action resembling that of clonidine, with an initial transient rise in blood pressure followed by a gradual decrease (Savola *et al.*, 1986). In pithed rats, its vasoconstrictor effect has been found to be antagonized by α_2-adrenoceptor, but not α_1-adrenoceptor blocking drugs (Savola *et al.*, 1986). Thus, it seems likely that the vasopressor response in WKY, as well as the smaller hypotensive response in SHR to the higher dose of medetomidine, are due to peripheral α_2-adrenoceptor-mediated vasoconstriction. This lack of hypotensive response to medetomidine in WKY rats is also in agreement with our previous study in which an analogue of medetomidine had a similar effect (Savola *et al.*, 1985). Finally, the SHR strain shows a hyperactivity of the sympathetic nervous system of central origin (Folkow, 1982), which may explain the observed enhanced hypotensive response to medetomidine in SHR rats.

To examine the capacity of the heart to release ANP and the role of α_2-adrenoceptors in the control of ANP secretion, we have compared here the effect of medetomidine infusion on plasma ANP levels in WKY and SHR rats. In normotensive WKY rats infusion of the selective α_2-agonist medetomidine resulted in an increase in the plasma concentration of IR-ANP, while a similar infusion of medetomidine in hypertensive rats did not cause an ANP response. Previously Baranowska *et al.* (1987a,b,c) have demonstrated that clonidine (10–50 μg i.v.) elicits a marked increase of plasma IR-ANP in conscious rats (20 fold elevation after the highest dose). They also noted that yohimbine, an α_2-adrenoceptor antagonist, decreased plasma IR-ANP levels and partially inhibited the increase of plasma IR-ANP induced by clonidine (Baranowska *et al.*, 1987b), suggesting that α_2-adrenoceptors are important for ANP release. However, they did not measure haemodynamic parameters, though they noted that mean arterial pressure increased by more than 30 mmHg after a dose of 50 μg of clonidine, while a dose of 25 μg of clonidine (i.v.) had no pressor effect and caused a 10 fold increase in plasma IR-ANP (Baranowska *et al.*, 1987a). Our present results show that the haemodynamic effects seem to explain the increase of ANP secretion in response to α_2-adrenoceptor stimulation in normotensive rats, because a significant increase in plasma IR-ANP levels during medetomidine infusion was accompanied by the pressor effect and increased right atrial pressure. In dogs, clonidine causes an elevation of left atrial pressure and left-ventricular end-diastolic pressure (Nayler *et al.*, 1968; Laubie & Schmitt, 1974).

Why medetomidine fails to cause ANP release in SHR is not clear. It is unlikely to be due to the impairment of right atrial stretch in SHR, because

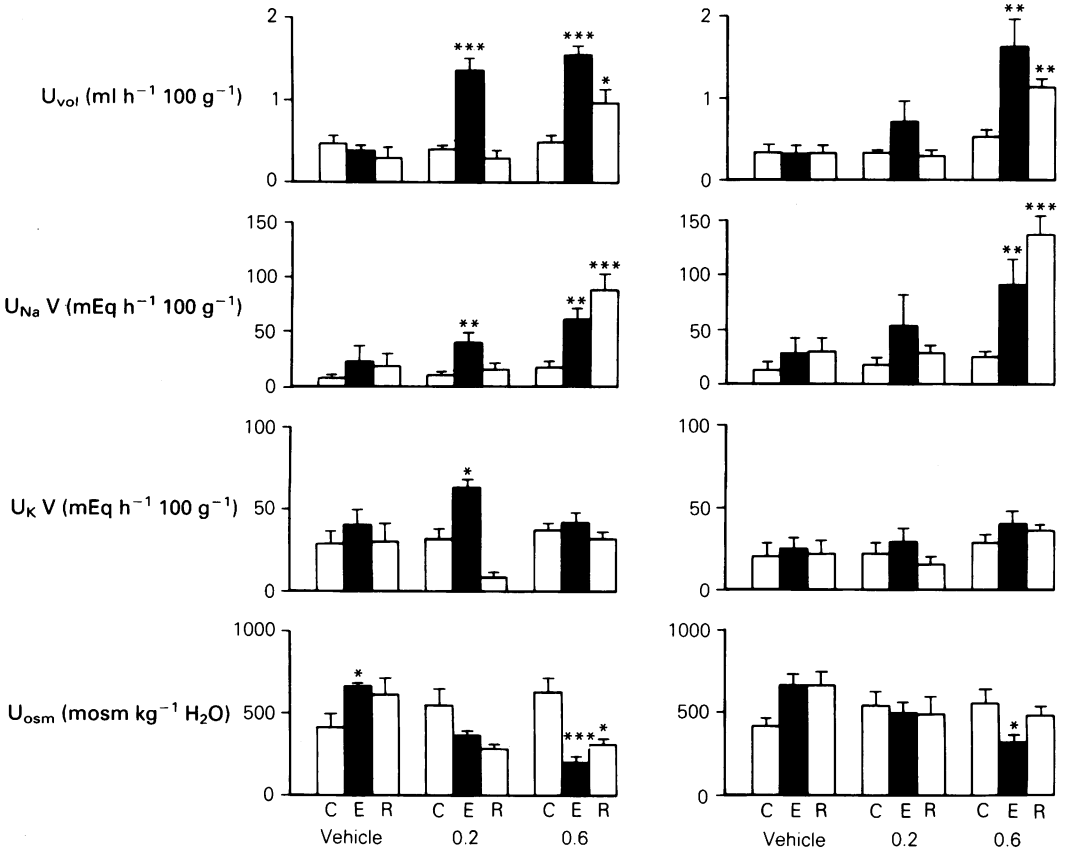

Figure 1 Effects of a 60 min infusion of the α_2-adrenoceptor agonist, medetomidine, on urinary excretory parameters in spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats. U_{vol} = urinary volume; $U_{Na}V$ = urinary sodium excretion; U_KV = urinary potassium excretion; U_{osm} = urinary osmolality. After a control period (C) (60 min), medetomidine was administered by a constant infusion (0.2 or 0.6 $\mu g\,kg^{-1}\,min^{-1}$) (E) for 60 min. One recovery period (R) (60 min each) was performed after the medetomidine infusion was stopped. Results are mean of values in 5–8 animals; vertical lines indicate s.e.mean. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (one-way-ANOVA followed by the Bonferroni t test).

medetomidine produced similar increases in right atrial pressure in both strains. In contrast, a different pattern of blood pressure response to medetomidine was observed in SHR and WKY rats. The decreased mean arterial pressure, as clearly demonstrated in each experiment in SHR, may explain the absence of an increased release of ANP in response to medetomidine in these animals. Previous haemodynamic data obtained in dogs suggest that a fall in blood pressure may counteract the increase in left atrial pressure induced by α_2-agonists (Nayler *et al.*, 1968). However, because hypotension induced by medetomidine did not decrease plasma IR-ANP levels in SHR, our results do not necessarily exclude the potential role of α_2-adrenoceptors in the modulation of ANP release in SHR.

Clonidine and other α_2-adrenoceptor agonists produce diuresis and increase the elimination of sodium in rats (Hoefke & Kobinger, 1966; Roman *et al.*, 1979; Bahr & Kauker, 1979; Miller, 1980; Gellai & Ruffolo, 1987; Gellai & Edwards, 1988). Their diuretic effect is not qualitatively influenced by the prior fluid and electrolyte status of the animal (Roman *et al.*, 1979), but the mechanism underlying the diuretic action of α_2-agonists is not fully understood. In the present study, medetomidine increased urine flow rate and sodium excretion in both strains. Potassium excretion was also slightly increased with the subpressor dose of medetomidine in WKY consistent with the previous observation by Gellai & Ruffolo (1987) using another selective α_2-agonist, BHT-933.

Based on *in vitro* studies, renal tubular α_2-adrenoceptors may be involved in modulating the water flow and urinary sodium excretion by inhibition of the tubular action of vasopressin and other hormones (Pettinger *et al.*, 1987; Gellai & Edwards, 1988). Experimental evidence from *in vivo* studies indicates that α_2-agonists decrease vasopressin secretion (Humpreys & Reid, 1975; Reid *et al.*, 1979; Roman *et al.*, 1979; Stranhoy *et al.*, 1982; Brooks *et al.*, 1986) as well as sympathetic nervous activity (DiBona, 1985); both of these actions probably contributing to the diuresis and urinary sodium excretion observed. In the present study, the diuretic action of medetomidine was associated with low levels of urine osmolality, maintained at 200 mosm kg^{-1} of H_2O in WKY rats, substantially less than plasma osmolality (290 mosm kg^{-1} of H_2O). The formation of urine hypotonic to plasma is indicative of a reduction in vasopressin-associated renal water reabsorption. However, further studies on the role of vasopressin are needed, since there is evidence that in conscious euvolemic animals administration of α_2-agonists does not inhibit vasopressin secretion (Olsen, 1976; Gellai & Edwards, 1988).

Baranowska *et al.* (1987c), in their study of the effect of clonidine on ANP secretion, showed that the increment of ANP correlated with the diuretic effect of clonidine in normally-hydrated, conscious normotensive rats. Our results on the effect of medetomidine on plasma IR-ANP and renal excretory functions in WKY rats support their observations and demonstrate that enhanced ANP release may be involved in the mechanisms of α_2-agonist-evoked diuresis. The increased plasma levels of ANP in response to α_2-adrenoceptor stimulation could also inhibit vasopressin release (Laragh, 1985; Brenner & Ballerman, 1986; Needleman & Greenwald, 1986) and thus enhance that diuretic effect. However, the present findings that medetomidine had a similar effect on the renal excretory functions in both strains, but did not affect plasma levels of IR-ANP in SHR rats suggest that changes in plasma ANP levels could not alone account for the diuretic and natriuretic effect of medetomidine in conscious SHR. Thus, the present study, together with previous results, suggest that the renal effects of α_2-agonists may be explained by hormonal effects (vasopressin, ANP) in combination with renal tubular and autonomic effects, depending on the rat strain and experimental conditions.

In conclusion, our results clearly show that α_2-adrenoceptor stimulation failed to increase plasma IR-ANP secretion in genetically hypertensive rats, but markedly increased ANP release in normotensive rats. This differing effect of medetomidine on ANP release in WKY and SHR rats was probably associated with differing mean arterial pressure responses. However, the lack of ANP response to α_2-adrenoceptor stimulation in SHR may also represent a generalized attenuation of ANP release in these animals.

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Evidence for a dihydropyridine-sensitive and conotoxin-insensitive release of noradrenaline and uptake of calcium in adrenal chromaffin cells

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1 It has been suggested that neuronal voltage-sensitive calcium channels (VSCC) may be divided into dihydropyridine (DHP)-sensitive (L) and DHP-insensitive (N and T), and that both the L and the N type channels are attenuated by the peptide blocker ω-conotoxin. Here the effects of ω-conotoxin on release of noradrenaline and uptake of calcium in bovine adrenal chromaffin cells were investigated.

2 Release of noradrenaline in response to 25 mM K^+, 65 mM K^+, 10 nM bradykinin or 10 μM prostaglandin E_1 was not affected by ω-conotoxin in the range 10 nM–1 μM.

3 $^{45}Ca^{2+}$ uptake stimulated by high K^+ and prostaglandin was attenuated by 1 μM nitrendipine and enhanced by 1 μM Bay K 8644; these calcium fluxes were not modified by 20 nM ω-conotoxin.

4 With superfused rat brain striatal slices in the same medium as the above cell studies, release of dopamine in response to 25 mM K^+ was attenuated by 20 nM ω-conotoxin.

5 These results show that in these neurone-like cells, release may be effected by calcium influx through DHP-sensitive but ω-conotoxin-insensitive VSCC, a result inconsistent with the suggestion that ω-conotoxin blocks both L-type and N-type neuronal calcium channels.

Introduction

The neuronal voltage-sensitive calcium channels (VSCC) act at the nerve terminals as transducers of electrical information into chemical signals and so play a central role in synaptic function. Neuronal VSCC have been characterized by Nowycky *et al.* (1985), using electrophysiological criteria, into three types (L, N and T); only L-type are responsive to the dihydropyridine (DHP) calcium channel drugs. A number of reports show that depolarization-evoked calcium flux and neurotransmitter release at the nerve terminal is largely or completely insensitive to DHP antagonists (e.g. Nachsen & Blaustein, 1979; Rampe *et al.*, 1984; Perney *et al.*, 1986) suggesting that L channels are not responsible for most of the calcium influx which causes neurotransmitter release upon depolarization. However, in some studies depolarization-evoked calcium influx and neurotransmitter release is partly sensitive to DHP blockers in the brain (Turner & Goldin, 1985; Herdon & Nahorski, 1988), and most substance P release from cultured dorsal root ganglion cells elicited by depolarizing concentrations of potassium can be blocked by the drugs (Perney *et al.*, 1986; Rane *et al.*, 1987; Holz *et al.*, 1988).

The peptide ω-conotoxin has been shown in certain preparations to attenuate depolarization-evoked release of neurotransmitter (Olivera *et al.*, 1984; Kerr & Yoshikama, 1984; Reynolds *et al.*, 1986; Rivier *et al.*, 1987; Sano *et al.*, 1987; Dooley *et al.*, 1987; Mohy El-Din & Malik, 1988; Maggi *et al.*, 1988; Herdon & Nahorski, 1988). On the basis of electrophysiological evidence it has been suggested that this toxin blocks both L and N type neuronal VSCC (McCleskey *et al.*, 1987; Cruz *et al.*, 1987), consistent with the suggestion that release is mediated principally by these two channel types (Miller, 1987). However neurotransmitter release has not always been found to be sensitive to ω-conotoxin, perhaps reflecting heterogeneity of VSCC between different types of neurone (Anderson & Harvey, 1987; Sano *et al.*, 1987; Maggi *et al.*, 1988). In these earlier studies ω-conotoxin insensitivity was apparent with depolarization-evoked transmitter release

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from preparations expected to be resistant to DHP antagonists. In the work described in this paper we used the cultured bovine adrenal chromaffin cell, a neurone-like cell in culture, and show that depolarization-evoked release and calcium influx which are known to be blocked by DHP antagonists are not blocked by ω-conotoxin. This suggests that some neuronal L-type VSCC are ω-conotoxin-insensitive.

Methods

Cells were prepared from fresh bovine adrenal glands by digestion with collagenase/protease (Marriott *et al.*, 1988) and purified by an adaptation of the differential plating procedure of Waymire *et al.* (1983) as described in Owen *et al.* (1988). Purified chromaffin cells were cultured at 0.5×10^6 cells per well in 24 well 'Primaria' plates in a complete medium with cell growth inhibitors (Owen *et al.*, 1988). Used after 3–7 days in culture, the monolayers were first washed twice with 1 ml of HEPES buffered balanced salt solutions (BSS) as follows (mM): NaCl 125, KCl 5.4, $NaHCO_3$ 16.2, N-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid 30, NaH_2PO_4 1, $MgSO_4$ 0.8, $CaCl_2$ 1.8 and glucose 5.5; gassed with 95% O_2:5% CO_2 and buffered to pH 7.4. A 12 min preincubation period preceded a 3 min stimulation period; calcium channel drugs, where appropriate, were present through both preincubation and stimulation period.

For estimation of noradrenaline release supernatants were removed, centrifuged and acidified to a final concentration of 0.1 M HCl. The remaining monolayer was extracted into 0.1 M HCl, and noradrenaline content of both supernatant and cell extract was estimated by high pressure liquid chromatography followed by electrochemical detection, essentially as described by Mefford (1981). Results are expressed as release of noradrenaline as a percentage of cell content, each figure being the mean of quadruplicates.

For estimations of calcium uptake incubations were in 0.5 ml BSS in the presence of 0.037 MBq $^{45}Ca^{2+}$; after 3 min the medium was aspirated and the monolayer was rapidly washed with 2 ml of cold BSS three times. Finally the monolayer was extracted into 10% trichloroacetic acid and the radioactivity determined by liquid scintillation counting. Results, expressed as c.p.m. per well, are the mean of quadruplicates.

To investigate release of dopamine from rat brain, 300 μm cross chopped slices of striatum were preincubated for 1 h in the BSS described above with three changes of medium. The slices were then packed into a four chamber perfusion apparatus and perfused at 1 ml min^{-1} for 20 min before starting the collection of 1 min fractions of the perfusate. Conotoxin, where appropriate, was introduced into the perfusing medium as the fraction collection was started and was present throughout the rest of the procedure. Stimulation of the slices was effected by raising the concentration of the potassium in the perfusing medium to 25 mM. Dopamine content of the fractions was measured by high performance liquid chromatography followed by electrochemical detection. Assessment of significant differences was by two tailed Student's *t* test.

Materials

Tissue culture medium, plastics and supplements were from GIBCO (Paisley, Scotland) except for Primaria plates (Falcon), which were from Becton-Dickinson, Oxford. $^{45}Ca^{2+}$ was from Amersham Corp. Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate) was a gift from Bayer (Leverkusen, F.R.G.). Synthetic ω-conotoxin GVIA was from Peptide and Protein Research Institute, Osaka, Japan, DHPs were kept as a stock solution in dimethylsulphoxide, the final concentration of which was 0.01%, at which concentration it was present in appropriate controls.

Results

Figure 1 shows the effect of 10 nM and 100 nM ω-conotoxin on basal release of noradrenaline and on release stimulated by 65 mM K^+ or 10 nM brady-

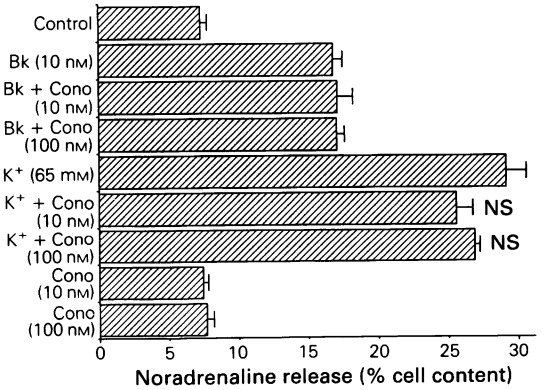

Figure 1 Effect of ω-conotoxin (Cono, 10 and 100 nM) on bradykinin (Bk) and high potassium stimulated release of noradrenaline from chromaffin cells. Bradykinin 10 nM or 65 mM potassium were present for the 3 min stimulation period. Conotoxin was present through this and the preceding 12 min preincubation period. Error bars are s.e.mean ($n = 4$). NS indicates no significant effect of the presence of ω-conotoxin.

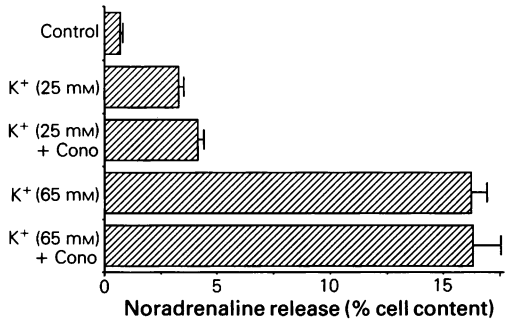

Figure 2 Effect of 1 μM ω-conotoxin (Cono) on release from chromaffin cells stimulated by 25 mM or 65 mM potassium. Error bars are s.e.mean ($n = 4$).

kinin. It can be seen that ω-conotoxin had no effect on either basal or stimulated release (the small apparent decrement in K^+-stimulated release in the presence of ω-conotoxin was not significant nor reproducible over a number of similar experiments). In order to see whether a smaller degree of depolarization, or a higher concentration of ω-conotoxin, would reveal an effect we tested 1 μM ω-conotoxin with either 25 mM or 65 mM K^+. As can be seen in Figure 2 the toxin still had no effect on noradrenaline release.

Figure 3 shows the effect of nitrendipine and ω-conotoxin on both noradrenaline release and calcium uptake stimulated by 65 mM K^+: nitrendipine reduced both stimulated noradrenaline release (down to 34% of control values, Figure 3a) and stimulated calcium uptake (to 52% of controls, Figure 3c). Neither release nor calcium uptake were affected by ω-conotoxin (Figure 3b and d).

Stimulation of calcium uptake by submaximal concentrations of extracellular K^+ (25 mM) was itself insensitive to 20 nM ω-conotoxin, but it could be enhanced by the DHP calcium channel agonist, Bay K 8644. This agonist-enhanced calcium uptake was itself insensitive to 20 nM conotoxin (data not shown).

Prostaglandin E_1 stimulated the release of noradrenaline from chromaffin cells, an effect powerfully potentiated by the calcium channel activator Bay K 8644 at 1 μM. Both the release stimulated by prostaglandin E_1 alone and in the presence of Bay K 8644 were insensitive to the presence of ω-conotoxin (Figure 4).

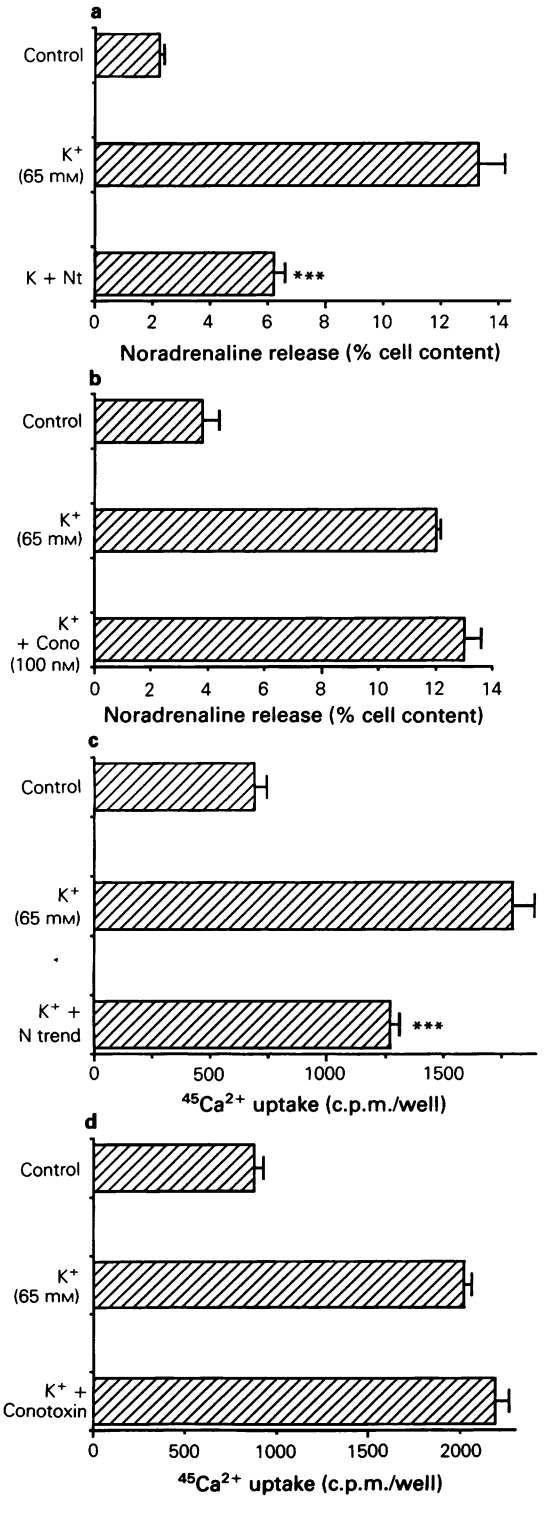

Figure 3 Effect of nitrendipine or conotoxin on noradrenaline release (a and b) and calcium uptake (c and d) stimulated by high potassium, with chromaffin cells. Nitrendipine (Nt) was 1 μM in (a) and (c), ω-conotoxin (Cono) 100 nM in (b) and 20 nM in (d). Error bars are ±s.e.mean ($n = 4$), ****$P < 0.005$ compared to potassium stimulation with no nitrendipine.

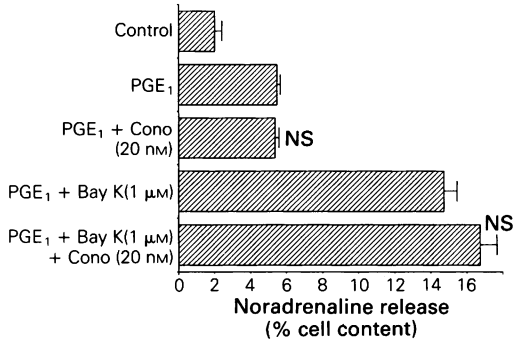

Figure 4 Effect of 1 μM Bay 8644 (Bay K) and 20 nM ω-conotoxin (Cono) on release of noradrenaline from chromaffin cells stimulated by 10 μM prostaglandin E_1 (PGE_1). Error bars are s.e.mean ($n = 4$). NS indicates no significant effect of the presence of ω-conotoxin.

To demonstrate that the toxin is active under conditions used here, we perfused rat striatal slices with the same BSS, stimulated with high K^+ in the presence and absence of ω-conotoxin and measured dopamine content of the perfusate. One of three similar experiments, each with a four channel perfusion system, is shown in Figure 5 where it can be seen that the presence of 20 nM conotoxin caused a substantial attenuation of dopamine release.

Discussion

In the studies described here, much of the depolarization-induced release of noradrenaline from chromaffin cells was found to be sensitive to 1 μM nitrendipine, consistent with earlier observations showing inhibition of release by DHPs in a stereospecific manner (Boarder *et al.*, 1987; Fonteriz *et al.*, 1987). Voltage and DHP sensitivity of calcium channels is characteristic of L type channels (Nowycky *et al.*, 1985). We have recently shown that bradykinin-stimulated release from chromaffin cells is largely dependent on extracellular calcium but is insensitive to DHP blockers (Owen *et al.*, 1988). Since ω-conotoxin has been reported to block both L and N types of neuronal channel (McCleskey *et al.*, 1987), we initiated experiments such as that shown here in Figure 1 with the expectation that K^+ depolarization-stimulated release would be sensitive to ω-conotoxin. We wished to see if bradykinin-stimulated release was attenuated by this peptide toxin. The evidence presented in this paper clearly shows however that high K^+-stimulated noradrenaline release and calcium uptake is not conotoxin-sensitive. This demonstrates that DHP-sensitive VSCC may be insensitive to conotoxin. This is clearly different from some recent reports of insensi-

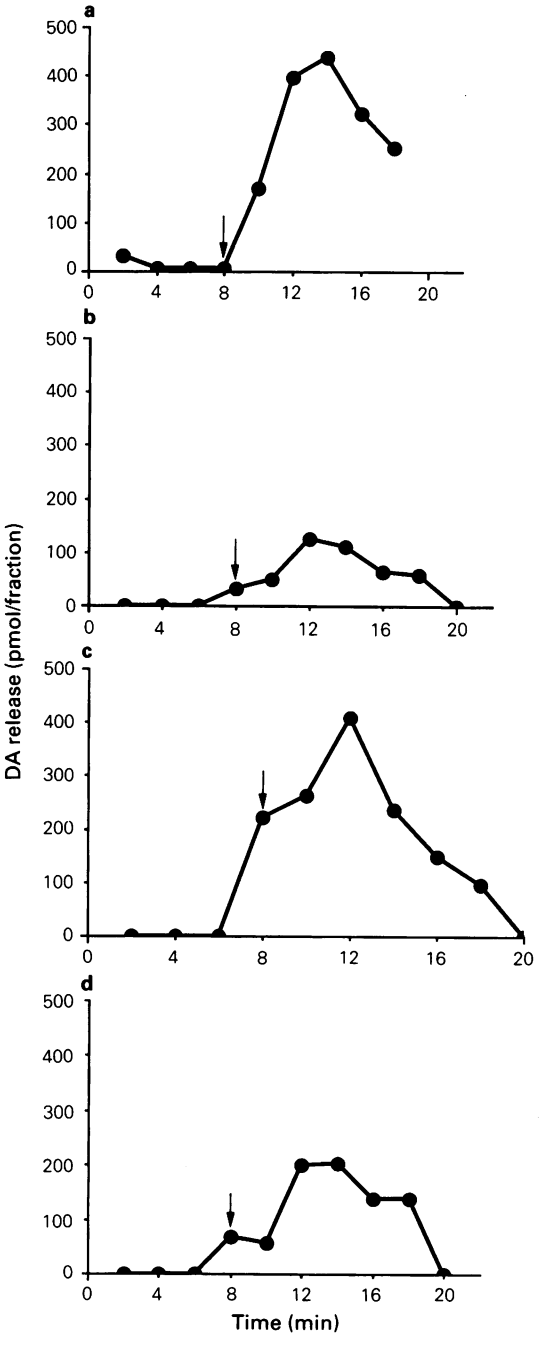

Figure 5 Release of dopamine (DA) from perfused slices of rat brain striatum. Slices were perfused in the presence (b and d) and absence (a and c) of 20 nM ω-conotoxin for the time shown, and the slices stimulated with 25 mM potassium introduced when indicated by the arrow, and present for the rest of the procedure.

tivity of neurotransmitter release to ω-conotoxin in preparations which would not be expected to be sensitive to DHP antagonists (Anderson & Harvey, 1987; Sano *et al.*, 1987; Maggi *et al.*, 1988).

A possible explanation for the failure to block the DHP-sensitive channels in the chromaffin cells is that ω-conotoxin is effective at N channels but not at neuronal L channels, contrary to the conclusion reached by others. The most substantial evidence for the effectiveness of conotoxin at neuronal L type channels is the electrophysiological data of McCleskey *et al.* (1987) obtained with a variety of patch clamp configurations. While this study provides evidence for action at the two channel types, other studies are not so unequivocal. For example Reynolds *et al.* (1986) using microspectrofluorimetry of fura-2 loaded cells showed that depolarization-induced calcium fluxes in cell bodies are sensitive to conotoxin as well as nitrendipine. However, rather than because these two drugs are acting on the same channels, this may be a reflection of channel heterogeneity in the cell bodies, with both N and L types being present. Militating against action at both channel types for ω-conotoxin is a study published as the work reported in this paper was being concluded, showing in two neuronal like cell lines (a human neuroblastoma cell line and the phaeochromocytoma-derived PC12 line) that ω-conotoxin was capable of modifying only those depolarization-induced calcium fluxes that are DHP-insensitive (Sher *et al.*, 1988). In agreement with the results presented here, this leads to the conclusion that ω-conotoxin is not active against neuronal L type channels.

This conclusion may be modified by considering L type channel heterogeneity. Perhaps different neurones and neurone-related cells will contain different channels of this type, all DHP-sensitive but only some ω-conotoxin-sensitive. Heterogeneity of L type channels with respect to ω-conotoxin-sensitivity has always been recognised in the comparison between neurones and muscle cells. No conotoxin binding or calcium channel activity is apparent on smooth, cardiac or skeletal muscle (Yeager *et al.*, 1987; McCleskey *et al.*, 1987; Cruz *et al.*, 1987; Barhanin *et al.*, 1988; Mohy El-Din & Malik, 1988) despite the presence of DHP-sensitive L type channels. Indeed Cruz *et al.* (1987) have proposed two types of L channel, L_n (neuronal and ω-conotoxin-sensitive) and L_m (ω-conotoxin-insensitive). The present results and those of Sher *et al.* (1988) are clearly in conflict with such a scheme, but may be reconciled with the work of McCleskey *et al.* (1987) by proposing that some neurones have mainly ω-conotoxin-sensitive L type channels while some have predominantly ω-conotoxin-insensitive L type channels. In considering this hypothesis it should be noted that McCleskey *et al.* (1987) investigated a number of neuronal cell types, including sympathetic postganglionic cells closely related to the chromaffin cells investigated here and the PC12 cells used by Sher *et al.* (1988) and reported no evidence for such heterogeneity. It is possible that neurone-like cells of adrenal origin differ from true neurones with respect to their calcium channel complement. It is also possible that ω-conotoxin sensitivity is more apparent under the conditions of patch clamp studies than with the potassium depolarization of populations of whole cells reported here. However, the prostaglandins also stimulate release from these cells in a DHP-dependent (Koyama *et al.*, 1988; and our unpublished observations) but ω-conotoxin-insensitive manner. Regardless of the mechanism of prostaglandin enhancement of calcium influx and catecholamine release, it is important in the present context in that it shows ω-conotoxin insensitivity of DHP-sensitive channels is not limited to the conditions of channel opening by high extracellular K^+.

Bradykinin-stimulated release is clearly by a different mechanism from prostaglandin-stimulated release: release in response to bradykinin is largely dependent on external calcium, but the route of calcium entry is different as shown by the lack of DHP-sensitivity (Owen *et al.*, 1988). The data presented here show that the route of calcium entry stimulated by bradykinin is also not sensitive to conotoxin, and therefore is presumably not via N-type VSCC.

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Noradrenaline activates a calcium-activated chloride conductance and increases the voltage-dependent calcium current in cultured single cells of rat portal vein

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1 Membrane responses were recorded by a patch pipette technique in cultured cells isolated from rat portal vein. Using the whole-cell mode, pressure ejections of noradrenaline evoked depolarization (current clamp) and inward current (voltage clamp) at membrane potentials of −60 to −70 mV. The noradrenaline-induced response was reversibly blocked by prazosin indicating that the response was mediated by α_1-adrenoceptors.

2 The ionic mechanism of the noradrenaline-induced inward current was investigated in potassium-free caesium-containing solutions. Alteration of the chloride equilibrium potential produced similar changes in the reversal potential of the noradrenaline-induced current, indicating that noradrenaline opened chloride-selective channels. There was no evidence implicating sodium or calcium as the charge-carrying ion.

3 Caffeine applied in the bathing solution also induced a transient increase in chloride conductance but the noradrenaline-induced response was lost after application of caffeine. This is interpreted to mean that the increase in chloride conductance induced by noradrenaline and caffeine can occur as a consequence of a rise in intracellular calcium concentration depending on release of calcium from the same intracellular stores.

4 In the presence of caffeine, noradrenaline increased both the voltage-dependent calcium and chloride membrane conductances during application of repetitive depolarizing pulses. It is concluded that in isolated cells of the rat portal vein the depolarization in response to noradrenaline is mediated by an increase in chloride conductance depending on both the calcium release from intracellular stores and the increase of the voltage-dependent calcium current.

Introduction

Stimulation of α_1-adrenoceptors in portal vein smooth muscle produces contraction that can be dependent on an increase in both calcium influx through voltage-dependent channels and release of calcium from intracellular stores (Mironneau & Gargouil, 1979; Dacquet *et al.*, 1987; Pacaud *et al.*, 1987). However, there are recent results concerning the ionic mechanisms underlying the electrophysiological early response to noradrenaline in veins which indicate that the noradrenaline-induced depolarization is mainly mediated by an increase in membrane chloride conductance (Byrne & Large, 1988a, b; Van Helden, 1988). Two other ionic mechanisms involved in noradrenaline action have been described: an increase in a non specific cation channel (Byrne & Large, 1988b) and a decrease in potassium conductance (Suzuki, 1981; Van Helden, 1988). Furthermore, noradrenaline increases the cytoplasmic calcium concentration as measured with fluorescent indicators (Satoh *et al.*, 1987).

In smooth muscle cells isolated from rat portal vein, a chloride current activated by the inward calcium current can modulate the action potential duration (Pacaud *et al.*, 1989). In the present study we have investigated the ionic mechanisms underlying the depolarization to noradrenaline (Nanjo, 1984) in cultured single cells isolated from rat portal vein using whole-cell recordings with patch-pipettes.

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The major finding is that noradrenaline increases the membrane conductance to chloride which is mediated by a rise in intracellular concentration of calcium dependent on both a release of internal calcium and an increase in the amplitude of the voltage-dependent calcium current.

Methods

Single cell isolation and short-term primary culture

Portal veins from Wistar rats (150 g) were dissected free of connective tissue and single cells were obtained by a dispersal procedure similar to that described previously (Loirand *et al.*, 1986). The cells were plated on collagen-coated glass cover slips in medium M199 (Flow Laboratories, Puteaux, France) containing 10% foetal bovine serum, 2 mM glutamine, 20 u ml^{-1} penicillin and 20 $\mu g\,ml^{-1}$ streptomycin (Gibco, Paisley, UK) and kept in an incubator gassed with 95% O_2: 5% CO_2 at 37°C. The cells were transferred in a saline solution (reference solution) 15 min before the electrophysiological experiments.

Electrophysiological recordings in single cells

Isolated cells in short-term primary culture were fully viable for whole-cell recordings for up to 2 days. Cells attached on glass cover slips were fixed in a small experimental chamber which was mounted on the stage of an inverted microscope (Nikon Diaphot, Tokyo, Japan). Whole-cell membrane currents were measured by use of standard patch-clamp technique (Hamill *et al.*, 1981). Calcium current characteristics such as the potentials for threshold and maximal activation, the apparent reversal potential and the time to half-inactivation remained unchanged from 5 to 30 h of culture ($n = 10$). Patch pipettes had resistances of 2–5 MΩ and series resistances of 5–6 MΩ were measured. Thus, even at the maximal membrane current (300–600 pA), errors in potential measurement due to series resistance were within 5% and no correction was applied. Data were filtered at 1 kHz (−3 dB frequency) by a 8 pole Bessel low pass filter (Frequency Devices, Haverhill, USA) and analyzed with a Plessey 6220 microcomputer; illustrations were drawn with an X–Y plotter.

The extracellular medium (reference solution) contained (mM): NaCl 130, KCl 5.6, $CaCl_2$ 5, $MgCl_2$ 0.24, glucose 11, HEPES 8, pH 7.4 at 35 ± 1°C. The basic pipette solution contained (mM): KCl 130, HEPES 10, pH 7.3 with KOH. In order to block outward potassium currents, CsCl or NaCl were used instead of KCl in both external and pipette solutions and pH was adjusted with CsOH or NaOH, respectively. The following test solutions were used: barium solution, prepared by substituting $BaCl_2$ for $CaCl_2$; low chloride solution, prepared by substituting Na-aspartate for NaCl. In some experiments, 10 mM EGTA was added to the pipette solution in order to control the free calcium concentration.

Cobalt chloride was used as an inhibitor of calcium conductance. Chemicals used were: caffeine, noradrenaline, prazosin and propranolol. Noradrenaline (10^{-5} M) was applied from a glass pipette for 100–500 ms with a pressure ejector.

The values given in the text are the mean ± s.e. of mean with n the sample size.

Results

Responses to noradrenaline in potassium-containing solution

In isolated cells of the rat portal vein maintained in short-term primary culture, the resting membrane potential was usually between −60 and −70 mV when recorded with patch pipettes filled with KCl. At these holding potentials the common response to micro-ejections of noradrenaline (10^{-5} M) in a solution containing 2.5 mM cobalt was a transient depolarization (Figure 1a), the membrane potential reaching a value of -30.8 ± 3.4 mV ($n = 6$). In potassium-containing solution, noradrenaline-induced responses were observed in 90% of the cells ($n = 23$). In responding cells, the amplitude of the noradrenaline-induced depolarization was slightly reduced on repeated administration but the effect was small and accurate estimates of reversal potential were obtained readily (e.g. Figure 2a). At depolarized holding potentials (Figure 1a), the noradrenaline-induced response was composed of an early hyperpolarizing component followed by a reduced depolarization. At holding potentials between −10 and 0 mV, the depolarization progressively decreased whereas the amplitude of the hyperpolarization increased. In the absence of external cobalt, action potentials were generally superimposed on the noradrenaline-induced depolarization (Figure 1c). In the same cell the noradrenaline-induced currents were recorded under voltage-clamp conditions (Figure 1b). Two components of currents were clearly identified the amplitude and direction of which were dependent on the holding potential. The time course and duration of both the membrane potential variations and the underlying membrane currents were similar when recorded from the same cell. The action of noradrenaline was blocked in the presence of prazosin (10^{-6} M) for 5 min while it remained unchanged in the presence of propranolol

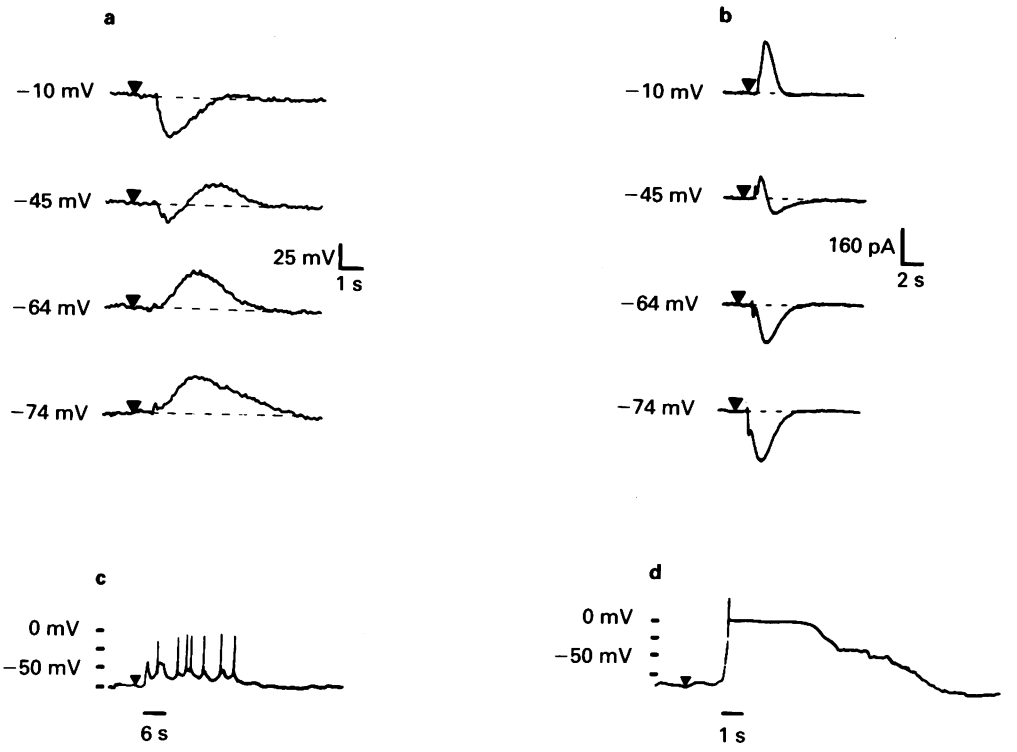

Figure 1 Membrane responses to noradrenaline (10^{-5} M) in isolated cells of rat portal vein in primary culture. (a) Depolarizations and hyperpolarizations to micro-ejections of noradrenaline (▼) recorded using current-clamp at different holding potentials in potassium-containing solution. (b) Membrane currents to noradrenaline recorded using voltage-clamp at different holding potentials in potassium-containing solution. (c) Burst of action potentials superimposed on the noradrenaline-induced depolarization in potassium-containing solution. (d) In potassium-free caesium-containing solution, noradrenaline induced a membrane depolarization to 0 mV which was maintained for 2–4 s. Noradrenaline (▼) was ejected for 100–500 ms at a distance of about 100 μm from the cell. The value of the holding potential (mV) is noted near the baseline.

(10^{-6} M). After washing out the α_1-adrenoceptor antagonist the noradrenaline-evoked current was similar to control. These results suggest that the responses to noradrenaline are mediated by α_1-adrenoceptors.

As hyperpolarization and outward membrane current induced by noradrenaline were abolished in potassium-free caesium-containing solution (Figure 2) it would seem that noradrenaline can increase the potassium conductance in addition to the membrane mechanism responsible for depolarization and inward current. Consequently, experiments to identify the ions responsible for the inward current were carried out in potassium-free caesium-containing bathing and pipette solutions. Under these experimental conditions, noradrenaline induced a membrane depolarization to 0 mV which was maintained for 2–4 s before repolarization occurred (Figure 1d).

Ionic basis of the noradrenaline-induced inward current

In order to determine the ionic basis of the noradrenaline-induced inward current, changes in the reversal potential of the current were studied with various ions in the bathing and pipette solutions. The reversal potential was estimated by measuring the responses to noradrenaline at different holding potentials. In caesium-containing solution (Figure 2a), the noradrenaline-evoked current decreased in amplitude with depolarization, was null at 0 mV and reversed at positive membrane potentials. There was a delay between application of noradrenaline and the onset of the response. The mean latency of the first noradrenaline-induced current was 980 ± 260 ms ($n = 27$) and the duration of the response was 1.7 ± 0.7 s ($n = 27$).

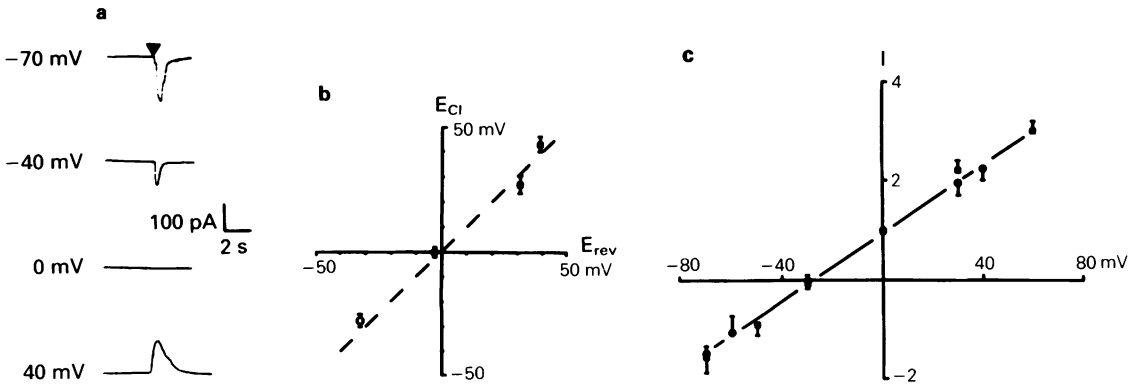

Figure 2 Effects of ionic substitution on the noradrenaline-induced current in caesium-containing solution. (a) Membrane current responses to ejections of noradrenaline (▼) at different holding potentials (noted near the current traces). The external chloride concentration was 146 mM, and the pipette concentration was 130 mM. (b) Reversal potential (E_{rev}) of the noradrenaline-induced current as a function of the equilibrium potential (E_{Cl}), for reference chloride (146 mM : 130 mM) gradient (●), low external chloride (■) and low pipette chloride concentrations (○). Dashed line predicts the reversal potential for a pure chloride conductance. Each point represents the mean of 3–15 cells; vertical lines show s.e.mean. (c) Current-voltage relationship of the noradrenaline-induced currents in caesium-containing solutions (■) and in sodium-containing solutions (●) with similar chloride (146 mM : 40 mM) gradients (substituted with aspartate). In caesium-containing solutions, the external Cs and Na concentrations were 5.6 and 130 mM, respectively. In sodium-containing solutions, the external and internal Na concentrations were 130 mM. Each point represents the mean of 7–10 cells; vertical lines show s.e.mean.

As the relationship between the amplitude of the noradrenaline-induced current and clamp potential was linear (Figure 2c), the reversal potentials were determined when the chloride electrochemical gradient was altered by replacing chloride with aspartate. Figure 2b illustrates the plot of the mean reversal potential against the calculated equilibrium potential for chloride ions. The experimental points were closely distributed along a straight line with a slope of 1, as expected for a pure chloride conductance. Figure 2c shows experiments in which the reversal potentials were determined in caesium-containing solutions (■, -27.5 ± 2.3 mV, $n = 7$) and in sodium-containing solutions (●, -29.2 ± 3.5 mV, $n = 10$). These values are close to the calculated E_{Cl} (−32 mV) and far from E_{Na} (respectively 0 and +67 mV). The absence of change in reversal potential of the noradrenaline-induced current produced by replacing caesium by sodium ions with similar anion gradients suggests that noradrenaline does not open a cation channel in smooth muscle cells isolated from rat portal vein.

It has to be noted that noradrenaline-induced currents were obtained in 45% of the cells in caesium-containing solution ($n = 16$). The percentage of responding cells was reduced to 36% in sodium-containing solution ($n = 11$) although there was no apparent difference in the response to noradrenaline in both cases.

Calcium-dependence of the noradrenaline-induced inward current

The role of calcium ions was examined first by comparing the noradrenaline-induced responses in barium and calcium solutions. As shown in Figure 3a, no responses were observed when the cells were perfused for 20 min in 5 mM barium solution. After replacement of barium with calcium for 5 min, inward currents could be evoked by noradrenaline applications. When the pipette solution contained 10 mM EGTA, the responses to noradrenaline were never observed even in the presence of external calcium (Figure 3b). In contrast, the noradrenaline-induced current was little altered (if at all) in the presence of 3 mM cobalt applied for 10 min. These results suggest that the noradrenaline-induced depolarization was not carried by calcium ions flowing through voltage-dependent calcium channels.

It is well known that caffeine displaces calcium from intracellular stores in portal vein smooth muscle (Nanjo, 1984) and we studied the effects of bath-applied caffeine in caesium-containing solution. In an experiment where the external chloride concentration was 130 mM, caffeine (10 mM) evoked a large inward current when the cell was held at −70 mV (Figure 4a). The caffeine-induced current was null near 0 mV and became outward at positive holding potential (Figure 4b). When the extracellular

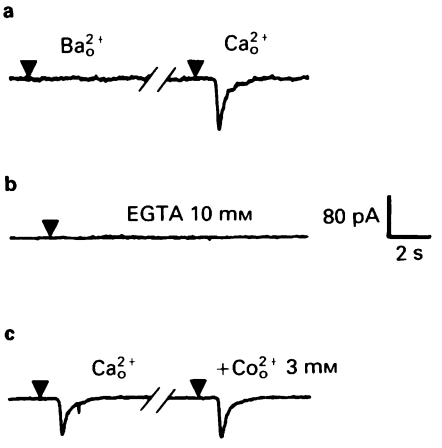

Figure 3 Calcium-dependence of the noradrenaline-induced current in caesium-containing solutions. (a) In 5 mM external barium solution for 20 min, no responses were observed to noradrenaline applications (▼). After reintroduction of 5 mM external Ca for 5 min, an inward current was recorded. (b) When the pipette solution contained 10 mM EGTA, no responses were induced by noradrenaline. (c) In the presence of 3 mM external cobalt for 10 min, the noradrenaline-induced current was unchanged.

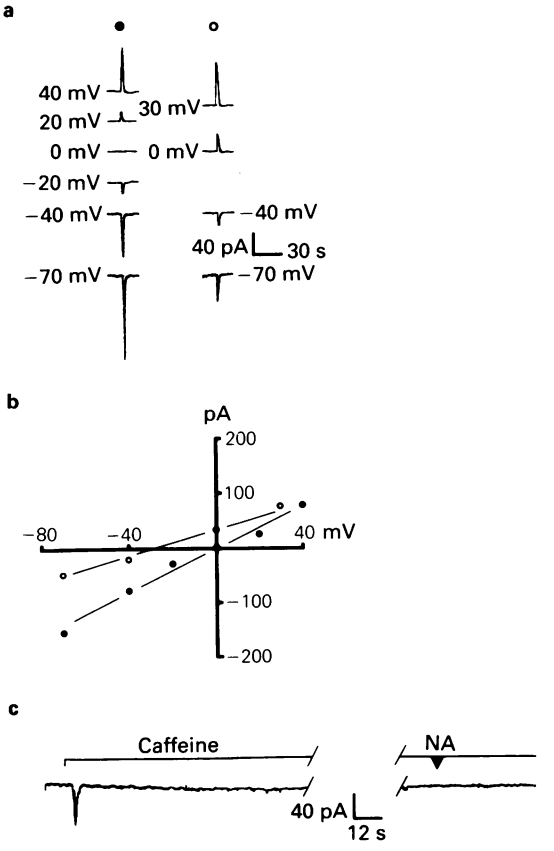

Figure 4 Membrane currents in response to bath-applied caffeine (10 mM) in caesium-containing solutions. (a) The external and pipette chloride concentrations were 146 mM and 130 mM (●) or 40 mM and 130 mM (○), respectively (substituted with aspartate). The value of the holding potential (mV) is noted near the current baseline. (b) Current-voltage relationships of the caffeine-induced current against the membrane potential in conditions as described in (a). (c) In the presence of caffeine (10 mM), there was no detectable action of noradrenaline (NA) (▼).

chloride concentration was reduced to 40 mM (substituted with aspartate), the caffeine-induced current reversed at more negative membrane potentials (-29.5 ± 2.5 mV, $n = 5$, Figure 4a–b). This value is close to the calculated E_{Cl} (−32 mV). As the reversal potential of the caffeine-induced current was similar to that found for noradrenaline with the same chloride concentrations, it appears that caffeine can induce a chloride conductance increase similar to that evoked by noradrenaline. In order to determine whether noradrenaline and caffeine displaced calcium from the same intracellular stores, microejections of noradrenaline were applied in the presence of 10 mM caffeine for 4 min. As shown in Figure 4c, we observed no detectable action of noradrenaline under these conditions. This observation favours the idea that the chloride conductance is activated by intracellular calcium release and that both noradrenaline and caffeine act on the same intracellular calcium stores.

Chloride conductance is activated by calcium release and calcium influx

It has been previously shown that noradrenaline increases the fast calcium current in rat portal vein smooth muscle cells (Pacaud *et al.*, 1987). To verify that the increased calcium influx could also activate the chloride conductance, we examined the change in membrane currents with time after pressure ejection of noradrenaline (Figure 5). In potassium-free caesium-containing bathing and pipette solutions, the current measured at the end of 150 ms depolarizing pulses was carried by chloride ions. Upon returning to −70 mV the chloride current was seen as a large inward tail current (Pacaud *et al.*, 1989). After stabilization of the membrane current in control for 10 min noradrenaline (10^{-5} M) was ejected in the vicinity of the cell. After the development of the noradrenaline-induced inward current (Figure 5a), repetitive depolarizing pulses from

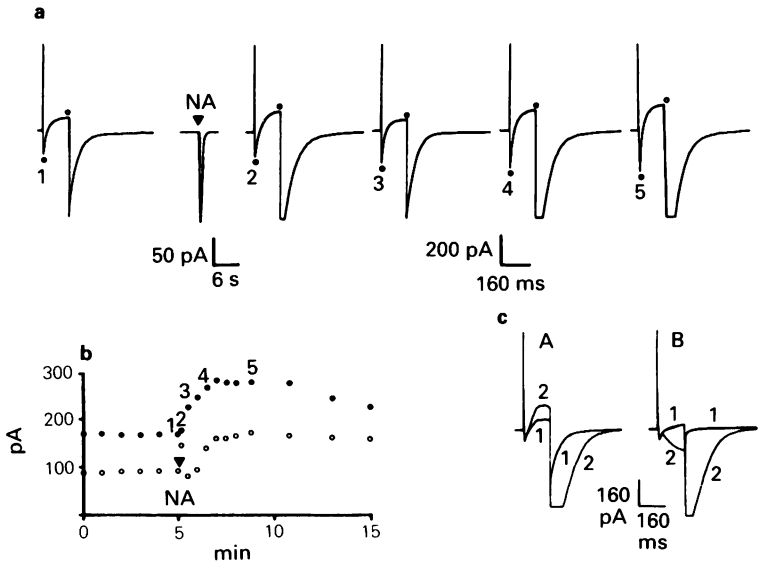

Figure 5 Change in membrane currents with time after pressure ejection of noradrenaline in caesium-containing solutions. (a) Currents were elicited from a holding potential of −70 mV to +10 mV in reference solution for 10 min (1). Ejection of noradrenaline (NA) (▼) produced the development of a transient inward current. Membrane currents in response to depolarizing pulses were recorded after 15 s (2), 30 s (3), 90 s (4) and 4 min (5). (b) Time course of the outward current measured at the end of the pulse (○) and the peak calcium current (●) before and after application of noradrenaline. (c) Superimposition of membrane currents elicited from a holding potential of −70 mV to +10 mV in the absence (1) and presence of noradrenaline (2). The external and pipette chloride concentrations were 146 mM and 130 mM (A) and 36 mM and 130 mM (B), respectively (substituted with aspartate). In potassium-free caesium-containing bathing and pipette solutions, the current measured at the end of the pulse is carried by chloride ions. Upon returning to −70 mV the chloride current is seen as a large inward tail current (Pacaud *et al.*, 1989).

−70 mV to +10 mV were applied. Figure 5b illustrates the typical time course of both the outward current measured at the end of the pulse (○) and the peak calcium current (●). About 15 s after noradrenaline ejection, both the outward current measured at the end of the pulse and the inward tail current upon returning to −70 mV were strongly increased (Figure 5a). After 30 s, the amplitude of the calcium current began to increase and finally, after 4–5 min, it reached an almost double stable amplitude (Figure 5b). It is obvious that the amplitude of the outward current measured at the end of the pulse increased with the same time course as that of the calcium current. The stimulation of the currents was not maintained. Obviously, 10 min after application of noradrenaline the calcium current remained increased to about 35% of control. At this time, the outward current measured at the end of the pulse remained almost at its maximal value. Changes in membrane currents obtained in control and after ejection of noradrenaline are illustrated in Figure 5a. The action of noradrenaline was completely blocked in the presence of 10^{-6} M prasozin. In order to verify that the chloride current did not overlap with the inward calcium current, an experiment was done with two different external chloride concentrations (146 and 36 mM). As shown in Figure 5c, the (outward or inward) noradrenaline-activated chloride current developed with a sufficient delay so that it did not interfere with the peak calcium current.

Activation of chloride conductance by internal calcium release and calcium influx can be separated by using successive applications of caffeine and noradrenaline (Figure 6). During bath application of caffeine (10 mM) a transient inward current was first recorded (Figure 4c) and then repetitive depolarizing pulses were applied as in Figure 5b to follow the time course of both the outward current measured at the end of the pulse (○) and the peak calcium current (●, Figure 6b). About 30 s after perfusion with caffeine, both the outward current measured at the end of the pulse and the tail inward current were strongly increased (Figure 6a) but the calcium current remained unchanged. In the presence of caffeine, pressure ejection of noradrenaline induced a strong increase in the amplitude of the calcium

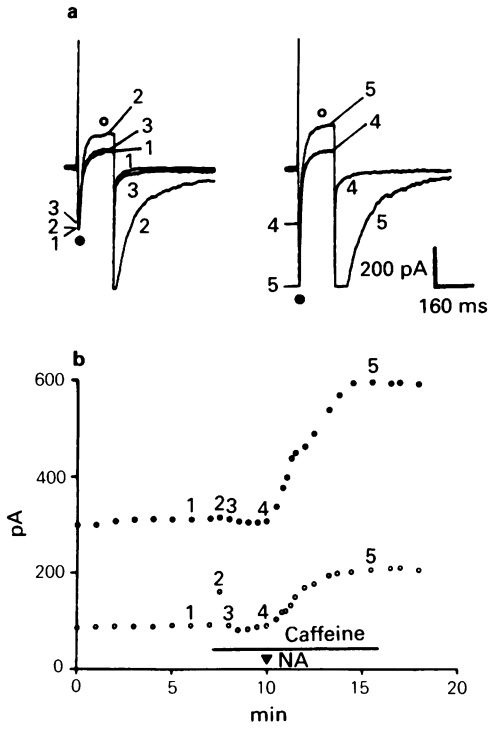

Figure 6 Changes in membrane currents with time in the presence of caffeine and noradrenaline in caesium-containing solutions. (a) Currents were elicited from a holding potential of −70 mV to +10 mV in reference solution (1), and during application of caffeine for 30 s (2), 1 min (3) and 3 min (4). Ejection of noradrenaline stimulated both the peak calcium current, the current measured at the end of the pulse and the tail current upon returning to −70 mV (5). (b) Time course of the outward current measured at the end of the pulse (○) and the peak calcium current (●) before and during application of caffeine and noradrenaline (NA) (▼). In potassium-free caesium-containing bathing and pipette solutions, the current measured at the end of the pulse is carried by chloride ions. Upon returning to −70 mV the chloride current is seen as a large inward tail current (Pacaud *et al.*, 1989).

current. Consequently, the amplitude of the outward current measured at the end of the pulse increased with the same time course as that of the calcium current. Changes in membrane currents obtained in control and after applications of caffeine and noradrenaline are illustrated in Figure 6a.

Discussion

The results from the present study provide evidence that in cultured single cells of the rat portal vein, noradrenaline activates two separate membrane conductance mechanisms. First, noradrenaline produces an increase in chloride conductance which leads to membrane depolarization and inward current. The reversal potential of the noradrenaline-induced inward current changes with external or internal chloride concentration as predicted by the Nernst relation. These results are in good agreement with those previously observed in short segments of guinea-pig mesenteric vein (Van Helden, 1988) and in isolated cells of rat anococcygeus muscle (Byrne & Large, 1987) and rabbit portal vein muscle (Byrne & Large, 1988b). However, the reversal potential of the noradrenaline-induced current does not change when different cations with similar anion gradients are used, suggesting that in rat portal vein noradrenaline does not open some non specific cation channels as shown in rabbit portal vein (Byrne & Large, 1988b). A mechanism involving only an increase in chloride conductance is consistent with ion flux studies which indicate that only a noradrenaline-induced efflux of chloride is recorded in muscle of rat portal vein (Wahlström, 1973). The finding that the noradrenaline-induced responses are maintained in the presence of external cobalt but can not be recorded in barium-containing solution or after addition of 10 mM EGTA in the pipette solution suggests that these responses can be mediated by intracellular calcium release activated by receptor stimulation. This is supported by a characteristic latency of about 1 s between the pressure ejection of noradrenaline and the onset of response. This long delay is not seen when external ATP is applied to isolated smooth muscle cells with the same pressure ejecter (P. Pacaud, G. Loirand, C. Mironneau and J. Mironneau, unpublished results). It is thought that the latency represents the time taken for intracellular mediators to link α-receptors binding to membrane channel opening. The involvement of internal calcium release is consistent with the observation that caffeine stimulates the chloride conductance in a manner similar to noradrenaline. A chloride conductance activated by the inward calcium current has also been observed in the smooth muscle cells of the rat portal vein (Pacaud *et al.*, 1989). The chloride current can be measured as either an outward current at the end of 150 ms depolarizing pulses or an inward tail current upon returning to −70 mV. The increase in chloride conductance produced by noradrenaline does not result from specific activation of a specialized subset of chloride channels as (i) the noradrenaline-induced response is lost after application of caffeine, and (ii) the increase in both depolarization-induced calcium and chloride currents during depolarizing pulses in response to noradrenaline is maintained in the presence of caffeine. Our results indicate that the chloride channels acti-

vated by noradrenaline belong to the general class of calcium-activated chloride channels.

Secondly, noradrenaline appears to open channels selective for potassium. Removal of potassium from the bathing and pipette solutions suppresses the noradrenaline-induced hyperpolarization and outward current. It is noted that, at the resting membrane potential, the increase in potassium conductance produced by noradrenaline is overwhelmed by the chloride inward current. From the present experiments it is not clear whether the outward current produced by noradrenaline is related to the opening of receptor-operated potassium channels or calcium-activated potassium channels.

After ejection of noradrenaline there is a discrepancy between the brief time course of chloride conductance activation (1–2 s) and the delayed increase in calcium current amplitude (5–10 min). As a similar observation is not seen during caffeine application, a possible membrane effect related to the increase in cytoplasmic calcium concentration may be discounted. A likely possibility is that the phospholipase C-protein kinase C pathway may be involved in the generation of the effects of noradrenaline on the calcium channels. More detailed experiments are needed to confirm this hypothesis.

In conclusion, both chloride and potassium channels in smooth muscle cells from rat portal vein are opened by noradrenaline in physiological conditions. This would result in the generation of an inward depolarizing current allowing repetitive electrical activity and contraction to occur.

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Regional haemodynamic effects of vasopressin infusion in conscious, unrestrained, Brattleboro rats

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1 Incremental rates of infusion (0.027–4.0 pmol min^{-1}) of exogenous vasopressin were given for 10 min periods to conscious, Brattleboro (i.e. vasopressin-deficient) rats, which had been chronically instrumented with pulsed Doppler probes and intravascular catheters (to permit assessment of regional haemodynamic changes).

2 There were no significant effects of the two lowest rates of infusion (0.027 and 0.105 pmol min^{-1}) but, after 10 min of vasopressin infusion at 0.313 pmol min^{-1}, superior mesenteric vasoconstriction had occurred unaccompanied by any other changes. Infusion of vasopressin at 0.687 pmol min^{-1} produced increases in blood pressure and in both mesenteric and hindquarters vascular resistance, but no change in renal vascular resistance or heart rate. These results do not indicate specific interactions between exogenous vasopressin and baroreflex mechanisms to offset the pressor effect of the neuropeptide.

3 The marked bradycardia, hypertension and renal, mesenteric and hindquarters vasoconstriction seen after 10 min infusion of vasopressin at 4.0 pmol min^{-1} were all abolished 10 min after administration of an antagonist of V_1-receptors ((+)-$(CH_2)_5$Tyr(Et)DAVP); additional antagonism of V_2-receptors (with the non-selective antagonist (+)-$(CH_2)_5$Tyr(Et)VAVP) had no effects on any measured variable. Hence, the present experiments provide no evidence for important vasodilator effects of vasopressin in conscious, Brattleboro rats.

Introduction

In spite of the fact that vasopressin has long been known as a vasoactive neuropeptide, its involvement in cardiovascular regulation is obscure (see Bennett & Gardiner, 1986a; Gardiner & Bennett, 1986). While this may be due to subtle interactions of vasopressin with cardiovascular regulatory mechanisms in dogs (see Cowley & Liard, 1988), the picture in rats has been made unnecessarily cloudy by investigators who have assumed results obtained in dogs are applicable to rats, with no evidence that this is so (see Bennett & Gardiner, 1986a; Gardiner & Bennett, 1986). It has become clear, however, that bolus doses of exogenous vasopressin may have regionally-selective effects on vascular resistances in several species, including conscious rats (Gardiner *et al.*, 1988a) and that these may be due to indirect actions of vasopressin in the renal vascular bed (e.g. Hofbauer *et al.*, 1983; Walker *et al.*, 1988). Furthermore, there are indications that vasopressin may exert systemic vasodilator actions in conscious rats (Walker, 1986) and in dogs (Liard, 1988). In the latter these effects appear to be mediated by V_2-receptors.

Previous experiments have dealt with the cardiovascular action of exogenous vasopressin in normal rats, i.e. under conditions in which circulating, endogenous vasopressin could have influenced responses to the administered peptide (Hofbauer *et al.*, 1983; Walker, 1986; Walker *et al.*, 1988). In the present work we avoided this problem by investigating the regional haemodynamic effects of vasopressin infusion in conscious Brattleboro (i.e. vasopressin-deficient) rats in an attempt to answer two questions: firstly, are there clear-cut indications that infusion of exogenous vasopressin can cause increases in regional vascular resistance, with no effect on arterial blood pressure? Secondly, does antagonism of V_1-receptors during vasopressin infusion reveal V_2-receptor-mediated haemodynamic effects in Brattleboro rats?

Some of these results have been presented in abstract form (Bennett *et al.*, 1988).

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Methods

Male, homozygous Brattleboro rats (350–400 g), from our own breeding colony, were studied. Under barbiturate anaesthesia (sodium methohexitone, Eli Lilly, 60 mg kg^{-1}, i.p.) miniaturized, pulsed Doppler probes (Haywood *et al.*, 1981) were implanted around the left renal artery, the superior mesenteric artery and around the distal abdominal aorta below the level of the ileocaecal artery. The probe wires were tunnelled subcutaneously and exteriorized at the back of the neck; they were soldered into a connector (Microtech Inc, Boothwyn, U.S.A.) held in a harness worn by the rat. Animals were left at least 7 days after operation with free access to water and food (Labsure 41 b). Thereafter, rats that were healthy and with good signals (signal:noise ratio > 20:1) from all three probes were briefly reanaesthetized and had catheters implanted in the distal abdominal aorta (via the caudal artery) and right external jugular vein. The following day continuous records were made of mean blood pressure, instantaneous heart rate and renal, mesenteric and hindquarters (i.e., distal aortic) Doppler shift signals (Crystal Biotech VFI Doppler flowmeter, Holliston, U.S.A.). The latter have been shown to be a good index of volume flow (Haywood *et al.*, 1981; Wright *et al.*, 1987), and are referred to as flows in the text. Changes in Doppler shift signals and vascular resistances were calculated as percentages relative to baseline (Haywood *et al.*, 1981; Gardiner *et al.*, 1988b).

Following a 30 min control period, an intravenous infusion of arginine vasopressin (Bachem, U.K.) was started. The catheter system was primed with the infusate before the experiment was begun so that when the pump was switched on the animal started to receive the vasopressin solution intravenously. The infusate was vasopressin (1 pmol ml^{-1}) in isotonic saline; the initial infusion rate was 0.027 μl min^{-1} (achieved with a 1 ml syringe mounted in an SRI constant slow infusion pump) i.e. delivering 0.027 pmol min^{-1} of vasopressin. This infusion was continued for 10 min, then the rate was increased to 0.105 μl min^{-1}. Thereafter, at 10 min intervals, the pump rate was increased sequentially to 0.313, 0.687 and 4 μl min^{-1}. From pilot experiments it was found that this protocol caused little change in mean blood pressure or heart rate except at the highest infusion rate, i.e. 4 pmol min^{-1} of vasopressin (infusion of isotonic saline alone at these rates had no cardiovascular effects).

After the initial 10 min period of infusion of vasopressin at 4 pmol min^{-1}, the infusion was continued while a V_1-receptor antagonist, ((+)-$(CH_2)_5$Tyr(Et)-DAVP; Manning & Sawyer, 1986; 10 μg kg^{-1}) was administered i.v. through a separate catheter. This dose of the V_1-receptor antagonist reduces by 80% the cardiovascular effects of bolus doses of 7 pmol of vasopressin for a period of at least 30 min, (Gardiner, Compton & Bennett, unpublished observations). While the vasopressin infusion was continued, and 10 min after administration of the V_1-receptor antagonist, an antagonist of the V_1- and V_2-receptor-mediated actions of vasopressin (i.e. (+)-$(CH_2)_5$Tyr(Et)VAVP, 100 μg kg^{-1}; Manning & Sawyer, 1986; Hofbauer *et al.*, 1986) was given.

For the purposes of analysis, measurements were made at the end of each 10 min period of vasopressin infusion and 1 and 10 min after the administration of the vasopressin antagonists.

The V_1-antagonist, (+)-$(CH_2)_5$Tyr(Er)DAVP, ([1-(β - mercapto - β,β - cyclopentamethylenepropionic acid), 2-O-ethyl-tyrosine, 8-D-arginine]vasopressin), was a gift from Professor Manning (Toledo Medical College, Ohio, U.S.A.) and the V_1,V_2-antagonist, (+)-$(CH_2)_5$Tyr(Er)VAVP, ([1-(β-mercapto-β,β-cyclopentamethylenepropionic acid)2-(O-ethyl)-D-tyrosine, 4-valine]arginine vasopressin), was a gift from Professor Brunner (Ciba Geigy, Basle). All drugs were made up in isotonic saline; the antagonists were initially dissolved in glacial acetic acid.

Results

The baseline values for cardiovascular variables are given in Table 1. Table 2 shows that infusion of vasopressin at 0.027 and 0.185 pmol min^{-1} had no significant effects on any measured or calculated variable. However, after 10 min infusion of vasopressin at 0.313 pmol min^{-1} there was a small but significant, superior mesenteric vasoconstriction which was not accompanied by any other significant changes. Following 10 min infusion of vasopressin at

Table 1 Baseline values for heart rate, blood pressure and regional vascular resistances in Brattleboro rats

Heart rate (beats min^{-1})	*Mean blood pressure* (mmHg)	*Doppler shift* (kHz)			*Vascular resistance* (mmHg kHz^{-1})		
		Renal	*Mesenteric*	*Hindquarters*	*Renal*	*Mesenteric*	*Hindquarters*
369 ± 11	104 ± 3	7.6 ± 0.9	9.5 ± 0.5	4.0 ± 0.3	15.2 ± 1.9	11.2 ± 0.6	26.9 ± 2.4

Values are mean ± s.e.mean (n = 8).

Table 2 Cardiovascular changes in Brattleboro rats receiving incremental infusions of vasopressin

	Vasopressin infusion								
	Doses (pmol min^{-1})					V_1-*antagonist*		V_1, V_2-*antagonist*	
	0.027	0.105	0.313	0.687	4.0	1 min	10 min	1 min	10 min
Heart rate (beats min^{-1})	3 ± 8	−4 ± 8	−11 ± 11	−19 ± 12	−89 ± 9*	−53 ± 11*	−13 ± 16	−1 ± 17	11 ± 14
Mean blood pressure (mmHg)	−1 ± 2	−1 ± 3	3 ± 4	13 ± 4*	32 ± 2*	24 ± 3*	0 ± 3	−2 ± 3	−4 ± 2*
Renal Doppler shift (%)	3 ± 2	0 ± 3	1 ± 4	6 ± 2	−1 ± 6	12 ± 5	6 ± 5	8 ± 5	0 ± 4
Mesenteric Doppler (shift) (%)	−3 ± 5	−3 ± 5	−4 ± 3	−11 ± 4*	−31 ± 7*	0 ± 6	−1 ± 4	−1 ± 4	−5 ± 3
Hindquarters Doppler shift (%)	−2 ± 8	8 ± 5	3 ± 4	−13 ± 9	−22 ± 7*	−11 ± 8*	−8 ± 8	−1 ± 7	2 ± 8
Renal vascular resistance (%)	−3 ± 3	−1 ± 4	3 ± 4	6 ± 4	37 ± 11*	12 ± 5*	−4 ± 5	−7 ± 5*	−3 ± 3
Mesenteric vascular resistance (%)	4 ± 6	4 ± 6	9 ± 5*	30 ± 11*	107 ± 26*	27 ± 9	1 ± 3	0 ± 4	3 ± 4
Hindquarters vascular resistance (%)	6 ± 9	−7 ± 5	2 ± 7	40 ± 16*	83 ± 22*	45 ± 12*	15 ± 11	2 ± 7	−1 ± 9

Each dose was infused for 10 min, with the exception of the highest dose (4.0 pmol min^{-1}). The latter infusion of vasopressin was continued, after the initial 10 min, together with administration of a V_1-receptor antagonist ((+)-$(CH_2)_5$Tyr(Et)DAVP; 10 μg kg^{-1}) for 10 min and, for a further 10 min after a V_1, V_2-receptor antagonist ((+)-$(CH_2)_5$ Tyr(Et)VAVP; 100 μg kg^{-1}) had been administered. Values are mean ± s.e.mean (n = 8).

* $P < 0.05$ for change relative to baseline (Friedman's test).

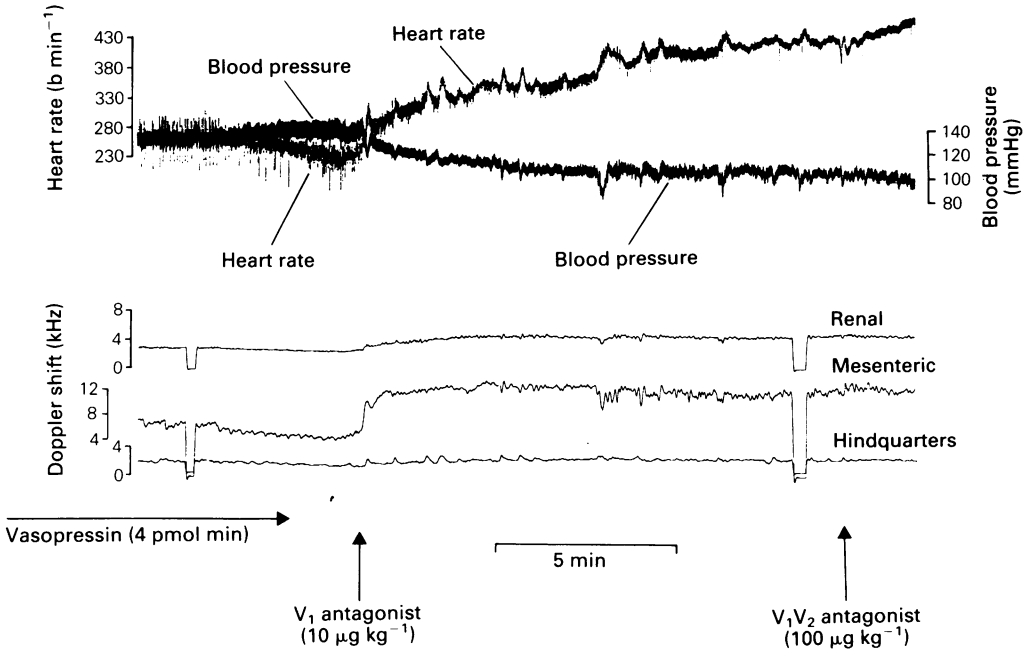

Figure 1 Continuous recording of cardiovascular variables in a conscious, unrestrained, Brattleboro rat. Initially the animal was given an infusion of exogenous vasopressin which was then followed by sequential, bolus injections of (+)-$(CH_2)_5$Tyr(Et)DAVP (a V_1-receptor antagonist) and (+)$(CH_2)_5$Tyr(Et)VAVP (a V_1- and V_2-receptor antagonist).

0.687 pmol min^{-1} there were significant reductions in superior mesenteric and hindquarters flows, accompanying an increase in mean arterial blood pressure. These changes were associated with constrictions in the superior mesenteric and hindquarters vascular beds, but there were no changes in renal vascular resistance or heart rate. Infusion of vasopressin at the highest rate (4 pmol min^{-1} for 10 min) produced a marked bradycardia accompanied by a rise in mean arterial blood pressure. There was no change in renal blood flow but there were reductions in flows in the superior mesenteric and the hindquarters vascular beds. Hence, renal, superior mesenteric and hindquarters vasoconstrictions were apparent, but the increase in renal vascular resistance was least marked (Table 2).

During the continued infusion of the highest dose of vasopressin, administration of the V_1-receptor antagonist caused a prompt reversal of all the effects of vasopressin (Figure 1), such that, after 10 min, all variables were at baseline levels. Additional administration of the V_1,V_2-receptor antagonist after a further 10 min caused no significant changes relative to the values measured at 10 min in the presence of the V_1-receptor antagonist (Figure 1). However relative to baseline, there were slight (but significant) reductions in renal vascular resistance and mean arterial blood pressure but these did not occur together (Table 2).

Discussion

An uncritical reading of the published literature on the cardiovascular effects of exogenous vasopressin could give the impression that this potent vasoconstrictor neuropeptide (Gardiner *et al.*, 1988a) does not exert pressor effects because any increase in peripheral resistance it causes is offset by a baroreflex-mediated reduction in cardiac output (see Bennett & Gardiner, 1986a; Gardiner & Bennett, 1986), together with activation of vasodilator systems that may be dependent on V_2-receptor-mediated mechanisms (Hofbauer *et al.*, 1983; Walker, 1986; Liard, 1988). The present study produced results germane to these points. Since the experiments were carried out on Brattleboro rats that are devoid of circulating, endogenous vasopressin (see Woods & Johnston, 1983) the findings would not have been

influenced by effects due to vasopressin receptor occupancy prior to administration of exogenous vasopressin.

At the two lowest infusion rates of exogenous vasopressin there were no significant changes in any measured or calculated cardiovascular variables. With an infusion rate of 0.313 pmol min^{-1} of vasopressin we found there was a significant (although small) mesenteric vasoconstriction that was not accompanied by a significant increase in mean arterial blood pressure. However, this could have been an autoregulatory response because mesenteric blood flow was not reduced below baseline. Since there were no vasodilatations in the renal or hindquarters vascular beds, it is possible that the lack of a significant increase in mean arterial blood pressure was due to a reduction in cardiac output, but there was no significant bradycardia. Thus any reduction in cardiac output during vasopressin infusion in rats is unlikely to have been baroreflex-mediated (see also Webb *et al.*, 1986).

Our results therefore provide no support for subtle interactions between exogenous vasopressin and baroreflex mechanisms acting to buffer the expected pressor effects of vasopressin-induced regional vasconstrictions, at least in Brattleboro rats. Indeed, elsewhere we have reported that, on a molar basis, vasopressin is a more potent pressor agent than either angiotensin II or neuropeptide Y in conscious, Long Evans (i.e. normal with respect to vasopressin secretion) and in Brattleboro rats (Gardiner *et al.*, 1988a).

Even when mean arterial blood pressure increased in company with superior mesenteric and hindquarters vasoconstrictions due to vasopressin infusion (at 0.687 pmol min^{-1} for 10 min), the pressor effect occurred in the absence of a significant fall in heart rate. Although it could be argued that this was due to Brattleboro rats having impaired cardiac baroreflex mechanisms (Imai *et al.*, 1983) we have found these animals have largely normal bradycardic responses to pressor challenges (Gardiner & Bennett, 1982; 1988; Bennett & Gardiner, 1986b; 1987; Harland *et al.*, 1988). In addition, it is unlikely that the heart rate response to vasopressin, particularly, is impaired in Brattleboro rats since it is similar to that seen in Long Evans rats and not different when pressor neuropeptides other than vasopressin are administered (Gardiner *et al.*, 1988a).

The potent cardiovascular effects of the highest dose infusion of vasopressin were characterized by a relatively weak renal vasoconstriction (possibly due to activation of vasodilator mechanisms, see below). All these effects were abolished within 10 min of administering a V_1-receptor antagonist. Hence, in the model studied in the present experiments there was no evidence for the occurrence of effects of vasopressin independent of V_1-receptors (Walker, 1986). This proposition is in line with the findings that additional blockade of V_2-receptors had no significant cardiovascular effects. However, under these conditions there was a slight, (although significant) transient reduction in calculated renal vascular resistance relative to the original baseline level. It is feasible this was due to the effects of some vasodilator mechanism activated by prior V_1- and/or V_2-receptor agonism (Walker, 1986; Liard, 1988). However, the small magnitude and brief duration of this effect make it unlikely that this phenomenon and that described by Walker (1986) represent the same event. Indeed, from the data in Table 2 it could be argued that any change in renal vascular resistance was autoregulatory since there were no significant alterations in renal blood flow at any stage in the experiment. However, the extent to which renal vasodilator prostanoids contributed to this picture (Walker *et al.*, 1988) needs to be determined.

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The effects of infusions of arginine vasopressin or 1-deamino-8-D-arginine vasopressin on common carotid vascular resistance in conscious, Long Evans rats

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1 Intravenous infusions of arginine vasopressin or 1-deamino-8-D-arginine vasopressin (DDAVP) were given to conscious, Long Evans rats chronically instrumented with bilateral, common carotid, pulsed Doppler probes and intravascular catheters.

