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Niguldipine discriminates between α_1-adrenoceptor-mediated second messenger responses in rat cerebral cortex slices

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The effect of both isomers of niguldipine, a highly selective α_1-adrenoceptor antagonist and dihydropyridine calcium channel blocker, on noradrenaline-stimulated inositol phosphate (IP) accumulation and adenosine 3':5'-cyclic monophosphate (cyclic AMP) potentiation was examined. Both isomers inhibited noradrenaline-stimulated IP accumulation. (+)-Niguldipine was 100 fold more potent than (−)-niguldipine. Potentiation of β-adrenoceptor-stimulated cyclic AMP by noradrenaline was only partially inhibited by both isomers. The dihydropyridine, israpidine, did not inhibit either second messenger response. This study provides further evidence that the α_1-adrenoceptors mediating IP accumulation and cyclic AMP potentiation are different.

Introduction In rat cerebral cortex slices noradrenaline activates two second messenger responses, inositol phospholipid hydrolysis and adenosine 3':5'-cyclic monophosphate (cyclic AMP) formation. The latter consists of a direct effect mediated via β-adrenoceptors and a potentiation of the direct affect by a 'non-β-adrenoceptor', which appears to have many of the pharmacological characteristics of an α_1-adrenoceptor, e.g. it is more potently inhibited by prazosin than by yohimbime.

Inositol phospholipid hydrolysis also appears to be linked to an α_1-adrenoceptor but there is evidence that this receptor is different from that responsible for the potentiation of cyclic AMP formation. Thus, although both responses are more potently inhibited by prazosin than yohimbine, the rank orders of potency of a range of α-antagonists are different for the two responses (Robinson & Kendall, 1989). On the basis of radioligand binding studies it has been proposed that there are two α_1-adrenoceptor subtypes (Morrow & Creese, 1986) and recently Boer *et al.* (1989) have shown that the 1,4-dihydropyridine niguldipine can readily discriminate between α_{1A}- and α_{1B}-receptors in various tissues.

We have therefore employed the enantiomers of niguldipine to see if this antagonist similarly distinguishes between α_1-adrenoceptor-mediated inositol phospholipid hydrolysis and the potentiation of cyclic AMP formation.

Since niguldipine is a potent calcium channel blocker (Boer *et al.*, 1989) we have also examined the effects of another dihydropyridine antagonist, isradipine (previously PN200-110).

Methods Agonist-stimulated accumulation of inositol phosphates in rat brain slices was measured by the method of Brown *et al.* (1984). Washed, cross-chopped slices (350 × 350 μm) of cerebral cortex were divided into aliquots (50 μl/tube) in polypropylene vials containing 0.3 μM [^{3}H]-inositol and 5 mM LiCl in a final volume of 300 μl Krebs-Henseleit buffer, (pH 7.5; 37°C). After a prelabelling period of 45 min, slices were stimulated by agonist for a further 45 min. Antagonists were added 20 min before agonists. Stimulation was terminated with 10% PCA and total [^{3}H]-inositol phosphates separated on Dowex-1-chloride columns. D.p.m. of total [^{3}H]-inositol phosphates were measured by liquid scintillation counting.

Cyclic AMP accumulation was measured by the tritiated adenine prelabelling technique of Shimizu *et al.* (1969). Washed, cross-chopped slices (350 μm × 350 μm) of rat cerebral cortex were prelabelled with [^{3}H]-adenine for 40 min then divided into aliquots (25 μl slices per tube) in vials containing a final volume of 300 μl Krebs-Henseleit buffer. Antagonists were added to tubes 20 min before agonists. Stimulation was stopped after 10 min with 1 M HCl. [^{3}H]-cyclic AMP was separated by Dowex 50 and alumina columns according to the double column method of Salomon *et al.* (1974). Samples were spiked with [^{14}C]-cyclic AMP to allow for recovery correction. D.p.m. of [^{3}H]- and [^{14}C]-cyclic AMP were determined by liquid scintillation counting.

Niguldipine enantiomers are highly lipophilic and absorb readily onto plastic (Boer *et al.* 1989). Therefore niguldipine dilutions (in dimethylsulphoxide, DMSO) were carried out in glass vials. Additions to slices were carried out using fresh pipette tips for each dilution.

8-[^{3}H]-adenine (26 Ci mmol^{-1}) and myo[^{3}H]-inositol (10–30 Ci mmol^{-1}) were obtained from Amersham Radiolabelled Chemicals. [^{14}C]-cyclic AMP (45 mCi mmol^{-1}) was obtained from New England Nuclear. Isoprenaline and noradrenaline were obtained from Sigma. Isradipine was kindly supplied by Sandoz A.G. Basel. (+)- and (−)-niguldipine were the gift of Byk Gulden Pharmazeutika, F.R.G.

Results Noradrenaline (100 μM) produced a five fold increase over the basal [^{3}H]-inositol phosphate ([^{3}H]-IP) accumulation. This response was potently inhibited by (+)-niguldipine with a K_i value of 3.5 ± 0.7 nM and Hill slope of 0.80 ± 0.11 (n = 3; see Figure 1a). The isomer, (−)-niguldipine was 100 fold less potent (K_i = 343 ± 24 nM, nH 0.86 ± 0.04; n = 3). Noradrenaline (NA)-stimulated IP responses in the presence of the highest concentrations of (+)- or (−)-niguldipine were not significantly different from basal values (basal 1160 ± 280 d.p.m., NA + (+)-niguldipine (10^{-5} M) 1710 ± 309 d.p.m.; basal 1318 ± 234 d.p.m., NA + (−)-niguldipine (10^{-4} M) 2683 ± 459 d.p.m.). Hill slope values were not significantly less than 1 (unpaired t test). Isradipine (up to 100 μM) had no effect on noradrenaline-stimulated inositol phosphate accumulation.

Isoprenaline (10 μM) typically produced a 3 fold increase in cyclic AMP formation over basal. This was increased a further 3 fold by noradrenaline (100 μM). The difference in the response to isoprenaline plus noradrenaline ($\alpha + \beta$) and to isoprenaline (β) alone is taken to represent the α-component of the cyclic AMP response. Neither enantiomer of niguldipine had any effect on the response to isoprenaline alone. The α-component of the response was significantly reduced to 65 ± 4% in the presence of (+)-niguldipine (1 μM) ($P < 0.001$, paired t test). Higher concentrations of (+)-niguldipine caused no further reduction in cyclic AMP accumulation. (−)-Niguldipine was even less potent. (−)-Niguldipine 1 μM, caused a 21 ± 17% (n = 3) reduction in the α-component of the cyclic

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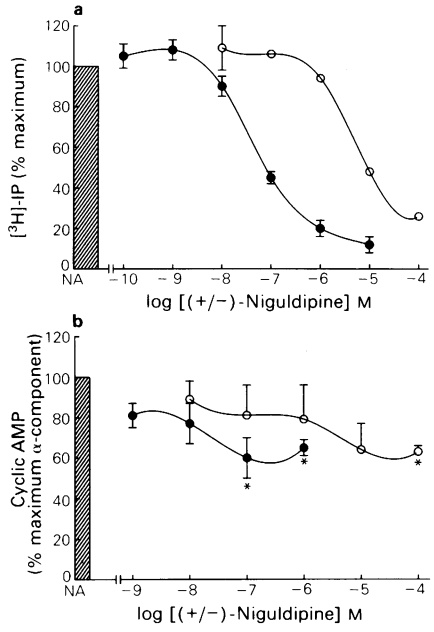

Figure 1 Effect of (+)-niguldipine (●) and (−)-niguldipine (○) on (a) noradrenaline (100 μM)-stimulated inositol phospholipid hydrolysis and (b) the α-component of noradrenaline (100 μM)-mediated potentiation of isoprenaline stimulated cyclic AMP in rat cortical slices. Data are means of 3–10 experiments each of which was performed on quadruplicate samples (*indicates significant difference from response to agonist alone, $P < 0.01$, paired t test); s.e.mean shown by vertical bars.

AMP response. This was not statistically significant. The maximum inhibition observed was 36 ± 13% ($n = 3$, $P < 0.01$, paired t test) in the presence of 10 μM (−)-niguldipine. Higher concentrations (up to 100 μM) had no further effect (see Figure 1b). Isradipine did not inhibit noradrenaline-stimulated cyclic AMP but tended to increase the response to isoprenaline alone and isoprenaline with noradrenaline (data not shown).

Discussion The inositol phospholipid response to noradrenaline was potently inhibited in an apparently competitive and stereoselective manner by niguldipine. This inhibition is probably unrelated to the action of niguldipine on dihydropyridine-sensitive (L-type) calcium channels because another potent dihydropyridine antagonist, isradipine, did not inhibit the response.

In contrast, niguldipine was much less effective in reducing the potentiation of β-adrenoceptor-stimulated cyclic AMP formation with a maximum inhibition of 35–40% of the response. However, although the data presented do not allow calculation of an inhibition constant for niguldipine with regard to cyclic AMP potentiation it is clear that niguldipine is very potent with a maximum effect evident at a concentration of 10 nM (+)-niguldipine, i.e. approximately 200 fold more potent than for the inhibition of inositol phosphate accumulation. It appears that the niguldipine-insensitive component of the cyclic AMP potentiation response is α_1-adrenoceptor-related since it was abolished by 10 nM prazosin (in the presence of 1 μM (+)-niguldipine; data not shown).

Taken together with previous reports of different adrenoceptor antagonist affinity profiles (Johnson & Minneman, 1986; Robinson & Kendall, 1989) and our recent observation that the potentiation of cyclic AMP formation but not the hydrolysis of inositol phospholipids due to noradrenaline is modulated by glucocorticoids (Robinson and Kendall, 1990), it does not seem possible that the two responses could be mediated by the same receptor.

This raises the problem of classification of the different adrenoceptors involved. There have been several attempts to classify α_1-adrenoceptor subtypes on the basis of binding studies and functional data (Morrow & Creese, 1986; Han *et al.*, 1987; Minneman *et al.*, 1988). Binding sites with high affinity for WB4101 (2',6'-dimethoxyphenoxy-ethylaminomethylbenzo-1,4-dioxan) and insensitivity to the irreversible alkylating agent chlorethylclonidine (CEC) have been designated α_{1a}-, and α_{1b}-receptors; they are characterized by a low affinity for WB4101, are sensitive to CEC and may be linked to inositol phospholipid hydrolysis (Han *et al.*, 1987; Minneman *et al.*, 1988).

In addition, the recent binding studies with niguldipine (Boer *et al.*, 1989) suggest the classification of α_{1A} for those receptors with high affinity and stereoselectivity for (+)-niguldipine, and α_{1B} for those sites with low affinity and little stereoselectivity for the isomers of niguldipine.

There is an inherent circularity in attempts to classify the α-adrenoceptor responses described in this study when linkage to second messenger systems is one of the classification criteria. However, if the discriminating ability of niguldipine was used as the major independent criterion, the data presented could be interpreted thus; the cyclic AMP potentiation consists of two components, a minor (α_{1A}) component blocked with very high affinity by (+)-niguldipine and a major component, insensitive to niguldipine but sensitive to prazosin and other α_1-adrenoceptor antagonists (Robinson & Kendall 1989). The inositol phospholipid response to noradrenaline is predominantly mediated by a different α_1-receptor with lower affinity but functional stereoselectivity for niguldipine (α_{1B}?). Thus, niguldipine appears to demonstrate some degree of stereoselectivity in functional studies that is not apparent in binding studies.

Although the identity of the α-adrenoceptor mediating the major component of the cyclic AMP potentiation response and its molecular mechanism of action remain unresolved, it is clear that niguldipine is an important tool for use in the unravelling of α_1-adrenoceptor classification.

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Calcium antagonizes the magnesium-induced high affinity state of the hepatic vasopressin receptor for the agonist interaction

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1 The present study describes the role of Ca^{2+} in the regulation of the hepatic vasopressin V_1 receptor. With low concentrations of Ca^{2+}, there was a small increase in [^{3}H]-arginine vasopressin ([^{3}H]-AVP) binding, but above 10 mM, Ca^{2+} decreased the binding of this agonist. In contrast, low concentrations of Mg^{2+} were associated with a dramatic concentration-dependent increase in [^{3}H]-AVP binding, reaching a maximal effect of 650% above control at concentrations ranging between 1–5 mM. At higher concentrations of Mg^{2+}, the stimulatory effect of this cation was less pronounced, falling to 210% of control at 100 mM Mg^{2+}. Strikingly, Ca^{2+}-inhibited the stimulatory effect of Mg^{2+} in a concentration-dependent fashion.

2 Saturation binding data revealed that Ca^{2+} (2 to 10 mM) *per se* promotes the high affinity conformation of the V_1 receptor for the agonist binding with the K_D decreased from a control value of 2.3 nM to 0.5 nM in the presence of 10 mM Ca^{2+}. This effect was attenuated with an increase in Ca^{2+} above 10 mM. With an increase in Ca^{2+} to 20 mM, however, the B_{max} for [^{3}H]-AVP binding was decreased. Ca^{2+} also decreased the high affinity/high capacity state (K_D 100 pM) of the receptor induced by 1 mM Mg^{2+} for agonist interaction.

3 [^{3}H]-V_1 antagonist binding was inhibited by both Ca^{2+} and Mg^{2+}. The IC_{50} values (mean ± s.e.mean) for Ca^{2+} and Mg^{2+} were 32 ± 8 and 53 ± 9 mM respectively. Maximal inhibition achieved at 100 mM was 29% for Ca^{2+} and 42% for Mg^{2+}. Both cations decreased the affinity and increased the capacity of the V_1 receptor for the antagonist.

4 The results suggest that the divalent metal ion binding site(s) modulated by Mg^{2+} is also accessible to Ca^{2+}. Although Ca^{2+} opposes the powerful stimulatory effects of Mg^{2+} on agonist binding, the effects of Ca^{2+} and Mg^{2+} on the B_{max} of [^{3}H]-AVP binding were different, suggesting that the divalent cations may bind to two different sites, thereby regulating the affinity and the capacity characteristics of the V_1 receptor.

Introduction

The vasoconstrictor and the antidiuretic actions of arginine vasopressin (AVP) are mediated by the activation of V_1 and V_2 receptors, respectively (Michell *et al.*, 1979). Recent evidence also supports the view that AVP enhances hepatic glycogenolysis by the activation of vascular type V_1 receptors located on liver plasma membranes (Stubbs *et al.*, 1976; Michell *et al.*, 1979; Cantau *et al.*, 1980). Several studies including recent work from our laboratory have characterized *in vitro* the hepatic V_1 receptor in rat liver membrane and intact hepatocytes (Cantau *et al.*, 1980; Fahrenholz *et al.*, 1985; Fishman *et al.*, 1985; Gopalakrishnan *et al.*, 1986; 1988; Cornett & Cate, 1989). A general conclusion emerging from these investigations supports the presence of a homogeneous population of V_1 receptors in rat liver. Further, we (Gopalakrishnan *et al.*, 1988) documented a marked increase in the agonist binding affinity induced by Mg^{2+} (1 mM) such that the estimated K_D values (~100 pM) for [^{3}H]-AVP were in the range that agreed well with the reported half-maximal concentrations of AVP in promoting hepatic glycogenolysis (Keppens & De Wulf, 1975; Stubbs *et al.*, 1976; Cantau *et al.*, 1980; Mauger *et al.*, 1984). In addition, we also demonstrated a decrease in the antagonist binding affinity caused by Mg^{2+}. Such differential modulation of agonist versus antagonist binding affinities provided an important insight indicating that the divalent cations may indeed regulate the actions of AVP on target cells. Further support for this concept can be found in the studies that showed an absolute requirement for Mg^{2+} in the contractile action of oxytocin and AVP (Bentley, 1965; Somlyo *et al.*, 1966; Altura, 1975).