2 During infusion of vasopressin at 0.3 nmol min^{-1} there was an increase in common carotid vascular resistance with no change in mean blood pressure or heart rate. Following infusion there was a common carotid vasodilatation.

3 During infusion of vasopressin at 3.0 nmol min^{-1} there were increases in mean arterial blood pressure and in common carotid vascular resistances, accompanied by bilateral reductions in flow and in heart rate. Administration of (+)-$(CH_2)_5$Tyr(Et)DAVP (a V_1-receptor antagonist), during the continued infusion of vasopressin, reversed the effects of the latter on mean blood pressure and heart rate; under these conditions there were increases in common carotid blood flows above baseline, in company with bilateral vasodilatations. The latter effects persisted after cessation of vasopressin infusion.

4 Infusions of DDAVP were without significant effects on any measured cardiovascular variable.

5 The results do not provide straightforward support for the claim that vasopressin acts to promote cerebral perfusion, at least when V_1-receptor effects are unopposed. Furthermore, it seems likely that the vasodilator influence of vasopressin on the common carotid vascular bed is not due to stimulation of V_2-receptors.

Introduction

In vitro observations on the responses of canine basilar artery preparations to vasopressin have shown these vessels can respond with an endothelium-dependent relaxation (Katusic *et al.*, 1984) and it has been suggested that vasopressin-induced vasoconstriction elsewhere could thus favour cerebral perfusion during haemorrhage or septic shock (Katusic *et al.*, 1984). However, subsequent studies with common carotid artery preparations from rats showed these vessels responded to vasopressin with an endothelium-independent vasoconstriction (Katusic & Krstic, 1987), although this effect was less marked than those seen in the aorta and renal artery. Katusic & Krstic (1987) suggested, therefore, that their observations were consistent with the hypothesis that elevated circulating levels of vasopressin would favour perfusion of the cerebral circulation. Since there have been no studies of the effects of vasopressin on common carotid vascular resistances in conscious rats we addressed this question in the present work and also investigated the effects of the V_2-receptor agonist, 1-deamino-8-D-arginine vasopressin (DDAVP). In addition, we assessed the effects of V_1-receptor antagonism on the carotid vascular responses to vasopressin.

Methods

Male, Long Evans rats (350–450 g) were anaesthetized (sodium methohexitone, Eli Lilly, 60 mg kg^{-1} i.p., supplemented as required to maintain abolition of the toe-pinch reflex) and had pulsed Doppler flow probes (Haywood *et al.*, 1981) implanted around left and right common carotid arteries. After the vessels had been exposed through a mid-line incision, they were gently separated from

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Table 1 Baseline heart rate, blood pressure, Doppler shift and carotid vascular resistances in Long Evans rats

Heart rate (beats min^{-1})	*Mean blood pressure* (mmHg)	*Doppler shift* (kHz)		*Vascular resistance* (mmHg kHz^{-1})	
		Left carotid	Right carotid	Left carotid	Right carotid
351 ± 12	99 ± 2	3.1 ± 0.4	2.8 ± 0.4	34 ± 4	41 ± 7

Values are mean ± s.e.mean (n = 8).

the accompanying nerve trunks, so that suturing the probes (Crystal Biotech, Holliston, U.S.A.) in position did not damage the nerve trunks, as assessed by intact bradycardic or tachycardic responses respectively to hypertensive and hypotensive stimuli and the absence of ptosis. The wires from the probes were tunnelled subcutaneously and were exteriorized at the back of the neck where they were soldered into a microconnector held in a harness worn by the rat (Microtech, Inc., Boothwyn, U.S.A.). Animals were allowed to recover for at least 7 days, after which time those with acceptable signals from both probes (signal:noise ratio, >20:1) were briefly re-anaesthetized (sodium methohexitone, 40 mg kg^{-1}) and had an intra-arterial (abdominal aorta via caudal artery) and 3 intravenous (left external jugular) catheters implanted. The catheters were tunnelled subcutaneously and were exteriorized at the same point as the probe wires. The catheters ran through a flexible spring (attached to the harness) supported by a counterbalanced gantry. Animals were left to recover overnight with free access to food and water.

The following day continuous recordings were made of mean blood pressure, heart rate and carotid Doppler shift signals. Although the latter do not give an absolute measure of volume flow, changes in the Doppler shift signal are linearly related to changes in volume flow (Haywood *et al.*, 1981; Wright *et al.*, 1987), and are referred to as flow in the text. Changes (%) in vascular resistances were calculated from the changes in mean blood pressure and Doppler shift signals, relative to baseline (Haywood *et al.*, 1981; Gardiner *et al.*, 1988).

On the basis of pilot experiments we found that vasopressin administered at 0.3 nmol min^{-1} had no significant effects on mean blood pressure or heart rate whereas a 10 fold higher dose affected both these variables. Therefore, animals received vasopressin (60 nmol ml^{-1}, 0.3 ml h^{-1}, i.e., 0.3 nmol min^{-1}) for 60 min, followed by a 60 min recovery period. Thereafter vasopressin (600 nmol ml^{-1}, 0.3 ml h^{-1} i.e., 3 nmol min^{-1}) was administered for 20 min (by which time variables were steady) before (+)-(CH$_2$)$_5$Tyr(Et)DAVP (a V$_1$-receptor antagonist, Manning & Sawyer, 1986) was given (10 μg kg^{-1}, bolus) while the vasopressin infusion was continued. This dose of the V$_1$-receptor antagonist blocked the pressor actions of vasopressin (10 pmol) for at least 30 min. After a further 20 min, the vasopressin infusion was stopped and recordings continued for an additional 20 min.

The protocol for DDAVP administration involved 60 min infusions followed by 60 min recovery periods with the lower dose (0.3 nmol min^{-1}) being given before the higher dose (3 nmol min^{-1}). Animals were randomized so they received vasopressin on one day and DDAVP on the other experimental day.

From preliminary examination of the experimental traces, measurement points for analysis were chosen at 10, 30, and 60 min during and after vasopressin infusion at 0.3 nmol min^{-1} and for both doses of DDAVP. For vasopressin infusion at 3 nmol min^{-1}, measurements were made at 5 and

Table 2 Changes in cardiovascular variables during and after infusion of vasopressin (0.3 nmol min^{-1}) in conscious, Long Evans rats

	Vasopressin infusion					
	10 min	30 min	60 min	10 min	30 min	60 min
Heart rate (b min^{-1})	6 ± 9	−11 ± 9	−5 ± 10	−5 ± 9	−4 ± 9	0 ± 10
Mean blood pressure (mmHg)	2 ± 1	1 ± 2	2 ± 2	0 ± 1	−2 ± 2	−1 ± 2
Left carotid flow (%)	−6 ± 5	−4 ± 7	−7 ± 3	3 ± 6	14 ± 10	11 ± 7
Right carotid flow (%)	1 ± 7	2 ± 6	−11 ± 4	6 ± 8	20 ± 8	15 ± 10
Left carotid resistance (%)	10 ± 5	9 ± 9	13 ± 7	−2 ± 5	−9 ± 7	−8 ± 5
Right carotid resistance (%)	4 ± 6	3 ± 7	17 ± 5*	−1 ± 7	−15 ± 6*	−10 ± 7*

Values are mean ± s.e.mean (n = 8).
* $P < 0.05$ for change relative to baseline (Friedman's test).

20 min after the start of the infusion, 5 and 20 min after administration of the V_1-receptor antagonist and 5 and 20 min following cessation of the infusion. In addition, attention was paid to the acute changes associated with any intervention (see results). The substances used (vasopressin, Bachem, U.S.A.; DDAVP, Ferring, Sweden and (+)-$(CH_2)_5$-Tyr(Et)DAVP [1-(β-mercaptr-β,β-cyclopentamethylenepropionic acid),2-O-ethyl-tyrosine,8-D-arginine]vasopressin, Peninsula Laboratories, U.S.A.) were dissolved in isotonic saline; in the case of the V_1-receptor antagonist the initial solution was made in glacial acetic acid.

Data were subjected to non-parametric analysis of variance (Theodorsson-Norheim, 1987). Where effects are mentioned in the text, then the differences are statistically significant at $P < 0.05$.

Results

Table 1 shows baseline values for the measured cardiovascular variables. The lower rate of infusion of vasopressin (0.3 nmol min^{-1}) had no effects on mean blood pressure, heart rate or common carotid flows (Table 2). However, there was a tendency for calculated common carotid vascular resistance to increase, and this reached significance in the right common carotid vascular bed (Table 2). There was a vasodilatation in this same vascular bed following cessation of vasopressin infusion (Table 2).

Infusion of vasopressin at the higher rate (3 nmol min^{-1}) caused changes in all measured and calculated variables (Table 3). The decreases in common carotid flows were coincident with the increase in mean arterial blood pressure (Figure 1). Administration of the V_1-receptor antagonist caused a rapid abolition of the effects of vasopressin on mean blood pressure and heart rate (Figure 2, Table 3), but under these conditions there were increases in common carotid blood flows (above baseline) accompanied by vasodilatations (Table 3). These effects were persistent, such that 40 min after the administration of the V_1-receptor antagonist (i.e., 20 min after cessation of vasopressin infusion) there were still reductions in common carotid vascular resistances, at that time accompanied by a small fall in mean arterial blood pressure (Table 3).

Neither the lower nor the higher rate of infusion of DDAVP had any significant effects on the measured cardiovascular variables.

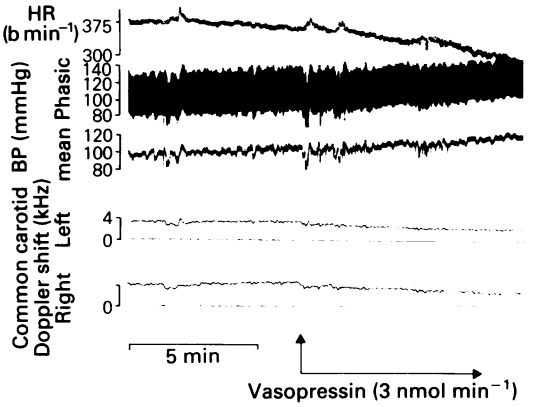

Figure 1 Cardiovascular changes at the onset of infusion of vasopressin (3 nmol min^{-1}) in a conscious, Long Evans rat instrumented with pulsed Doppler probes around both common carotid arteries.

Discussion

In the present study we found that during infusion of vasopressin, increases in common carotid blood flow were only seen in the presence of a V_1-receptor

Table 3 Changes in cardiovascular variables during and after infusion of vasopressin (3.0 nmol min^{-1}) in conscious, Long Evans rats

			V_1-receptor antagonist ↓			
	Vasopressin infusion					
	5 min	20 min	25 min	40 min	5 min	20 min
Heart rate (b min^{-1})	−38 ± 13*	−77 ± 15*	4 ± 10	−1 ± 12	−5 ± 13	−4 ± 11
Mean blood pressure (mmHg)	14 ± 4*	26 ± 4*	0 ± 3	−7 ± 3*	−3 ± 2	−6 ± 3*
Left carotid flow (%)	−17 ± 5*	−24 ± 7*	21 ± 5*	21 ± 9*	10 ± 5	14 ± 5*
Right carotid flow (%)	−22 ± 7*	−30 ± 8*	31 ± 17*	20 ± 19	20 ± 15	23 ± 16*
Left carotid resistance (%)	46 ± 18*	77 ± 22*	−18 ± 5*	−21 ± 7*	−11 ± 3*	−17 ± 4*
Right carotid resistance (%)	59 ± 12*	98 ± 21*	−17 ± 7*	−12 ± 9	−12 ± 8	−15 ± 9*

Twenty min into the infusion a V_1-receptor antagonist ((+)-CH_{25}Tyr(Et)DAVP) was administered. Values are mean ± s.e.mean ($n = 8$).

* $P < 0.05$ for change relative to baseline (Friedman's test).

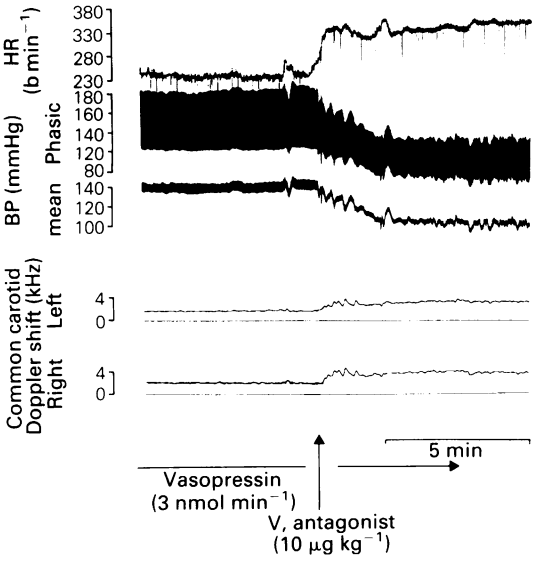

Figure 2 Cardiovascular effects of administering a V_1-receptor antagonist ((+)-$(CH_2)_5$Tyr(Et)DAVP) in a conscious, Long Evans rat that had been infused with vasopressin (3 nmol min^{-1} for 20 min).

antagonist. In addition, increases in common carotid blood flow following vasopressin infusion were only seen when there had been decreases during the infusion. Common carotid vasodilatation was never seen during or after infusion of DDAVP (a V_2-receptor agonist).

Although we infused doses of vasopressin sufficiently low as to cause no change in mean blood pressure or heart rate, only decreases in common carotid blood flow were observed during the infusions. These observations do not lend support to the proposition that 'increased levels of vasopressin during hemorrhage and septic shock could favour the redistribution of blood from the periphery to the cerebral circulation and help to maintain cerebral blood flow' (Katusic *et al.*, 1984). Since Katusic & Krstic (1987) subsequently found that vasopressin caused endothelium-independent contractions of common carotid arteries from rats, they modified the earlier proposal (Katusic *et al.*, 1984) by suggesting that a greater contractile response of renal than of common carotid arteries to vasopressin would favour cerebral perfusion when circulating vasopressin levels were raised. However, even under conditions in which sufficient vasopressin has been infused to cause substantial changes in cardiovascular variables (Bennett *et al.*, 1988) we observed only a modest reduction in renal blood flow possibly due to concurrent activation of renal vasodilator mechanisms (Hofbauer *et al.*, 1983; Walker *et al.*, 1988). Moreover, in our previous study, although the changes in blood pressure and heart rate were similar to those seen with infusion of the higher dose of vasopressin in the present experiments, the increase in renal vascular resistance was only about half the increase in common carotid vascular resistance seen here.

While we know of no evidence from *in vivo* studies in conscious rats that increases in circulating levels of vasopressin favour cerebral perfusion, it is theoretically possible that monitoring common carotid blood flow could be misleading, since these vessels supply both intracranial and extracranial vascular beds. Thus, if vasopressin caused concurrent but directionally opposite, changes in internal and external carotid vascular resistances, for example, this peptide might favour brain perfusion, specifically. At first sight the finding that vasopressin causes constriction of basilar arteries of the rat (Hermsmeyer & Rusch, 1984; Webb *et al.*, 1987) is against this possibility. But it is feasible that differential changes in more distal resistances could augment brain perfusion, even in the face of constriction of the basilar artery, in a way analogous to changes in the proportional perfusion of cortical and medullary portions of the kidney accompanying renal arterial constriction. Indeed, Kozniewska & Skolasinska (1982) found that vasopressin could increase cerebral blood flow in anaesthetized rats. However, these investigators administered vasopressin as bolus doses (into the internal carotid artery) and measured cerebral blood flow (by ^{133}Xe clearance) between 3–4 min later, i.e., at a time when mean blood pressure had returned to control levels. Thus it is possible there had been reductions in cerebral blood flow during the pressor phase following vasopressin administration, and the cerebral vasodilatation observed by Kozniewska & Skolasinska (1982) could represent the same phenomenon as the common carotid vasodilatation, seen in the present study, following infusion of the lower dose of vasopressin, or during infusion of the higher dose of vasopressin in the presence of a V_1-receptor antagonist.

The serendipitous observation that significant vasodilatation following the end of the infusion of the lower dose of vasopressin only occurred on the same side as that showing significant vasoconstriction during the infusion could indicate that the vasodilator mechanism was triggered by the prior vasoconstriction, or was initially coincident with the latter, but more persistent. The marked common carotid hyperaemia and vasodilatation seen during and after infusion of the higher dose of vasopressin in the presence of a V_1-receptor antagonist means these phenomena were not dependent on continued V_1-receptor stimulation, but these observations do

not permit distinction between changes in cerebral metabolism (Kozniewska & Skolasinska, 1982) or persistence of V_1-receptor-triggered, or non-specific, vasodilator mechanisms as possible causes. While further analysis of these phenomena will require complex experimentation, it is clear from the present results that the most striking effects of vasopressin on common carotid blood flow were seen when its V_1-receptor actions were antagonized. A similar conclusion, but regarding the effects of vasopressin on total peripheral resistance, was reached by Walker (1986). He demonstrated that a systemic vasodilator effect of vasopressin was apparent even if changes in mean blood pressure were prevented by pretreatment with a V_1-receptor antagonist. Furthermore, Walker (1986) showed the vasodilatation was not influenced by inhibition of cyclo-oxygenase, although he did not exclude the possible involvement of V_2-receptors. From the present results, showing that the V_2-receptor agonist, DDAVP, did not cause common carotid vasodilatation, it seems unlikely that V_2-receptors mediated the vasodilator effects seen with infusion of the higher dose of vasopressin in the presence of V_1-receptor antagonism. Hence the latter effect seems to be different from the systemic V_2-receptor-mediated vasodilatation reported in conscious dogs (Liard, 1988).

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Reactive blue 2 selectively inhibits P_{2y}-purinoceptor-stimulated surfactant phospholipid secretion from rat isolated alveolar Type II cells

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1 The effect of reactive blue 2 on adenosine 5'-triphosphate (ATP), 12-O-tetradecanoylphorbol 13-acetate (TPA) and terbutaline-induced surfactant phospholipid secretion from rat isolated alveolar Type II cells was studied.

2 Reactive blue 2 significantly inhibited ATP-induced surfactant phospholipid secretion, but was without effect on C-kinase agonist (TPA) or β-adrenoceptor agonist (terbutaline)-stimulated surfactant phospholipid secretion. The IC_{50} for inhibition of ATP-induced surfactant secretion was 1.5×10^{-4} M.

3 These data are consistent with a P_{2y}-purinoceptor regulating surfactant phospholipid secretion from isolated Type II cells and support previous work suggesting reactive blue 2 is a specific inhibitor at P_{2y}-purinoceptors.

Introduction

Type II cells synthesize and secrete surfactant phospholipid in response to a variety of secretagogues (Hollingsworth & Gilfillan, 1984). Adenosine 5'-triphosphate (ATP) remains one of the most potent secretagogues for surfactant phospholipid secretion identified to date (Rice & Singleton, 1986; Gilfillan & Rooney, 1987). While the precise mechanism of ATP-induced surfactant phospholipid secretion is not known, previous work demonstrated calcium mobilization in association with binding of exogenous ATP to a receptor, and based on the rank order of agonist potency suggested a P_{2y}-subtype purinoceptor was responsible for mediating ATP-induced surfactant phospholipid secretion (Rice & Singleton, 1987). Burnstock & Kennedy (1985) had previously suggested the division of P_2- purinoceptors into P_{2x} and P_{2y} subtypes, based on rank order of agonist potency, and further work provided data suggesting reactive blue 2 was a specific antagonist of P_{2y}-purinoceptor-mediated responses (Burnstock & Warland, 1987; Houston *et al.*, 1987). Reactive blue 2, which does not affect responses mediated by P_{2x}-purinoceptors, has previously been proposed as an ATP antagonist (Kerr & Krantis, 1979; Choo, 1981). In the present work, to support further the hypothesis that ATP-induced surfactant phospholipid secretion is mediated via a P_{2y}-purinoceptor, we tested the effect of reactive blue 2 on ATP-induced surfactant phospholipid secretion compared to the effect of reactive blue 2 on C-kinase agonist 12-O-tetradecanoylphorbol 13-acetate (TPA)- and β-adrenoceptor agonist (terbutaline)-stimulated surfactant phospholipid secretion.

Methods

Animals

Pathogen-free male Sprague-Dawley rats (200–250 g) were obtained from Charles River (Wilmington, MA).

Isolation and culture of Type II cells

Type II cells were isolated from rat lungs by a modification of the method described by Dobbs *et al.* (1986). Rats were maintained under sterile guard hooded cages and allowed food and water *ad libitum* before the experiment. Rats were then anaesthetized with sodium pentobarbitone and lungs perfused via the pulmonary artery with buffer A (NaCl 125 mM, KCl 5 mM, Na_2HPO_4 2.5 mM, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES) 17 mM,

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glucose 1 mg ml^{-1}, gentamycin 10 μg ml^{-1}, penicillin 20 u ml^{-1}, pH 7.4) containing 3% bovine serum albumin (w/v). Lungs were then lavaged as previously described (Rice & Singleton, 1986) and incubated with elastase solution (40 ml, 0.4 units ml^{-1} in buffer A) for 20 min at 37°C (Dobbs *et al.*, 1986). Tissue was minced and filtered through progressively smaller Nitex gauze as described and washed in buffer A. Cells were then resuspended in Dulbecco's Modified Eagle's medium and incubated on 100 mm bacteriological plastic Petri dishes which had been precoated with 500 μg ml^{-1} IgG for 3 h at room temperature. Following a 1 h incubation at 37°C in 5% CO_2 in air, the plates were 'panned' as described previously (Dobbs *et al.*, 1986) and the unattached cells removed and collected by centrifugation. Isolated cells were resuspended in Dulbecco's Modified Eagle's medium (GIBCO, Grand Island, NY, U.S.A.) containing 10% foetal calf serum and utilized for experiments following 18 h in culture at 37°C in 5% CO_2. This procedure routinely yielded 40 × 10^6 cells per rat. For release experiments, cells were diluted in plating medium to 0.5 × 10^6 cells ml^{-1}. Cell suspension (1 ml) was placed in each well of a 24 well tissue culture plate which had been precoated with collagen (Collaborative Research, Lexington, MA, U.S.A.) and prelabelled by addition of 1 μCi of [^{3}H]-choline, (specific activity, 80.0 Ci mmol^{-1}). The plating efficiency at 18 h was generally 40–50%. Non-adherent cells were removed from the wells by washing before the assay. Cultures contained 89 ± 2% viable Type II cells as determined by fluorescence staining with phosphine 3R.

Secretion of phosphatidylcholine

Secretion of phosphatidylcholine (PC) by cultured Type II cells was determined as previously described (Rice & Singleton, 1986). Briefly, cells incubated overnight were washed 3 times with Dulbecco's Modified Eagle's medium containing 40 mM HEPES and 3 mg ml^{-1} bovine serum albumin, pH 7.4, 37°C (buffer B). Cells were allowed to equilibrate for 30 min and at the end of this time, agents were added and [^{3}H]-PC released was determined after 3 h. Medium was aspirated and the cells were washed with 0.5 ml of fresh medium. The two samples of medium were then combined and centrifuged at 9000 *g* for 5 min to pellet the cells. Supernatant was removed and lipid extracted according to Folch with addition of 1 mg of egg-PC as a carrier (Folch *et al.*, 1975).

Cells remaining in the wells were extracted with 1 ml ethanol × 2 and fractions obtained as for the medium. Samples containing lipid were dried overnight and radioactivity determined with a β-scintillation counter after addition of 5.0 ml of Scintiverse II to each sample. Dipalmitoyl [^{14}C]-phosphatidylcholine was used as an internal standard to follow recoveries of [^{3}H]-PC which were generally 95 ± 1%. The amount of [^{3}H]-PC secretion was calculated as the percentage of total [^{3}H]-PC present in the medium relative to the amount present in cells (i.e., c.p.m. in medium/(c.p.m. in medium + c.p.m. in cells)). The amount of [^{3}H]-PC released following the 30 min preincubation was subtracted for all samples.

Lactate dehydrogenase activity was determined in each sample as a measure of cytotoxicity by adding an aliquot (0.3 ml) to 0.5 ml of 250 μM 3-[N-morpholino]propane sulphonic acid, pH 7.0, and 0.1 ml of freshly prepared 10 mM NADH. The reaction was initiated by adding 0.1 ml of sodium pyruvate and the enzyme activity determined by following the decrease in absorbance at 340 nm. Total activity was determined by treating sample plates with 0.1% Triton X-100.

Statistical analyses

Kruskall-Wallis one-way analysis of variance by ranks (Kruskall & Wallis, 1952) or Friedman two-way analysis of variance by ranks (Friedman, 1937) for non-parametric data were utilized as appropriate. Analysis of variance for both parametric and non-parametric data was performed on an IBM PC XT microcomputer using commercially available statistical packages.

Materials

Methyl [^{3}H]-choline chloride and dipalmitoyl [^{14}C]-phosphatidylcholine were purchased from New England Nuclear (Boston, MA, U.S.A.). Chloroform, methanol and Scintiverse II were from Fisher Chemical Co. (Cincinnati, OH, U.S.A.). ATP, TPA and terbutaline were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Egg phosphatidylcholine (PC) was obtained from Avanti Polar Lipids (Birmingham, AL, U.S.A.). Elastase was obtained from Cooper Biomedicals (Malvern, PA, U.S.A.).

Results

ATP potently stimulated [^{3}H]-phosphatidylcholine secretion, as previously observed. This ATP-stimulated [^{3}H]-phosphatidylcholine secretion was inhibited by reactive blue 2 in a dose-dependent fashion (Figure 1). The IC_{50} (concentration producing 50% of the maximal inhibition) for inhibition of ATP-induced [^{3}H]-PC secretion by reactive blue 2 was ~1.5 × 10^{-4} M.

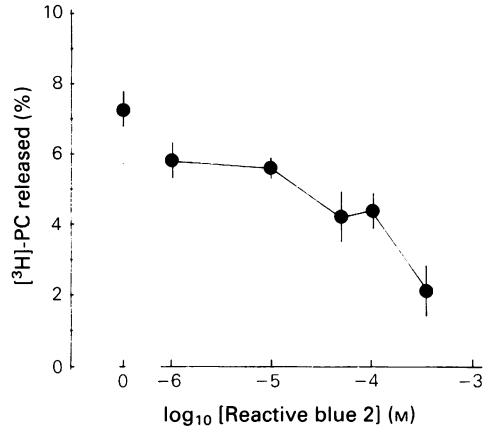

Figure 1 The effect of reactive blue 2 on ATP-induced [^{3}H]-phosphatidylcholine ([^{3}H]-PC) release. Release of [^{3}H]-PC was determined after a 3 h exposure of Type II cells to ATP (10 μM). This concentration of ATP was previously shown to give maximal stimulation of [^{3}H]-PC release (Rice & Singleton, 1986). Reactive blue 2 was present at the indicated concentrations for 30 min before the addition of ATP. Release of [^{3}H]-PC is expressed as stated in Methods. Data represent the mean (n = 6 experiments, 3 replicates in each) and vertical lines show the s.e.mean. Reactive blue 2 significantly inhibited ATP-induced [^{3}H]-PC release (P = 0.0001).

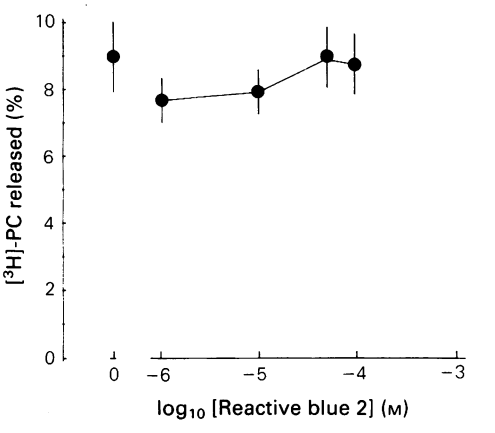

Figure 2 Effect of reactive blue 2 on 12-O-tetradecanoylphorbol 13-acetate (TPA)-induced [^{3}H]-phosphatidylcholine ([^{3}H]-PC) release. Release of [^{3}H]-PC was determined after a 3 h exposure of Type II cells to TPA (100 nM). This concentration of TPA was previously shown to give maximal stimulation of [^{3}H]-PC release (Dobbs & Mason, 1978). Release of [^{3}H]-PC is expressed as stated in Methods. Data represent the mean (n = 4 experiments, 3 replicates in each). Vertical lines show the s.e.mean. Reactive blue 2 did not have a significant effect on TPA-induced [^{3}H]-PC release at the concentrations tested.

To examine the specificity of the reactive blue 2 inhibition, we also tested reactive blue 2 for its ability to inhibit β-adrenoceptor agonist (terbutaline)-induced surfactant phospholipid secretion and 12-O-tetradecanoylphorbol 13-acetate (TPA)-induced surfactant secretion mediated by C-kinase. Reactive blue 2 had no significant effect on TPA-induced surfactant secretion (Figure 2) nor did it significantly inhibit terbutaline-induced surfactant secretion (Figure 3).

None of the agents utilized for these experiments produced a statistically significant release of lactate dehydrogenase above control levels which were 1–2% of total cellular lactate dehydrogenase released after a 3 h incubation.

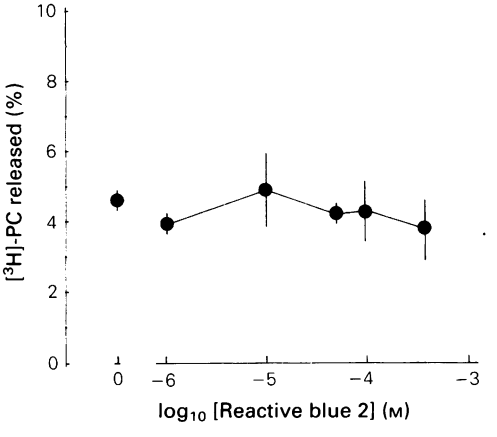

Figure 3 The effect of reactive blue 2 on terbutaline-induced [^{3}H]-phosphatidylcholine ([^{3}H]-PC) release. Release of [^{3}H]-PC was determined after a 3 h exposure of Type II cells to terbutaline (10 μM). This concentration of terbutaline was previously shown to give maximal stimulation of [^{3}H]-PC release (Dobbs & Mason, 1979). Reactive blue 2 was present at the indicated concentrations for 30 min before the addition of terbutaline. Data represent the mean (n = 4 experiments, 3 replicates in each). Vertical lines show s.e.mean. Reactive blue 2 had no significant effect on terbutaline-induced [^{3}H]-PC secretion at the concentrations tested.

Discussion

Exogenous ATP is a potent stimulus for surfactant phospholipid secretion from isolated alveolar Type II cells (Rice & Singleton, 1986; Gilfillan & Rooney, 1987). Previous work suggested the receptor on the Type II cell mediating this effect was a P_{2y}-purinoceptor based on rank order of agonist potency (Rice & Singleton, 1987). The present work supports this hypothesis.

Reactive blue 2 has been purported to be a specific inhibitor of P_{2y}-purinoceptors in rabbit and rat portal vein (Reilly *et al.*, 1987), rabbit mesenteric

artery (Burnstock & Warland, 1987), canine endothelium (Houston *et al.*, 1987) and rat caecum (Manzini *et al.*, 1986). The present work supports the specificity of reactive blue 2 at P_{2y}-purinoceptors, since reactive blue 2 potently inhibited surfactant phospholipid secretion induced by ATP, while reactive blue 2 did not have an effect on surfactant phospholipid secretion induced by the phorbol ester TPA or the β-agonist terbutaline which act via protein kinase C (Sano *et al.*, 1985) and cyclic AMP-dependent protein kinase (Rice *et al.*, 1985), respectively. The IC_{50} which we observed for reactive blue 2 inhibition of ATP-induced surfactant secretion was 1.5×10^{-4} M. This compares favourably with an IC_{50} of 4×10^{-4} M previously obtained for inhibition of junction potentials of the rat caecum by reactive blue 2 (Manzini *et al.*, 1986).

While inhibition of ATP-induced surfactant phospholipid secretion by reactive blue 2 could be the result of cytotoxicity, this does not appear probable for two reasons. Reactive blue 2 at similar concentrations had no effect on surfactant phospholipid secretion induced by other secretagogues (terbutaline and TPA) and release of a cytosolic marker (lactate dehydrogenase) was no different in control cells compared to cells exposed to various concentrations of reactive blue 2. The concentrations of reactive blue 2 utilized for the present study are also less than concentrations used in previous studies when cytotoxicity was also not noted (Manzini *et al.*, 1986; Reilly *et al.*, 1987; Burnstock & Warland, 1987).

In conclusion, we demonstrated that reactive blue 2 is a specific inhibitor of ATP-induced surfactant phospholipid secretion. Previous work supports the hypothesis that ATP-induced surfactant secretion is mediated via a P_{2y}-purinoceptor (Rice & Singleton, 1987), consistent with the present data. The present work also supports the previous observations of Burnstock and colleagues (Manzini *et al.*, 1986; Reilly *et al.*, 1987; Burnstock & Warland, 1987; Houston *et al.*, 1987) suggesting reactive blue 2 is a specific inhibitor of P_{2y}-purinoceptors. Further work to design a more potent antagonist of P_{2y}- purinoceptors based on the structure of reactive blue 2 may prove useful.

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Calcium mobilization and response recovery following P_2-purinoceptor stimulation of rat isolated alveolar type II cells

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1 The effect of adenosine 5'-triphosphate (ATP) on surfactant phospholipid secretion, calcium mobilization, and the time course for recovery of the response system was studied in isolated alveolar Type II cells of the rat.

2 ATP (10 μM) stimulated a biphasic intracellular Ca^{2+} transient monitored by changes in Fura-2 fluorescence, from a basal level of 126 $\pm$ 9 nM, to a rapid peak of 391 $\pm$ 1 nM, followed by a prolonged plateau 26 $\pm$ 4 nM above baseline (mean $\pm$ s.e.mean, $n = 26$).

3 ATP-stimulated surfactant phospholipid secretion and peak Ca^{2+} levels had similar EC_{50}s (1×10^{-6} M), and were unaffected by chelation of extracellular Ca^{2+}. However, the prolonged plateau phase was abolished by chelation of extracellular Ca^{2+}.

4 There was a 15 min refractory period before full recovery of the Ca^{2+}-response to ATP. Recovery was dependent on extracellular Ca^{2+}, was accelerated by removing extracellular agonist and was prolonged following stimulation with the poorly hydrolyzed ATP analogue, ATP-γ-S.

5 While the Type II cell was capable of multiple ATP-induced Ca^{2+} transients following recovery, no additional surfactant phospholipid was released with sequential stimulation.

6 These findings suggest initial exposure of Type II cells to ATP mobilizes intracellular Ca^{2+}, stimulates phospholipid secretion and rapidly desensitizes the cell to further stimulation by ATP. Recovery of the ATP-induced Ca^{2+}-response depends on presence of extracellular Ca^{2+} and removal of agonist.

Introduction

Exogenous adenosine 5'-triphosphate (ATP) interacts with P_2-purinoceptors to produce cellular responses in a variety of tissue types (Gordon, 1986). Exogenous ATP stimulates glycogenolysis in hepatocytes (Haussinger *et al.*, 1987), increases insulin secretion from the pancreas (Chapal & Loubatières-Mariani, 1981), augments amylase secretion from parotid acinar cells (Gallacher, 1982), and stimulates surfactant phospholipid secretion from alveolar Type II cells in primary culture (Rice & Singleton, 1986; Gilfillan & Rooney, 1988). P_2-purinoceptor stimulation generates second messengers from the breakdown of membrane phospholipids. In hepatocytes, stimulation of P_2-purinoceptors leads to hydrolysis of phosphatidylinositol 4,5-bisphosphate and generation of myoinositol 1,4,5-triphosphate (IP_3) (Prpić *et al.*, 1982; Charest *et al.*, 1985). IP_3 acts on non-mitochondrial stores to release Ca^{2+} (Dubyak, 1986), and elevation of intracellular Ca^{2+} is associated with secretion in many cell types including pancreatic islet cells and alveolar Type II cells (Zawalich *et al.*, 1983; Dobbs *et al.*, 1986b). P_2-purinoceptor stimulation of Type II alveolar cells leads to an elevation of intracellular Ca^{2+} in association with surfactant phospholipid release (Rice & Singleton, 1987). Further work with Type II cells demonstrated rapid densensitization of the Ca^{2+}-response upon restimulation with ATP (Dorn & Rice, 1988). Desensitization of agonist-induced response is common in biological systems, but the mechanism by which desensitization occurs in various cell types is diverse (Lefkowitz & Caron, 1986). Desensitization of the β-adrenoceptor-stimulated response in astrocytoma cells occurs by receptor sequestration (Toews *et al.*, 1984). In avian erythrocytes the response to β-

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adrenoceptor stimulation is modified by phosphorylation of the receptor and uncoupling of the GTP-dependent signalling protein (Sibley & Lefkowitz, 1985). In other cell types, desensitization is due to depletion of substrates necessary for the cellular response to agonist. Muscarinic stimulation of astrocytoma cells leads to depletion of an intracellular Ca^{2+} pool, desensitizing the cell until this calcium pool is refilled (McDonough *et al.*, 1988). Little is known about purinoceptor desensitization in any cell type, so the time course for response recovery in ATP-stimulated Type II cells and the factors which influence purinoceptor desensitization were investigated.

Methods

Isolation of Type II cells

Alveolar Type II cells were isolated from rat lungs by elastase digestion and selective plating on IgG as described previously (Dobbs *et al.*, 1986a). With this method, 30–40 $\times$ 10^6 Type II cells were routinely obtained per rat. Cell viability was approximately 90% as measured by trypan blue exclusion. Cell purity determined by phosphine 3R staining was $89 \pm 2\%$. Isolated cells were either used fresh or plated 18 h in Dulbecco's Modified Eagle medium (DMEM) with 10% foetal calf serum at 37°C in 5% CO_2.

Measurement of intracellular calcium

Freshly isolated or cultured (18 h) cells were suspended in medium (DMEM + 40 mM HEPES + 3 mg ml^{-1} bovine serum albumin with a final Ca^{2+} concentration of 1.4 mM) at a concentration of 15 $\times$ 10^6 cells ml^{-1} and incubated with Fura-2AM (5 μM in dimethylsulphoxide, DMSO) at 37°C for 10 min. Cells were washed with fresh medium and incubated for an additional 10 min at 37°C. To terminate Fura-2AM loading, cells were washed three times with medium (4°C) and stored on ice until use. With this protocol, cells were uniformly labelled with Fura-2, without evidence of organellar sequestration as determined by fluorescence microscopy. Calcium sensitive fluorescence was measured with a fluorometer (excitation = 339 nM, emission = 500 nm), equipped with a thermostatted (37°C), magnetic stirrer. For each run, the concentration of intracellular Ca^{2+} in Fura-2 loaded cells was calculated as previously described (Rice & Singleton, 1987). For some time courses, cells were placed in a bath at 37°C shielded from light. In long time courses, signal drift from dye leakage was accounted for and amounted to < 10% of incorporated dye.

Phospholipid secretion

Secretion of phosphatidylcholine (PC) from cultured Type II cells was determined by the methods described previously (Rice & Singleton, 1986). Briefly, freshly isolated cells were labelled by addition of 1 μCi [^{3}H]-choline (sp. act. 80.0 Ci mM^{-1}) and plated overnight at 37°C in 5% CO_2. Following 3 washes with fresh medium and a 30 min equilibration period, ATP was added to the plated cells. At 3 h, medium was aspirated and centrifuged to pellet loose cells. Lipid was extracted from the supernatant according to Folch *et al.* (1957). The remaining plated cells were extracted with methanol and the lipid fraction was obtained as for the medium. Lipid samples were dried overnight and radioactivity determined with a β scintillation counter after addition of Scintiverse II to the samples. The amount of [^{3}H]-PC secreted was calculated as the percentage of [^{3}H]-PC in the medium relative to the amount present in the cells and medium. The amount of [^{3}H]-PC released during the 30 min preincubation was subtracted from all samples.

Lactate dehydrogenase release

Lactate dehydrogenase (LDH) activity was determined as a measure of cytotoxicity by methods described previously (Rice *et al.*, 1984). Release of LDH from ATP-treated, freshly isolated, Fura loaded cells and plated cells (18 h) was not significantly different from control values (0–4% of total LDH present in cells).

Statistical analysis

Analysis of variance was performed on an IBM-PC using commercially available statistical packages. $P < 0.05$ was considered statistically significant. All experiments were performed in triplicate unless noted.

Materials

Pathogen-free male Sprague-Dawley rats (200–250 g) were obtained from Charles River Laboratories (Wilmington, MA, U.S.A.). Elastase (porcine pancreas) was purchased from Cooper Biomedicals (Malvern, PA, U.S.A.), Fura-2-acetoxymethyl ester (Fura-2 AM) from Molecular Probes (Junction City, OR, U.S.A), methyl [^{3}H]-choline chloride and [^{14}C]-dipalmitoyl phosphatidylcholine from New England Nuclear (Boston, MA, U.S.A.), egg phosphatidylcholine (PC) from Avanti Polar Lipids (Birmingham, AL, U.S.A.), adenosine 5'-O-(3-thiotriphosphate) (ATP-γ-S) from Boehringer-Mannheim Biochemicals (Indianapolis, IN, U.S.A.), and Dul-

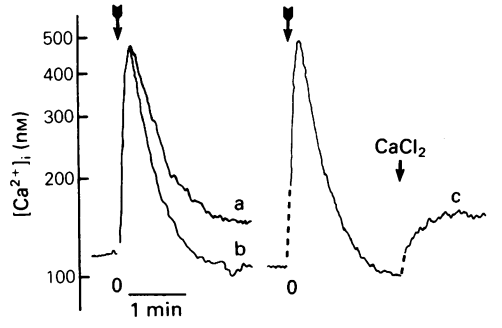

Figure 1 Effect of ATP on intracellular calcium levels in freshly isolated Type II cells. Type II cells were loaded with Fura 2 as described in Methods. ATP (1×10^{-5} M) was added at the arrow in the absence (a), or presence (b) of EGTA (2×10^{-3} M). (c) ATP treatment of cells in presence of EGTA at time zero, then at the time noted, $CaCl_2$ (2×10^{-3} M) was added. Intracellular calcium levels were determined as described in methods. The results presented are representative of 26 separate experiments.

becco's Modified Eagle Medium (DMEM) from Gibco (New York, NY, U.S.A.). Chloroform, methanol and Scintiverse II were purchased from Fisher Chemical (Cincinnati, OH, U.S.A.). IgG (rat), DNAse, adenosine 5′-triphosphate (ATP), 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES), ethylene glycol *bis* (β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA) and bovine serum albumin were obtained from Sigma (St. Louis, MO, U.S.A.).

Results

Treatment of freshly isolated Type II cells with ATP (10 μM) resulted in a rapid peak of intracellular Ca^{2+}, followed by a prolonged plateau phase (Figure 1a). Resting intracellular Ca^{2+} was 126 ± 9 nM while the peak Ca^{2+} was 391 ± 10 nM and the plateau was 26 ± 4 nM above baseline (mean ± s.e.mean, $n = 26$). Peak levels were unaffected by chelation of extracellular Ca^{2+} with EGTA, but the plateau phase was abolished (Figure 1b). In the absence of extracellular Ca^{2+}, the plateau phase could be reestablished with addition of $CaCl_2$ (2 mM) after the rapid phase transient (Figure 1c). These data suggest the peak phase represents Ca^{2+} mobilized from intracellular stores while the plateau phase is dependent on the influx of extracellular Ca^{2+}. Examination of Type II cells maintained in primary culture for 18–20 h also revealed a biphasic Ca^{2+}-response to ATP stimulation, but the magnitude and duration of both phases was diminished (not shown).

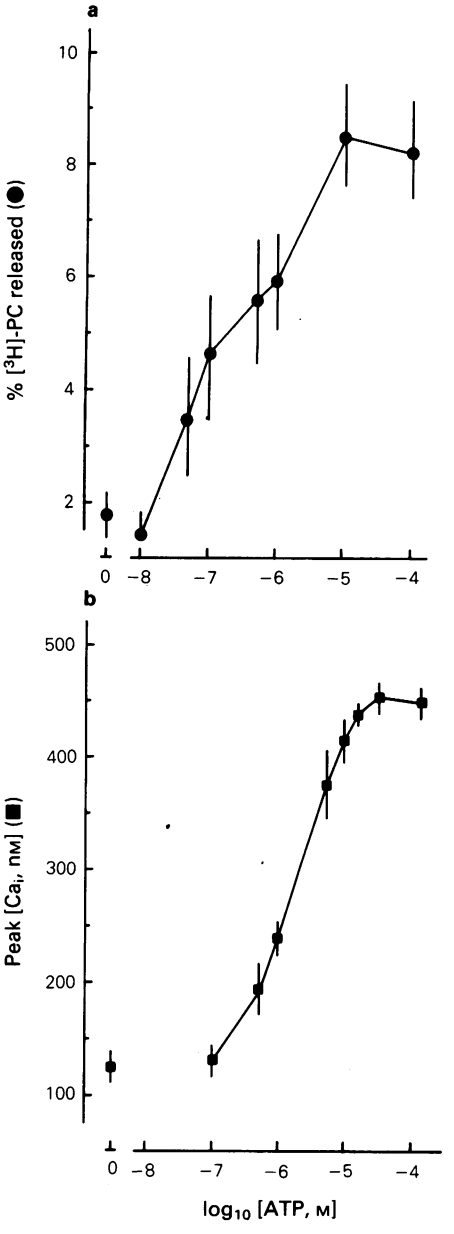

Figure 2 Dose-response for ATP-stimulated Ca^{2+} mobilization and [^{3}H]-phosphatidylcholine ([^{3}H]-PC) secretion: Type II cells were either incubated with [^{3}H]-choline (18 h) (a) or loaded with Fura 2 after isolation (b) after being suspended by gentle scraping with a rubber policeman. Cells were then exposed to various concentrations of ATP and Ca^{2+} mobilization or [^{3}H]-PC release were determined as described in Methods. Results represent the mean of 3 separate experiments done in triplicate and vertical lines show s.e.mean.

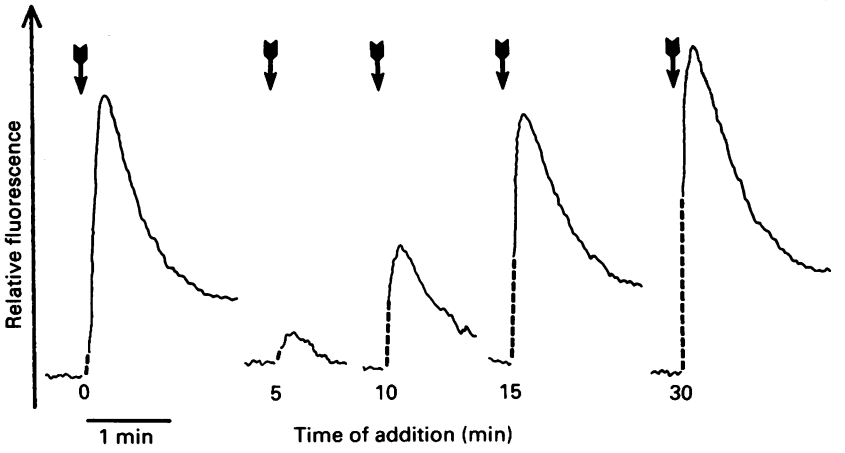

Figure 3 Recovery of ATP-stimulated calcium mobilization response: freshly isolated Type II cells were loaded with Fura 2 as noted in Methods then exposed to ATP (1×10^{-5} M) at time 0. At 5, 10, 15, or 30 min a second identical dose of ATP was added. The results shown are representative of 11 separate experiments.

Comparison of dose-response curves for ATP-induced Ca^{2+} mobilization and ATP-stimulated surfactant phospholipid secretion demonstrated similar EC_{50}s (1×10^{-6} M, Figure 2). To determine which phase of Ca^{2+} mobilization was associated with surfactant phospholipid release, EGTA (2 mM) was added to the incubation media 30 min before stimulation with ATP. Basal [^{3}H]-PC secretion ($1.2 \pm 0.2\%$ of total [^{3}H]-PC-labelled) was not significantly altered with the addition of EGTA ($2.2 \pm 0.7\%$). ATP significantly elevated [^{3}H]-PC secretion to $6.5 \pm 0.3\%$, and the addition of EGTA had no significant effect on ATP-stimulated [^{3}H]-PC secretion ($6.1 \pm 1.6\%$) (values represent mean $\pm$ s.e.mean of three experiments done in triplicate). These data argue against association of the plateau phase of Ca^{2+} mobilization with [^{3}H]-PC secretion.

Restimulation of freshly isolated cells following the initial Ca^{2+}-response revealed rapid desensitization of the purinoceptor system. There was

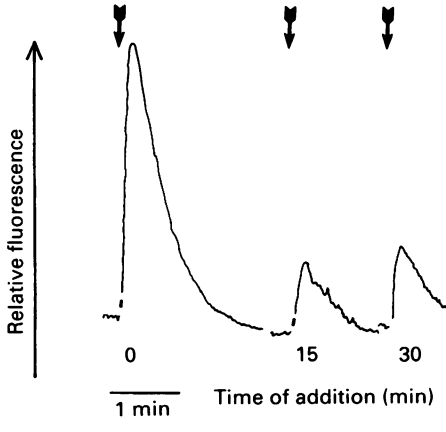

Figure 4 Effect of EGTA on response recovery at ATP-stimulated calcium mobilization: freshly isolated Fura 2 loaded Type II cells were treated with ATP (1×10^{-5} M) in the presence of 2 mM EGTA. At 15 or 30 min, a second identical ATP dose was added. Results shown are representative of 3 separate experiments.

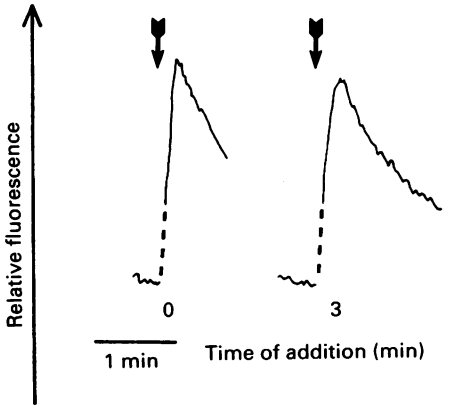

Figure 5 The effect of agonist removal on response recovery: Fura 2 loaded Type II cells were exposed to ATP (1×10^{-5} M) at time 0. During the plateau phase the cell suspension was centrifuged, at 9000 g × 30 s, supernatant medium containing ATP was removed and cells were resuspended in fresh medium. At 3 min, ATP (1×10^{-5} M) was again added. The results shown are representative of 3 separate experiments.

diminished Ca^{2+}-response to restimulation with ATP at 5 or 10 min after the first exposure (Figure 3). However, by 15 to 30 min following the initial exposure to ATP, the response system was fully recovered (Figure 3). Chelation of extracellular Ca^{2+} with EGTA suppressed recovery at least 30 min (Figure 4). These findings suggest complete recovery from ATP-induced desensitization requires extracellular Ca^{2+}.

To investigate the role of ATP in desensitization, we tested the effect of agonist removal on Ca^{2+}-dependent-response recovery. Following the first ATP-induced Ca^{2+} transient, cells were quickly washed to remove exogenous ATP and resuspended in fresh medium. Re-exposure to ATP 3 min after initial stimulation demonstrated prompt recovery of the Ca^{2+}-response (Figure 5). However, cells initially stimulated and resuspended in medium containing EGTA, demonstrated a blunted Ca^{2+}-response to restimulation with ATP (Figure 6). These findings suggest the presence of agonist desensitizes the Ca^{2+}-response in Type II cells, and removal of agonist results in complete recovery of the response if extracellular Ca^{2+} is present.

Exogenous ATP is removed *in vivo* by cell surface ectoATPases which hydrolyze ATP. Therefore, we tested the hypothesis that a poorly hydrolyzed agonist would prolong the densitization period. The ATP analogue, ATP-γ-S, stimulated a biphasic Ca^{2+}-response similar to ATP. However, in the continued presence of this poorly hydrolyzed agonist, cells were refractory to ATP simulation for more than 30 min (Figure 7). These data support the hypothesis that continued presence of agonist desensitizes the Type II cells.

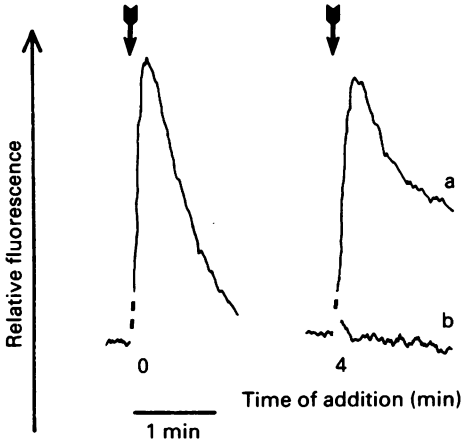

Figure 6 Effect of agonist removal and EGTA on response recovery: freshly isolated Fura 2 loaded Type II cells were exposed to ATP (1×10^{-5} M) in the presence of 2 mM EGTA at time 0. When the fluorescence signal had returned to baseline the cell suspension was centrifuged, supernatant removed, and cells were resuspended in fresh Ca^{2+}-containing medium (a), or fresh medium with 2 mM EGTA (b). At 4 min, a second ATP (1×10^{-5} M) dose was added. The results shown are representative of 3 separate experiments.

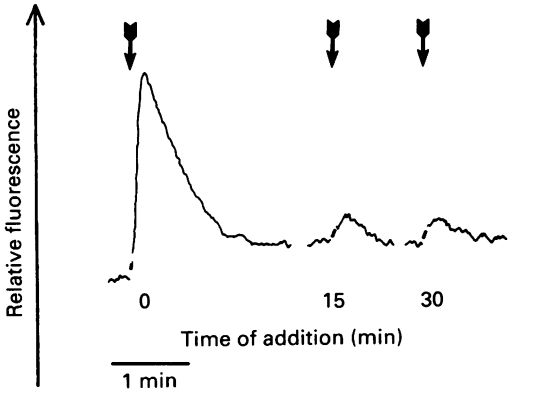

Figure 7 Calcium mobilization and response recovery, effect of adenosine 5'-O-(3-thiotriphosphate) (ATP-γ-S): Fura 2 loaded fresh Type II cells in medium containing Ca^{2+} were exposed to ATP-γ-S (1×10^{-5} M) at time 0. At 15 or 30 min following this initial exposure, the cells were exposed to ATP (1×10^{-5} M). The results shown are representative of 3 separate experiments.

Although ATP-induced Ca^{2+} mobilization was associated with surfactant phospholipid secretion and the Type II cell was capable of multiple peak Ca^{2+} transients, there was no significant difference in surfactant [^{3}H]-PC release following stimulation with a single 10 μM dose of ATP, five 10 μM pulses of ATP 30 min apart or a single 50 μM ATP dose (Table 1). This suggests an initial ATP-induced Ca^{2+} transient is associated with maximal surfactant phospholipid secretion, since repeated calcium transients are not associated with further surfactant phospholipid secretion.

Discussion

Extracellular ATP stimulated biphasic intracellular Ca^{2+} transients in freshly isolated and cultured alveolar Type II cells. The initial rapid phase was not dependent on extracellular Ca^{2+}, suggesting agonist-stimulated release of Ca^{2+} from intracellular stores. The plateau phase was dependent on extracellular Ca^{2+} levels. These findings are consistent

Table 1 Effect of multiple doses of ATP on [^{3}H]-phosphatidylcholine ([^{3}H]-PC) secretion

Agonist dosage	*% [^{3}H]-PC released*
Control	1.3 ± 0.5
10 μM ATP	4.6 ± 0.6
50 μM ATP	5.5 ± 0.9
10 μM ATP × 5 (30 min intervals)	5.9 ± 1.3

Values are means ± s.e.mean, $n = 4$.
Release of [^{3}H]-PC from prelabelled Type II cells was determined after exposure to 10 μM ATP, 50 μM ATP, or 5 doses of 10 μM ATP at 30 min intervals.
[^{3}H]-PC release was determined as described in Methods, 3 h after initial exposure to agonist.

with ATP-stimulated Ca^{2+} mobilization in smooth muscle cells and Ehrlich ascites tumor cells (Tawada *et al.*, 1987; Dubyak, 1986), where a peak and plateau phase was also noted. The reason for the diminished response of cultured (18 h) cells is unknown, but may represent biochemical changes since Type II cells are known to lose Type II cell morphology following isolation (Cott *et al.*, 1987; Dobbs *et al.*, 1988).

The link between agonist binding and Ca^{2+} release is thought to be myo-inositol 1,4,5 triphosphate (IP_3) created by receptor-mediated hydrolysis of membrane phosphatidyl inositol 4,5-bisphosphate (Williamson, 1986). ATP stimulation of hepatocytes and Ehrlich ascites tumour cells leads to cytosolic elevation of IP_3 (Charest *et al.*, 1985; Dubyak, 1986), and treatment of permeabilized cells with IP_3 mobilizes Ca^{2+} from intracellular stores (Joseph *et al.*, 1984; Dubyak, 1986). Although production of IP_3 continues in the presence of bound agonist (Ambler *et al.*, 1987; Nakahata & Harden, 1987), the transient nature of the Ca^{2+} peak results from the combined effect of Ca^{2+} extrusion by plasma membrane, Ca^{2+}, Mg^{2+}-dependent ATPase, uptake by other cytoplasmic constituents, and Ca^{2+} pool depletion (Putney, 1986; Merritt & Rink, 1987).

The plateau phase seen in Type II cells is dependent on extracellular Ca^{2+}. In Ca^{2+}-free medium, ATP stimulated a rapid Ca^{2+} transient which returned to basal levels within 90 s. In the continued presence of agonist, the plateau phase could be re-established by the addition of 2 mM Ca^{2+} to the medium. This is consistent with the Ca^{2+}-response in carbachol-treated parotid cells (Merritt & Rink, 1987). Moreover, Merritt & Rink (1987) found displacement of carbachol with atropine before the addition of Ca^{2+} prevented the reestablishment of the plateau phase. This suggests the plateau phase is not only dependent on extracellular Ca^{2+}, but also depends on receptor occupation by agonist. While it is generally accepted that the plateau phase depends on extracellular Ca^{2+}, the mechanism and route of Ca^{2+} influx remains in question. Studies with sea-urchin eggs and from T-lymphocytes suggest a direct influx of Ca^{2+} through transmembrane channels activated by IP_3 (Kuno & Gardner, 1987) and IP_4 (Irvine & Moor, 1986). Putney (1986) and Merritt & Rink (1987) have proposed a more direct pathway between the plasma membrane and endoplasmic reticulum for Ca^{2+} entry to the cytosol. Agonist-stimulated IP_3 production empties the endoplasmic reticulum Ca^{2+} pool. This drop in endoplasmic reticulum Ca^{2+} concentration opens the plasma membrane/endoplasmic reticulum pathway allowing influx of Ca^{2+} directly from the extracellular space to the endoplasmic reticulum, then cytosol, through the IP_3-activated Ca^{2+} channels (Putney, 1986; Merritt & Rink, 1987). This plateau phase of Ca^{2+} influx continues as long as agonist-stimulated IP_3 production continues. Regardless of pathway, the plateau phase in isolated Type II cells requires extracellular Ca^{2+} and receptor occupancy by agonist.

In ATP-stimulated Type II cells, we found a 15 min refractory period before the Ca^{2+}-response recovered fully. Chelation of extracellular Ca^{2+} suppressed recovery at least 30 min, suggesting that complete recovery from ATP-induced desensitization of the Ca^{2+}-response requires refilling of intracellular Ca^{2+} pools from an extracellular source. This process is similar to that described in smooth muscle (Den Hertog *et al.*, 1985).

The poorly hydrolyzed ATP analogue, ATP-γ-S, produced a refractory period greater than 30 min, suggesting receptor occupancy blocks response recovery. Consistent with this hypothesis, removal of extracellular ATP following the initial transient resulted in prompt response recovery. Similar experiments in the absence of extracellular Ca^{2+} demonstrated refilling of the Ca^{2+} pool occurred within 60 s after Ca^{2+} addition in the absence of agonist. These findings are similar to those in Ehrlich ascites tumour cells (Dubyak, 1986) and hepatocytes (Charest *et al.*, 1985). Charest *et al.* (1985) found an even more rapid recovery of Ca^{2+}-response in hepatocytes compared to Type II cells. Within 3 min, a second ATP dose produced an identical Ca^{2+} transient with less than 20% of the initial ATP remaining after 20 s. These findings suggest the rate limiting step to Ca^{2+}-response recovery is ATP hydrolysis. The difference between hepatocyte response recovery and Type II cell recovery may reflect differences in number of ectoATPases, the rate of ATP hydrolysis by the ATPases, or ATP receptors on each cell type.

The relationship between ATP stimulated Ca^{2+} mobilization and phospholipid secretion in isolated alveolar Type II cells was examined. We found a similar EC_{50} for intracellular Ca^{2+} mobilization and surfactant phospholipid secretion. This is consistent with the findings of Rice & Singleton (1987) where the order of potency among methylene, thio and imido substituted ATP analogues was similar for phospholipid release and Ca^{2+} mobilization in cultured Type II cells. These findings suggest Ca^{2+} mobilization is associated with ATP-stimulated surfactant phospholipid release. While the Type II cell was capable of multiple ATP-induced Ca^{2+} transients following purinoceptor recovery, there was no additional surfactant phospholipid release with sequential ATP stimulation. Extracellular Ca^{2+} depletion abolished the plateau phase of ATP-stimulated Ca^{2+} transients, but had no effect on rapid phase Ca^{2+} peak transients nor surfactant phospholipid secretion. These data suggest a single ATP-stimulated peak Ca^{2+} transient is associated with maximal surfactant phospholipid secretion from Type II cells and may reflect the presence of a preformed pool of lamellar bodies which must be regenerated prior to the next secretory stimulus. Alternatively, other steps in the signal pathway may be down-regulated following the initial secretory stimulus.

Although investigations with phorbol esters have shown elevation of cytoplasmic free Ca^{2+} is not a necessary event in surfactant phospholipid secretion (Sano *et al.*, 1985), a synergistic effect on secretion has been demonstrated between phorbol esters and A23187 which elevates intracellular Ca^{2+} in Type II cells (Dobbs *et al.*, 1986b). This suggests complementary pathways for surfactant phospholipid secretion in Type II cells. Evidence in other cell types suggests ATP may mobilize intracellular Ca^{2+} and activate protein kinase C. In hepatocytes, ATP stimulates hydrolysis of phosphatidyl inositol 4,5 bisphosphate (PIP_2) to generate IP_3 (Charest *et al.*, 1985). The other product of agonist-stimulated PIP_2 hydrolysis is diacylglycerol (DAG), a potent activator of protein kinase C (Berridge, 1987). Although it has yet to be demonstrated in Type II cells, the ATP-induced generation of both IP_3 and DAG may create the secretory synergism of Ca^{2+} mobilization and protein kinase C activation seen in previous studies of surfactant phospholipid release.

In summary, while the final common pathway for surfactant phospholipid secretion from Type II cells is not known, initial binding of extracellular ATP appears sufficient to mobilize intracellular Ca^{2+} and stimulate phospholipid secretion. Recovery of the P_2-purinoceptor-stimulated Ca^{2+} transient is dependent on agonist removal and presence of extracellular Ca^{2+}.

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Antagonism of Paf-induced oedema formation in rabbit skin: a comparison of different antagonists

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1 Eight platelet activating factor (Paf) antagonists were evaluated as inhibitors of oedema formation in rabbit skin induced by intradermal injection of Paf plus prostaglandin E_2 (PGE_2). Antagonists were tested by both intradermal (i.d.) and intravenous (i.v.) routes.

2 Intradermal injection of two antagonists structurally-related to Paf (SRI 63-675 and CV-3988) resulted in a partial inhibition of Paf-induced oedema formation but at high doses of antagonist, marked agonist activities were detected. CV-3988 administered i.v. inhibited Paf-induced plasma leakage by 73–80%; however, oedema responses to a range of other inflammatory mediators were also reduced, albeit to a lesser extent (40–60%). SRI 63-675 administered i.v. did not significantly inhibit Paf-induced oedema.

3 The antagonist 48740 RP administered either i.d. or i.v. showed partial, but selective, inhibition of Paf-induced oedema formation, although the doses required were high when compared with other antagonists.

4 BN 52021 was a weak Paf antagonist when injected i.d., but following i.v. administration the responses to Paf were inhibited by 63–71%. Responses to all other mediators tested were unaffected.

5 Kadsurenone and its synthetic derivatives, L-652,731 and L-659,989 all blocked responses to Paf in the skin. L-659,989 was the most potent, achieving almost total inhibition when injected i.d. and i.v.; moreover, it was selective for Paf. L-652,731 was more potent than kadsurenone.

6 WEB 2086 given i.d. and i.v. showed similar activity to L-659,989 and it was also selective for Paf-induced oedema formation.

7 These results illustrate that in rabbit skin not all Paf antagonists are selective for Paf, some showing agonist-like activity which can mask antagonist properties. It is suggested that before ascribing a role for endogenous Paf in an inflammatory reaction based on results with antagonists, the activity of the antagonists in the model under investigation should be rigorously established.

Introduction

Intradermal injection of platelet activating factor (Paf) in the rabbit and other species induces an increase in local microvascular permeability and synergism with vasodilators, such as prostaglandin E_2 (PGE_2), to produce marked oedema formation has been observed (Wedmore & Williams, 1981a; Humphrey *et al.*, 1982; Morley *et al.*, 1983; McGivern & Basran, 1984; Pirotzky *et al.*, 1984; Hellewell & Williams, 1986). Paf appears to act directly to induce permeability changes since its actions are not reduced in animals depleted of circulating neutrophils (Wedmore & Williams, 1981a) or platelets (Pirotzky *et al.*, 1984).

Since the initial description of CV-3988 as a Paf antagonist (Terashita *et al.*, 1983), a large number of structurally unrelated compounds have been reported as selective Paf antagonists (for reviews, see Braquet & Godfroid, 1986; Braquet *et al.*, 1987; Saunders & Handley, 1987). These have been characterized primarily on the basis of inhibition of Paf-induced platelet aggregation and may be grouped as follows: antagonists structurally-related to Paf; natural products and synthetic analogues; novel synthetic compounds; and products belonging to well-known pharmacological classes (Braquet &

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Godfroid, 1986). Based on the activities of Paf and the actions of these antagonists, Paf has been implicated as a contributory mediator in a wide range of models of inflammation (Braquet *et al.*, 1987). The mechanisms underlying inflammation are complex and it is likely that many mediators interact to produce the final tissue response. Therefore, it is important to establish the selectivity and efficacy of the antagonist in the model under investigation before ascribing a role to Paf in an inflammatory reaction. For example, we have previously reported that the Paf antagonist L-652,731 (Hwang *et al.*, 1985) reduced oedema formation in an Arthus reaction in rabbit skin (Hellewell & Williams, 1986), a reaction that is dependent on generation of the complement peptide C5a (Cochrane & Janoff, 1974; Jose *et al.*, 1983). However, to exclude the possibility that the antagonist was working via a mechanism other than Paf antagonism, we established that L-652,731 was selective for Paf in rabbit skin and did not antagonize C5a or modify blood flow.

In the present study, we have investigated the activities of eight structurally diverse Paf antagonists on oedema formation in rabbit skin induced by the intradermal injection (i.d.) of Paf. Examples of Paf-related compounds tested were CV-3988 (Terashita *et al.*, 1983) and SRI 63-675 (Handley *et al.*, 1987); of natural products were BN-52021 (Braquet *et al.*, 1985) and kadsurenone (Shen *et al.*, 1985), and synthetic derivatives L-652,731 (Hwang *et al.*, 1985) and L-659,989 (Ponpipom *et al.*, 1988); of synthetic compounds was 48740 RP (Sedivy *et al.*, 1985); and of pharmacological agents was WEB 2086 (Casals-Stenzel *et al.*, 1987). The antagonists were tested by both the i.d. and intravenous (i.v.) routes.

Methods

Animals

Male New Zealand White specific pathogen-free rabbits (2.5–3.5 kg) were purchased from Hacking and Churchill, Huntingdon, Cambridgeshire.

Solubilization of antagonists

For i.d. and i.v. injection, CV-3988 was dissolved in saline (62 mg ml^{-1} and 10 mg ml^{-1} respectively) and diluted appropriately. SRI 63-675 was dissolved in saline for both i.d. and i.v. injection (6.91 mg ml^{-1} and 10 mg ml^{-1} respectively). Similarly, 48740 RP was dissolved in saline at 28 mg ml^{-1} and 50 mg ml^{-1} for i.d. and i.v. administration, respectively. For i.d. injection, BN 52021 was dissolved at 84.88 mg ml^{-1} in dimethylsulphoxide (DMSO) and further diluted in saline; for i.v. administration, 10 mg of BN 52021 was solubilized in 1 ml 0.1 N NaOH and adjusted to pH 7.4 with 1 N HCl (approximately 0.1 ml). Kadsurenone, L-652,731 and L-659,989 were dissolved in DMSO at concentrations of 34 mg ml^{-1}, 40 mg ml^{-1} and 48 mg ml^{-1} respectively for i.d. injection; further dilutions were made in warmed (50°C) saline. Of these latter compounds, only L-659,989 was tested i.v. and it was dissolved in dimethylformamide at 50 mg ml^{-1}. Finally, WEB 2086 was dissolved in saline (4.56 mg ml^{-1}) for i.d. injection; for i.v. administration, 10 mg was solubilized in 0.3 ml 0.1 N HCl, neutralized with 0.23 ml 0.1 N NaOH and made up to 1 ml with saline.

For i.d. injection, the local concentration of DMSO did not exceed 0.5%. Above this concentration, non-specific effects of the vehicle were detected.

Measurement of local oedema formation in rabbit skin

Oedema formation was measured as the local accumulation of i.v. injected ^{125}I-human serum albumin into skin sites. Rabbits were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}), the fur on the dorsal skin shaved and 1.5 μCi kg^{-1} of ^{125}I-albumin mixed with a solution of Evans blue dye in sterile saline (10 mg dye kg^{-1}) was injected i.v. via a marginal ear vein. Agents under test were freshly prepared in sterile saline from stock solutions and 0.1 ml injected i.d. in six replicates per treatment according to a balanced site injection plan. After 30 min, a 10 ml cardiac blood sample was taken into heparin and plasma prepared. The animal was then killed with sodium pentobarbitone and the dorsal skin removed. Injection sites were punched out with a 17 mm diameter punch and counted together with three 1 ml plasma samples in a 12 head gamma counter (LKB, Wallac 1260 Multigamma). The amount of plasma protein that had accumulated in each skin site was calculated in terms of μl plasma by dividing the ^{125}I-count rate in each skin site by the ^{125}I-count rate in 1 μl plasma.

When Paf antagonists were tested i.d., they were mixed before injection at the appropriate concentrations with combinations of inflammatory agents under test. For testing of antagonists via the i.v. route, experiments were performed with pairs of animals, one rabbit receiving the antagonist, the other receiving an appropriate volume of vehicle. After 5 min pretreatment, each rabbit was injected i.d. with the same combination of inflammatory mediators and killed 30 min later and oedema formation assessed as described above.

Statistical analysis

Data are presented as the mean ± s.e.mean and have been analysed with Student's paired t test after i.d.

Table 1 Effect of local administration of eight Paf antagonists on Paf-induced oedema formation in rabbit skin

Antagonist	*Dose* (mol per site)	% *inhibition*	P
SRI 63-675	10^{-8}	45 ± 5 (5)	<0.001
CV-3988	10^{-7}	36 ± 4 (4)	<0.05
48740 RP	10^{-6}	57 ± 7 (4)	<0.01
BN 52021	10^{-7}	37 ± 6 (9)	<0.001
Kadsurenone	5×10^{-8}	51 ± 6 (5)	<0.02
L-652,731	5×10^{-8}	65 ± 6 (16)	<0.001
L-659,989	5×10^{-8}	90 ± 2 (5)	<0.0001
WEB 2086	10^{-7}	96 ± 2 (6)	<0.0001

Plasma exudation was induced by i.d. injection of a fixed dose of Paf (10^{-9} mol per site) mixed with prostaglandin E_2 (3×10^{-10} mol per site) and the antagonists at the doses shown. Number of rabbits given in parentheses.

administration of antagonists, and with Student's *t* test after i.v. administration of antagonists. A *P* value of <0.05 was considered statistically significant. The background response to PGE_2 alone has been subtracted from all data before calculating the percentage inhibition.

Drugs and materials

Prostaglandin E_2 (PGE_2), bradykinin, N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) and zymosan were purchased from Sigma Chemical Co., Poole, Dorset; histamine and Evans blue dye were from BDH Chemicals, Poole, Dorset; 1-O-hexadecyl-2-acetyl-*sn*-glycero-3-phosphorylcholine (Paf) was from Bachem, Saffron Walden, Essex; sodium pentobarbitone was from May and Baker, Dagenham, Essex; sterile 0.9% saline (Steriflex) was from the Boots Company, Nottingham, Nottinghamshire; ^{125}I-human serum albumin (50 μCi ml^{-1}, 20 mg albumin ml^{-1}) was from Amersham International plc, Amersham, Buckinghamshire. Leukotriene B_4 was a gift from Dr S.J. Foster, I.C.I., Pharmaceuticals Division, Macclesfield, Cheshire.

Zymosan-activated plasma (ZAP) was used as a source of C5a des Arg. Briefly, rabbit heparinized (10 u ml^{-1}) plasma was incubated with zymosan (5 mg ml^{-1}) for 30 min at 37°C followed by centrifugation, twice for 10 min at 3000 *g*, to remove the zymosan. Aliquots of ZAP were stored at −25°C.

The following Paf antagonists were obtained as gifts: CV-3988 (RS-2-methyl-3-(octadecylcarbamoyloxy)-propyl-2-(3-thiazolo)ethyl phosphate) from Dr K. Nishikawa, Takeda Chemical Industries Ltd, Osaka, Japan; SRI 63-675 (*cis*-(±)-1-[2-[hydroxy[[tetrahydro-2,5-dimethyl-5-[(ocyadecylaminocarbonyl)-oxy]methyl]furan-2-yl]methoxy-phosphinyloxy]ethyl]-quinolinium hydroxide, inner salt) from Dr D.A. Handley, Sandoz Research Institute, East Hanover, New Jersey, U.S.A.; 48740 RP (3-(3-pyridyl)-1H,3H-pyrollo[1,2-c]-thiazole-7-carboxamide) from Dr P. Sedivy, Rhône-Poulenc Santé, Vitry sur Seine, France; BN 52021 (3-t-butyl-hexahydro-4,7b,11-trihydroxy-8-methyl-9H-1,7a-epoxymethano-1H,6aH-cyclopenta-[c]furo[2,3-b]furo[3',2':3,4]cyclopenta[1,2-d]furan-5,9,12(4H)trione) from Dr P. Braquet, Institut Henri Beaufour, Le Plessis Robinson, France; kadsurenone (from Piper futokadsura), L-652,731 (*trans*-2,5-bis(3,4,5-trimethoxyphenyl)tetrahydrofuran) and L-659,989 (*trans*-2-(3-methoxy-5-methylsulphonyl-4-propoxyphenyl)-5-(3,4,5-trimethoxyphenyl)tetrahydrofuran) from Drs J.C. Chabala and T.Y. Shen, Merck Sharp & Dohme Research Laboratories, Rahway, New Jersey, U.S.A.; WEB 2086 (3-[4-(2-chlorophenyl)-9-methyl-6H-thieno(3,2f)(1,2,4)-triazolo-(4,3-a)(1,4)-diazepine-2-yl]-1-(4-morpholinyl)-1-propanone) from Dr H. Heuer, Boehringer Ingelheim KG, Ingelheim am Rhein, FRG.

Results

The effects of local administration of eight Paf antagonists on oedema formation induced by intradermal injection of Paf + PGE_2 in rabbit skin are summarized in Table 1. The doses of antagonists quoted were either the maximal doses that could be achieved because of limited solubility, or were the doses that gave maximal inhibition for each compound. Aspects of these data are presented in detail below.

SRI 63-675 and CV-3988

Figure 1 demonstrates the synergism between Paf and PGE_2 in producing oedema formation in rabbit skin (Wedmore & Williams, 1981a; Hellewell & Williams, 1986). Responses to either agent injected alone were only slightly above the background value obtained after injection of saline vehicle alone. However, when injected together there was marked oedema formation.

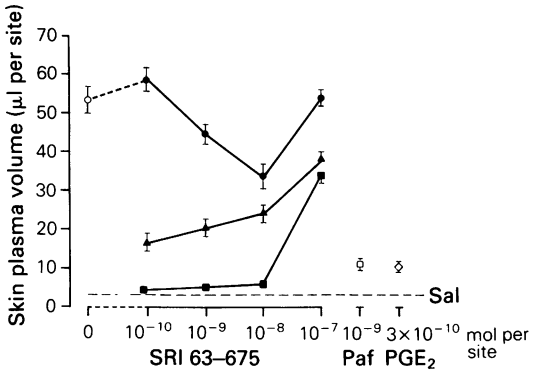

Figure 1 Effect of i.d. injection of SRI 63-675 on oedema formation in rabbit skin induced by i.d. injection of a fixed dose of Paf (10^{-9} mol per site) + prostaglandin E_2 (PGE_2) (3×10^{-10} mol per site). SRI 63-675 was injected i.d. at the doses shown. The symbols represent: oedema responses to Paf (□), PGE_2 (◇), Paf + PGE_2 (○); responses to Paf + PGE_2 + SRI 63-765 (●); responses to SRI 63-675 + PGE_2 (▲); responses to SRI 63-675 alone (■). The dashed line shows the control value obtained after i.d. injection of saline. Data represent 6 replicate injections.

Local injection of SRI 63-675 produced a dose-related inhibition of Paf-induced oedema formation up to 10^{-8} mol per site (Figure 1). At 10^{-7} mol per site SRI 63-675 however, no inhibition was observed. As the data illustrate in Figure 1, this reversal of inhibition can be explained by an intrinsic permeability-increasing activity of SRI 63-675. Thus, although responses to SRI 63-675 injected alone (10^{-10}–10^{-8} mol per site) were small, synergism with PGE_2 to produce marked oedema formation was observed. Since PGE_2 did not produce oedema formation, the implication was that SRI 63-675 possessed permeability-increasing activity which masked the full antagonist properties of the molecule. At 10^{-7} mol per site of SRI 63-675, addition of PGE_2 did not potentiate the oedema responses. This effect of a high dose of SRI 63-675 may be a detergent-like effect on skin cells and another Paf antagonist which virtually abolishes the actions of Paf (WEB 2086, 10^{-7} mol per site; see below), was unable to block the agonist-like activity of SRI 63-675 (data not shown). In view of the non-specific effects of SRI 63-675 when given i.d., which would limit its usefulness in investigating models of inflammation, its selectivity for Paf by this route was not investigated.

When tested i.v. up to $10\,mg\,kg^{-1}$, SRI 63-675 showed no significant inhibition of Paf-induced oedema formation. Similar observations have been made in the guinea-pig with this antagonist (D.A. Handley, personal communication) and related compounds (Deacon *et al.*, 1986). The reason for this lack of activity is unclear, but may relate to rapid inactivation.

When injected i.d., CV-3988 showed similar agonist-like activity to SRI 63-675 (data not shown); thus the percentage inhibition at 10^{-7} mol per site was the maximal inhibition obtained with this compound (Table 1). At higher doses, substantial plasma leakage was found with the antagonist alone and in combination with PGE_2. Therefore, we did not investigate the selectivity of CV-3988 given by this route. When injected i.v. at $10\,mg\,kg^{-1}$, CV-3988 produced an effective inhibition of oedema formation induced by intradermal injection of Paf + PGE_2 (Figure 2). In animals treated with CV-3988, responses to Paf at 10^{-9} and 10^{-10} mol per site were inhibited by 73 ± 6% ($P < 0.001$) and 80 ± 6% ($P < 0.005$) respectively ($n = 6$). However, CV-3988 inhibited responses to all other mediators tested by 40–60%. For example, the response to bradykinin (10^{-10} mol per site) + PGE_2 was reduced by

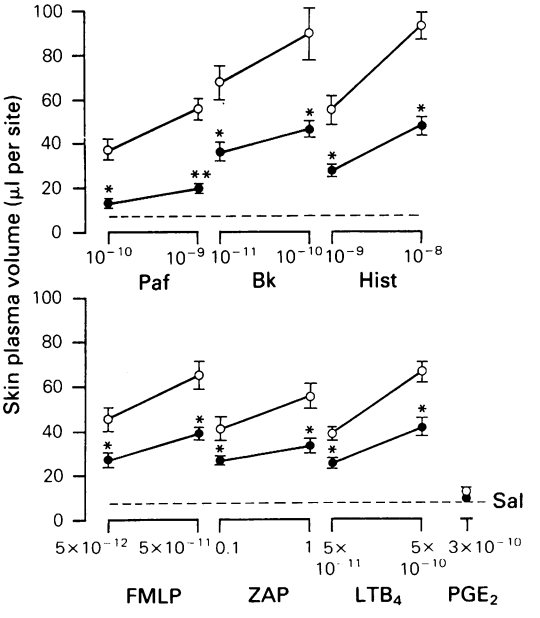

Figure 2 Effect of i.v. administration of CV-3988 on oedema responses in rabbit skin. Responses were obtained to the agents indicated, mixed with prostaglandin E_2 (PGE_2), in control rabbits (○) (saline, $1\,ml\,kg^{-1}$) and in rabbits treated with CV-3988 ($10\,mg\,kg^{-1}$) (●). Doses of agents are given in mol per site except for zymosan-activated plasma (ZAP) which is given as a dilution. PGE_2 was injected at 3×10^{-10} mol per site. Bk = bradykinin; Hist = histamine. The dashed line shows the control value obtained after i.d. injection of saline (Sal). Data represent 6 rabbits per group. $^*P < 0.05$, $^{**}P < 0.005$ compared to control.

47 ± 7% ($P < 0.01$). In addition, the oedema response to i.d. zymosan (300 μg per site; Wedmore & Williams, 1981b) was significantly ($P < 0.01$) reduced from 42 ± 7 μl to 16 ± 2 μl ($n = 6$).

48740 RP

We have previously observed that i.d. administration of 48740 RP antagonizes Paf-induced oedema formation in rabbit skin (Hellewell & Williams, 1985; 1987) and summary data from 4 experiments is shown in Table 1. Figure 3 shows the effect of i.v. injection of 48740 RP (50 mg kg^{-1}) on Paf-induced oedema formation. The response to 10^{-9} mol per site Paf was inhibited by 54 ± 7% ($n = 5$, $P < 0.02$). Even at the high dose of 48740 RP required to produce the partial suppression of Paf-induced oedema formation, responses to all other mediators were not significantly affected (Figure 3).

BN 52021

As shown in Table 1, local administration of BN 52021 antagonized Paf-induced oedema formation, yet it was weak, showing similar activity to CV-3988. The compound possessed no agonist-like activity (data not shown). When tested i.v. at 10 mg kg^{-1}, BN

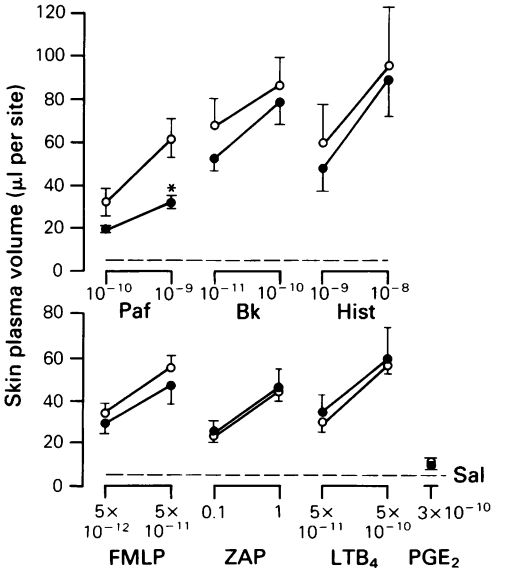

Figure 3 Effect of i.v. administration of 47840 RP on oedema responses in rabbit skin. Responses were obtained in control rabbits (○) (saline, 1 ml kg^{-1}) and in 48740 RP-treated rabbits (●) (50 mg kg^{-1}). See Figure 2 for details of doses of inflammatory agents. The dashed line shows the control value obtained after i.d. injection of saline (Sal). Data are from 4 rabbits per group. *$P < 0.02$.

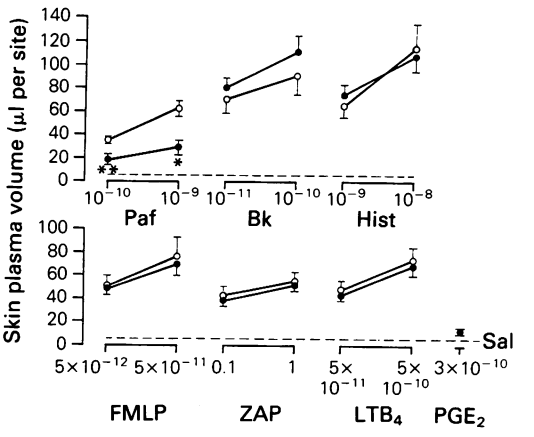

Figure 4 Effect of i.v. administration of BN 52021 on oedema responses in rabbit skin. Responses were obtained in control rabbits (○) (injected with 0.1 N NaOH neutralized with HCl, 1 ml kg^{-1}) and in BN 52021-treated rabbits (●) (10 mg kg^{-1}). See Figure 2 for details of doses of inflammatory agents. The dashed line shows the control value obtained after i.d. injection of saline (Sal). Results from 5 rabbits per group. *$P < 0.01$, **$P < 0.002$.

52021 showed selective inhibition of plasma leakage induced by Paf + PGE_2 (Figure 4). The degree of inhibition was incomplete, but substantial; responses to 10^{-9} mol and 10^{-10} mol Paf were reduced by 63 ± 9% ($P < 0.01$) and 71 ± 7% ($P < 0.002$) respectively ($n = 5$). In view of the unusual structure of BN 52021 (Braquet *et al.*, 1985) and its effectiveness when administered i.v., we wondered whether access of the compound to the venular endothelium (the site of plasma leakage) was restricted when given i.d. and this may have explained its weak activity. We therefore pretreated skin sites with BN 52021 (10^{-7} mol per site) before injecting Paf (10^{-9} mol per site) + PGE_2. In 3 rabbits, co-administration of BN 52021 with Paf inhibited the oedema response by 44 ± 8%, compared to 43 ± 12% after 5 min pretreatment.

Kadsurenone, L-652,731 and L-659,989

All 3 of these antagonists inhibited Paf-induced oedema formation (Table 1). L-652,731 was more potent than kadsurenone (approximately 10 times from dose-response curves, data not shown), and in turn, L-659,989 was 30–100 times more active than L-652,731, almost abolishing the effect of Paf (Figure 5). From the data in Figure 5, 50% inhibition of the response to Paf + PGE_2 was obtained with approximately 10^{-8} mol per site L-652,731 (similar to our previous observations; Hellewell & Williams, 1986) and with approximately 2×10^{-10} mol per site

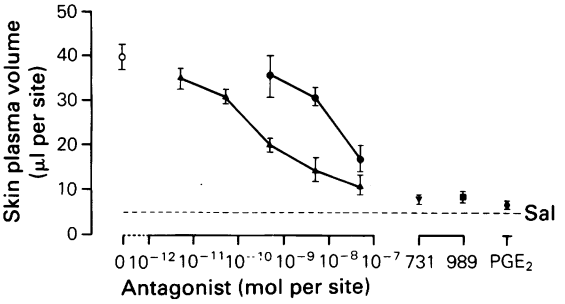

Figure 5 Comparison of the ability of L-652,731 and L-659,989 administered i.d. to inhibit oedema formation in rabbit skin induced by a fixed dose of Paf (10^{-9} mol per site) + prostaglandin E_2 (PGE_2) (3×10^{-10} mol per site). The symbols represent: oedema response to Paf + PGE_2 alone (○); responses to Paf + PGE_2 + L-652,731 (●); responses to Paf + PGE_2 + L-659,989 (▲); response to L-652,731 (5×10^{-8} mol per site) + PGE_2 (▼); response to L-659,989 (5×10^{-8} mol per site) + PGE_2 (■); response to PGE_2 (◆). The dashed line shows the control value obtained after i.d. injection of saline. Data from 6 replicate injections.

L-659,989. At the doses shown in Table 1, none of these antagonists displayed agonist activity; data for L-652,731 and L-659,989 are shown in Figure 5. Kadsurenone did not affect oedema responses induced by bradykinin (10^{-10} mol per site) + PGE_2 (data not shown), and we have published evidence

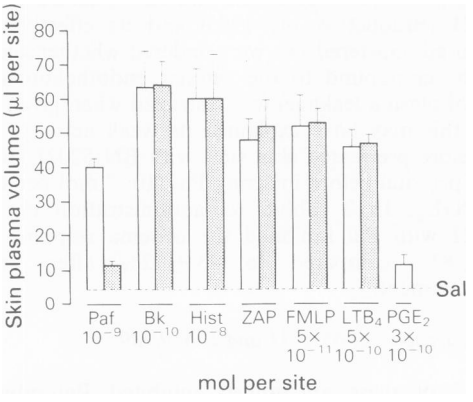

Figure 6 L-659,989 administered i.d. is selective for Paf-induced oedema formation in rabbit skin. Open columns represent responses to the agents indicated mixed with prostaglandin E_2 (PGE_2) (3×10^{-10} mol per site) and the stippled columns show the effect of adding L-659,989 (5×10^{-8} mol per site). Agents were used at the doses shown, except for zymosan-activated plasma (ZAP) which was used undiluted (0.1 ml per site). The dashed line shows the control value obtained after i.d. injection of saline (Sal). Data from 6 replicate injections.

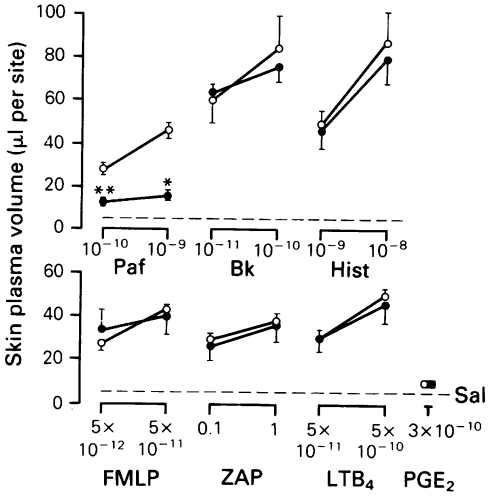

Figure 7 Effect of i.v. administration of L-659,989 on oedema responses in rabbit skin. Responses were obtained in control rabbits (○) (dimethylformamide vehicle, 0.1 ml kg^{-1}) and in L-659,989-treated rabbits (●) (5 mg kg^{-1}). See Figure 2 for details of doses of inflammatory agents. The dashed line shows the control value obtained after i.d. injection of saline (Sal). Data from 5 rabbits per group. $*P < 0.0001$, $**P < 0.001$.

elsewhere that L-652,731 is a selective Paf antagonist (Hellewell & Williams, 1986). Figures 6 and 7 demonstrate the selectivity of L-659,989 when given i.d. and i.v. respectively. In Figure 6, the oedema response to Paf + PGE_2 was inhibited by 96% (i.e. reduced almost to the leakage level seen with PGE_2 alone), while responses to all other mediators tested were unaffected. In Figure 7, the leakage responses to 10^{-9} and 10^{-10} mol Paf were inhibited by $83 \pm 2\%$ ($P < 0.0001$) and $82 \pm 6\%$ ($P < 0.001$)

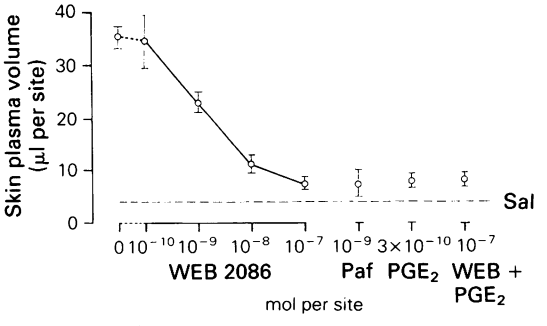

Figure 8 Effect of i.d. administration of WEB 2086 on oedema formation in rabbit skin induced by i.d. injection of a fixed dose of Paf (10^{-9} mol per site) + prostaglandin E_2 (PGE_2) (3×10^{-10} mol per site). The dashed line shows the control value obtained after i.d. injection of saline (Sal). Data from 4 rabbits.

respectively by L-659,989 (5 mg kg^{-1}, i.v., $n = 5$). When tested at a lower dose (2 mg kg^{-1}, i.v.), L-659, 989 inhibited the oedema response to Paf (10^{-9} mol per site) + PGE_2 by approximately 70% (2 rabbits) compared to PGE_2 alone.

WEB 2086

Local administration of WEB 2086 produced a dose-related inhibition of Paf-induced oedema formation (Figure 8), with 50% inhibition of the response obtained with approximately 10^{-9} mol per site. In addition, the compound displayed no agonist activity at 10^{-7} mol per site (Figure 8). Local and i.v. administered WEB 2086 displayed selectivity for Paf (Figures 9 and 10); at 10^{-7} mol per site WEB 2086, only the leakage response induced by Paf was inhibited (Figure 9), and similarly, at 10 mg kg^{-1} i.v. (Figure 10). At 10^{-9} and 10^{-10} mol per site Paf, responses were reduced by 91 ± 3% ($P < 0.002$) and 86 ± 7% ($P < 0.005$) respectively ($n = 5$).

Discussion

In this study we have tested eight reported Paf antagonists in the same *in vivo* model of Paf-induced oedema formation in rabbit skin. Each of the compounds displayed activity as an inhibitor of Paf-induced plasma leakage, but there was considerable variability in activity and selectivity. The compounds most problematic in terms of non-specific effects

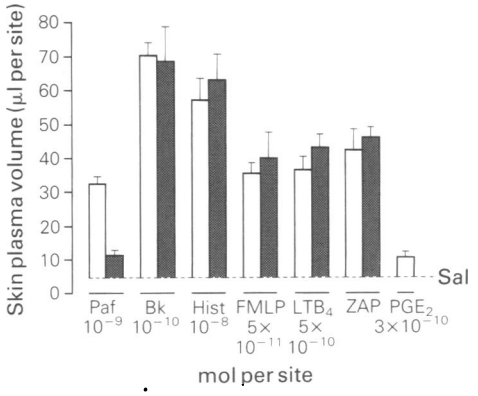

Figure 9 WEB 2086 administered i.d. is selective for Paf-induced oedema formation in rabbit skin. Open columns represent responses to the agents indicated mixed with prostaglandin E_2 (PGE_2, 3×10^{-10} mol per site) and the stippled columns show the effect of adding WEB 2086 (10^{-7} mol per site). Agents were used at the doses shown, except for zymosan-activated plasma (ZAP) which was used undiluted (0.1 ml per site). The dashed line shows the control value obtained after i.d. injection of saline (Sal). Data from 6 replicate injections.

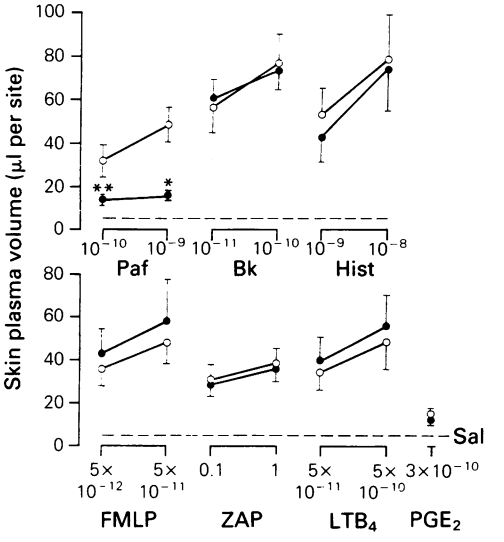

Figure 10 Effect of i.v. administration of WEB 2086 on oedema responses in rabbit skin. Responses were obtained in control rabbits (○) (vehicle, 1 ml kg^{-1}) and in WEB 2086 (10 mg kg^{-1}) treated rabbits (●). See Figure 2 for details of doses of inflammatory agents. The dashed line shows the control value obtained after i.d. injection of saline. Data from 5 rabbits per group. *$P < 0.002$, **$P < 0.005$.

were the lipid-like Paf-related antagonists, CV-3988 and SRI 63-675. When administered i.d., both compounds displayed antagonist properties but these were masked at higher doses by agonist-like activities. These agonist effects may result from non-specific lipid interactions with cells in the skin as indicated by the failure of WEB 2086 to inhibit the response (our unpublished data). Intravenous SRI 63-675 failed to antagonize the effects of i.d. Paf, but this may be related to rapid inactivation of the compound in the circulation. In guinea-pigs and primates, the half-life of SRI 63-675 after i.v. injection is approximately 9–12 min (Handley *et al.*, 1987) but the half-life in rabbits has not been reported. We allowed 5 min to elapse between i.v. injection of antagonist (10 mg kg^{-1}) and i.d. injection of Paf and this may have been sufficient time for inactivation to occur. CV-3988 injected i.v. produced effective inhibition of Paf-induced oedema formation, although this action was not selective for Paf; responses to all other agents tested also being inhibited, albeit to a lesser extent. One possible explanation for this non-selectivity is that this Paf-related compound lowers systemic blood pressure. When administered i.v. in rabbits Paf is known to cause rapid hypotension (Pinckard *et al.*, 1982) and a decrease in arterial pressure has been shown to be associated with decreased

oedema formation in skin in response to a variety of agents (Rampart & Williams, 1986). However, following an i.v. bolus injection of CV-3988 ($10\,mg\,kg^{-1}$) we found a transient (< 3 min) decrease in systemic blood pressure of approx. 30% in anaesthetized rabbits, and it is thus unlikely that this would explain the observations of non-selectivity. Agonist activity of CV-3988 (10^{-4} M) has been reported following its infusion into the pulmonary circulation of the rat (Voelkel *et al.*, 1986). CV-3988 has been used by other investigators to implicate Paf as a mediator of various types of inflammation in animal models, including the rabbit (Issekutz & Szpejda, 1986). We question the use of the compound for this purpose because of its non-selective action.

The antagonist 48740 RP displayed antagonism of Paf-induced oedema formation. Although $50\,mg\,kg^{-1}$ i.v. produced only a 54% inhibition of the response to Paf, the antagonist appeared to be selective at this dose. We did not try higher doses of 48740 RP in the rabbit and do not know if total inhibition of the Paf response could be obtained.

Of interest was the observation that BN 52021 was a moderately effective and selective inhibitor when injected i.v., despite producing weak inhibition of Paf-induced oedema when injected i.d. The reason for this may relate to restricted access of the antagonist to endothelial cell Paf receptors when injected i.d., although our pretreatment studies do not support this concept. BN 52021 has been tested in man for its ability to inhibit skin and platelet responses to Paf. At the maximal dose tested (120 mg orally), partial suppression of Paf-induced weal and flare and *ex vivo* platelet aggregation were observed (Chung *et al.*, 1987).

The *in vivo* potencies of kadsurenone, L-652,731 and L-659,989 compare favourably with potencies *in vitro* on inhibition of Paf binding to human and rabbit platelet membranes (Ponpipom *et al.*, 1988). We have previously observed that L-652,731 inhibited oedema formation in the Arthus reaction (Hellewell & Williams, 1986; 1987), but not oedema associated with a passive cutaneous anaphylactic reaction (Hellewell & Williams, 1987; Hellewell *et al.*, 1987). The former observation has also been shown by others using the same antagonist in the rat (Mandel *et al.*, 1988). We have also tested L-659,989 on both oedema formation and neutrophil accumulation in the Arthus reaction. Local administration of L-659,989 (5×10^{-8} mol per site) reduced oedema formation by approximately 60% and neutrophil accumulation was also inhibited, but to a lesser extent (Hellewell, 1989; Hellewell *et al.*, unpublished observations). We found similar results with i.v. administered L-659,989 ($5\,mg\,kg^{-1}$). In these earlier experiments, and those described in the present study, responses to C5a, which has been presumed to be a major mediator in the Arthus reaction, were unaffected by the antagonist. In addition, L-659,989 does not interfere with binding of C5a to its receptor on the neutrophil (Ponpipom *et al.*, 1988).

Finally, WEB 2086 also proved to be a potent Paf antagonist *in vivo* in the rabbit, showing similar activity to L-659,989. At 10^{-9} mol per site, Paf-induced oedema formation was inhibited by 50%, which is similar to observations made in rat skin (Casals-Stenzel *et al.*, 1987). This is 5 times the dose of L-659,989 required to cause a similar inhibition, and comparing Figures 5 and 8, it appears that at the lower doses of antagonist, L-659,989 was more potent than WEB 2086. WEB 2086 produced a slightly greater inhibition of Paf-induced oedema at the top doses of antagonist tested, but we were able to test WEB 2086 at a higher concentration than L-659,989. When injected i.v., WEB 2086 was more effective than L-659,989 but, again for reasons of limited solubility of L-659,989, we were able to test WEB 2086 at a higher dose. The dose of WEB 2086 required i.v. to inhibit Paf-induced responses in the rabbit skin is high when compared with that needed to block responses to i.v. Paf in the guinea-pig. For example, $10\,\mu g\,kg^{-1}$ i.v. was required to achieve total inhibition of plasma leakage in the airways induced by i.v. Paf in the guinea-pig (Evans *et al.*, 1988), although in the same species $1\,mg\,kg^{-1}$ i.v. was required to block the decrease in respiratory flow and hypotension observed following intratracheal instillation of Paf (Casals-Stenzel *et al.*, 1987). In addition, $20\,mg\,kg^{-1}\,h^{-1}$ i.v. of WEB 2086 (equivalent to $10\,mg\,kg^{-1}$ over 30 min as carried out in our experiments) was needed to attenuate the pulmonary changes induced by i.v. Paf and endotoxin in sheep (Purvis *et al.*, 1988). One possibility is that in the rabbit and sheep, WEB 2086 is inactivated more rapidly than in the guinea-pig and also, the antagonist may be more effective when Paf is administered by the same route.

The results of this study indicate that not all reported Paf antagonists behave as selective Paf antagonists *in vivo* in rabbit skin, and the same compound can show different potencies depending on the route of administration. Taken together, these observations suggest that before ascribing a role for endogenous Paf in mediating a particular inflammatory reaction *in vivo* based on the inhibitory properties of a given antagonist, it is important to establish that the antagonist is selective in that model.

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Characteristics of oedema formation induced by N-formyl-methionyl-leucyl-phenylalanine in rabbit skin

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1 The characteristics of N-formyl-methionyl-leucyl-phenylalanine (FMLP)-induced oedema formation were investigated *in vivo* in rabbit skin.

2 FMLP injected intradermally alone induced a small increase in plasma leakage, but marked synergism with prostaglandin E_2 (PGE_2) in producing oedema responses was observed. In the presence of PGE_2, FMLP was equiactive with C5a des Arg and 100–1000 times more active than histamine in terms of permeability-increasing activity. The response to FMLP was not dependent on endogenous histamine release.

3 FMLP-induced responses were of long duration ($t_{1/2} \sim 40$–50 min) when compared with bradykinin ($t_{1/2} \sim 4$–5 min).

4 The activity of a range of N-formyl peptides in increasing vascular permeability in skin correlated well with their activity as neutrophil stimulants *in vitro*.

5 Intravenous infusion of zymosan-activated plasma (ZAP) resulted in transient neutropenia and inhibition of oedema formation induced by FMLP and C5a des Arg in the skin. Responses to bradykinin were unaffected by the infusion of ZAP.

6 Intravenous injection of the non-steroidal antiinflammatory drug, ibuprofen, resulted in an inhibition of FMLP-induced, but not histamine-induced, oedema formation. This effect was independent of cyclo-oxygenase inhibition and the drug did not induced neutropenia.

7 Intravenous injection of the microtubule blocking agent colchicine inhibited FMLP-induced oedema formation. Responses to bradykinin were unaffected. When colchicine was administered after intradermal FMLP, subsequent plasma leakage was abolished.

8 The inference that receptors have evolved to bacterial secretions (i.e. FMLP) and products of the interaction of bacterial cell walls with tissue fluid (i.e. C5a des Arg), is consistent with the hypothesis that oedema formation is fundamentally a functional process concerned with regulating microbial lysis and opsonisation in an infected tissue.

Introduction

Peptides such as N-formyl-methionyl-leucyl-phenylalanine (FMLP) are powerful chemotactic substances for neutrophils (Schiffmann *et al.*, 1975; Showell *et al.*, 1976; Marasco *et al.*, 1984). Although originally described as prokaryotic in origin (Schiffmann *et al.*, 1975), chemotactic N-formyl peptides have also been reported to be released from damaged mitochondria of eukaryotic cells (Carp, 1982). In addition to being chemotactic *in vitro*, FMLP induces leukocyte accumulation following intradermal (i.d.) injection in skin (Issekutz, 1981; Colditz & Movat, 1984). Although weak in inducing oedema when injected alone into rabbit skin, FMLP is potent in inducing oedema in the presence of potentiating doses of prostaglandins (Wedmore & Williams, 1981). In hamster lung, FMLP-induced neutrophil accumulation was observed to be accom-

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panied by a small permeability change which it was suggested might be due to histamine release (Desai *et al.*, 1979). Furthermore, histamine release from human basophil-rich leukocytes was stimulated by N-formyl peptides (Hook *et al.*, 1976), but it is unclear whether endogenous histamine is released in rabbit skin in response to FMLP.

Unlike increased permeability induced by i.d. injection of mediators such as bradykinin and histamine, leakage responses induced by FMLP are dependent on circulating neutrophils. Thus, injection of FMLP induces a rapid interaction (detected within 6 min of injection) between circulating neutrophils and venular endothelium that leads to increased vascular permeability (Wedmore & Williams, 1981). The nature of this interaction remains to be determined, but it results in a protracted increase in permeability ($t_{1/2} \sim 50$ min) when compared with bradykinin and histamine ($t_{1/2} \sim$ 4–5 min) (Wedmore & Williams, 1981). In an attempt to investigate the underlying mechanisms, we used the microtubule blocking agent colchicine to determine whether functionally active neutrophils were required for the full duration of the permeability-increasing action of FMLP.

In the initial experiments of Wedmore & Williams (1981) which investigated the neutrophil dependency of oedema formation induced by FMLP, C5a and leukotriene B4 (LTB_4) in rabbit skin, nitrogen mustard was administered to rabbits 3 days before the experiment to induce neutropenia. In the current investigation we wished to establish techniques for inducing rapid inhibition of neutrophil-dependent oedema. In other studies, i.v. infusion of chemotactic factors (C5a or FMLP) or activators of the complement system *in vivo* in rabbits resulted in a profound but transient neutropenia (McCall *et al.*, 1974; O'Flaherty *et al.*, 1977). In the experiments described here, we infused zymosan-activated plasma (as a source of C5a des Arg) into rabbits, and measured oedema responses to FMLP before and after this treatment. Additionally, recent reports have indicated that the non-steroidal antiinflammatory compound ibuprofen can inhibit certain aspects of neutrophil function independent of cyclo-oxygenase inhibition (Maderazo *et al.*, 1984; Abramson *et al.*, 1985; Nielsen & Webster, 1987). Furthermore, ibuprofen has been shown to inhibit C5a-induced oedema formation *in vivo* (Rampart & Williams, 1986a). Thus, we also investigated the effects of this drug on oedema formation induced by FMLP. In further experiments we investigated the effects of intravenous colchicine on FMLP-induced oedema. With this drug, we were especially interested to determine whether inhibition could be obtained when it was administered after intradermal injection of FMLP.

Methods

Animals

Male New Zealand White specific pathogen-free rabbits (2.5–3.5 kg) were purchased from Hacking and Churchill, Huntingdon, Cambridgeshire.

Endotoxin determination

The amount of endotoxin in solutions for i.v. administration was determined by use of *Limulus* amoebocyte lysate and a chromogenic substrate (Coatest, Kabivitrum, Stockholm, Sweden). The limit of detection was approximately 0.01 ng endotoxin ml^{-1}.

Measurement of local oedema formation in rabbit skin

Oedema formation was measured as the local accumulation of i.v. injected ^{125}I-human serum albumin into skin sites as described (Hellewell & Williams, 1989). Agents under test were freshly prepared in sterile phosphate buffered saline (PBS) from stock solutions and 0.1 ml injected i.d. in six replicates per treatment according to a balanced site injection plan. After 30 min, a 10 ml cardiac blood sample was taken into heparin and the plasma prepared. The animal was then killed and plasma leakage determined as described by Hellewell & Williams (1989).

In the experiments designed to observe the effects of systemic administration of zymosan-activated plasma (ZAP), colchicine or ibuprofen on FMLP-induced oedema formation, the procedure was designed so that each animal served as its own control. Test agents (C5a des Arg + PGE_2, FMLP + PGE_2, bradykinin + PGE_2 or histamine + PGE_2) were injected i.d. after i.v. ^{125}I-albumin and Evans blue dye, and plasma protein accumulation was allowed to proceed for 30 min. ZAP, colchicine or ibuprofen was then injected i.v. and 5 min later the remaining skin sites were injected with the same combination of test agents. After a further 30 min, the animals were killed and local oedema formation assessed as described above. ZAP was filtered (0.22 μm, Millex) and infused i.v. at 6 ml min^{-1} for 30 s, then at 0.5 ml min^{-1} for the remainder of the experiment. Colchicine and ibuprofen were dissolved in sterile saline at 1 mg ml^{-1} and 20 mg ml^{-1} respectively and injected i.v. (1 ml kg^{-1}). Control animals received saline alone (1 ml kg^{-1}).

For total and differential leukocyte counts, samples of peripheral blood were taken from a central ear artery at intervals before and after systemic administration of compounds. Total leukocytes were determined in a Coulter counter (type ZBI) and May-Grunwald-Giemsa stain was used for differential counts on at least 200 cells.

Statistical analysis

Data are presented as the mean ± s.e.mean and have been analysed by Student's paired t test for within animal treatments and Student's t test for between animal comparison. A P value of <0.05 was considered statistically significant. Before calculating percentage inhibition of a response, the background value for i.d. injection of PGE_2 alone was subtracted from the data.

Drugs and materials

Prostaglandin E_2 (PGE_2) arachidonic acid (AA), bradykinin, zymosan, N-formyl-methionyl-leucyl-phenylalanine (FMLP), N-formyl-norleucyl-phenylalanine (FNLP), N-formyl-methionyl-phenylalanine (FMP) and N-formyl-methionine (FM) were purchased from the Sigma Chemical Co., Poole, Dorset; N-formyl-methionyl-methionyl-phenylalanine (FMMP) was from Miles Laboratories, Stoke Poges, Buckinghamshire; histamine and Evans blue dye were from BDH Chemicals, Poole, Dorset; sodium pentobarbitone was from May and Baker, Dagenham, Essex; ^{125}I-human serum albumin (50 μCi ml^{-1}, 20 mg albumin ml^{-1}) was from Amersham International plc, Amersham, Buckinghamshire; sterile 0.9% saline (Steriflex) was from the Boots Company, Nottingham, Nottinghamshire, U.K.; sterile phosphate buffered saline (PBS) was from the Clinical Research Centre media department. Zymosan-activated plasma (ZAP) was used as a source of C5a des Arg (Hellewell & Williams, 1989).

The following were gifts; purified rabbit C5a, in its physiologically more stable form C5a des Arg, was from Dr P.J. Jose in our laboratory (Williams & Jose, 1981; Jose *et al.*, 1983); N-formyl-methionyl-leucyl-phenylalanyl-phenylalanine (FMLPP) was from Dr R.J. Freer, Medical College of Virginia, Richmond, Virginia, U.S.A.; sodium ibuprofen dihydrate was from Dr I.M. Hunneyball, the Boots Company; mepyramine maleate was from May and Baker Ltd.

Results

Characteristics of FMLP-induced oedema

Figure 1 shows that i.d. injection of FMLP alone induced a small but significant increase in leakage of ^{125}I-albumin over a 30 min period when compared with sites injected with PBS. Similarly, injection of histamine alone induced a small increase in ^{125}I-albumin leakage. However, as Figure 1 also illustrates, when the vasodilator PGE_2 (3×10^{-10} mol) was mixed with either FMLP or histamine before i.d. injection, a potentiation of the response was observed. The potentiation occurs as a result of synergism between increased microvascular permeability, induced by FMLP or histamine, and the increased microvascular blood flow induced by PGE_2. Injection of PGE_2 alone induced little oedema formation. When tested in the presence of PGE_2, FMLP was 100–1000 times more active than histamine in increasing microvascular permeability (Figure 1) although, FMLP was equiactive with purified rabbit C5a des Arg (data not shown).

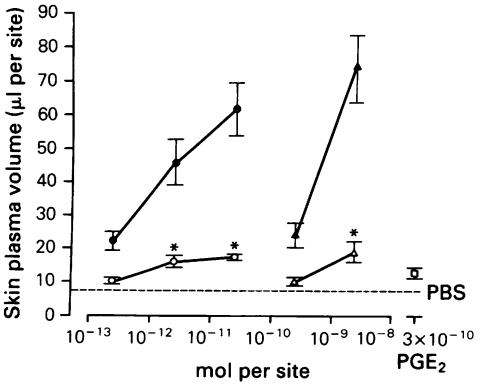

Figure 1 Oedema responses in rabbit skin induced by i.d. injection of FMLP and histamine, showing synergism with prostaglandin E_2 (PGE_2). The symbols represent oedema responses to FMLP alone (○); responses to FMLP + PGE_2 (3×10^{-10} mol) (●); responses to histamine alone (△); responses to histamine + PGE_2 (▲). The dashed line shows the control value obtained after i.d. injection of PBS. Data from 6 rabbits, $^*P < 0.05$ with respect to PBS controls.

In some experiments we measured the accumulation of i.v. injected ^{125}I-albumin over a 4 h period and found that FMLP elicited responses which were significantly greater than those measured over 30 min. In 6 rabbits, responses to i.d. injection of FMLP (5×10^{-11} mol) were 6 ± 2 μl at 30 min and 17 ± 3 μl at 4 h ($P < 0.05$; results subtracted for PBS control value). Neither of these responses was affected by the local administration of indomethacin (10^{-8} mol, a dose previously demonstrated to inhibit the cyclo-oxygenase in rabbit skin; Williams & Peck, 1977); corresponding leakage values were 5 ± 2 μl and 18 ± 3 μl respectively.

Permeability responses to FMLP were independent of histamine release. In 6 rabbits, oedema responses to injection of a mixture of histamine (2.5×10^{-8} mol) + PGE_2 (3×10^{-10} mol) were suppressed by $84 \pm 3\%$ after the local administration of 3×10^{-9} mol mepyramine ($P < 0.005$). The suscepti-

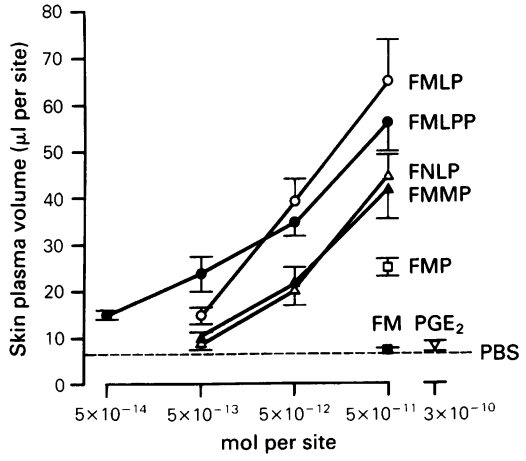

Figure 2 Oedema responses in rabbit skin induced by i.d. injection of a range of N-formyl peptides. All peptides were injected with PGE_2 (3×10^{-10} mol). The dashed line shows the control value obtained after i.d. injection of PBS. Data from 6 replicate injections. For abbreviations, see text.

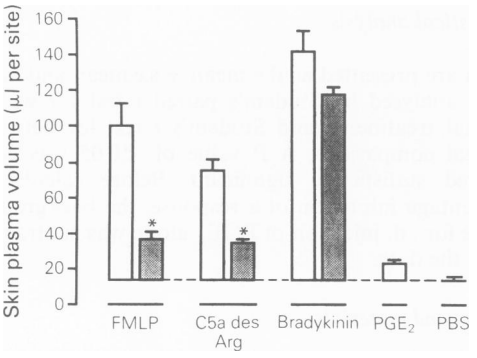

Figure 3 Effect of i.v. infusion of zymosan-activated plasma (ZAP) on oedema responses in rabbit skin induced by FMLP + prostaglandin E_2 (PGE_2), C5a des Arg + PGE_2 and bradykinin + PGE_2. The open columns represent responses obtained before, and stippled columns represent responses after infusion of ZAP. FMLP and C5a were injected at 5×10^{-11} mol, bradykinin at 10^{-10} mol and PGE_2 at 3×10^{-10} mol. The dashed line shows the control value after i.d. injection of PBS. Data from 6 replicate injections, $^*P < 0.05$, compared with control.

bility of oedema responses induced by FMLP (2.5×10^{-11} mol) + PGE_2 to inhibition by local administration of mepyramine showed some inter-animal variation; however, in the same group of animals described above, the effect of mepyramine was minimal; $18 \pm 6\%$ suppression ($P > 0.05$).

Figure 2 shows the results of an experiment in which we compared the ability of six formyl-peptides to increase microvascular permeability in rabbit skin. In the presence of PGE_2 (3×10^{-10} mol), the peptide most potent in increasing permeability was FMLPP; significant oedema responses ($P < 0.05$) being obtained with 5×10^{-14} mol. At higher doses, FMLPP showed similar activity to FMLP. The analogues FNLP and FMMP were approximately 10 times less active than FMLP, while FMP was considerably weaker (Figure 2). We were unable to detect any permeability-increasing activity of FM, i.e. the response to i.d. injection of FM + PGE_2 was no different to that obtained with PGE_2 alone.

Effect of intravenous infusion of zymosan-activated plasma

Figure 3 shows the effect of infusion of ZAP on oedema responses to FMLP + PGE_2, C5a des Arg + PGE_2 and bradykinin + PGE_2. Responses to FMLP and C5a des Arg were reduced by 81% and 78% respectively, while the leakage to bradykinin was only partially reduced (20% suppression) (Figure 3). When the same experiment was repeated in 3 rabbits, the inhibitions were $84 \pm 2\%$, $82 \pm 4\%$ and $15 \pm 7\%$ for FMLP, C5a des Arg and bradykinin respectively. Thus, infusion of ZAP resulted in a selective suppression of neutrophil-dependent oedema responses. This is probably due to a transient neutropenia and neutrophil sequestration in vascular beds induced by the infusion of ZAP. Circulating white cell numbers ($\times 10^6$ ml^{-1}, $n = 3$) before ZAP were 6.6 ± 0.9, 5 min after initiation of ZAP infusion 3.2 ± 0.6 ($P < 0.05$) and 35 min after ZAP (i.e. the end of the experiment) were 4.6 ± 0.7 ($P < 0.05$). Corresponding neutrophil numbers ($\times 10^6$ ml^{-1}) were 2.3 ± 0.4, 0.1 ± 0.04 ($P < 0.05$) and 0.8 ± 0.3 ($P < 0.05$) respectively.

Suppression of FMLP-induced oedema formation by ibuprofen

The effects of i.v. administration of either saline (1 ml kg^{-1}) or ibuprofen (20 mg kg^{-1}) on oedema responses to FMLP + PGE_2, histamine + PGE_2, FMLP + arachidonic acid (AA) and histamine + AA are shown in Figure 4.

Figure 4a shows that administration of saline had no significant effect on oedema responses to all combinations of agents tested (i.e. responses over 35–65 min were the same as those measured 0–65 min). In contrast, Figure 4b shows that systemic treatment with ibuprofen resulted in significant suppression of leakage responses to FMLP and histamine when they were potentiated by AA, due to inhibition of cyclo-oxygenase and reduction of vasodilator prosta-

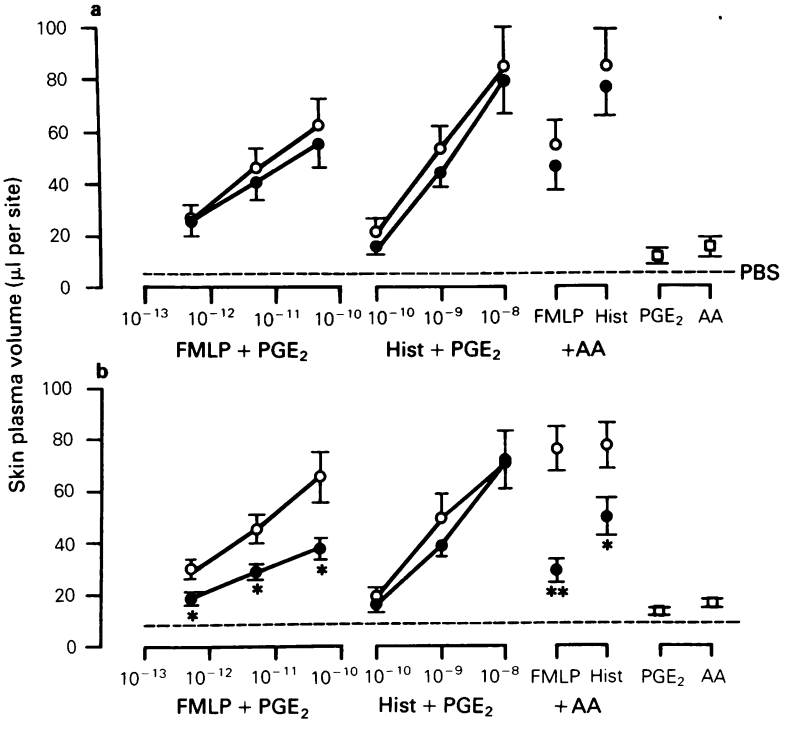

Figure 4 Oedema responses in rabbit skin induced by i.d. injection of mixtures of FMLP + prostaglandin E_2 (PGE_2), histamine (Hist) + PGE_2, FMLP + arachidonic acid (AA) and histamine + AA to show the cyclo-oxygenase-dependent and independent action of ibuprofen. In (a) responses before (○) and after (●) i.v. saline (1 ml kg^{-1}) and in (b) responses before (○) and after (●) i.v. ibuprofen (20 mg kg^{-1}). When injected with PGE_2 (3×10^{-10} mol), FMLP and histamine were tested at the doses shown. When injected with AA (3×10^{-9} mol), FMLP and histamine were injected at their top doses, i.e. 5×10^{-11} mol and 10^{-8} mol respectively. The dashed line shows the control value obtained after i.d. injection of PBS. Data from 6 rabbits, $*P < 0.05$, $**P < 0.01$, compared with controls.

glandin production. However, when leakage responses to FMLP and histamine were potentiated by the simultaneous injection of PGE_2 to override cyclo-oxygenase inhibition, only the oedema induced by FMLP + PGE_2 was significantly suppressed by the systemic administration of ibuprofen. Responses to histamine + PGE_2 were unaffected by ibuprofen treatment.

The level of endotoxin in the ibuprofen solution (20 mg ml^{-1}) was 0.8 ng ml^{-1} which we considered to be an insufficient amount to account for the observed effects of the drug on oedema responses to FMLP + PGE_2 (Haslett *et al.*, 1987). Total leukocyte numbers before, 5 and 35 min after ibuprofen were 5.6 ± 1.4, 4.9 ± 1.3 and $5.5 \pm 1.3 \times 10^6$ cells ml^{-1}, respectively. Neutrophil counts at the same time were 2.0 ± 0.5, 1.9 ± 0.4 and $2.1 \pm 0.5 \times 10^6$ cells ml^{-1} respectively ($n = 6$).

Effect of colchicine on FMLP-induced oedema formation

In earlier experiments designed to investigate the neutrophil dependency of oedema formation induced by FMLP, C5a and LTB_4, nitrogen mustard was administered to rabbits 3 days before the experiment (Wedmore & Williams, 1981). In the current investigation we wished to establish a technique for inducing rapid inhibition of neutrophil-dependent oedema.

Figure 5 shows the effect of i.v. administration of colchicine (1 mg kg^{-1}) on oedema formation induced by mixture of FMLP + PGE_2 and bradykinin + PGE_2 in rabbit skin. Internally-controlled experiments were performed in 4 rabbits as described in the previous section. Responses to FMLP + PGE_2 were virtually abolished in the presence of colchicine,

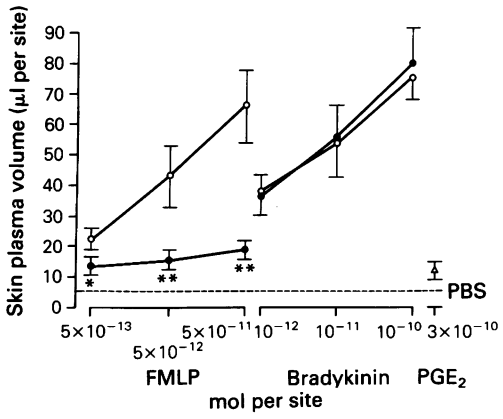

Figure 5 Oedema responses in rabbit skin induced by i.d. injection of mixtures of FMLP + prostaglandin E_2 (PGE_2) and bradykinin + PGE_2 to show the selective effect of i.v. colchicine. The symbols represent: responses before colchicine (○); responses after i.v. cochicine (1 mg kg^{-1}) (●), FMLP and bradykinin were injected at the doses shown mixed with PGE_2 (3×10^{-10}) mol. The dashed line shows the control value obtained after i.d. injection of PBS. Data from 4 rabbits. $*P < 0.05$, $**P < 0.02$ compared with controls.

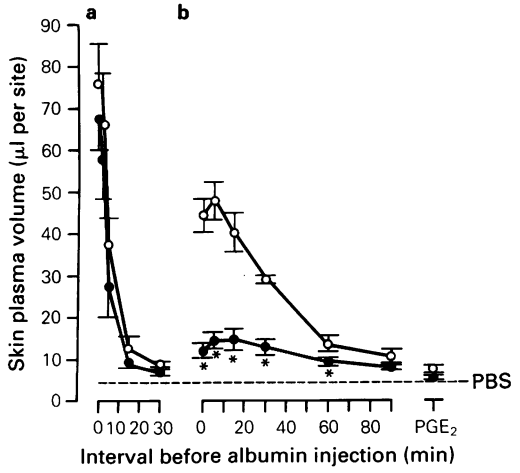

Figure 6 Effect of colchicine on the duration of action of (a) bradykinin and (b) FMLP in rabbit skin. FMLP (5×10^{-10} mol) and bradykinin (10^{-10} mol) were injected i.d. at various intervals before an i.v. injection of ^{125}I-albumin and Evans blue dye and either saline (1 ml kg^{-1}) or colchicine (1 mg kg^{-1}) at $t = 0$. PGE_2 (3×10^{-10} mol) was injected into all skin sites and oedema formation assessed after 30 min. In (a) responses to bradykinin and in (b) responses to FMLP in saline- (○) and colchicine-treated rabbits (●). The dashed line shows the control value obtained after i.d. injection of PBS. Data from 6 rabbits, $*P < 0.05$ with respect to saline-treated rabbits.

while those to bradykinin + PGE_2 were unaffected. The amount of endotoxin in the colchicine solution (1 mg ml^{-1}) was 0.05 ng ml^{-1}. In the control animals which received 1 ml kg^{-1} saline i.v., oedema responses to FMLP + PGE_2 and bradykinin + PGE_2 were unaffected, the results (not shown) being similar to those shown in Figure 4a.

Circulating leukocyte counts before, 10 and 40 min after colchicine were 5.9 ± 0.9, 4.8 ± 0.6 and $3.8 \pm 0.6 \times 10^6$ cells ml^{-1}. Neutrophil counts at the same time points were 2.8 ± 0.8, 1.2 ± 0.3 ($P < 0.05$) and 0.7 ± 0.2 ($P < 0.05$) $\times 10^6$ cells ml^{-1} respectively. Thus, injection of colchicine resulted in a slow decline in both total number of leukocytes and the number of neutrophils. The mechanism for this is unknown but may relate to sequestration in vascular beds such as the lung (Flick *et al.*, 1981).

One of the characteristic features of plasma leakage induce by the neutrophil chemoattractants is the long duration of action of the responses when compared with directly-acting mediators such as bradykinin and histamine (Wedmore & Williams, 1981). The duration of the permeability-increasing activity of bradykinin and histamine is approx. 4 min and that of FMLP is approx. 50 min. The mechanism for this protracted response is unknown; indeed the mechanism by which the rapid interaction between neutrophils and venular endothelium induced by extravascular chemoattractant leads to increased plasma leakage also remains to be established.

We were interested to determine whether colchicine could inhibit FMLP-induced oedema if administered after FMLP injections. Figure 6 shows the effect of colchicine administration (1 mg kg^{-1}) on the duration of action of bradykinin and FMLP in rabbit skin. Colchicine or saline was injected i.v. at the times indicated after i.d. injection of bradykinin and FMLP. Animals were also pretreated with mepyramine (3 mg kg^{-1} i.v.) to block the action of any endogenous histamine that may have been released by FMLP. Saline (1 ml kg^{-1}) or colchicine (1 mg kg^{-1}) was injected i.v. together with ^{125}I-albumin and Evans blue dye and responses measured over 30 min. A potentiating dose of PGE_2 was injected into all sites at the beginning of the measurement period. As shown in Figure 6a, colchicine had no effect on the duration of action of bradykinin, the response half-life in controls being 5 ± 1 min, compared with 4 ± 1 min in the colchicine group. In contrast, the long duration of action of FMLP (response half-life of 36 ± 3 min) was signifi-

cantly reduced at all but one time point in the presence of colchicine (Figure 6b).

Discussion

Oedema formation in response to intradermal injection of FMLP was difficult to detect unless a vasodilator prostaglandin (PGE_2) was present, and in the presence of PGE_2, FMLP showed similar permeability-increasing activity to rabbit C5a des Arg. The response to FMLP was independent of endogenous histamine release and FMLP was 100–1000 times more potent than histamine. Oedema formation was dependent on circulating neutrophils (Figure 3) and the activity of a range of N-formyl peptides in increasing vascular permeability correlated well with their activity as neutrophil chemoattractants and inducers of lysosmal enzyme secretion *in vitro* (Showell *et al.*, 1976; Freer *et al.*, 1982).

Intravenous infusion of ZAP was found to be an effective method of inducing transient neutropenia in rabbits and this resulted in selective inhibition of oedema formation induced by FMLP and C5a des Arg (neutrophil-dependent), but not that induced by bradykinin (neutrophil-independent). It was necessary to infuse ZAP for the duration of the experiment to maintain the neutropenia, although even under these conditions there was some return of cells to the circulation within 30 min. Upon cessation of ZAP infusion, we observed a transient neutrophilia and responses to C5a des Arg and FMLP were restored (data not shown). We also attempted analogous experiments infusing FMLP i.v., and while this resulted in a major fall in circulating neutrophil counts as found by O'Flaherty *et al.* (1977), there was also a fall in systemic blood pressure which resulted in significant inhibition of responses to not only FMLP and C5a des Arg, but also to bradykinin. The mechanism for this is unclear, but falls in systemic blood pressure have previously been shown to result in a generalized suppression of oedema responses to both neutrophil-dependent and neutrophil-independent mediators (Rampart & Williams, 1986b).

Ibuprofen administered i.v. to rabbits resulted in a selective reduction of oedema responses induced by FMLP + PGE_2 (neutrophil-dependent), but not oedema induced by histamine + PGE_2 (neutrophil-independent). Systemic ibuprofen did not reduce circulating neutrophil counts and the results suggest that the drug can interfere with the interaction between circulating neutrophils and venular endothelial cells, which is the initial step in oedema formation induced by FMLP. Certainly, ibuprofen can inhibit several aspects of neutrophil function *in vitro* including adherence to surfaces (Nielsen & Webster, 1987). One intriguing possibility is that ibuprofen inhibits the FMLP-induced increased expression of the CD11/CD18 adhesion glycoproteins on the neutrophil (Wallis *et al.*, 1986); however, this remains to be investigated. We have found that i.v. ibuprofen selectively inhibits neutrophil-dependent oedema formation in rat skin (Hellewell *et al.*, unpublished) and the drug also inhibits oedema formation induced by C5a des Arg in the rabbit by a cyclo-oxygenase-independent method (Rampart & Williams, 1986a). Whatever the mechanism of action of ibuprofen, it may relate to the observations that ibuprofen suppresses experimentally-induced myocardial infarction, a reaction characterized by neutrophil accumulation (Kirlin *et al.*, 1982; Flynn *et al.*, 1984).

The experiments with colchicine suggest that oedema formation induced by FMLP can be virtually abolished by this compound. The mechanism for this inhibition is unknown but is presumably related to suppression of neutrophil function. That it is not related to an inhibition of endothelial function is indicated by the observation that oedema responses to bradykinin were unaffected. However, at a higher dose of colchicine ($3\,mg\,kg^{-1}$), we found that while responses to FMLP remained inhibited, those to bradykinin were enhanced, perhaps indicating an effect of the drug at this dose on the endothelium (data not shown). Colchicine is known to interfere with several aspects of neutrophil function *in vitro* such as chemotaxis and chemokinesis (Malech *et al.*, 1977; Valerius, 1978), and aggregation (Lackie, 1974; Cunningham & Smith, 1982). *In vivo* in the rat, colchicine inhibits neutrophil migration into inflammatory lesions (Higgs *et al.*, 1983; Simmons *et al.*, 1983) and suppresses oedema formation in the Arthus reaction (Chang, 1975). We found that intravenous colchicine abolished subsequent plasma protein leakage when administered at different times after intradermal FMLP injection. This suggests that continuing interactions (whatever these may be) between neutrophils and endothelial cells are necessary for the protracted plasma protein leakage induced by this chemoattractant.

The results of this study are consistent with the concept that receptors have evolved to bacterial secretions (i.e. FMLP) and also to products of the interaction of bacterial cell walls with tissue fluid (i.e. C5a des Arg). These observations support the hypothesis that oedema formation is fundamentally a functional process concerned with regulating microbial lysis and opsonisation in an infected tissue.

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TRH-Induced blood flow and mean arterial pressure changes in the rabbit are not dependent on the anaesthetic used

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1 The effects of thyrotropin releasing hormone (TRH) on regional cerebral blood flow were studied in rabbits anaesthetized with pentobarbitone or ketamine. The blood flow was determined with the labelled microsphere method before and after the i.v. administration of either $50\,\mu g\,kg^{-1}$ or $2\,mg\,kg^{-1}$ TRH.

2 In order to measure the cerebral O_2 consumption the arteriovenous difference in oxygen saturation in the brain (CAVOD) was measured before and after the administration of $2\,mg\,kg^{-1}$ TRH.

3 In animals under pentobarbitone anaesthesia $50\,\mu g\,kg^{-1}$ TRH elicited an increase in mean arterial blood pressure (MAP) of about 1 kPa and $2\,mg\,kg^{-1}$ TRH elevated the MAP by about 2 kPa. With ketamine as the anaesthetic the corresponding values were 0.5 kPa and 7 kPa, respectively. TRH induced significant vasoconstriction in several peripheral tissues.

4 The total cerebral blood flow (CBF_{tot}) increased from 54 ± 4 to $78 \pm 5\,g\,min^{-1}\,100\,g^{-1}$ after the administration of $50\,\mu g\,kg^{-1}$ TRH in pentobarbitone-anaesthetized animals. An even greater effect was elicited by $2\,mg\,kg^{-1}$ TRH, from 48 ± 6 to $113 \pm 19\,g\,min^{-1}\,100\,g^{-1}$. In ketamine-anaesthetized rabbits, $50\,\mu g\,kg^{-1}$ TRH tended to enhance the CBF_{tot} and $2\,mg\,kg^{-1}$ increased it from 71 ± 6 to $141 \pm 19\,g\,min^{-1}\,100\,g^{-1}$.

5 In animals anaesthetized with pentobarbitone, the CAVOD decreased from $47.3 \pm 1.7\%$ to $35.1 \pm 2.2\%$ at 3 min after TRH delivery, and then gradually increased to the control level. In animals under ketamine anaesthesia the CAVOD decreased from $63.3 \pm 2.0\%$ to $45.2 \pm 7.4\%$ after the administration of $2\,mg\,kg^{-1}$ TRH.

6 It is concluded that TRH elicits cerebral vasodilatation in excess of that required by the change in cerebral metabolism which may have taken place. The pattern of responses was similar to that produced in rabbits under urethane anaesthesia.

Introduction

The tripeptide thyrotropin releasing hormone (TRH, pyroglutamyl-histidyl-proline amide) was originally studied and isolated because of its adenohypophyseal action to stimulate the release of thyroid stimulating hormone (TSH) (Guillemin *et al.*, 1963; Schally *et al.*, 1966). However, an extra endocrine role for TRH has since been suggested; see Morley (1979), Yarbrough (1979), Horita *et al.* (1986). The development of radioimmunoassay and immunohistochemical techniques resulted in the demonstration of a widespread distribution of TRH-like immunoreactive material (Hökfelt *et al.*, 1975; Morley *et al.*, 1977; Leppäluoto *et al.*, 1978; Parker & Capdevila, 1984) and of receptors for TRH (Burt & Snyder, 1975; Taylor & Burt, 1982) throughout the neuroaxis of mammals, including man. In addition, the presence of TRH has been demonstrated in invertebrates which lack a pituitary gland (Jackson & Reichlin, 1974; Grimm-Jorgensen *et al.*, 1975). In fact, only about one third to one quarter of the total content of TRH in the brain is found in the hypothalamus (Yarbrough, 1979). TRH is also present in peripheral organs (Morley *et al.*, 1977; Leppäluoto *et al.*, 1978).

TRH injected i.v., intracerebroventricularly or intracisternally gives rise to a number of physiologi-

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cal effects such as release of TSH and prolactin, changes in behaviour, a blood pressure response and changes in EEG activity (Yarbrough, 1979; Horita *et al.*, 1986). TRH has been shown to reduce not only the duration of ethanol- and barbiturate-induced narcosis but also the hypothermia provoked by these anaesthetics (Breese *et al.*, 1974; 1975). It was recently reported that TRH causes both sympathetic activation, resulting in vasoconstriction in extracranial and other peripheral tissues and, at the same time, cerebral vasodilatation in rabbits under urethane anaesthesia (Koskinen & Bill, 1984; Koskinen, 1985; 1986a). This cerebral vasodilatation was not caused by an increase in O_2- or glucose-consumption of the brain (Koskinen & Bill, 1984; Koskinen & Sperber, 1986). Subsequently the cerebral vasodilator effect was demonstrated in the cat (Mirzoyan *et al.*, 1985) and rat (Koskinen, 1986b; 1987; Helke & Phillips, 1988; Inanami *et al.*, 1988). Interestingly, in the conscious rabbit TRH caused sympathetic activation but no significant change in cerebral blood flow (CBF) (Koskinen, 1986a).

As the anaesthetic used may modify physiological and pharmacological responses, the present study was performed in order to evaluate the effects of TRH on cerebral blood flow, on O_2 supply to the brain and on blood pressure, with two entirely different anaesthetics, namely pentobarbitone and ketamine. The results were presented in part at the New York Academy of Science Congress on 'Recent Advances in the Biological Significance of TRH', November 1987 (Hugoson-Seligsohn & Koskinen, 1989).

Methods

New Zealand albino rabbits, of either sex, weighing 1.5–2.8 kg were used: 21 animals were anaesthetized with an initial dose of pentobarbitone sodium (Membumal; ACO, Solna, Sweden), 40 mg kg^{-1}, injected into a marginal ear vein; additional doses were given when the corneal reflex so indicated. In another 12 animals, anaesthesia was induced with 0.5 ml kg^{-1} fluanisone-fentanyl (Hypnorm, Janssen, Beerse, Belgium) given intramuscularly, followed by ketamine chloride (Ketalar; Parke-Davis, Barcelona, Spain) i.v. after 45–60 min. In these animals a continuous infusion of about 1 mg min^{-1} of ketamine chloride with a syringe pump was used to maintain surgical anaesthesia.

Both femoral arteries were cannulated with polyethylene catheters. One was used for determination of mean arterial blood pressure (MAP) and heart rate with a PDCR 75/1 transducer (Druck Ltd, Groby, England) and a Servogor 460 recorder (BBC-Goerz Metrawatt, Vienna, Austria). The other catheter was used for withdrawal of blood during microsphere injections. One femoral vein was cannulated and used for drug injections. The animal was tracheotomized and ventilated by a Palmer pump. Arterial P_{O_2}, P_{CO_2} and pH were determined with an ABL 2 or an ABL 300 acid-base analyzer (Radiometer, Copenhagen, Denmark). Sodium bicarbonate was given i.v. if needed in order to correct deviations in pH. Heparin (KabiVitrum, Stockholm, Sweden), 500 iu kg^{-1}, was used as the anticoagulant. Pancuronium bromide, (Pavulon; Organon, Oss, Holland) 0.05–0.2 mg kg^{-1} was given i.v. in order to induce skeletal muscle relaxation. Body temperature was determined with a rectal thermistor and maintained at about 38°C with a heating pad.

Pentobarbitone-anaesthetized animals

In 9 rabbits the arteriovenous O_2 saturation difference in the brain (CAVOD) was measured in order to investigate whether or not the O_2 demand of the brain was altered by TRH. The animal was placed in a stereotaxic instrument (David Kopf Instrument, Tujunga, CA, U.S.A.). A 1 × 0.5 cm opening was drilled in the midline of the skull between the coronal and the lamboid suture. Indomethacin (Sigma Chemicals Co., St Louis, MO., U.S.A.) was administered i.v. in a dose of 20 mg kg^{-1} in order to inhibit the cyclo-oxygenase activity and thus reduce the adhesion of platelets to the vessel wall. The superior sagittal sinus was cannulated under visual control with a polyethylene catheter. The arterial and venous O_2 saturation were determined with an oxymeter (American Optical Company, Buffalo, NY., U.S.A.) before and after the i.v. administration of 2 mg kg^{-1} TRH. In 3 rabbits both arterial and venous P_{O_2}, P_{CO_2}, pH and O_2 saturation were measured with the ABL 2 acid-base analyzer.

In a second series of experiments, the influence of TRH on regional blood flow was measured. Blood flow determinations were made a few minutes before, and 5 and 15 min after the injection of either 50 μg kg^{-1} ($n = 6$) or 2 mg kg^{-1} ($n = 6$) TRH. In order to elucidate possible effects of the sympathetic nerves on cerebral blood flow the cervical sympathetic chain was sectioned on one side about 1 cm below the upper cervical ganglion.

Ketamine-anaesthetized animals

In the first series of experiments ($n = 5$), the blood gases and the O_2 saturation were determined with the ABL 300 acid-base analyzer.

In a second series of animals, blood flow determinations were made before and 5 min after the i.v. administration of 50 μg kg^{-1} TRH ($n = 7$). After an

additional 10 min, 2 mg kg^{-1} was injected and the third microsphere injection was made 5 min later.

Blood flow measurements

The regional blood flow was determined by the tracer microsphere method (Wagner *et al.*, 1969; Alm & Bill, 1972). For injection of the microspheres a polyethylene catheter was inserted into the left ventricle via the left brachial artery. Spheres, 15 μm in diameter (NEN, Boston, Mass., U.S.A.), labelled with ^{103}Ru, ^{113}Sn and ^{141}Ce were used. The free flow method was used for reference blood sampling (Alm & Bill, 1972). Cardiac output was calculated as $Q_r \times$ c.p.m.$_i \times$ c.p.m.$_r^{-1}$, where Q_r is the reference blood flow, c.p.m.$_r$ is the radioactivity in the blood sample and c.p.m.$_i$ is the injected radioactivity.

Evan's blue (Kebo AB, Stockholm, Sweden) was injected i.v. into some of the animals in order to visualize any damage to the bloodbrain barrier caused by the elevation of the blood pressure. No extravasation of the dye was found in any part of the brain.

After the experiment, the animal was killed by injection of anaesthetic followed by saturated KCl. Tissue samples were removed and placed in preweighed plastic tubes. All of the brain was removed and samples included grey matter from the frontal and occipital cortex, white matter, hippocampal region, caudate nucleus, thalamic region, hypothalamic region, collicles, pons-mesencephalon, medulla oblongata, cerebellum and spinal cord (C_1–C_3) were taken. All remaining parts of the brain were placed in separate plastic tubes to be included in the calculation of the total cerebral blood flow. The total cerebral blood flow (CBF_{tot}) was calculated to include all regions except the medulla oblongata, the cerebellum and the spinal cord. The radioactivity of blood and tissue samples were determined by gamma spectrometry. Blood flows were calculated according to Alm & Bill (1972).

The results were evaluated statistically by the two-tailed Student's t test for paired observations, or the Wilcoxon signed rank test. The results are expressed as means ± s.e.mean.

Results

Pentobarbitone-anaesthetized animals

The CAVOD decreased from 47.3 ± 1.7% to 35.1 ± 2.2% ($P < 0.001$) during the first 3 min after TRH administration, and then gradually returned to the control level (Figure 1). There were no significant changes in the blood gases although minor increases in P_aCO_2 and P_vO_2 were noted (Table 1). A pressor

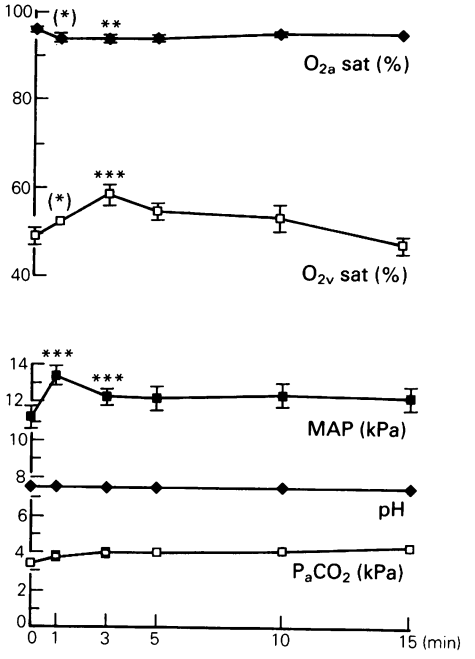

Figure 1 Arterial and venous O_2 saturation in the brain, mean arterial pressure (MAP), pH and $PaCO_2$ before and after the i.v. administration of 2 mg kg^{-1} TRH in pentobarbitone-anaesthetized animals ($n = 9$). * $P < 0.02$. ** $P < 0.01$; *** $P < 0.001$.

effect of about 2 kPa ($P < 0.001$) was elicited by TRH (Figure 1).

In the animals where the blood flow determinations were performed, the administration of 50 μg kg^{-1} TRH caused no significant change in MAP. Heart rate, P_aO_2 and P_aCO_2 were also unchanged (Table 2). The change in cardiac output from 376 ± 13 to 408 ± 20 g min^{-1} was not statistically significant. A marked increase in MAP from 10.7 ± 0.2 to 14.6 ± 0.5 kPa ($P < 0.001$) was elicited by 2 mg kg^{-1} TRH while P_aO_2 and P_aCO_2 were not significantly altered (Table 2).

In several peripheral tissues there was vasoconstriction similar to that previously described (Koskinen & Bill, 1984).

Table 1 Blood gas values (kPa) before and after the administration of 2 mg kg^{-1} TRH ($n = 3$)

		After TRH	
	Control	*3 min*	*10 min*
P_aCO_2	3.42 ± 0.10	3.98 ± 0.20	4.09 ± 0.16
P_aO_2	11.36 ± 0.07	10.98 ± 0.26	10.37 ± 0.01
P_vCO_2	5.32 ± 0.10	5.38 ± 0.50	5.38 ± 0.20
P_vO_2	4.14 ± 0.08	4.42 ± 0.36	4.85 ± 0.16

Table 2 Arterial blood gas values (kPa) mean arterial pressure (MAP) (kPa) and heart rate (HR) (min^{-1}) before and after the administration of $50\,\mu g\,kg^{-1}$ TRH(a) ($n = 6$) and $2\,mg\,kg^{-1}$ TRH(b) ($n = 6$) in pentobarbitone-anaesthetized animals; in ketamine-anaesthetized animals ($n = 7$), both $50\,\mu g\,kg^{-1}$ TRH(c) and $2\,mg\,kg^{-1}$ TRH(d) were administered to the same individuals

	P_aO_2	P_aCO_2	*pH*	*MAP*	*HR*
Control	10.71 ± 0.20	3.69 ± 0.10	7.48 ± 0.02	10.7 ± 0.3	308 ± 9
TRH(a)	10.30 ± 0.30	3.76 ± 0.30	7.48 ± 0.02	11.2 ± 0.4	332 ± 13
Control	11.49 ± 0.38	3.83 ± 0.07	7.46 ± 0.02	10.7 ± 0.2	304 ± 16
TRH(b)	10.45 ± 0.38	4.22 ± 0.17	7.45 ± 0.03	14.6 ± 0.5***	295 ± 9
Control	11.07 ± 0.65	4.37 ± 0.16	7.45 ± 0.02	7.4 ± 0.6	220 ± 12
TRH(c)	10.89 ± 0.68	4.38 ± 0.12	7.42 ± 0.02	7.9 ± 0.7*	216 ± 8
TRH(d)	10.36 ± 0.75*	4.53 ± 0.09	7.39 ± 0.02*	14.3 ± 0.8***	225 ± 13

* $P < 0.05$; *** $P < 0.001$.

No influence of the sympathetic nerves on the regional cerebral blood flow was observed either in the controls or after TRH administration. Therefore, only the values for the side with intact sympathetic supply are presented. Cerebral vasodilatation was elicited by $50\,\mu g\,kg^{-1}$ TRH (Figure 2a). The increase in blood flow was statistically significant in almost all regions. CBF_{tot} increased from 54 ± 4 to $78 \pm 5\,g\,min^{-1}\,100\,g^{-1}$ ($P < 0.001$). The greatest increase, about 50%, was observed in the caudate nucleus. An even greater effect was elicited by $2\,mg\,kg^{-1}$ TRH. The CBF_{tot} increased from 48 ± 6 to $113 \pm 19\,g\,min^{-1}\,100\,g^{-1}$ ($P < 0.05$). The most prominent effect was observed in the cortical grey matter, caudate nucleus and thalamic and hypothalamic regions, where the blood flow increased by more than 100%. In the other parts of the brain the increment ranged between 50–100% (Figure 2b).

Ketamine-anaesthetized animals

The CAVOD in the brain decreased from 63.3 ± 2.0% to 45.2 ± 7.4% ($P < 0.02$) 1 min after TRH delivery and then gradually increased to the control level (Figure 3). The control values for MAP and heart rate were reduced as compared with pentobarbitone-anaesthetized animals. There was no change in cardiac output after the administration of $50\,\mu g\,kg^{-1}$ TRH and the change after $2\,mg\,kg^{-1}$ TRH from 338 ± 36 to 294 ± 25 was not statistically significant. As Table 2 shows, the lower dose of TRH caused a minor rise in blood pressure of about 0.5 kPa. A marked pressor response was elicited by $2\,mg\,kg^{-1}$ TRH.

The lower dose of TRH did not cause any significant change in CBF. As shown in Table 3, $2\,mg\,kg^{-1}$ TRH elicited an increase in both the CBF_{tot} and in the regional cerebral blood flows.

The pattern of increment in regional cerebral blood flow after $2\,mg\,kg^{-1}$ of TRH resembled that observed in rabbits under pentobarbitone anaesthesia.

Discussion

Previous studies have shown that TRH affects the cardiovascular system in several ways (Beale *et al.*, 1977; Yarbrough, 1979). Although there was a marked rise in MAP after the injection of $2\,mg\,kg^{-1}$ TRH and a minor one after $50\,\mu g\,kg^{-1}$, there were no significant changes in cardiac output or heart rate, indicating an increase in peripheral vascular resistance. Thus, the present results confirm those of Koskinen & Bill (1984) and Koskinen (1985, 1986a, 1986c).

Ketamine has been found to increase selectively the blood flow in the cerebral hemisphere (Dhasmana *et al.*, 1984). However, in our study the baseline cerebral blood flow was lower than in conscious rabbits (Koskinen & Bill, 1984; Koskinen, 1986a) and comparable with the cerebral blood flow in urethane-anaesthetized rabbits. Thus, in our experimental model no marked cerebrovasodilator effects of ketamine were shown. TRH caused a marked increase in regional cerebral blood flow. The cortical grey matter, caudate nucleus, thalamus and hypothalamus were the most sensitive regions.

The arteriovenous difference in O_2 saturation decreased after TRH administration, indicating that the vasodilatation was not wholly due to an increased O_2 demand of the brain. It was recently reported that the regional glucose metabolism in the rabbit brain was unaffected by TRH (Koskinen & Sperber, 1986). Thus, the cerebral vasodilatation elicited by TRH in the present study is similar to that in animals under urethane anaesthesia (Koskinen & Bill, 1984).

TRH has been found to disturb pentobarbitone-induced sleep, when administered either i.v. or intracisternally (Breese *et al.*, 1975). The turnover of

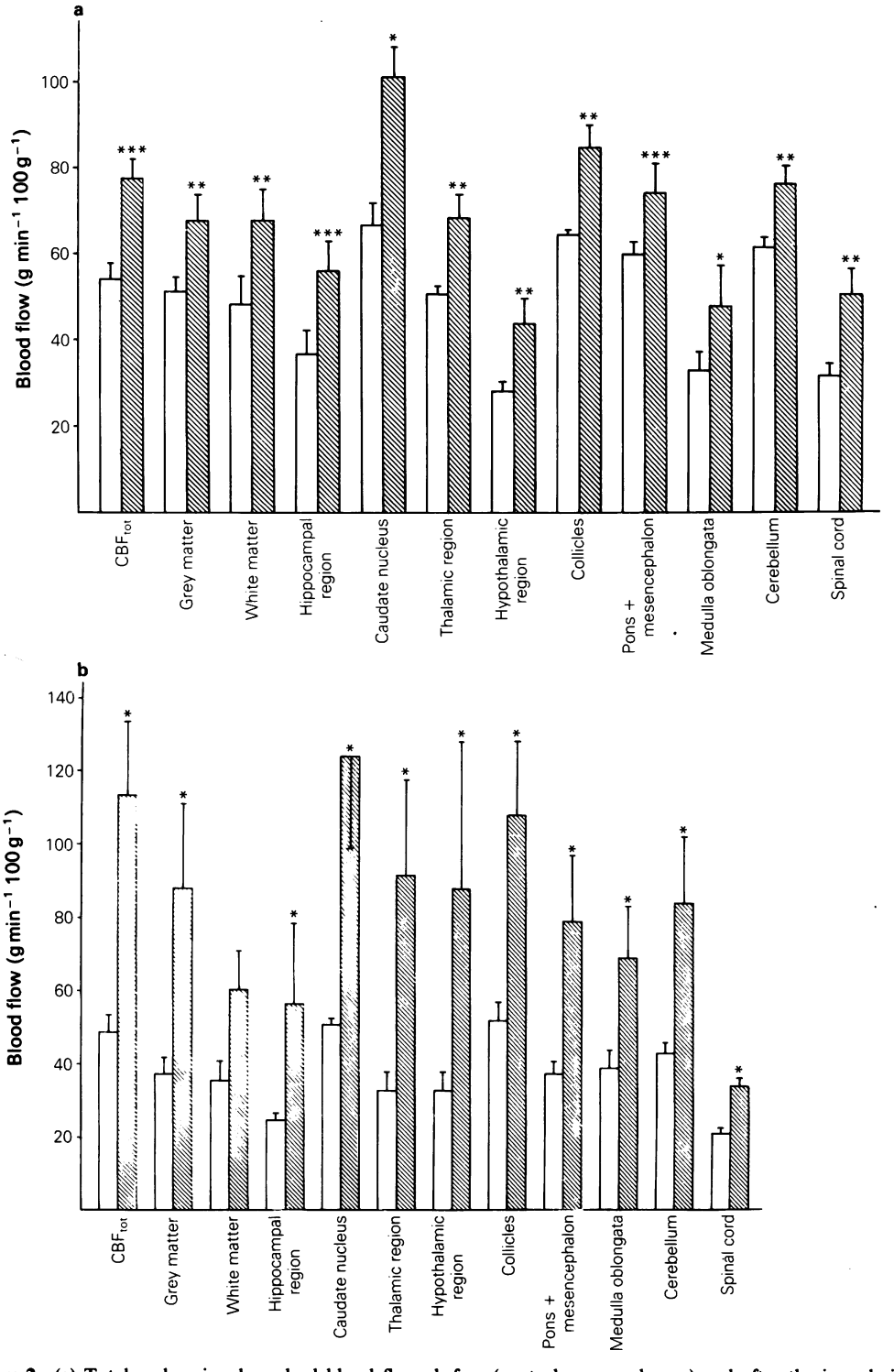

Figure 2 (a) Total and regional cerebral blood flows before (control, open columns) and after the i.v. administration of $50\,\mu g\,kg^{-1}$ TRH (hatched columns) in pentobarbitone-anaesthetized animals ($n = 6$). * $P < 0.02$, ** $P < 0.01$, *** $P < 0.001$ (Student's t test). (b) Total and regional cerebral blood flow before (control, open columns) and after the i.v. administration of $2\,mg\,kg^{-1}$ TRH (hatched columns) in pentobarbitone-anaesthetized animals ($n = 6$). * $P < 0.05$ (Wilcoxon's signed rank test).

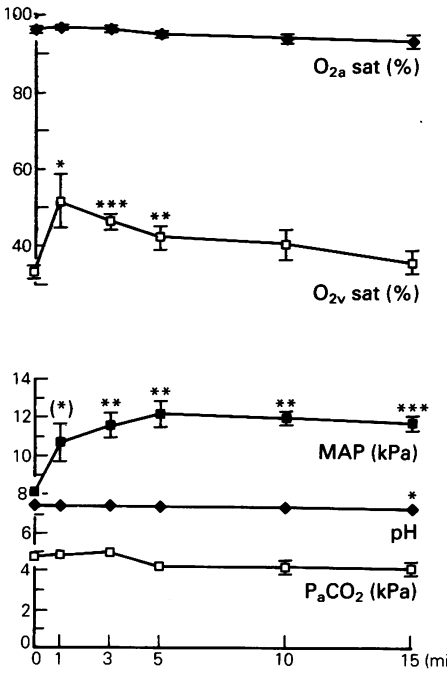

Figure 3 Arterial and venous O_2 saturation in the brain, mean arterial pressure (MAP), pH and P_aCO_2 before and after the i.v. administration of $2\,mg\,kg^{-1}$ TRH in ketamine-anaesthetized animals ($n = 5$). * $P < 0.02$, ** $P < 0.01$, *** $P < 0.001$.

pentobarbitone does not seem to be affected by TRH (Kraemer *et al.*, 1976) providing some support for the belief that the cerebral vasodilatation is not due to an altered blood concentration of the anaesthetic. It has also been shown that TRH has no effect on the anaesthesia induced by $1\,g\,kg^{-1}$ of urethane (Smith *et al.*, 1976). The duration of narcosis induced by ketamine seems to be diminished only when TRH is given prior to ketamine (Smith *et al.*, 1976). The analeptic effect of TRH has been shown to be reduced by muscarinic blockade, suggesting that a cholinergic mechanism is involved in this effect (Breese *et al.*, 1975). However, the vasodilator effect of TRH is not markedly impaired by muscarinic blockade (Koskinen, 1988). Therefore, it seems unlikely that most of the cerebrovascular action of TRH is due to an analeptic effect.

Table 3. Total (CBF_{tot}) and regional cerebral blood flow before and after the administration of $2\,mg\,kg^{-1}$ TRH in ketamine-anaesthetized animals ($n = 7$)

	Control	*TRH*
CBF_{tot}	71 ± 6	141 ± 19*
Grey matter	78 ± 12	173 ± 48*
White matter	57 ± 7	107 ± 6*
Hippocampal region	38 ± 13	115 ± 79
Caudate nucleus	120 ± 20	389 ± 68*
Thalamic region	64 ± 3	137 ± 12*
Hypothalamic region	37 ± 3	59 ± 9*
Collicles	67 ± 5	92 ± 9*
Pons-mesencephalon	49 ± 3	68 ± 7*
Medulla oblongata	41 ± 3	52 ± 5*
Cerebellum	59 ± 4	75 ± 6*
Spinal cord	25 ± 3	32 ± 4*

Values in $g\,min^{-1}\,100\,g^{-1}$ tissue. * $P < 0.05$ (Wilcoxon's signed rank test).

It has been suggested that the increase in cerebral blood flow is independent of the pressor effect of TRH (Koskinen & Bill, 1984; Inanami *et al.*, 1988). Furthermore, as in the present study, low doses of TRH administered i.v., with a minor effect on blood pressure, elicit cerebral vasodilatation (Koskinen, 1986a). The mechanism by which TRH produces cerebral vasodilatation is not fully understood. There is evidence that the increase in cerebral blood flow elicited by TRH is due to an activation of an intrinsic, cerebral vasodilator pathway (Koskinen, 1986c; Inanami *et al.*, 1988).

In conclusion, TRH elicits cerebral vasodilatation to the same extent in both pentobarbitone- and ketamine-anaesthetized, and urethane-anaesthetized rabbits. The effect of TRH on regional blood flow cannot be explained by the interaction with a particular anaesthetic agent.

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Potentiation by TRH of the effect of antidepressants in the forced-swimming test, involvement of dopaminergic and opioid systems

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1 It has been shown that thyrotropin releasing hormone (TRH) can potentiate the effects of the antidepressant, imipramine, as measured by the mouse forced-swimming test. This potentiation is not associated with an increase of effective levels of noradrenaline in the synaptic clefts, but depends upon the integrity of opioid systems. The present study was designed to investigate: (a) the potentiation by TRH of the effects of other antidepressants, using the same test; (b) the possible involvement of other neuronal systems, such as the 5-hydroxytryptaminergic and dopaminergic systems; (c) the contribution of the opioid and dopaminergic systems to the potentiation of the actions of other antidepressants by TRH.

2 The effects of nortriptyline, amineptine, maprotiline, nomifensine and mianserin, but not that of clomipramine, were potentiated by TRH ($2\,mg\,kg^{-1}$, i.p.). The inhibitor of 5-hydroxytryptamine synthesis, *p*-chlorophenylalanine (PCPA), did not prevent the effect induced by imipramine plus TRH. Blockade of dopaminergic systems by γ-butyrolactone (GBL) ($37.5\,mg\,kg^{-1}$, i.p.), α-methyl-*p*-tyrosine (AMPT) ($125\,mg\,kg^{-1}$, i.p.) and apomorphine ($0.025\,mg\,kg^{-1}$, i.p.) antagonized the effects induced by various antidepressants alone (at high, effective doses) or at lower ineffective doses in association with TRH. The effect induced by imipramine plus TRH was also blocked by sulpiride ($16\,mg\,kg^{-1}$, i.p.). Pretreatment with the opioid antagonist, naloxone, inhibited the effects induced by nomifensine plus TRH or mianserin plus TRH but not those induced by nortriptyline plus TRH, maprotiline plus TRH or amineptine plus TRH. When high active doses of the antidepressants were used alone, only the clomipramine effect was blocked by naloxone.

3 These data indicate that TRH is able to potentiate the effect not only of imipramine but of other antidepressants in the mouse forced-swimming test, although these other antidepressants act in various ways on cerebral amines. The antagonism of the effects induced by the antidepressants alone or in association with TRH after blockade of dopaminergic systems may indicate a reversal of the effect of the antidepressants by blockade of dopaminergic systems. Hence, the same mechanisms would be involved in the effects induced by antidepressants alone or in association with TRH, with respect to dopaminergic systems. However, different mechanisms of action seem to be responsible for the potentiation of TRH of the effect of the various antidepressants, since the involvement of the opioid systems varies according to the antidepressant tested.

Introduction

There is now considerable evidence that the tripeptide thyrotropin releasing hormone (TRH), which is widely distributed throughout the central nervous system, is both a neurohormone and a centrally-acting neuropeptide (see Horita *et al.*, 1986). Although the antidepressant action of TRH was observed as early as 1972 by Prange *et al.* and it was shown to potentiate the effect of imipramine in imipramine-resistant depressed patients (Plotnikoff & Kastin, 1976), this action remains controversial.