Other divalent cations including cobalt (Co^{2+}), manganese (Mn^{2+}), nickel (Ni^{2+}) and zinc (Zn^{2+}) have been shown to influence the receptor binding of AVP (Pletscher *et al.*, 1985; Lariviere & Schiffrin, 1987; Thibonnier *et al.*, 1987; Gopalakrishnan *et al.*, 1988) and of oxytocin (see review by Soloff, 1979) in a fashion similar to that of Mg^{2+}. In contrast, calcium (Ca^{2+}), surprisingly, did not affect [^{3}H]-AVP binding to platelets (Thibonnier *et al.*, 1987), membrane fractions from vascular smooth muscle (Lariviere & Schiffrin, 1987), and liver (Gopalakrishnan *et al.*, 1988). However, these studies examined the influence of Ca^{2+} with only a fixed concentration of radioligands. Furthermore, the possibility that Ca^{2+} could compete for the metal ion binding site regulated by Mg^{2+} had not been considered in these studies. We have now obtained evidence that Ca^{2+} does regulate the hepatic V_1 receptor by interacting with the metal ion binding site accessible to Mg^{2+}. Such an effect of Ca^{2+} in the regulation of AVP receptors has not yet been documented.

Methods

Plasma membrane isolation

Twelve week old male Sprague-Dawley rats (200–250 g) fed *ad libitum* were decapitated with a guillotine. The livers were rapidly removed, cleaned of adhering connective tissues and blood, weighed quickly and placed in an ice-cold homogenizing buffer (0.3 M sucrose, 20 mM glycylglycine, 0.5 mM $CaCl_2$, pH 7.5). The livers were minced finely with scissors and homogenized in the buffer (10% w/v) by 30 passes with a tight fitting homogenizer. The homogenate was then centrifuged at 1500 *g* for 10 min and the resulting pellet was suspended in the isolation medium. The nuclear and the plasma

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membrane fractions were separated by the sucrose density gradient technique (Sulakhe & Lautt, 1987). The presence of Ca^{2+} in the buffer ensured effective separation of nuclear and plasma membrane fractions. The final plasma membrane layer collecting on top of the gradient was carefully aspirated, diluted with a buffer containing 20 mM glycylglycine, 2 mM disodium EDTA (pH 7.5) and centrifuged at 40,000 *g* for 30 min. The plasma membrane pellet obtained was suspended in 20 mM glycylglycine, pH 7.5, homogenized by hand and centrifuged at 40,000 *g* for 30 min. This buffer was essentially free of divalent cations, achieved by passing the buffer through a column of Chelex-100 (50–100 mesh, sodium form) cation exchange resin. The membrane was resuspended in an appropriate volume of the same buffer to yield the desired concentration of membrane protein (1.0 to 1.6 mg ml^{-1}).

Binding assay

The methodology is described in detail elsewhere (Gopalakrishnan *et al.*, 1988). Briefly, the assay was conducted in glycylglycine (20 mM) buffer (pH 7.5) with 0.2% bovine serum albumin in a final volume of 0.5 ml. It was initiated by the addition of plasma membrane protein (50–80 μg) and conducted for 90 min at 23°C, which ensured equilibrium binding. The assay contents were filtered on GF/C filter strips by rapid filtration under vacuum, followed by three washes of 3 ml of ice-cold 0.9% saline using a 24-hole cell harvester system (Brandel, Gaithersburg, Maryland U.S.A.). The filters were dried and counted by liquid scintillation spectrometry. Non specific binding was determined by the addition of 2 μM of unlabelled AVP or deamino$(CH_2)_5$ Tyr (Me) AVP (a V_1 receptor selective antagonist). Three types of assays were performed. First, the concentration-related interactions of Ca^{2+} and Mg^{2+} were assessed by use of a fixed concentration of [^{3}H]-AVP (100 pM) or [^{3}H]-V_1 antagonist (50 pM). Ca^{2+} and Mg^{2+} were employed as their chloride salts (certified reagent grade) covering a concentration-range of 1 μM to 100 mM. Secondly, the saturation binding assays included a concentration range of 10 pM to 10 nM for [^{3}H]-AVP and 1 pM to 2 nM for the [^{3}H]-V_1-antagonist. Finally, the competition experiments between varying concentrations of either unlabelled AVP or V_1 antagonist and a fixed concentration of their radiolabelled counterpart were performed in the presence as well as the absence of both the divalent cations. Other details are described in the legends to the appropriate figures and tables. Protein was determined with crystalline bovine serum albumin used as the standard (Lowry *et al.*, 1951). All assays were performed in duplicate on the same day as the preparation of the plasma membrane.

Analysis of data

Equilibrium binding data were analyzed by the computer programme EBDA (McPherson, 1983). The initial estimate of the equilibrium dissociation constant (K_D), the maximal binding sites (B_{max}) and Hill slope (n_H) obtained under EBDA was subjected to further best fit analysis of either single or multiple binding sites using the LIGAND programme (Munson & Rodbard, 1980). Statistical significance of differences between means for different conditions was estimated by analysis of variance and evaluation by the Peritz F test (Harper, 1984).

Drugs and chemicals

Radiolabelled agonist [^{3}H]-AVP (specific activity 58–80 Ci $mmol^{-1}$) and the selective V_1-antagonist, deamino-$(CH_2)_5$ Tyr (Me)-[^{3}H]-AVP (specific activity 56.2 Ci $mmol^{-1}$), were obtained from Dupont (Montreal, Quebec, Canada). Unlabelled AVP and V_1 antagonist were from Bachem Laboratories (Torrance, California, U.S.A.). Glycylglycine and crystalline bovine serum albumin were from Sigma Chemical Co. Missouri, U.S.A. Chelex-100 sodium form cation exchange resin (50–100 mesh) was obtained from Biorad Laboratories, California, U.S.A. Na_2EDTA and chloride salts of divalent cations were all of analytical grade obtained from either Sigma or BDH (Dartmouth, Nova Scotia, Canada).

Results

Influence of Ca^{2+} on the binding of [^{3}H]-arginine vasopressin to hepatic plasma membrane

A concentration-dependent decrease in the specific binding of radiolabelled AVP was evident with increasing concentrations of Ca^{2+} above 10 mM (Figure 1). At concentrations less than 10 mM, there was either no change in [^{3}H]-AVP binding or a small increase which is not significant ($P > 0.05$). The concentration required for half-maximal inhibition was 40 mM and a maximal reduction to the extent of 75% of specific binding was attained at 100 mM Ca^{2+} in the assay medium.

In order to explore the interaction between Ca^{2+} and Mg^{2+}, the effect of varying amounts of Mg^{2+} was examined in the presence of various fixed concentrations of Ca^{2+} (Figure 2). In the absence of Ca^{2+}, Mg^{2+} increased [^{3}H]-AVP binding; this stimulatory effect of Mg^{2+} was concentration-dependent reaching a maximal effect (6.5 fold) at 1–3 mM. Increases in Mg^{2+} beyond this range were associated with a diminution in the stimulatory effect of this cation. Inclusion of Ca^{2+} at concentrations greater than 1 mM resulted in the diminution of the stimulatory effect of Mg^{2+} on [^{3}H]-AVP specific binding. Again, this inhibitory effect of Ca^{2+} was concentration-dependent (Figure 2). The two highest concentrations of Ca^{2+} (20 and 50 mM) inhibited binding such that lower concentrations of Mg^{2+} failed to stimulate [^{3}H]-AVP binding, suggesting that Ca^{2+} competes for the metal ion binding site accessible to Mg^{2+}. The inhibitory effects seen either with Ca^{2+} or Mg^{2+} were not due to a non-specific influence of altered ionic strength of the assay medium, since choline chloride or NaCl (up to 150 mM) failed to exert any inhibitory effect on [^{3}H]-AVP binding assayed in the presence of 1 mM Mg^{2+} (unpublished finding).