We have shown that when given intraperitoneally (i.p.) or intracerebroventricularly (i.c.v.) TRH can potentiate the effect of imipramine in the mouse (forced-swimming test). The potentiation of the imipramine effect, as assessed by a simple test, allowed the mechanisms of this potentiation to be studied. The potentiation does not seem to be associated with an increase in noradrenaline levels in the synaptic clefts, since pretreatment with α_1- and α_2-adrenoceptor antagonists (phenoxybenzamine,

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phentolamine) or an α_1-adrenoceptor antagonist (prazosin) did not prevent potentiation. In contrast, the α_2-adrenoceptor antagonist, yohimbine, blocked the TRH effect. Imipramine potentiation by TRH is also blocked by pretreatment with an opioid antagonist (naloxone) and decreased in opioid (morphine)-tolerant mice, suggesting an interaction between TRH and opioid systems (Reny-Palasse & Rips, 1985).

However, these studies have concerned only α-adrenergic and opioid systems, and there have been no systematic studies of the influence of TRH on other antidepressants using a simple test such as the mouse forced-swimming test. The present study was therefore designed to examine the effect of TRH on the action of other antidepressants, the possible involvement of 5-hydroxytryptaminergic and/or dopaminergic systems in the potentiation of imipramine by TRH, and the role of the opioid and dopaminergic systems in the potentiation of imipramine and other antidepressants by TRH.

The mouse forced-swimming test was used to study the influence of TRH upon the following antidepressants: nortriptyline, a tricyclic antidepressant which preferentially inhibits noradrenaline uptake (Carlsson *et al.*, 1969a); clomipramine, a tricyclic antidepressant which preferentially inhibits 5-hydroxytryptamine (5-HT) uptake (Carlsson *et al.*, 1969b); amineptine, a tricyclic antidepressant which stimulates dopaminergic systems and inhibits dopamine uptake (Samanin *et al.*, 1977); maprotiline, a tetracyclic antidepressant which specifically inhibits noradrenaline uptake (Maitre *et al.*, 1971); nomifensine, which stimulates noradrenaline and dopamine release (Braestrup & Scheel-Kruger, 1976) and inhibits their uptake (Hunt *et al.*, 1974), this drug has only a small effect on 5-HT uptake (Samanin *et al.*, 1975); mianserin, an atypical antidepressant which blocks presynaptic α_2-adrenoceptors (Bauman & Maitre, 1977) and increases noradrenaline turnover (Kafoe *et al.*, 1976; Maj, 1981, this drug also blocks 5-HT_2- and histamine (H_1)-receptors (Peroutka & Snyder, 1981).

Methods

Animals

Male OF1 mice (Iffa-Credo, France) weighing 25–30 g on the day of the experiment were used. All animals were kept at an ambient temperature of 22 ± 1°C and a day/night cycle of 12 h/12 h. Experiments were carried out between 9 h 00 min and 13 h 00 min.

Forced-swimming test

The procedure used was that described by Porsolt *et al.* (1977b). Naive mice were forced to swim for 6 min inside a vertical plexiglass cylinder from which they could not escape (height = 25 cm, diameter = 10 cm) containing water 6 cm deep at 22 ± 1°C. The total duration of immobility during the last 4 min was recorded. A mouse was judged to be immobile whenever it remained floating in the water, in an upright position making only very small movements necessary to keep its head above water.

Experimental procedures

Modulation of the effects of antidepressants by TRH Control experiments were carried out to evaluate the effects of each antidepressant on the mouse forced-swimming test and to establish the minimum effective dose of each drug (i.e., the dose producing a significant decrease in the mouse immobility time). A second group of tests determined the lowest dose of antidepressant which, with TRH ($2\,mg\,kg^{-1}$, i.p.), induced a significant decrease in the mouse immobility time. The TRH dose used ($2\,mg\,kg^{-1}$, i.p.) was selected as this was found to be the lowest dose which increased the effect of an otherwise ineffective dose of imipramine ($2\,mg\,kg^{-1}$, i.p.) (Reny-Palasse & Rips, 1985). Antidepressants were given 54 min before testing and TRH was given 6 min before testing.

Involvement of 5-hydroxytryptaminergic systems The possible implication of 5-HTergic systems in the potentiation of imipramine by TRH was investigated by use of PCPA, which blocks 5-HT synthesis through inhibition of tryptophan hydroxylase (Koe & Weissman, 1966). Doses of $300\,mg\,kg^{-1}$ i.p. were given 48 and 24 h before the test. The group treated with PCPA was compared with the relevant controls (vehicle alone). The group treated with imipramine ($2\,mg\,kg^{-1}$ i.p.) + TRH ($2\,mg\,kg^{-1}$ i.p.) + PCPA was compared to the relevant controls and to the group treated with imipramine ($2\,mg\,kg^{-1}$, i.p.) + TRH ($2\,mg\,kg^{-1}$, i.p.) + vehicle.

Involvement of dopaminergic systems Four dopaminergic system inhibitors were used. They were: GBL, which blocks nerve impulse at dopaminergic neurones (Walters & Roth, 1976), at a dose of $37.5\,mg\,kg^{-1}$, i.p., given 18 min before testing; AMPT, a tyrosine hydroxylase inhibitor which decreases noradrenaline and dopamine synthesis (Corrodi & Hanson, 1966), at a dose of $125\,mg\,kg^{-1}$, i.p. given 3 h before testing; apomorphine, a dopamine receptor agonist which, at low doses ($0.025\,mg\,kg^{-1}$, i.p., given 5 min before testing), produces a decrease in dopamine release (Martres *et al.*, 1977; Kendler *et al.*, 1982); sulpiride, a dopamine D_2

receptor antagonist (see Creese *et al.*, 1983), at a dose of 16 mg kg^{-1}, i.p., given 54 min before testing.

The forced-swimming test was used to evaluate the effects of: (1) GBL, AMPT, apomorphine and sulpiride on the response to a combination of imipramine (2 mg kg^{-1}, i.p.) and TRH (2 mg kg^{-1}, i.p.). (2) GBL, AMPT and apomorphine on the response to imipramine alone (high, effective dose). (3) GBL, AMPT and apomomorphine on the response to a combination of antidepressant (low, ineffective dose) and TRH (2 mg kg^{-1}, i.p.). (4) GBL, AMPT and apomorphine on the response to each of the other antidepressants alone (high, effective dose).

Details of exact doses of all drugs are given in Figures 2, 3, 4, 5, 6 and 7. The results for each group were compared with the relevant controls (vehicle alone) and with the appropriate group in which vehicle was substituted for the drug affecting the dopaminergic system.

Involvement of opioid systems The opioid receptor antagonist, naloxone (1 mg kg^{-1}, i.p., given 18 min before testing), was used in a series of experiments to evaluate opioid systems involvement. The following drugs were given: naloxone + antidepressant (low dose) + TRH (2 mg kg^{-1}, i.p.); naloxone + antidepressant (high, effective dose).

The results for each group were compared with the relevant controls (vehicle alone) and with the appropriate group in which vehicle was substituted for naloxone.

Drugs and injections

TRH (Bachem, Budendorf, Switzerland), nortriptyline hydrochloride (Lilly), clomipramine (Ciba), amineptine (Servier), nomifensine acid maleate (Hoechst), maprotiline (Ciba), mianserin (Organon), *p*-chlorophenylalanine ethyl ester hydrochloride (PCPA) (Aldrich), DL-α-methyl-*p*-tyrosine (AMPT) (Aldrich), γ-butyrolactone (GBL) (Sigma), apomorphine hydrochloride (Sigma), sulpiride (Delagrange) were dissolved in distilled water or suspended in 2% w/v Tween 80 in water. Imipramine hydrochloride (Geigy) was taken from commercially available ampoules and diluted with distilled water. The injections were given i.p. in a volume of 0.1 ml 10 g^{-1} body wt. Control animals received the vehicle alone. The experiments were conducted blind and in each experiment the treatments were administered randomly.

Statistical analysis

The results are expressed as the mean ± s.e.mean or as a percentage relative to the respective controls. The significance of the results was calculated by the Kruskal-Wallis test followed by the Mann-Whitney test.

Results

Modulation of the effects of antidepressants by TRH

The results are summarized in Table 1. All the antidepressants tested, used at a sufficient dose (except mianserin), induced a significant decrease in the duration of immobility compared to controls given vehicle alone ($P < 0.01$). A significant decrease in the duration of immobility was observed after administration of nortriptyline (2.5 mg kg^{-1}, i.p.), amineptine (2.5 mg kg^{-1}, i.p.), maprotiline (20 mg kg^{-1}, i.p.), nomifensine (0.625 mg kg^{-1}, i.p.) or mianserin (3.75 mg kg^{-1}, i.p.) in combination with TRH (2 mg kg^{-1}, i.p.). In contrast, there was no significant decrease in the duration of immobility after administration of clomipramine used at an inactive dose (5 mg kg^{-1}, i.p.) in combination with TRH (2 mg kg^{-1}, i.p.). The different antidepressants at the doses used in combination with TRH were ineffective on their own on the forced-swimming test.

Involvement of 5-hydroxytryptaminergic systems (Figure 1)

PCPA alone (2 × 300 mg kg^{-1}, i.p.) caused no change in the duration of immobility and did not induce any significant modification in the duration of immobility induced by imipramine (2 mg kg^{-1}, i.p.) + TRH (2 mg kg^{-1}, i.p.).

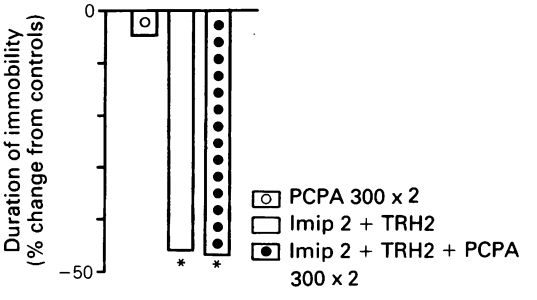

Figure 1 Involvement of 5-hydroxytryptaminergic systems in the potentiation by TRH of imipramine (Imip) effect (forced-swimming test in the mouse). *p*-Chlorophenylalanine (PCPA) was administered 48 and 24 h before the test. TRH was administered 6 min before the test. Doses are expressed as mg kg^{-1}, i.p. The number of mice per group was 10. The duration of immobility was recorded over a 4 min period. *: $P < 0.05$, comparison with the relevant controls, Kruskal-Wallis test followed by the Mann-Whitney test.

Table 1 Potentiation by thyrotropin releasing hormone (TRH) of the effects of various antidepressants in the mouse forced-swimming test

Drugs	*Doses* ($mg\,kg^{-1}$, i.p.)	*Number of mice per experiment*	*Duration of immobility (s) drug/vehicle*	*% change from controls*
Nortriptyline	20	20	73 ± 13/167 ± 12	↓56**¶
Nortriptyline	2.5	20	148 ± 15/158 ± 13	↓ 6
Nor + TRH	2.5 + 2	20	88 ± 14/158 ± 13	↓44**¶**†
Clomipramine	10	20	74 ± 20/156 ± 15	↓53**¶
Clomipramine	5	20	133 ± 16/136 ± 15	↓ 2
Clomi + TRH	5 + 2	20	133 ± 13/136 ± 15	↓ 2
Amineptine	5	10	88 ± 14/191 ± 12	↓54**¶
Amineptine	2.5	20	113 ± 12/139 ± 12	↓19
Ami + TRH	2.5 + 2	20	80 ± 13/139 ± 12	↓42**¶*†
Maprotiline	40	10	36 ± 13/154 ± 19	↓77**¶
Maprotiline	20	20	102 ± 19/157 ± 13	↓35
Mapro + TRH	20 + 2	20	73 ± 14/157 ± 13	↓54**¶*†
Nomifensine	2.5	10	49 ± 16/141 ± 20	↓65**¶
Nomifensine	0.625	20	145 ± 10/157 ± 9	↓ 8
Nomi + TRH	0.625 + 2	20	102 ± 15/157 ± 9	↓35**¶*†
Mianserin	15	20	122 ± 18/160 ± 14	↓24
	30	20	157 ± 14/163 ± 11	↓ 4
Mianserin	3.75	20	123 ± 14/132 ± 14	↓ 7
Mians + TRH	3.75 + 2	20	62 ± 11/132 ± 14	↓53**¶*†

Determination of the minimal effective dose of each antidepressant (except mianserin found inactive) and of the lowest dose of antidepressant which, in association with TRH, induced a significant effect. The antidepressants and TRH were administered 54 and 6 min before the test, respectively. The duration of immobility was recorded over a 4 min period (values are mean ± s.e.mean). *$P < 0.05$; **$P < 0.01$, ¶comparison with the relevant controls, †comparison with the group treated with the antidepressant at low dose, Kruskal-Wallis test followed by the Mann-Whitney test.

Involvement of dopaminergic systems

None of the dopaminergic system modulators, when given alone at the doses indicated in Figure 2, modified the forced-swimming test immobility times from those of controls. Pretreatment with GBL, AMPT, apomorphine and sulpiride, followed by imipramine plus TRH, produced immobility times significantly different from those obtained with imipramine plus TRH alone, indicating that the dopaminergic system modulators antagonized the effects of imipramine plus TRH. GBL, AMPT and apomorphine, also significantly ($P < 0.01$) antagonized the effect of imipramine alone.

In the same way, pretreatment with GBL, AMPT or apomorphine, significantly ($P < 0.05$ or 0.01) antagonized the decrease in immobility times induced by nortriptyline ($2.5\,mg\,kg^{-1}$, i.p.), amineptine ($2.5\,mg\,kg^{-1}$, i.p.), maprotiline ($20\,mg\,kg^{-1}$, i.p.), nomifensine ($0.625\,mg\,kg^{-1}$, i.p.) and mianserin ($3.75\,mg\,kg^{-1}$, i.p.) associated with TRH ($2\,mg\,kg^{-1}$, i.p.) (Figures 3, 4, 5, 6, 7). These same pretreatments significantly ($P < 0.05$ or $P < 0.01$) antagonized the action of effective doses of all the antidepressants when given alone, including clomipramine whose effect was not potentiated by TRH. However, although the amineptine effect was antagonized by apomorphine, this change was not statistically significant (Figures 3, 4, 5, 6, 7).

Involvement of opioid systems

Naloxone alone ($1\,mg\,kg^{-1}$, i.p.) caused no change in the forced-swimming test results. Pretreatment with naloxone significantly ($P < 0.01$) antagonized the decrease in the duration of immobility induced by nomifensine ($0.625\,mg\,kg^{-1}$, i.p.) and mianserin ($3.75\,mg\,kg^{-1}$, i.p.) associated with TRH ($2\,mg\,kg^{-1}$, i.p.), but not those induced by nortriptyline ($2.5\,mg\,kg^{-1}$, i.p.), maprotiline ($20\,mg\,kg^{-1}$, i.p.) or amineptine ($2.5\,mg\,kg^{-1}$, i.p.) associated with TRH ($2\,mg\,kg^{-1}$, i.p.) (Figure 8). Tested with effective doses of the antidepressants alone, only the clomipramine ($10\,mg\,kg^{-1}$, i.p.) effect was antagonized by naloxone (Figure 8).

Discussion and conclusions

The mouse forced-swimming test has been used to show that TRH ($2\,mg\,kg^{-1}$, i.p.) not only potentiates

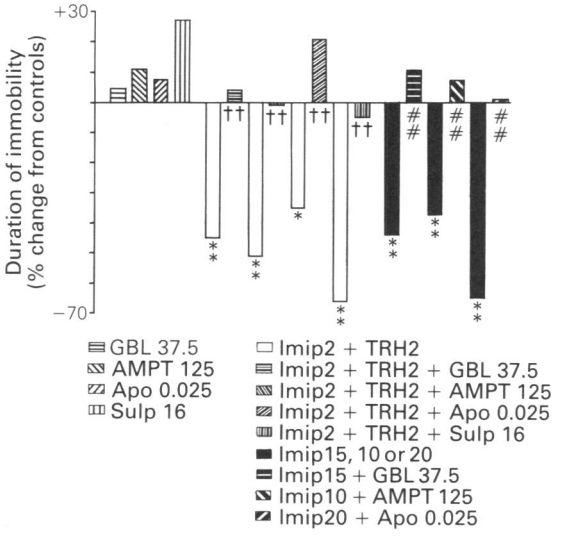

Figure 2 Involvement of dopaminergic systems in the potentiation by TRH of imipramine (Imip) effect (forced-swimming test in the mouse). α-Methyl-*p*-tyrosine (AMPT), apomorphine (Apo) and γ-butyrolactone (GBL) were administered 3 h, 6 min and 18 min before the test, respectively. Imipramine and sulpiride (Sulp) were administered 54 min before the test. TRH was administered 6 min before the test. Doses are expressed as mg kg^{-1}, i.p. The number of mice per group was 10, 20 or 30. The duration of immobility was recorded over a 4 min period. $*P < 0.05$; $**P < 0.01$, comparison with the relevant controls; $\dagger\dagger P < 0.01$, comparison with the group treated with (imipramine + TRH); $\#\#P < 0.01$, comparison with the group treated with imipramine alone; Kruskal-Wallis test followed by the Mann-Whitney test.

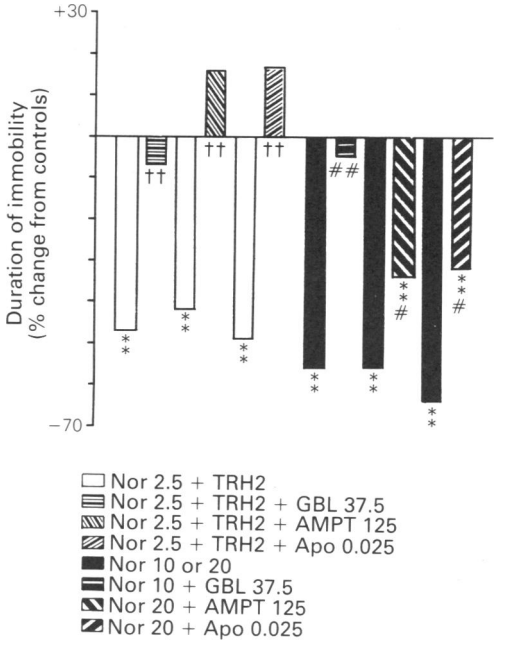

Figure 3 Involvement of dopaminergic systems in the potentiation by TRH of nortriptyline (Nor) effect (forced-swimming test in the mouse). Nortriptyline and TRH were administered 54 and 6 min before the test, respectively. Doses are expressed as mg kg^{-1}, i.p. The number of mice per group was 20. The duration of immobility was recorded over a 4 min period. $**P < 0.01$, comparison with the relevant controls; $\dagger\dagger P < 0.01$, comparison with the group treated with (nortriptyline + TRH); $\#P < 0.05$; $\#\#P < 0.01$, comparison with the group treated with nortriptyline alone; Kruskal-Wallis test followed by the Mann-Whitney test. For other abbreviations see legend to Figure 2.

the effect of imipramine but also those of the other antidepressants tested (nortriptyline, amineptine, maprotiline, mianserin and nomifensine). The only antidepressant the effect of which was not modified by TRH was clomipramine. Thus, TRH activity does not depend on the type of amine modified by the antidepressant, since the antidepressants whose effects are potentiated by TRH act by different ways on aminergic systems. No activity was observed in the forced-swimming test after administration of mianserin alone at the doses of 1.88, 3.75, 7.5, 15 or 30 mg kg^{-1}, i.p., although Porsolt *et al.* (1977a, b) found that this antidepressant was active in both rats and mice, using the forced-swimming test. In contrast, a significant decrease ($P < 0.01$) in the duration of immobility was observed when a low dose of mianserin (3.75 mg kg^{-1}, i.p.) was given with TRH. The ratio of the minimal effective doses of the antidepressants in the absence or in the presence of TRH (2 mg kg^{-1}, i.p.) were: not calculable (mianserin), 10 (imipramine), 8 (nortriptyline), 4 (nomifensine), 2 (amineptine) and 2 (maprotiline).

We have previously shown that potentiation of the effect of imipramine by TRH does not seem to be associated with an increase of effective levels of noradrenaline in the synaptic clefts, and have suggested that there is an interaction with opioid systems (see Reny-Palasse & Rips, 1985). The results of the present study indicate that 5-HTergic systems are not involved in this potentiation, since the effect induced by imipramine plus TRH is not modified by pretreatment with the inhibitor of 5-HT synthesis, PCPA.

Concerning the involvement of dopaminergic systems in the activity of the antidepressants on the forced-swimming test, our results confirm and extend the data of Borsini *et al.* (1984, 1985) showing reversal of effects of antidepressants after blockade of

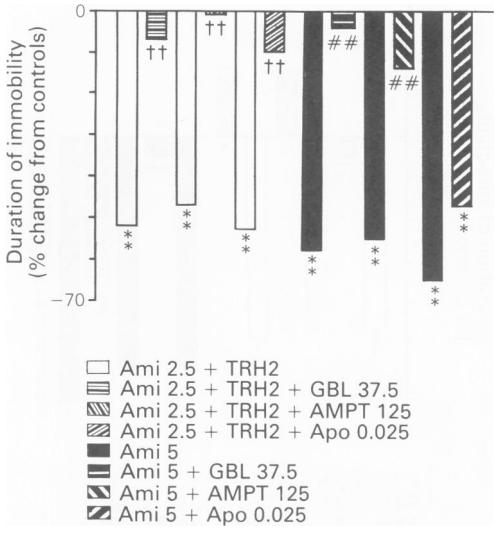

Figure 4 Involvement of dopaminergic systems in the potentiation by TRH of amineptine (Ami) effect (forced-swimming test in the mouse). Amineptine and TRH were administered 54 and 6 min before the test, respectively. Doses are expressed as mg kg^{-1}, i.p. The number of mice per group was 20. The duration of immobility was recorded over a 4 min period. $^{**}P < 0.01$, comparison with the relevant controls; $\dagger\dagger P < 0.01$, comparison with the group treated with (amineptine + TRH); $\#\#P < 0.01$, comparison with the group treated with amineptine alone; Kruskal-Wallis test followed by the Mann-Whitney test. For other abbreviations see legand to Figure 2.

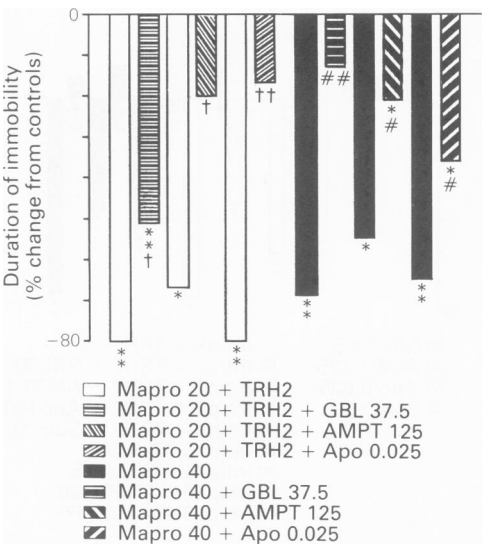

Figure 5 Involvement of dopaminergic systems in the potentiation by TRH of maprotiline (Mapro) effect (forced-swimming test in the mouse). Maprotiline and TRH were administered 54 and 6 min before the test, respectively. Doses are expressed as mg kg^{-1}, i.p. The number of mice per group was 20. The duration of immobility was recorded over a 4 min period. $^{*}P < 0.05$; $^{**}P < 0.01$, comparison with the relevant controls; $\dagger\dagger P < 0.01$, comparison with the group treated with (maprotiline + TRH); $\#\#P < 0.01$, comparison with the group treated with maprotiline alone; Kruskal-Wallis test followed by the Mann-Whitney test. For other abbreviations see legend to Figure 2.

dopaminergic systems. Those authors observed that both sulpiride (100 mg kg^{-1}, i.p.) and haloperidol (0.5 mg kg^{-1}, i.p.) antagonized the effect induced by chronic administration of mianserin, iprindole, amineptine and nomifensine, in the rat, and that the effect of desipramine was antagonized only by sulpiride. Using three different methods to block the dopaminergic systems (reduction of impulse flow by GBL, decrease in dopamine synthesis by AMPT or decrease in dopamine release by low dose apomorphine), we found an antagonism of the effects induced by the different antidepressants alone used at an effective dose. These data confirm the involvement of dopaminergic systems in the effect induced by amineptine, nomifensine and mianserin and demonstrate the involvement of these systems in the effect induced by other antidepressants such as imipramine, nortriptyline, maprotiline and clomipramine.

In the same way, blockade of dopaminergic systems by GBL, AMPT or apomorphine antagonized the effects induced by imipramine, nortriptyline, amineptine, maprotiline, nomifensine and mianserin (low doses) in association with TRH. Moreover, the effect induced by imipramine (low dose) in association with TRH, was prevented by sulpiride (D_2 dopamine receptor blocker).

According to these results, it appears that dopaminergic system blockers may counter the effect of antidepressants. Thus, antidepressants alone become inactive, and in the case of antidepressants in association with TRH (TRH alone being ineffective in this test) no effect can be observed. The action of the different antidepressants in association with TRH may depend on the same mechanisms as those of the antidepressants acting alone, with respect to dopaminergic systems. Moreover, these results indicate that dopaminergic systems may play a primary role in reducing the duration of immobility. In fact, Porsolt *et al.* (1979) described an increase of the duration of immobility, in the rat, after administration of the dopamine receptor antagonists, haloperidol (0.1 mg kg^{-1}, i.p.) or pimozide (2 mg kg^{-1}, i.p.) and a

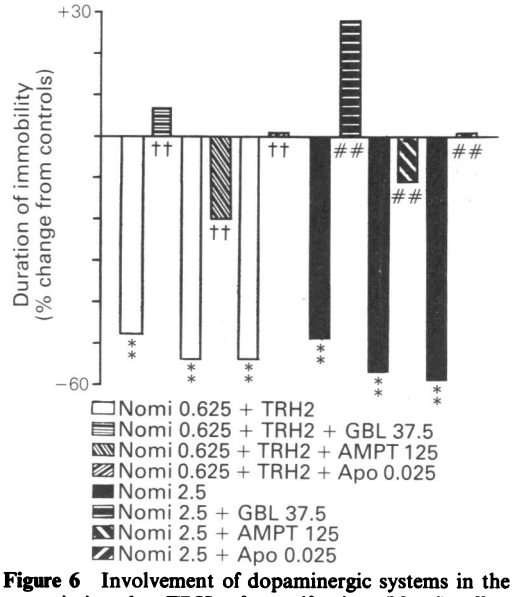

Figure 6 Involvement of dopaminergic systems in the potentiation by TRH of nomifensine (Nomi) effect (forced-swimming test in the mouse). Nomifensine and TRH were administered 54 and 6 min before the test, respectively. Doses are expressed as $mg\,kg^{-1}$, i.p. The number of mice per group was 20. The duration of immobility was recorded over a 4 min period. $^{*}P < 0.05$, $^{**}P < 0.01$, comparison with the relevant controls; $\dagger\dagger P < 0.01$, comparison with the group treated with (nomifensine + TRH); $\#\#P < 0.01$, comparison with the group treated with nomifensine alone; Kruskal-Wallis test followed by the Mann-Whitney test. For other abbreviations see legend to Figure 2.

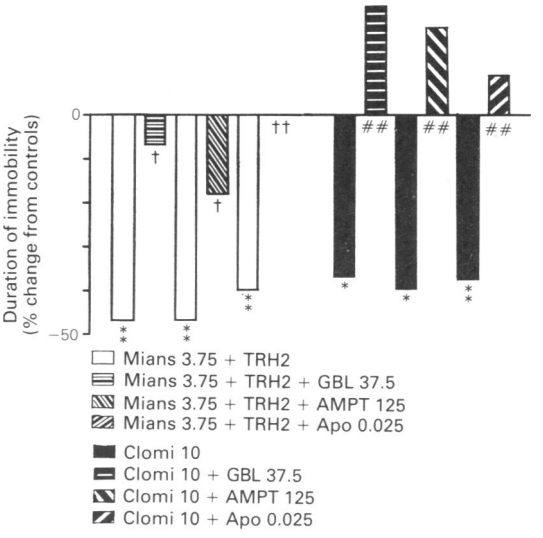

Figure 7 Involvement of dopaminergic systems in the potentiation by TRH of mianserin (Mians) effect, and in the clomipramine (Clomi) effect (forced-swimming test in the mouse). Mianserin and clomipramine were administered 54 min before the test. TRH was administered 6 min before the test. Doses are expressed as $mg\,kg^{-1}$, i.p. The number of mice per group was 20. The duration of immobility was recorded over a 4 min period. $^{**}P < 0.01$, comparison with the relevant controls; $\dagger P < 0.05$, $\dagger\dagger P < 0.01$, comparison with the group treated with (mianserin + TRH); $\#\#P < 0.01$, comparison with the group treated with clomipramine alone; Kruskal-Wallis test followed by the Mann-Whitney test.

decrease of the duration of immobility after administration of dopamine receptor agonists, apomorphine (0.5 or $2\,mg\,kg^{-1}$, i.p.) or bromocriptine ($5\,mg\,kg^{-1}$, i.p.). We have observed (not described here) a significant increase in the duration of immobility after administration of dopaminergic system blockers at doses higher than those used to investigate dopaminergic system involvement: GBL ($75\,mg\,kg^{-1}$, i.p.), AMPT ($250\,mg\,kg^{-1}$, i.p.) and sulpiride ($64\,mg\,kg^{-1}$, i.p.), and after haloperidol (0.25 or $0.5\,mg\,kg^{-1}$, i.p.).

Concerning the involvement of opioid systems in the activity of the antidepressants on the forced-swimming test, our results are also in agreement with previous studies. Devoize *et al.* (1984) found that naloxone antagonizes the effects of clomipramine and desipramine, but not those of nomifensine, mianserin or pargyline. We have observed that, among all the antidepressants tested, only the effect of clomipramine was prevented by naloxone. Thus opioid systems may not be involved in the effects induced by imipramine, nortriptyline, amineptine, maprotiline, nomifensine or mianserin. Clomipramine was the only antidepressant tested which was not potentiated by TRH. This difference between clomipramine and the other antidepressants may be at the origin of the lack of TRH effect on clomipramine activity.

As in the case of imipramine, naloxone was found to antagonize the effect induced by nomifensine and mianserin associated with TRH. Thus, the opioid systems would seem to be implicated in the effect induced by imipramine, nomifensin and mianserin associated with TRH.

These data suggest that not only imipramine may be of potential use in man, but also other antidepressants, particularly mianserin the activity of which in the forced-swimming test could be demonstrated only in the presence of TRH. Overall, these results indicate that potentiation by TRH of the effect of the different antidepressants does not act via a simple mechanism, as there are differences in opioid system involvement depending on which antidepressant is used.

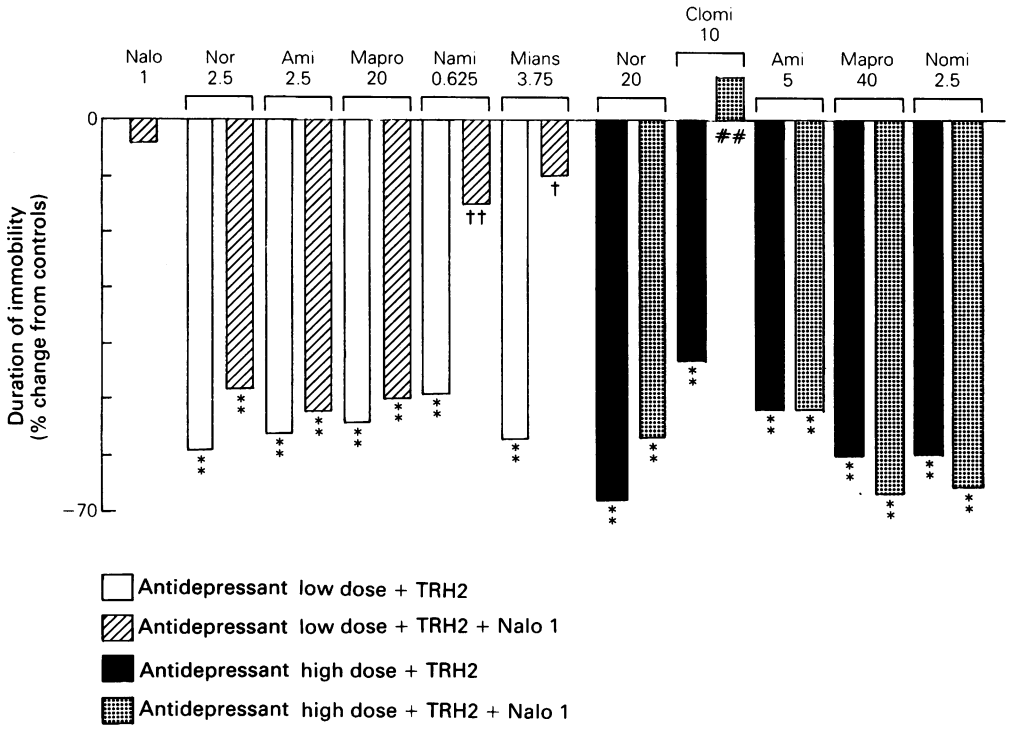

Figure 8 Involvement of opioid systems in the potentiation by TRH of the effect of different antidepressants and in the effect of different antidepressants alone (forced-swimming test in the mouse). The different antidepressants, naloxone (Nalo) and TRH were administered 54, 18 and 6 min before the test, respectively. Doses are expressed as $mg\,kg^{-1}$, i.p. The number of mice per groups was 20. The duration of immobility was recorded over a 4 min period. $^{**}P < 0.01$, comparison with the relevant controls; $\dagger P < 0.05$, $\dagger\dagger P < 0.01$, comparison with the group treated with (antidepressant + TRH); $\#\#P < 0.01$, comparison with the group treated with antidepressant alone; Kruskal-Wallis test followed by the Mann-Whitney test. For other abbreviations see legend to Figure 2 and text.

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An *in vivo* dialysis and behavioural study of the release of 5-HT by *p*-chloroamphetamine in reserpine-treated rats

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1 Reserpine (2.5 mg kg^{-1} i.p.) decreased rat brain 5-hydroxytryptamine (5-HT) by 86% 24 h later but most components of the 5-HT-dependent behavioural syndrome induced by *p*-chloroamphetamine (PCA, 5 mg kg^{-1} i.p.) or 5-methoxy-N,N-dimethyltryptamine (5-MeODMT, 5 mg kg^{-1} i.p.) over 1 h after administration were unaffected. However, Straub tail was increased after giving PCA or 5-MeODMT and head weaving was decreased after giving 5-MeODMT.

2 Frontal cortex extracellular 5-HT concentrations of vehicle pretreated rats before injection of PCA, as calculated from dialysate 5-HT concentrations, were about 1/1000th of corresponding brain values. Extracellular 5-hydroxyindoleacetic acid (5-HIAA) and brain values were comparable with each other. Dialysate 5-HT increased after PCA with peak values at 20–40 min.

3 Reserpine pretreatment reduced dialysate 5-HT concentration before PCA was given but the net increase (AUC) over the 1 h after PCA did not differ significantly from that seen in animals pretreated with vehicle. Dialysate 5-HIAA values slowly decreased after PCA injection in both reserpine and vehicle pretreated groups.

4 The results suggest that PCA causes the 5-HT syndrome by releasing 5-HT from the neuronal cytoplasm but that physiological release of 5-HT occurs from vesicular stores.

Introduction

The activation of brain 5-hydroxytryptamine (5-HT) receptors induces the 5-HT syndrome in rats, the main components of which are head weaving, forepaw treading, hind limb abduction, flat body posture, Straub tail and wet-dog shakes (Grahame-Smith, 1971; Jacobs, 1976; Sloviter *et al.*, 1978). Some or all of these components can be elicited by drugs which selectively release neuronal 5-HT, such as *p*-chloroamphetamine (PCA; Trulson & Jacobs, 1976; Kuhn *et al.*, 1985), as well as by 5-HT receptor agonists (Hjorth *et al.*, 1982; Tricklebank *et al.*, 1985a,b; Smith & Peroutka, 1986).

The PCA-induced 5-HT behavioural syndrome has been suggested to result from the release of neuronal 5-HT from a small non-vesicular compartment, since it still occurs after pretreatment with reserpine (Kuhn *et al.*, 1985) which disrupts vesicular storage (Shore & Giachetti, 1978). It has also been shown that PCA releases [^{3}H]-5-HT from synaptosomes prepared from the brains of reserpine-treated rats (Ross & Kelder, 1977). More detailed studies of components of the 5-HT syndrome showed that reserpine did not prevent the induction of forepaw treading or flat body posture by the 5-HT agonists 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) and 5-methoxy-N,N-dimethyltryptamine (5-MeODMT), but drastically reduced the head weavings elicited by these drugs (Tricklebank *et al.*, 1985a,b).

The 5-HT behavioural syndrome has usually been scored by methods based on rating scales for its components (Andrews *et al.*, 1982; Tricklebank *et al.*, 1985a,b; Smith & Peroutka, 1986) or by 'all or none' procedures (Trulson & Jacobs, 1976; Sloviter *et al.*, 1978) which do not quantify either the intensity or the duration of each component. As Kuhn *et al.* (1985) investigated the effect of reserpine pretreatment on the 5-HT syndrome elicited by PCA by an 'all or none' method, the interpretation of their findings is uncertain. Also, while *in vivo* intracerebral dialysis shows that PCA releases 5-HT (Sharp *et al.*, 1986) and that the time course of release and the induced 5-HT syndrome parallel each other (Hutson & Curzon, 1989), the effect of reserpine pretreatment NaCl containing BRIJ 35/polyoxyethylene lauryl

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on release is unknown. It is conceivable that reserpine both reduces the release of 5-HT by PCA and increases the response of receptors to it so that the behavioural effect is unaltered.

We have now reinvestigated the behavioural effect of PCA in rats pretreated with reserpine by scoring the frequency or duration of each behavioural component (Dourish *et al.*, 1985). This procedure avoids the disadvantages of previous scoring methods. Also, the release of brain 5-HT was monitored by *in vivo* dialysis and the responsiveness of the 5-HT receptors mediating the syndrome was studied by measuring its components when induced by the 5-HT agonist 5-MeODMT.

Methods

Male Sprague-Dawley rats (Charles River, U.K.) weighing 250–350 g were used in all experiments. They were housed individually under a 12 h light-dark cycle with food and water available at all times.

Behavioural experiments

Rats were given reserpine ($2.5\,mg\,kg^{-1}$, i.p.) or vehicle 24 h before the administration of either PCA ($5\,mg\,kg^{-1}$, i.p.) or 5-MeODMT ($5\,mg\,kg^{-1}$, i.p.). All animals pretreated with reserpine showed ptosis at the time of injection. The dose of PCA was chosen because it caused submaximal expression of the major components of the 5-HT syndrome in normal rats (Fernando & Curzon, 1981; Kennett *et al.*, 1985). After PCA or 5-MeODMT injection, rats were placed in individual perspex cages ($25 \times 25 \times 21$ cm) with grid floors and behaviours videotaped for 1 h. The frequency of head weaving, forepaw treading, wet-dog shakes and rearing, and the duration of hind limb abduction, flat body posture, Straub tail, locomotion and grooming were scored over 1 h using a microcomputer (Donohoe *et al.*, 1987).

Intracerebral dialysis

Concentric dialysis probes made essentially as described by Hutson *et al.* (1985) were fitted with 36G steel microcannulae (Brain Research Instruments Co, Princeton, U.S.A.). Cuprophan hollow fibres (Enka AG, Wuppertal, F.R.G.) were used for the dialysis membranes. Before implantation, the recoveries of 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) by the probes were determined as follows. Probes were perfused with artificial cerebrospinal fluid (CSF) (composition in mM; NaCl 125, KCl 2.5, $MgCl_2$ 1.18, $CaCl_2$ 1.26) at $0.25\,\mu l\,min^{-1}$ using a microlitre syringe pump (Harvard Apparatus Ltd, South Natick, U.S.A.) and immersed in a beaker containing $10\,ng\,ml^{-1}$ of each indole compound. Successive 20 min (5 μl) samples of perfusate were collected in tubes containing 10 μl of mobile phase (see below) and analysed by high-performance liquid chromatography (h.p.l.c.). Percentage recoveries were 57 ± 1 for 5-HT and 51 ± 2 for 5-HIAA ($n = 10$, mean $\pm$ s.e.mean).

Rats were given reserpine ($2.5\,mg\,kg^{-1}$, i.p.) or vehicle 24 h before injecting PCA ($5\,mg\,kg^{-1}$, i.p.) as in the behavioural experiments. Following anaesthesia with sodium pentobarbitone ($60\,mg\,kg^{-1}$, i.p.), dialysis probes were implanted in frontal cortex (3.2 mm anterior to bregma, 6.0 mm ventral to it and 2.5 mm lateral to the midline) according to Paxinos & Watson (1982). Animals were left to recover for approximately 18 h and artificial CSF then perfused through the probe as described. Dialysis samples were collected for 1 h before giving PCA and for 4 h 40 min subsequently.

Biochemical measurements

Immediately after collection, each dialysis sample was assayed for 5-HT and 5-HIAA by h.p.l.c. with electrochemical detection. The mobile phase consisted of 0.15 M NaH_2PO_4, 0.01 mM octyl sodium sulphate, 0.5 mM EDTA (pH 3.8 adjusted with phosphoric acid) and 12.5% methanol. Indoles were separated on an Altex ultrasphere 3 μm ODS column (4.6 mm × 7.5 cm) (Beckman Ltd, U.K.). The electrochemical detector was an ESA Coulochem model 5100A (Severn Analytical Ltd) with a dual electrode analytical cell (model 5011). The conditioning cell was set at −0.04 V, electrode 1 at +0.10 V and electrode 2 at +0.30 V with respect to palladium reference electrodes.

In a separate experiment, rats were given either reserpine or vehicle as before, killed 24 h later and their brains removed for determination of 5-HT, 5-HIAA and also dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). The brains were homogenized in 0.4 M perchloric acid containing 0.1% sodium metabisulphite, 0.01% EDTA, 0.1% cysteine and centrifuged at $3000 \times g$ for 20 min. Aliquots of supernatants were then filtered through 0.45 μm filters (Gelman) and analysed by h.p.l.c. as described, except for the mobile phase which was made up of 0.1 M KH_2PO_4, 1 mM octyl sodium sulphate, 0.1 mM EDTA (pH 2.75) and 18% methanol.

Drugs

Reserpine, PCA and 5-MeODMT were purchased from Sigma. DL-PCA hydrochloride was dissolved in 0.9% NaCl and 5-MeODMT suspended in 0.9%

ether (0.005%). Reserpine was dissolved in a few drops of glacial acetic acid and diluted in distilled water. Drugs were injected in volumes of 1 ml kg^{-1} body weight.

Statistics

Differences between the behavioural effects of drugs on vehicle and reserpine pretreated rats were assessed using the two-tailed Mann-Whitney U-test or Duncan's multiple range test. The effects of reserpine on whole brain levels of indoles and catechols were analysed by Student's *t* test. In the *in vivo* intracerebral dialysis study the mean of the four values immediately before PCA injection was taken as the basal concentration for each indole compound. The effects of PCA and reserpine on extraneuronal levels of 5-HT and 5-HIAA were assessed by two-way analysis of variance followed by Newman-Keuls' multiple range test.

Results

As shown in Table 1 reserpine depleted whole brain levels of 5-HT and dopamine very substantially and increased 5-HIAA and HVA.

Results in Table 2 and Figure 1 show that the overall patterns of the behavioural effects of PCA and 5-MeODMT were largely unaffected by pretreatment with reserpine, major components of the behaviour not being significantly altered. However, head weaving frequency was significantly decreased (after 5-MeODMT) and Straub tail behaviour was significantly increased after giving either drug.

Table 1 Effect of reserpine (2.5 mg kg^{-1}, i.p.) on whole brain levels of 5-hydroxytryptamine (5-HT), 5-hydroxyindole-acetic acid (5-HIAA), dopamine (DA), dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA)

	Vehicle ($n = 6$)	*Reserpine* ($n = 6$)
5-HT	5.04 ± 0.49	0.69 ± 0.27**
5-HIAA	2.03 ± 0.10	2.62 ± 0.29*
DA	8.88 ± 1.09	0.44 ± 0.12**
DOPAC	0.76 ± 0.08	0.68 ± 0.09
HVA	0.45 ± 0.03	0.73 ± 0.14**

Reserpine or vehicle was injected 24 h before killing. Values (mean ± s.e.mean) are expressed as pmol mg^{-1} wet wt tissue. *$P < 0.01$, **$P < 0.001$ compared with vehicle-injected rats (Student's *t* test).

In the absence of PCA or 5-MeODMT, the above components of the 5-HT syndrome were, at the most, marginally present in the vehicle and reserpine-treated groups, but appreciable amounts of normal locomotor, rearing and grooming behaviour occurred and was comparable in both groups (Figure 1). PCA significantly increased locomotion and almost totally inhibited rearing and grooming. These effects of PCA were unaltered by reserpine pretreatment. 5-MeODMT had a negligible effect on locomotion in both vehicle and reserpine-treated rats, decreased rearing significantly and comparably in both of these groups but significantly decreased grooming only in reserpine pretreated rats.

Before injection of PCA, extraneuronal 5-HT was measurable in dialysates from rats pretreated with

Table 2 Effects of reserpine on stereotyped behaviour and motor activity induced by *p*-chloroamphetamine (PCA 5 mg kg^{-1}, i.p.) or 5-methoxy-N,N-dimethyltryptamine (5-MeODMT 5 mg kg^{-1}, i.p.)

	Vehicle + PCA	*Reserpine + PCA*	*Vehicle + 5-MeODMT*	*Reserpine + 5-MeODMT*
Head weaving (frequency)	209.5 ± 24.3	150.2 ± 24.1	26.7 ± 6.3	9.2 ± 1.2*
Forepaw treading (frequency)	305.5 ± 39.4	322.0 ± 52.6	859.3 ± 75.0	802.0 ± 112.2
Hind limb abduction (min)	53.6 ± 1.2	54.5 ± 1.4	29.6 ± 2.6	31.2 ± 5.6
Flat body posture (min)	51.3 ± 4.4	55.0 ± 1.6	28.0 ± 3.5	30.2 ± 6.0
Straub tail (min)	0 ± 0	0.2 ± 0.1**	1.0 ± 0.2	2.2 ± 0.4*
Wet-dog shakes (frequency)	8.7 ± 2.8	16.2 ± 3.5	0.7 ± 0.2	1.0 ± 0.3

Reserpine or vehicle was injected 24 h before PCA or 5-MeODMT. Values are means ± s.e.mean of 6 rats per group. *$P < 0.05$, **$P < 0.02$ with respect to corresponding vehicle-injected rats (Mann-Whitney U test).

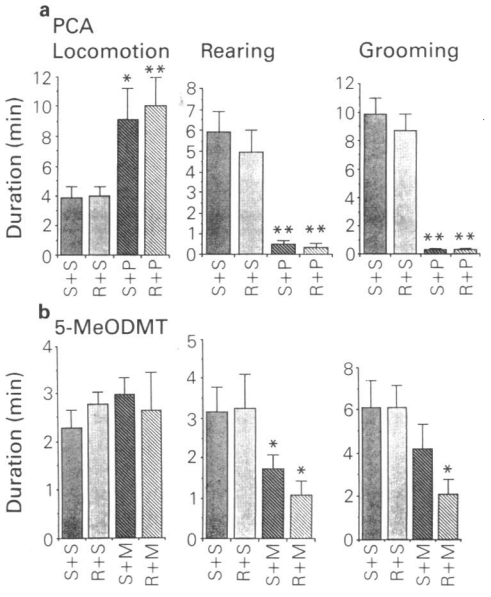

Figure 1 Locomotion, rearing and grooming in rats pretreated with vehicle or reserpine (2.5 mg kg^{-1}, i.p.) and given 0.9% NaCl, *p*-chloroamphetamine (PCA, 5 mg kg^{-1}, i.p.) (a) or 5-methoxy-N,N-dimethyltryptamine (5-MeODMT, 5 mg kg^{-1}, i.p.) (b) 24 h later. S = vehicles, R = reserpine, P = PCA, M = 5-MeODMT. Values are mean durations (min) of each behavioural component for 6 rats; vertical lines indicate s.e.mean. $*P < 0.05$, $**P < 0.01$ with respect to values for appropriate control rats not given PCA or 5-MeODMT (Duncan's multiple range test).

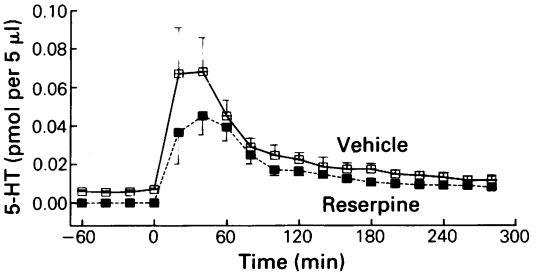

Figure 2 Effect of reserpine (2.5 mg kg^{-1}, i.p.) on the dialysate 5-hydroxytryptamine (5-HT) concentration in frontal cortex before and after *p*-chloroamphetamine (PCA, 5 mg kg^{-1}, i.p. at zero time). Values are uncorrected for recovery. Two way ANOVA showed significant effects of time ($F [14, 209] = 16.22$ and reserpine $F [1,209] = 22.71$; both $P < 0.001$). Basal 5-HT levels were taken as the mean of the four values before PCA. The mean ± s.e.mean was 0.0059 ± 0.0007 pmol per 5 µl ($n = 8$) for vehicle-injected rats and < 0.002 pmol per 5 µl (detection limit of 5-HT) for all reserpine-treated rats ($n = 10$). These values differ significantly ($P < 0.05$). Increases after PCA were significant ($P < 0.05$) over 1 and 4 h in the vehicle and reserpine pretreated groups. Net 5-HT release (increase of area under the curve) over 1 h after giving PCA (i.e. the period over which behaviour was measured) was 0.164 ± 0.047 and 0.122 ± 0.028 pmol ml^{-1} h^{-1} for the vehicle and reserpine pretreated groups, respectively (difference NS). Vertical lines indicate s.e.mean.

vehicle but not from animals pretreated with reserpine, the difference between the two groups being significant (Figure 2). However, dialysate 5-HT rose substantially on giving PCA to either group, peaking at 20–40 min after injection and then declining, but remaining significantly elevated more than 1 and 4 h later for the vehicle and reserpine pretreated groups, respectively. Mean increases over appropriate base-line values were less for the reserpine pretreated rats than for the vehicle pretreated controls at 20 and 40 min after PCA injection and significantly so at 20 min ($P < 0.05$). However, the area under the curve (AUC) for increase of 5-HT over the 1 h after giving PCA (i.e. the period over which behaviour was assessed) was not significantly different for the reserpine and vehicle-pretreated rats, though it was 26% smaller in the former group. Increases of dialysate 5-HT for both groups were similar at all times greater than 40 min after PCA injection. Figure 3 shows the individual peak values for the two groups. It can be seen that the spread of

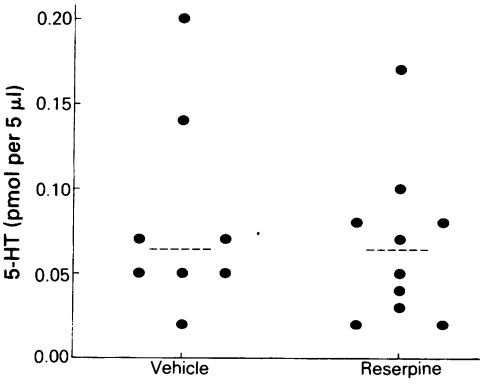

Figure 3 Individual peak values for dialysate 5-hydroxytryptamine (5-HT) after *p*-chloroamphetamine (PCA). General details as described in Figure 1. Values for reserpine and vehicle pretreated groups did not differ significantly. Mean peak times were 30 ± 4 (s.e.mean) and 38 ± 4 min for vehicle and reserpine pretreated groups, respectively (difference NS). Median peak values are shown as broken lines.

values was considerable but the median concentrations were almost identical.

Dialysate 5-HIAA values are shown in Figure 4. After injection of PCA both vehicle and reserpine pretreated groups showed a slow decline in dialysate 5-HIAA concentrations to about 2/3 of base-line values.

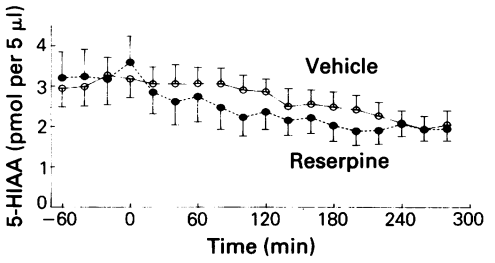

Figure 4 Effect of reserpine (2.5 mg kg^{-1}, i.p.) on dialysate 5-hydroxyindoleacetic acid (5-HIAA) concentration before and after *p*-chloroamphetamine (PCA, 5 mg kg^{-1}, i.p.). General details as described in Figure 1. Overall, reserpine significantly reduced 5-HIAA in dialysates (F [1,213] = 3.96, $P < 0.05$).

Discussion

Base-line dialysate 5-HT levels and increases after giving PCA were much lower than previously found (Sharp *et al.*, 1986; Hutson & Curzon, 1989), possibly because dialysates were previously collected within a few hours of inserting the probe in the earlier studies but a day later in the present experiments. Westerink *et al.* (1987) were only able to detect 5-HT in cortical dialysates on the first day and suggest that this largely came from platelets accumulating around the recently implanted probe. It might also have derived from terminals damaged by insertion of the probe. Our measurements were made at a time which was probably sufficient for healing of damage but not long enough for gliosis to alter the environment of the probe (Benveniste & Diemer, 1987; Benveniste *et al.*, 1987).

The base-line extracellular 5-HT concentration in the region of the dialysis probe as calculated from Figure 2 and *in vitro* 5-HT recovery values was 2.0 pmol ml^{-1}. Using a frontal cortex 5-HT concentration of 1.7 nmol g^{-1} = 2.1 nmol ml^{-1} water (assuming 80% water) previously obtained in our laboratory (Kennett *et al.*, 1986), a tissue/extracellular space ratio for 5-HT of 1050 can be calculated which is comparable with data on other transmitters (Westerink *et al.*, 1987). The corresponding ratio of 1.4 for 5-HIAA is consistent with the ratios of about 1 obtained for transmitter metabolites (Westerink *et al.*, 1987).

After reserpine treatment, although brain 5-HT was decreased by 86% and 5-HT was no longer detectable in cortical dialysates, the components of the 5-HT syndrome induced by the 5-HT releaser PCA were not significantly altered (Table 2), apart from the appearance of slight Straub tail behaviour. These results supplement the data of Kuhn *et al.* (1985) that reserpine-treated rats still showed at least three behavioural components of the response to PCA. One possible explanation is that depletion of 5-HT stores by reserpine and resultant decrease of physiological release of 5-HT (as indicated by the inability to detect it in cortical dialysates) increases postsynaptic receptor sensitivity so that the response to PCA is unaltered. However, despite findings that reserpine enhanced responses to agonists acting at 5-HT (Nakamura & Fukushima, 1978) and dopamine (Arnt, 1985) receptors, it did not alter most components of the response to the 5-HT agonist 5-MeODMT. An exception was the Straub tail which (in agreement with findings on PCA treatment) was increased. Also, head weaving frequency was decreased, in agreement with Tricklebank *et al.* (1985a,b).

The above results, as a whole, argue against reserpine increasing the responses of postsynaptic 5-HT receptors. The increased Straub tail behaviour may result from depletion of dopamine by reserpine as dopamine inhibits this behaviour (Bedard & Pycock, 1977; Andrews *et al.*, 1982; Dickinson & Curzon, 1983; Dickinson *et al.*, 1983). Conversely, the decrease of head weaving agrees with its requirement of dopamine as well as 5-HT (Andrews *et al.*, 1982; Tricklebank *et al.*, 1985a,b). However, forepaw treading and hindlimb abduction respectively require and are inhibited by dopamine (Andrews *et al.*, 1982; Dickinson *et al.*, 1983; Tricklebank *et al.*, 1985a,b) but were unaltered by reserpine, which suggests that residual dopamine was sufficient for their expression. Some availability of catecholamines is also consistent with the finding that reserpine did not alter locomotion, rearing or grooming in vehicle, PCA or 5-MeODMT-treated animals, although 50% decreases of locomotion were observed 24 h after giving 2 and 5 mg kg^{-1} of reserpine (Faith *et al.*, 1968; Geyer & Segal, 1973).

The lack of effect of reserpine treatment on most components of the 5-HT syndrome induced by PCA is largely explicable by the finding that release of neuronal 5-HT by PCA in the 1 h period of behavioural assessment did not decrease significantly. As reserpine depletes vesicular 5-HT (Shore & Giachetti, 1978), from which its physiological release probably occurs (Sanders-Bush & Martin, 1982), these results imply that PCA releases 5-HT by a mechanism which is not of physiological importance.

Although reserpine prevents vesicular storage of 5-HT, it does not alter its synthesis under conditions similar to those of the present study (Carlsson & Lindqvist, 1978), but the new 5-HT molecules remain in a small rapidly turning over cytoplasmic pool from which they are presumably released by PCA. Somewhat similar to our findings with PCA, reserpine has been shown to decrease spontaneous dopamine release but not amphetamine-induced release (Niddam *et al.*, 1985; Butcher *et al.*, 1988).

Unlike reserpine, a range of doses of the 5-HT synthesis inhibitor *p*-chlorophenylalanine (PCPA) attenuated forepaw treading and head weaving induced by PCA in parallel with the degree of depletion of brain 5-HT (Dickinson & Curzon, 1983). Under these circumstances newly synthesized 5-HT molecules are probably less available for release by PCA because of avid uptake by the partially emptied vesicles where, as indicated by high 5-HT/5-HIAA ratios (Curzon *et al.*, 1978; Dickinson & Curzon, 1983), they are protected from metabolism. In contrast, reserpine disrupts vesicular storage so that newly synthesized 5-HT remains in the neuronal cytoplasm and is readily metabolised, as indicated by low 5-HT: 5-HIAA ratios (Table 1), but is available for release by PCA.

Kuhn *et al.* (1985) suggest that the cytoplasmic 5-HT is the 'functional' transmitter pool. We agree inasmuch as we find that PCA releases cytoplasmic 5-HT with behavioural consequences. However, as reserpine without PCA markedly reduced dialysate 5-HT, physiological release of 5-HT probably occurs from vesicular stores, perhaps from a specific small pool of recently synthesized 5-HT (Shields & Eccleston, 1972) distinct from the cytoplasmic pool.

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Effect of 5-hydroxytryptamine on [^{3}H]-acetylcholine release from guinea-pig striatal slices

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1 The effect of 5-hydroxytryptamine (5-HT) on spontaneous and electrically-evoked tritium efflux was studied in guinea-pig caudate nucleus slices preloaded with [^{3}H]-choline.

2 5-HT, 10–300 μmol l^{-1}, temporarily increased the spontaneous tritium efflux (as well as the endogenous acetylcholine (ACh) release) and, after 15 min perfusion, inhibited it. The facilitatory effect of 5-HT on spontaneous efflux was increased while the inhibitory effect did not occur in slices taken from dopamine-depleted guinea-pigs.

3 The increase in spontaneous tritium efflux by 5-HT was blocked by methiothepin, methysergide (pA_2 8.7) and by the selective 5-HT$_2$ antagonist, ritanserin (pA_2 6.7).

4 The inhibition of spontaneous tritium efflux by 5-HT was prevented by methysergide and methiothepin but not by ritanserin and (−)-propranolol.

5 5-HT, 100 μmol l^{-1}, inhibited the electrically-evoked tritium efflux and this effect was unchanged in dopamine-depleted slices.

6 The inhibition of electrically-evoked tritium efflux by 5-HT was blocked by methiothepin and methysergide but not by (−)-propranolol or ritanserin.

7 These results suggest that 5-HT may exert a rapid and transient (excitatory) and a more prolonged (inhibitory) control over striatal cholinergic neurones.

Introduction

There is a great deal of evidence to suggest that 5-hydroxytryptamine (5-HT) exerts an inhibitory effect on the cholinergic system. In fact, in the whole animal, 5-HT or 5-HT-mimetic drugs increase acetylcholine (ACh) content in different brain areas (Envrard *et al.*, 1977; Samanin *et al.*, 1978; Consolo *et al.*, 1980), probably by inhibiting cholinergic neurone activity. Accordingly, 5-HT and 5-HT-releasing drugs reduce the ACh efflux from rat nucleus accumbens slices (De Belleroche & Gardiner, 1982) and from rat hippocampal synaptosomes (Maura & Raiteri, 1986).

However, the views concerning the striatum are conflicting. Some authors have reported that 5-HT reduces the activity of striatal cholinergic neurones (Vizi *et al.*, 1981; Gillet *et al.*, 1985), others have claimed that neurones of the raphe nucleus do not modulate these cholinergic cells (Robinson, 1983) while Butcher *et al.* (1976) demonstrated that dorsal raphe neurones may exert an excitatory effect on striatal cholinergic interneurones. No doubt the influence of 5-HT on the cholinergic system is very complex: for instance it has been found that i.c.v. injection of 5-HT increased ACh outflow from the exposed cortex of freely moving guinea-pigs but inhibited it in methiothepin-pretreated animals (Bianchi *et al.*, 1986).

Starting from these discrepancies, experiments have been performed to re-examine the effect of 5-HT on ACh release from guinea-pig caudate nucleus slices kept at rest or electrically-stimulated.

A preliminary account of this study has been presented (Beani *et al.*, 1985).

Methods

Tritium efflux experiments

Superfusion of guinea-pig caudate nucleus slices preincubated with [^{3}H]-choline was performed as previously described (Beani *et al.*, 1984). The cerebral tissue was sliced with a vibratome-like apparatus and the slices (0.4 μm thick) were incubated at

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37°C for 30 min in 2 ml Krebs solution containing 0.1 μmol l^{-1} [^{3}H]-choline (80 Ci $mmol^{-1}$). The slices were then rinsed and transferred to superfusion chambers and superfused at 0.5 ml min^{-1} with Krebs solution containing hemicholinium-3 (HC-3) (mmol l^{-1}: NaCl 118.5, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11, HC-3 0.01), which was bubbled with 95% O_2 and 5% CO_2.

Tritium efflux at rest The basal release of tritium was determined during a period of 5 min and the effect of the drugs on resting release was evaluated by calculating the ratio between postdrug and predrug release rates. The predrug value was taken at the 55th min and the postdrug value was taken at the 60th min (i.e. 5 min after the drug was added) and at the 80th min (i.e. 25 min after the drug was added).

The effect of 5-HT was studied by adding the amine to the perfusate either for 2 min at 56th min or at the 56th min continuously until the end of the experiment.

Electrically-evoked tritium efflux The slices were electrically stimulated on two occasions for periods of 2 min with rectangular pulses of alternate polarity (5 ms duration, 0.2 Hz, 30 mA cm^{-2}). The stimulation frequency of 0.2 Hz was chosen since at this low frequency, the evoked tritium efflux as a percentage of tritium content remained constant (S_2/S_1 ratio near 1). This was done at the 45th (S_1) and 85th (S_2) min of perfusion. Drug effect on electrically-evoked release was estimated as the ratio (S_2/S_1) of the net extra release (Beani *et al.*, 1984). This was calculated by subtracting resting release from the total electrically-evoked release.

The effect of 5-HT was tested by adding the amine either for 2 min at S_2 or 15 min before S_2 and continuing until perfusion ended. Drugs were added to the superfusion medium 15 min before S_2 to check for their effect on tritium efflux or at the beginning of superfusion to test their influence on 5-HT effects.

In some experiments the basal and electrically-evoked tritium efflux was studied in slices taken from dopamine-depleted guinea-pigs which had been given reserpine 5 mg kg^{-1} i.p. plus α-methyl-*p*-tyrosine 100 mg kg^{-1} i.p. 18 h before the experiment as well as a further 200 mg kg^{-1} i.p. of the latter drug 2 h before the experiment.

Tritium determination

At the end of the experiment the tissue was removed from the chambers, weighed and solubilized with 1 ml of Protosol (New England Nuclear, Boston, MA, U.S.A.). This tissue sample and the 5 min samples of Krebs solution were added to scintillation liquid for tritium determination (beta spectrometer Beckman LS 1800). The tissue tritium at each sampling time was calculated by adding the final tissue tritium of the slices to the whole amount of tritium released by the tissue from that time until the end of the superfusion.

All the results were given as mean ± s.e.mean of tritium efflux expressed as a percentage of tissue tritium (fractional rate) in a given time interval.

Endogenous acetylcholine release

In another set of experiments the endogenous ACh release was studied according to the method previously described (Beani *et al.*, 1978) by perfusing the slices with Krebs solution containing physostigmine sulphate 30 μmol l^{-1} and choline 20 μmol l^{-1}. The ACh content of the samples was bioassayed on tetrodotoxin-pretreated guinea-pig ileum in the presence not only of cyproheptadine and morphine (Beani *et al.*, 1978) but also methysergide (0.02 μmol l^{-1}) to prevent 5-HT-mediated responses of the ileum.

Statistical analysis

The differences between control and treated slices were determined by analysis of variance, Student's *t* test for paired and non paired data and the Wilcoxon two sample test. The apparent pA_2 values of the antagonists, tested at three concentrations, were calculated according to Schild (1947). Linear regression lines and correlation coefficient were calculated to quantify the effect of treatment.

Drugs

The drugs used were: [^{3}H]-choline (80 Ci $mmol^{-1}$, NEN), Protosol (NEN), 5-hydroxytryptamine creatinine sulphate and methysergide maleate (Sigma), methiothepin maleate (Hoffmann La Roche), (−)-propranolol HCl (ICI), ritanserin (prepared as a hydrochloride, Janssen), hemicholinium-3 (Sigma), tetrodotoxin (Sankyo), paroxetine hydrochloride (Beecham). Freshly prepared solutions were used for each experiment.

Results

Tritium efflux under control conditions

The basal overflow of tritium from guinea-pig caudate nucleus slices kept in Krebs solution containing HC-3 (10 μmol l^{-1}) was 1.18 ± 0.03% of tritium content in 5 min sample (48 expts). In the slices perfused with a solution in which the Ca^{2+}

Table 1 Effect of 5-hydroxytryptamine (5-HT) on tritium efflux from caudate nucleus slices electrically stimulated at 0.2 Hz for 2 min

Experimental conditions	*No. of expts*	*Efflux of tritium in S_1 as % of tritium content*	S_2/S_1	% *changes*
Normal guinea-pigs:				
$[Ca^{2+}]$ 2.5 mmol l^{-1}				
Without paroxetine:				
Controls	14	7.9 ± 0.31	0.91 ± 0.01	100
5-HT 100 μmol l^{-1}	14	7.5 ± 0.30	0.72 ± 0.04*	82 ± 4
With paroxetine:				
Controls	23	6.7 ± 0.24	0.81 ± 0.02	100
5-HT 100 μmol l^{-1}	20	6.01 ± 0.37	0.67 ± 0.02*	82 ± 3
$[Ca^{2+}]$ 1.25 mmol l^{-1}				
With paroxetine:				
Controls	15	2.63 ± 0.15†	0.92 ± 0.02	100
5-HT 100 μmol l^{-1}	15	2.90 ± 0.19	0.76 ± 0.02*	80 ± 2
Dopamine-depleted guinea-pigs:				
$[Ca^{2+}]$ 2.5 mmol l^{-1}				
With paroxetine:				
Controls	9	8.02 ± 0.42††	0.79 ± 0.02	100
5-HT 100 μmol l^{-1}	9	8.16 ± 0.35	0.67 ± 0.03*	85 ± 3

5-HT was added 15 min before S_2. Paroxetine was present at 3.2 μmol l^{-1}.
The difference with respect to corresponding controls is statistically significant *$P < 0.01$, according to the Wilcoxon two sample test. The difference with respect to 2.5 mmol l^{-1} $[Ca^{2+}]$ is statistically significant †$P < 0.01$, according to Student's t test for non paired data. The difference with respect to normal guinea-pigs is statistically signifcant ††$P < 0.01$, according to Student's t test for non paired data.

concentration was reduced to half the normal amount or in the presence of tetrodotoxin, 0.5 μmol l^{-1}, the tritium outflow was significantly reduced to 0.97 ± 0.03% with reduced Ca^{2+} concentration (27 expts) and to 1.05 ± 0.05% with tetrodotoxin (20 expts, $P < 0.05$ with respect to basal values in normal Krebs solution). The electrically (0.2 Hz)-evoked tritium efflux (expressed as net extra

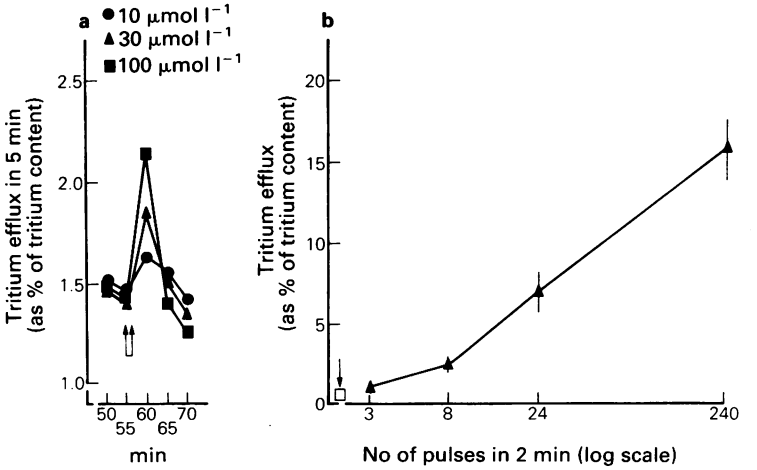

Figure 1 Effect of 5-hydroxytryptamine (5-HT) on tritium efflux from guinea-pig caudate nucleus slices. (a) A typical example of the effect of 5-HT, applied as 2 min pulse (arrows), on tritium efflux of unstimulated slices. (b) Relationship between the number of electrical pulses, applied in a 2 min period and stimulus-induced net extra efflux as % of tritium content. At the arrow, the net extra efflux in 2 min caused by 5-HT (100 μmol l^{-1}) in unstimulated slices. Each point is the mean of at least 5 experiments.

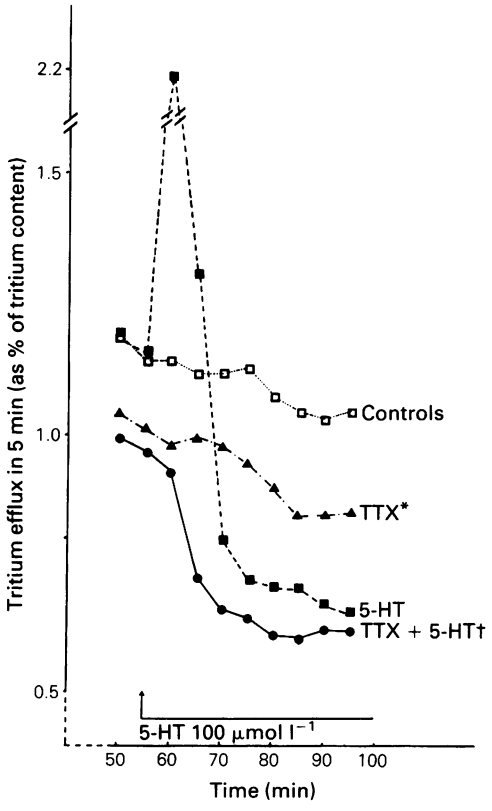

Figure 2 Effect of 5-hydroxytryptamine (5-HT, 100 μmol l^{-1}) (arrow) on tritium efflux from unstimulated caudate nucleus slices. The effect of tetrodotoxin (TTX) 0.5 μmol l^{-1}, added from the beginning of the experiment is also shown. Each point is the mean of at least 10 expts. Error bars, less than 10% of the respective means were omitted. Significantly different from control, *$P < 0.05$, Wilcoxon two sample test; significantly different from TTX alone †$P < 0.05$, Student's t test for non paired data.

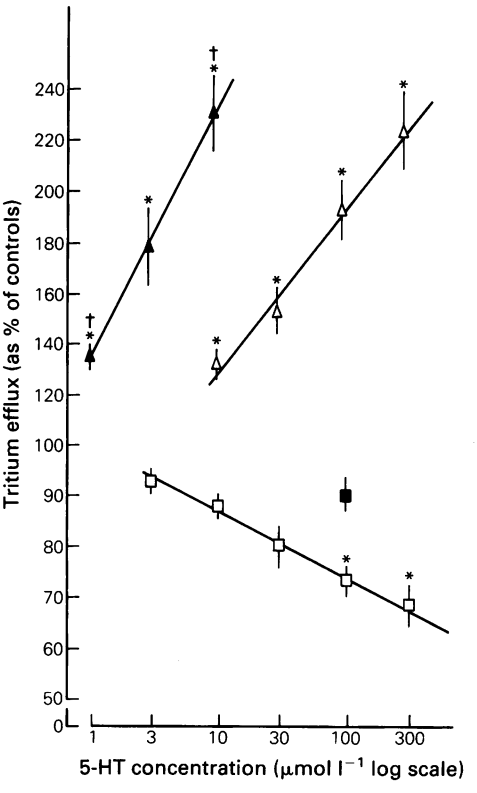

Figure 3 Dual effect of 5-hydroxytryptamine (5-HT) on tritium overflow from guinea-pig caudate nucleus unstimulated slices. Each point, as percentage of predrug value, represents the mean of 10–20 experiments; vertical lines show s.e.mean. The 5-HT-induced increase in tritium efflux from normal (△) [$r = 0.65$, $P < 0.01$] and dopamine-depleted slices (▲) [$r = 0.90$, $P < 01$] was estimated at the 60th min (i.e. 5 min after starting 5-HT perfusion). The 5-HT-induced reduction in tritium efflux from normal (□) [$r = 0.55$, $P < 0.01$] and dopamine-depleted slices (■) was estimated at the 80th min i.e. 25 min after starting 5-HT perfusion. Significantly different from the corresponding control (*$P < 0.05$) according to the Wilcoxon two sample test. Significantly different from normal guinea-pigs (†$P < 0.05$) according to Student's t test for non paired data.

release in 2 min) was 6.7 ± 0.2% of the radioactivity present in the tissue at the start of the stimulation (Table 1). If the Ca^{2+} concentration was halved, the outflow was significantly reduced (Table 1). The electrically-evoked efflux was completely abolished by tetrodotoxin.

Effect of 5-HT on tritium efflux under rest conditions

5-HT, 10–100 μmol l^{-1}, added to the superfusion medium for 2 min temporarily increased spontaneous tritium efflux in a dose-dependent manner (Figure 1a). The increase, expressed as net extra efflux in 2 min, was very small (5-HT 10 μmol l^{-1} 0.41 ± 0.09%, 5-HT 30 μmol l^{-1} 0.63 ± 0.11%, 5-HT 100 μmol l^{-1} 0.84 ± 0.13% of tritium content, 6 expts). In order to quantify the 5-HT-induced increase in tritium outflow, some slices were submitted to a different number of electrical pulses applied over a 2 min period. Figure 1b shows that 5-HT, applied for 2 min, caused a net extra efflux of tritium which was lower than that produced by applying 3 electrical pulses (5-HT 100 μmol l^{-1}: 0.84 ± 0.13; 3 electrical pulses

1.21 ± 0.012, see also Figure 1b). If the amine were present in the Krebs solution until the end of the experiment, the increase in tritium efflux vanished within a few min and after 15 min the tritium outflow was significantly reduced (Figure 2). Both the facilitatory and inhibitory effects were dose-dependent (see Figure 3) and calcium-sensitive. Tetrodotoxin, 0.5 μmol l^{-1} prevented only the facilitation leaving the inhibition of tritium efflux unchanged (Figure 2).

5-HT, 10 μmol l^{-1}, also increased the basal release of endogenous ACh from 0.39 ± 0.05 pmol g^{-1} min^{-1} to 0.54 ± 0.07 pmol g^{-1} min^{-1} (142 ± 2%) after 5 min and to 0.87 ± 0.14 pmol g^{-1} min^{-1} (224 ± 14%) after 10 min (5 expts). The facilitatory effect of 5-HT on endogenous ACh release was more persistent than the effect on tritium efflux overflow.

Effect of 5-HT antagonists on tritium efflux under resting conditions

None of the antagonists tested modified the tritium efflux at rest. Figure 4 shows that methysergide, methiothepin and ritanserin, all at 1 μmol l^{-1}, antagonized the facilitatory effect caused by 5-HT on tritium efflux. In the presence of methysergide and ritanserin, the concentration-effect curves shifted to the right. The pA_2 values were 8.7 and 6.7, respectively. In contrast, (−)-propranolol, up to 1 μmol l^{-1}, was unable to prevent the action of 5-HT on tritium overflow (Figure 4a). Ritanserin, 1 μmol l^{-1}, also prevented the 5-HT-mediated facilitation of endogenous ACh release from unstimulated slices (5 expts).

In order to examine the effect of 5-HT antagonists on 5-HT (100 μmol l^{-1})-induced inhibition, tetrodotoxin, 0.5 μmol l^{-1}, was added from the beginning of the perfusion (see Figure 2). Only methysergide, 3 μmol l^{-1}, and methiothepin, 1 μmol l^{-1}, antagonized the inhibition of resting transmitter overflow induced by 5-HT, whereas (−)-propranolol, 1 μmol l^{-1}, and ritanserin, 3 μmol l^{-1}, were ineffective both alone (Figure 4b) and when added together (data not shown).

Possible involvement of other transmitters in 5-HT effect

Bicuculline, 100 μmol l^{-1}, and naloxone, 1 μmol l^{-1}, did not change the facilitatory or inhibitory action of 5-HT on tritium efflux (Table 2). Similarly, atropine, 0.15 μmol l^{-1}, did not modify the increase in tritium overflow induced by 5-HT, 30 μmol l^{-1}, but it prevented the reduction of the efflux induced by 5-HT 100 μmol l^{-1}. Haloperidol, 0.25 μmol l^{-1}, almost completely antagonized both effects of 5-HT. Since this dopamine antagonist has an important anti-5-HT action (Leysen *et al.*, 1983), some experiments were performed in caudate nucleus slices taken from dopamine-depleted guinea-pigs. As shown in Figure 3, in depleted slices 5-HT displayed a facilitatory

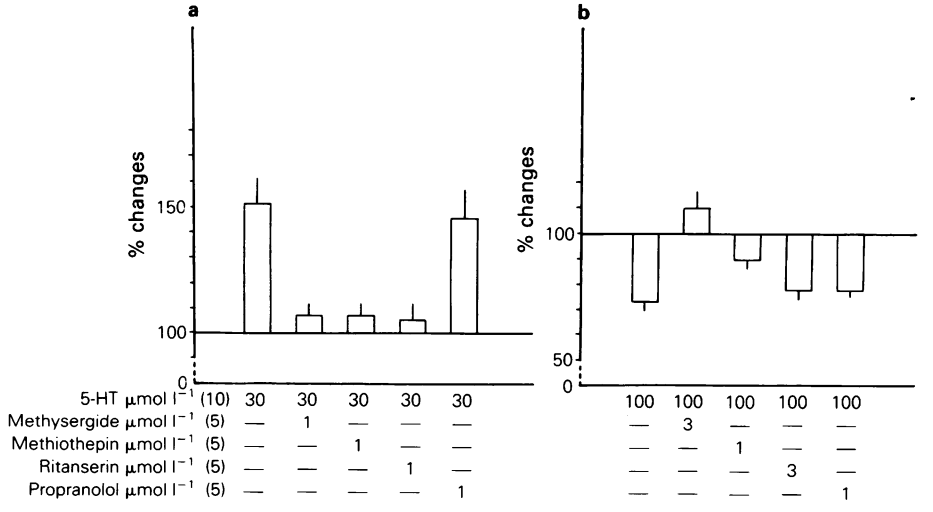

Figure 4 Effect of 5-hydroxytryptamine (5-HT) antagonists on the 5-HT-induced facilitation (a) and inhibition (b) of tritium efflux. The antagonists were added to Krebs solution at the start of perfusion. Two different sets of experiments were performed: with 5-HT 30 μmol l^{-1} to induce facilitation and with 5-HT 100 μmol l^{-1} (in the presence of tetrodotoxin 0.5 μmol l^{-1}) to induce inhibition. The data represent the percentage changes with respect to predrug efflux. Mean values are shown with s.e.mean indicated by vertical lines. Number of experiments in parentheses.

Table 2 Effect of different antagonists on spontaneous tritium efflux induced by 5-hydroxytryptamine (5-HT) from guinea-pig caudate nucleus slices

Experimental conditions	*No. of expts.*	*% of tritium efflux* (basal value = 100) *Increase by 5-HT* ($30\,\mu mol\,l^{-1}$)	*Decrease by 5-HT* ($100\,\mu mol\,l^{-1}$)
No pretreatment	15	149 ± 10*	73 ± 3*
Bicuculline $100\,\mu mol\,l^{-1}$	5	164 ± 7*	70 ± 6.3*
Naloxone $1\,\mu mol\,l^{-1}$	5	159 ± 2.5*	72 ± 2.4*
Atropine $0.15\,\mu mol\,l^{-1}$	5	198 ± 28*†	92 ± 1.7†
Haloperidol $0.25\,\mu mol\,l^{-1}$	5	108 ± 6.3†	90 ± 4.6†

Data represent the % changes with respect to predrug efflux. 5-HT $100\,\mu mol\,l^{-1}$ was tested in the presence of tetrodotoxin $0.5\,\mu mol\,l^{-1}$.
The antagonists were added to the Krebs solution from the beginning of the experiments.
The difference from predrug value is statistically significant $*P < 0.05$, according to the Wilcoxon two sample test.
The difference from 5-HT alone (no pretreatment group) is statistically significant $\dagger P < 0.05$, Student's t test for non paired data.

effect greater than that observed in normal tissue. The concentration-effect curve was shifted to the left so that the threshold dose needed to cause an appreciable increase was reduced more than ten fold (Figure 3). In contrast, the late inhibition of resting overflow caused by 5-HT, $100\,\mu mol\,l^{-1}$, was prevented (Figure 3).

Effect of 5-HT on tritium efflux induced by electrical pulses

5-HT applied during a 2 min period of stimulation (S_2) did not change the S_2/S_1 ratio (controls: 0.92 ± 0.05; 5-HT $100\,\mu mol\,l^{-1}$ 0.93 ± 0.06, 5 expts). Conversely, when 5-HT was added 15 min before starting the second cycle of stimulation (S_2), the tritium efflux was significantly reduced (Table 1). This inhibitory effect was unchanged in slices perfused with paroxetine or with halved calcium concentration. Methysergide, $1\,\mu mol\,l^{-1}$, and methiothepin, $3\,\mu mol\,l^{-1}$, completely prevented inhibition of stimulated overflow by 5-HT but ritanserin and (−)-propranolol, up to $3\,\mu mol\,l^{-1}$ and $1\,\mu mol\,l^{-1}$ respectively, were ineffective (data not shown).

To ascertain whether the 5-HT-induced inhibition of the electrically-evoked tritium efflux was dopamine-mediated, some experiments were performed in dopamine-depleted slices. The results showed that (i) the net extra-overflow was significantly increased with respect to control slices reaching 8.02 ± 0.42% of tritium content and (ii) 5-HT maintained its inhibitory effect unchanged (Table 1). Methysergide, $1\,\mu mol\,l^{-1}$, and methiothepin, $3\,\mu mol\,l^{-1}$, antagonized this effect of 5-HT.

Discussion

The data in this study demonstrate that 5-HT can both increase and inhibit the tritium efflux from guinea-pig caudate nucleus slices preloaded with [^{3}H]-choline. Similar results were obtained by Kilbinger & Pfeuffer-Friederich (1985) in the guinea-pig myenteric plexus. On the other hand, Gillet *et al.* (1985) reported that 5-HT was ineffective on basal tritium efflux in the rat striatum. Our unpublished data obtained in rat caudate nucleus slices also agree with this last finding. This discrepancy might be due to the animal species used which can display different responsiveness to 5-HT (de Fatima Campos & das Chagas Rodriguez, 1987; Moser *et al.*, 1988). Moreover, it has recently been reported that no spontaneous synaptic activity was observed in rat neostriatal neurones (Galarraga *et al.*, 1987). This is in contrast to previous results obtained by us (Bianchi *et al.*, 1982) showing that in guinea-pig caudate nucleus slices a large part of the resting ACh release is tetrodotoxin-sensitive.

Effect of 5-HT on tritium efflux at rest

Facilitation Most of the experiments were performed by measuring tritium efflux, assuming that the released radioactive material for the most part represented [^{3}H]-ACh. Previous experiments carried out by different authors (Richardson & Szerb, 1974; Wikberg, 1977; Hertting *et al.*, 1980; Lehmann & Scatton, 1982) have, in fact, demonstrated that tritium recovered from superfused slices preloaded with [^{3}H]-choline and in the presence of HC-3 is a faithful tracer of the endogenous transmitter actually

released and makes it possible to estimate the influence of drugs on the release process.

The release of endogenous ACh as well as the efflux of tritium from unstimulated caudate nucleus slices partly represent the release of transmitter from the cholinergic nerve-endings at rest and partly must be ascribed to nerve-mediated activity. In fact, in guinea-pig caudate nucleus slices, tetrodotoxin reduces the endogenous ACh release to about 1/3 and the tritium efflux to about 75% (present results, see also Bianchi *et al.*, 1982; Beani *et al.*, 1984). Since cholinergic interneurones are present in this brain area, one can infer that their firing rate consistently contributes to the so-called spontaneous ACh (or tritium) efflux. Consequently the effect of drugs can depend on changes in the basal release from the nerve endings at rest and/or in the firing rate of the cholinergic interneurones. Tetrodotoxin treatment makes it possible to discriminate between these two components (Lehmann & Scatton, 1982). The facilitation of spontaneous tritium efflux induced by 5-HT vanished in a few minutes, was dose-dependent and was prevented by tetrodotoxin. This pattern of action suggests that 5-HT promotes the repetitive firing of cholinergic cells present in caudate nucleus slices. The short-lived effect may reflect the refractoriness of cholinergic neurones to 5-HT facilitation, although exhaustion of tritium stores cannot be ruled out. In fact change in the specific activity of the released material could occur in response to 5-HT agonists and/or antagonists. Thus the reliability of the response found with the tritium efflux method was verified by measuring the endogenous ACh release. These control experiments, limited to the relatively new aspect of 5-HT action i.e. to the facilitation, confirmed the findings obtained with tritium efflux studies. Obviously a quantitative comparison of the results achieved with the two approaches is unreliable (Beani *et al.*, 1984).

The receptor involved in the increase of tritium efflux by 5-HT seems to be of 5-HT_2 type. In fact, not only methysergide and methiothepin (effective at 5-HT_1 and 5-HT_2 sites) but above all ritanserin, a specific ligand of the 5-HT_2 sites (Janssen, 1985; Bradley *et al.*, 1986), antagonized 5-HT facilitation. In contrast (−)-propranolol which is a ligand of 5-HT_1 sites (Nahorski & Willcocks, 1983), failed to prevent the increase of tritium release by 5-HT.

The experiments performed to ascertain whether 5-HT directly or indirectly increased the spontaneous tritium efflux are in favour of a direct action, although other neurotransmitters or modulators, different from those tested in this study, must be considered.

The antagonism by haloperidol of the dual effect of 5-HT may be related to the anti-5-HT action of butyrophenone derivatives (Leysen *et al.*, 1983). On the other hand, the increase in tritium efflux induced by 5-HT in dopamine-depleted slices, was potentiated as if a tonic inhibitory control by dopamine on the cholinergic interneurones had been removed (Bianchi *et al.*, 1979; Hertting *et al.*, 1980).

Inhibition 5-HT, applied for a long time, decreased tritium efflux at rest in a concentration-dependent manner. The effect was evident even in the presence of tetrodotoxin; therefore its dependence on the preceding facilitation, as well as the involvement of voltage-dependent sodium channels, can be excluded. The inhibition is long lasting (see Figure 2) at variance with the rapid disappearance of 5-HT facilitation found even with low 5-HT doses, unable to cause inhibition.

Attempts to identify the putative transmitters possibly involved in this inhibition demonstrate that bicuculline (a GABA_A receptor antagonist) and naloxone (an opioid receptor antagonist) were ineffective. In contrast, 5-HT, tested in dopamine-depleted slices failed to inhibit tritium efflux at rest. Therefore 5-HT does not seem to exert its inhibitory effect directly but by releasing dopamine which, in turn, inhibits ACh release (Bianchi *et al.*, 1979; Hertting *et al.*, 1980).

Another point that must be considered is the antagonism displayed by atropoine versus 5-HT inhibition. Probably the increase in Ca^{2+} influx induced by atropine (Nordstrom *et al.*, 1981), as reflected by the increased tritium efflux (see Table 2), could have obscured the 5-HT effect. This interpretation agrees with what has been reported by Pedata *et al.* (1983) who demonstrated that, in the presence of atropine, adenosine did not reduce ACh release. This would suggest that heteromodulation of ACh release is effective only when the autoreceptors are operative.

Among the antagonists tested, ritanserin and (−)-propranolol were totally ineffective while methysergide and methiothepin, although at high concentrations, prevented 5-HT inhibition at rest. These results do not allow for identification of the receptor involved. In fact, ritanserin, a specific ligand of 5-HT_2 receptor sites (Janssen, 1985; Bradley *et al.*, 1986), and (−)-propranolol which stereospecifically displaces [^{3}H]-5-HT from 5-HT_1, sites (Nahorski & Willcocks, 1983) were both ineffective. The antagonism by methysergide and methiothepin of the inhibition of tritium efflux caused by 5-HT may be due to stimulation of 5-HT_1 or 5-HT_2 sites other than those classified or recognized to date.

Effect of 5-HT on electrically-evoked tritium efflux

The present data concerning the effect of 5-HT on tritium efflux induced by electrical stimulation shows

that, in accordance with previous findings (Vizi *et al.*, 1981; Gillet *et al.*, 1985), 5-HT inhibits tritium outflow and that methysergide or methiothepin significantly reduce its action. However, the effect is evident only at high doses of 5-HT ($100\,\mu mol\,l^{-1}$) and is not changed even by a reuptake inhibitor such as paroxetine (Table 1). This pattern of action differs from that reported by other authors (De Belleroche & Gardiner, 1982; Gillet *et al.*, 1985) who demonstrated that 5-HT even at low concentrations of 1–$10\,\mu mol\,l^{-1}$, inhibited tritium efflux induced by potassium ($25\,mmol\,l^{-1}$) in rat brain slices. The different animal species (guinea-pig versus rat) and the different kind of stimulation (electrical stimulation vs KCl depolarization) are probably responsible for this discrepancy. Since the inhibition of electrically evoked tritium outflow is unchanged in slices obtained from dopamine-depleted guinea-pigs, it is reasonable to assume that 5-HT directly inhibits the tritium efflux, as already described by Gillet *et al.* (1985). It is worth noting, however, that in the dopamine-depleted slices the net extra release increases, suggesting a tonic control by the amine of the cholinergic cells (Bianchi *et al.*, 1979; Hertting *et al.*, 1980). In conclusion, 5-HT seems to exert a dual effect on the spontaneous tritium efflux: i.e. first a transient enhancement and, later, a dopamine-mediated inhibition of efflux. Thus, the physiological effects of 5-HT might at first be to activate and then inhibit the cholinergic neurones. Clearly the prolonged release of endogenous 5-HT in the striatum inhibits the electrosecretory coupling of the cholinergic structures as previously reported by others.

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Characterization of pigment aggregating α_2-adrenoceptors of fish melanophores by use of different agonists after partial irreversible receptor inactivation

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1 The affinity for, and the intrinsic efficacy on, postsynaptic melanosome aggregating α_2-adrenoceptors of fish melanophores was studied for B-HT 920, clonidine, medetomidine, noradrenaline, phenylephrine and UK-14,304. Investigations were carried out by evaluating the effects of progressive, irreversible inactivation of the α_2-adrenoceptors by benextramine.

2 The double reciprocal plots of equieffective concentrations of B-HT 920, clonidine, noradrenaline and phenylephrine were linear, which indicated that these compounds exerted their effects, mainly, through interaction with one receptor site.

3 The affinity for the α_2-adrenoceptor selective agonist B-HT 920, was found to be about 1000 times higher than the affinity for the α_1-adrenoceptor selective agonist phenylephrine.

4 The corresponding plot of equieffective concentrations of medetomidine was not linear, which may indicate that this imidazole compound exerted its effect through more than one receptor site. However, when phenoxybenzamine was used in place of the more selective benextramine, a linear relationship was obtained.

Introduction

Spectacular colour changes of lower vertebrates are mainly due to motile activities of specialized integumetary pigment cells, the chromatophores. These cells are highly branched, and, upon appropriate stimulation, they either aggregate pigment granules centrally or disperse them throughout the cells. Among different types of chromatophores, the melanophores, which contain melanin granules called melanosomes, have been most widely studied due to the conspicuousness of the pigment and its permanence after preparation.

It was originally suggested (Fujii, 1961) that the sympathetic nerve-to-melanophore transmission in teleosts was mediated by α-adrenoceptors, since dibenamine, antagonized effectively the melanosome aggregating effect of adrenaline and noradrenaline, as well as those of neural stimuli.

Adrenoceptors have been divided into α_1- and α_2-subtypes on a functional basis (Langer, 1974) and on the basis of the potency order of a number of selective drugs (Berthelsen & Pettinger, 1977; Wikberg, 1978). Recently, we have suggested that the postjunctional α-adrenoceptors mediating pigment migration in the melanophores from several fish species, could be characterized as being of the α_2 subtype (Andersson *et al.*, 1984; Karlsson *et al.*, 1985; 1987; 1988). This suggestion is mainly based on the observation that the α_2-adrenoceptor selective antagonist, yohimbine, strongly inhibited both the nerve-induced and noradrenaline-induced pigment aggregation, whereas the α_1-selective antagonist, prazosin, failed to or only weakly inhibited these responses. The presence of α_2-adrenoceptors as mediators of the melanosome aggregation in teleosts has recently been confirmed by Morishita (1987).

To characterize further the postjunctional α-adrenoceptors mediating pigment migration in the melanophores of the cuckoo wrasse (*Labrus ossifagus*), we have used a procedure developed by Furchgott (1966). This procedure uses the effects of progressive alkylation of the melanophore α-adrenoceptors to determine the dissociation constants and the efficacy of different α-adrenoceptor agonists.

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Methods

Isolation of scales

Scales from the cuckoo wrasse (*Labrus ossifagus* L.) were removed from the dark areas of the dermis and immediately transferred to a saline buffer solution containing (mM): NaCl 150, KCl 5.2, $CaCl_2$ 2.9, $MgSO_4$ 1.8, Na_2HPO_2 2.4, $NaHCO_3$ 17.9 and glucose 5.6 (Nilsson, 1971; Andersson *et al.*, 1984). The saline buffer was equilibrated with 5% CO_2, 95% O_2 and kept at 20°C at a pH of 7.3.

Evaluation of the α-adrenoceptor-induced pigment aggregation

The scales were placed with the dermal side down on glass microscope slides and suspended in 50 µl of the saline buffer solution. The effects of different drugs on the aggregating response of the melanophores were studied through a light microscope (Leitz SM Lux) using a magnification of × 100. The effects were evaluated according to the melanophore index (Hogben & Slome, 1931; Andersson *et al.*, 1984; Karlsson *et al.*, 1987). All drugs used in the experiments were dissolved in the saline buffer solution.

Irreversible blockade of postsynaptic α-adrenoceptors by benextramine

Concentration-response curves for different α-adrenoceptor agonists (i.e. B-HT 920, clonidine, medetomidine, noradrenaline, phenylephrine and UK-14,304) were performed after pretreatment with benextramine (Melchiorre, 1981) for 30 min. The scales were then washed 3 times in saline buffer solution for 20 min to remove non-reacted benextramine. Control scales were run in parallel. In some experiments, phenoxybenzamine, was used in place of benextramine.

Statistical and mathematical evaluation

Furchgott (1966) has developed the following equation to describe the relationship between concentration-response curves of agonists obtained in the absence, and after partial irreversible receptor inactivation

$$1/[A] = 1/q[A'] + (1 - q)/qK_A$$

where [A] and [A'] are corresponding equieffective concentrations of agonist before and after partial irreversible receptor inactivation; K_A is the equilibrium dissociation constant of the agonist and q is the fraction of receptors *not* alkylated by the treatment (see Furchgott, 1966; Furchgott & Bursztyn, 1967; Ruffolo, 1982). If [A] and [A'] are assumed to be equal to the concentration of agonist in the bathing medium, plotting the reciprocals of the concentrations of agonist before receptor inactivation (i.e. 1/[A]) against the reciprocals of equieffective concentrations of agonists after partial receptor inactivation (i.e. 1./[A']), will yield a straight line, from which the slope and the intercept may be used to calculate the dissociation constant of the agonist (K_A). The fraction of receptors not alkylated by the treatment, q, is obtained as the reciprocal of the slope (i.e. 1/slope). Concentration-response curves were evaluated by fitting the experimental data to the logistic function by non-linear regression (Parker & Waud, 1971) using a computer and digital plotter. All straight lines were fitted by linear regression.

All values are given as mean ± s.e.mean of 5 experiments, which are indicated by vertical lines on the figures.

Drugs

The following drugs were used: benextramine tetrahydrochloride (Sigma Chemical Co., St. Louis, Mo., U.S.A.), B-HT 920 (5-allyl-2-amino-5,6,7,8-tetrahydrothiazolo = [4,5-d]azepine-dihydrochloride; Thomae, Biberach, Germany), clonidine hydrochloride (Boehringer-Ingelheim, Ingelheim, Germany), cocaine chloride (ACO, Solna, Sweden), medetomidine hydrochloride (Farmos Group Ltd, Turku, Finland), (−)-noradrenaline bitartrate (Sigma Chemical Co.), phenoxybenzamine hydrochloride (Smith Kline & French Research Ltd, Welwyn, Hertfordshire), (−)-phenylephrine hydrochloride (Sigma Chemical Co.) and UK 14,304 ((5-bromo-6-[2 - imidazolin - 2 - ylamino] - quinoxaline)tartrate; Pfizer Central Research, Sandwich, Kent). Stock solutions were prepared daily in demineralized water and kept refrigerated. Shortly before use, they were diluted in the saline buffer solution.

Results

Effects of benextramine on the melanophore-aggregating response induced by different α-adrenoceptor agonists

Concentration-response curves for the pigment aggregating effects of B-HT 920 (Figure 1a), clonidine (Figure 1b), medetomidine (Figure 1c), noradrenaline (Figure 1d) and phenylephrine (Figure 1e), are presented in Figure 1. In order to eliminate neuronal uptake of noradrenaline and phenylephrine, 10^{-5} M cocaine was included. The α_2-adrenoceptor selective agonist, medetomidine, was by far the most potent, whereas the α_1-adrenoceptor selective agonist, phenylephrine,

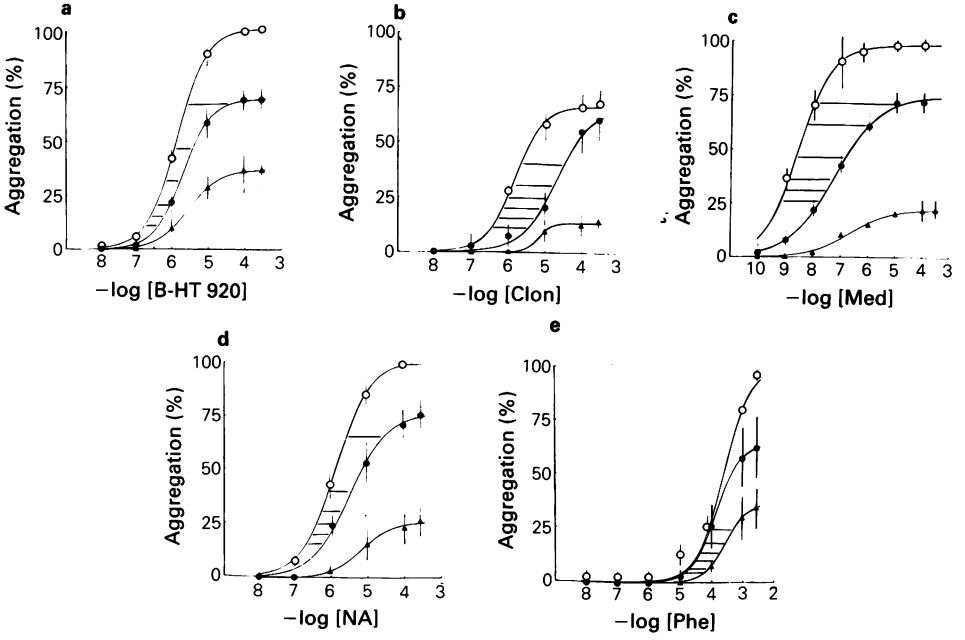

Figure 1. Concentration-response curves for the melanosome aggregating effects of B-HT 920 (a), clonidine (b), medetomidine (c), noradrenaline (d) and phenylephrine (e), following pretreatment with various concentrations of benextramine (1×10^{-6}–1×10^{-5} M; ●, ▲). Each point is the mean of observations on 5 different scales. Vertical lines indicate s.e.mean. The horizontal lines connecting controls (○) and the benextramine-treated curves represent the equieffective concentrations of the different agonists used in the double reciprocal analyses presented in Figure 2.

was a very weak agonist. Clonidine, in accordance with the result of a previous study (Andersson *et al.*, 1984), was found to be a partial agonist. Figure 1 also illustrates the progressive alkylation of α-adrenoceptors. Treatment with benextramine produces the characteristic pattern of irreversible receptor alkylation (i.e., nonparallel, rightward displacements of the concentration-response curves, with progressive reduction in the maximal effect).

The clonidine analogue UK-14,304 was found to be completely without stimulant effect on the *L. ossifagus* melanophores.

Calculation of dissociation constants

The double reciprocal plots of equieffective concentrations of B-HT 920 (Figure 2a), clonidine (Figure 2b), noradrenaline (Figure 2d) and phenylephrine (Figure 2e) seem to be linear. In the case of medetomidine (Figure 2c), a non-linear relationship was obtained, which may indicate that medetomidine acts through more than one binding site. However, a linear relationship was obtained after partial receptor inactivation with the alkylating compound, phenoxybenzamine (3×10^{-6}–1×10^{-5} M; not shown). Table 1 presents the calculated dissociation constants. In the table, and in the following, the dissociation constant of medetomidine is approximated to the value obtained by calculating equieffective concentrations before and after partial inactivation with phenoxybenzamine. It must be stressed that this value is only an approximation, since the treatment with benextramine revealed a non-linear relationship. Nevertheless, it is evident from this table, that the α_2-adrenoceptor selective agonists B-HT 920, clonidine and medetomidine have a high affinity to the binding sites, whereas the α_1-adrenoceptor selective agonist phenylephrine has a low affinity.

Calculation of efficacies

The intrinsic efficacies were calculated from the slopes of the double reciprocal plots of the equieffective agonist concentrations after treatment with different concentrations of benextramine (or phenoxybenzamine). From the analyses of progressive inactivation of the α-adrenoceptor pool with benextramine it was possible to plot the effect of a given agonist as a function of the fraction of α-adrenoceptors remaining intact (Figure 3). In accordance with Furchgott & Bursztyn (1967), the relative efficacies of a series of agonists on a given response

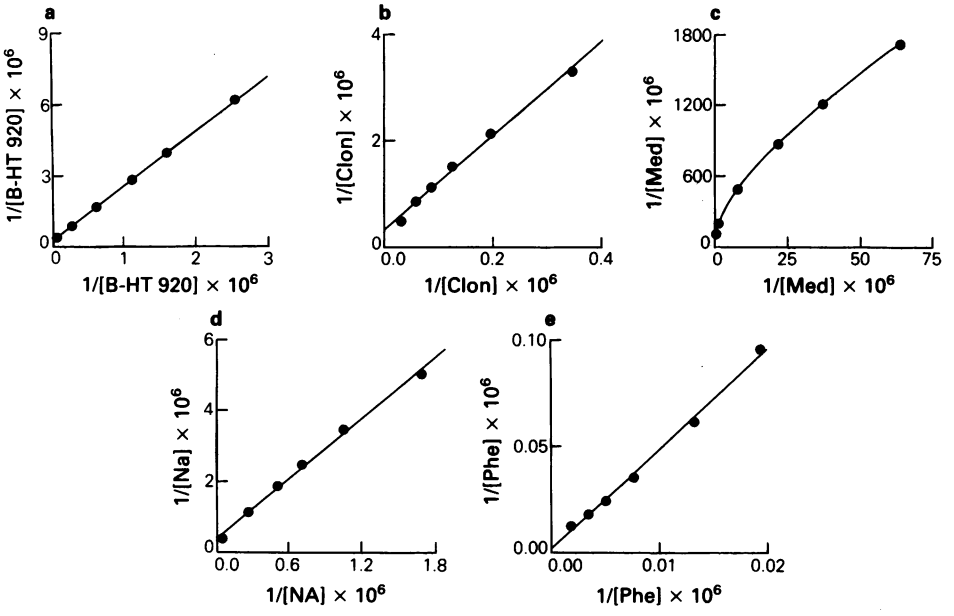

Figure 2 The reciprocals of equieffective concentrations of B-HT 920 (a), clonidine (b), medetomidine (c), noradrenaline (d) and phenylephrine (e) regarding the aggregating effects on untreated and benextramine-treated melanophores. Each point is the mean of observations on 5 different scales. The particular equieffective concentrations used in the reciprocals are indicated by horizontal lines in Figure 1. The slope and the intercept are used to calculate the dissociation constant of the agonists (K_A) (see Methods). The fraction of receptors not alkylated by benextramine (q) is obtained as the reciprocal of the slope (i.e. 1/slope).

could be estimated. In order to do this, the ratios between the receptors that must be occupied by the agonist with the highest efficacy and the fraction of receptors that must be occupied by another agonist for the same response, need to be determined. From the data shown in Figure 3, it was possible to determine the fraction of receptors needed to produce half-maximal response to the different agonists. For the compounds in Figure 3, relative efficacies are given in Table 1.

Table 1 Dissociation constants (K_A) and relative efficacy for different α-adrenoceptor agonists in respect to their melanosome aggregating effect within the melanophores of the cuckoo wrasse

Agonists	K_A (M)[a]	*Relative efficacy*[b]
B-HT 920	5.1×10^{-6}	0.04
Clonidine	2.7×10^{-5}	0.09
Medetomidine[c]	3.0×10^{-6}	1.00
Noradrenaline	4.8×10^{-6}	0.28
Phenylephrine	2.9×10^{-3}	0.08

[a] The dissociation constants (K_A) are determined, in accordance with the method of Furchgott (1966), from the double reciprocal plots of equieffective concentrations of the different agonists on untreated and benextramine-treated melanophore-containing scales

[b] Half maximal response expressed as a fraction of the half maximal response obtained with medetomidine

[c] K_A is determined after receptor inactivation with phenoxybenzamine

Discussion

Upon appropriate stimulation, the mammalian α_2-adrenoceptors are known to lower the cyclic AMP levels via an inhibiting action on adenylate cyclase (Ui, 1984; Limbird, 1988). In previous investigations, we have shown that the α_2-adrenoceptors of the teleost melanophores also act via a negative control of adenylate cyclase.

The present investigation reveals that the α-adrenoceptors within the melanophores of the cuckoo wrasse have a high affinity for certain α_2-adrenoceptor agonists ($K_A \approx 10^{-6}$ M), whereas they have a low affinity for the α_1-adrenoceptor agonist phenylephrine ($K_A \approx 10^{-3}$ M). This is in agreement with our previous findings concerning the ability of selective α_1- and α_2-adrenoceptor antagonists (prazosin and yohimbine) to inhibit nerve-induced and noradrenaline-induced melanosome aggregation. Although the potency of phenylephrine

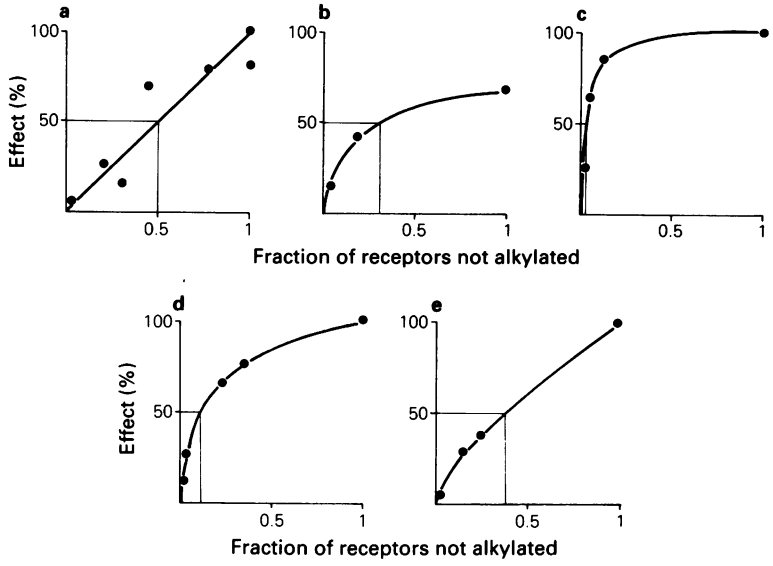

Figure 3 Relationship between maximum melanosome aggregating effects of B-HT 920 (a), clonidine (b), medetomidine (c), noradrenaline (d) and phenylephrine (e), and the fraction of receptors not alkylated by the irreversible α-adrenoceptor antagonist benextramine (in the case of medetomidine (c), phenoxybenzamine was used).

was only about 1/1000 of that observed for the α_2-adrenoceptor selective agonists, it functioned as a full agonist with a comparatively high intrinsic efficacy. Therefore, the low potency of phenylephrine seems to depend solely on its very low affinity for the α_2-adrenoceptor.

Two distinct classes of chemical compounds have been shown to interact with presynaptic α-adrenoceptors (Starke *et al.*, 1975); namely, imidalzolines and phenylethylamines. Ruffolo *et al.* (1977) suggested that imidazolines and phenylethylamines may interact at different sites on the α-adrenoceptors, based on the lack of cross-desensitization between these two classes of compounds.

Although a relatively novel class of azepine derivatives, such as B-HT 920, is not comprised of phenylethylamines or imidazolines, pharmacologically, the activity of the derivatives parallels that of imidazolines (Ruffolo, 1984). The two classes of α-adrenoceptor agonists seem to bind to and/or activate α-adrenoceptors in a different manner, regardless of the subtype they activate.

According to Furchgott (1966), a linear relationship of the double reciprocal plot of equieffective concentrations of an agonist, before and after a partial and irreversible inactivation of a receptor, is only obtained when the irreversible antagonist occupies the same active site of the receptor as does the agonist. Thus, a non-linear relationship of the double reciprocal plot indicates more than one binding site. The novel imidazole compound medetomidine, which has been shown to possess a very high α_2-adrenoceptor selectivity and potency (Savola *et al.*, 1986), possibly interacts with two, kinetically different binding sites in the melanophores. The non-linear relationship of the double reciprocal plot of equieffective concentrations of medetomidine, before and after receptor inactivation with benextramine, probably reflects different sensitivities of the binding sites to benextramine. In accordance with previous findings (Mottram, 1983a,b; Vizi *et al.*, 1983; Mottram & Thaker, 1984), it is proposed that pharmacologically, these sites could be classified as being of an imidazoline-binding type and of a phenylethylamine-binding type.

In contrast to the effect of benextramine on the medetomidine-induced melanosome aggregation, it was found that phenoxybenzamine resulted in a linear relationship. This difference presumably reflects a non-selective action of phenoxybenzamine (Furchgott, 1966).

Benextramine-inactivation of the responses elicited by B-HT 920, clonidine, noradrenaline and phenylephrine, resulted in linear relationships, indicating that these compounds interact mainly with a single receptor site, although not necessarily with the same site. We suggest that in the case of B-HT 920 and clonidine this binding site is of an imidazoline type. Furthermore, it is tempting to assume that noradrenaline and phenylephrine interact with a phenylethylamine-binding site within the melanophores. Medetomidine presumably interacts with

both of these two sites, which may explain both the high potency and the very high intrinsic efficiency of that particular imidazole compound.

Although a great degree of uncertainty concerning the affinity of medetomidine exists, it is interesting to compare the affinity and the intrinsic efficacy of medetomidine to those of the other α_2-adrenoceptor agonist B-HT 920. Such a comparison reveals that the high potency of medetomidine, is, to a great extent, due to the extremely high intrinsic efficacy of this particular compound. Medetomidine has to occupy only 2% of the receptors to elicit a 50% aggregation response of the melanophores, whereas B-HT 920 must occupy 50% of the receptors for a comparable response. The response of B-HT 920 seems to be directly proportional to the stimulus generated by the drug-receptor complex.

It has been suggested that α-adrenoceptor agonists with high intrinsic efficacy activate several concurrent signalling mechanisms, whereas α-adrenoceptor agonists with low intrinsic efficacy activate only a single signalling mechanism (Nichols & Ruffolo, 1988). A number of physiological findings suggest that attenuation of cyclic AMP production is not sufficient to serve as the only signal for eliciting certain α_2-adrenoceptor-mediated effects (Limbird, 1988). Investigations are under way at our laboratory to test the possibility that α_2-adrenoceptor agonists with high intrinsic efficacy, in addition to inhibiting the adenylate cyclase of the melanophores, also activate other concurrent signalling mechanisms.

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Lipomobilizing effects of procaterol and yohimbine in the conscious dog: comparison of endocrinological, metabolic and cardiovascular effects

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1 Lipid mobilization during a hypocaloric diet may be enhanced by a pharmacological approach using β_2-adrenoceptor agonists or α_2-adrenoceptor antagonists. Studies were undertaken in the dog, an animal model presenting fat cell antilipolytic α_2- and lipolytic β-adrenoceptors, in order, firstly, to demonstrate the presence of β_2 subtype adrenoceptors on adipocytes and, secondly, to compare the effects of procaterol (β_2-adrenoceptor agonist) and of yohimbine (α_2-adrenoceptor antagonist) on metabolic, endocrinological and cardiovascular parameters.

2 Procaterol strongly stimulates lipolysis in dog adipocytes *in vitro*. The utilisation of selective β_1- and β_2-adrenoceptor antagonists (bisoprolol and ICI 118,551) in both lipolysis and binding studies (displacement of [^{3}H]-dihydroalprenolol binding) demonstrated the presence of the two β-adrenoceptor subtypes in dog fat cells.

3 Infusion of either yohimbine or procaterol (10 and 0.4 nmol min^{-1} kg^{-1}, respectively for 30 min). provoked an equivalent increase in plasma non-esterified fatty acids (+100%). Procaterol, but not yohimbine, induced hyperglycaemia (+120%). Plasma insulin was weakly enhanced by yohimbine (+120%) as compared to the increase given by procaterol (+500%).

4 Both drugs stimulated sympathetic nervous system activity, as indicated by the increased plasma noradrenaline concentration, but only yohimbine increased the plasma adrenaline level.

5 Cardiovascular measurements indicated that procaterol strongly enhances heart rate and transiently decreases mean blood pressure. Yohimbine exhibits a weaker effect on heart rate and slightly increases mean blood pressure.

6 The present work clearly indicates that lipid mobilization is enhanced during fasting in the dog by selective β_2-adrenoceptor stimulation or by α_2-adrenoceptor blockade. This enhanced lipolytic effect may result either from a direct action of the drugs on the adrenoceptors of fat cells or from an activation of the sympathetic nervous system. Procaterol suffers major limitations since it strongly increases heart rate, immunoreactive insulin and glycaemia. On the other hand, yohimbine induces only minor modifications both in cardiovascular and endocrinological parameters.

Introduction

Lipid mobilization is accelerated during the earlier part of a period of energy restriction or fasting in man and animals. It is likely that the decrease in plasma insulin levels is the major factor involved in the induction of lipid mobilization since insulin is a potent antilipolytic hormone (Cahill *et al.*, 1966; Lilavivathana *et al.*, 1978; Jensen *et al.*, 1987). Moreover, some endocrinological modifications, consisting of a reduction in plasma thyroid hormones (Suda *et al.*, 1978; Stockholm *et al.*, 1987) and a probable decrease in sympathetic activity (Landsberg & Young, 1978), could also be partly responsible for the weight-loss resistance usually observed during obesity therapy. However, data concerning the activity of the nervous system during caloric restriction are controversial. It seems probable that in man (James *et al.*, 1981; Scheen *et al.*, 1981) and in the rat (Young & Landsberg, 1977)

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starvation is accompanied by a lowering of the level of adrenergic neuronal activity. Finally, it has also been shown that both the number of β-adrenoceptors and the response to catecholamines are decreased in subcutaneous adipose tissues during fasting (Östman et al., 1984). This latter effect could enhance the antilipolytic action of catecholamines mediated by α_2-adrenoceptors (Berlan & Lafontan, 1985) and heighten the weight-loss resistance. On the whole, these observations support the idea that several adaptive mechanisms preserve energy mobilization by reducing the metabolic rate.

Pharmacological approaches aimed at the improvement of lipid mobilization could have potential interest in the treatment of obesity. Two major approaches may be proposed for enhancement of lipolysis. First, it should be possible to stimulate directly fat cell β-adrenoceptors with β-adrenoceptor agonists (Ricks et al., 1984). The use of a non-specific β-adrenoceptor agonist (e.g. isoprenaline) or physiological catecholamines should be avoided since these agents possess powerful cardiovascular effects (Stanton et al., 1969) and are rapidly eliminated after administration. A selective β_2-adrenoceptor agonist with a high degree of selectivity would theoretically be more suitable in minimising cardiac effects (O'Donnell & Wanstall, 1979). Moreover, β_2-adrenoceptor agonists should be efficient as lipolytic agents since the human fat cell possesses lipolytic mechanisms linked to β_2-adrenoceptors (Mauriège et al., 1988).

The use of α_2-adrenoceptor antagonists may also offer potential interest for lipid mobilizing strategies. These drugs could act by blockade of the antilipolytic α_2-adrenoceptor on fat cell membranes (Arner & Östman, 1976; Fain & Garcia-Sainz, 1983; Berlan & Lafontan, 1985; Mauriège et al., 1987). They could also activate sympathetic tone either through actions on the central nervous system (Papeschi & Theiss, 1975) or indirectly by blockade of the presynaptic α_2-adrenoceptors located on the sympathetic nerve endings (Starke et al., 1975; Langer, 1980) or by a mixture of both actions. These mechanisms could be additive in the improvement of lipid mobilization. This strategy for lipid mobilization has recently been applied by our group in man (Galizky et al., 1988).

The comparative evaluation of lipid mobilization together with endocrinological and cardiovascular responses induced by α_2-adrenoceptor antagonist and β_2-adrenoceptor agonist compounds is of importance for the therapeutic utilization of these drugs. Such a comparative approach is presented in this paper, using the dog as a model. The properties of dog fat cell α- and β-adrenoceptors have been defined in previous studies. In dog adipocytes the pharmacological properties of the β- and α_2-adrenoceptor binding sites were found to be very similar to those defined in human adipocytes (Milavec-Krisman & Wagner, 1978; Berlan et al., 1982; Taouis et al., 1987), although the presence of β_2-adrenoceptors still has not been clearly demonstrated.

The following studies were undertaken. Firstly, to explore the presence of β_2-adrenoceptor subtypes in dog fat cells and to establish the validity of the model. Secondly, to compare *in vivo* the relative potencies of yohimbine, an α_2-antagonist and procaterol, a highly selective β_2-agonist (Yabuuchi, 1977; O'Donnell & Wanstall, 1985) with special attention being paid to their side-effects on the cardiovascular system and to various endocrinological and metabolic indices such as plasma glucose, insulin and catecholamines.

Methods

Adipose tissue was obtained from female mongrel dogs, weighing 15 to 20 kg which had been fasted overnight before omentum biopsy taken immediately after the induction of general anaesthesia by 30 mg kg^{-1} pentobarbitone (i.v.).

Preparation of dog adipocyte membranes

The isolated adipocytes, obtained as previously described (Mauriège et al., 1987), were washed four times in a hypotonic lysing medium to elicit total cell breakage and recover fat cell ghosts. The lysing medium was composed of $MgCl_2$ 3.5 mM, $KHCO_3$ 1 mM, Tris HCl 2 mM, pH 7.5 and the following protease inhibitors: leupeptin (5 μg ml^{-1}), EGTA (3 mM), benzamidine (10 μM) and phenylmethylsulphonyl fluoride (100 μM). Homogenization had to be performed at 20–22°C to minimize trapping of plasma membranes in the coalescing fat cake. Crude adipocyte ghosts were pelleted by centrifugation (40,000 *g*, 10 min) at 4°C, washed twice in the same buffer and pelleted in similar conditions. At the end of the washing procedure, they were suspended in the lysing buffer at a final concentration of 2–2.5 mg protein ml^{-1} and immediately frozen. The membrane preparation was stored at −80°C and generally used within 1 week for binding analysis. Protein was assayed by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Radioligand binding studies

[^{3}H]-dihydroalprenolol was used, as previously described (Mauriège et al., 1987), for the identification of β-adrenoceptor binding sites. Thawed frozen membranes were rehomogenized with four pestle strokes in a Potter homogenizer and washed once

with the buffer used in the binding studies. Binding assays were performed in a final volume of 400 μl as previously described (Lafontan *et al.*, 1983; Berlan & Lafontan, 1985) with only minor modifications. Incubations were carried out at 25°C in a water bath for 20 min under constant shaking at 120 cycles min^{-1}. At the end of the incubation, duplicate 400 μl aliquots of medium were diluted in 4 ml of ice-cold incubation buffer (50 mM Tris-HCl, 0.5 mM $MgCl_2$, pH 7.5) and vacuum filtered immediately through Whatman GF/C glass fibre filters placed on a Millipore manifold. The filters were washed twice with 10 ml portions of ice-cold incubation buffer, placed in minivials containing 4 ml of liquid scintillation medium (Ready-Solv MP, Beckman Instruments Ltd) and counted in a Packard scintillation spectrometer at an efficiency of 40%. Specific binding was taken as the amount of radioactivity bound to the membranes and defined as the difference between the total binding and binding in the presence of 10 μM unlabelled ligand (propranolol). Specific binding ranged between 70–80% of the total binding for [^{3}H]-dihydroalprenolol at a final concentration of 5 nM. For competition experiments, various adrenoceptor antagonists were dissolved in the binding buffer, diluted, and added to the assay (100 μl) just before experimentation.

Lipolysis measurements

The lipolytic activity was analysed on isolated adipocytes prepared according to the technique of Rodbell (1964) with minor modifications. Krebs-Ringer bicarbonate buffer containing bovine serum albumin (3.5%) and glucose (6 mM), adjusted to pH 7.4 with 1N NaOH just before use, was utilized as previously described (Berlan & Lafontan, 1985). After collagenase action (1 mg ml^{-1} in Krebs-Ringer bicarbonate buffer), isolated fat cells were washed three times and the packed cells were brought to a suitable dilution in Krebs-Ringer bicarbonate buffer. The cells were incubated in plastic vials (1 ml incubation medium containing 10,000–15,000 cells) with gentle shaking in a water bath under an air phase at 37°C. After 90 min, the incubation tubes were placed in an ice bath, the adipocytes were separated from the buffer and 200 μl of the underlying fluid were removed for the enzymatic determination of glycerol which was taken as an index of the lipolytic rate. Total lipids were evaluated gravimetrically after extraction. Pharmacological agents were added just before the beginning of the incubation in 10 μl volume of vehicle to obtain the final concentration. The lipolytic activity of the isolated fat cells was tested using isoprenaline (a non-selective β-adrenoceptor agonist) and procaterol (a new highly selective β_2-adrenoceptor agonist) and various selective β_2- and β_1-adrenoceptor antagonists (respectively ICI 118,551, O'Donnell & Wanstall, 1980; and bisoprolol, Kaumann & Lemoine, 1985).

In vivo *studies*

Twelve normal female mongrel dogs (15–20 kg) were used for the *in vivo* studies. Several days before each experiment, the dogs were trained to stand still for 3 to 4 h on a Pavlov table and accustomed to intravenous infusion and blood sampling. Systolic and diastolic blood pressure were recorded by means of a catheter introduced into the abdominal aorta via the left femoral artery under local anaesthesia (xylocaine 5%) and connected to a Gould P23ID transducer on one channel of a Honeywell recorder. Mean blood pressure was measured by automatic electrical integration of systolic and diastolic blood pressure. Heart rate was obtained using a heart period meter triggered by blood pressure. At 09 h 00 min, after 24–36 h fasting, the dogs were placed on the Pavlov table. Another catheter was placed in the antecubital vein for drug perfusion. After that, the dog remained quiet until the beginning of the experiment. Two blood samples were taken before drug infusion (times −15 min and 0 min). Then yohimbine or procaterol were infused for 30 min. Other blood samples were taken 5 min, 15 min and 30 min after the beginning of infusion and every 15 min after the end of infusion.

Blood samples were collected in heparinized tubes and immediately centrifuged; plasma was collected and frozen at −20°C until use. Blood glucose was determined with a glucose-oxidase technique (Biotrol kit, Paris, France) and insulin by radioimmunoassay (commercial radioimmunoassay kit, Institut Pasteur, Paris). Glycerol was determined by an enzymatic procedure and non-esterified fatty acids (NEFA) by the Wako enzymatic method (commercial radioimmunoassay kit, Biolyon, Lyon).

For catecholamine determinations, blood was collected on lithium heparin with 10 mM sodium metabisulphite and centrifuged at 5000 *g* at 0°C; the plasma was stored at −80°C. Catecholamines were selectively isolated from the plasma by adsorption on activated alumina, then eluted with 0.1 M perchloric acid. Dihydroxybenzylamine was used as internal standard to monitor recovery from the extraction step. Catecholamines were assayed by high performance liquid chromatography using electrochemical (amperometric) detection (Waters h.p.l.c. system) (Valet *et al.*, 1988).

Data analysis

All experiments were performed in duplicate. Values are means ± s.e.mean. Student's paired *t* test was

used for comparisons between matched pairs, differences were considered significant when P was less than 0.05. The Mann Whitney U test was used for analysis of unpaired data. The determination of the relative affinity of adrenoceptor compounds for β-adrenoceptor binding sites was carried out by computer-assisted calculations (Barlow, 1983).

Drugs and chemicals

[^{3}H]-dihydroalprenolol (specific activity 75 Ci mmol^{-1}) was obtained from Amersham (Amersham, U.K.). Yohimbine hydrochloride, isoprenaline and propranolol were purchased from Sigma Chemical Company (St Louis, MO, U.S.A.). The following drugs were obtained from the companies mentioned: procaterol hydrochloride (5-(1-hydroxy-2-isopropylaminobutyl)-8-hydroxycarbostyril), Warner-Lambert; ICI 118,551 (erythro-DL-(7 methylindan-4-yloxy)-3-isopropylaminobutan-2-ol); (−)-bisoprolol hemifumarate, Merck (Darmstadt, F.R.G.). Collagenase, bovine serum albumin and enzymes for glycerol assays came from Boehringer Mannheim (F.R.G.). All other chemicals and organic solvents were of reagent grade.

Results

In vitro *studies*

Lipolytic studies on dog fat cells The first aim of the study was to determine the properties of the β-adrenoceptor controlling the lipolytic processes in dog fat cells. Comparative lipolytic responses to isoprenaline and procaterol in the same batch of dog fat cells are shown in Figure 1. An increase in glycerol production was observed in the range 10^{-9}–10^{-5} M for the two β-adrenoceptor agonists. The maximal lipolytic action of procaterol was 80–85% of the maximal effect of isoprenaline. Figure 1b shows the dose–response curves with the responses expressed as a percentage of the maximal effect given by each agent at 10^{-5} M. The mean EC_{50} value (agonist concentration required for half maximal stimulation of lipolysis) for procaterol was not significantly different from that of isoprenaline (62.2 ± 21.3 nM and 27.2 ± 13.5 nM, respectively, $n = 5$).

Figure 2 shows the antagonism of isoprenaline with the highly selective β_2- (ICI 118,551) and β_1-adrenoceptor (bisoprolol) antagonists on dog fat cell lipolysis. There is no significant difference in the

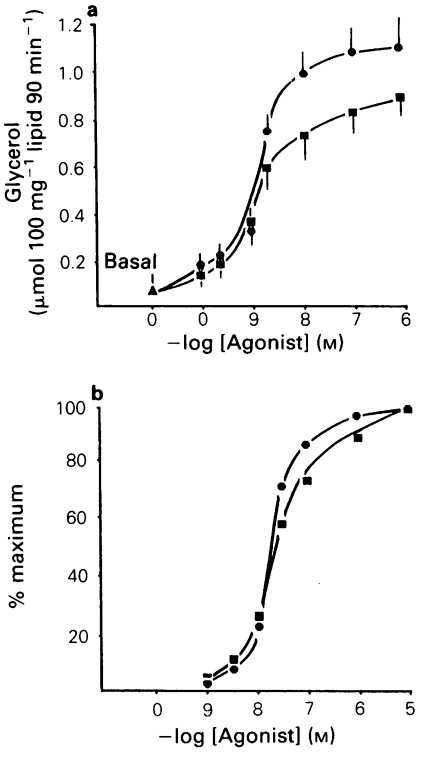

Figure 1 Effect of isoprenaline (●) and procaterol (■) on glycerol release by dog omental fat cells. Fat cells (15–20 mg total lipid) were incubated in 1 ml of Krebs-Ringer bicarbonate solution containing 3.5% bovine albumin and 6 mM glucose as described in Methods. (a) Lipolysis is expressed as μmol of glycerol released by the cells. Values are the mean, with vertical lines indicating s.e.mean, from 5 animals. (b). Lipolysis is expressed as a percentage of the maximal lipolytic response initiated by each agent (10^{-5} M).

potency of the two selective β-adrenoceptor antagonists. The IC_{50}s (concentrations required to suppress 50% of the lipolytic effect of isoprenaline) were both in the micromolar range. Then, to confirm the presence of β_2-adrenoceptors in dog fat cells, we compared the antagonistic effect of these selective β-adrenoceptor antagonists on procaterol-induced lipolysis (Figure 2b). The selective β_2-adrenoceptor antagonist ICI 118,551 was a more potent inhibitor of lipolysis than bisoprolol in such conditions, the IC_{50}s were 86.3 ± 29.6 nM and 36,900 ± 10,600 nM, respectively. This result strongly supports the presence of a β_2-adrenoceptor in dog fat cells. However, it should be noticed that the incubation medium used for lipolysis contains albumin (3.5 g% buffer)

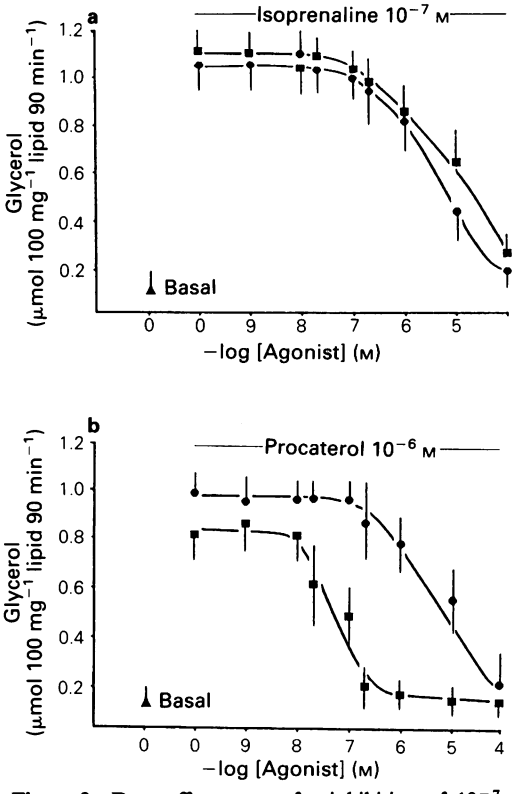

Figure 2 Dose–effect curves for inhibition of 10^{-7} M isoprenaline (a)- or 10^{-6} M procaterol (b)-stimulated lipolysis in the presence of the β_1-adrenoceptor selective antagonist bisoprolol (●) or the β_2-adrenoceptor selective antagonist ICI 118,551 (■). Lipolysis is expressed in μmol of glycerol released by the cells. Values are the mean, with vertical lines indicating s.e.mean, from 5 animals.

and it is not possible to exclude the possibility that adrenoceptor antagonists binding to albumin (or their relative lipid solubility) could introduce some discrepancies in the measurement of their relative affinities. So, in order to improve the characterization of β-adrenoceptor subtypes in dog adipocytes, binding studies were carried out on fat cell membranes.

Comparative binding of specific β_1- or β_2- adrenoceptor compounds on β-adrenoceptors of dog fat cell membranes In order to characterize the mixed population of β_1- and β_2-adrenoceptors in dog fat cell membranes, [^{3}H]-dihydroalprenolol binding competition studies were performed with the selective β_1- and β_2-adrenoceptors antagonists previously used in the lipolysis experiments.

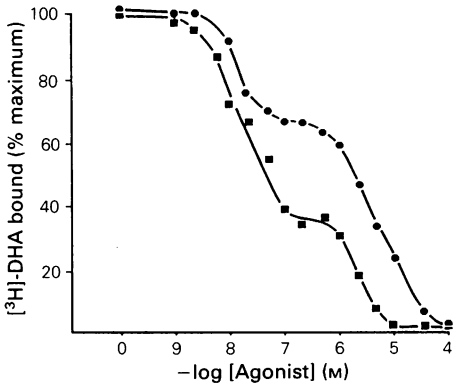

Figure 3 A typical example of the inhibition by either bisoprolol (●) (β_1-adrenoceptor selective antagonist) or ICI 118,551 (■) (β_2-adrenoceptor selective antagonist) of specific [^{3}H]-dihydroalprenolol ([^{3}H]-DHA) binding to dog fat cell membranes.

The dissociation of [^{3}H]-dihydroalprenolol specific binding (75 to 85% of the total binding) in the presence of 10^{-4} M ICI 118,551 or bisoprolol was rapid in both cases ($t_{1/2}$ for dissociation was 10.7 min and 7.6 min, respectively) and represented a first order process. The calculated first order rate constants for the displacement of 5 nM [^{3}H]-dihydroalprenolol (slope of the semilogarithmic plot calculated by regression analysis) by ICI 118,551 (0.0021 min^{-1}) and bisoprolol (0.0019 min^{-1}) were not significantly different. The displacement studies showed that both ICI 118,551 and bisoprolol suppressed 80–90 of the specific [^{3}H]-dihydroalprenolol binding on dog fat cell membranes.

Typical competition curves for two compounds are depicted in Figure 3. Bisoprolol and ICI 118,551 completely displaced the binding of [^{3}H]-dihydroalprenolol in dog fat cell membranes. The competition curves were shallow and a shoulder was observed between 10^{-8} M and 10^{-6} M. The results clearly showed two competition phases for each compound indicating the presence of two β-adrenoceptor subtypes. The relative proportions of β_1- and β_2-adrenoceptor subtypes were evaluated by a computed-aided curve fitting technique. The results, depicted in Table 1 indicate that the β_2-subtype represents about 60–70% of the total number of [^{3}H]-dihydroalprenolol binding sites whatever the specificity of the β-adrenoceptor antagonist used for subtype definition. In contrast, the non-selective β-adrenoceptor antagonist, propranolol, gave steep displacement curves with a mean K_i of 4.0 ± 0.1 nM and a Hill coefficient of 1; the competition curves could be modelled by a single homogeneous class of binding site.

Table 1 Relative percentages of β_1- and β_2-adrenoceptor binding sites in dog fat cell membranes: characteristics of competition between [^{3}H]-dihydroalprenolol binding and ICI 118,551 (β_2-adrenoceptor antagonist) or bisoprolol (β_1-adrenoceptor antagonist)

	(n)	%	β_1 K_D (nM)	%	β_2 K_D (nM)	*Selectivity ratio*
ICI 118,551	(5)	34 ± 5	3795 ± 640	66 ± 5	44 ± 12	104 ± 25
Bisoprolol	(5)	32 ± 3	74 ± 36	68 ± 3	7724 ± 2490	252 ± 85

%: percentages of β_1- or β_2-sites evaluated from [^{3}H]-dihydroalprenolol competition curves (as indicated in Figure 3). The Selectivity ratio is calculated from the formula: $K_D\ \beta_1/K_D\ \beta_2$ for ICI 118,551 and $K_D\ \beta_2/K_D\ \beta_1$ for bisoprolol. Non-linear analysis of the competition curves by the method of Barlow (1983) yielded the two dissociation constants and the percentages of the receptor subtypes. Each evaluation is the mean ± s.e.mean of *n* experiments.

In vivo *studies*

As expected, a 24–36 h fasting period induced an increase in plasma NEFA concentrations in the dog (283 ± 35 nM and 602 ± 52 nM before and after fasting).

Metabolic and endocrinological effects of yohimbine or procaterol infusion In a preliminary study, we compared the effects of the infusion of various increasing doses of yohimbine and procaterol on plasma NEFA levels in normal fasted dogs. Yohimbine provoked an increase in plasma NEFA concentrations, the maximal effect was obtained with the dose rate of 10 nmol min^{-1} kg^{-1} and persisted after the end of the infusion (Figure 4). The lowest procaterol dosing rate tested (0.08 nmol min^{-1} kg^{-1}) slightly increased plasma NEFA levels only at 5 min after the beginning of the infusion (Table 2) while a higher dose of procaterol (4 nmol min^{-1} kg^{-1}) promoted an increase in plasma NEFA concentrations which was equivalent to that induced by the dose of yohimbine selected for this study (Figure 4).

The time-course of plasma NEFA concentration increments induced by these two agents was different (Figure 4). A significant NEFA mobilizing effect with yohimbine only appeared 15 min after the beginning of the infusion and the maximal effect was reached within 30 min (a longer infusion period was without any additional effect). Moreover, the increment in plasma NEFA levels persisted until the end of the experimental period. However, the lipomobilizing action of procaterol was rapid, the increase in plasma NEFA levels was significant 5 min after the beginning of the infusion and the maximal effect was reached after 15 min. A progressive decrease was observed after cessation of the infusion.

Modifications in various metabolic and endocrinological parameters during and after the infusion periods of the drugs are presented in Figure 4 and Table 2. At the dose used, yohimbine did not significantly modify plasma glucose levels. Plasma immunoreactive insulin concentrations were transiently increased 15 to 30 min after the beginning of yohimbine infusion while post-infusion plasma immunoreactive insulin levels quickly returned towards the

Table 2 Effect of an infusion of procaterol (0.08 nmol min^{-1} kg^{-1}) on plasma non-esterified fatty acids (NEFA) and glucose concentrations, immunoreactive insulin (IRI) and heart rate in the conscious dog

Time (min)	*−15*	*0*	*5*	*15*	*30*	*45*	*60*
NEFA (μM)	802 ± 129	833 ± 136	*938 ± 152	1004 ± 179	780 ± 160	517 ± 131	*507 ± 119
Glucose (mM)	5.5 ± 0.3	5.6 ± 0.3	*6.3 ± 0.4	*7.6 ± 0.5	*8.5 ± 0.8	*7.3 ± 0.6	*6.1 ± 0.6
IRI (μu ml^{-1})	24 ± 6	21 ± 7	*89 ± 46	*155 ± 50	*126 ± 47	41 ± 14	25 ± 8
Heart rate (beats min^{-1})	94 ± 4	95 ± 4	*138 ± 3	*163 ± 11	*175 ± 16	*144 ± 7	*130 ± 8

Values are the mean ± s.e.mean from 5 animals.
* Significantly different ($P < 0.05$) compared to the values measured at time 0.

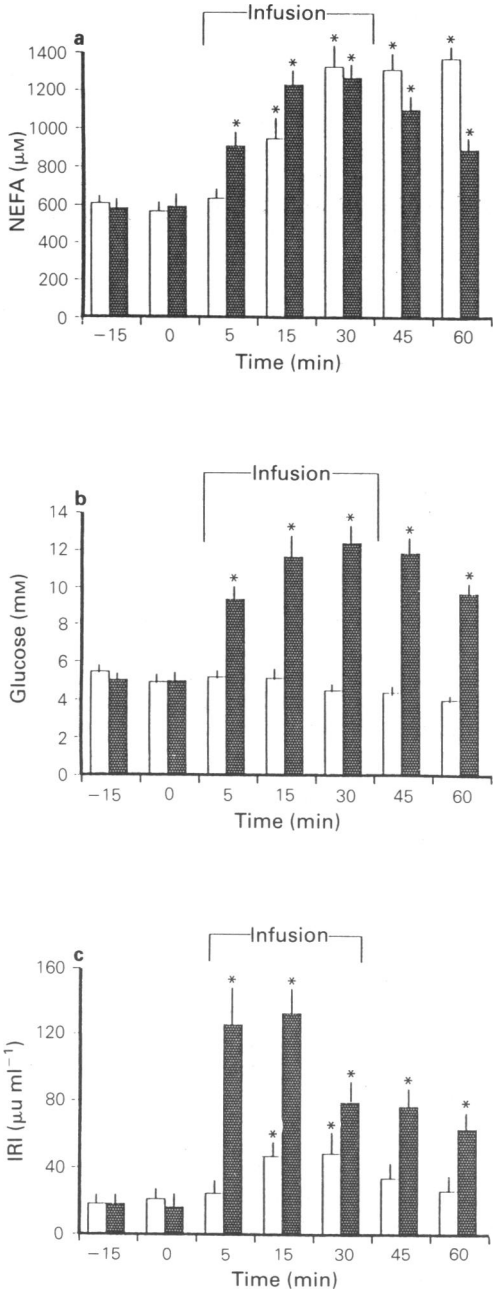

Figure 4 Comparative effects of i.v. infusion of yohimbine (10 nmol min^{-1} kg^{-1}, open columns) and procaterol (0.4 nmol min^{-1} kg^{-1}, stippled columns) on dog plasma levels of (a) non-esterified fatty acids (NEFA), (b) glucose (c) immunoreactive insulin (IRI). Values are the mean, with vertical lines indicating s.e.mean, from 5 animals. * Significantly different ($P < 0.05$) as compared to the values measured before drug infusion.

Table 3 Effect of 30 min i.v. infusions of procaterol or yohimbine on plasma noradrenaline and adrenaline levels in dog

	Noradrenaline (pg ml^{-1})	*Adrenaline* (pg ml^{-1})
Resting values	341 ± 63	188 ± 47
	Procaterol (0.4 nmol min^{-1} kg^{-1})	
A	1065 ± 203*	149 ± 30
B	904 ± 119*	229 ± 26
	Yohimbine (10 nmol min^{-1} kg^{-1})	
A	1349 ± 194*	578 ± 97*
B	1517 ± 258*	755 ± 170*

Resting values: before drug infusion.
A: at the end of drug infusion
B: 30 min after the end of drug infusion.
Values are the mean ± s.e.mean from 6 animals.
* Significantly different ($P < 0.05$) as compared to the values measured before drug infusion (resting values).

pre-infusion values. Plasma noradrenaline and adrenaline levels (basal values: 341 ± 63 pg ml^{-1} and 188 ± 47 pg ml^{-1}, respectively) increased during the infusion period and remained elevated after the infusion was stopped (Table 3).

With regard to the effects of procaterol, an early increase in plasma glucose levels was observed; the hyperglycaemia persisted during the infusion and the effect was dose-dependent (Table 2 and Figure 4). It was noticeable that the lower dose of procaterol increased plasma glucose levels but was ineffective on NEFA mobilization. Procaterol strongly stimulated insulin secretion with the two doses used in this study (500% whatever the dose). Plasma noradrenaline levels increased during the infusion and remained elevated after the infusion. Plasma adrenaline levels, however, were unchanged during the whole experimental period (Table 3).

Cardiovascular effects of yohimbine and procaterol Yohimbine infusion induced a progressive and significant rise in blood pressure until 15 min (Figure 5). The maximal effect occurred at the end of infusion and persisted until the end of the experimental period. The heart rate also increased, maximal values were measured at the end of the infusion.

Procaterol induced a significant and transient decrease in mean blood pressure. The maximal effect appeared at 5 min from the start of the infusion (Figure 5). After that, a return to normal pressure was observed during the rest of the infusion period.

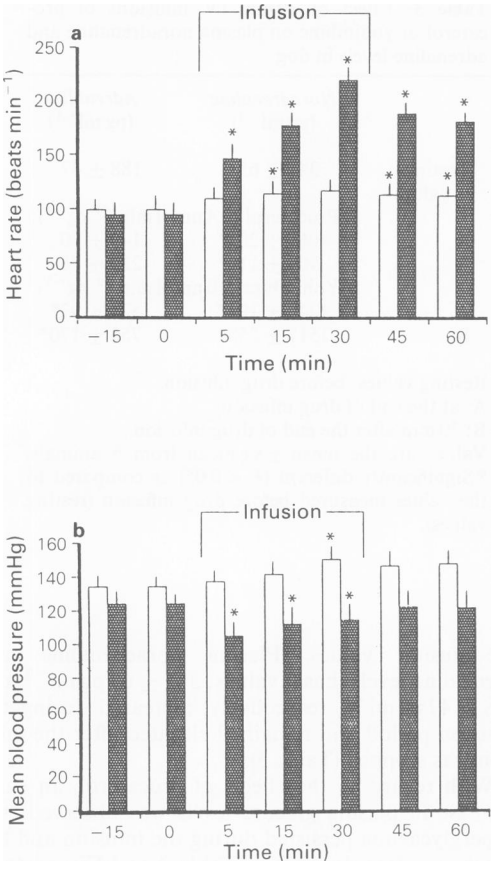

Figure 5 Comparative effects of i.v. infusion of yohimbine (10 nmol min^{-1} kg^{-1}, open columns) and procaterol (0.4 nmol min^{-1} kg^{-1}, stippled columns) on heart rate (a) and mean blood pressure (b). Values are mean, with vertical lines indicating s.e.mean, from 5 animals. *Significantly different ($P < 0.05$) compared to the values measured before drug infusion.

Procaterol induced a strong increase in heart rate which was dose-dependent (Table 2 and Figure 5), the lower dose used in this study provoked an 84% increase while the higher dose induced a 133% increase in heart rate.

Discussion

The present results demonstrate firstly that the dog is a suitable model for the exploration of the lipid-mobilizing effects of various compounds active at adrenoceptors, since the properties of dog fat cell β- and α_2-adrenoceptors (present study and Taouis *et al.*, 1987) are similar to those of man (Lafontan & Berlan, 1980; Mauriège *et al.*, 1988). Secondly, β_2-adrenoceptor agonists and α_2-adrenoceptor antagonists are both useful tools for induction of lipid mobilization. Thirdly, in the dog, the α_2-adrenoceptor antagonist yohimbine has fewer side-effects (endocrino-metabolic, cardiovascular) than the β_2-adrenoceptor agonist, procaterol.

Previous studies on dog fat cell membranes showed that the specific binding of [^{3}H]-dihydroalprenolol, a non-selective β-adrenoceptor antagonist, was saturable and of high affinity. Scatchard analysis defined a population of binding sites which was characterized by an equilibrium dissociation constant (K_D) of 2.2 ± 0.6 nM and a maximum binding of 378 ± 76 fmol mg^{-1} protein (Taouis *et al.*, 1987). The first part of the present work was devoted to the demonstration that dog fat cells possess a typical β_2-adrenoceptor. Lipolytic measurements and binding studies using either a highly specific β_2-agonist procaterol (O'Donnell & Wanstall, 1985; Brodde *et al.*, 1988) or antagonist ICI 118,551 (O'Donnell & Wanstall, 1980) clearly demonstrate the existence of β_2 adrenoceptors in dog adipocytes. The stimulation with the β_2-adrenoceptor agonist enhanced lipolysis with the same efficiency as the non-selective β adrenoceptor agonist isoprenaline. It can be concluded that stimulation of the β_2-adrenoceptor (which constitutes 60–70% of the total population of the β-adrenoceptors) is able to provoke the maximal lipolytic rate in fat cells. The presence of a mixed population of β-adrenoceptors is also supported by the analysis of the dose-response relations of ICI 118,551 and bisoprolol on isoprenaline-induced lipolysis. The antagonistic effect of these drugs only appeared at concentrations around 10^{-6} M when the selectivity of the compounds is lost. The weaker concentrations of each selective compound were without any effect on isoprenaline-enhanced lipolysis. Moreover, in binding studies, bisoprolol and ICI 118,551 displaced 60–70% and 40–30% of [^{3}H]-dihydroalprenolol specific binding, respectively, at concentrations lower than 10^{-7} M. So this last result and previous findings on α_2-adrenoceptors in dog adipocytes (Taouis *et al.*, 1987) led us to conclude that the dog is a valuable model for performing *in vivo* pharmacological studies in this field. The β-adrenoceptors of fat cells of the most commonly used species, the rat, are atypical β-adrenoceptors (Harms *et al.*, 1982; Bojanic & Nahorski, 1983). The adipocytes of other species have generally been less extensively studied (Harms *et al.*, 1982; Mersmann, 1984).

Another important point is that the dog shows early and late endocrino-metabolic adaptations to fasting that are quite similar to those observed in

man. De Bruijne (1979) obtained an increase in NEFA and a decrease in insulin levels in the dog. A concomitant reduction of thyroid function, as reflected by a progressive reduction of plasma triiodothyronine and thyroxine levels during fasting, was also observed (De Bruijne *et al.*, 1981).

Two possible strategies can be proposed with the aim of improving lipid mobilization during fasting or hypocaloric diet. Arner *et al.* (1981) showed that the increased NEFA levels during fasting could be enhanced by an infusion of noradrenaline. However, this strategy cannot be currently used in obesity therapy since noradrenaline induces large increments in systolic and diastolic blood pressures. The use of β-adrenoceptor agonists has also been proposed in order to stimulate fat cell lipolysis and thermogenesis (Henny *et al.*, 1987). An approach based on the use of a selective β_2-agonist is of putative interest in order to minimize side-effects on the cardiovascular system. The recent characterization of β_2-adrenoceptors on human fat cells by our group (Mauriège *et al.*, 1988) supports this idea. The second approach involves the use of α_2-adrenoceptor antagonists which can act directly on fat cell antilipolytic α_2-adrenoceptors and indirectly stimulate lipolysis by sympathetic nervous system activation. This strategy has recently been tested in obese patients by Berlin *et al.* (1986) and Zahorska-Markiewicz *et al.* (1986) but their results exhibit some discrepancies.

The present data demonstrate that yohimbine and procaterol infusions promote an increment of plasma NEFA levels. In the dog, as in the rat (Pfister & Keeton, 1988) and in man (Galitzky *et al.*, 1988), yohimbine enhances sympathetic activity as indicated by the increased plasma catecholamine levels. This effect can be attributed to a peripheral action either on sympathetic nerve endings (blockade of presynaptic α_2-adrenoceptors) or to a central action (Wemer *et al.*, 1979; Hedler *et al.*, 1981), or both. Procaterol increases plasma noradrenaline levels while leaving adrenaline concentration unchanged. This differential action suggests a direct effect of the drug on the presynaptic β-adrenoceptors located on sympathetic nerve endings (Majewski, 1983; Rump & Majewski, 1987); these presynaptic β-adrenoceptors seem to belong to the β_2-subtype (Brodde *et al.*, 1988). The lipid mobilizing effect of yohimbine may be explained through sympathetic activation, α_2-adrenoceptor blockade on fat cells, or both. The effect of procaterol may consist of both direct stimulation of the β_2-adrenoceptor of adipocytes and noradrenaline release.

Thus, the present data indicate both pharmacological approaches are satisfactory with regard to induction of lipid mobilisation. However, the two drugs provoked various endocrino-metabolic side-effects and also altered cardiovascular functions. For a comparison of the undesirable effects of the drugs, we selected yohimbine and procaterol doses inducing equivalent lipomobilization.

Procaterol administration increased plasma insulin levels by acting on the β_2-adrenoceptors located on the pancreatic β cells (Loubatières *et al.*, 1971) and also raised plasma glucose levels. In addition it provoked strong tachycardia. This chronotropic effect of procaterol on the heart could be attributed to a direct effect of the drug on cardiac β_2-adrenoceptors (Hedberg *et al.*, 1980; Johansson & Persson, 1983; O'Donnell & Wanstall, 1985; Arnold *et al.*, 1985). Moreover, it is noticeable that a five fold lower dose of procaterol increases plasma insulin and glucose levels and the heart rate without any major effect on NEFA levels. The drug also induced a weak and transient hypotensive action which was probably counteracted during the infusion by baroreflex tachycardia, by the increase in noradrenaline levels or both. Similar effects have been described in man by Henny *et al.* (1987) with a new type of β-adrenoceptor agonist (RO 16-8714), the selectivity of which is still undefined. Even with the highly selective β_2-adrenoceptor agonist used in the present experiments there is no reduction of side-effects. The strong stimulation of heart rate, the hyperglycaemia and hyperinsulinaemia consequent upon procaterol infusion represent limitations for the use of β_2-adrenoceptor agonists as lipomobilizing agents.

As compared to procaterol, yohimbine had a weaker effect on some of these parameters (its effect on heart rate and plasma insulin levels represent only 14% and 26% of the increase induced by procaterol). Plasma glucose concentration was not modified by yohimbine administration. As opposed to procaterol, yohimbine weakly increased the mean blood pressure in spite of an increment of both catecholamines in the plasma. This lack of major hypertensive effect is not surprising since the blockade of vascular α_2- and also probably of α_1-adrenoceptors by yohimbine (a weakly selective adrenoceptor antagonist) could minimize the vascular responses consecutive to catecholamine release. However, the hypertensive effect could represent a potential limitation for the use of this compound in man. Nevertheless, in a previous study (Galitzky *et al.*, 1988), we showed that acute oral administration of yohimbine ($0.2\,mg\,kg^{-1}$) in normal fasting volunteers increased NEFA levels without any major impact on heart rate or blood pressure.

In summary, these results demonstrate firstly, that the dog fat cell β-adrenoceptors are composed of two subtypes that can be interpreted in terms of classical β_1- and β_2-adrenoceptors, as demonstrated by competition studies conducted with highly selective β_1- and β_2-adrenoceptor antagonists. Secondly, that

both yohimbine and procaterol induce a lipomobilizing effect in the dog. Procaterol stimulates β_2-, while yohimbine blocks α_2-adrenoceptors in fat cells. These effects are enhanced by the activation of the sympathetic nervous system (as indicated by the increase in plasma catecholamine levels). Owing to their side-effects, selective β_2-adrenoceptor agonists (like procaterol) may have major limitations in their use as lipomobilizing agents since they strongly enhance heart rate, insulin secretion and glucose production. In contrast, α_2-adrenoceptor antagonist compounds (like yohimbine) could offer interesting possibilities for the improvement of lipid mobilization since they have limited side-effects.

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Electrophysiological effects of adenosine and adenosine triphosphate on sheep Purkinje fibres under normal and simulated ischaemic conditions

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1 The electrophysiological effects of adenosine and adenosine triphosphate (ATP) were examined in sheep Purkinje fibres, superfused *in vitro* with either a normal or a hypoxic, hyperkalaemic and acidotic physiological salt solution (PSS). The ability of adenosine to modify the effects of noradrenaline on action potential characteristics was also investigated.

2 The only statistically significant effects of adenosine (10^{-6}–10^{-4} M) and of ATP (10^{-6}–10^{-4} M) on normal action potential characteristics were a slight dose-dependent shortening of the action potential by adenosine and a depolarization by ATP, 10^{-4} M.

3 Superfusion with a hypoxic, hyperkalaemic and acidotic PSS caused marked reductions in resting membrane potential, upstroke and duration of the action potential.

4 Both adenosine and ATP attenuated the reduction in the rate of rise of the upstroke and the amplitude of the action potential caused by the modified PSS.

5 Adenosine did not alter the noradrenaline-induced effects on automaticity or on action potentials of normal or depressed Purkinje fibres.

6 Adenosine and ATP had electrophysiological effects on Purkinje fibres, exposed to conditions *in vitro* that mimic mild myocardial ischaemia, that were different from those observed on normally polarized fibres.

Introduction

It has been known for many years that adenosine and adenosine triphosphate (ATP) exert pronounced electrophysiological effects on the normal mammalian heart (Drury & Szent-Gyorgi, 1929). These effects include slowing of atrioventricular conduction (Urthaler & James, 1972), shortening of atrial action potential duration (Hollander & Webb, 1957) and modulation of the cardiac electrophysiological effects of catecholamines on Purkinje and ventricular tissue (Belardinelli *et al.*, 1982; Rardon & Bailey, 1984). Furthermore, in experimental animals, both adenosine (Fagbemi & Parratt, 1984; Parratt & Wainwright, 1985) and ATP (G. Boachie-Ansah, unpublished observations) protect the heart against ischaemia-induced ventricular arrhythmias. The mechanism(s) underlying this antiarrhythmic action of adenosine and ATP is not known, yet only one study appears to have examined the electrophysiological actions of adenosine on ischaemic cardiac tissue (Rosen *et al.*, 1983).

The aim of this work was to investigate the electrophysiological effects of adenosine and of ATP on sheep cardiac Purkinje cells exposed *in vitro* to conditions that mimic mild myocardial ischaemia. It has been shown that, during mild myocardial ischaemia *in vivo*, depressed Na^+-dependent action potentials occur (Downar *et al.*, 1977) and that these can be simulated *in vitro* by superfusion with a hypoxic, hyperkalaemic and acidotic physiological salt solution (Gilmour & Zipes, 1980). Therefore, the effects of adenosine and ATP on action potential characteristics of sheep Purkinje fibres superfused with such a modified PSS were examined and compared with those on normal fibres. In addition, the ability of adenosine to modify the cardiac electrophysiological effects of noradrenaline (Rardon & Bailey, 1984) was examined both under normal and simulated 'ischaemic' conditions. A preliminary account of these findings has been published (Boachie-Ansah *et al.*, 1986).

Methods

Action potential recording

Sheep hearts were obtained from a local abattoir and delivered in physiological salt solution (PSS) to the laboratory within 30 min of excision. Purkinje

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fibres were pinned to the silastic base of the recording chamber and superfused at a rate of 5 ml min^{-1} with a normal PSS equilibrated with 95% O_2:5% CO_2. An equilibration period of about 1 h in normal PSS was allowed before beginning the experimental protocol. The composition of the normal PSS was as follows (mM): NaCl 125, $NaHCO_3$ 25, NaH_2PO_4 1.2, $MgCl_2$ 1.0, KCl 5.4, $CaCl_2$ 1.8 and glucose 5.5. That of the modified solution was as follows (mM): NaCl 141.5, $NaHCO_3$ 8.5, NaH_2PO_4 1.2, $MgCl_2$ 1.0, KCl 8.0, $CaCl_2$ 1.8, and glucose 5.5. The modified PSS was gassed with 95% N_2:5% CO_2, and this yielded a Po_2 and a pH in the organ bath of 33.9 ± 1 mmHg and 6.8 ± 0.01 u, respectively, compared with values in normal PSS of 419 ± 31 mmHg and 7.31 ± 0.02 u. The bath temperature was maintained at 36.5 ± 0.5°C.

The preparations were stimulated at a frequency of 1.5 Hz by rectangular pulses, 1 ms in duration and twice threshold voltage, delivered through a bipolar silver electrode. Transmembrane action potentials were recorded by conventional microelectrode techniques. The variables measured were as follows: resting membrane potential (RMP); action potential amplitude (APA); the maximum rate of depolarization of phase 0 (MRD), which was determined by an electronic differentiating circuit; and the action potential duration at 50 and 90% repolarization levels (APD_{50} and APD_{90}).

Experimental protocol

To observe drug effects on normal preparations, 8–10 action potentials were recorded before and 30–40 min after cumulative addition of the drug, dissolved in reservoirs of gassed normal PSS to obtain final bath concentrations detailed below. In a different set of fibres, the effects of modified PSS alone were measured; multiple action potentials were recorded before and at 30, 60, 90 and 120 min following exposure to the modified PSS. Drug-induced effects in the presence of modified PSS were examined following two protocols. In one, action potentials were recorded before and 30 min after superfusion with modified PSS alone and subsequently following the administration of two cumulative concentrations of the drug dissolved in modified PSS. Action potentials were again recorded 30 min after the addition of each concentration of the drug. In another series of experiments, drugs were administered prior to the introduction of the modified PSS. In this case, action potentials were recorded before and 30 min after the addition of one concentration of the drug dissolved in normal PSS and subsequently at 30 min intervals, over a 2 h period, following a changeover to the modified PSS containing the same concentration of the drug.

In order to study any adenosine-noradrenaline interaction, noradrenaline (10^{-5} M) was superfused for 30 min under normal or 'ischaemic' conditions followed by superfusion of adenosine (10^{-4} M) in the continued presence of noradrenaline. Action potential recordings were taken before, in the presence of noradrenaline and in the presence of both adenosine and noradrenaline. The frequency of spontaneous action potential initiation in the preparations was also measured in these experiments.

Drugs and concentrations

The following were used: adenosine (Sigma) 10^{-6}, 10^{-5} and 10^{-4} M; adenosine 5'-triphosphate (vanadium free) (ATP) (Sigma), 10^{-6}, 10^{-5} and 10^{-4} M; (−)-noradrenaline bitartrate (Sigma) 10^{-5} M.

Analysis of data

The mean values of measurements from each set of 8–10 multiple impalements were obtained and used to represent the data from each preparation. Data are reported either as mean values ± s.e.mean or mean percentage change from control values ± s.e.mean and are derived from 4–7 experiments. Multiple treatment and control mean values were analysed by a one-way Analysis of Variance and where the F-value permitted further analysis, individual treatment means were compared with respective control values by a Modified t test. For all other comparisons a two-tailed Student's t test was employed. $P < 0.05$ was considered to be statistically significant.

Results

Effects of adenosine and of ATP on normal action potential characteristics of sheep Purkinje cells

In the five preparations subjected to adenosine, control measurements of the variables were as follows: resting membrane potential -86.9 ± 0.3 mV, action potential height 109 ± 1.5 mV, maximum rate of depolarization of phase 0 (MRD) 427 ± 23 V s^{-1}, APD_{50} 155.2 ± 10.1 ms and APD_{90} 232.0 ± 9.3 ms. Adenosine (10^{-6}, 10^{-5} and 10^{-4} M) caused no statistically significant changes in resting membrane potential, action potential amplitude or in MRD. A slight concentration-dependent shortening of action potential duration, measured at both 50 and 90% repolarization, was observed following exposure to adenosine. The respective percentage reductions from the control APD_{90} were 3 ± 0.8, 6 ± 1.2 and 11 ± 0.5 ($P < 0.05$) following adenosine 10^{-6}, 10^{-5} and 10^{-4} M. Similar percentage reductions in APD_{50} were observed.

Table 1 The effects of the 'ischaemic' (i.e. hypoxic, hyperkalaemic, and acidotic) physiological salt solution on action potential characteristics of sheep Purkinje fibres

	Control	*Time post-ischaemia* (min)			
		30	60	90	120
RMP (mV)	−87.2 ± 0.8	−78.3 ± 0.4*	−78.1 ± 0.5*	−78.1 ± 0.3*	−77.5 ± 0.4*
MRD (Vs^{-1})	523 ± 51	261 ± 27*	231 ± 17*	196 ± 16*	195 ± 18*
APA (mV)	119.4 ± 1.4	86.6 ± 3.8*	82.4 ± 3.4*	79.9 ± 3.8*	80.0 ± 3.9*
APD_{50} (ms)	158.0 ± 10.6	106.1 ± 11.5*	98.1 ± 12.5*	96.8 ± 11.6*	97.6 ± 11.4*
APD_{90} (ms)	234.0 ± 15.6	170.1 ± 13.7*	165.5 ± 14.5*	164.3 ± 14.9*	165.2 ± 14.7*

*$P < 0.05$ significantly different from control value; $n = 7$
RMP, resting membrane potential; MRD, maximum rate of depolarization of phase 0; APA, action potential amplitude; APD, action potential duration at 50% repolarization level; APD_{90}, action potential duration at 90%, repolarization level

In five preparations exposed to ATP, control measurements similar to those detailed above were obtained. The only statistically significant effect observed of ATP (10^{-6}–10^{-4} M) was a slight depolarization which was significant at 10^{-4} M ATP (−84.4 ± 0.4 mV compared with a control value of −87.9 ± 0.4 mV). ATP-induced effects on action potential duration were inconsistent in that in 3/5 preparations a slight shortening was observed but in the other 2, ATP marginally prolonged APD.