Influence of Ca^{2+} on the binding of [^{3}H]-deamino $(CH_2)_5$ Tyr (Me) AVP (V_1 antagonist)

The binding of the antagonist, in contrast to the agonist, was inhibited by both Ca^{2+} and Mg^{2+} in a concentration-dependent manner (Figure 3), but the degree of inhibition was

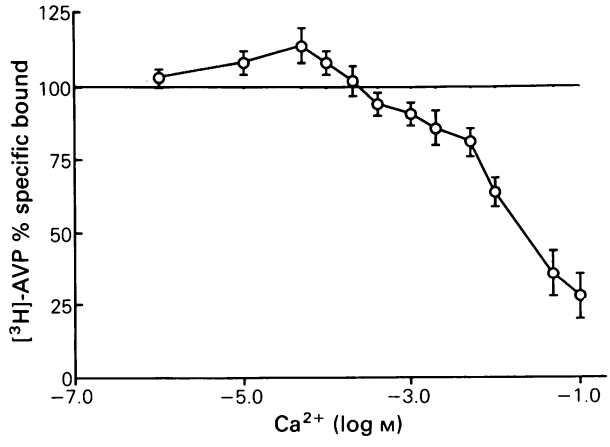

Figure 1 Relationship of the increasing concentrations of Ca^{2+} (○) and the specific binding of [^{3}H]-arginine vasopressin ([^{3}H]-AVP) to rat liver plasma membrane. [^{3}H]-AVP (100 pM) was incubated with 60–80 μg membrane protein in a final volume of 500 μl at 23°C for 90 min. Each data point is mean of four separate experiments; s.e.mean shown by vertical bars. The horizontal line denotes specific binding in the absence (control) of Ca^{2+}.

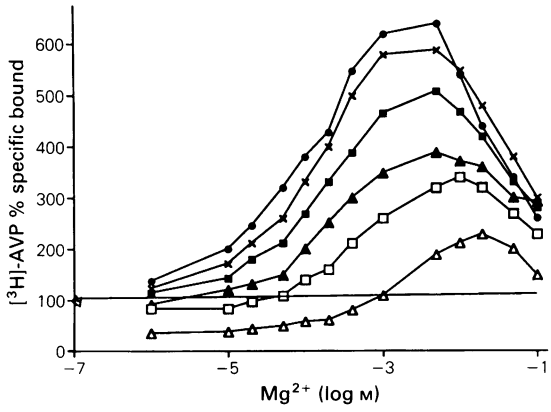

Figure 2 Relationship of the increasing concentrations of Mg^{2+} and the specific binding of [^{3}H]-arginine vasopressin ([^{3}H]-AVP, 100 pM) to rat liver plasma membrane (60–80 μg) under equilibrium conditions (23°C for 90 min) in the presence and absence of fixed concentration(s) of Ca^{2+} in the incubation medium: (●) 0 Ca^{2+} i.e. control; (×) 2 mM Ca^{2+}; (■) 5 mM Ca^{2+}; (▲) 10 mM Ca^{2+}; (□) 20 mM Ca^{2+}; (△) 50 mM Ca^{2+}. Values are means of 5 separate experiments. Calculated s.e.mean values for each data point was less than 6%. The horizontal line denotes specific binding in the absence (control) of Ca^{2+} and Mg^{2+}.

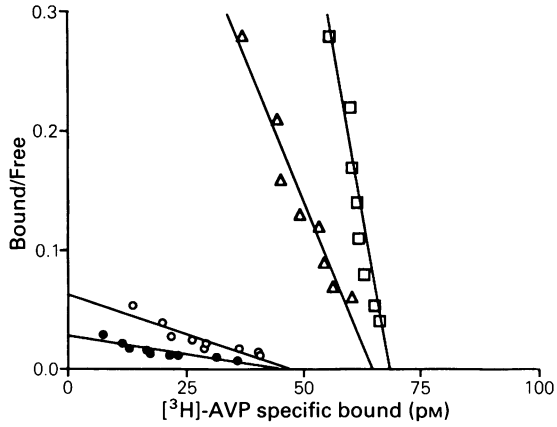

Figure 4 Scatchard plots of a representative saturation binding experiment with [^{3}H]-arginine vasopressin ([^{3}H]-AVP) performed under equilibrium conditions (23°C for 90 min) with rat liver plasma membrane (54 μg) in the absence of divalent cations (control ●) or the presence of 2 mM Ca^{2+} (○), 1 mM Mg^{2+} (□), or a combination (△) of both Mg^{2+} (1 mM) and Ca^{2+} (2 mM) in the incubation medium. Concentrations of the radioligand ranged from 10 pM to 10 nM. The following parameters were calculated: Control—K_D 1582 pM, B_{max} 392 fmol mg^{-1}; 2 mM Ca^{2+}—K_D 628 pM, B_{max} 406 fmol mg^{-1}; 1 mM Mg^{2+}—K_D 46 pM, B_{max} 612 fmol mg^{-1}; Ca^{2+} (2 mM) + Mg^{2+} (1 mM) combination $-K_D$ 350 pM, B_{max} 602 fmol mg^{-1}.

more marked with Ca^{2+} than Mg^{2+}. Indeed, lower concentrations of Mg^{2+}, actually enhanced the antagonist specific binding under these conditions. IC_{50} values (mean ± s.e.mean) for both Ca^{2+} and Mg^{2+} were 32 ± 8 and 53 ± 9 mM respectively. The maximal inhibitions attained by 100 mM Ca^{2+} and Mg^{2+} were 71% and 58% respectively.

Effect of Ca^{2+} and Mg^{2+} on saturation binding of radiolabelled agonist and antagonist

Figures 4 and 5 show the Scatchard plots of the results obtained in representative equilibrium binding experiments with the agonist and the antagonist radioligands respectively. In agreement with our previous work (Gopalakrishnan *et al.*, 1988), the present results confirm the presence of a single population of agonist and antagonist binding sites (n_H = 0.90–1.10). Previously, we had also shown that equilibrium binding was attained after 40 min and maintained thereafter for up to 120 min (Gopalakrishnan *et al.*, 1988) even when the lowest concentrations (20 pM [^{3}H]-AVP and 5 pM [^{3}H]-V_1 antagonist) were employed. In the present study 90 min incubation time was used which ensured equilibrium binding under various conditions. Both Ca^{2+} (2 mM) and Mg^{2+} (1 mM) enhanced [^{3}H]-AVP binding affinity (Figure 4) with the effect of Mg^{2+} being much greater relative to Ca^{2+}. Whereas Mg^{2+} significantly enhanced the B_{max} of the agonist, Ca^{2+} failed to increase the B_{max}. Interestingly, the combination of the same concentrations of Ca^{2+} and Mg^{2+} did not result in further enhancement in the affinity. Instead, a decrease in the affinity was noted, which was also accompanied by a decrease in the B_{max}. Either Mg^{2+} (1 mM) or Ca^{2+} (2 mM) decreased the antagonist binding affinity with marked increases in the B_{max} (Figure 5). The decrease in the affinity by Ca^{2+} was of greater magnitude, which was not influenced by inclusion of Mg^{2+}.

The representative plots shown in Figures 4 and 5 were performed using fixed concentrations of Ca^{2+} (2 mM) and Mg^{2+} (1 mM). Table 1 summarizes the results of the experiments in which assays were conducted with varying amounts of either

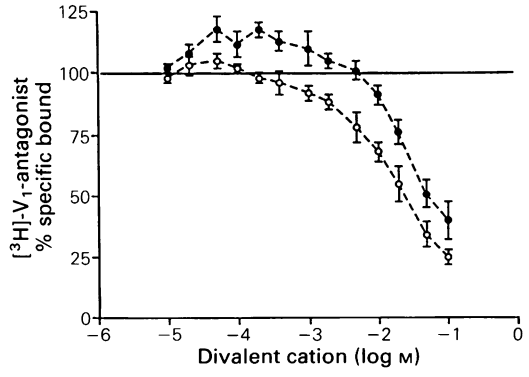

Figure 3 Relationship of the concentrations of Mg^{2+} (●) and Ca^{2+} (○) and the specific binding of [^{3}H]-deamino $(CH_2)_5$ Tyr (Me) AVP to rat liver plasma membrane. The radiolabelled antagonist (50 pM) was incubated in a final assay volume of 500 μl with 60–80 μg membrane protein at 23°C for 90 min. Values are the mean of four separate experiments; s.e.mean shown by vertical bars. The horizontal line denotes specific binding in the absence (control) of Ca^{2+} and Mg^{2+}.