Effects of altered physiological salt solution alone

Table 1 summarises the effects of the modified PSS on action potential characteristics of sheep Purkinje cells measured over a 2 h exposure period. By 30 min of exposure, the hypoxic, hyperkalaemic and acidotic PSS had reduced the resting membrane potential and there were concomitant reductions in action potential height and in MRD. The action potential duration was also markedly reduced. Although resting membrane potential was stable throughout the remainder of the observation period, MRD and action potential height continued to fall slightly between 30 and 120 min of exposure to the modified PSS.

The excitability of the preparations was depressed by the conditions that mimicked ischaemia. Two out of nine fibres exposed to the modified PSS became completely inexcitable and in 50% of the remainder the stimulation voltage had to be increased by approximately two fold.

Effects of adenosine, given during or before exposure to modified PSS

Figure 1 shows the effects of adenosine (10^{-5} and 10^{-4} M) when given after the first 30 min of exposure to the modified PSS. In contrast to its effects on normal Purkinje action potentials, adenosine significantly modified MRD and action potential amplitude. Thus the 'ischaemia'-induced fall in both of these variables was attenuated by adenosine, 10^{-5}

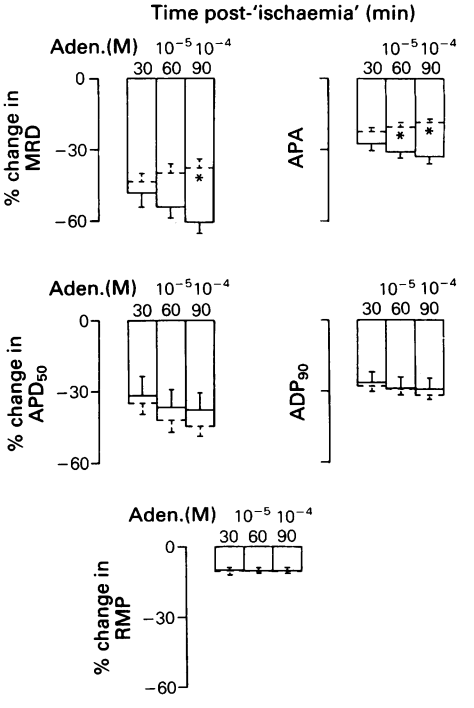

Figure 1 Percentage changes from control values in sheep Purkinje action potentials induced by the modified PSS alone (unbroken line) and modified PSS plus adenosine, 10^{-5} and 10^{-4} M (broken line). *$P < 0.05$ significantly different from value in modified PSS alone. $n = 6$–7. For abbreviations see text.

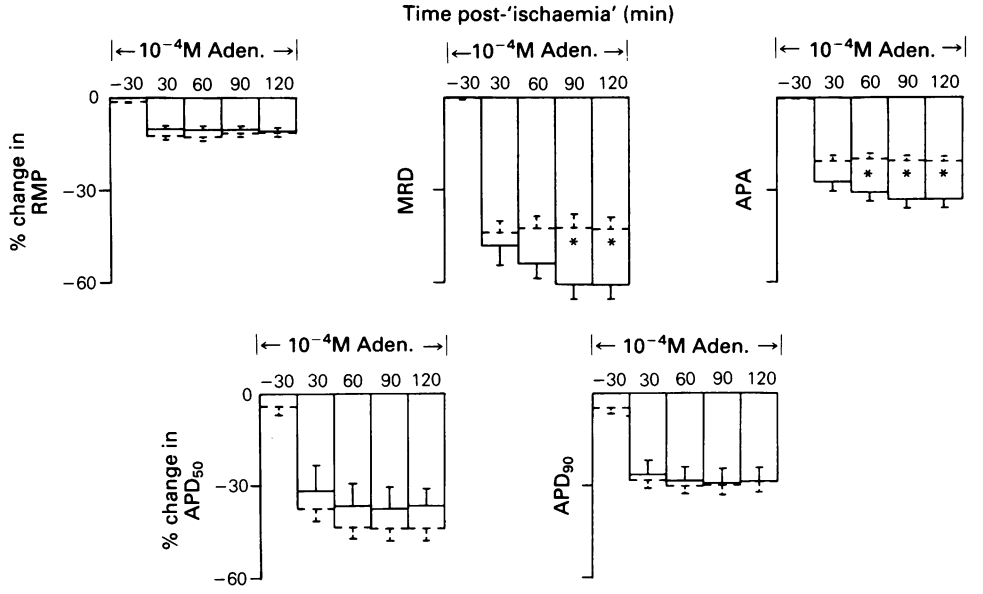

Figure 2 Percentage changes from control values in sheep Purkinje action potential characteristics induced by modified PSS alone (unbroken line) and adenosine before (−30 min) and during superfusion with modified PSS (broken line). *$P < 0.05$ significantly different from value in modified PSS alone. $n = 6$–7. For abbreviations see text.

and 10^{-4} M, without any concomitant effect on the resting membrane potential. Action potential duration was not significantly different in fibres exposed to the modified PSS alone and that containing adenosine.

When adenosine (10^{-4} M) was given prior to (for 30 min) and throughout exposure to the modified PSS, it did not modify the marked and rapid (within 30 min) changes observed in all of the variables (Figure 2). However, adenosine did abolish the further fall in MRD and in action potential amplitude that occurred between 30 and 120 min of superfusion with the modified PSS. The adenosine-induced shortening of APD_{50}, but not that of APD_{90}, persisted throughout the exposure to the 'ischaemic' solution.

Table 2 The effects of noradrenaline (NA) and noradrenaline together with adenosine (Aden) on action potential characteristics of sheep Purkinje fibres exposed to 'ischaemic' conditions

	RMP (mV)	*MRD* (Vs^{-1})	*APA* (mV)	APD_{50} (ms)	APD_{90} (ms)
Control	−83 ± 0.6	488 ± 31	113.4 ± 1.8	137.8 ± 11.9	234.7 ± 11.2
Modified PSS	−75.4 ± 0.4*	178 ± 45*	80.2 ± 1.5*	102.9 ± 9.1*	181.2 ± 9.3*
NA 10^{-5} M in mod. PSS	−76.2 ± 0.3*	161 ± 39*	82.6 ± 2.6*	173.7 ± 10.2†	269.4 ± 15.7†
NA 10^{-5} M + Aden 10^{-4} M in mod. PSS	−75.6 ± 0.7*	175 ± 40*	85.7 ± 2.5*	163.1 ± 11.0†	259.7 ± 13.2†

* $P < 0.05$ significantly different from control
† $P < 0.05$ significantly different from modified PSS alone. $n = 4$

Effects of adenosine on noradrenaline-induced electrophysiological actions

A detailed description of the effects of noradrenaline on sheep Purkinje cells superfused with normal or modified PSS is given by Boachie-Ansah *et al.* (1989). Noradrenaline caused a prolongation of action potential duration which was particularly marked under conditions that simulate myocardial ischaemia (Table 2). On normal action potentials, adenosine (10^{-4} M) administered concurrently with 10^{-5} M noradrenaline slightly but significantly attenuated the noradrenaline-induced prolongation of action potential duration at both 50% and 90% repolarization (by $21.0 \pm 3.5\%$ and $22.1 \pm 7.8\%$ reductions in APD_{50} and in APD_{90}, respectively). However, no suppression of noradrenaline-induced automaticity by adenosine (10^{-4} M) was observed. The spontaneous rate of beating of preparations exposed to noradrenaline and noradrenaline plus adenosine was 32 ± 8 and 32 ± 9 beats min^{-1}, respectively.

In modified PSS, a similar response was observed in that adenosine slightly reduced the prolongation of action potential duration induced by noradrenaline (Table 2). The % reductions in APD_{50} and APD_{90} induced by adenosine were 16.5 ± 6.7 and 10.7 ± 2.4, respectively. Noradrenaline did not induce automaticity in the presence of the modified PSS.

Effects of ATP, given during and before exposure to modified PSS

Figure 3 illustrates the effects of ATP, 10^{-5} and 10^{-4} M, (given 30 min after the start of superfusion with modified PSS) on depressed Purkinje action potentials. In contrast to its effects on normal Purkinje fibres and in a similar (but less marked) manner to adenosine, ATP significantly attenuated the fall in MRD and in action potential amplitude observed with the longer periods of exposure to the modified PSS. The changes in the other measured variables were not altered significantly by the presence of ATP, although action potential duration tended to be longer.

When ATP (10^{-4} M) was given before (30 min) and during exposure to modified PSS, it failed to modify the early (within 30 min) changes in action potential characteristics induced by the solution mimicking ischaemia. However, ATP tended to ablate the gradual subsequent fall in MRD and in action potential amplitude. Thus the % reductions in MRD and in action potential amplitude under ischaemic conditions were 60.6 ± 4.8 and 33.1 ± 3.0 respectively, compared with % reductions of 53.1 ± 4.0 and 25.8 ± 0.8 in the presence of ATP (10^{-4} M). ATP did not modify resting membrane potential of the depressed action potentials and once again although it tended to lengthen the action potential duration, this effect was not statistically significant.

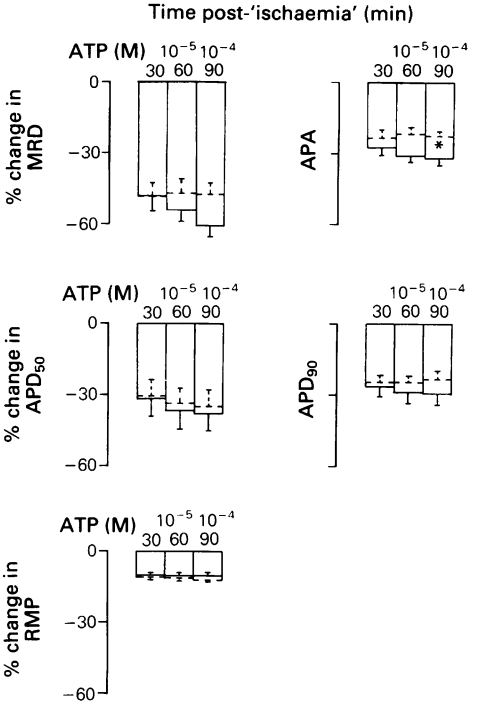

Figure 3 Percentage changes from control values in sheep Purkinje action potential characteristics induced by the modified PSS alone (unbroken line) and modified PSS plus ATP (broken line). * Significantly different from value in modified PSS alone. $n = 5$–7. For abbreviations see text.

Discussion

On normal paced sheep Purkinje fibres, adenosine (10^{-6} to 10^{-4} M) caused a concentration-dependent shortening of action potential duration without influencing any of the other measured variables. These results agree with the recent findings of Wesley & Belardinelli (1985) on guinea-pig isolated Purkinje cells but differ from those on canine Purkinje cells (Rosen *et al.*, 1983; Rardon & Bailey, 1984) in which adenosine was shown to have no effect on action potential duration. This suggests

that the action of adenosine on action potential duration is species-dependent. On atrial muscle, the adenosine-induced abbreviation of the action potential can be attributed either to an increase in K^+ conductance (Belardinelli & Isenberg, 1983; Nawrath & Jochem, 1983) or to a decrease in the inward Ca^{2+} current (Grossman & Furchgott, 1964; Schrader *et al.*, 1975). However, the mechanism underlying this effect of adenosine in Purkinje cells has not been studied. No consistent effect of ATP on the duration of the normal sheep Purkinje action potential was observed, which is in agreement with results obtained on canine Purkinje cells, using a stable analogue of ATP (Rosen *et al.*, 1983).

In contrast to their actions on normal Purkinje tissue, both adenosine and ATP, under conditions that mimic mild myocardial ischaemia, modified the upstroke of the action potential. The 'ischaemia'-induced reductions in the action potential amplitude and in the rate of rise of phase zero that occurred between 30 and 120 min of exposure to the modified PSS were attenuated by adenosine and to a lesser extent by ATP. Neither of the adenyl compounds, when given before exposure to the modified PSS, influenced the fall in resting membrane potential, amplitude and rate of rise of the action potential that occurred within the first 30 min of exposure.

The ionic mechanism underlying the protective effects of adenosine and ATP on the amplitude and rate of rise of the action potential during the 30–120 min post-ischaemic period remain unknown. Since these effects were not associated with any changes in the resting membrane potential, it would appear that the adenyl compounds may protect against the fall in the inward current that underlies phase 0 depolarization. Experiments carried out in this laboratory have indicated that the reduction in the resting membrane potential and the concommitant decreases in the amplitude and rate of rise of the action potential induced by the modified PSS within the first 30 min of exposure are mainly a consequence of the elevation in extracellular K^+ concentration (Boachie-Ansah *et al.*, 1989) whereas the continued decline in the upstroke and rate of rise is hypoxia and/or acidosis-mediated (D. Pacini, unpublished observations). The ionic mechanisms responsible for this effect of hypoxia and acidosis are also unknown but they may possibly involve induced increases in cytosolic Na^+ as a result of partial inhibition of the Na^+/K^+ pump and/or an induced shift in the membrane responsiveness curve (i.e., the relationship between membrane potential and amplitude of the inward Na^+ current) to more negative potentials. Adenosine and ATP may, in turn, attenuate these effects but such suggestions are purely conjectural and emphasise the need for further electrophysiological analysis of the effects of these drugs under ischaemic conditions. It would also be of interest to ascertain if the adenyl compounds are acting extra- or intra-cellularly and, furthermore, if the action of ATP depends upon enzymatic breakdown to adenosine. It is possible that this attenuation of the ischaemia-induced depression of the upstroke of the action potential by the adenyl compounds may, if it occurs *in vivo*, improve conduction and eliminate areas of unidirectional block. Such an action might explain the ability of adenosine and ATP to protect against ischaemia-induced arrhythmias in experimental animals (Fagbemi & Parratt, 1984; Parratt & Wainwright 1985; G. Boachie-Ansah, unpublished observations).

Neither adenosine nor ATP had a marked effect on APD under conditions that simulate mild myocardial ischaemia. Thus ATP, given extracellularly, is unable to protect against the abbreviation of the action potential that is, in part, induced by hypoxia (D. Pacini, unpublished work). Since it has been shown that intracellular injection of ATP does attenuate the action potential shortening caused by metabolic inhibitors (Taniguchi *et al.*, 1983), it is most likely that our results reflect the rapid breakdown of ATP and lack of uptake into the cytoplasm.

In addition to its direct electrophysiological effects, adenosine has been shown to modulate the effects of catecholamines on Purkinje tissue. For instance, adenosine reduces adrenaline-induced automaticity (Rosen *et al.*, 1983) and antagonizes the action potential shortening induced by isoprenaline (Rardon & Bailey, 1984). In our experiments, there was no evidence of any interaction between the effects of adenosine and noradrenaline, the neurotransmitter at sympathetic nerve endings. On normal sheep Purkinje fibres, adenosine did not modify noradrenaline-induced automaticity. It did reduce the noradrenaline-mediated prolongation of APD in both normal and 'ischaemic' fibres, but this effect could be explained by the ability of adenosine alone to shorten the action potential. The reason for this lack of interaction between adenosine and noradrenaline may be that adenosine specifically antagonises β- but not α-adrenoceptor-mediated catecholamine effects (Endoh & Yamashita, 1980; Hattori & Levi, 1984). At least part of the electrophysiological effects of noradrenaline on sheep Purkinje fibres is α-adrenoceptor-mediated (Boachie-Ansah *et al.*, 1989).

In summary, adenosine caused a concentration-dependent shortening of the action potential duration in normally-polarized sheep Purkinje fibres. Both adenosine and ATP attenuated the fall in the maximum rate of rise of phase 0 and the action potential amplitude in fibres exposed to combined hypoxia, hyperkalaemia and acidosis. No interaction between the effects of adenosine and noradrenaline

on action potential characteristics of normal or 'ischaemic' sheep Purkinje fibres was observed.

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5-HT3 receptor antagonists injected into the area postrema inhibit cisplatin-induced emesis in the ferret

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1 The purpose of the present study was to identify and investigate the role of 5-hydroxytryptamine$_3$ (5-HT$_3$) receptors in the area postrema in the control of cisplatin-induced emesis in the ferret.

2 Homogenate binding and autoradiography experiments using the high affinity 5-HT$_3$ receptor ligand, [^{3}H]-GR65630, identified the presence of a high concentration of 5-HT$_3$ receptors in the area postrema of the ferret.

3 Intraperitoneal injection of the 5-HT$_3$ receptor antagonists, GR38032F, GR65630A and MDL72222, at doses of 1, 0.1 and 1 mg kg^{-1} respectively, inhibited emesis induced by cisplatin, 9 mg kg^{-1} i.p.

4 Discrete injection of low doses of the 5-HT$_3$ receptor antagonists directly into the area postrema region also inhibited cisplatin-induced (9 mg kg^{-1} i.p.) emesis. The dose ranges used were: GR38032F, 0.01–1 μg; GR65630A, 0.001–0.1 μg; MDL72222, 0.1–10 μg.

5 Cisplatin-induced emesis was not inhibited by discrete injection of ketanserin (30 μg) or methiothepin (30 μg) into the area postrema. Injection of the 5-HT$_3$ receptor agonist, 2-methyl-5-HT, directly into the area postrema produced an incomplete emetic response.

6 These results confirm a role of 5-HT, and in particular 5-HT$_3$ receptors, in the control of cisplatin-induced emesis, and show that at least one functional site for these receptors in modulating the emetic response is the area postrema, the locus of the chemoreceptor trigger zone.

Introduction

It is well documented that the selective 5-hydroxytryptamine$_3$ (5-HT$_3$)-receptor antagonist, GR38032F (Butler *et al.*, 1988), inhibits emesis induced by cytotoxic drugs in man (Cunningham *et al.*, 1987) and in experimental animals (Stables *et al.*, 1987). Experiments in ferrets have revealed that cisplatin-induced emesis is associated with intestinal damage and changes in the levels of 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) present in the intestinal mucosa (Gunning *et al.*, 1987), and that abdominal vagotomy ameliorates such emesis (Hawthorn *et al.*, 1988). These latter studies have led to the proposition that cytotoxic drugs cause 5-HT release from the enterochromaffin cells of the gastrointestinal mucosa to induce stimulation of 5-HT$_3$ receptors on the afferent vagal fibres (Ireland & Tyers, 1987). Stimulation of 5-HT$_3$ receptors then induces abnormal discharge of the vagal fibres which evokes the vomiting reflex (Hawthorn *et al.*, 1988).

However, emesis induced by cytotoxic drugs appears to involve centrally, as well as peripherally, located mechanisms. For example, it is known that ablation of the area postrema, the site of the chemoreceptor trigger zone (CTZ) causes inhibition of cisplatin-induced emesis (McCarthy & Borison, 1984). In addition, the involvement of 5-HT mechanisms in the area postrema in the control of emesis is implicated by the observations that the area postrema contains a large number of 5-HT-containing neurones (Pickel & Armstrong, 1984), and that depletion of 5-HT from the area postrema totally inhibits cisplatin-induced emesis (Barnes *et al.*, 1988).

The latter observations on 5-HT in the central nervous system, together with the proposed central role of the area postrema in controlling emesis (Borison *et al.*, 1984), prompted us to undertake studies to investigate directly the role of 5-HT$_3$ receptors in this medullary structure in the emetic response to cisplatin. To this end, we have carried out experiments in the ferret to (i) identify 5-HT$_3$ receptor binding sites in the area postrema, using the

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techniques of homogenate receptor binding and receptor autoradiography, and (ii) determine the effects of discrete injections of GR38032F and other 5-HT_3 receptor antagonists directly into the area postrema on cisplatin-induced emesis.

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

Methods

Animals

Fitch and albino ferrets of either sex weighing 0.5–2 kg were used. The ferrets were allowed free access to food and water before the experiment. Unless stated otherwise each animal was used for one experiment only.

Surgery

Ferrets were anaesthetized with pentobarbitone (30–40 mg kg^{-1} i.p.) and a guide cannula (23 gauge stainless steel tube, 13 mm length) was lowered vertically so that the tip was 9 mm below the skull surface (midline) and fixed to the skull with dental cement and two holding screws. The coronal co-ordinate used was a cross-suture located approximately 2 mm from the posterior edge of the skull. These co-ordinates sited the guide cannula tip 3–5 mm directly above the area postrema. Following treatment with antibiotic (50 mg Streptopen injection) the ferrets were returned to holding pens for a period of 24–96 h before experimentation.

Experimental protocol in conscious ferrets

Emesis was induced in all ferrets by intraperitoneal injection of cisplatin, 9 mg kg^{-1} (dose volume 4.5 ml kg^{-1}). For intraperitoneal administration the 5-HT_3 receptor antagonists were injected immediately after the dose of cisplatin. When injected directly into the area postrema the 5-HT_3 receptor antagonists were administered immediately after the first vomiting episode induced by cisplatin. Following intraperitoneal injection of compounds the ferrets were observed for 3 h, and following central injection they were observed for 1 h and the total number of vomits and retches were recorded. In some experiments the ferrets received two central injections into the area postrema (2-methyl-5-HT followed by apomorphine) which were separated by at least 5 h. All central drug injections were made at a point approximately 1 mm below the area postrema into the hindbrain using a 30 gauge injection cannula. During injection the needle passed between the two rostral projections of the area postrema and thus did not damage the structure. The injection volume of 2 μl was slowly infused over 2 min with the ferret fully conscious.

Histological verification of central injection site

At the end of each experiment, the ferrets were anaesthetized with pentobarbitone (30–40 mg kg^{-1} i.p.) and 2 μl of Indian ink injected into the area postrema via the indwelling cannula. Ten minutes later the ferrets were decapitated, the hindbrain including the cerebellum removed, and sectioned on a freezing microtome. Any ferrets showing no ink penetration into the area postrema were excluded from the study.

Homogenate binding of [^{3}H]-GR65630

Ferrets were decapitated under pentobarbitone anaesthesia, their brains removed and dissected. Tissue samples were homogenised (ultra turrax, 15 s) in 10 volumes of HEPES buffer (50 mM, pH 7.4, 4°C) and centrifuged at 48,000 *g* and 4°C (Sorvall RC5C). The pellet was retained, the homogenisation and centrifugation repeated and the final pellet resuspended in 10 volumes of HEPES buffer.

For the binding assay, tubes (in triplicate) contained 100 μl [^{3}H]-GR65630 (81 Ci $mmol^{-1}$; final concentration 0.2 nM) in HEPES buffer, 50 μl of metoclopramide (30 μM, to define non-specific binding) or vehicle (HEPES buffer) and 100 μl of tissue homogenate (0.2–0.4 mg protein). Tubes were incubated for 30 min at 37°C in a shaking water bath. The reaction was terminated by rapid vacuum filtration through Whatman GF/B filters and extensive washing (5 × 2.5 ml; HEPES buffer) using a Brandel cell harvester. Filters were placed with 10 ml of Picofluor 30 and left overnight before radioactivity counting in a Packard CA2000 scintillation spectrometer. Tissue protein was assayed by the method of Bradford (1976).

Autoradiography

Ferret brains were frozen in liquid nitrogen and mounted in Tissue-Tek O.C.T. embedding medium. Thin sections (20 μm) were cut using an Anglia AS600 cryotome (−15°C). Sections were thaw-mounted on to gelatin-coated glass slides.

Slide-mounted brain sections were incubated with [^{3}H]-GR65630 (0.2 nM) in HEPES buffer (50 mM, pH 7.4, 37°C) in the presence or absence of metoclopramide (30 μM, to define non-specific binding) for 30 min. Sections were washed twice in an excess of HEPES buffer (pH 7.4, 20°C) for 30 s followed by a 2 s wash in distilled water. Sections were dried in a stream of cool air. The slide-mounted brain sections

Table 1 The effect of intraperitoneal injection of 5-HT$_3$ receptor antagonists on cisplatin-induced emesis in the ferret

Compound and dose (mg kg^{-1} i.p.)	n	*No. of vomits*	*No. of retches*	*Latency to emesis* (min)
Control	9	14.3 ± 1.5	123.7 ± 11.9	75.4 ± 2.9
GR38032F (1)	4	0*	0*	313.3 ± 18.2*
GR65630A (0.1)	4	0*	1.0 ± 1.0*	256.7 ± 25.7*
MDL72222 (1)	4	0.25 ± 0.25*	4.3 ± 3.6*	227.5 ± 69.7

Results expressed as means ± s.e.mean.
Dose of cisplatin = 9 mg kg^{-1} i.p. Test compounds were injected immediately after the injection of cisplatin. Vomiting and retching were measured for 3 h following injection of cisplatin. Latencies were measured up to 6 h following injection of cisplatin. *$P < 0.05$ vs controls (Mann-Whitney 'U'-test).

were then opposed to photographic film (Amersham Hyperfilm) for 3 months. Film was developed using Kodak D-19 developer.

Compounds

The 5-HT$_3$ receptor antagonists used were GR38032F (1,2,3,9-tetrahydro-3-[(methyl-imidazol-1-yl)methyl]-9-methyl-4H-carbazol-4-one hydrochloride, $2H_2O$), GR65630A (3-(5-methyl-1H-imidazol-4-yl)-1-(1-methyl-1H-indol-3-yl)-1-propanone maleate) and MDL72222 (1αH,3α,5αH-tropan-3yl-3,5-dichlorobenzoate), and the selective 5-HT$_3$ receptor agonist used was 2-methyl-5-HT; these compounds were synthesized in our own laboratories. The compounds described above and cisplatin (Lederle) were dissolved in saline immediately before use. Methiothepin maleate (Roche) and ketanserin (Janssen) were dissolved in minimal quantities of 1 M HCl and 0.1% tartaric acid respectively, made up to volume with distilled water and adjusted to pH 5 with sodium bicarbonate. Apomorphine hydrochloride (Sigma) was dissolved in saline containing

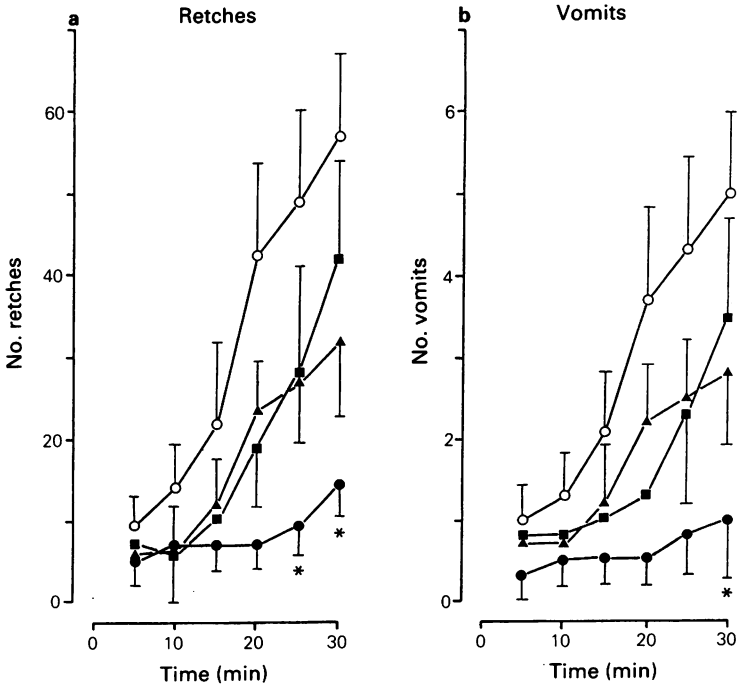

Figure 1 The effect of an injection of GR38032F into the area postrema on cisplatin-induced emesis in the ferret. GR38032F was injected at doses of 0.01 (▲, $n = 6$), 0.1 (■, $n = 4$) and 1 (●, $n = 4$) μg. (○) Shows vehicle control with cisplatin, 9 mg kg^{-1} i.p. ($n = 7$). Results are expressed as cumulative (a) retches/(b) vomits over the appropriate time period. Each point is the mean, and vertical lines s.e.mean, for n observations. *$P < 0.05$.

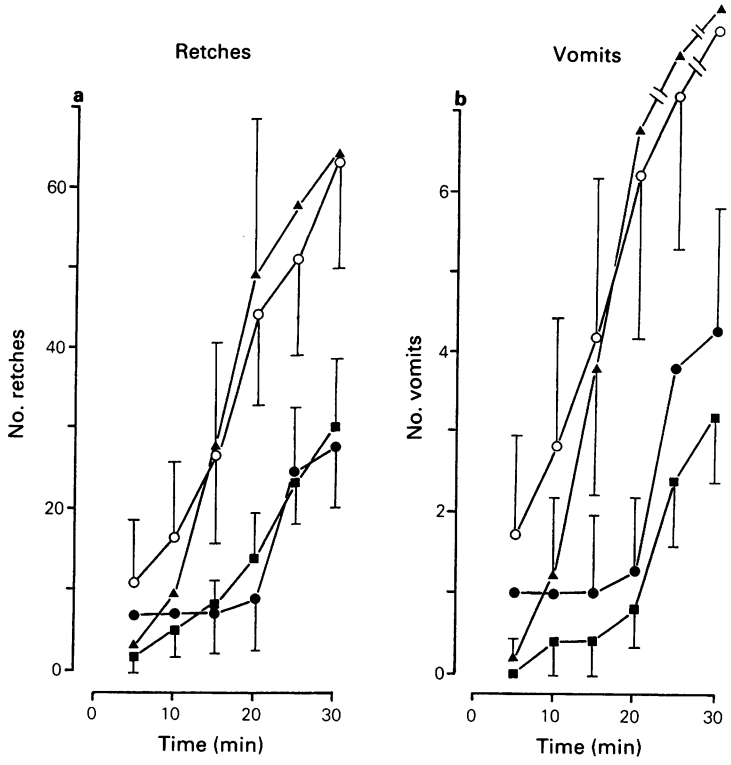

Figure 2 The effect of injection of GR65630A into the area postrema on cisplatin-induced emesis in the ferret. GR65630A was injected at doses of 0.001 (▲, $n = 5$), 0.01 (■, $n = 5$) and 0.1 (●, $n = 4$) μg. (○) Shows vehicle control with cisplatin, 9 mg kg^{-1} i.p. ($n = 6$). Results are expressed as cumulative (a) retches/(b) vomits over the appropriate time period. Each point is the mean and vertical lines s.e.mean for n observations.

200 mg l^{-1} ascorbate. Control solutions for each drug were prepared with the appropriate vehicle.

Doses of drugs are expressed as that of the free base.

Expression of data

Retches and vomits are expressed as cumulative means ± s.e.mean over the specified time period following drug injection. Differences between drug treatments and vehicle pretreated controls were analysed by use of the Mann-Whitney 'U' test.

Results

The effect of intraperitoneal injection of 5-HT$_3$ antagonists on cisplatin-induced emesis

Before commencing the studies on the central injection of compounds, some preliminary experiments were carried out using intraperitoneal injections to establish the anti-emetic actions of the compounds; the results are shown in Table 1. All three 5-HT$_3$ receptor antagonists, given intraperitoneally, were anti-emetic in the ferret.

The effect of 5-HT$_3$ receptor antagonists injected into the area postrema on cisplatin-induced emesis

In these experiments the 5-HT$_3$ receptor antagonists were injected into the area postrema immediately after the first emetic episode, the latency of which did not vary between groups. Within 5 min of microinjection into the area postrema, both GR38032F (0.01–1 μg, Figure 1) and GR65630A (0.001–0.1 μg, Figure 2) produced some inhibition of the number of cisplatin-induced retches and vomits by comparison with controls. This antagonism was particularly marked between 5–20 min after dosing with almost complete inhibition in all animals at 1 μg GR38032F (vomits 5–20 min post dosing: vehicle 2.7 ± 0.8, GR38032F 1 μg 0.3 ± 0.3; $P < 0.05$) and 0.01–0.1 μg

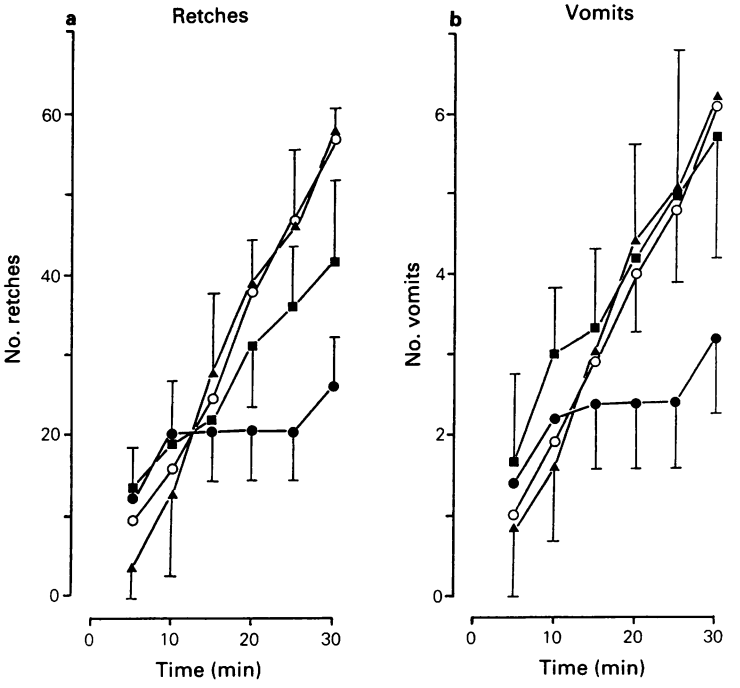

Figure 3 The effect of injection of MDL72222 into the area postrema on cisplatin-induced emesis in the ferret. MDL72222 was injected at doses of 0.1 (▲, $n = 5$), 1 (■, $n = 5$) and 10 (●, $n = 4$) μg. (○) Shows vehicle control with cisplatin, 9 mg kg^{-1} i.p. ($n = 6$). Results are expressed as cumulative (a) retches/(b) vomits over the appropriate time period. Each point is the mean and vertical lines s.e.mean for n observations.

GR65630A (vomits 5–20 min post dosing: vehicle 4.5 ± 0.8, GR65630A 0.1 μg 0.3 ± 0.3; $P < 0.05$). After this time, particularly with GR65630A, the mean emetic response began to return towards control levels. MDL72222 (10 μg) also produced a marked inhibition of cisplatin-induced emesis (Figure 3). However, this compound had a slower rate of onset compared with GR38032F and GR65630A, blockade of emesis being particularly marked 10–25 min after dosing (vomits 10–25 min post dosing: vehicle 2.9 ± 0.8, MDL 72222 10 μg 0.2 ± 0.2; $P < 0.05$).

Following 5-HT$_3$ antagonist treatment, the ferrets also displayed fewer signs of nausea, i.e. searching behaviour, backwards walking, licking of lips and yawning were reduced. Instead, the ferrets tended to lie quietly on the cage floor; reactivity and co-ordination were not impaired as assessed by visual observation.

Effect of methiothepin and ketanserin injected into the area postrema on cisplatin-induced emesis

Neither methiothepin (30 μg) nor ketanserin (30 μg) injected into the area postrema, significantly modified the emetic response induced by cisplatin (Figure 4). The ferrets treated with methiothepin, but not ketanserin, showed some sedation and a flattened posture approximately 10 min after administration.

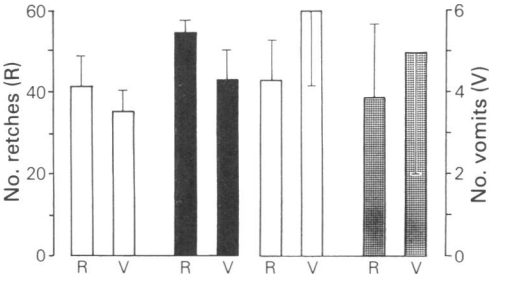

Figure 4 Effects of ketanserin or methiothepin injected into the area postrema against cisplatin-induced emesis in the ferret. Cisplatin was administered at a dose of 9 mg kg^{-1} i.p. Open columns, vehicle control ($n = 3$ to 4). Solid columns, methiothepin, 30 μg ($n = 3$). Stippled columns, ketanserin, 30 μg ($n = 3$). Both retches (R) and vomits (V) are the cumulative total obtained 0–30 min following injection of the test compound. Results are expressed as means and vertical bars show s.e.mean.

Table 2 Effect of 2-methyl-5-HT following injection into the area postrema of the ferret

	Dose (μg)	n	*Vomit latency*	*Behavioural effects*	*Emesis*	*Other changes* (>10 min)
2-Me-5-HT	1	2	>30 min	NSE	0	NSE
	3	2	>30 min	Search, back walk, licking lips, sl. salivation	0	NSE
	10	2	>30 min	As above 1/2 retching	0	Flat posture LMA markedly reduced, hypotension?
	30	5	>30 min	As above 1/5 retching	0	As above
Apomorphine	10	4	0.3 ± 0.1 min[a]	Search, back walk, licking lips	23.3 ± 7.8 ret.[a] 2.8 ± 0.9 vom.[a] 4/4 responder	Effects gone >15 min

Ferrets had no prior treatment with cisplatin. Dose volume = 2 μl.
[a] Data are means ± s.e.mean.
NSE = no significant effect.

Effect of 2-methyl-5-HT and apomorphine injected into the area postrema

The 5-HT_3 receptor agonist 2-methyl-5-HT (1–30 μg) injected into the area postrema induced signs of 'nausea' (salivation, searching, licking of lips, backwards walking, some retching) following doses of 3–30 μg, although at 10 μg and above marked sedation was apparent approximately 10 min after injection (Table 2). However, at no dose was any emesis observed within 30 min of treatment.

Apomorphine (10 μg) produced a full emetic response within 0.5 min of dosing in ferrets that failed to respond to 2-methyl-5-HT (Table 2). Both retching and vomiting were observed in all animals. These effects had a duration of approximately 15 min, after which time the behaviour of the ferrets returned to normal.

Homogenate binding and autoradiography

Specific [^{3}H]-GR65630 binding was evident in homogenates of ferret brain. This binding was discretely localised (Table 3). The highest level of specific binding was in homogenates of the area postrema. Other brain areas showing high levels of specific binding were the striatum, nucleus accumbens/olfactory tubercle, septum and amygdala.

Autoradiographic analysis of [^{3}H]-GR65630 binding to sections of ferret brain confirmed the observation of high specific binding in the area postrema (Figure 5). No specific binding was evident in areas surrounding the area postrema.

Discussion

Intraperitoneal injection of the 5-HT_3 receptor antagonists, GR38032F, GR65630A and MDL72222, inhibited cisplatin-induced emesis in the

Table 3 The specific binding of [^{3}H]-GR65630 to homogenates of discrete areas of ferret brain

Brain region	*Bound* (fmol mg^{-1} protein)
Entorhinal cortex	0.4 ± 0.1
Retrosplenic cortex	0.1 ± 0.04
Frontal cortex	0.4 ± 0.1
Cingulate cortex	0.3 ± 0.1
Temporal cortex	0.3 ± 0.1
Occipital cortex	0.2 ± 0.05
Parietal cortex	0.3 ± 0.1
Amygdala	1.0 ± 0.4
Hippocampus	0.5 ± 0.2
Nucleus accumbens/ olfactory tubercle	2.8 ± 0.7
Septum	1.4 ± 0.3
Thalamus	0.4 ± 0.02
Hypothalamus	0.8 ± 0.2
Striatum	2.9 ± 0.11
Cerebellum	0.1 ± 0.03
Area postrema	37.1 ± 12.3

Results are the means ± s.e.mean of three separate experiments. Non-specific binding was determined by the inclusion of metoclopramide (30 μM). The specific proportion of total binding varied between 15% and 90% depending on brain area. Protein was assayed by the method of Bradford (1976).

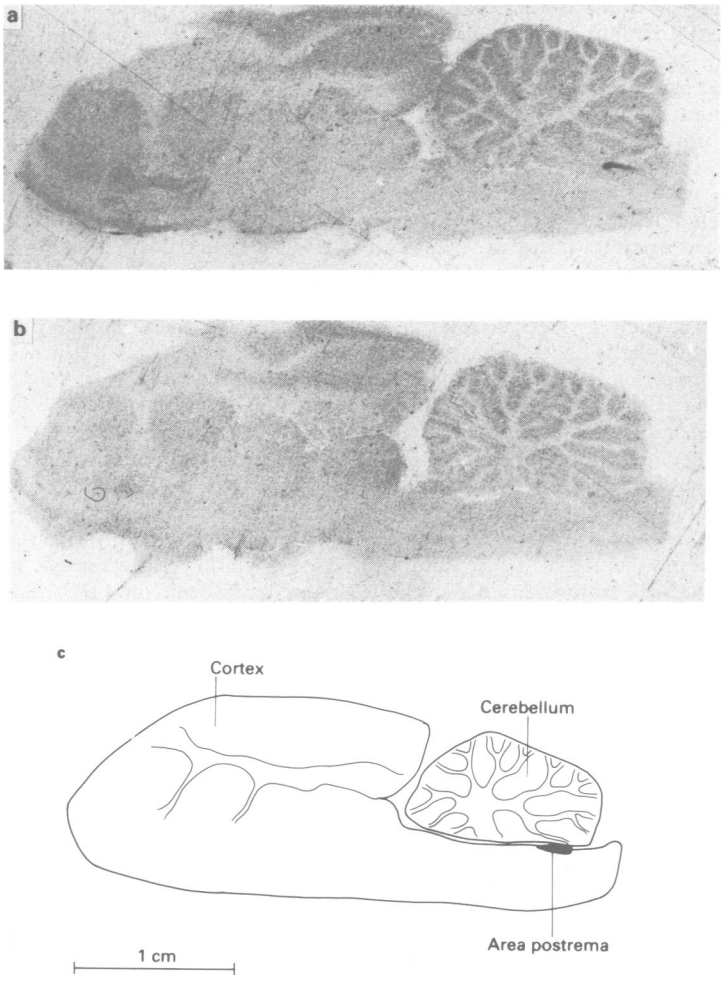

Figure 5 The autoradiography of (a) total and (b) non-specific [^{3}H]-GR65630 binding to longitudinal sections (20 μm) of ferret brain. Darker regions represent areas with high receptor density. Sections shown are approximately on the midline. (c) Line drawing of ferret brain corresponding to the autoradiographs (a) and (b). The area postrema is a flat medullary structure lying just beneath the cerebellum.

ferret. Similar doses of GR38032F and MDL72222 to those used intraperitoneally in the present study have previously been shown to be anti-emetic against cisplatin in the ferret when administered by the intravenous route (Miner & Sanger, 1986; Costall *et al.*, 1987). Like GR38032F, GR65630A at the dose used produced an almost complete inhibition of cisplatin-induced emesis over the 3 h observation period.

Although GR65630A has been shown previously to bind specifically to several discrete areas of rat brain (Kilpatrick *et al.*, 1987), the area postrema was not included in that study. However, as pointed out above, a number of observations led us to investigate the specific 5-HT$_3$ receptor binding in the area postrema, the most important being that this medullary structure is the site of the chemoreceptor trigger zone (CTZ). Also, it was necessary to carry out these experiments on ferret tissue since this species was used to investigate emesis. The results obtained with experiments on both receptor binding and autoradiography showed that the area postrema contains a high concentration of 5-HT$_3$ receptors.

Having established the presence of 5-HT$_3$ receptors in the area postrema it was important to investigate their role, if any, in the control of emesis. This was done by examining the effect of discrete injections of 5-HT$_3$ receptor antagonists into the area

postrema on cisplatin-induced emesis. All three of the compounds tested inhibited emesis with a rank order of potency of GR65630A > GR38032F > MDL72222, which agrees well with their affinities for 5-HT_3 receptors in rat vagus nerve (Kilpatrick *et al.*, 1987). Also, when administered directly into the area postrema the anti-emetic dose of each compound was considerably smaller than when given by intraperitoneal or intravenous injection, for instance, the minimal effective intravenous doses of GR38032F and MDL72222 against cisplatin-induced emesis in the ferret are 0.01 mg kg^{-1} and 2 × 0.05 mg kg^{-1}, respectively (Miner & Sanger, 1986; Stables *et al.*, 1987). Thus it is unlikely that the anti-emetic response following injection into the area postrema was due to diffusion of the compound from the injection site into the general circulation. Administration of methiothepin or ketanserin into the area postrema had no effect on emesis, indicating that other 5-HT receptor types, 5-HT_1-like and 5-HT_2, are unlikely to be involved. In the light of the results obtained with the 5-HT_3 receptor antagonists, the failure of the agonist 2-methyl-5-HT to evoke emesis readily was surprising and contrasted sharply with the powerful emetic effect of apomorphine. A possible explanation for this is that 5-HT_3 receptors in the area postrema have a permissive role in emesis so that their activation does not evoke emesis, but is necessary for emesis to occur. Alternatively, since 2-methyl-5-HT is a basic hydrophilic compound insufficient penetration into the area postrema may account for the absence of a full emetic response. A third explanation is that the sedative effect of 2-methyl-5-HT may have overcome any underlying emetic response. This, however, is unlikely because methiothepin also caused sedation yet it was not able to inhibit cisplatin-induced emesis. Finally, at present we have no knowledge as to the intrinsic activity of 2-methyl-5-HT at area postrema 5-HT_3 receptors. Thus, 2-methyl-5-HT may function as a partial agonist at this site with insufficient efficacy to evoke a full emetic response. Clearly further studies are necessary to clarify this interesting result.

The data in Figures 1, 2 and 3 reveal two important points. First, the 5-HT_3 receptor antagonists did not produce an immediate effect since some emesis occurred up to 5 min after their central injection. Second, following intravenous injection even the minimally effective doses of GR38032F (0.01 mg kg^{-1}) and MDL72222 (2 × 0.5 mg kg^{-1}) must exhibit a duration of approximately 60–90 min since this is the latency to emesis following administration of cisplatin (Miner & Sanger, 1986; Stables *et al.*, 1987). However, when the 5-HT_3 receptor antagonists were injected into the area postrema emesis started to recur only 20–30 min after injection. The area postrema is a 'V' shaped structure and the injections were made between the two rostral projections so as to avoid tissue damage. The short delay in the onset of the anti-emetic effect may be attributable to the time required for the compounds to diffuse to all regions of the CTZ within the area postrema. In addition, the area postrema has a rich blood supply and is continuously being flushed with cerebrospinal fluid, and these factors would increase the rate of elimination of compounds from the site of injection and so limit their duration of action.

It has previously been shown that injection of the 5-HT_3 receptor antagonist, zacopride, into the fourth ventricle inhibits cisplatin-induced emesis (Smith *et al.*, 1988). However, the present study is believed to be the first to demonstrate inhibition of cisplatin-induced emesis by discrete injections of a 5-HT_3 receptor antagonist into the area postrema, the region containing the CTZ. The precise mechanisms affected by the antagonists are unclear at present, although some clues may be derived from previous work with cisplatin. Intravenous injection of cisplatin induces emesis with a delay of approximately 60 min and 100 min in the ferret and cat respectively (Stables *et al.*, 1987; Smith *et al.*, 1988), whereas injection of cisplatin directly into the fourth ventricle of the cat caused emesis within 4 min (Smith *et al.*, 1988). Since the area postrema is outside the blood–brain barrier, intravenously injected cisplatin would be expected to make rapid contact with this structure. Nevertheless, the emetic response to intravenous cisplatin is delayed (see above), and it therefore seems improbable that cisplatin exerts its emetic effect principally through a mechanism located solely in the area postrema. It is more likely that cisplatin activates a peripheral mechanism, probably involving the gastro-intestinal (GI) tract. Indeed, such a contention is supported by the observation that cisplatin accumulates (Rosenberg, 1985), causes degenerative changes (Vermorken & Pinedo, 1982) and affects the levels of 5-HT and 5-HIAA (Gunning *et al.*, 1987) in the GI tract. Also, sectioning of the visceral afferent nerves in the ferret completely blocks cisplatin-induced emesis (Hawthorn *et al.*, 1988). Why then, if cisplatin induces emesis mainly through an action on the GI tract, do 5-HT_3 receptor antagonists inhibit such emesis when injected into the area postrema? Three observations are pertinent to this discussion: first, the area postrema contains large numbers of 5-HT-containing neurones (Pickel & Armstrong, 1984); second, it contains a high concentration of 5-HT_3 receptors (this study); and third, it receives visceral afferents from the GI tract (Andrews & Hawthorn, 1988). Thus, is it possible that the area postrema acts as a relay between the visceral afferents and the vomiting centre identified in the reticular formation, and that disruption of

5-hydroxytryptaminergic pathways, by injection of 5-HT$_3$ receptor antagonists into the area postrema, inhibits emesis.

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(+)-[^{3}H]-PN 200-110 binding to cell membranes and intact strips of portal vein smooth muscle: characterization and modulation by membrane potential and divalent cations

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1 Specific binding of the calcium-antagonist dihydropyridine derivative, (+)-[^{3}H]-PN 200–110 (isradipine), to cell membranes of equine portal vein smooth muscle was compared with binding to intact strips isolated from rat portal veins.

2 Specific binding to vascular smooth muscle membranes was of high affinity, saturable and reversible. The dissociation constant obtained from association and dissociation kinetics of (+)-[^{3}H]-PN 200-110 was similar to that obtained from equilibrium binding and competition experiments.

3 Specific binding of (+)-[^{3}H]-PN 200-110 was completely displaced by unlabelled dihydropyridines. Among other calcium antagonists, D888 and (+)-*cis*-diltiazem partially inhibited the binding at 25°C. At 37°C, only (+)-*cis*-diltiazem stimulated the binding. $LaCl_3$, $CdCl_2$, $NiCl_2$, $CoCl_2$ had inhibitory effects, whereas KCl and NaCl had no effect.

4 When intact strips of portal vein were incubated in high external potassium concentrations for 30 min, the K_d was lowered to 0.04 ± 0.01 nM from the control value of 0.14 ± 0.02 nM ($n = 5$), thereby indicating that (+)-[^{3}H]-PN 200-110 bound to voltage-dependent calcium channels, with a higher affinity, in the depolarized state.

5 When external Ca^{2+} was removed or substituted with Ba^{2+} or Sr^{2+}, K_d values increased suggesting that the dihydropyridine binding to intact strips was modulated by binding of Ca^{2+} ions to voltage-dependent calcium channels.

Introduction

A better understanding of the voltage-dependent calcium channels in smooth muscles can be obtained by use of the three methodologies described below. (1) The voltage clamp method studies both activation and inactivation parameters of calcium channels responding to depolarization, and to the effects of calcium antagonists on calcium currents (Tsien, 1983; Dacquet *et al.*, 1988). Calcium channels may be in one of at least three states: resting, open, or inactivated and each state is determined by the membrane potential. Binding of a calcium antagonist to a site located within the channel is influenced by the state of the channel. (2) In contraction expériments, the voltage-dependence of calcium channel blockade is indicated by a shift to the left of the dose-inhibition curves measured in physiological solution or in K^+-rich depolarizing solution (Dacquet *et al.*, 1987). (3) The third method is to use radioligand binding techniques. With radiolabelled calcium antagonists, the binding studies revealed the affinities and maximal binding capacities of the calcium antagonists, and the interactions with various drugs and polyvalent cations (Fosset *et al.*, 1983; Fosset & Lazdunski, 1987). As binding studies are generally carried out using cell membranes, demonstration of a correlation with binding to calcium channels under

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physiological conditions is difficult to obtain. Accordingly, methods for binding of calcium antagonists to isolated cells or intact tissue preparations have been developed (Kokubun *et al.*, 1986; Morel & Godfraind, 1987; Sumimoto *et al.*, 1988). We describe the properties of the high affinity (+)-[^{3}H]-PN 200-110 binding to vascular smooth muscle membranes. The findings are compared with the binding to intact smooth muscle strips which is modulated by both the membrane potential and the ionic environment.

Methods

Membrane preparation

Microsomes from fresh equine portal vein were prepared in the presence of 1 mM iodoacetamide and 0.1 mM phenylmethylsulphonylfluoride to minimize proteolytic degradation (Dacquet *et al.*, 1988). Protein was determined with use of gamma-globulins as standard (Bradford, 1976).

Measurement of (+)-[^{3}H]-PN 200-110 binding to membranes

Membrane proteins (0.08 to 0.15 mg ml^{-1}) were incubated for 45 min at 25°C with various concentrations of (+)-[^{3}H]-PN 200-110 in 1 ml of 20 mM HEPES buffer (pH 7.4) containing 0.1% bovine serum albumin (Dacquet *et al.*, 1988). Non specific binding was defined as the amount of radioligand bound in the presence of 2 μM unlabelled nifedipine and subtracted from the total binding. All binding experiments were performed under a dim light.

Measurement of (+)-[^{3}H]-PN 200-110 binding to rat portal vein strips

Studies of binding to intact strips of rat portal vein (0.5–2 mg wet weight) were performed under conditions similar to those described for mesenteric arteries (Morel & Godfraind, 1987). Strips were incubated for 90 min at 37°C in a physiological solution (mM): NaCl 130, KCl 5.6, $CaCl_2$ 2, $MgCl_2$ 0.24, HEPES 8.3, glucose 11, at pH 7.4, (polarized veins) or for 60 min in physiological solution followed by 30 min in a 135 mM K^+ depolarizing solution obtained by substituting NaCl for KCl in equimolar amounts (depolarized veins). At the end of the incubation time, each strip was dried on filter paper, and then weighed. Radioactivity was revealed after dissolving the veins in 100 μl of a mixture of perchloric acid and H_2O_2 (1 : 1).

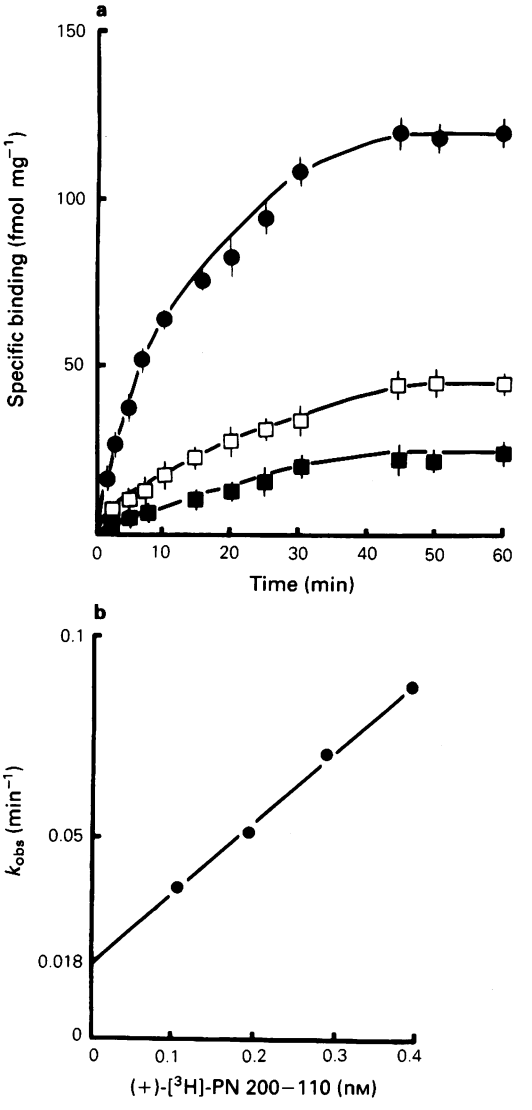

Figure 1 Kinetic constants of [^{3}H]-PN 200-110 binding to vascular smooth muscle membranes. Cell membranes (0.08 mg ml^{-1}) were incubated with different concentrations of (+)-[^{3}H]-PN 200-110 at 25°C in 1 ml buffer containing 1 mM $CaCl_2$, 20 mM HEPES NaOH (pH 7.4). (a) Association time course with three different concentrations of (+)-[^{3}H]-PN 200-110: (■) 0.015 nM, (□) 0.190 nM and (●) 0.393 nM for different times. Each point represents the mean response of 4 experiments with s.e.mean shown by vertical lines. (b) The association (k_1) and dissociation (k_{-1}) rate constants were determined by linear regression of the k_{obs} value versus (+)-[H]-PN 200-110 concentration. The k_1 and k_{-1} values were 0.178×10^9 M^{-1} min^{-1} and 0.018 min^{-1}, respectively. The equilibrium dissociation constant K_d was $k_{-1}/k_1 = 0.101$ nM, $n = 2$.

Table 1 Inhibition of (+)-[^{3}H]-PN 200-110 binding to membranes of portal vein smooth muscles in the presence of calcium channel antagonists

	IC_{50} or K_i	n Hill	Maximal inhibition (%)
Nitrendipine	0.45 ± 0.05 nM	1.08	100
D888	0.25 ± 0.02 μM	0.50	55 ± 5
(+)-*cis*-Diltiazem	50.00 ± 5.00 μM	1.25	65 ± 7
La^{3+}	10.00 ± 2.00 μM	0.55	100
Cd^{2+}	25.00 ± 4.00 μM	0.75	100
Ni^{2+}	1.00 ± 0.50 mM	0.42	70 ± 8
Co^{2+}	2.00 ± 0.80 mM	0.40	70 ± 7

Each result is mean ± s.e.mean of 5 experiments.
K_i values were calculated for competitive inhibitors from inhibition curves of (+)-[^{3}H]-PN 200-110 specific binding according to the equation of Cheng & Prussoff (1973).

Drugs

(+)-[^{3}H]-PN 200-110 (specific activity 84 Ci mmol^{-1}) was obtained from Amersham (France). (+)-PN 200-110 (isradipine), was from Sandoz (France); (±)-nifedipine and (±)-nitrendipine were from Bayer (F.R.G.); (+)-*cis*-diltiazem was from Synthelabo (France) and (−)-D888 (desmethoxy verapamil) and (±)-D600 (methoxyverapamil) were from Knoll (F.R.G.).

Statistical analysis

Scatchard plots for binding of (+)-[^{3}H]-PN 200-110 were obtained by a non linear least-square ligand programme (Munson & Rodbard, 1980). The experimental results were expressed as mean ± s.e.mean and significance was tested by Student's t test.

Results

Association and dissociation kinetics of (+)-[^{3}H]-PN 200-110 binding to membranes

Specific binding of (+)-[^{3}H]-PN 200–110 was determined as a function of time for different concentrations of labelled dihydropyridine (Figure 1a). Association reached a plateau after 40–50 min incubation at 25°C. The data for association kinetics were plotted according to a pseudo first order equation (Weiland & Molinoff, 1981). The slope of the plot, k observed (k_{obs}), was calculated for each radioligand concentration and plotted as a function of the (+)-[^{3}H]-PN 200-110 concentration (Figure 1b). This allowed a direct determination of both association rate constant (0.178×10^9 M^{-1} min^{-1}) and dissociation rate constant (0.018 min^{-1}). After 60 min incubation of membranes with 0.4 nM (+)-[^{3}H]-PN 200-110, the rate of dissociation of the complex was measured after addition of 2 μM unlabelled nifedipine. The data, plotted according to first order kinetics, gave a rate constant for dissociation (k_{-1}) of 0.014 min^{-1} that was similar to that obtained from Figure 1b. The dissociation constant from kinetic data $K_d = k_{-1}/k_1$, was estimated to be 0.08–0.10 nM. This value is similar to the dissociation constant obtained from equilibrium binding data (0.09 ± 0.03 nM, $n = 7$; Dacquet *et al.*, 1988).

Effects of organic calcium antagonists and polyvalent cations on the high affinity binding sites of (+)-[^{3}H]-PN 200-110 to membranes

Increasing concentrations of unlabelled drugs gradually inhibited the (+)-[^{3}H]-PN 200-110 binding (Figure 2a). The concentration producing half-maximal inhibition (IC_{50}) or the inhibition constant (K_i) and the slope factor of inhibition curves are listed in Table 1. The increase in (+)-[^{3}H]-PN 200-110 binding by (+)-*cis*-diltiazem was observed only at 37°C. To elucidate the mode of binding action by these drugs, Scatchard analysis of the (+)-[^{3}H]-PN 200-110 binding was carried out in the presence or absence of calcium antagonists (Table 2). Nitrendipine (2–4 nM) increased the K_d of [^{3}H]-PN 200-110 binding without affecting the B_{max} suggesting that nitrendipine behaved as an apparent competitive inhibitor. A change in both K_d and B_{max} values was observed when D888 (50–100 nM) was added. This was a typical result showing that inhibition of (+)-[^{3}H]-PN 200-110 binding was not of the competitive type. At 37°C, (+)-*cis*-diltiazem (50 μM) increased B_{max} with no significant change in K_d. It was noted that temperature had no significant effect on the (+)-[^{3}H]-PN 200-110 binding in the absence of (+)-*cis*-diltiazem as well as on the binding inhibi-

Table 2 Parameters of (+)-[^{3}H]-PN 200-110 binding to membranes in the presence of calcium channel antagonists

	K_d (nM)	B_{max} (fmol mg^{-1} protein)
Control	0.09 ± 0.01	55 ± 10
Nitrendipine (2 nM)	0.44* ± 0.02	50 ± 4
D888 (100 nM)	0.22* ± 0.02	35* ± 2
(+)-*cis*-Diltiazem (50 μM)	0.11 ± 0.03	33* ± 4
La^{3+} (10 μM)	0.10 ± 0.02	32* ± 3
Cd^{2+} (10 μM)	0.11 ± 0.01	45* ± 2
Ni^{2+} (2 mM)	0.10 ± 0.01	38* ± 3
Co^{2+} (5 mM)	0.11 ± 0.01	40* ± 4

* Significant change ($P < 0.05$) caused by calcium antagonists. Each result is mean ± s.e.mean of 5 experiments.

tion induced by 100 nM (−)-D888. These results indicate that (+)-*cis*-diltiazem behaves as a non competitive inhibitor of (+)-[^{3}H]-PN 200-110 binding to vascular smooth muscles membranes.

Both tri- and divalent cations dose-dependently decreased the binding of (+)-[^{3}H]-[PN 200-110 obtained in the presence of 1 mM $CaCl_2$ (Figure 2b). The concentrations producing half maximal inhibition (IC_{50}) and the slope factor of inhibition curves are listed in Table 1. In contrast, monovalent cations such as Na^+ and K^+ were without effect on the binding of (+)-[^{3}H]-PN 200-110, whatever the concentrations used. Scatchard representations of the effects of inorganic calcium entry antagonists on (+)-[^{3}H]-PN 200-110 binding obtained in the presence of 1 mM $CaCl_2$ showed that calcium antagonists lowered the B_{max} with no significant change of the K_d, indicating that they acted as non competitive inhibitors (Table 2).

Effects of membrane depolarization on specific binding of (+)-[^{3}H]-PN 200-110 to intact portal vein strips

Figure 3a illustrates the equilibrium binding of (+)-[^{3}H]-PN 200-110 to polarized intact portal vein strips (5 mM external K^+) at various concentrations of labelled PN 200-110 in the absence or presence of 2 μM unlabelled nifedipine. The non specific binding increased linearly with the concentration of (+)-[^{3}H]-PN 200-110. The specific binding accounted for 75–80% of the total binding at a concentration close to the K_d value. Scatchard analysis of the specific binding of (+)-[^{3}H]-PN 200-110 resulted in a linear plot, thereby indicating specific binding to a single class of sites (Figure 3b). The K_d and B_{max} values were 0.14 ± 0.02 nM and 12.0 ± 1.5 fmol mg^{-1}

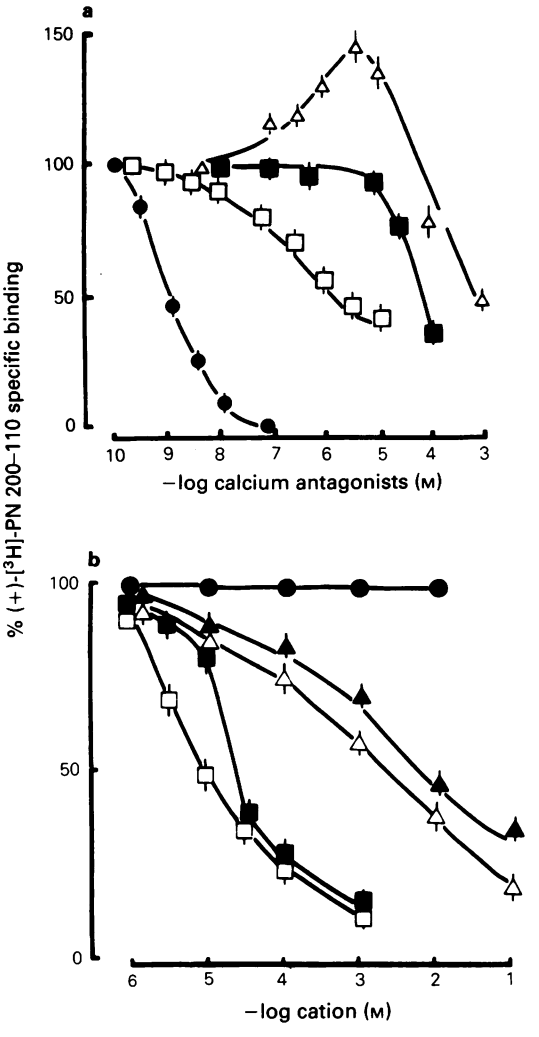

Figure 2 Effects of organic calcium channel antagonists and polyvalent cations on (+)-[^{3}H]-PN 200-110 binding to vascular smooth muscle membranes. (a) Membranes (0.1 mg ml^{-1}) were incubated with 0.15 nM (+)-[^{3}H]-PN 200-110 for 45 min in the absence or presence of unlabelled nitrendipine (●), (−)-D888 (□), and (+)-*cis*-diltiazem at 25°C (■) and 37°C (△). Results are expressed as a percentage of specific binding obtained in the absence of unlabelled drugs, and are means of 4 experiments with s.e.mean shown by vertical lines. (b) Specific bindings (+)-[^{3}H]-PN 200-110 (0.1 nM) to membranes (0.1 mg ml^{-1}) was measured under equilibrium conditions in presence of 1 mM Ca^{2+} and after addition of indicated concentrations of La^{3+} (□), Cd^{2+} (■), Ni^{2+} (△), Co^{2+} (▲) and K^+ (●). Binding is expressed as a percentage of that occurring in the presence of 1 mM $CaCl_2$. Each point represents the mean response of 4–6 experiments with s.e.mean shown by vertical lines.

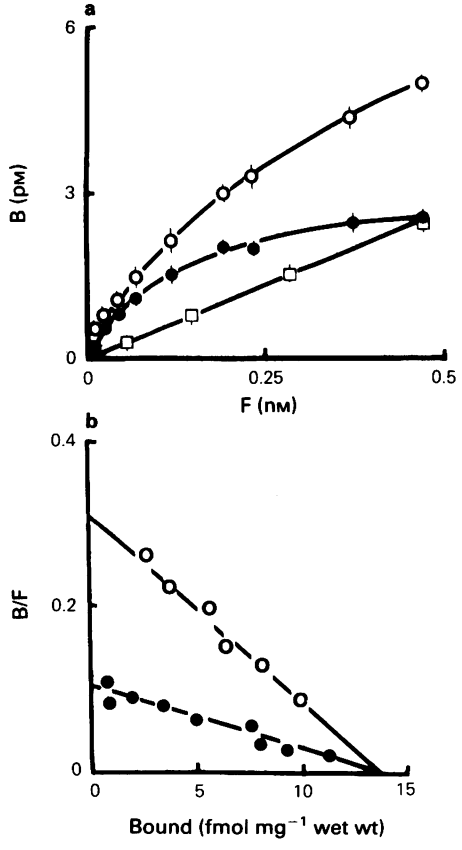

Figure 3 Specific binding of (+)-[^{3}H]-PN 200-110 to polarized and depolarized intact strips from rat portal vein. (a) Saturation curve of (+)-[^{3}H]-PN 200-110 binding to polarized rat portal vein. Saturation binding experiments were carried out on portal strips (0.5 to 1.2 mg) with increasing concentrations of (+)-[^{3}H]-PN 200-110 for 90 min at 37°C. Non specific binding was defined with 2 μM nifedipine. (○) Total binding, (●) specific binding and (□) non specific binding. Each point represents the mean response of 5 experiments with s.e.mean shown by vertical lines. (b) Scatchard analysis of specific binding to polarized strips in 5 mM K^+ (●) and depolarized strips in 135 mM K^+ solution (○). For depolarized strips, a first incubation for 60 min is 5 mM K^+ was made in presence of radioligand followed by a 30 min incubation in 135 mM K^+ with the same concentration of radioligand. To obtain high K^+ solution, NaCl was replaced with an equimolar concentration of KCl. Similar estimates were obtained from 5 separate experiments; duplicate estimates were used for each point.

wet weight ($n = 5$), respectively. The inhibition induced by various calcium antagonists was similar to that reported with cell membranes. In depolarized (135 mM external K^+) strips (Figure 3b) the Scatchard analysis showed that the K_d was reduced to 0.04 ± 0.01 nM ($P < 0.01$, $n = 5$) but the B_{max} was unchanged. As (+)-PN 200-110 blocks the calcium channels with a higher affinity in a more depolarized state (Kokubun *et al.*, 1986; Dacquet *et al.*, 1988), these results indicate that (+)-[^{3}H]-PN 200-110 preferentially binds to inactivated calcium channels rather than to rested channels. Affinities of (+)-PN 200-110 obtained from electrophysiological, mechanical and binding experiments on both cell membranes and muscle strips are summarized in Table 3. It can be observed that the relative potency of (+)-PN 200-110 against rested slow calcium channels and against inactivated slow calcium channels ranged between 3.5 and 5.

Effects of divalent cations on (+)-[^{3}H]-PN 200-110 binding to polarized or depolarized intact strips

Figure 4a shows that in 5 mM external K^+, the K_d value increased from 0.14 ± 0.02 nM in 2 mM Ca^{2+} solution to 0.26 ± 0.01 nM when external $CaCl_2$ was replaced by $BaCl_2$ or $SrCl_2$ and when $CaCl_2$ was removed in the presence of 0.1 mM EGTA ($P < 0.001$, $n = 5$–15) without significant variations in the B_{max} values (between 11.5 and 14.5 fmol mg^{-1} wet weight, $P > 0.05$). When intact strips were bathed in 135 mM external K^+ (Figure 4b), the K_d value was increased from 0.04 ± 0.01 nM to 0.11 ± 0.02 nM after removal of external calcium (in the presence of 0.1 mM EGTA) and when $CaCl_2$ was replaced by $BaCl_2$ or $SrCl_2$ ($P < 0.01$, $n = 5$–15), without significant variations in the B_{max} values ($P > 0.05$).

Discussion

Several studies have suggested that the high affinity binding site identified in radioligand binding experiments is the receptor for the pharmacological action of the dihydropyridine calcium channel antagonists in skeletal (Fosset *et al.*, 1983), cardiac (Lee *et al.*, 1984), and smooth muscles (Sarmiento *et al.*, 1984). Using membranes of portal vein smooth muscle, we demonstrated that the (+)-[^{3}H]-PN 200-110 binding was of high affinity, saturable and reversible, and correlated well with the pharmacological activities determined from inhibition of the mechanical and electrical responses (Dacquet *et al.*, 1987; 1988). In portal vein smooth muscle strips, the affinity of (+)-[^{3}H]-PN 200-110 increased as the membrane depolarized suggesting that (+)-[^{3}H]-PN 200-110 bound to calcium channels in the inactivated state with a higher affinity than in the rested state. The effect of membrane depolarization in decreasing the

Table 3 Binding affinities and pharmacological potencies of PN 200-110 in rat portal vein

	K_d (nM)		IC_{50}** (nM)	
	Microsomes	*Strips*	*Ca current*	*Contraction*
Membrane potential (mV)	0	0	0	−20
Inactivated Ca channels	0.09 ± 0.03	*0.04 ± 0.01	*0.03 ± 0.01	*0.03 ± 0.01
Membrane potential (mV)	—	−45	−50	−50
Resting Ca channels	—	0.14 + 0.02	0.15 ± 0.02	0.13 ± 0.02
Relative potency		3.52	5.00	4.33

Results were obtained from binding, electrophysiological and contraction experiments. Relative potency is expressed as the ratio of affinities against polarized preparations and against depolarized preparations.
* Significant change ($P < 0.01$) caused by membrane depolarization with respect to normal resting potential.
Each result is mean ± s.e.mean of 5–8 experiments.
** From Dacquet *et al.* (1987, 1988).

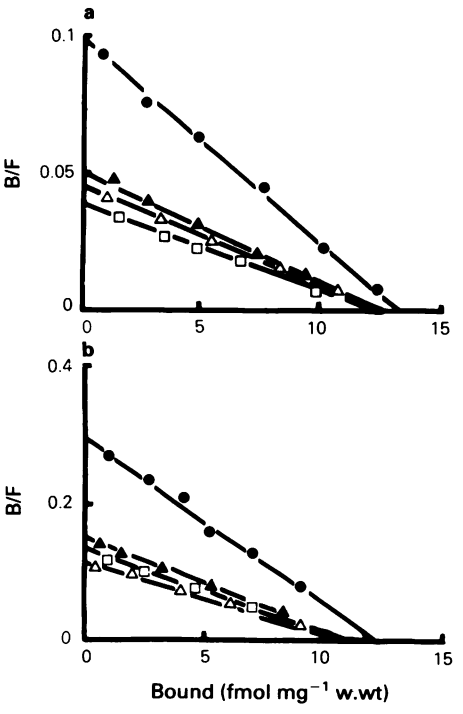

Figure 4 Modulation of (+)-[^{3}H]-PN 200-110 affinity by divalent cations and membrane depolarization to intact strips from rat portal vein. (a) Scatchard analysis of specific binding to polarized strips in 5 mM K^+ in the presence of 2 mM $CaCl_2$ (●), 2 mM $BaCl_2$ (▲), 2 mM $SrCl_2$ (△), after removal of external Ca^{2+} and addition of 0.1 mM EGTA (□). (b) Scatchard analysis of specific binding to depolarized strips in 135 mM K^+ in the different ionic conditions as described in (a). Similar estimates were obtained from 5–7 separate experiments; duplicate estimates were used for each point.

K_d values of intact strips suggests that the K_d values in cell membranes are lower than those in intact polarized strips, because the calcium channels are in an inactivated state in cell membranes. Similar results showing that depolarization enhances the affinity of dihydropyridine binding have been reported in cardiac (Kokubun *et al.*, 1986) and vascular myocytes (Morel & Godfraind, 1987; Sumimoto *et al.*, 1988). The relative potencies of (+)-PN 200-110 calculated from the affinity against polarized preparations and against depolarized preparations were similar using binding and contraction responses in intact portal vein strips and calcium current recordings in isolated cells (Table 3).

The inhibition of (+)-[^{3}H]-PN 200-110 binding to smooth muscle membranes induced by various calcium antagonists (nitrendipine, D888 and (+)-*cis*-diltiazem) suggest that there might be several sites for these structurally unrelated calcium antagonists and that they were allosterically regulated. Inorganic polyvalent cations are known to block the voltage-dependent calcium channels. In our results, the polyvalent cations inhibited (+)-[^{3}H]-PN 200-110 binding to smooth muscle membranes with a rank order of potency: $La^{3+} > Cd^{2+} > Ni^{2+} > Co^{2+}$, similar to that obtained from electrophysiological experiments (Jmari *et al.*, 1986a). The cations behaved as apparent non competitive inhibitors of (+)-[^{3}H]-PN 200-110 binding suggesting that they did not bind at the same site on the calcium channel as (+)-PN 200-110.

In intact smooth muscle strips, the maximal binding capacity of (+)-[^{3}H]-PN 200-110 was not affected by calcium removal (in the presence of 0.1 mM EGTA) or when $CaCl_2$ was replaced by $BaCl_2$ or $SrCl_2$ while the dissociation constant was significantly increased. The reduction in binding

affinity was observed whatever the membrane potential (polarized or depolarized preparations). Thus, the affinity of (+)-[^{3}H]-PN 200-110 binding was increased by both membrane depolarization and addition of external calcium. These observations can be correlated to electrophysiological studies demonstrating that inactivation of calcium channels in smooth muscles is mediated by both calcium-dependent and membrane potential-dependent mechanisms (Jmari *et al.*, 1986b; Ohya *et al.*, 1988).

In conclusion, our studies on binding of (+)-[^{3}H]-PN 200-110 to membranes and intact strips of portal vein smooth muscle indicate that the dihydropyridine binding site may be associated with the voltage-dependent calcium channel and modulated by binding of calcium ions to binding sites such as those which regulate calcium-dependent inactivation.

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An investigation into the mechanisms of inhibition of calcium channel currents in cultured sensory neurones of the rat by guanine nucleotide analogues and (−)-baclofen

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1 The mechanism of inhibition of calcium channel currents by the guanine nucleotide analogue guanosine 5'-O-3 thiotriphosphate (GTP-γ-S) and by the $GABA_B$ agonist (−)-baclofen has been studied in cultured dorsal root ganglion neurones of the rat. The inhibition by GTP-γ-S is particularly characterized by an abolition of the transient component of calcium channel currents carried either by Ba^{2+} (I_{Ba}) or by Ca^{2+} (I_{Ca}).

2 The effect of agents increasing intracellular cyclic AMP levels has been examined. Neither internal cyclic AMP nor forskolin prevented the inhibition of I_{Ba} by baclofen. Neither forskolin nor pretreatment of cells with cholera toxin prevented the inhibition of the transient component of I_{Ba} by GTP-γ-S. However, both these treatments increased the amplitude of the sustained I_{Ba} in the presence of GTP-γ-S. The ATP analogue adenosine imido-diphosphate which inhibits many ATP requiring enzymes did not prevent the effect of GTP-γ-S although it reduced the amplitude of I_{Ba}.

3 Baclofen (100 μM) produced a 22 ± 2% increase in inositol phosphate production in 30 s, whereas the increase produced by bradykinin (1 μM) was 70 ± 14%. However, unlike baclofen, bradykinin did not inhibit I_{Ba} or I_{Ca} in these cells.

4 The effect of protein kinase C inhibitors was examined. Polymixin B (20 μM in patch pipette) had no effect on the inhibition of I_{Ba} by baclofen or GTP-γ-S. A higher concentration (100 μM) alone inhibited I_{Ba} and no further inhibition by baclofen was observed. Neither H7 (50 μM) nor staurosporine (100 nM), applied extracellularly, prevented the response to GTP-γ-S.

5 The protein kinase C activator di-octanoyl glycerol (20 μM) did not inhibit I_{Ba}. Arachidonic acid (100 μM) also produced no inhibition of I_{Ba}.

6 In conclusion we have obtained no evidence that a second messenger system mediates the inhibition of calcium channel currents by GTP-γ-S or baclofen in dorsal root ganglion neurones. These results support the hypothesis that $GABA_B$ receptors are directly coupled to calcium channels by G proteins.

Introduction

Voltage-activated calcium channel currents recorded from cultured dorsal root ganglion neurones may be inhibited by a variety of neurotransmitters including γ-aminobutyric acid (GABA) acting at $GABA_B$ receptors (Holz *et al.*, 1986; Scott & Dolphin, 1986) and neuromodulators such as adenosine acting at A_1-receptors (Dolphin *et al.*, 1986; MacDonald *et al.*, 1986). A common biochemical feature of these receptors is that they are known to couple to guanine nucleotide binding or G proteins (for review see Gilman, 1987), which are sensitive to the bacterial toxin pertussis toxin (PTX) (Ui *et al.*, 1985; Dolphin, 1987) and by this mechanism to inhibit adenylate cyclase in several cell types (Dolphin, 1984). We have observed that the inhibition of calcium channel currents by the $GABA_B$ agonist baclofen and the adenosine agonist 2-choloroadenosine is also prevented by PTX. In addition, the receptor-mediated inhibition of these currents can be enhanced by inclusion of the guanosine 5'-triphosphate (GTP) analogue guanosine 5'-O-3 thiotriphosphate (GTP-γ-S) in the patch pipette, and diminished by the guanosine 5'-

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diphosphate (GDP) analogue guanosine 5'-O-2 thiodiphosphate (GDP-β-S) (Scott & Dolphin, 1986; Dolphin & Scott, 1987). Further experiments showed that these two guanine nucleotide analogues alone influence the amplitude and characteristics of calcium channel currents. GTP-γ-S and the related GTP analogue guanylyl imidodiphosphate reduce the amplitude, particularly of the transient component of the current, and slow the kinetics of activation and inactivation of the residual sustained Ca channel current (Dolphin & Scott, 1987). GDP-β-S, like PTX, increases the amplitude of the transient component of the calcium channel current (Dolphin & Scott, 1987).

The activation of G-proteins by GTP analogues may inhibit calcium currents in a number of ways. Firstly, the activated α subunit of the G protein involved (probably α_i or α_o, since it is PTX-sensitive), or possibly the liberated $\beta\gamma$ subunits may directly inhibit the calcium current. Secondly, activated G protein may interact with an enzyme, such as adenylate cyclase or phospholipase C, either increasing or decreasing the activity. The finding that the G protein is sensitive to PTX initially indicated a possibility that inhibitory modulation of adenylate cyclase activity might be involved (for review see Dolphin, 1984). However, in some cell types (e.g. neutrophils, mast cells and adipocytes) the activation of phospholipase C by receptors is also sensitive to PTX (Cockcroft & Gomperts, 1985); although in most cells this is not the predominant G-protein subtype which is responsible for coupling receptors to the inositol phosphate/diacyglycerol second messenger system (for review see Taylor & Merritt, 1986). It has also been reported by Burch *et al.* (1986) that receptor-mediated phospholipase A_2 activation is sensitive to PTX, although less is known about the coupling of this enzyme to receptors.

In the present study, we have investigated the possible mechanisms by which G protein activation either by the $GABA_B$ receptor agonist (−)-baclofen or by GTP analogues may inhibit calcium channel currents.