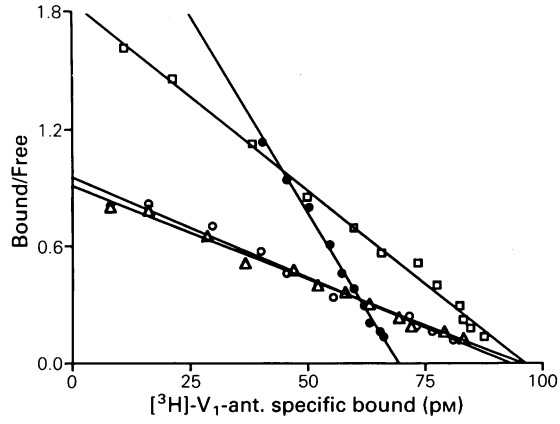

Figure 5 Scatchard plots of a representative saturation binding experiment of [^{3}H]-deamino $(CH_2)_5$ Tyr (Me) AVP performed under equilibrium conditions (23°C for 90 min) with rat liver plasma membrane (54 μg) in the absence (control, ●) or the presence of 2 mM Ca^{2+} (○), or 1 mM Mg^{2+} (□), or a combination (△) of both Mg^{2+} (1 mM) and Ca^{2+} (2 mM) in the incubation medium. Concentrations of the radioligand ranged from 1 pM to 2 nM. The following parameters were calculated: Control − K_D 22 pM, B_{max} 612 fmol mg^{-1}; 2 mM Ca^{2+} − K_D 44 pM, B_{max} 760 fmol mg^{-1}; 1 mM Mg^{2+}: $-K_D$ 54 pM, B_{max} 732 fmol mg^{-1}; Ca^{2+} (2 mM) + Mg^{2+} (1 mM) combination: $-K_D$ 93 pM, B_{max} 867 fmol mg^{-1}.

Table 1 Equilibrium binding characteristics of [^{3}H]-arginine vasopressin ([^{3}H]-AVP) and [^{3}H]-V_1-antagonist to rat liver plasma membrane fraction: analysis of Ca^{2+} and Mg^{2+} effects

Incubation conditions		*Agonist-[^{3}H]-AVP*		*[^{3}H]-V_1-subtype antagonist*	
Ca^{2+} (mM)	Mg^{2+}	K_D (pM)	B_{max} (fmol mg^{-1})	K_D (pM)	B_{max} (fmol mg^{-1})
—	—	2278 ± 428	543 ± 53	45 ± 9	671 ± 38
1	—	1288 ± 596	520 ± 41	62 ± 11	578 ± 28
2	—	565 ± 82*	474 ± 45	85 ± 19	800 ± 26
10	—	418 ± 115*	460 ± 39	126 ± 22*	1007 ± 64*
20	—	2253 ± 243	362 ± 20*	205 ± 32**	1005 ± 64*
—	1	98 ± 12**	780 ± 64*	74 ± 10	943 ± 73
2	1	155 ± 14**†	692 ± 50	64 ± 7	954 ± 38*
10	1	198 ± 21**†	634 ± 45	128 ± 17*	978 ± 68*
20	1	551 ± 244*†	554 ± 25†	190 ± 24*†	1040 ± 80*
—	10	201 ± 18**	914 ± 52**	85 ± 13	1040 ± 89*
2	10	298 ± 29**	860 ± 48**	105 ± 8*	1027 ± 76*
10	10	420 ± 25**†	805 ± 53**	132 ± 10*	1082 ± 98*
20	10	895 ± 266*†	620 ± 36†	167 ± 18*†	1030 ± 75*

Each value is the mean ± s.e.mean (n of 5 to 7 experiments)
* $P < 0.05$ compared to respective control.
** $P < 0.01$ compared to respective control.
† $P < 0.05$ compared to its respective subgroup value in the presence of Mg^{2+}.

or both divalent cations. In the absence of Mg^{2+}, Ca^{2+} (2 to 10 mM) produced significant ($P < 0.05$) decreases in the K_D for [^{3}H]-AVP and a large increase in K_D at 20 mM. The B_{max} of [^{3}H]-AVP was decreased significantly only at the highest concentration of Ca^{2+} ($P < 0.05$). As expected, and previously established (Gopalakrishnan *et al.*, 1988), Mg^{2+} (1 mM) maximally enhanced the affinity for [^{3}H]-AVP. Increasing concentrations of Ca^{2+} (with 1 mM Mg^{2+}) attenuated the Mg^{2+}-induced changes in the agonist binding characteristics. Higher concentration of Mg^{2+} (10 mM) increased the B_{max} for [^{3}H]-AVP and it modestly increased the K_D relative to that seen with 1 mM Mg^{2+}. Again, Ca^{2+} opposed the effect of 10 mM Mg^{2+} on [^{3}H]-AVP binding (both K_D and B_{max}). Antagonist binding, on the other hand, showed a quite different pattern in which both the K_D and the B_{max} values increased with increasing concentrations of Ca^{2+} and Mg^{2+}. Additivity in the actions of these cations was evident at subsaturating concentrations such that at 10 mM Ca^{2+}, there was no further effect of Mg^{2+} or *vice versa*.

Effect of Ca^{2+} and Mg^{2+} on competitive inhibition of [^{3}H]-arginine vasopressin binding

Since the shift in the K_D values by inclusion of Mg^{2+} and Ca^{2+} were more marked on the agonist binding, assays were performed with increasing concentrations of unlabelled AVP in competition with a fixed but appropriate concentration of its radiolabelled counterpart. Monophasic inhibitory curves with n_H values close to 1.0 were always obtained. Inclusion of Ca^{2+} (2 mM) resulted in a significant leftward shift in the curve when the assays were performed with a concentration (2.0 nM) of the radiolabelled agonist near its K_D value. Increasing the concentration of Ca^{2+} to 20 mM led to a diminution in this leftward shift while a concentration of 50 mM Ca^{2+} resulted in a rightward shift compared to the control curve. Thus, Ca^{2+} displayed a concentration-dependent dual effect on agonist binding. However, when these competition curves were obtained in combination with 1 mM Mg^{2+}, utilizing 100 pM [^{3}H]-AVP, Ca^{2+} produced only rightward shifts in the inhibition curves with increasing concentrations of Ca^{2+}. Table 2 summarizes the mean K_i values for AVP calculated for several inhibition curves under various incubation conditions with these divalent cations. These values are reasonably in good agreement with the K_D values determined by the saturation analysis (Table 1).

Table 2 Effect of Ca^{2+} and Mg^{2+} on the competitive inhibition of [^{3}H]-arginine vasopressin ([^{3}H]-AVP) binding to rat liver plasma membrane fraction by unlabelled AVP

Divalent cations (mM)		*K_i values* (pM) 0 Mg^{2+}	1 mM Mg^{2+}
Ca^{2+}	0	3200 ± 480	120 ± 24
	2	720 ± 80**	198 ± 18*
	10	560 ± 55**	320 ± 27**
	20	1840 ± 185*	440 ± 21**
	50	5020 ± 860	980 ± 58**

Rat liver plasma membrane fractions (50–60 μg) were incubated with 100 pM of [^{3}H]-AVP in the presence of (1 mM) Mg^{2+} and 2000 pM [^{3}H]-AVP in the absence of Mg^{2+} for 90 min at 23°C with increasing concentrations of unlabelled AVP. Each value shown is a mean ± s.e.mean of four separate determinations. K_i values were determined by the LIGAND programme.
* $P < 0.05$ compared to control.
** $P < 0.01$ compared to control.

Discussion

The regulation of AVP and oxytoxin actions or binding by divalent cations, Ca^{2+} and Mg^{2+}, has remained a topic of intense study for several years (Bentley, 1965; Somlyo *et al.*, 1966; Altura, 1975; Pearlmutter & Soloff, 1979; Soloff, 1979; Pletscher *et al.*, 1985; Gopalakrishnan *et al.*, 1986; 1988). The general consensus is that Ca^{2+} does not affect the interaction of these peptide hormones to their receptors, whereas it is necessary in the steps distal to the receptor activation. In addition to being the final mediator in the signal transduction pathway (Michell *et al.*, 1979; Exton, 1988), it is also involved in the activation of phospholipase C for the hydrolysis of phosphoinositides (Exton, 1988). Mg^{2+}, on the other hand, is a critical divalent cation necessary for promoting the initial step of binding of these neuropeptides to their receptors (Altura, 1975; Soloff, 1979; Pletscher *et al.*, 1985; Gopalakrishnan *et al.*, 1988; Antoni & Chadio, 1989). With a single fixed concentration of [^{3}H]-AVP, it was suggested that Ca^{2+} does not influence agonist binding to hepatic (Gopalakrishnan *et al.*, 1986; 1988), vascular (Lariviere & Schiffrin, 1987) and platelet (Thibonnier *et al.*, 1987) V_1 receptors. The possibility that Ca^{2+} may have opposing actions on the B_{max} and K_D for [^{3}H]-AVP was not considered in the above studies. In contrast to the agonist, in our previous study (Gopalakrishnan *et al.*, 1988), we did observe that both Ca^{2+} and Mg^{2+} inhibited [^{3}H]-V_1-antagonist binding, and interestingly, Ca^{2+} was more potent than Mg^{2+}. This preliminary finding prompted

us to examine the direct role of Ca^{2+} in the regulation of the hepatic V_1 receptor by including it in saturation binding assays.

In the presence of Ca^{2+} (<10 mM), the increase in the affinity for [^{3}H]-AVP was considerably lower than in the presence of Mg^{2+} (Table 1). This is consistent with the view that Ca^{2+} in low concentrations has the ability to induce the high affinity agonist binding state of the hepatic V_1 receptor. The smaller increase in the agonist binding affinity induced by Ca^{2+} as compared to Mg^{2+}, reflects the differences in the abilities of these two cations to promote the association between G protein(s) and V_1 receptor. In a previous study (Gopalakrishnan *et al.*, 1988), we observed a decrease in the affinity of [^{3}H]-AVP towards the hepatic receptor by GTPγS which supported the view that the hepatic V_1 receptor is coupled to a G protein and that the guanine nucleotide decreased the agonist binding by stimulating the dissociation of the receptor/G protein complex. Additionally, the increase in [^{3}H]-AVP binding affinity induced by Mg^{2+}, described in this and previous studies (Gopalakrishnan *et al.*, 1988), can also be accounted in part by stimulatory action of Mg^{2+} for the association between the receptor and the G protein. The presence of Mg^{2+} interaction sites on the G proteins has been recognized recently (Higashijima *et al.*, 1987). Whether such sites on G proteins can bind Ca^{2+}, however, is not clear. It may be that Ca^{2+} binding is unable to promote (in quantitative terms) the association between the receptor and the G protein to the same extent as Mg^{2+}, thus, partly accounting for the smaller changes in the K_D for [^{3}H]-AVP.

The observed antagonism of Mg^{2+}-induced alterations in the K_D for [^{3}H]-AVP by inclusion of Ca^{2+} indicates the competition between the two cations for binding to the sites, of which some are probably present on the G proteins. Pearlmutter & Soloff (1979) have shown that Ca^{2+} (0.2 mM to 0.6 mM) opposed Mg^{2+} (5 mM)-induced high affinity state of the oxytocin receptor for the agonist binding, and that a concentration of Ca^{2+} > 1 mM decreased the capacity. The present study confirms that a similar effect of Ca^{2+} is observed with the hepatic V_1 receptor. The degrees of antagonism may be dependent on the affinities of such sites towards these two cations and the concentrations employed in the experiments. Similar types of antagonism have been previously recognized in other systems including brain adenylate cyclase (Bradham, 1972; Sulakhe, 1985) and actomyosin ATPase (Potter & Gergely, 1975). Similarly, Mg^{2+} antagonism of the Ca^{2+}-mediated contractile response of smooth muscle is also known (Altura *et al.*, 1987).

The observed biphasic nature of the effect of either Ca^{2+} and Mg^{2+}, described for the first time for the agonist binding to the hepatic V_1 receptor (Figures 1 and 2), was seen in the presence of a fixed (100 pM) concentration of [^{3}H]-AVP held close to its K_D value seen with 1 mM Mg^{2+}. A similar type of observation was made by Hulme *et al.*, (1983) for muscarinic receptors. However, there are interesting differences in the modulatory effects of Mg^{2+} and Ca^{2+} on high affinity agonist binding to the hepatic V_1 and muscarinic receptors. For example, Mg^{2+} (1 mM) increased the affinity for [^{3}H]-AVP binding to the hepatic receptor (Table 1) while Mg^{2+} failed to influence the affinity for [^{3}H]-oxotremorine-M binding of the rat myocardial muscarinic receptors (see Table 5 in Hulme *et al.*, 1983). Further, the Mg^{2+}-induced increase in the B_{max} of the hepatic [^{3}H]-AVP binding is of much greater magnitude (about 200%) than is the [^{3}H]-oxotremorine-M binding to myocardial membranes (about 25%). However, in the EDTA-treated medulla-pons preparations, Mg^{2+} was shown to increase significantly the B_{max} for [^{3}H]-oxotremorine-M binding along with a modest increase in the affinity. Hulme *et al.* (1983) did not examine whether the inhibitory effect of Ca^{2+} or Mg^{2+} on muscarinic receptor agonist binding was associated with alterations in the affinity or B_{max}. We have observed in this work that 20 mM Ca^{2+} decreased the B_{max} without any discernible action on [^{3}H]-AVP binding affinity (Table 1). This and the fact that higher concentrations of Mg^{2+} also inhibited [^{3}H]-AVP binding are suggestive of the presence of additional metal ion interaction sites, which are of low affinity and are probably present on structures distinct from the G proteins whose identity remains unclear. While such inhibition was seen at rather high concentrations of either Ca^{2+} and Mg^{2+}, which are clearly not within the physiological range for either cation, the observed inhibition was not the result of altered ionic strength of incubation media since addition of up to 100 mM NaCl or choline chloride failed to mimic this effect.