Methods

Tissue culture

Dorsal root ganglia from 2 day old rats were dissociated using the method of Winter (1987), and grown in defined F14 medium (Imperial) containing glutamine 4 mM, $NaHCO_3$ 14 mM, insulin (5 μg ml^{-1}), transferrin (5 μg ml^{-1}), progesterone 20 nM, putrescine 100 μM and selenium 30 nM, on either collagen or polyornithine and laminin coated coverslips, or for biochemical experiments on 35 mm 'Primaria' dishes (Falcon). Cells maintained for up to 3 weeks in culture were used, and were treated with cytosine arabinoside (10 μM) for 48–60 h, approximately 48 h after plating, to reduce to a minimum the contamination with dividing non-neuronal cells.

Electrophysiological experiments

Recordings were made from small-medium spherical dorsal root ganglia with small diameter neurites, under voltage clamp with an Axoclamp 2 switching voltage clamp amplifier operated at 15–20 kHz, using the whole cell recording technique (Hamill *et al.*, 1981). Patch electrodes of 1–3 MΩ resistance were used. Drugs were applied either in the bathing medium or by low pressure ejection from a broken micropipette (tip diameter 10 μm) placed 50–100 μm from the cell. Calcium and barium currents were elicited by 100 ms depolarising steps from a holding potential (V_H) of −80 mV (unless otherwise stated). Analysis was performed using a PDP 11/23 computer following digitization at 2 kHz. All calcium channel currents are shown following subtraction of scaled linear leakage and capacitance currents. The standard bathing medium contain (mM): NaCl 130, KCl 3, $MgCl_2$ 0.6, HEPES 10, glucose 4, tetrodotoxin 0.001, tetraethylammonium chloride 25, and either $CaCl_2$, or $BaCl_2$ 2.5. The pH was 7.4 and the osmolarity 320 mosmol (adjusted with sucrose). Patch electrodes were filled with a solution containing (mM): Cs acetate 140, $CaCl_2$ 0.1, EGTA 1.1, $MgCl_2$ 2, ATP-Na 2, and HEPES 10. The pH was 7.2 and the osmolarity 310 mosmol. When present, GTP-γ-S (500 μM; Boehringer-Mannheim) was dissolved directly in the patch solution. In some experiments adenosine imido-diphosphate (AppNHp, Sigma) replaced ATP in the patch solution. Cells were allowed to equilibrate with the contents of the patch pipette for 5 min before recording was begun. Forskolin was a gift from Dr David Hill and PTX was obtained from Dr L. Irons, Porton Down.

Biochemical experiments

All experiments were carried out in defined F14 medium which contained 2 mM Ca^{2+}. Adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels were determined by prelabelling the intracellular ATP pool with [^{3}H]-adenine for 3 h, as previously described by Weiss *et al.* (1985). Cells were then incubated at 37°C with the phosphodiesterase inhibitor isobutylmethylxanthine (1 mM) for 10 min either alone or with other agents. Cyclic AMP formation was expressed as % conversion of [^{3}H]-ATP to cyclic AMP.

Total inositol phosphates was determined by prelabelling cells with [^{3}H]-inositol for 24 h, before

stimulating with either bradykinin or baclofen for 30 s. The method for determination of inositol phosphate production was essentially that of Downes & Michell (1981). Inositol phosphates were calculated as d.p.m. mg^{-1} protein, which was determined by scraping the cells from the culture dish, and estimated using the method of Lowry *et al.* (1951).

Polymixin B (Sigma) and H7 (1-(5-isoquinolinylsulphonyl)-2-methyl piperazine; Sigma) were made up as a 10 mM stock solution in water and diluted on the day of use. Staurosporine (gift from Dr G. Burgess, Sandoz Institute, London) was stored in the dark as a 2 mM solution in DMSO and diluted on the day of use.

Results are expressed as the mean ± s.e.mean, of the number of determinations given in parentheses. The statistical significance of the differences between results was determined by use of Student's *t* test.

Results

Possible involvement of changes in intracellular cyclic AMP levels in the response to GTP-γ-S and baclofen

The inclusion of GTP-γ-S in the patch pipette induces a characteristic response which can be observed if calcium channel currents are recorded during the first 5 min after establishing whole cell clamp. It abolishes the transient component of the calcium channel current and slows the kinetics of activation (Figure 1a). This effect was not prevented by several means of increasing intracellular cyclic AMP. Inclusion of 10 μM forskolin in the patch pipette (Figure 1b) or 1–10 μM forskolin in the bathing medium (results not shown) was without effect on the response to GTP-γ-S. For a series of experiments on the same batch of cells in the presence and absence of forskolin and GTP-γ-S, the amplitude of the maximum calcium channel currents and the respective percentage inactivation during the 100 ms step command are given in Table 1. Both 1 and 10 μM forskolin significantly increased adenylate cyclase activity (approximately 3 and 8 fold respectively) in these cells. Pretreatment of cells with cholera toxin (0.5 μg ml^{-1} for 16 h) did not affect the response to GTP-γ-S (Figure 1c, Table 1). This treatment was sufficient to increase basal adenylate cyclase activity approximately 4 fold in these cells. Similarly, intracellular cyclic AMP (100 μM) had no effect on the response of calcium channel currents to GTP-γ-S (results not shown). However it is clear from the results given in Table 1 that both cholera toxin and forskolin increased the amplitude of the component of the calcium channel currents carried by Ba^{2+} (I_{Ba}), particularly in GTP-γ-S-containing cells.

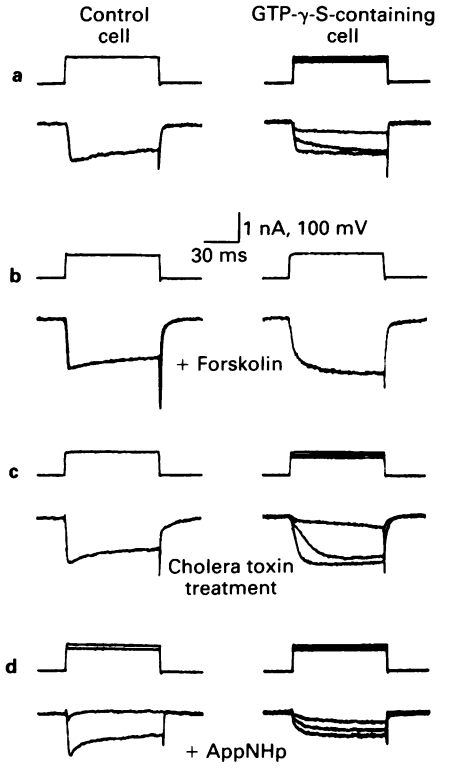

Figure 1 The effect of GTP-γ-S on I_{Ba}. I_{Ba} is not prevented by agents increasing cyclic AMP or by inhibition of phosphorylation. Either maximum currents or a maximal and submaximal current activated from a holding potential (V_H) of −80 mV are shown for control cells (left hand panel) and cells recorded with 500 μM GTP-γ-S in the patch pipette (right hand panel), under each condition. (a) Control conditions; (b) in the presence of 10 μM forskolin in the patch pipette; (c) in cells pretreated for 18 h at 37°C with cholera toxin (0.5 μg ml^{-1}); (d) in cells in which ATP was absent from the patch pipette, and substituted by 2 mM adenosine imido-diphosphate (AppNHp).

The substitution of the ATP analogue AppNHp (2 mM) for ATP in the patch pipette solution, to inhibit phosphorylation reactions, was also unable to prevent the effect of GTP-γ-S (Figure 1d, Table 1). Calcium channel currents recorded in the presence of AppNHp alone were reduced in peak amplitude by 29% compared to controls ($P < 0.05$), although there was no difference in the percentage inactivation during the 100 ms voltage step (Table 1). In addition, the time-dependent run-down of I_{Ba} appeared to be more rapid when AppNHp was substituted for ATP, although this was not investigated in detail.

Neither 50 μM cyclic AMP in the pipette solution

Table 1 The effect of internal GTP-γ-S (500 μM) on control calcium channel currents and those in the presence of various agents

	Control		+GTP-γ-S	
	I_{Ba} (or I_{Ca}) (nA)	% *inactivation*	I_{Ba} (or I_{Ca}) (nA)	% *inactivation*
Control	2.5 ± 0.2 (14)	27.7 ± 6.5 (14)	1.1 ± 0.2 (11)	0
Forskolin (10 μM in pipette)	2.8 ± 0.3 (14)	21.0 ± 3.1 (14)	1.7 ± 0.2* (14)	0
Cholera toxin pretreatment	3.1 ± 0.5 (9)	32.1 ± 6.5 (9)	2.1 ± 0.4** (8)	4.4 ± 3.5 (8)
AppNHp (2 mM in pipette)	1.8 ± 0.3* (9)	26.4 ± 4.0 (9)	1.3 ± 0.3 (9)	8.0 ± 7.1 (9)
Ca^{2+} (5 mM)	2.4 ± 0.4 (17)	55.6 ± 7.9** (17)	1.3 ± 0.2 (5)	16.6 ± 9.2 (5)

The peak amplitude of the maximum I_{Ba} or I_{Ca} was measured and the percentage inhibition of the current during the 100 ms voltage step was determined. *$P < 0.05$; **$P < 0.01$, compared to control.

(Figure 2a) nor 1 μM forskolin in the bath (Figure 2b) reduced the ability of the $GABA_B$ agonist baclofen to inhibit calcium channel currents. In parallel with this finding, in biochemical experiments using intact cells grown in culture for 1 week, 100 μM baclofen had no effect either on basal cyclic AMP accumulation for 10 min or on the enhanced cyclic AMP accumulation in the presence of 100 μM isoprenaline or 10 μM forskolin (Figure 3). However, in preliminary experiments, using the same method which demonstrated baclofen-mediated inhibition of adenylate cyclase in brain membranes (Hill & Dolphin, 1984), baclofen (100 μM) inhibited adenylate cyclase activity in membranes prepared from dorsal root ganglion neuronal cultures by about 20%.

Possible involvement of phospholipase C activation in the response to GTP-γ-S and baclofen

The effect of baclofen was compared to that of bradykinin, which is known activate receptors coupled to phospholipase C. Bradykinin (1 μM) stimulated total inositol phosphate production by 70 ± 14% ($n = 14$) in 30 s, in 1 week old dorsal root ganglion neurones (Figure 4a). In the same batch of cells, 30 s stimulation with baclofen (100 μM) caused a 22 ± 2% increase in total inositol phosphate accumulation (Figure 4a). However, whereas baclofen substantially inhibited calcium channel currents using either 2.5 mM Ba^{2+} (Figure 4b) or 5 mM Ca^{2+} (Figure 4c) as charge carrier, bradykinin (1 μM) did not inhibit calcium channel currents under either of these conditions (Figure 4b, c), and in 3/5 cells examined produced a substantial increase in I_{Ba}.

Since phospholipase C is a calcium-dependent enzyme, and can be activated by Ca^{2+} influx, the ability of GTP-γ-S to exert its effects on calcium channel currents was also examined in medium containing 5 mM Ca^{2+} and compared to 2.5 mM Ba^{2+}.

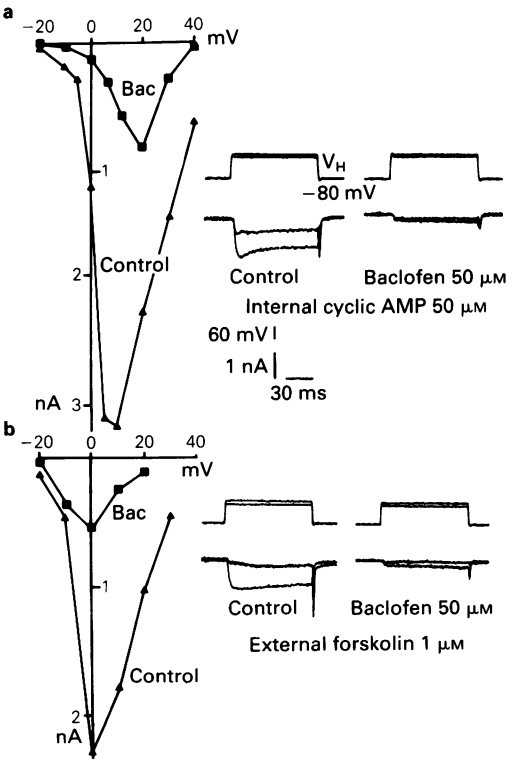

Figure 2 Baclofen inhibition of I_{Ba} is not prevented by cyclic AMP or forskolin. (a) Cyclic AMP (50 μM) was included in the patch pipette solution and the effect of baclofen (Bac) was examined on I_{Ba} by performing a current/voltage (I/V) relationship from a V_H of −80 mV before and during application of baclofen (50 μM). The I/V plot is shown on the left and examples of the current are shown on the right. (b) Forskolin was included in the bathing solution at 1 μM and the effect of baclofen (50 μM) was examined on I_{Ba}, as in (a). For key to abbreviations used see Figure 1 legend.

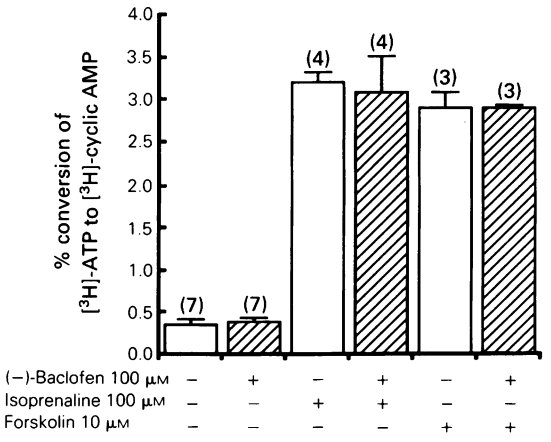

Figure 3 Lack of effect of baclofen on cyclic AMP levels in intact dorsal root ganglion neurones. The formation of cyclic AMP in the presence of 1 μM isobutyl methylxanthine was examined as described in the Methods. Dishes of cells were exposed for 4 min to the combinations of drugs indicated, and the cyclic AMP estimated according to Weiss *et al.* (1985). The open columns represent determinations made in the absence of baclofen and the hatched columns are results obtained in the presence of 100 μM baclofen. The numbers of determinations are given in parentheses; vertical bars show s.e.mean.

When Ca^{2+} was used as charge carrier, the control calcium channel current showed a greater degree of inactivation than that carried by Ba^{2+} (e.g. Figure 4c, compared to Figure 4b). Although the peak amplitude was similar (Table 1), I_{Ca} showed a 55.6 ± 7.9% inactivation during the voltage step, compared to I_{Ba} in the same batch of cells which showed 27.7 ± 6.5% inactivation ($P < 0.01$). Whereas GTP-γ-S (500 μM) abolished the inactivating component of I_{Ba}, it did not completely inhibit that of I_{Ca} (Table 1), leaving an inactivating component of about 16%.

Possible involvement of protein kinase C on the response to GTP-γ-S and baclofen

The protein kinase C (PKC) inhibitor polymixin B was included either in the bathing medium or in the patch pipette to examine the ability of baclofen and GTP-γ-S to inhibit calcium channel currents in its presence. In this series of experiments, control I_{Ba} was 3.1 ± 0.5 nA (7), and inhibition by 50 μM baclofen was 25.3 ± 5.8%. With 20 μM polymixin B in the patch pipette, I_{Ba} was 2.2 ± 0.5 nA (5) and the same concentration of baclofen produced a 55.9 ± 14% inhibition of the current (Figure 5a). The presence of 100 μM polymixin B in the patch pipette substantially inhibited I_{Ba} which was 1.2 ± 0.4 nA (5) and baclofen

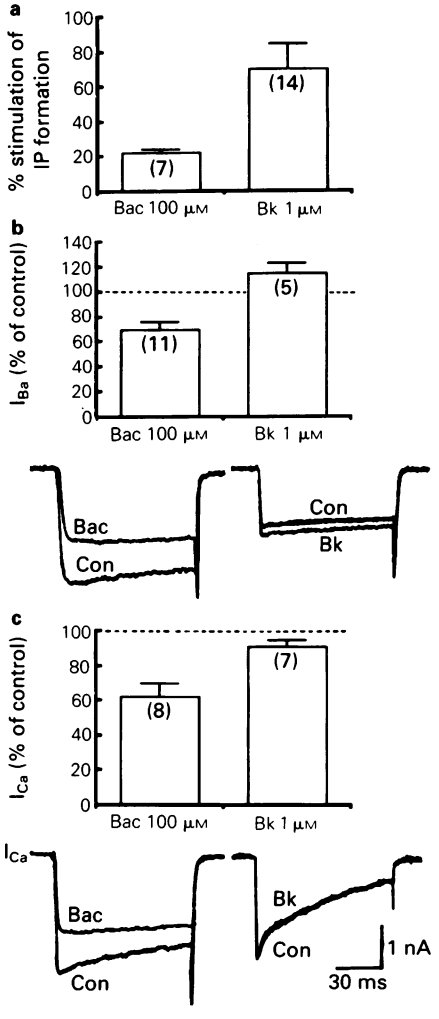

Figure 4 Effect of baclofen (Bac) and bradykinin (Bk) on inositol phosphate production, and on I_{Ba} and I_{Ca}. (a) Shows the % stimulation of formation of total inositol phosphates (IP) due to 30 s exposure to either baclofen 100 μM or bradykinin (1 μM). Estimation of IP production is described in Methods. (b) Shows the effect of baclofen and bradykinin on I_{Ba}, expressed as % of the control current in each cell. Examples of the response to the two agonists are shown beneath the histogram. (c) Shows the effect of baclofen and bradykinin on I_{Ca} (5 mM Ca^{2+} present in the medium, substituted for Ba^{2+}). Examples of I_{Ca} and the response to the two agonists are shown beneath the histogram. Note the greater inactivation of I_{Ca} compared to I_{Ba}. Con = control.

subsequently produced no further inhibition. Bath application of 10 μM polymixin B also reduced I_{Ba} which was 1.2 ± 0.1 nA (5), again with negligible inhibition by baclofen (Figure 5b). Like polymixin B,

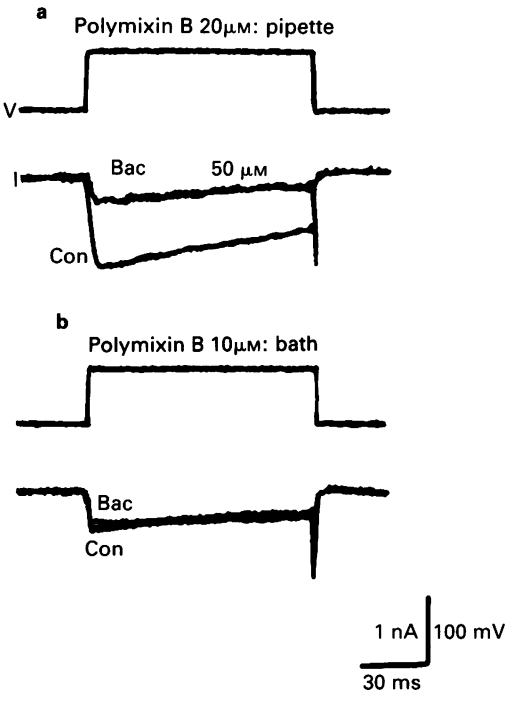

Figure 5 Effect of polymixin B on I_{Ba} and its inhibition by baclofen (Bac). (a) The effect of 50 μM baclofen on I_{Ba} in a cell recorded with 20 μM polymixin B in the patch pipette. (b) The lack of effect of 50 μM baclofen on I_{Ba} in a cell recorded in the presence of 10 μM polymixin B in the bathing medium.

H7 also reduced the amplitude of the control I_{Ba} (results not shown) but did not affect its inactivation. The effect of baclofen was thus not examined further.

The effect of a series of protein kinase C inhibitors (polymixin B, H7 and staurosporine) was tested on the ability of internal GTP-γ-S to inhibit the transient component of I_{Ba} (Figure 1a). In this batch of cells, I_{Ba} in the presence of internal GTP-γ-S, was 2.4 ± 0.7 nA (4) and non-inactivating. This effect of GTP-γ-S (Figure 6a) was not prevented by 20 μM polymixin B in the patch pipette solution (Figure 6b). In these cells the current was 1.8 ± 0.3 nA (6), and was also slowly activating, with no inactivation during the voltage step (Figure 6b). Similarly, H7 (50 μM) and staurosporine (100 nM) applied in the bathing medium did not influence the response of I_{Ba} to GTP-γ-S. In GTP-γ-S containing cells the current amplitude in the presence of H7 was 1.2 ± 0.2 nA (5) significantly less than in the absence of H7, although it remained slowly activating and non-inactivating (Figure 6c). In the presence of staurosporine, I_{Ba} was 2.1 ± 0.5 nA (5) in GTP-γ-S-containing cells, and it showed $2.4 \pm 2.6\%$ inactivation during the voltage step (Figure 6d).

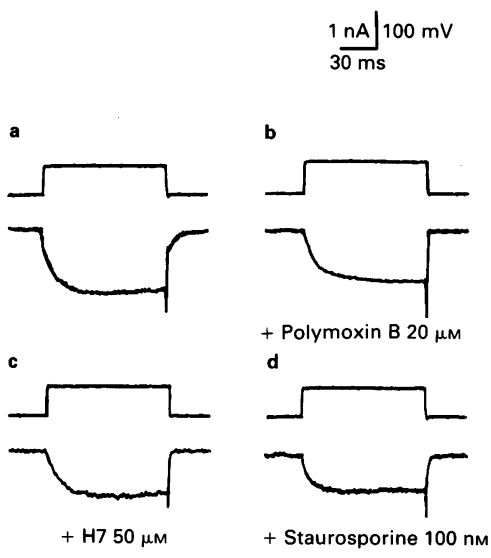

Figure 6 Inability of protein kinase C (PKC) inhibitors to prevent the effect of GTP-γ-S. Results from 4 different cells are illustrated. When the effect of bath applied PKC inhibitors was examined, cells were preincubated with the inhibitor for 30 min before beginning the experiment. (a) The effect on internal GTP-γ-S (50 μM) in a control cell. The maximum current is illustrated, activated from V_H of -80 mV. (b) The lack of effect of polymixin B (20 μM) in the patch pipette on the response to GTP-γ-S. (c) The lack of effect of H7 (50 μM) included in the bathing medium, on the response to GTP-γ-S. (d) The lack of effect of staurosporine (100 nM) included in the bathing medium on the response to GTP-γ-S.

The effect of protein kinase C activation by diacylglycerol was examined to determine whether it might mimic the effect of GTP-γ-S. Local application of di-octanoylglycerol (diC8) by low pressure ejection at a concentration of 20 μM had little effect on either the amplitude of the maximum I_{Ba} or on its inactivation during the voltage step. I_{Ba} was $109 \pm 11\%$ (6) of its control amplitude following 30 s application of diC8, and an increase in I_{Ba} was observed in 4/6 cells.

Possible involvement of arachidonic acid as a second messenger in the response of calcium channel currents to GTP-γ-S

Arachidonic acid (100 μM) applied by low pressure ejection for up to 5 min had no effect on maximum calcium channel currents. I_{Ba} was 3.0 ± 0.2 nA (8) before application of arachidonic acid, with 36.9% inactivation during the voltage step (Figure 7a). The current was not significantly reduced after 30 s application of arachidonic acid (Figure 7b), with no

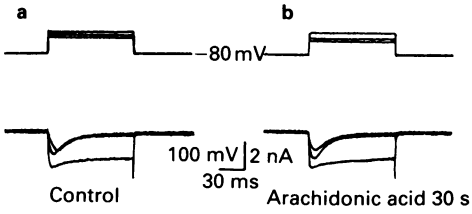

Figure 7 Lack of effect of arachidonic acid on I_{Ba}. (a) Shows the activation of I_{Ba} in a control cell from a V_H of −80 mV, and (b) shows the activation of I_{Ba} after 30 s application of arachidonic acid (100 μM). Arachidonic acid (Sigma) was made up and used on the same day.

further reduction after continued application for 5 min in 4 of the cells.

Discussion

The response of calcium channel currents to GTP-γ-S in rat dorsal root ganglion neurones may represent a direct activation of the G protein which couples receptors to voltage activated calcium channels, and mediates inhibition of calcium currents. In dorsal root ganglion neurones and certain other cell types, receptor agonists differentially reduce the transient component of the whole cell Ca channel current (Tsien *et al.*, 1988; and see Figures 4 and 5). This effect is mimicked by GTP-γ-S (Scott & Dolphin, 1986; Lewis *et al.*, 1986) although baclofen also inhibited the sustained component of the current (Scott & Dolphin, 1986; Dolphin & Scott, 1987). From our own results the response to GTP-γ-S, like the response to agonists can be prevented by pretreatment of cells with PTX, which interferes with the activation of its substrate G proteins by receptors (Katada & Ui, 1981), and also reduces the rate of G protein activation by GTP and its analogues (Jakobs *et al.*, 1984). This indicates that the G proteins involved are of the G_i/G_o family, of which several more members are now known (Gilman, 1987).

There is evidence that PTX-sensitive G proteins may couple receptors directly to K channels in cardiac myocytes (for review see Brown & Birnbaumer, 1988), but as yet there is no comparable single channel evidence for the direct association of Ca^{2+} channels with these G proteins. Although reconstitution experiments have been performed (Hescheler *et al.*, 1987; Ewald *et al.*, 1988), which show α_o to be particularly effective in restoring the coupling between opiate and neuropeptide Y (NPY) receptors and Ca channels in PTX treated neuroblastoma-glioma hybrids and dorsal root ganglion cells respectively, such experiments have only been done in intact cells, and not using isolated patches. The alternative possibility is that G protein-induced activation of an enzyme and a subsequent change in the concentration of a second messenger is responsible for the reduced calcium channel current. The candidate enzymes whose activity is likely to be modified by agonists or internal GTP-γ-S include adenylate cyclase, phospholipase C, and possibly phospholipase A_2. We have investigated agents which either mimic or ihhibit receptor activation of these second messenger systems, or the subsequent response to the second messengers. In parallel experiments we have examined the ability of several agents to prevent the effect of the agonist baclofen.

All the receptor agonists known to be associated with inhibition of calcium channel currents in dorsal root ganglia are also associated in various tissues with inhibition of adenylate cyclase. Elevation or reduction of the cyclic AMP levels in cardiac myocytes is associated respectively with enhancement or inhibition of the calcium currents in these cells (Reuter, 1983; Hartzell & Fischmeister, 1987). However, none of the means used to activate maximally adenylate cyclase (forskolin, cyclic AMP, cholera toxin) mimicked the effect of GTP-γ-S on calcium channel currents in dorsal root ganglion neurones, nor did they prevent the effect of GTP-γ-S or baclofen. If GTP-γ-S were acting on calcium channel currents by inhibiting adenylate cyclase, then this effect should be abolished if cyclic AMP levels were maximally activated by any of these means. Similarly, substitution of AppNHp for ATP in the patch solution, an analogue which inhibits many ATP requiring enzymes at the substrate site, will both inhibit cyclic AMP production and non-specifically inhibit all kinases, reducing levels of phosphorylation inside the cell (Kameyama *et al.*, 1985). Internal AppNHp did not interfere with the response to GTP-γ-S, suggesting that a change in the phosphorylation state of a substrate protein is not responsible for the effects of GTP-γ-S. Nevertheless, the agents increasing intracellular cyclic AMP levels produced an enhancement of calcium channel currents and AppNHp produced a small reduction, suggesting that cyclic AMP-dependent phosphorylation may be important in regulating neuronal as well as heart calcium channels. Similar results were obtained by Fedulova *et al.* (1985) who concluded that cyclic AMP-dependent protein phosphorylation reduces rundown of calcium currents in sensory neurones. It has been observed that there is a rapid loss of high threshold single channel activity when patches are isolated from these neurones (Carbone & Lux, 1987; Kostyuk *et al.*, 1988).

It has been shown that phorbol esters and DAG inhibit calcium channel currents in mouse and chick dorsal root ganglion neurones (Rane & Dunlap, 1986; Gross & MacDonald, 1988), and subsequently prevent further inhibition by the agonist noradrena-

line (Rane & Dunlap, 1986), suggesting that protein kinase C (PKC) is responsible for mediating the inhibition of Ca channel currents. Similarly, down-regulation of PKC activity by chronic phorbol ester treatment reduced the ability of NPY to inhibit the sustained component of the current in these cells. This was taken as evidence that PKC activation by DAG inhibits L-type currents (Ewald *et al.*, 1988). In addition, a correlation between the distribution of immunoreactivity for the PTX-sensitive G protein G_o and phorbol ester binding in brain has also been taken as evidence for such a relationship (Worley *et al.*, 1986). However, a complicating factor is that PKC activation by phorbol esters also phosphorylates and inactivates pertussis toxin-sensitive G proteins and some receptors (Jakobs *et al.*, 1985; Nishizuka, 1988). Although in most neuronal tissues the activation of phospholipase C to produce DAG and inositol triphosphate (IP_3) is not under the control of the PTX-sensitive G protein, and this is also the case for bradykinin-stimulated inositol phosphate production in dorsal root ganglion cells (G. Burgess, personal communication), it is clear that production of DAG may also occur from non-inositol-containing phospholipids (Irving & Exton, 1987).

We have not found the various PKC inhibitors used, polymixin B, H7 and staurosporine to prevent the effect of GTP-γ-S. However, both H7 and polymixin B reduced the amplitude of I_{Ba}, possibly because they are not specific inhibitors. For example, H7 also inhibits other protein kinases and will have a similar effect to AppNHp (Garland *et al.*, 1987). The effect of polymixin B was also examined on the ability of baclofen to inhibit calcium channel currents. No inhibitory effect was observed, except at a concentration which alone inhibited calcium channel currents, possibly as a result of polymixin B binding to the N type calcium channel (Knaus *et al.*, 1987). In addition the PKC activator DiC8 which is one of the more water soluble DAGs that has not been found to have detergent actions, unlike oleoyl-acetylglycerol (Lapetina *et al.*, 1985), had little effect on I_{Ba} and did not mimic the effect of GTP-γ-S, although it clearly mimics the action of other PKC activators when applied to intact cells (Lapetina *et al.*, 1985). These results are in agreement with those of several other authors, including Wanke *et al.* (1987), who investigated the effect of acetylcholine (ACh) on I_{Ca} in superior cervical ganglion cells, and did not find the response to ACh to be mimicked by phorbol esters. Similarly, phorbol esters do not inhibit calcium currents in NG 108-15 neuroblastoma-glioma hybrid cells (Docherty & McFazdean, personal communication), and increase the activity of single calcium channels in sympathetic neurones (Lipscombe *et al.*, 1988).

Phospholipase C activation, in addition to producing DAG, also causes polyphosphoinositide breakdown to IP_3, and it is possible that release of intracellular Ca^{2+} by IP_3 might result in inhibition of calcium channel currents. This hypothesis was tested by examining the ability of bradykinin, which is known to stimulate PIP_2 breakdown in these cells (Burgess *et al.*, 1988) (a result confirmed in these studies), to inhibit calcium channel currents. No significant inhibition was observed, even on I_{Ca} where sufficient extracellular Ca^{2+} would be available to replenish intracellular stores, which might become depleted in Ba^{2+}-containing medium. In contrast, baclofen significantly inhibited both I_{Ba} and I_{Ca} producing a differential inhibition of the transient component. Although baclofen produced a small increase in inositol phosphate turnover, this was much less than that induced by bradykinin. It is now the subject of further investigation, to determine the nature of the $GABA_B$ receptor involved. In addition, the finding that GTP-γ-S was similarly effective in reducing the transient component of the Ca^{2+} channel current when Ca^{2+} rather than Ba^{2+} was the charge carrier, also indicates that a rise in intracellular Ca^{2+} is unlikely to be responsible for its effects. The small degree of inactivation still present in GTP-γ-S-containing cells with 5 mM external Ca^{2+} is likely to represent Ca-dependent inactivation of the calcium current, since it was not present when 2.5 mM Ba^{2+} was used as the charge carrier.

In some systems it has been proposed that $GABA_B$ receptors activate phospholipase A_2 (Duman *et al.*, 1986). Activation of this enzyme leads to arachidonic acid formation which has been implicated as a second messenger in several systems (Piomelli *et al.*, 1987; Axelrod *et al.*, 1988), and can activate one of the subtypes of PKC (Murakami & Routtenberg, 1985; for review see Nishizuka, 1988). Phospholipase A_2 is a Ca-dependent enzyme, which also appears in some cases to be under the direct control of a receptor via a G protein. The possibility that GTP-γ-S might act on calcium channel currents by stimulating this enzyme was examined by the application of arachidonic acid itself, since it has been shown that it can act intracellularly following extracellular application (Piomelli *et al.*, 1987). However, application of arachidonic acid by this means had no clear effect on the amplitude of the maximum calcium channel current, and in no case mimicked the effect of GTP-γ-S.

In conclusion, we have obtained no evidence in these whole cell clamp experiments that second messengers are involved in the response of calcium channel currents to either G protein or receptor activation. This is in agreement with the conclusion of Forscher *et al.* (1986) who found that noradrenaline,

while reducing the whole cell calcium channel current in dorsal root ganglion neurones, had no effect on the calcium channels in a cell-attached patch when applied externally to the patch pipette. This suggested that a diffusible second messenger was not involved in the effect of noradrenaline. Similar results have also been obtained for the effect of noradrenaline on sympathetic neurones (Lipscombe *et al.*, 1988), and of baclofen on dorsal root ganglion neurones (Green & Cottrell, 1988). In the case of cardiac myocytes and purified skeletal muscle calcium channels it is clear that L-type calcium channels respond to phosphorylation by a cyclic AMP-dependent protein kinase with an increased probability of opening (Hymel *et al.*, 1988; for review see Tsien *et al.*, 1988), although it is unclear whether the channel must be phosphorylated to be functional (Hymel *et al.*, 1988). In the present experiments non-phosphorylating conditions (AppNHp) reduced the amplitude of I_{Ba} to a small extent, but there was only a small enhancement by agents which raised internal cyclic AMP. An enhancement was particularly observed of the sustained current in the presence of GTP-γ-S. Either L channels in dorsal root ganglion neurones are different from those in heart, and do not require phosphorylation to be functional, or they are more completely phosphorylated under the conditions of the present experiments.

It has recently been shown from single channel studies that L-type calcium channels from cardiac ventricular cells are directly influenced by activation of the cholera toxin substrate G protein G_s (Yatani *et al.*, 1988). An effect of agonists such as isoprenaline in isolated patches could be demonstrated to be independent of adenylate cyclase activation. This is the first evidence that these calcium channels are associated directly with a G_s-protein, although there remains no evidence for any direct interaction of a PTX-sensitive G protein with calcium channels in cardiac myocytes. The present results indicate that in dorsal root ganglion neurones a direct coupling may exist between G proteins and calcium channels. In our present and previous studies we have observed that whereas GTP-γ-S differentially inhibited the transient component of the calcium channel current particularly, baclofen often caused complete inhibition of the current, especially when applied to GTP-γ-S-containing cells. This suggests that the channels underlying the transient and the sustained calcium channel currents may be associated with different G proteins with different basal off-rates for GDP (Dolphin *et al.*, 1988). Taken together with our previous data implicating a PTX-sensitive G protein in the modulation of neuronal L-type calcium channel currents by calcium channel ligands (Scott & Dolphin, 1987; 1988), the possibility is opened that, like the catalytic subunit of adenylate cyclase, Ca channel activity is regulated by several classes of G protein, which may interact directly with the channel.

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Facilitation by tachykinins of neurotransmission in guinea-pig pulmonary parasympathetic nerves

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1 The effect of tachykinins on cholinergic neurotransmission was studied in an innervated tracheal tube preparation isolated from guinea-pigs anaeshetized with urethane. The tracheal tube was bathed in Krebs-Henseleit solution containing 5 μM indomethacin.

2 Neurokinin A (NKA), eledoisin (El) and substance P (SP) caused concentration-dependent increases in intraluminal pressure (ILP), with an order of potency NKA > El $\gg$ SP.

3 Low concentrations of tachykinins, that had little effect on ILP, caused an increase in the contractions elicited by stimulation of the preganglionic vagal nerve fibres and by postganglionic (transmural) stimulation. The order of potency was NKA $\geqslant$ El > SP. Contractions induced by exogenous acetylcholine (ACh) were not increased by the tachykinins.

4 The magnitude of the tachykinin-induced augmentation of responses to nerve stimulation was inversely related to stimulation voltage and frequency.

5 These results suggest that tachykinins act on NK_2 receptors, both on the trachealis muscle and on postganglionic pulmonary parasympathetic nerve terminals. Activation of the neuronal receptors may increase the probability of transmitter release from the nerve terminals.

Introduction

Substance P (SP) and neurokinin A (NKA) are endogenous tachykinins which are encoded by two genes and expressed in neurones. SP is present in afferent nerves in the airways of several species, including guinea-pigs and man (Nilsson *et al.*, 1977; Lundberg *et al.*, 1984; Polak & Bloom, 1986) and SP-immunoreactive neurones have been identified in the vagus nerve (Gamse *et al.*, 1979). SP and other tachykinins have been shown to cause contraction of human and guinea-pig airways *in vitro* (Mizrahi *et al.*, 1982; Lundberg *et al.*, 1983) and *in vivo* (Andersson & Persson, 1977; Goel & Biggs, 1986). These responses are unaffected by atropine or mepyramine, and are probably due to a direct effect of the peptides on a specific sub-population of tachykinin receptors on the airway smooth muscle cells. This notion is supported by the demonstration by means of an autoradiographic technique that a high density of SP-receptors exists on guinea-pig airways smooth muscle (Carstairs & Barnes, 1986).

In the guinea-pig myenteric plexus, SP stimulates the release of acetylcholine (ACh) from cholinergic nerves (Yau & Youther, 1982) and in the rabbit trachea, SP enhances the cholinergic contractions induced by electrical field stimulation (Tanaka & Grunstein, 1986). This suggests that SP may have a neuromodulatory role and may facilitate neurotransmission at postganglionic cholinergic nerve terminals. SP-immunoreactive neurones have also been identified in airway ganglia (Lundberg *et al.*, 1984; Polak & Bloom, 1986) and it is therefore possible that SP may modulate airway ganglionic neurotransmission.

The rank orders of potency of SP and related peptides on different tissues suggests the presence of at least three distinct sub-populations of tachykinin receptor (Lee *et al.*, 1986; Buck & Burcher, 1986). In guinea-pig trachea, NKA and eledoisin are more potent than SP in causing smooth muscle contraction *in vitro* (Karlsson *et al.*, 1984; Uchida *et al.*, 1987; Grandordy *et al.*, 1988) and bronchoconstriction *in vivo* (Hua *et al.*, 1984) suggesting the presence of NK_2 (SP-E) receptors on airway smooth muscle cells. It is not known, however, whether the putative tachykinin receptors on airway cholinergic nerve terminals are of the same sub-type as those on the smooth muscle.

We have now investigated the effects of three tachykinins on cholinergic neurotransmission at postganglionic nerve terminals, using an innervated

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guinea-pig tracheal tube preparation in which preganglionic stimulation of the vagus nerve and postganglionic (transmural) stimulation were possible (Blackman & McCaig, 1983). Using rank orders of potency of NKA, eledoisin and SP, we have been able to classify the tachykinin receptor subtype involved in the effects. A preliminary study has been communicated to the British Pharmacological Society (Barnes *et al.*, 1987).

Methods

Innervated tracheal tube preparation

Guinea-pigs (350–450 g) of the Dunkin-Hartley strain (Graystoke, Hampshire) were anaesthetized with urethane (1.5 g kg^{-1} i.p.). The trachea was removed with the right vagus and recurrent laryngeal nerves attached, as described by Blackman & McCaig (1983). The trachea was tied onto an electrode holder to form a sealed tube filled with Krebs-Henseleit solution (KHS; composition mM: NaCl 118.4, KCl 4.7, $NaHCO_3$ 2.50, glucose 11.1, KH_2PO_4 1.16, $MgSO_4 \cdot 7H_2O$ 1.19 and $CaCl_2$ 2.6).

The tracheal tube was cannulated and connected to a Statham transducer (P23AC) for measurement of intraluminal pressure (ILP). Trachea and electrode were suspended in a 40 ml bath of KHS, containing 5 μM indomethacin to abolish spontaneous tone (as described by Farmer & Coleman, 1970). Both reservoir and bath were bubbled with a 95% O_2/5% CO_2 mix and the bath temperature was maintained at 37°C.

The vagus nerve was drawn through a silver–silver chloride ring electrode to enable preganglionic nerve stimulation (30 Hz, 0.2 ms for 5 s at 45 s intervals). Transmural stimulation (using the same parameters) was performed using a separate pair of electrodes, one positioned inside the tube and the other in the organ bath. Stimuli were delivered using a Grass S44 stimulator. In experiments using transmural stimulation, propranolol (1 μM) was added to the KHS to abolish relaxant responses due to simultaneous excitation of sympathetic nerve fibres (Farmer & Coleman, 1970). Both types of electrical stimulation, or addition of acetylcholine (ACh), caused an atropine-sensitive increase in ILP due to contraction of the trachealis muscle.

In order to check the pre- and post-ganglionic nature of the two types of electrical stimuli, hexamethonium (25 μM) was added to the organ bath (at the end of each experiment) to abolish the response to preganglionic vagal nerve stimulation, followed by atropine (0.5 μM) to abolish the response to transmural stimulation.

Experimental protocol

After a 1.5 h equilibration time in the presence of 5 μM indomethacin, a stable base-line ILP and constant responses to nerve stimulation were obtained (control values). Tachykinins were added directly to the organ bath in volumes of not more than 0.4 ml and left in contact with the tissue for 8 min. The effects of tachykinins, both on ILP and on responses to nerve stimulation had been previously shown to equilibrate within this time period. After each dose, the tissue was washed and all readings allowed to return to control values before subsequent additions.

In those experiments involving nerve stimulation, the voltage-response relationship was determined at the beginning and checked at the end of each such experiment. The term V_{50} describes the voltage which, in any given preparation, elicited 50% of the maximal response to nerve stimulation. Where stimulation frequency was varied, the number of impulses in the train was kept constant (at 150) by increasing the train length as the frequency decreased.

Drugs and chemicals

Drugs used were as follows: urethane and indomethacin (Sigma), propranolol hydrochloride (ICI), histamine acid phosphate, acetylcholine bromide and atropine sulphate (BDH), hexamethonium bromide (Koch-Light), neurokinin A, eledoisin and substance P (Peninsula Laboratories).

ACh, hexamethonium, histamine and atropine were dissolved in physiological saline. Indomethacin (1 mg ml^{-1}) was made up in buffer (KH_2PO_4 19.76 mM and Na_2HPO_4 118.34 mM; pH adjusted to 7.8 with NaOH) before addition to the KHS. The precautions for handling peptides outlined by Stewart (1983) were followed throughout. Where possible, polyethylene or polypropylene tubes were used, and all glass tubes and the organ bath were treated before use with dimethyldichlorosilane (Sigma).

Concentrated solutions of the peptides were prepared in distilled water, divided into aliquots of 100 μl each and stored at −70°C until used. Freshly made solutions of peptides were diluted in saline and stored on ice throughout the experiments. Peptide concentrations were calculated from the manufacturer's data for purity and molecular weight.

Results

Effect of tachykinins on trachealis muscle contraction

Addition of tachykinins to the organ bath caused slowly-developing, concentration-dependent in-

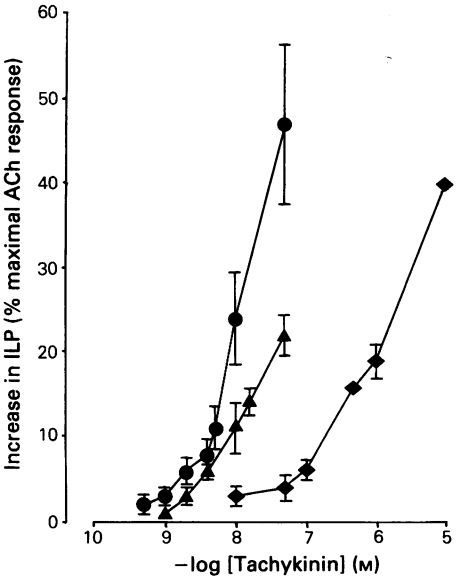

Figure 1 Effects of neurokinin A (●), eledoisin (▲) and substance P (◆) on intraluminal pressure (ILP) of guinea-pig isolated tracheal tube preparation. Increases in ILP are expressed as a % of maximal response to acetylcholine (ACh). Points represent means of 5–9 experiments (except SP 10 μM; $n = 4$); vertical lines show s.e.mean. Indomethacin (5 μM) was present throughout all experiments.

creases in the ILP, reflecting contraction of the trachealis muscle. There was no evidence of tachyphylaxis in our experiments. The effect of the tachykinins on the ILP was not reduced by 0.5 μM atropine, a concentration which totally inhibited the response to preganglionic nerve stimulation, and these results therefore confirm the findings of other workers (Mizrahi *et al.*, 1982; Lundberg *et al.*, 1983).

In each tissue, the contractions elicited by the tachykinins were expressed as a % of the maximal increase in ILP caused by ACh. NKA was more potent than eledoisin, which was more potent than SP (Figure 1). Consideration of expense became a limiting factor at high concentrations of tachykinins, and maximal contractile responses were therefore not measured for the peptides.

Effect of tachykinins on cholinergic neural responses

Preganglionic vagal nerve stimulation, or transmural stimulation in the presence of 1 μM propranolol, caused a rapid, brief increase in ILP due to contraction of the trachealis muscle. The response to vagal stimulation could be abolished by hexamethonium

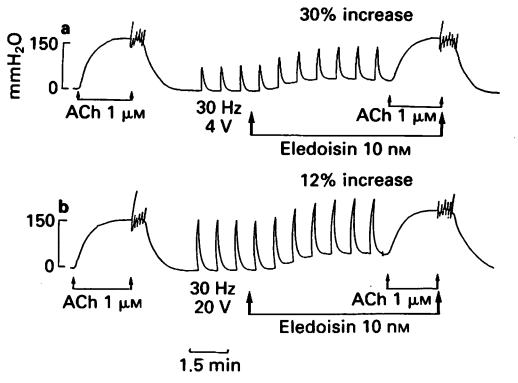

Figure 2 Effect of 10 nM eledoisin on responses of the guinea-pig isolated tracheal tube preparation to preganglionic vagal nerve stimulation at two different voltages (30 Hz, 0.2 ms for 5 s at 45 s intervals). Eledoisin caused a 30% increase in the response to stimulation at 4 V (a) and a 12% increase in response at 20 V (b). The rise in intraluminal pressure produced by 10 nM eledoisin was similar after both challenges, and the pressor responses to 1 μM acetylcholine (ACh) were unaltered by the presence of the tachykinin. Maximal response to ACh was 450 mmH_2O. Indomethacin (5 μM) was present throughout.

(25 μM), indicating that stimulation was preganglionic. Atropine (0.5 μM) abolished the responses to both vagal nerve stimulation and transmural stimulation, indicating that only cholinergic pathways were involved. Responses to both types of electrical stimulation were increased by addition of tachykinins to the organ bath. However, no direct comparison between pre- and post-ganglionic effects was made. The augmenting effect of the tachykinins could be demonstrated at low concentrations, which had little effect on ILP. This is illustrated in Figure 2, which shows the results of an experiment using eledoisin. Eledoisin (10 nM) augmented the responses to nerve stimulation, but caused a rise in ILP equivalent to only 10% of the maximal response to ACh. This figure also shows that the response to 1 μM ACh was unaltered by the presence of the tachykinin, suggesting that the augmenting effect of the eledoisin on neurally-mediated responses involves a prejunctional mechanism. This lack of effect of the peptides on responses to exogeneous ACh was subsequently confirmed for NKA and SP.

The degree of augmentation of neurally-mediated responses was dependent on the stimulation voltage used. This is also illustrated in Figure 2a, which shows that responses to preganglionic vagal nerve stimulation at 4 V were increased by 30% on addition of 10 nM eledoisin to the organ bath. However, at 20 V the same concentration of eledoisin increased

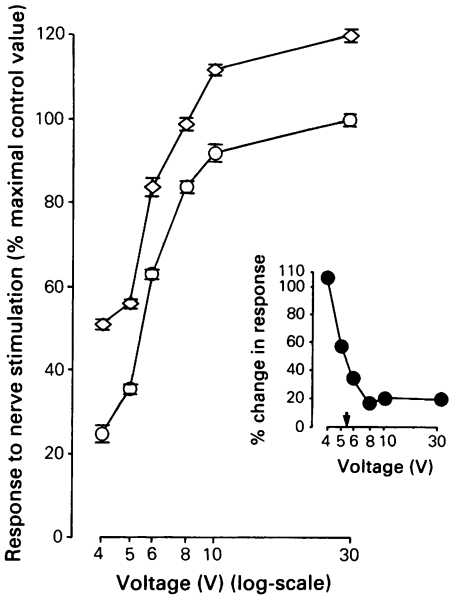

Figure 3 Effect of 5 nM neurokinin A (NKA, ◇) on responses of the guinea-pig isolated tracheal tube preparation to preganglionic vagal nerve stimulation at different voltages (30 Hz, 0.2 ms for 5 s at 45 s intervals). Controls (○) in absence of NKA. Each point represents mean of 5 measurements; vertical lines show s.e.mean. The % change in response at different voltages, calculated from these data, is shown in the inset. Indomethacin (5 μM) was present throughout.

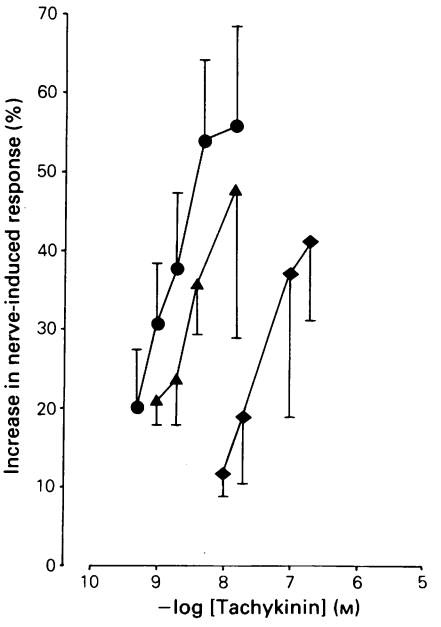

Figure 4 Effect of neurokinin A (●), eledoisin (▲) and substance P (◆) on responses of the guinea-pig isolated tracheal tube preparation to preganglionic vagal stimulation (V_{50}, 30 Hz, 0.2 ms for 5 s at 45 s intervals). Effect is expressed as a % increase in response with respect to control values before addition of the tachykinin. Points represent means of 5–7 experiments; vertical lines indicate s.e.mean. Indomethacin (5 μM) was present throughout all experiments.

the responses by only 12% (Figure 2b), although the small rise in ILP produced by 10 nM eledoisin was similar for both applications. A similar effect of voltage was subsequently confirmed for augmentation by NKA and SP.

Augmentation of responses to vagal stimulation produced by NKA at different voltages is shown graphically in Figure 3. The control curve shows the effect of voltage on the responses elicited by preganglionic vagal nerve stimulation. NKA (5 nM) caused an upward shift of the voltage-response curve, but the % change in response in the presence of the tachykinin was greater at submaximal voltages. Stimulation at very low voltages produced inconsistent responses, and subsequent experiments were performed at V_{50}, which was a voltage low enough to allow a measurable effect of the tachykinins to be observed, yet high enough to produce relatively consistent responses to nerve stimulation.

All 3 tachykinins tested augmented the responses to preganglionic vagal nerve stimulation at V_{50} in a concentration-dependent manner (Figure 4). In any one experiment, the order of potency was NKA > eledoisin > SP. However, the % increase caused by a given concentration of tachykinin varied considerably between preparations. It is probable that this was due to the steep voltage-effect relationship (Figure 3), which meant that any slight deviation from the V_{50} would produce a large difference in % increase between preparations. A comparison of Figures 1 and 4 again illustrates that the augmentation caused by the tachykinins could be seen at concentrations that produced very little rise in ILP.

The magnitude of the augmenting effect of the tachykinins was inversely related to stimulation frequency. Figure 5 illustrates this for SP. Addition of 1 M SP to the organ bath caused a 31% increase in the response to preganglionic vagal stimulation at 30 Hz (Figure 5a), but the same concentration of SP produced a 76% increase in response to stimulation at 5 Hz (Figure 5b). Both SP challenges caused comparable small rises in ILP.

The effect of stimulation frequency on the response to nerve stimulation in the presence and absence of NKA is shown graphically in Figure 6. The control frequency–response curve was obtained

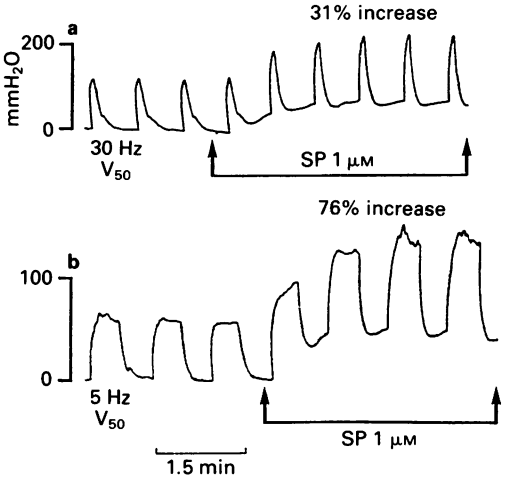

Figure 5 Effect of 1 μM substance P (SP) on responses of the guinea-pig isolated tracheal tube preparation to preganglionic vagal nerve stimulation at two different frequencies (V_{50}; 0.2 ms pulse width). SP caused a 31% increase in the response to stimulation at 30 Hz (a) and a 76% increase in response at 5 Hz (b). The rise in intraluminal pressure produced by 1 μM SP was similar after both challenges. The number of impulses in the train was kept constant (at 150) by increasing the train length as the frequency was decreased. Indomethacin (5 μM) was present throughout.

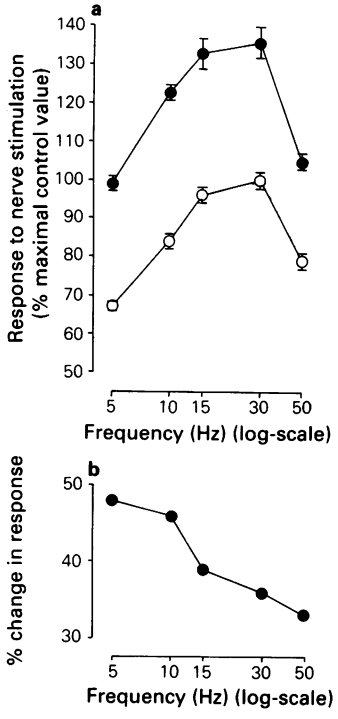

Figure 6 (a) Effect of 5 nM neurokinin A (NKA, ●) on responses of the guinea-pig isolated tracheal tube preparation to preganglionic vagal nerve stimulation at different frequencies (V_{50}; 0.2 ms pulse width). Controls (○) in the absence of NKA. Each point represents the mean of 5 measurements; vertical lines indicate s.e.mean. (b) The % change in response at different frequencies, calculated from the data in (a). Indomethacin (5 μM) was present throughout.

by making step-wise increases in stimulation frequency in the absence of NKA. NKA 5 nM caused an upward shift of the frequency–response curve, but the % change in response in the presence of the tachykinin was greatest at low frequencies.

Discussion

The present results demonstrate that tachykinins cause concentration-dependent contractions of the guinea-pig isolated tracheal tube preparation. Previous studies (mostly with SP) have generally indicated that this effect is due to a direct action of the peptide(s) through receptors located on the trachealis muscle cells (Nilsson *et al.*, 1977; Pernow, 1983). There is good evidence for this hypothesis, since the SP-induced contractions in human and guinea-pig isolated airway preparations are unaltered by atropine or mepyramine (Mizrahi *et al.*, 1982; Lundberg *et al.*, 1983), histamine antagonists, α-adrenoceptor antagonists or β-adrenoceptor antagonists (Malo *et al.*, 1986). However, there have also been at least two accounts of an effect of atropine on SP-induced contractions of airway-smooth muscle (Tanaka & Grunstein, 1984; Malo *et al.*, 1986). In the experiments described in this paper, we found no effect of atropine on the tachykinin-induced contractions, which suggests a direct action of the peptides on the tracheal smooth muscle. The order of potency NKA > eledoisin $\gg$ SP, observed in our experiments, is indicative of an NK_2 receptor, and these findings are consistent with previous studies of guinea-pig tracheal rings *in vitro* (Karlsson *et al.*, 1984; Uchida *et al.*, 1987; Grandordy *et al.*, 1988).

Airway epithelium may attenuate the contractile response to tachykinins and with intact epithelium, as in our tracheal tube preparations, there may be a marked blunting of the response (Tschirhart & Landry, 1986; Grandordy *et al.*, 1988). This may be due to the release of an epithelial relaxant factor (Cuss & Barnes, 1987), but a more likely explanation is that the major metabolising enzyme for tachykinins, neutral metallo-endopeptidase, or enkephalinase, is present in airway epithelial cells (Sekizawa *et al.*, 1987; Frossard *et al.*, 1988).

The major finding in our study was the concentration-dependent enhancement by tachykinins of contractile responses of the tracheal tube preparation to electrical stimulation of cholinergic nerves. This effect was observed even at very low concentrations of trachykinins, where there was little or no direct effect on ILP. The responses both to preganglionic vagal stimulation and to transmural stimulation (in the presence of propranolol) were increased by the tachykinins, but no direct comparison between pre- and post-ganglionic effects was made. On the basis of our study, it is therefore not possible to speculate on a neuromodulatory role for the peptides in the parasympathetic ganglia.

Our experiments showed no effect of tachykinins on the contractile response to exogenous ACh, endorsing the findings of Tanaka & Grunstein (1986), who showed that SP did not affect the cumulative methacholine dose-response curve in the rabbit. This suggests that the augmentation of neurally-mediated responses by the tachykinins involves a prejunctional rather than a postjunctional mechanism, and is probably due to facilitation of ACh release from the nerve terminals. The rank order of potency for the augmentation of responses was NKA $\geqslant$ eledoisin > SP, and this indicates that the neural effect is also mediated via an NK_2 receptor. This correlates with findings in the guinea-pig gastrointestinal tract, in which an NK_2 receptor is involved in modulating ACh release from the mesenteric plexus (Featherstone *et al.*, 1986).

We have also shown that stimulation frequency and voltage affect the magnitude of augmentation by tachykinins of neurally-mediated contractions. A greater enhancement was observed at low stimulation frequencies, and this is consistent with the findings of Tanaka & Grunstein (1986) for SP in the rabbit trachea. The stimulation voltage had a very marked effect on the degree of augmentation observed. When supramaximal voltages were used, as is conventional in these studies, augmentation by tachykinins was small, but increased sharply as voltage was reduced. It may be relevant that, in the study of rabbit trachea (Tanaka & Grunstein, 1986) in which a marked augmentation by SP on responses to electrical field stimulation was observed, the stimulation voltage was probably submaximal. The mechanism for greater enhancement at submaximal voltages is not clear, but one possible explanation may be that tachykinins facilitate the spread of impulses and increase the probability of neurotransmitter release from cholinergic nerve varicosities. In sympathetic nerves, facilitation of transmitter release appears to be due to an increase in the probability of release without any change in the shape or propagation of the nerve action potential, suggesting that it may be due to alteration in stimulus-secretion coupling mechanisms in the invaded varicosity (Brock & Cunnane, 1987). It could be postulated that tachykinins, by activating NK_2 receptors on cholinergic nerve terminals, lead to some intracellular biochemical change in the nerve terminal which increases the probability of ACh secretion when the membrane is depolarized. Several neurotransmitters and neuropeptides influence cholinergic neurotransmission in guinea-pig airways, and therefore may contribute to the regulation of bronchomotor tone (Grundstrom *et al.*, 1981; Fryer & Maclagan, 1984; Maclagan, 1987; Stretton & Barnes, 1988).

The effect of tachykinins on cholinergic neurotransmission in guinea-pig airways may have physiological relevance. The release of NKA from afferent nerves in guinea-pig airways (Uchida *et al.*, 1987) may enhance bronchial tone and may facilitate cholinergic reflex bronchoconstriction, thereby increasing the 'gain' of these reflexes. Whether our findings are relevant to human airways is not yet certain. Axon reflex mechanisms have been implicated in asthma (Barnes, 1986) and, if tachykinins are released, this may lead to enhanced neurally-mediated bronchoconstriction.

We are grateful to the Medical Research Council and Asthma Research Council for support.

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The effect of cromakalim on the smooth muscle of the guinea-pig urinary bladder

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1 The actions of cromakalim were studied on the detrusor muscle from guinea-pig urinary bladder. Cromakalim reduced the frequency and amplitude of spontaneous contractile activity of the smooth muscle of the guinea-pig urinary bladder at 5×10^{-8} M and abolished the activity at concentrations above 5×10^{-7} M.

2 Electrophysiological experiments demonstrated that cromakalim increased membrane conductance, caused a dose-dependent hyperpolarization of the cell membrane and loss of spike activity. These events are consistent with the opening of K^+ channels.

3 The effects of 10^{-6} M and 10^{-5} M cromakalim on the contractile responses to carbachol, potassium and transmural nerve stimulation were studied. Cromakalim did not prevent the detrusor from responding to these agents, although it significantly reduced the contractile response to K^+ at concentrations below 70 mM.

4 Uptake and efflux experiments using $^{86}Rb^+$ were unable to demonstrate any significant effect on transmembrane movement produced by cromakalim (10^{-5} M).

5 $^{43}K^+$ efflux showed a dose-dependent increase in the rate constant on addition of cromakalim. The difference in the selectivity for K^+ over Rb^+ was confirmed in dual label uptake experiments.

6 Substitution experiments in which the K^+ ions in the tissue were gradually replaced by Rb^+ demonstrated that cromakalim had a progressively decreasing effect on spontaneous activity as internal K^+ was lowered. When all the K^+ was replaced by Rb^+, cromakalim no longer inhibited spontaneous activity, confirming that the channel opened by cromakalim appears relatively impermeant to Rb^+.

Introduction

Cromakalim (BRL 34915) is one of the new class of potassium channel activating drugs that has recently received much attention. It has been shown to hyperpolarize the membranes of various smooth muscles (vascular smooth muscle, Hamilton *et al.*, 1986; taenia coli, Weir & Weston, 1986a; Allen *et al.*, 1986). In these tissues the drug is thought to produce its effect by opening K^+ channels in the smooth muscle membranes, so moving the membrane potential towards the potassium equilibrium potential. Evidence for this was provided by experiments in which $^{86}Rb^+$ was used as a tracer for K^+. This has become accepted protocol due to the expense and short half life of potassium isotopes ($^{42}K^+$, $t_{1/2}$ = 11 h; $^{43}K^+$, $t_{1/2}$ = 23 h) compared with the relatively inexpensive and longer acting $^{86}Rb^+$ ($t_{1/2}$ = 19 days). The use of $^{86}Rb^+$ as a marker for K^+ has been investigated by many workers. (see e.g. Widdicombe, 1977; Mauger *et al.*, 1978; Imaizumi & Watanabe, 1981; Bolton & Clapp, 1984).

Cromakalim is a potent relaxant of vascular and tracheal smooth muscles, and the potential use of such drugs in the control of hypertension and asthma has been postulated. We are interested in functional abnormalities of the urinary bladder, and in particular the condition of detrusor instability in which the smooth mucle becomes hyperexcitable. In this condition the potassium channel activating drugs have a potential use in directly decreasing the excitability of bladder smooth muscle cells. The effects of cromakalim on the contractile responses, electrical activity and transmembrane K^+ and Rb^+ fluxes of normal guinea-pig bladder smooth muscle have therefore been examined.

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Methods

Contractile studies

Tension experiments were carried out on bladder smooth muscle strips measuring 1 × 1 × 7 mm. These were dissected from the detrusor under a binocular microscope. They were mounted in a 0.2 ml organ bath (Brading & Sibley, 1983) and continuously superfused with Krebs (35–37°C) solutions at a flow rate of approximately 1 ml min^{-1}. Recessed platinum ring electrodes in the organ bath were used for the stimulation of the intrinsic nerves (50 V, 0.05 ms, 5 s train, variable frequency). The apparatus allowed six strips to be studied simultaneously; the tension was measured isometrically on home mounted Akers transducers and the output was recorded on a six channel Watanabe pen recorder. Initially strips were placed under a 1 g resting tension and then allowed to equilibrate for at least 1 h. Contractile responses were expressed as a percentage of a control response to a 10 s application of 10^{-4} M carbachol, applied at the beginning of the experiment. For construction of dose-response curves agonists were applied and then washed off, leaving 5–10 min between doses.

Electrophysiology

Electrical activity was recorded either intracellularly with microelectrodes, or extracellularly, using the double sucrose-gap apparatus. For microelectrode studies, strips of smooth muscle 10 × 1 × 1 mm were dissected from the dome of the bladder, and mounted in a 2 ml organ bath. Tissues were superfused at a rate of 2–3 ml min^{-1} with warmed (36°C) Krebs solution made hypertonic by addition of 12 g sucrose to 100 ml Krebs to reduce tissue movement. Microelectrodes were filled with 3 M KCl, and had a tip resistance of between 40 and 70 mΩ. Potentials were displayed on a pen-writing recorder (Gould Brush 2200).

For the double sucrose-gap method, a strip of smooth muscle 15 mm by 1.5 mm was mounted in a recording chamber similar to the one described by Goto *et al.* (1974). The chamber consisted of five compartments separated by four rubber membranes with holes in the centre, through which the specimen was pulled. Both ends of the tissue were connected to threads, one fixed, and the other connected to a mechano-transducer for simultaneous tension measurement. The central compartment (0.5 mm width) was superfused with warmed (36°C) isotonic Krebs solution, and the adjacent chambers (2 mm) with isotonic sucrose solution. The two outer chambers (1 ml capacity) were filled with isotonic KCl solution. Electrical activity was recorded between the central and outer chamber with a Ag-AgCl electrode and a plate electrode. Electrical and mechanical activity were displayed on a pen-writing recorder (Gould Brush 2200). Electrical stimulation was applied via a Ag-AgCl electrode in the central chamber. Test solutions were applied to the central chamber.

Radio-tracer ion flux analysis

Slightly larger strips of detrusor were used for flux studies; they were mounted on stainless steel holders and allowed to equilibrate in normal Krebs solution for at least an hour before the experiment. For efflux experiments, the tissues were equilibrated with warmed Krebs solution containing 1.5–3 μCi ml^{-1} $^{43}K^+$ or 8–12 μCi ml^{-1} $^{86}Rb^+$ or both. To wash out, the holder and tissue were first dipped into a non-radioactive bath of Krebs to remove adhering radioactivity and then inserted into a superfusion apparatus. The superfusate was collected in 2 min samples and subsequently counted in a gamma counter. The last sample contained the tissue (Brading, 1967). $^{43}K^+$ and $^{86}Rb^+$ loss was analysed in terms of the rate coefficient. This was calculated by dividing the counts leaving the tissue during a 2 min interval by the mean counts in the tissue during that interval. In uptake experiments tissues were exposed for 10 min either to $^{43}K^+$ and $^{86}Rb^+$ simultaneously (dual label experiment), or to just one isotope (single label). They were then washed in ice cold Krebs for 5 min to remove extracellular tracer, (Aickin & Brading, 1983) before counting. It was possible to distinguish between the two isotopes because of their differing half lives.

Statistics

Results are expressed as the mean ± s.e. (except where s.d. is indicated) (n), and their significance was assessed by a paired t test. Values of P less than 0.05 are shown with a single *, and less than 0.001 with two **.

Solutions

The composition of the Krebs solution was (mM): NaCl 120, KCl 5.9, $NaHCO_3$ 15.4, $MgCl_2$ 1.2, NaH_2PO_4 1, $CaCl_2$ 2.5 and glucose 11. Cromakalim (BRL 34915, (±)-6-cyano-3,4-dihydro-2,2-dimethyl-*trans*-4-(2-oxo-1-pyrrolidyl)-2H-benzo[b]pyran-3-ol), was a gift from Beechams U.K. It was made up as a stock solution of 10^{-2} M with 70% ethanol, and diluted appropriately with Krebs solution. Carbachol was supplied by Sigma. This was made up as a stock solution of 10^{-2} M in distilled water. In experiments where high potassium Krebs solutions were used, NaCl was replaced isosmotically with

KCl. In experiments where internal K^+ was replaced by Rb^+, RbCl was substituted for KCl in the above Krebs solution. All solutions were equilibrated with 97% O_2, 3% CO_2.

Results

Mechanical activity

The majority of guinea-pig detrusor strips exhibited spontaneous mechanical activity during the course of an experiment (81%, $n = 74$). In Figure 1 the effect of increasing concentrations (10^{-8}–5×10^{-5} M) of cromakalim on this spontaneous activity is shown: 10^{-8} M appeared to have no effect on this activity. In 2 out of 6 strips 5×10^{-8} M reduced the frequency of the spontaneous contractions with variable effects on the amplitude. Concentrations above 5×10^{-7} M invariably caused cessation of the spontaneous mechanical activity with the time taken for reappearance of the activity being concentration-dependent. After washout the spontaneous activity reappeared and a transient potentiation in the frequency and amplitude of myogenic activity was observed. This lasted for approximately 10 min before the spontaneous activity returned to resting level.

Due to the very phasic nature of bladder smooth muscle it was impossible to achieve a maintained degree of tension against which an inhibitory dose-response curve for cromakalim could be constructed, and so the effect of the compound on the response to agonists was investigated to observe whether or not it was preventing the muscle from reacting to its normal excitatory agonist (muscarinic stimulation), potassium depolarization, and intrinsic nerve activation.

Figure 2 shows that cromakalim (10^{-5} M) did not significantly inhibit the ability of the detrusor to

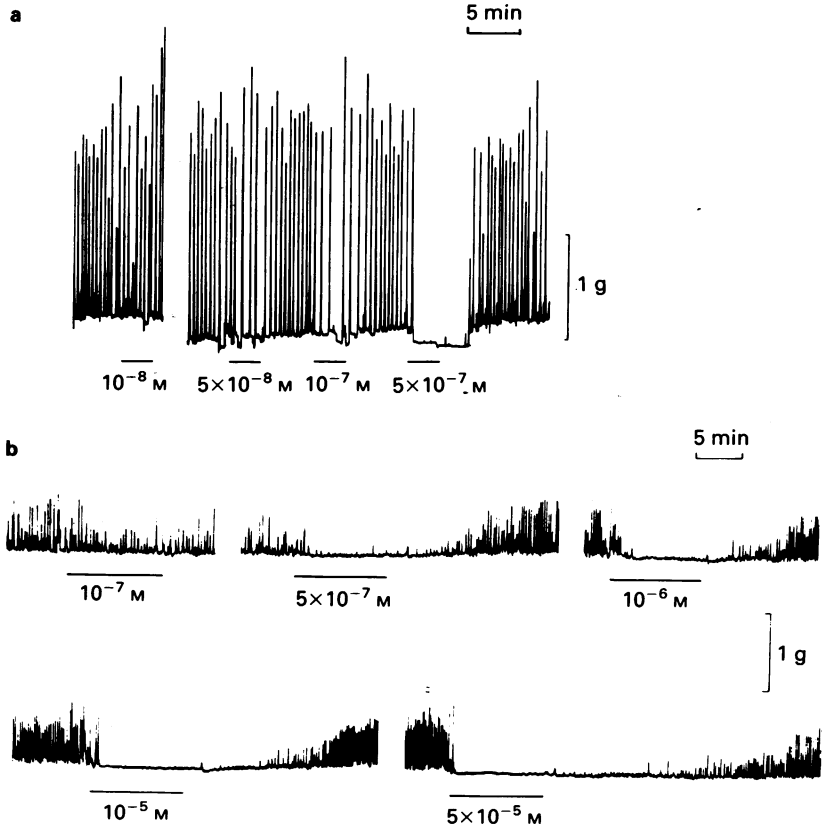

Figure 1 The effect of cromakalim on spontaneous activity from guinea-pig detrusor muscle. Little effect is seen at 10^{-8} M cromakalim, while 5×10^{-8} M reduces the frequency of the spontaneous activity. At 5×10^{-7} M, cromakalim totally inhibits the spontaneous contractions. There is little maintained tone in these strips. (a) and (b) are tissues from separate experiments.

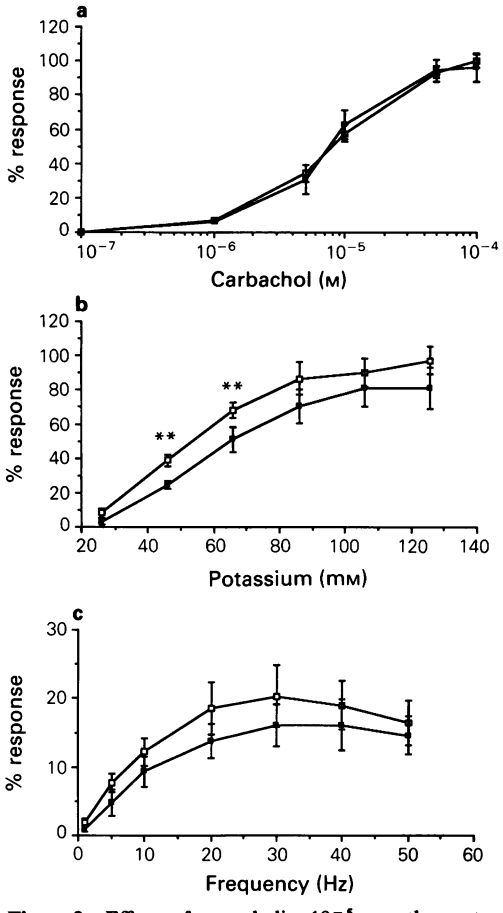

Figure 2 Effects of cromakalim 10^{-5} M on the contractile responses of guinea-pig detrusor. (a) Dose-response curve to 10 s applications of carbachol. (b) Dose-response curve to 4 min applications of K^+. (c) Frequency-response curve to transmural nerve stimulation (5 s train, 50 V, 0.05 ms). Controls (□); in the presence of cromakalim (■). Results are expressed as means with s.e.mean shown by vertical bars ($n = 12$).

Table 1 The effect of cromakalim on the frequency of spontaneous electrical activity and the associated hyperpolarization of the cell membrane

Cromakalim (M)	*Spike frequency* (min^{-1})	*Hyperpolarization* (mV)
Control	22.4 ± 3.9	0
10^{-7}	23.3 ± 1.5	0
5×10^{-7}	13.0 ± 3.0	0
10^{-6}	0	5.8 ± 1.9
5×10^{-6}	0	12.2 ± 3.0
10^{-5}	0	22.0 ± 5.0

respond to these agents, except at the lower concentrations of potassium where there was some small depression of the dose-response curve.

Electrical activity

The detrusor smooth muscle cells had a mean resting potential of −60.6 ± 3.2 mV (mean ± s.d., $n = 20$, 10 animals). Most cells showed spontaneous spike activity, either continuously, or in periodic bursts. Table 1 shows the effects of cromakalim on the membrane potential and spike frequency. At concentrations above 5×10^{-7} M, cromakalim caused a dose-dependent hyperpolarization, which at 10^{-5} M was such that the membrane potential must have been close to the potassium equilibrium potential. At a concentration of 5×10^{-7} M, the spike frequency was reduced, with no apparent change in the membrane potential. Higher concentrations of the drug abolished spike activity. These results are illustrated in Figure 3.

Figure 4 shows the effect of increasing the extracellular potassium to 20 mM after the membrane had been hyperpolarized by 5×10^{-6} M cromakalim and spike activity abolished. This concentration of K^+ was calculated to bring the potassium equilibrium potential close to the normal resting potential and, as can be seen, the spontaneous electrical activity returned in the continuous presence of cromakalim.

Figure 5 shows the effects of cromakalim (10^{-6}–10^{-5} M) on a strip of muscle mounted in the double sucrose gap apparatus. Alternate hyperpolarizing and depolarizing current pulses were applied to the node. Depolarizing pulses caused spike activity in the cells (not well recorded with this method, due to lack of synchronization of the spikes in the various muscle bundles in the node), and a phasic contraction. The size of the electrotonic potentials allowed assessment of changes in the membrane resistance. Cromakalim hyperpolarized the tissue, reduced the phasic tension responses and also decreased the membrane resistance. It was also found that cromakalim had little effect on the excitatory junction potentials evoked by brief current pulses. For example, in one experiment in the presence of 10^{-6} M cromakalim, the amplitude was reduced to 94.8 ± 3.84% of the control amplitude, and in 10^{-5} M to 77.6 ± 2.22% ($n = 10$). A reduction in amplitude would be expected even if transmitter release is unaffected, because of the decreased membrane resistance.

Flux studies

Although cromakalim had very consistent and repeatable effects on the tension and electrical

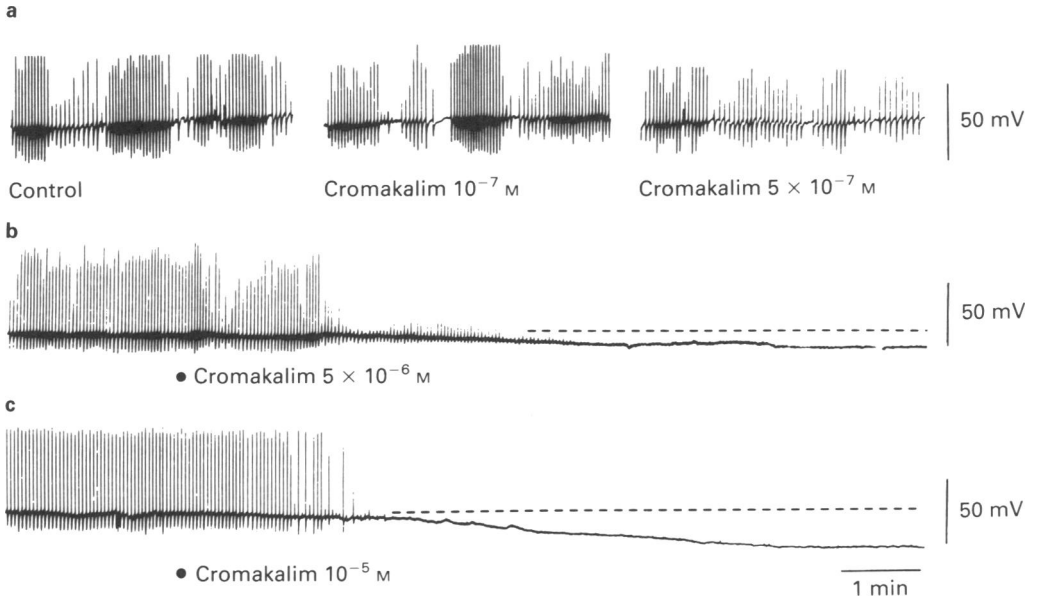

Figure 3 The effects of cromakalim on membrane potential and spontaneous spike activity (micro-electrode recording). (a) Drug concentrations up to 5×10^{-7} M had no effect on the resting potential (−60 mV) but reduced spike frequency at 5×10^{-7} M. (b and c) Higher concentrations hyperpolarized the membrane (by 12 mV at 5×10^{-6} M and 25 mV at 10^{-5} M) and abolished spontaneous activity. In 10^{-5} M cromakalim, the membrane potential was close to the estimated E_K (−85 mV).

properties of guinea-pig bladder smooth muscles, the effects of Rb^+ and K^+ fluxes were much more variable.

Efflux experiments When $^{86}Rb^+$ was used as a tracer for K^+, cromakalim at 10^{-5} M had no effect on the rate of loss of $^{86}Rb^+$ from 8 strips from five guinea-pigs, but in another 4 strips from three different guinea-pigs, there was a very slight increase in the efflux rate. In comparison, when $^{43}K^+$ was used as the tracer, out of 20 strips from 14 guinea-pigs, only 3 strips from 2 particular guinea-pigs did not respond to cromakalim at this concentration. Some strips responded to lower concentrations. Examining all the results to date, 47 strips from 14 guinea-pigs responded to concentrations of cromakalim between 5×10^{-6} M and 10^{-4} M with an obvious (but variable) increase in the rate of loss of K^+. Figure 6 illustrates an experiment in which strips from the same animal were loaded in Krebs solutions containing either $^{43}K^+$ or $^{86}Rb^+$ as tracer, washed out in normal Krebs solution, and exposed to 10^{-5} M cromakalim for 10 min. In this experiment the drug caused a clear increase in the rate of loss of $^{43}K^+$, but had no effect on the loss of $^{86}Rb^+$. Figure 7 shows the results of an experiment with strips from three bladders, in which the effect of varying the concentration of cromakalim was studied on the rate of

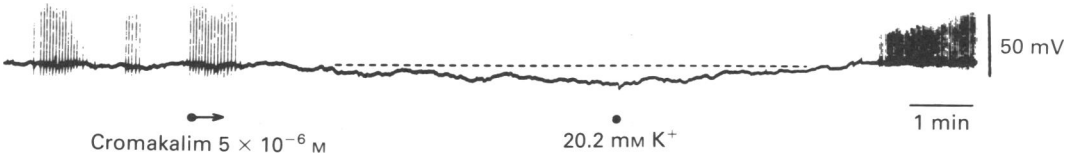

Figure 4 The effects of raising extracellular K^+ concentration ($[K^+]_o$) on the action of cromakalim, (microelectrode recording). Raising $[K^+]_o$ to 20.2 mM reversed the membrane hyperpolarization and the spontaneous activity returned, suggesting that under the influence of cromakalim, the membrane potential moves toward E_K (E_K in 20.2 mM $[K^+]_o$ is about −55 mV, close to the resting membrane potential).