Both divalent cations also modulated the binding of the V_1 antagonist. Such modulation (Table 1) involved influences on the affinity (decrease) as well as on the B_{max} (increase). The results further indicate that 10 mM or higher concentrations of Ca^{2+} were necessary to produce such modulation (decreased affinity along with increased B_{max} for the binding of the V_1 antagonist). Further, there was no additivity in the influences of Mg^{2+} and Ca^{2+} on the affinity or the B_{max}, when either cation was present in concentrations above 5 mM. This is consistent with the idea that both cations bind to common sites and the binding of either cation leads to essentially similar alterations in the antagonist binding properties of the hepatic V_1 receptor. The location of divalent cation binding site(s), however, cannot be stated with certainty. Previously, Soloff (1979) has proposed the possibility of two distinct metal ion binding sites or a single site with two different regions to regulate the capacity and affinity characteristics of the ligands interacting at oxytocin receptors. The alterations in the K_D and B_{max} of the hepatic V_1 receptor by divalent cations supports this possibility. It is also likely that the binding sites may be located on G proteins as mentioned earlier and divalent cation(s) binding to such sites produce opposite effect on the antagonist binding affinity compared to that seen with agonist binding affinity. The modulatory role of G proteins on the antagonist binding affinity (e.g. increased affinity in the presence of guanine nucleotides) has been documented in the case of other G protein coupled receptors, notably muscarinic receptors from the heart (e.g. see Hulme *et al.*, 1983). We have also seen a modest increase in the affinity for the V_1 antagonist binding to the hepatic plasma membranes in the presence of GTPγS (unpublished observation). This supports the view that antagonist-occupied V_1 receptors are also capable of interaction with G proteins just as are agonist-occupied receptors, a view also supported by findings with cardiac muscarinic receptors (Burgisser *et al.*, 1982; Martin *et al.*, 1984). Thus, it seems that the observed decrease by divalent cations of the V_1 antagonist affinity is in part via modulation of the receptor/G protein interaction. It follows then that G protein coupled V_1 receptor state (in the presence of divalent cations) shows high affinity – high capacity agonist binding/low affinity – high capacity antagonist binding while the uncoupled ('free') receptors detected in the absence of divalent cations show low affinity – low capacity agonist binding/high affinity – low capacity antagonist binding and that guanine nucleotides and divalent cations differentially influence the proportions of coupled versus uncoupled V_1 receptors of the membrane fraction. However, further studies with solubilized receptor (either in a coupled or uncoupled state to the G protein) are required to resolve these issues.

It is evident from the present study that Ca^{2+} can exert a modulatory effect on the agonist binding to the hepatic V_1 receptor and that such modulation is seen in the absence and presence of Mg^{2+}. At the same time the clarification of the location of divalent cation binding sites, whether these are present on the G proteins, V_1 receptors or both, as well as the precise physiological significance of the observed divalent cation modulation of the interactions between the hepatic V_1 receptor and agonist/antagonist requires additional work. The high affinity conformation for the agonist exists only over an optimal concentration for either divalent cation. The results with regard to Mg^{2+} and Ca^{2+} are consistent with the concept that Mg^{2+} (1 mM) is a full activator of the agonist binding whereas Ca^{2+} is a partial activator antagonizing the

effects of the full agonist Mg^{2+}. In our future work, we are planning to examine these issues by studying the divalent cation-dependent modulations of the binding of agonists/antagonists to isolated hepatocytes and whether such modulations influence the coupling between the V_1 receptors to a phospholipase C effector system by varying the cation concentrations in the medium over an optimal range of 0.4 mM to 2.4 mM.

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NK_1-receptors mediate the proliferative response of human fibroblasts to tachykinins

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1 The effect of synthetic tachykinin selective receptor agonists was studied on the growth of cultured human skin fibroblasts (HF).

2 Human fibroblasts were grown in serum-free conditions in the presence of natural tachykinins (substance P and neurokinin A) and of three synthetic agonists, [β-Ala^4, Sar^9, Met$(O_2)^{11}$]-SP(4-11), [β-Ala^8]-NKA(4-10) and [MePhe^7]-NKB selective for NK_1-, NK_2- and NK_3-receptors respectively. Cell proliferation was measured by percentage increase in cell number and by [^{3}H]-thymidine uptake following 48 h exposure to agents compared to baseline condition.

3 Neurokinin A (NKA) and substance P (SP) significantly increased cell proliferation the threshold concentrations being 10^{-12} and 10^{-11} M, respectively. Addition of thiorphan to culture conditions enhanced the effect of SP but not of NKA.

4 The selective NK_1-receptor agonist produced a dose-dependent increase in cell proliferation as judged by total cell number and [^{3}H]-thymidine uptake. No significant effect was observed with NK_2- and NK_3-receptor agonists.

5 These data indicate that the effect of SP on fibroblast proliferation is mediated by interaction with a NK_1-receptor type and local metabolism can interfere with the full expression of this effect of SP on cell proliferation.

Introduction

Tachykinins (TKs) are a family of peptides which share the common C-terminal sequence, Phe-X-Gly-Leu-Met-MetNH_2. In mammals, several TKs have been isolated and characterized and for three of them, substance P (SP), neurokinin A (NKA) and neurokinin B (NKB), a transmitter role has been proposed, both in the central and peripheral nervous system. A major neuronal localization of TKs in mammals is within a specific subset (about 20% of the total) of primary sensory neurones (Hokfelt *et al.*, 1975) which are sensitive to the stimulant and toxic action of capsaicin (see Holzer, 1988 for review). These sensory neurones play a dual, sensory and 'efferent' function (Szolcsanyi, 1984; Maggi & Meli, 1986; 1988), the latter being determined by transmitter release from peripheral nerve endings at the time of sensory receptor stimulation. Strong pharmacological and physiological evidence indicates that TKs release in the periphery takes place during the well-known phenomenon of 'neurogenic inflammation' (Lembeck & Holzer, 1979; see Chahl, 1988 and Holzer, 1988 for review). Nilsson *et al.* (1985) reported that TKs exert a potent stimulant action on proliferation of human skin fibroblasts and, on this basis, a possible role of endogenous TKs on wound healing was suggested (see also Maggi *et al.*, 1987a; Kjartansson *et al.*, 1987).

Pharmacological evidence indicates that at least three receptors (termed NK_1, NK_2 and NK_3) mediate the biological effects of TKs in mammalian tissues (Regoli *et al.*, 1986; 1988; Lee *et al.*, 1986). Natural TKs have some preferential activity at one of these three receptors, e.g. SP, NKA and NKB stimulate preferentially NK_1-, NK_2- and NK_3-receptors, respectively (Regoli *et al.*, 1988). Nilsson *et al.* (1985) reported that NKA (previously known as substance K), is about 100 times more potent than SP in inducing DNA synthesis in human fibroblasts, which may suggest an involvement of NK_2-receptors. However the selectivity of natural TKs for the three receptors is quite limited, each one of them being fully effective at NK_1-, NK_2- or NK_3-sites, at least at high concentrations (Regoli *et al.*, 1988). Recently the task of characterizing TK receptors which mediate a particular response has been greatly facilitated by the development of synthetic TK analogues which possess strong or even absolute selectivity for one particular TK receptor (Dion *et al.*, 1987; Drapeau *et al.*, 1987; Regoli *et al.*, 1988; Rovero *et al.*, 1989). These synthetic analogues have been fruitfully employed to characterize specific TK receptors in various bioassays (Giuliani *et al.*, 1988; Devillier *et al.*, 1988; Maggi *et al.*, 1989a,b,c; Patacchini *et al.*, 1989). The aim of this study was to characterize the receptors mediating the proliferative response of human skin fibroblasts to TKs (Nilsson *et al.*, 1985) by using receptor-selective synthetic TK analogues.

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Methods

Maintenance of human fibroblasts

Human fibroblasts (HF) were isolated from human skin fragments of healthy donors. Cells were grown in 75 cm^2 plastic tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with glucose 1 g l^{-1}, HEPES 25 mM (pH 7.4), penicillin 100 u ml^{-1}, streptomycin 100 μg ml^{-1} and 10% Foetal Calf Serum (FCS) and kept in a humidified incubator at 37°C in 5% CO_2. Cells were subcultured once a week at a ratio of 1:2.

Proliferation test

Confluent cell monolayers were treated with trypsin and 100 μl of cell suspension (2×10^3) in DMEM with 1% FCS added were plated into 96-well flat-bottomed tissue culture plates. After 2 h of incubation to favour cell adhesion, medium was removed and replaced with assay substances dissolved in DMEM with 0.1% bovine serum albumin (BSA). Fibroblast proliferation was compared to cells growing in the presence of DMEM supplemented with 0.1% BSA alone or with 10% FCS (optimal growth conditions). After 48 h incubation, the supernatant was removed and the cells were fixed with methanol at 4°C and stained with Diff-Quik. Cell proliferation was quantified by the number of cells counted in 5 random fields

in each well with the aid of an ocular grid at 100 times magnification. Numbers represent the percentage increase in cell proliferation compared to cells growing in 0.1% BSA (100%).