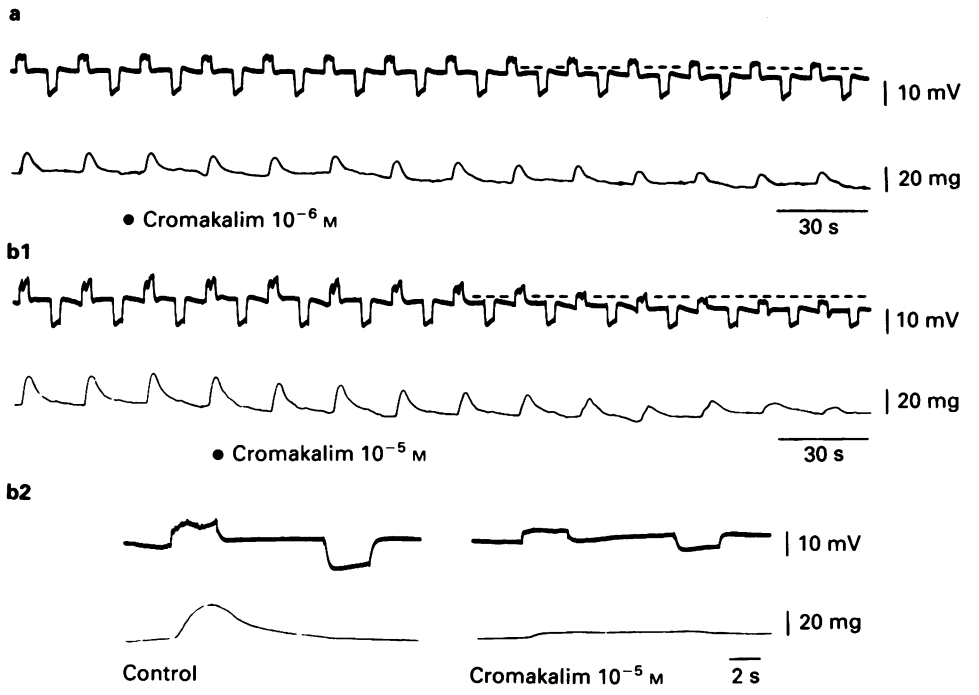

Figure 5 The effects of cromakalim on membrane potential and membrane conductance (double sucrose gap recording). In each pair, the upper trace shows electrical activity and the lower trace mechanical activity. Electrotonic potentials were evoked by inward and outward current pulses (3 s, 0.1 Hz). Depolarizing electrotonic potentials lead to contractions. (a and b1) Cromakalim 10^{-6} M (a) and 10^{-5} M (b1), hyperpolarized the membrane, increased the conductance and subsequently reduced the contraction. (b2) Electrotonic potentials (faster time scale) before and during exposure to cromakalim (10^{-5} M).

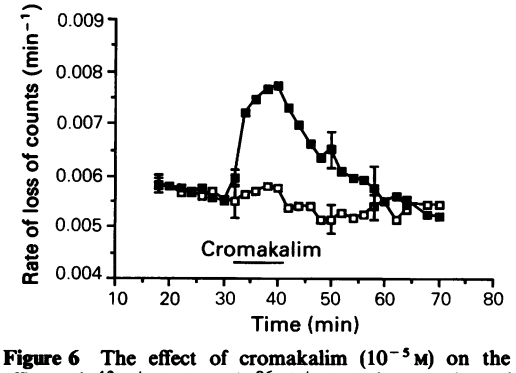

Figure 6 The effect of cromakalim (10^{-5} M) on the efflux of $^{43}K^+$ (■) and $^{86}Rb^+$ (□) from strips of guinea-pig detrusor muscle. The results shown are the means with s.e.mean shown by vertical bars ($n = 8$ for each curve). These results were obtained in the same experiment, under the same conditions using the same solutions. The channels opened by cromakalim appear to be selective for K^+ over Rb^+.

loss of $^{43}K^+$. In this case there was a clear dose-dependency of the effect.

Uptake experiments We used 10 min exposure to the radioactive label to assess the effect of cromakalim on the uptake of $^{43}K^+$ or $^{86}Rb^+$, since this time is on the linear part of the uptake curve, and is long enough to get significant uptake, but sufficiently short to prevent much backflux of tracer. Cromakalim (10^{-5} M) had little effect on the uptake of $^{86}Rb^+$, but normally caused a significant increase in the uptake of $^{43}K^+$, although the effect was rather variable. The largest increase seen in one experiment was 43%, and in another experiment there was no significant increase. In a group of dual label experiments, in which the uptake baths contained both $^{43}K^+$ and $^{86}Rb^+$, there was no significant difference in the uptake of the two isotopes in normal Krebs solution, (the tissue took up 73.2 ± 3.0% ($n = 24$ tissues) of the activity of an equivalent volume of the bathing solution), but if this is taken as 100%, in the presence

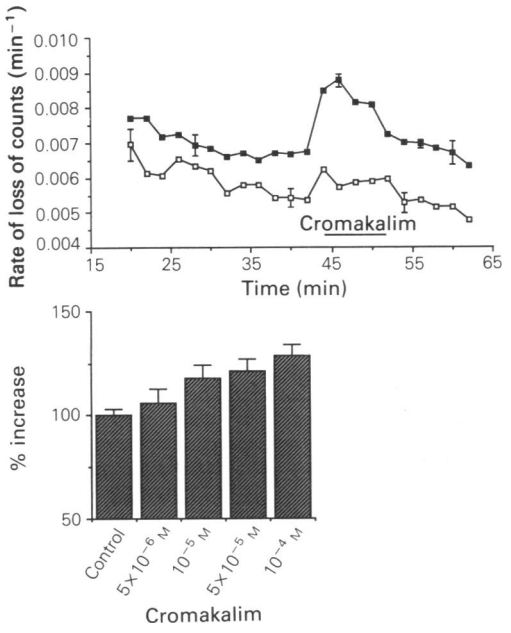

Figure 7 The effect of different concentrations of cromakalim on the $^{43}K^+$ efflux from stips of guinea-pig detrusor muscle ($n = 3$ for each concentration shown). Full efflux curves of the mean (with s.e.mean shown by vertical bars) are given showing the effect of 5×10^{-6} M (□) and 5×10^{-5} M (■). The histograms show the mean increase (s.e.mean shown by vertical bars) in efflux rate during 10 min exposure to the drug, expressed as a percentage of the control (the average of the previous 5 values).

of 10^{-5} M cromakalim there was an increase in the uptake of $^{43}K^+$ to $114.2 \pm 3.1\%$ (52) whereas the $^{86}Rb^+$ uptake ($103.6 \pm 2.5\%$ (30)) was not significantly increased.

Experiments on Rb tissues

Since the flux experiments suggest that the channels opened by cromakalim are significantly less permeant to Rb^+ than to K^+, we performed a series of experiments on tissues which had been exposed for 5–7 h to K^+-free Krebs, in which the KCl had been replaced with RbCl. Figure 8 shows the time course of uptake of Rb^+ in tissues bathed in K^+-free (Rb^+) solution, with $^{86}Rb^+$ as a tracer. Extrapolation of the graph suggests that the tissues will be virtually fully loaded with Rb^+ in about 7 h.

Contractile studies Figure 9 shows how the effect of cromakalim (10^{-5} M) on spontaneous mechanical activity varies with time of exposure to K^+-free (Rb^+) solution. As can be seen, there is a progressive

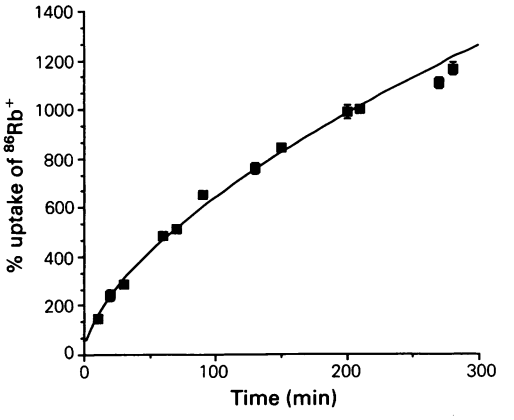

Figure 8 Graph to show the time course for replacement of cell K^+ with Rb^+ in strips bathed in zero potassium (Rb^+) Krebs solution. Means of $n = 7$; s.e.mean shown by vertical bars.

decrease in the inhibitory effect of cromakalim; this effect was seen in every tissue we tried (16). The response to cromakalim was virtually abolished after 7 h. Significant recovery of the effect occurred after an hour of bathing in normal Krebs solution.

Electrophysiology A similar reduction in the effect of cromakalim was seen on membrane potential and spike activity. This is shown in Figure 10. During

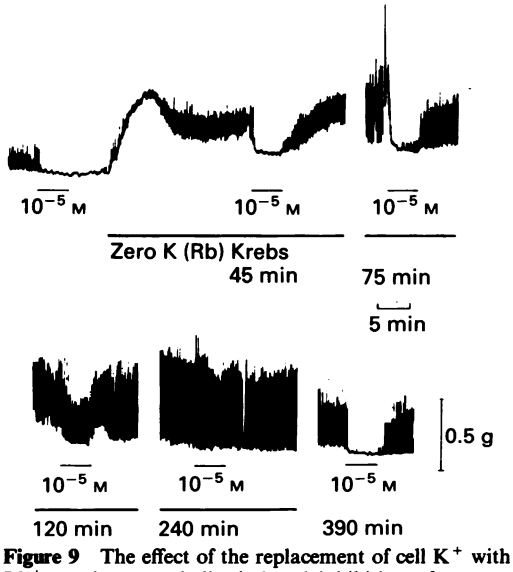

Figure 9 The effect of the replacement of cell K^+ with Rb^+ on the cromakalim-induced inhibition of spontaneous mechanical activity. After 7 h of exposure to zero K^+ (Rb^+) Krebs cromakalim had no effect. After 150 min perfusing with normal Krebs the response to cromakalim returns.

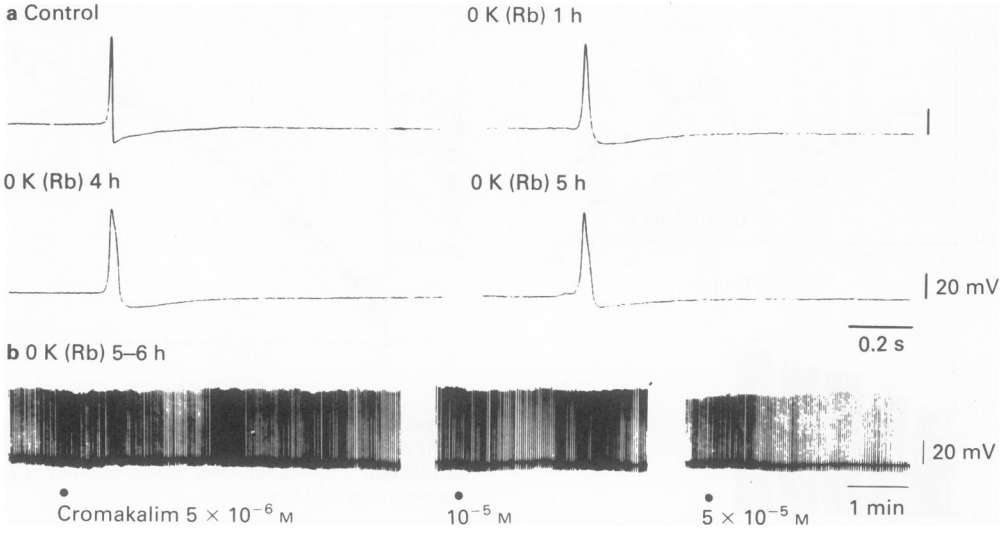

Figure 10 The effects of zero K^+ (Rb^+) solution (microelectrode recording) on the shape of the action potential, and the effects of cromakalim on spike frequency. During exposure to zero K^+ the spike duration was progressively prolonged (a), and after 5–6 h the effect of cromakalim was greatly reduced (b).

exposure to K^+-free (Rb^+) solution there was a progressive lengthening of the duration of the spikes (due to a reduction in the rate of spike repolarization). In one experiment the time taken for spike repolarization (from peak to maximum after-hyperpolarization), increased from 10–11 ms in the control, to 32–40 ms after 2 h in K^+-free (Rb^+) Krebs, and 40–44 ms after 4 h in K^+-free (Rb^+) solution. After 5–6 h in this solution the effects of cromakalim on the membrane potential and spike activity were virtually abolished.

Flux studies Figure 11 shows the results from an experiment in which the effect of cromakalim on $^{86}Rb^+$ efflux was studied using Rb^+ tissues (which had been for 5.5 h in K^+-free (Rb^+) solution at the start of the washout) and compared with its effects on the $^{43}K^+$ efflux from normal tissues. As can be seen, in this experiment there was no significant effect of cromakalim (10^{-5} M) on the K^+ flux from normal tissues, but there was a significant decrease in the rate of loss of Rb^+ from the Rb^+ tissues. The effect of cromakalim on $^{43}K^+$ and $^{86}Rb^+$ uptake into Rb^+ tissues is shown in Figure 12. In this case cromakalim increased the uptake of $^{43}K^+$, but not of $^{86}Rb^+$.

Discussion

The evidence presented in this paper is consistent with the view that cromakalim opens potassium channels in the membranes of smooth muscle cells of the guinea-pig detrusor. Electrophysiological experiments demonstrate a clear dose-related hyperpolarization and increase in membrane conductance. The hyperpolarization is reversed when the potassium equilibrium potential is altered to a value close to the original membrane potential. The loss of spike activity induced by cromakalim correlates with the

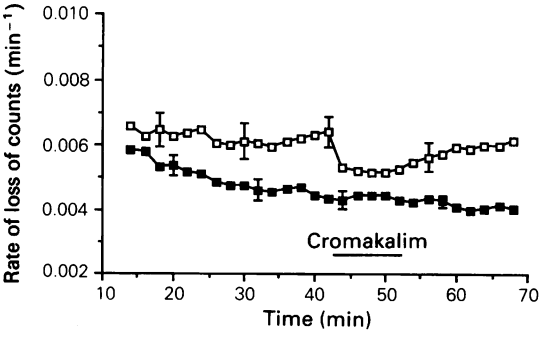

Figure 11 The effect of cromakalim (10^{-5} M) (■) on the efflux of $^{43}K^+$ from normal tissues; the results from tissues in which most of the K^+ had been replaced by Rb^+ (exposed to zero K^+ for 5 h) shown by (□). The results are the means with s.e.mean shown by vertical bars ($n = 8$ for each curve). Cromakalim decreased the rate of loss of Rb^+ from Rb^+ tissues but did not affect K^+ loss from normal tissues.

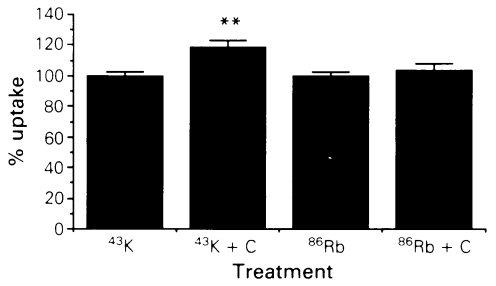

Figure 12 The effect of cromakalim (10^{-5} M) on the 10 min uptake of $^{43}K^+$ and $^{86}Rb^+$ (dual label experiment) by Rb^+ tissues (5.5 h in $0K^+$ (Rb^+) Krebs). Uptake is measured as c.p.m./mg of wet wt. as a percentage of the counts in the uptake solution (c.p.m./mg). Results are the mean with s.e.mean shown by vertical bars ($n = 6$).

reduction of the spontaneous mechanical activity recorded from tissue strips.

The effects of cromakalim on the contractile responses of the tissue to increasing extracellular K^+, application of carbachol and stimulation of intrinsic excitatory nerves are also consistent with this mode of action of cromakalim. The responses to increasing K^+ concentration were reduced significantly in the lower range of K^+ concentrations. In this range, the membrane potential is still probably significantly less negative than the potassium equilibrium potential (as has been shown in other smooth muscles, e.g. Casteels & Kuriyama 1966), and cromakalim will still be able to hyperpolarize the membrane, whereas at the higher K^+ concentrations cromakalim would have no significant effect on the membrane potential.

There was no significant effect of cromakalim on the contractile response to carbachol. This was a little surprising, since in many smooth muscles, muscarinic receptor activation leads to a marked depolarization (Bolton, 1979), which would have been reduced by the opening of extra potassium channels. However, recent work in our laboratory (Fujii, 1987; 1988) has shown that in the guinea-pig bladder, muscarinic receptor activation, even at maximally effective concentrations of carbachol or acetylcholine, has relatively little effect on the membrane potential, the contraction being mediated almost entirely through pharmaco-mechanical coupling.

Cromakalim caused a slight reduction in the response to intramural nerve stimulation. The results with muscarinic agonists suggest that this diminished response is unlikely to be due to effects on cholinergic transmission. This possibly demonstrates an effect on the non-adrenergic, non-cholinergic transmission that is responsible for about 70% of the response to transmural nerve stimulation in the guinea-pig bladder (Brading, 1987). Fujii (1987, 1988) has shown that the excitatory junction potentials (e.j.ps) that occur on intrinsic nerve stimulation are unaffected by muscarinic antagonists, but abolished after treatment with the P_2 receptor-desensitizing drug α,β-methylene ATP. Cromakalim does not abolish these excitatory junction potentials but does reduce their size, probably because of the lowered input resistance of the cell membrane following the opening of the K^+ channels. This would account for the slight reduction in the response to transmural nerve stimulation. It seems therefore that cromakalim is probably not significantly affecting the release of the excitatory transmitter responsible for the e.j.ps, although our data are not sufficiently precise to exclude a presynaptic effect of the drug.

Some of the more interesting observations we have made, come from the analysis of the effects of cromakalim on the uptake and efflux of Rb^+ and K^+ isotopes, and the effects of replacing intracellular K^+ with Rb^+. Many workers have used $^{86}Rb^+$ as a tracer for K^+ ions, as it is a more convenient isotope (half-life 18 days) and cheaper than the available K^+ isotopes $^{42}K^+$ and $^{43}K^+$ which have half-lives of only 11 and 23 h. It has also been shown that cromakalim increases the efflux of Rb^+ from rat (Weir & Weston, 1986b) and guinea-pig (Quast, 1987) blood vessels. Initial experiments were therefore carried out on the efflux of $^{86}Rb^+$ from strips of detrusor but there was little if any effect of cromakalim. Theoretically, an increase in K^+ permeability of the membrane leading to hyperpolarization will have two opposing actions on the efflux of cations that can pass through the channels. An increase in the number of ions leaving the cell might occur because of the larger permeability, but also there will be a decrease in the driving force due to the hyperpolarization of the cell membrane, which would attenuate the increase in efflux. Both factors work in the same direction however when examining the uptake of tracer. For this reason the subsequent experiments examined the effect of cromakalim on the uptake of $^{86}Rb^+$, but again there were no significant effects.

Further investigations were then undertaken using K^+ isotopes, and significant increases in both uptake and efflux were recorded. However, the concentrations of cromakalim required to produce these effects were high: we found no significant effects at less than 5×10^{-6} M cromakalim, whereas spontaneous electrical and mechanical activity is inhibited at 10^{-7} M. Another problem with these experiments has been the variability of the size of the response to cromakalim. Cromakalim 10^{-5} M was routinely used for the majority of experiments, and its effect on K^+ fluxes varied markedly from experiment to experiment.

There are several possible reasons for this variability. The normal procedure was to distribute tissues from each bladder used in an experiment through all the protocol groups. The means of the results from each group were used to compare treatments. In a recent experiment we compared the effects of cromakalim on the uptake of tissues using controls from the same bladder, in order to compare two guinea-pigs of different sizes. We found a very large difference in the uptake of K^+ in normal Krebs, the smaller guinea-pig had almost double the uptake per unit tissue weight compared with the larger. Tissues from the smaller animal were also less sensitive to cromakalim. Large inter-animal variation could have masked the effects of the drug in the pooled experiments. This variation could be due to differences in the proportions of the various K^+ channels that occur in these membranes, and also to differences in the amount of spontaneous electrical activity occurring (which involves activation of various K^+ channels).

When Rb^+ and K^+ fluxes were examined in both single and dual labelled experiments, the results confirmed that cromakalim has a much greater effect on the K^+ fluxes than Rb^+ fluxes. This suggests strongly that the channels opened by this drug are much more permeable to K^+ than to Rb^+. The differential permeability of the cromakalim-activated channels to K^+ and Rb^+ has also been suggested by Quast (1988) although the selectivity in the rat aorta and guinea-pig portal vein was not as great as in the detrusor.

When Rb^+ was used as a tracer, the total amount of Rb^+ in the intra and extracellular solutions was very small, and interactions between unlabelled K^+ and $^{86}Rb^+$ could have occurred. We therefore carried out an experiment on $^{86}Rb^+$ efflux from Rb^+ tissues, in which much of the intracellular K^+ had been replaced by Rb^+, and compared this with $^{43}K^+$ efflux from normal tissues. In this experiment, cromakalim had no significant effect on normal tissues, but caused a reduction of the efflux of $^{86}Rb^+$ in the Rb^+ tissues. This result could be explained if there were still enough K^+ in the Rb^+ tissues for cromakalim to cause hyperpolarization (E_K would be very negative, as there is no external K^+), and if Rb^+ was unable to penetrate the channels opened by cromakalim. In this case both the hyperpolarization and the possible shutting of voltage-sensitive K^+ channels through which Rb^+ could pass, would act to reduce the Rb^+ efflux. In normal tissues the lack of effect of cromakalim would be due to the balancing effects of factors tending to reduce the efflux and the increased K^+ efflux that would occur through the channels opened by the drug.

Further support for the idea that Rb^+ ions do not pass easily through the channels opened by cromakalim comes from the time-dependent decrease in the effectiveness of cromakalim on the spontaneous mechanical activity after the external K^+ had been replaced by Rb^+. The inhibitory effect of cromakalim is totally abolished at a time when all the intracellular K^+ would be replaced by Rb^+. As expected, this was also seen with electrical activity. However the unexpected result here was the progressive lengthening of the action potential that occurred, suggesting that some of the channels responsible for the repolarization phase of the spike are also more permeable to K^+ than to Rb^+.

In conclusion, an attempt has been made to correlate the effect of cromakalim on spontaneous activity, electrical activity and ionic fluxes. As low concentrations of cromakalim have an effect on spontaneous electrical activity without causing any hyperpolarization it is a possibility that the drug might also be effective in blocking pacemaker mechanisms. Also at concentrations which increase potassium permeability there is a selectivity for the channel opened by cromakalim for K^+ over Rb^+, a fact which has also been noted, to a lesser extent, in the rat aorta and guinea-pig portal vein (Quast, 1988).

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Further characterization of the 5-HT receptor mediating vascular relaxation and elevation of cyclic AMP in porcine isolated vena cava

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1 5-Hydroxytryptamine (5-HT) and 5-carboxamidotryptamine (5-CT) produce both smooth muscle relaxation and elevation of tissue adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels in isolated rings of neonatal porcine vena cava. We now present studies attempting to characterize in more detail the 5-HT receptor mediating these responses.

2 Both 5-HT and 5-CT relaxed porcine isolated vena cava rings (EC_{50} values 200 nM and 4 nM respectively) and elevated tissue cyclic AMP levels (EC_{50} values 1500 nM and 16 nM respectively). For both responses 5-CT was approximately 50–100 fold more potent than 5-HT.

3 Both 5-CT-induced smooth muscle relaxation and cyclic AMP elevation were potently and specifically antagonized to a similar extent by methiothepin, methysergide and spiperone.

4 At concentrations up to 1 μM, 8-hydroxy-2-(di-n-propylamino) tetralin, buspirone, ipsapirone, n,n-dipropyl-5-CT, cyanopindolol, RU24969, ketanserin, GR38032 and GR43175 were devoid of both agonist and antagonist activity for both responses.

5 These findings suggest that the same 5-HT_1-like receptor mediates both smooth muscle relaxation and elevation of cyclic AMP. This receptor is unlike any known 5-HT_1 ligand binding site or adenylate cyclase-coupled 5-HT receptor in brain tissues.

Introduction

Functional 5-hydroxytryptamine (5-HT) receptors comprise at least 3 classes, designated '5-HT_1-like', 5-HT_2 and 5-HT_3 (Bradley *et al.*, 1986). 5-HT_1-like receptors are characterized by a high sensitivity to the agonists 5-HT and 5-carboxamidotryptamine (5-CT), and to the antagonist methiothepin, and are insensitive to 5-HT_2 and 5-HT_3 receptor antagonists, such as ketanserin and GR38032 (1,2,3,9-tetrahydro-9-methyl-3[(2-methyl-1H-imidazol-1-yl) methyl]4H-carbazol-4-one), respectively. However, it has been claimed that 5-HT_1-like receptors (as opposed to 5-HT_1 binding sites) can be subdivided (Humphrey *et al.*, 1988). This study is part of our continued work to characterize further the putative subtypes within the 5-HT_1-like receptor class.

Relaxant responses to 5-HT and 5-CT, associated with an activation of adenylate cyclase, have previously been described in neonatal porcine isolated abdominal vena cava ring preparations (Trevethick *et al.*, 1984; 1986). Both the relaxant and adenosine 3':5'-cyclic monophosphate (cyclic AMP) responses to 5-CT were antagonized by methysergide (pA_2 values 7.88 and 7.95, respectively) and on the basis of the available data, appeared to be mediated by 5-HT_1-like receptors. Since then, a new generation of drug tools have emerged which show a differential selectivity for various 5-HT receptors and ligand binding sites (Fozard, 1987). In particular, compounds such as the 5-HT_{1A} ligands, 8-hydroxy-2(di-n-propyl-amino) tetralin (8-hydroxy-DPAT), buspirone, ipsapirone and n,n-dipropyl-5-CT, the non-selective 5-HT_{1A} and 5-HT_{1B} ligands, cyanopindolol and RU24969, the 5-HT_3 receptor antagonist GR38032 (Butler *et al.*, 1988) and the novel 5-HT_1-like receptor agonist, GR43175 (Humphrey *et al.*, 1988), have fueled the interest in 5-HT receptor subtypes and their characterization. Indeed, the advent of such compounds has led to a proliferation in the number of 5-HT_1 binding sites identified, and four subtypes are currently recognised, denoted 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C} and 5-HT_{1D} (Heuring & Peroutka, 1987). In addition, renewed interest in the

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transducing mechanisms for these binding sites has led to studies describing 5-HT_{1A} responses that are both positively and negatively coupled to adenylate cyclase (Roth & Chuang, 1987), and more recently, to accounts of 5-HT_{1D} receptor-mediated inhibition of adenylate cyclase activity (Hoyer & Schoeffter, 1988).

We have therefore re-examined and attempted to characterize comprehensively the 5-HT receptors mediating the relaxant and cyclic AMP responses in porcine vena cava ring preparations. Some of these results have previously been communicated to the British Pharmacological Society (Sumner *et al.*, 1987).

Methods

Measurement of vascular relaxation

Lengths of abdominal vena cava (~25 mm) were removed from neonatal pigs (large white variety, 1–20 days of age, supplied by Glaxo animal services) that were killed by captive-bolt pistol and exsanguination. No attempts were made to remove the endothelial cell layer from these vessels.

Each vena cava was dissected to obtain 4 rings of 1–2 mm in length which were individually mounted in 10 ml organ baths for isometric recording of tension changes using a Dynamometer UF1 isometric transducer (±57 g range) connected to a Lectromed 8-channel pen recorder. The preparations were maintained at 37°C in a gassed (95% O_2, 5% CO_2), modified Krebs solution (Apperley *et al.*, 1976), under a resting tension of 0.2–0.5 g. Throughout the experiment a rest period of 60 min (with changes of Krebs solution at 15 min intervals) was allowed between successive treatments of the tissue.

After an initial 60 min equilibration period, a cumulative concentration-effect curve for the contractile agonist (spasmogen) was established, successive concentrations being applied when the response to the previous concentration had plateaued. From this curve, the concentration of spasmogen required to attain 50–70% of its own maximal response was established, and this concentration was used to induce tone throughout the experiment.

After a rest period, the preparations were contracted to a constant tone (0.5–1 g) with the predetermined concentration of spasmogen. In all preparations, a cumulative concentration-effect curve to 5-HT was established, successive concentrations being applied when the response to the previous dose had reached a plateau. When two consecutive concentrations of 5-HT produced no greater relaxation (usually maximal at 10 μM), the tissues were washed with fresh Krebs solution to remove both 5-HT and the spasmogen.

Agonist potency measurements

After another rest period, the preparations were again contracted to a constant tone. On one ring (control) a cumulative concentration-effect curve to 5-HT was repeated to assess changes in sensitivity of the tissue to 5-HT (mean agonist concentration-ratio 0.97 ± 0.46, $n = 22$). On the remaining three rings, the relaxant effects of test agonists were examined. In each case a cumulative concentration-effect curve to the agonist was constructed up to a maximum of 10 μM. A 5 min interval was employed if a particular concentration of agonist failed to relax the preparation. At the end of the curve for a test agonist, a single concentration of 10 μM 5-HT was applied to ascertain the maximal relaxant response to 5-HT in the presence of the first agonist.

Relaxant responses were expressed as a percentage relaxation of spasmogen-induced tone, this being the difference between the resting and sustained tensions (0.5–0.75 g); 5-HT and 5-CT usually produced 100% relaxation of this tone. The concentration of agonist required to produce 50% relaxation of this tone (the EC_{50}) was calculated for 5-HT and the test compound in each preparation, together with the maximum relaxation evoked by the test compound as a percentage of that evoked by 10 μM 5-HT. The equipotent concentration-ratio (ECR) for the test compound relative to 5-HT (5-HT = 1) was calculated by dividing the EC_{50} value for the compound by the EC_{50} value for 5-HT. Where quoted, the pEC_{50} value is the negative logarithm_{10} of the EC_{50} value.

In most cases, EC_{50} and equipotent concentration-ratio values were obtained by data analysis using the logistic curve-fitting programme ALLFIT (De Lean *et al.*, 1978).

Antagonist potency measurements

Potential antagonists of the relaxant response were tested by examining their ability to antagonize the relaxation induced by 5-CT in preparations contracted with either histamine or the stable thromboxane A_2 mimetic U-46619 (Coleman *et al.*, 1981).

For these experiments, four ring preparations were set-up as above, and a suitable concentration of spasmogen was determined as described. Cumulative concentration-effect curves to 5-CT were then obtained in each preparation. After washing, three rings were exposed to vehicle or antagonist (up to a maximal concentration of 10 μM), whilst the fourth remained untreated (control). After 30 min the prep-

arations were contracted to constant tone, and a cumulative concentration-effect curve to 5-CT was repeated in all four preparations. After washing, the three test preparations were exposed to higher concentrations of antagonists before generating another concentration–effect curve to 5-CT. Usually four 5-CT concentration-effect curves (one control and three curves in the presence of increasing concentrations of antagonist) were generated per ring preparation.

After correcting for any sensitivity changes for successive 5-CT concentration-effect curves, concentration-ratios were calculated by dividing the EC_{50} for 5-CT in the presence of the antagonist by the EC_{50} for 5-CT in the absence of antagonist. In control preparations, the mean concentration-ratio over four consecutive 5-CT concentration-effect curves was 1.23 ± 0.51 ($n = 16$). Concentration-ratio values were then used to calculate pA_2 and slope values by linear regression analyses of Schild plots (Arunlakshana & Schild, 1959). Compounds were considered to be inactive as antagonists of 5-CT if they produced concentration-ratio values of less than 2.0 at 1 μM.

Measurement of cyclic AMP formation

Rings of 2–3 mm in length of freshly isolated vena cava were incubated for 30 min at 37°C in gassed (95% O_2, 5% CO_2), modified Krebs solution (Apperley *et al.*, 1976) containing the cyclic AMP phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (100 μM), and test antagonists, if appropriate. The rings were then exposed to vehicle control or test agonist for a further 2 min, after which they were rapidly frozen in liquid nitrogen to terminate cyclic AMP formation.

The cyclic AMP was extracted (mean efficiency 95%) by homogenisation in absolute ethanol using a glass-glass tissue grinder (Jencons Scientific). The extracts were centrifuged at 15,000 *g* for 2 min and the supernatants contained in the ethanol removed and dried under a gentle stream of nitrogen gas. The remaining extracted pellets were dissolved in sodium hydroxide (1 M) for the subsequent determination of protein contents by the method of Lowry *et al.* (1951). When dry, the ethanol extracts were redissolved in a buffer (pH 7.5) comprising Tris-HCl (50 mM) and EDTA (4 mM), and were assayed for cyclic AMP content using a commercially available competitive binding-assay kit (Amersham International plc).

Results are expressed as pmol cyclic AMP formed mg^{-1} tissue protein. The small number of rings available from each vessel (maximum of 10) dictated a protocol in which parts of agonist concentration-effect curves were generated in different experiments, and these data were then subsequently pooled to generate full concentration-effect curves. For this reason, points on these curves were obtained from between 20 and 60 experiments. In order to calculate equipotent concentration-ratios (ECR) relative to 5-HT, the mean EC_{50} (concentration of agonist producing 50% of its own maximum response) of the test agonist was divided by the mean EC_{50} value for 5-HT. These EC_{50} values were obtained by 'ALLFIT' analysis of the data.

In order to obtain approximate pA_2 values for antagonists, two point concentration-effect curves for 5-CT were generated in the absence and presence of two antagonist concentrations, and concentration-ratios were estimated at an arbitrary point drawn approximately midway through the concentration-effect curves. The concentration-ratios were calculated by dividing the concentration of 5-CT required to produce a response at this point in the presence of antagonist by that required for 5-CT in the absence of antagonist. These estimates were used to calculate pA_2 and slope values, which were subsequently meaned. In the case of methiothepin, pA_2 values were estimated from a single ratio at a methiothepin concentration of 10 nM, since the higher concentration produced a non-parallel shift in the 5-CT concentration–effect curve.

Drugs

5-Hydroxytryptamine creatine sulphate, 3-isobutyl-1-methylxanthine (IBMX) and histamine hydrochloride (Sigma), spiperone and ketanserin (Janssen), forskolin (Calbiochem), buspirone hydrochloride (Bristol-Myers), ipsapirone hydrochloride (Troponwerke), prostaglandin E_2 (PGE_2, Upjohn), methiothepin maleate (Hoffman La Roche), metergoline (Farmitalia), methysergide hydrogen maleate (Sandoz) and mesulergine (a kind gift from Dr G. Engel, Sandoz). The following compounds were synthesized by Chemistry Research, Glaxo Group Research, Ware, U.K.: 5-carboxamidotryptamine maleate, α-methyl-5-HT maleate, 2-methyl-5-HT sulphate, n,n-dipropyl-5-CT, 8-hydroxy-DPAT (8-hydroxy-2-(di-n-propylamino) tetralin hydrobromide), RU24969 (5-methoxy-3 (1,2,3,6-tetrahydro- 4-pyridinyl)1H indole), (±)-cyanopindolol, U-46619 (11, 9, epoxymethano - PGH_2), GR43175 (3 - (2 - di - methylamino)ethyl-N-methyl - 1H - indole - 5 - methane sulphononamide) and GR38032 (1,2,3,9-tetrahydro-9-methyl-3[(2-methyl-1H-imidazol-1-yl)methyl] 4H - carbazol-4-one).

Drug stocks were prepared in distilled water or Krebs buffer, except for RU24969, cyanopindolol, ketanserin, spiperone and GR38032 (dissolved in 2 M acetic acid), and forskolin (dissolved in dimethylacetamide). Stock solutions were diluted in

Krebs buffer, and equivalent vehicle controls were included where appropriate.

Statistics

Values are expressed as mean ± s.e.mean of n observations, which is also the number of animals used. Student's unpaired t test was used to assess the significance of differences between mean values.

Results

Relaxant response

5-HT and 5-CT produced a concentration-dependent relaxation (pEC_{50} values of 6.7 and 8.4, respectively) of preparations contracted with α-methyl-5-HT (Figure 1a). This relaxation was endothelial-independent since endothelial removal by balloon catheterisation abolished relaxant responses to acetylcholine, but did not significantly alter the pEC_{50} value for 5-HT (7.1 ± 0.3, $n = 4$) or 5-CT (8.5 ± 0.2, $n = 4$). When either histamine or U-46619 were used as the spasmogen, 5-HT (but not 5-CT) elicited a more complex relaxant response (Figure 1b), and hence relaxant agonist potencies relative to 5-HT were determined in α-methyl-5-HT-contracted preparations (Figure 2 and Table 1). Under these conditions, 2-methyl-5-HT (pEC_{50} 5.2) and n,n-dipropyl-5-CT (pEC_{50} 5.0) were weak agonists. The weak relaxant activity of 2-methyl-5-HT was not antagonized by the 5-HT_3 antagonist GR38032 (agonist concentration-ratio at 10 μM = 0.87 ± 0.21, $n = 3$). RU24969, 8-hydroxy-DPAT, buspirone and ipsapirone showed little or no agonist activity at concentrations up to 10 μM and, at a concentration of 1 μM, failed to antagonize the re-

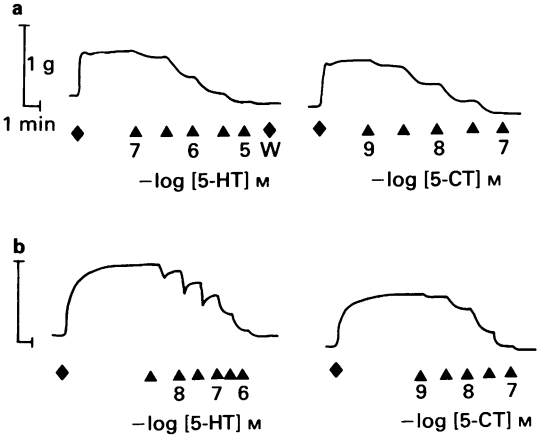

Figure 1 Relaxant responses to 5-hydroxytryptamine (5-HT) and 5-carboxamidotryptamine (5-CT) in endothelium-intact porcine vena cava ring preparations contracted with α-methyl-5-HT (10^{-7} M, a) or U-46619 (10^{-8} M, b). The symbol (◆) indicates application of contractile agent and washout at the end of the cumulative concentration-response curve to 5-HT or 5-CT.

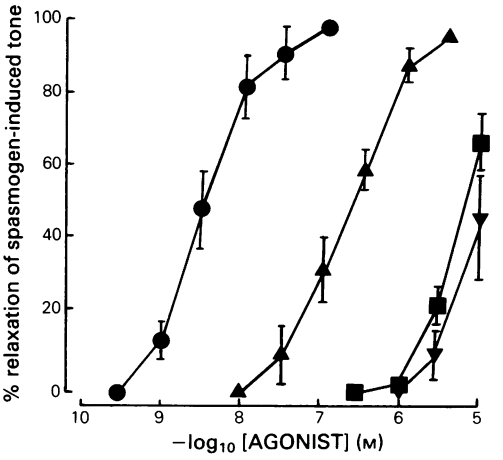

Figure 2 Tryptamine agonist-induced relaxation of porcine isolated vena cava ring preparations contracted with α-methyl-5-hydroxytryptamine (α-methyl-5-HT). Results are means ± s.e.mean of at least four determinations. Data shown for 5-carboxamidotryptamine (5-CT, ●), 5-HT (▲), 2-methyl-5-HT (■) and n,n-dipropyl-5-CT (▼).

Table 1 Relaxant activity in isolated ring preparations of neonatal porcine vena cava contracted with α-methyl-5-HT

Compound	pEC_{50}	*Equipotent concentration-ratio*
5-HT	6.7 ± 0.3	1.0
5-CT	8.4 ± 0.3	0.03 ± 0.01
2-methyl-5-HT	5.2 ± 0.1	52 ± 2
n,n-dipropyl-5-CT	5.0 ± 0.3	51 ± 11
8-hydroxy-DPAT	< 5	> 100
RU24969	< 5	> 100
Buspirone	< 5	> 100
Ipsapirone	< 5	> 100
GR43175	< 4	> 500
IBMX	5.8 ± 0.4	8.0 ± 5
Forskolin	7.4 ± 0.3	0.36 ± 0.12

Results are means ± s.e.mean of the negative logarithm$_{10}$ of the EC_{50} value (pEC_{50}) obtained from at least three experiments. Equipotent concentration-ratios are expressed relative to 5-HT (see Methods).

Table 2 Antagonism of 5-carboxamidotryptamine (5-CT)-induced relaxation and elevation of tissue cyclic AMP levels in piglet vena cava ring preparations

Compound	Response			
	Relaxation		Cyclic AMP	
	pA_2	Slope	pA_2	Slope
Methysergide	7.9 ± 0.2	0.95 ± 0.11	7.9 ± 0.3	1.03 ± 0.23
Spiperone	7.5 ± 0.3	0.87 ± 0.11	7.7 ± 0.3	1.07 ± 0.13
Methiothepin	9.0 ± 0.2	1.50 ± 0.85*	8.8 ± 0.3**	
Metergoline	8.3 ± 0.3	0.89 ± 0.32		
Mesulergine	7.1 ± 0.1	0.96 ± 0.17		

Concentration-effect curves for 5-CT were obtained in the absence and presence of three (relaxant responses) or two (cyclic AMP responses) concentrations of antagonist. Concentration-ratios, estimated at the EC_{50} level where possible, were used to calculate pA_2 and slope values. Results are means ± s.e.mean of at least 3 determinations.
* Significantly different from unity ($P < 0.05$ see Figure 3.).
** Calculated only at the lower concentration of antagonist due to non-parallelism of the agonist concentration-effect curve at the higher antagonist concentration (See Figure 7).

laxant activity of 5-CT (agonist concentration-ratios of 1.73 ± 0.37, 1.92 ± 0.62, 2.10 ± 0.38 and 1.43 ± 0.65, respectively, $n \geq 3$). The novel 5-HT_1-like receptor agonist GR43175 displayed no significant agonist or antagonist (versus 5-CT) activity at concentrations up to 10 μM.

In order to avoid interference with the spasmogen, potential antagonists were assessed against 5-CT in preparations contracted with either U-46619 or histamine (the relaxant profile of 5-CT was identical for all spasmogens employed – see Figure 1a and b). Under these conditions, relaxant responses to 5-CT were antagonized competitively by methysergide, spiperone, metergoline and mesulergine (Table 2) and in an unsurmountable fashion by methiothepin (Figure 3). None of these compounds significantly antagonized (at 10 μM) relaxant responses to PGE_2 (concentration-ratios < 2). Relaxant responses to 5-CT were not affected by the 5-HT_2 antagonist ketanserin, the 5-HT_3 antagonist GR38032 or by the non-selective 5-HT_{1A} and 5-HT_{1B} ligand, cyanopindolol (agonist concentration-ratio estimates at 1 μM were 0.87 ± 0.14, 1.68 ± 0.22 and 1.64 ± 0.36 respectively, $n = 3$).

Cyclic AMP responses

The basal control level of cyclic AMP in isolated ring preparations of neonatal porcine vena cava was 6.3 ± 1.7 pmol mg^{-1} protein ($n = 73$).

The time-course for the elevation of tissue cyclic AMP levels evoked by 5-HT (10 μM) or 5-CT (1 μM) was similar to that required for relaxation and was comparable to that previously obtained (Trevethick *et al.*, 1984). Maximal elevation of tissue cyclic AMP levels was attained after a 2 min exposure to the agonists, and hence this exposure time was adopted throughout this study.

Both 5-HT (0.1–10 μM) and 5-CT (1–1000 nM) increased basal levels of cyclic AMP in a concentration-dependent manner with a maximum increase of about 4 fold (Figure 4). Forskolin (10 μM),

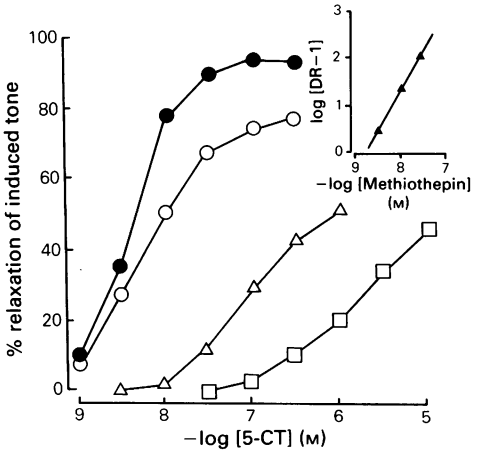

Figure 3 Antagonism of 5-carboxamidotryptamine (5-CT)-induced relaxation by methiothepin. Porcine vena cava rings contracted with histamine or U-46619 were exposed to 5-CT after pretreatment in the absence (●) and presence of methiothepin at concentrations of 3×10^{-9} M (○), 1×10^{-8} M (△) and 3×10^{-8} M (□). The inset shows the Schild plot obtained using concentration-ratios, necessarily in this case, estimated at the 40% relaxation level. These data are from a single representative experiment for which the calculated pA_2 and slope values were 8.7 and 1.70, respectively.

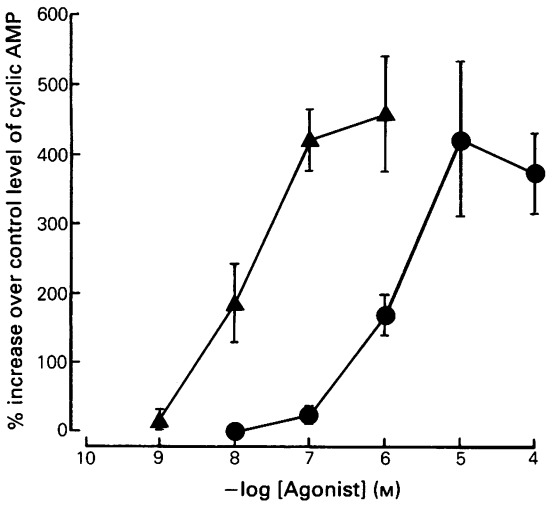

Figure 4 Concentration-effect curves for 5-carboxamidotryptamine (5-CT) and 5-hydroxytryptamine (5-HT)-induced elevation of porcine vena cava ring cyclic AMP levels. Porcine isolated vena cava rings were exposed to 5-HT (●) or 5-CT (▲) at various concentrations, and the cyclic AMP contents extracted and assayed. Data are expressed relative to control cyclic AMP levels of 7.13 (±2.8) pmol mg^{-1} tissue protein. Results are means, and vertical lines indicate s.e.mean, of 20–60 experiments, from which EC_{50} values for 5-HT and 5-CT were 1.5 ± 1.2 μM and 16 ± 8 nM, respectively.

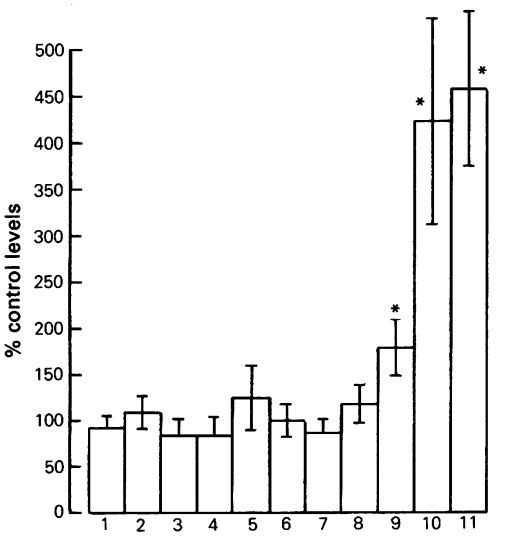

Figure 5 Effects of various agonists (10 μM) on porcine vena cava ring cyclic AMP levels. Rings were incubated with: (1) U-46619, (2) α-methyl-5-hydroxytryptamine (α-methyl-5-HT) (3) 2-methyl-5-HT, (4) 8-hydroxy-2-(di-n-propylamino) tetralin, (5) GR43175, (6) buspirone, (7) ipsapirone, (8) RU24969, (9) n,n-dipropyl-5-carboxamido-tryptamine (n,n-dipropyl-5-CT), (10) 5-HT or (11) 5-CT (1 μM). Results are means, and vertical lines indicate s.e.mean, from 6–20 determinations, and are compared with a control of 7.3 (±2.9) pmol mg^{-1} protein. * Significantly different from control ($P < 0.05$).

Table 3 Effects of 5-HT, 5-CT and GR43175 on forskolin and PGE_2-induced elevation of porcine vena cava ring cyclic AMP content

	Cyclic AMP content (pmol mg^{-1} protein) *in the presence of:*	
Treatment	*Forskolin* (10 μM)	*PGE_2* (10 μM)
Control	178 ± 33	22 ± 8
5-HT	380 ± 57*	35 ± 12
5-CT	415 ± 72*	41 ± 9*
GR43175	169 ± 43	32 ± 14

The effects of a single concentration (10 μM) of 5-hydroxytryptamine (5-HT), 5-carboxamidotryptamine (5-CT) and GR43175 on the elevation of tissue cyclic AMP levels evoked by forskolin (10 μM) or prostaglandin E_2 (PGE_2, 10 μM) were determined. Results are means ± s.e.mean of three experiments. The mean control level of cyclic AMP in the absence of stimulation was 6.3 ± 1.7 pmol mg^{-1} protein.
* Significantly different from the control ($P < 0.05$).

a direct activator of adenylate cyclase, elicited a 20–50 fold increase in cyclic AMP levels (data not shown).

Of the agonists examined, only 5-HT and 5-CT produced a marked elevation of cyclic AMP levels at concentrations up to 10 μM, although a significant elevation was produced by 10 μM n,n-dipropyl-5-CT (Figure 5). The equipotent molar concentration-ratio (ECR) for 5-CT (0.01) relative to 5-HT (1.0) was similar to that seen for the relaxant response (ECR 0.02). 5-Hydroxytryptamine, 5-CT or GR43175 did not produce any significant inhibition of forskolin- or PGE_2-stimulated adenylate cyclase activity at concentrations up to 10 μM (Table 3).

The elevation of cyclic AMP levels induced by 5-CT was prevented by 10 μM methiothepin, methysergide and spiperone, but not by 10 μM ketanserin, GR38032, cyanopindolol or 8-hydroxy-DPAT (Figure 6). Metergoline and mesulergine were not examined. We have attempted to provide pA_2 estimates for spiperone, methysergide and methiothepin (Table 2). These were based on concentration-ratios estimated from 2-point concentration-effect curves to

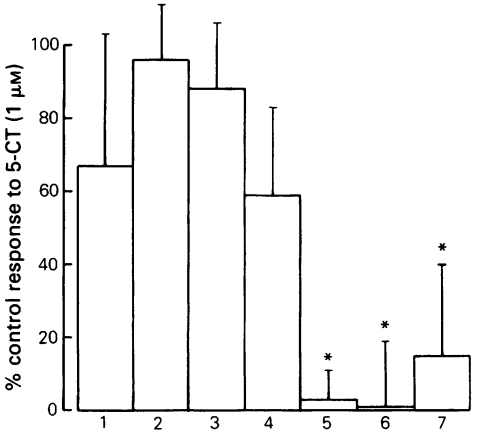

Figure 6 Antagonism of 5-carboxamidotryptamine (5-CT, 1 μM)-stimulated cyclic AMP accumulation in porcine vena cava rings treated with 10 μM antagonist (1) ketanserin, (2) GR38032, (3) cyanopindolol, (4) 8-hydroxy-2-(di-n-propylamino) tetralin, (5) methysergide, (6) methiothepin, (7) spiperone. Results are means, and vertical line indicate s.e.mean, from 6–8 determinations, for which the control and 5-CT-stimulated cyclic AMP levels were 6.3 ($\pm$1.7) and 26.5 ($\pm$6.3) pmol mg^{-1} protein, respectively. * Significantly different from control ($P < 0.01$).

5-CT in the absence and presence of two concentrations of antagonist (Figure 7), a protocol dictated by limited tissue supplies. As was seen in the 'relaxant' studies, spiperone and methysergide appeared to act in a competitive manner, whereas antagonism by methiothepin was clearly unsurmountable at a high concentration.

Discussion

The results of this study confirm and extend those of Trevethick *et al.* (1984, 1986), and provide evidence that a 5-HT$_1$-like receptor in porcine vena cava ring preparations mediates both the smooth muscle relaxation response and the elevation of cyclic AMP response seen with 5-HT and 5-CT. That these responses are mediated by a 5-HT$_1$-like receptor has been illustrated by the high sensitivity to the agonist 5-CT, the selective antagonism by methiothepin (albeit unsurmountable) and methysergide, and by a lack of sensitivity to the 5-HT$_2$ and 5-HT$_3$ antagonists ketanserin and GR38032 respectively (see Bradley *et al.*, 1986).

Surprisingly, 2-methyl-5-HT (an agonist normally considered to be selective for 5-HT$_3$ receptors) produced some relaxation of preparations contracted with α-methyl-5-HT, albeit at concentrations one

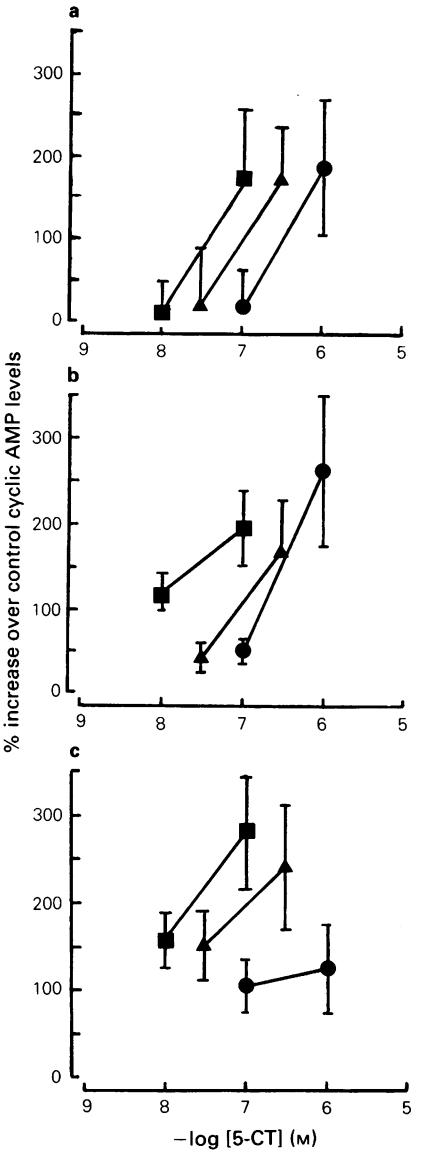

Figure 7 Antagonism of 5-carboxamidotryptamine (5-CT)-induced elevation of cyclic AMP levels in porcine vena cava ring preparations by methysergide (a), spiperone (b) and methiothepin (c). Two-point 5-CT concentration–effect curves were generated in the absence (■) and the presence of two concentrations of antagonist: methysergide 3×10^{-8} M (▲) and 3×10^{-7} M (●), spiperone 1×10^{-7} M (▲) and 1×10^{-6} M (●), and methiothepin 1×10^{-8} M (▲) and 1×10^{-7} M (●). Each point is the mean, and vertical lines indicate s.e.mean, of 6–8 determinations. pA_2 and slope values were estimated in each individual experiment, and the mean values are shown in Table 2.

thousand-times greater than those required for 5-CT. This action of 2-methyl-5-HT was not, however, antagonized by the 5-HT_3 antagonist GR38032, and may reflect weak agonist activity at this 5-HT_1-like receptor (the specificity of this response has not been investigated). No significant elevation in tissue cyclic AMP levels was seen with 2-methyl-5-HT. However, the fact that the concentration–effect curves for 5-HT and 5-CT for elevation of tissue cyclic AMP levels lay to the right of the corresponding curves for relaxation, suggests that cyclic AMP measurements may be a less sensitive index of agonist activity. Nevertheless, there was generally an excellent agreement between the pharmacology of the relaxant and cyclic AMP responses in terms of agonist and antagonist potencies, implying that a common receptor is involved.

A number of groups have described effects of 5-HT and 5-CT on adenylate cyclase activity in brain tissues. Both stimulant (Barbaccia *et al.*, 1983; Shenker *et al.*, 1983; 1987; Markstein *et al.*, 1986) and inhibitory (DeVivo & Maayani, 1986; Weiss *et al.*, 1986; Hoyer & Schoeffter, 1988) effects have been described and assigned to various types of 5-HT_1 binding site (for review see Roth & Chuang, 1987). Based upon the agonist potencies of 5-CT and 8-hydroxy-DPAT, together with antagonism by spiperone, many of these adenylate cyclase effects have been ascribed to activation of 5-HT_{1A} receptors. The cyclic AMP response that we have characterized in porcine vena cava appears to be distinct from these brain 5-HT receptors. Thus we exclude a role for 5-HT_{1A} receptors because, although spiperone antagonized the actions of 5-CT, other potent 5-HT_{1A} ligands, such as 8-hydroxy-DPAT, buspirone, ipsapirone and n,n-dipropyl-5-CT (Fozard, 1987) were either very weak or inactive as agonists or antagonists at concentrations up to 1 μM. In addition, we have obtained no evidence for a 5-HT receptor-mediated inhibition of forskolin- or PGE_2-activated adenylate cyclase activity in porcine vena cava preparations, thereby precluding a negatively-coupled 5-HT receptor. The non-selective 5-HT_{1B} ligands, cyanopindolol and RU24969 (Fozard, 1987), were similarly devoid of appreciable activity in our study. The possibility that 5-HT_{1C} or 5-HT_{1D} sites could mediate the effects of 5-HT, as might be suggested by the antagonist potencies of mesulergine and metergoline (Table 2), is excluded on the basis of the high affinity of spiperone for the porcine vena cava receptor as compared to its low affinity at 5-HT_{1C} and 5-HT_{1D} binding sites (Heuring & Peroutka, 1987).

The 5-HT_1-like receptor present in porcine vena cava preparations is very similar if not identical to that found to mediate smooth muscle relaxation in cat isolated saphenous vein and guinea-pig ileum preparations (Feniuk *et al.*, 1983; 1984; Kalkman *et al.*, 1986). 5-CT appears to be a relatively selective agonist for this receptor and is competitively antagonized by spiperone (pA_2 ~7.5) and methysergide (pA_2 ~7.8). However, vascular relaxation can also be mediated by an endothelial 5-HT receptor (Leff *et al.*, 1987; Martin *et al.*, 1987). At this receptor subtype, 5-HT and α-methyl-5-HT are more potent agonists than 5-CT, and are antagonized by methiothepin and methysergide, but not by spiperone. We have preliminary data that also suggest the presence of endothelial 5-HT receptors in porcine vena cava preparations (Sumner & Humphrey, 1988). Under the experimental conditions purposefully employed in this study, however, we have no evidence for a contribution of the endothelium to the relaxant activities of any of the compounds examined.

In addition to these receptors mediating smooth muscle relaxation, a 5-HT_1-like receptor which mediates smooth muscle contraction and inhibition of neuronal transmitter release has been described, and this receptor is selectively activated by the novel agonist GR43175 (Feniuk *et al.*, 1979; 1985; Humphrey *et al.*, 1988). Hence, the lack of effect of GR43175 in the porcine vena cava (as an agonist or antagonist) provides further evidence for the proposed sub-division of 5-HT_1-like receptors (Humphrey *et al.*, 1988).

In conclusion, this study has demonstrated that porcine vena cava contains a smooth muscle 5-HT_1-like receptor, activation of which leads to adenylate cyclase activation and smooth muscle relaxation. This receptor appears to be unlike any known 5-HT_1 binding site and is dissimilar to those 5-HT_1-like receptors activated by GR43175.

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1. Title page
2. Summary
3. Introduction
4. Methods
5. Results
6. Discussion and conclusions
7. Acknowledgements
8. List of references
9. Tables
10. Figures and captions

Each section must be typed in double spacing with margins of not less than 2.5 cm all round and each page should be numbered. **The original and one copy of the typescript should be supplied.**

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The title should normally contain no more than 150 characters and should not consist of a sentence (statement or conclusion) or be interrogative. There should be a separate title page giving the names of authors and their appropriate addresses. It should be made clear which address relates to which author. Authors' present addresses differing from those at which the work was carried out should be given as footnotes on the title page and referenced at the appropriate place in the author list by superscript numbers. A footnote may also be used to indicate the author to whom correspondence should be sent. The use of footnotes for any other reason is not allowed. If the address to which proofs should be sent is not that of the first mentioned author, clear instructions should be given in a covering note and not on the title page. The title page should be paginated as page 1 of the paper.

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Introduction

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Methods

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Drugs should be listed in a separate paragraph. Their names should be 'approved names' as published previously in British Approved Names, 1986 (HMSO). If a drug has no 'approved name' its chemical name must be used and the rules set out in

the current *Handbook for Chemical Society Authors* (London, Chemical Society) observed, or its structural formula given. Cumbersome chemical names should be suitably abbreviated for later reference in the paper.

The doses of drugs should be given as unit weight per body weight, e.g. $mmol\,kg^{-1}$ or $mg\,kg^{-1}$; concentrations should be given in terms of molarity, e.g. nM or μM.

Reference should be made to any statistical analyses that have been performed on the results in order, for example, to determine the significance of differences between results obtained under different experimental conditions.

Results

The description of the experimental results should be succinct but, nevertheless, in sufficient detail to allow the experiments to be repeated by others. Typical single experiments may be presented with a clear statement that *n* number of similar experiments had similar results. Where appropriate, however, the mean results with confidence limits or with standard errors of the means and the number of observations should be given. Statistical tests of significance should be performed where appropriate. The results of such tests should be stated as the numerical value of the probability (*P*) that is calculated, with any necessary clarification (e.g. one-tail or two-tail test).

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Acknowledgments

Acknowledgements should be brief but should include reference to sources of support. Sources of drugs not freely available commercially should be acknowledged.

References

In the text, references to other work should take the form: (Bolton & Kitamura, 1983) or, 'Bolton & Kitamura (1983) showed that . . .'. If there are more than two authors, the first author's name should be given followed by *et al.*

References to 'unpublished observations' or 'personal communications' should be mentioned in the text only, and not included in the list of references. Papers which have been submitted *and accepted* for publication, should be included in the list of references with the names of the periodical and 'in press'. **A photocopy should normally be submitted with the manuscript.** If this is not possible, authors should indicate whether the work cited is an abstract or a full paper. Papers in preparation or which have been submitted but not yet finally accepted for publication must not be included in the list of references.

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BOLTON, T.B. & KITAMURA, K. (1983). Evidence that ionic channels associated with the muscarinic receptor of smooth muscle may admit calcium. *Br. J. Pharmacol.*, **78**, 405–416.

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Each table should be given on a separate page, paginated as part of the paper. Tables should be numbered consecutively with arabic numericals and the number should be followed by a brief descriptive caption, occupying not more than two lines, at the head of the table. The proportions of the text area

should be borne in mind when designing the layout of tables. For the sake of clarity, tables should not have more than 85 characters to a line, with spaces between columns counted as four characters. The absolute maximum is 110 characters to a line. Each column should have a heading and the units of measurement should be given in parentheses in the heading. Except in special circumstances, tables should be self-explanatory; the necessary descriptions should be at the bottom of the table.

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Please note that unsatisfactory Figures will be returned to the Author for revision. The Journal reserves the right to reject a manuscript if the Figures are unacceptable.

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(a) The Authors' names and the Figure number must be indicated lightly *in pencil* on the back of each Figure; if necessary, use an adhesive label to avoid damage to the Figure.
(b) One set of Figures complete with lettering, numbering, arrows etc. must accompany *each* copy of the manuscript. An original set and one high quality photocopy will suffice.
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It is best to submit an original drawing (black ink on heavy white paper or faint blue graph paper) which has been prepared to conform with the style and convention of the Journal, because redrawing is expensive. The original drawing should be lettered in pencil and should be larger (up to two times as large) than the intended size in the Journal.

It is important that the printed symbols and lines should retain their clarity. To achieve this the symbols and lines in original drawings should be sharply defined and of an even density and breadth. Heavier (broader) lines should be used for curves than for the axes of graphs. The table below illustrates line widths and symbol sizes to be used together on a figure and the appropriate reductions in the final printed form.

Line width (axes)	Line width (graphs)	Symbol size	Figure will reduce to this percentage of the original size
———	———	△□○	100 (No reduction)
———	———	△□○	80
———	———	△□○	70
———	———	△□○	60
———	———	△□○	50
———	———	△□○	40

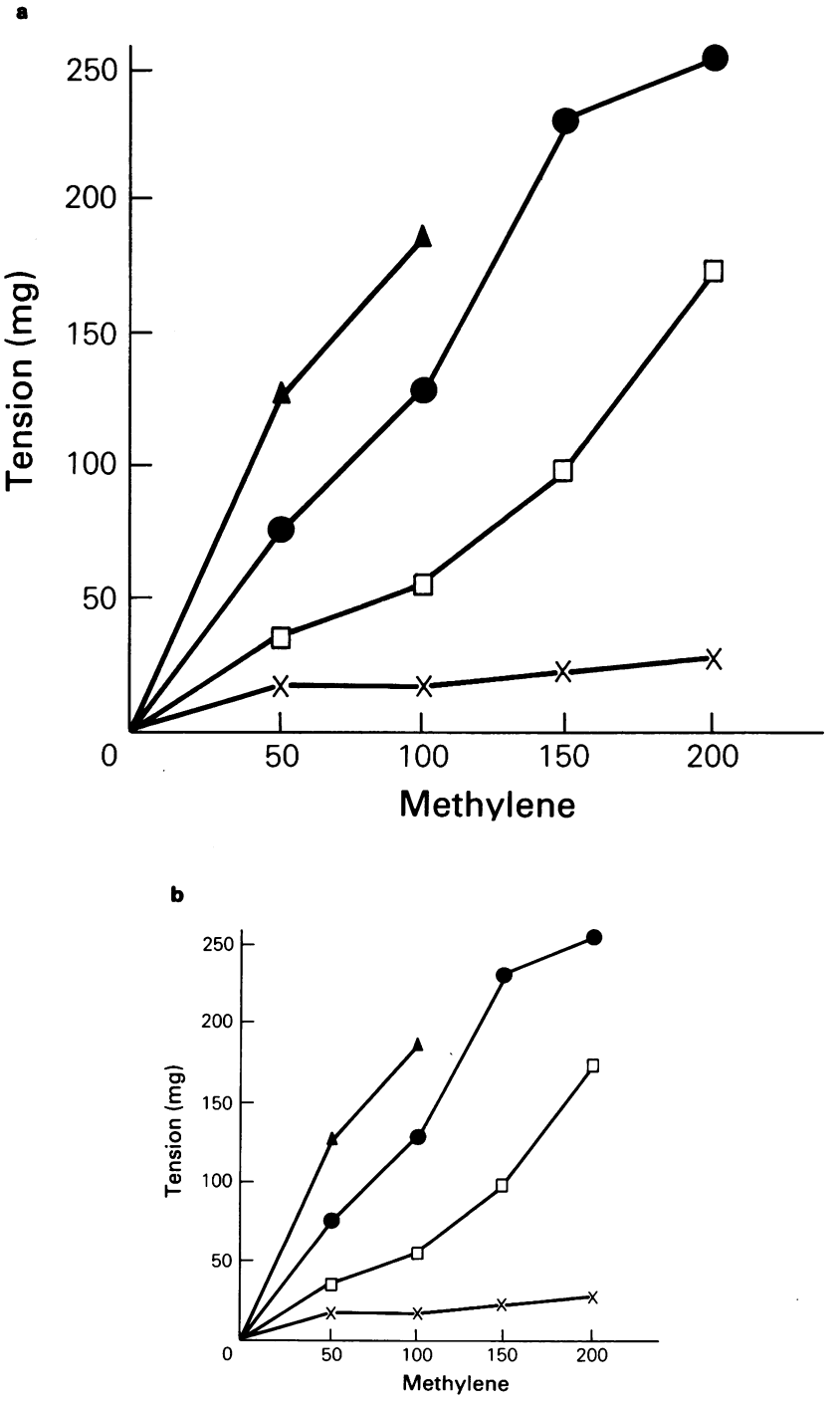

Figure 1. (a) Artwork as drawn. (b) Artwork reduced to 60 per cent of its original size for publication in the Journal.

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The preferred order to shading of histogram columns is: open (clear), closed (solid), cross-hatched, heavily stippled and other (if required).

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ABBREVIATIONS AND SYMBOLS

Physico-chemical quantities

The *British Journal of Pharmacology* uses the SI symbols for units. The following prefixes for multiples of units should be used:

Multiplier	*Prefix*	*Symbol*
10^{-1}	deci	d
10^{-2}	centi	c
10^{-3}	milli	m
10^{-6}	micro	μ
10^{-9}	nano	n
10^{-12}	pico	p
10^{-15}	femto	f
10^{-18}	atto	a

Multiplier	*Prefix*	*Symbol*
10^{3}	kilo	k
10^{6}	mega	M
19^{9}	giga	G
10^{12}	tera	T

Thus, micron = μm; ångstrom = 0.1 nm. Mixed prefixes are not permissible, thus mμg should be ng. The symbols d (10^{-1}) and c (10^{-2}) should be restricted to those occasions on which there is a strongly felt need for them (e.g. cm).

Use of the solidus

The solidus should be avoided as far as possible and the negative index substituted, e.g $mg\,kg^{-1}$ rather than mg/kg; $pmol\,mm^{-2}\,min^{-1}$ rather than pmol/mm^2/min.

Quantity	*Preferred unit*	*Symbol*
Amount (of substance)	mole	mol
Capacitance	farad	F
Concentration	moles per litre	M or mol l^{-1}
Current	ampere	A
Electrical conductance	siemens	S
Electromotive force	volt	V
Flow (blood or other liquid)	litres per second (or min)	$1\,s^{-1}$ or $1\,min^{-1}$
Flow (air or other gas)	litres per second (or min)	$1\,s^{-1}$ or $1\,min^{-1}$
Force	newton	N
Frequency of regular event	hertz	Hz
Length	metre	m
Mass	gram	g
Power	watt	W
Pressure (or partial pressure)	pascal*	Pa
Radioactivity	becquerel or curie	Bq (60 d.p.m.) or Ci (3.7×10^{10} Bq)
Resistance (electrical)	ohm	Ω
Temperature	degree celsius	°C
Time	second (preferred)	s
	minute	min
	hour	h
Volume (blood or other liquid)	litre	l
Volume (air or other gas)	litre	l
Work	joule	J

* mm of mercury (mmHg) are allowed if conventional, and if mercury manometer is used for calibration.

SYMBOLS

Symbols denoting physical quantities are usually printed as italic capitals (indicated by single underline in typescript). A dash over the symbol indicates a mean value; a dot over the symbol indicates a time derivative. Suffixes may be used to indicate 'where' and 'what'. They are printed as inferiors on the line. Multiple suffixes should be avoided if a simpler symbol adequately defined is unambiguous, but if necessary should be separated by commas e.g. P_{A,CO_2} denotes partial pressure of CO_2 alveolar air.

CHEMICAL AND BIOLOGICAL ABBREVIATIONS

The abbreviations listed may be used without definition *except* those for chemicals, drugs and enzymes which must be written in full at first mention in the title, summary and again in the text. At first mention they should be followed by the abbreviation in brackets. Subsequently, the abbreviation alone may be used.

The list of abbreviations for chemical, drug and enzyme names is clearly not comprehensive and includes only a few commonly used examples.

Use abbreviations sparingly as extensive use can make the text hard to follow.

acetylcholine	ACh
acetylcholinesterase	AChE
adenosine 3′ : 5′-cyclic monophosphate	cyclic AMP
adenosine 5′-phosphate	AMP
adenosine triphosphatase	ATPase
γ-aminobutyric acid	GABA
analysis of variants	F
adrenaline	Ad
analytical standard of reagent purity	A.R.
anhydrous	anhyd.
approximate(ly)	approx.
approximately equals	≈
aqueous	aq.
arg-vasopressin	AVP
boiling point	b.p.
bovine serum albumin	BSA

cardiovascular system	CVS
catechol-*O*-methyl transferase	COMT
central nervous system	CNS
cerebrospinal fluid	CSF
chi-squared (statistics)	χ^2
clearance	*c*
coenzyme A	CoA
concentrated	conc.
correlation coefficient	r
cubic	cu.
degree of freedom (statistics)	d.f.
deoxyribonucleic acid	DNA
deoxyribonuclease	DNase
dextro-(absolute configuration)	D-
dextro-(optical rotation)	(+)-
diameter	diam.
diameter, inside	i.d.
diameter, outside	o.d.
diffusion coefficient	*D*
3,4-dihydroxyphenylalanine	DOPA
3,4-dihydroxyphenylethylamine	dopamine
direct current	d.c.
disintegration per minute	d.p.m.
dissociation constant	K_D
dissociation constant, negative logarithm of	pK
distilled	dist.
dry ice	solid CO_2
edition	edn
editor(s)	ed.
effective concentration	EC_{50}
effective dose, median	ED_{50}
electrocardiogram	ECG
electrocorticogram	ECoG
electroconvulsive therapy	ECT
electroencephalogram	EEG
electromyogram	EMG
electron spin resonance	e.s.r.
endothelial-derived relaxing factor	EDRF
epithelial-derived relaxing factor	EpDRF
equilibrium constant	*K*
equivalent (general use)	equiv.
erythrocyte	r.b.c.
erythrocyte sedimentation rate	ESR
ethylenediaminetetracetic acid	EDTA
excitatory postsynaptic potential	e.p.s.p.
experiment	expt
experimental	exptl
fatty acids, nonesterified	NEFA
figure(s) (with reference number)	Figure(s)
figure (diagram)	figure
gas–liquid chromatography	g.l.c.
glomerular filtration rate	GFR
haemoglobin	Hb
half-life	$t_{1/2}$
high-frequency	h.f.
high performance liquid chromatography	h.p.l.c.
human serum albumin	HSA
hydrogen-ion concentration	$[H^+]$
hydrogen-ion activity, negative logarithm of (hydrogen-ion exponent)	pH
6-hydroxydopamine	6-OHDA
5-hydroxyindoleacetic acid	5-HIAA
5-hydroxytryptamine	5-HT
immunoglobulins	IgA, IgD, IgE, IgG, IgM
inhibitor constant	K_i
inhibitory concentration	IC_{50}
inhibitory postsynaptic potential	i.p.s.p.
insoluble	insol.
international unit	iu
intra-arterial	i.a.
intracellular fluid	ICF
intradermal	i.d.
intramuscular	i.m.
intraperitoneal	i.p.
intracerebroventricular	i.c.v.
intravenous	i.v.
isotope (atomic mass) e.g. iodine-131	^{131}I
isotopically substituted compounds e.g.	[^{14}C]-ethanol
laevo-(absolute configuration)	L-
laevo-(optical rotation)	(−)-
lethal dose, median	LD_{50}
leukotriene	LT
logarithm to base e	$\log_e$ or ln
logarithm to base 10	$\log_{10}$
maximum	max.
mean arterial pressure	MAP
mean value of (statistics)	$\bar{x}$
melting point	m.p.
meta	*m*-
Michaelis constant	K_M
minimum	min.
mobility (electrophoresis)	*m*
monoamine oxidase	MAO
noradrenaline	NA
nuclear magnetic resonance	n.m.r.
number	no. or No.
number of observations (statistics)	*n*
ortho	*o*-

packed cell volume	PCV
page/pages	p./pp.
para-	*p*-
paragraph	para. or ¶
parts per million	p.p.m.
per cent	%
posterior	post.
probability (significance level in a statistical test)	*P*
radioimmunoassay	RIA
rectus (configuration by the sequence rule)	**R**
red blood corpuscle	RBC
relative band speed to front (chromatography)	R_F
relative molecular mass	M_r
relative retention time (gas chromatography)	t_r
renal plasma flow	RPF
resistance (respiratory)	*R*
respiratory conductance	Sgaw
revolutions per minute	r.p.m.
ribonucleic acid	RNA
section	§
sedimentation coefficient (ultracentrifugation)	*s*
sinister (configuration by the sequence rule	**S**
soluble	sol.
solution	soln.
Spearman rank coefficient	r_s
standard deviation: (of observed sample)	s.d.
standard error (of estimate mean value)	s.e.mean
standard error (of sampling)	s.e.
standard temperature and pressure	STP
subcutaneous	s.c.
sum (statistical):	
of hypothetical population	Σ
of observed sample	S or Σ
temperature	temp.
thin layer chromatography	t.l.c.
time clock-24 h clock used e.g. 18 h 30 min	*t*
time constant	τ
2-amino-2-hydroxymethyl-propan-1,3-diol	Tris
ultraviolet	u.v.
unit	u
vacuum	vac.
valency	e.g. Fe^{2+}; Fe(II) protoporphyrin
volume by volume	v/v
wavelength	λ
weight	wt.
weight by volume	w/v

MEETINGS CALENDAR

8–12 May 1989

3rd International Conference on PAF and Structurally related Alkyl ether Lipids, Tokyo, Japan. (Professor S. Nojima, Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Tsukin, Kanagawa 199, Japan).

9–12 May 1989

International group for Tryptophan Research. 6th International Meeting, Baltimore, USA. (Dr R. Schwarcz, Maryland Psychiatric Research Center, PO Box 21247, Baltimore, MD 21228, U.S.A.)

21–23 May 1989

Current and Future Trends in Anticonvulsant, Anxiety and Stroke Therapy, Princeton, USA. (Dr J. Emmett, Dialogue in Science, Packhorse Place, Kensworth, Beds LU6 3QU, U.K.)

22–26 May 1989

Second Meeting of the International Neurotoxicology Association, Barcelona, Spain. (Dr. E. Rodriquez Farre, Dept. of Pharmacology & Toxicology, CSIC, Jorge Girona Salgado, 18–26, E-08034, Spain).

25–27 May 1989

4th International Symposium on Calcium Antagonists, Florence, Italy. (Secretariat, Fondazione Giovanni Lorenzini, Via Monte Napoleone 23, 20121 Milan, Italy).

1–2 June 1989

International Workshop on Sensory Neuropeptides, Florence, Italy. (Miss A. Giannini, Istituto Farmacobiologico Malesci, S.p.A., Dept. Pharmacology, Via Porpora 22, 50144 Florence, Italy).

1–3 June 1989

International Symposium on Kidney, Proteins & Drugs, Montecatani Terme, Italy. (Dr. Claudio Bianchi, Centro Nefrologico, Clinica Medica Generale 2, University of Pisa, 56100 Pisa, Italy).

28 June–2 July 1989

Ethnicity & Cardiovascular Diseases Conference, Nairobi, Kenya. (Ms. C. Cate, Int. Society on Hypertension in Blacks, 69 Butler Street, S. E., Atlanta, GA 30303, U.S.A.).

12–14 July 1989

British Pharmacological Society Summer Meeting. Leeds, U.K. (Closed meeting for members and guests)

16–21 July 1989

Vth International Congress of Toxicology, Brighton, U.K. (IUTOX '89, Congress House, 65 West Drive, Sutton, Surrey SH2 7NB, U.K.)

23–25 July 1989

New Strategies in Pain Control, Princeton, USA. (Dr J. Emmett, Dialogue in Science, Packhorse Place, Kensworth, Beds, LU6 3QU, U.K.)

7–12 August 1989

Therapy with Amino Acids and Analogues, Ist International Congress, Vienna, Austria. (G. Lubec, University of Vienna, Dept. of Paediatrics, A-1090 Vienna, Währinger Gürtel 18, Austria).

1–3 September 1989

Second International FIP Symposium on Disposition & Delivery of Peptide Drugs, Leiden, The Netherlands. (Dr. J. Verhoef, Centre for Bio-Pharmaceutical Sciences, PO Box 9502, 2300 RA Leiden, The Netherlands).

13–15 September 1989

British Pharmacological Society Autumn Meeting. Manchester, U.K. (Closed meeting for members and guests)

17–20 September 1989

1st International $GABA_B$ Symposium, Cambridge, U.K. (Professor N.G. Bowery, Department of Pharmacology, School of Pharmacy, 29/39 Brunswick Square, London WC1N 1AX.)

19–21 September 1989

Electrochemical Detection, HPLC and *in vitro* monitoring in the Biosciences, Nottingham, U.K. (Dr I.A. MacDonald, Department of Physiology & Pharmacology, Medical School, Queen's Medical Centre, Nottingham, U.K.)

20–21 September 1989

9th Annual Meeting of the Bayliss and Starling Society. Peptides: Physiology, Pharmacology and Therapeutics, Nottingham, U.K. (Professor T. Bennett, Dept. of Physiology & Pharmacology, Medical School, Queen's Medical Centre, Nottingham, U.K.)

28 September 1989

Leukotrienes. London, U.K. (Society for Drug Research, c/o Institute of Biology, 20 Queensbury Place, London SW7 2DZ, U.K.)

22–25 October 1989

Molecular and Cellular Biology of IL-1, TNF and Lipocortins in Inflammation and Differentiation. Siena, Italy. (L. Parente, Sclavo Research Centre, via Florentina 1, 53100 Siena, Italy).

8–11 November 1989

Tenth International Symposium on Drugs affecting Metabolism, Houston, USA. (Meeting Manager, 4550 Post Oak Place, Suite 248, Houston, Texas 77027, U.S.A.)

27–29 November 1989

Biological Actions of Extracellular ATP, Philadelphia, USA. (Conference Dept., New York Academy of Sciences, 2 East 63rd Street, New York, NY 10021, U.S.A.)

6–8 December 1989

Presynaptic Receptors — an Examination of Different Views, New York City, USA. (Conference Department, New York Academy of Sciences, 2 East 63rd Street, New York, NY 10021, U.S.A.)

3–5 January 1990

British Pharmacological Society Winter Meeting. London, U.K. (Closed meeting for members and guests)

1–6 April 1990

6th World Congress on Pain, Adelaide, Australia. (Int. Association for the Study of Pain, 909 NE 43rd Street, Suite 306, Seattle, WA 98105, U.S.A.)

18–20 April 1990

British Pharmacological Society Spring Meeting. Sheffield, U.K. (Closed meeting for members and guests)

29 May–1 June 1990

Fondazione Giovanni Lorenzini Conference on Prostaglandins and Related Compounds, Florence, Italy (Fondazione Giovanni Lorenzini, Via Monte Napoleone, 23, 20121 Milan, Italy).

27–30 June 1990

International Society for Heart Research XI European Section Meeting, Glasgow, UK. (Professor J.R. Parratt, Dept. of Physiology & Pharmacology, Royal College, University of Strathclyde, Glasgow G1 1XW).

1–6 July 1990

IUPHAR 11th International Congress of Pharmacology, Amsterdam, Netherlands (Contact to be advised).

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MEETINGS CALENDAR

Errata

Br. J. Pharmacol. (1988), **95**, 777–782

'Studies of the effects of subacute treatment with N-(cyclopropylmethyl)-19-isopentylnororvinol (M320) on timing of parturition in the rat' by R.G. Evans, G.E. Rice & J.E. Olley.

On page 781 (Discussion) of this paper, the third complete sentence in the right-hand column was misprinted. The correct version is as follows:

'Furthermore, subacute administration of M320 is not associated with reduced maternal OXY release at term (Evans *et al.*, 1987a), indicating that maternal OXY does not play a role in the effects of M320 on the timing of paturition.'