Autoradiography

A modification of the method described by Sholley *et al.* (1977) was used in these experiments. Confluent cells were removed by trypsin treatment, resuspended in medium with 5% FCS and seeded onto 8-chamber Lab-Tek Chamber Slides at a density of 5000 cells per well. Four hours later medium was removed and replaced by 200 μl of medium supplemented with the substance to be assayed. After 24 h incubation, [^{3}H]-thymidine (sp. act. 42 Ci mmol^{-1}; 0.5 μCi ml^{-1} final concentration) was added to each well and 24 h later, part of the media was removed and replaced with an equal volume of acetic acid-ethanol (1:3) and further processed for fixation. Gaskets and chambers were then removed and fixed slides were dipped in NTB-2 nuclear track emulsion and were exposed at 4°C for 7 or 10 days. Development was performed with half-strength Kodak D-19 developer and with Kodak Ektaflo fixer. The autoradiographs were stained with Diff-Quik. Cell proliferation was expressed as percentage of labelled nuclei over a mean of 300 cells counted at 100 times magnification in five fields per well. Counting was performed with the aid of a 21 mm square ocular grid.

Statistical analysis

Each experimental point was done in triplicate. Results are expressed as means ± standard error (s.e.) of % increase in proliferation of baseline value (100%) and as % labelled nuclei over 300 cell counted in each well. Statistical analysis was performed by means of analysis of variance or Student's *t* test for paired or unpaired data, when applicable. Differences with $P < 0.05$ were considered to be statistically significant.

Materials

[β-Ala^8, Sar^9, Met(O$_2$)^{11}]-SP(4-11) and [MePhe^7]-NKB were kindly provided by Dr D. Regoli, Department of Physiology and Pharmacology, University of Sherbrooke, Canada. [β-Ala^8]-NKA(4-10) was synthesized by Dr P. Rovero, Chemistry Department, A. Menarini Pharmaceuticals, Florence, Italy. Cells were grown on sterile plastic (Costar Europe, Ltd., The Netherlands). Lab-Tek chamber slides were purchased from Nunc (Mascia Brunelli, Milan, Italy).

Reagents were from the indicated companies: cell culture media and reagents (Gibco, Mascia Brunelli, Milan, Italy); Diff-Quik (Merz + Dade AG, Switzerland); photographic products for autoradiography (Eastman Kodak Company, Rochester, New York, U.S.A.); [^{3}H]-thymidine (Amersham, Buckinghamshire, England); BSA fraction V (BDH Biochemical Limited Poole, England); substance P (Boehringer-Mannheim, GmbH, Germany); neurokinin A (Peninsula Lab., Merseyside, England); thiorphan (Sigma, St. Louis, MI, U.S.A.).

Results

Effects of substance P and neurokinin A

We confirm (cf. Nilsson *et al.*, 1985) that both SP and NKA induce proliferation of cultured human skin fibroblasts in both the proliferation test and the [^{3}H]-thymidine incorporation assay. In the cell counting assay (Figure 1) the lowest concentration producing a significant effect was 10^{-12} M (130 ± 7% of control) and 10^{-11} M (115 ± 4% of control) for NKA and SP, respectively. The maximal proliferative response to NKA (150 ± 11% of control) at 10^{-9} M did not differ from that of SP (154 ± 7% at 10^{-8} M). The proliferative response of human skin fibroblasts to natural TKs was confirmed in the [^{3}H]-thymidine incorporation assay (Figure 2).

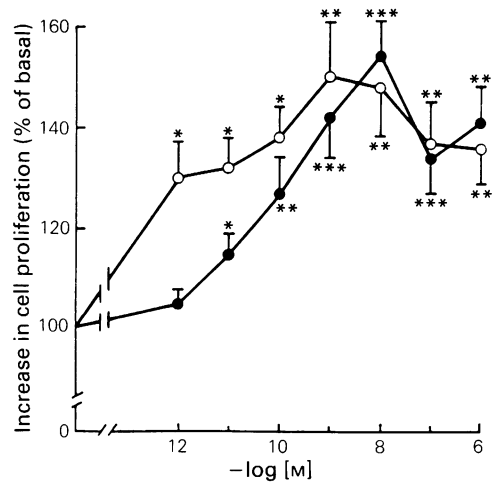

Figure 1 Effect of substance P (●) and neurokinin A (○) on the proliferation of human skin fibroblasts. Proliferation is expressed as the percentage increase in cell number compared to serum-free conditions (BSA 0.1%) and was obtained by counting stained cells in five different fields at 100 times magnification. Each point was done in triplicate. Values represent means obtained from 6 experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs BSA 0.1%.

The threshold concentration was 10^{-10} M both for SP (31.5 ± 2% labelled nuclei) and NKA (39 ± 3% labelled nuclei). Maximal activity for both SP and NKA was obtained at 10^{-8} M (44 ± 1 and 49 ± 0.6% labelled nuclei).

Effect of synthetic tachykinin analogues

The selective NK$_1$-receptor agonist [β-Ala^4, Sar^9, Met(O$_2$)^{11}]-SP(4-11) induced a marked proliferative response of human skin fibroblast in both cell counting and [^{3}H]-thymidine incorporation assays (Figure 3 and 4). A complete concentration-response curve to this agonist was not obtained because the lowest concentration tested (10^{-12} M) already produced a near maximal response (140 ± 9% of control), the maximal effect being at 10^{-11} M (150 ± 7%) (Figure 3). Higher concentrations still produced a significant proliferative response of progressively decreasing intensity. Therefore, when looking at the results of the cell counting assay, the NK$_1$-selective tachykinin receptor agonist was about 100 and 1000 times more potent than NKA or SP respectively, the maximal proliferative response being observed at 10^{-11}, 10^{-9} and 10^{-8} M for [β-Ala^4, Sar^9, Met(O$_2$)^{11}]-SP(4-11), NKA and SP, respectively.

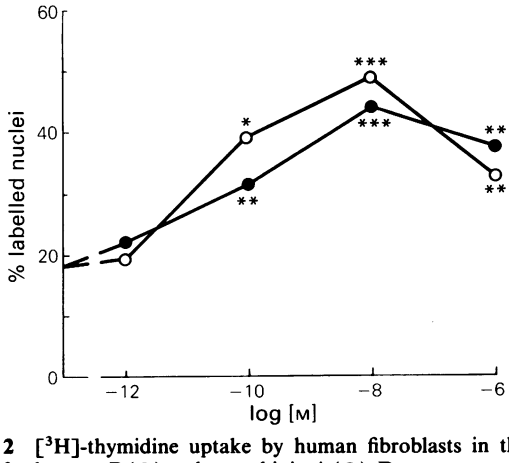

Figure 2 [^{3}H]-thymidine uptake by human fibroblasts in the presence of substance P (●) and neurokinin A (○). Data are expressed as percentage of labelled nuclei over a mean of 300 cells counted at 100 times magnification in 5 fields per well. Values represent means of 4 experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs BSA 0.1%; the coefficient of variation does not exceed 10%.

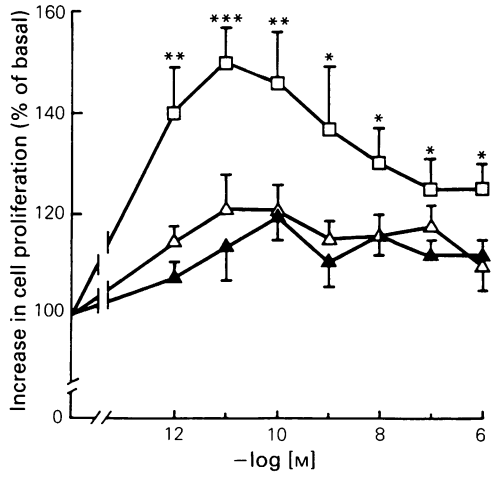

Figure 3 Effect of selective NK_1- (□), NK_2- (△) and NK_3- (▲) receptor agonists on the proliferation of human fibroblasts. Proliferation is expressed as the percentage increase in cell number compared to serum-free condition (BSA 0.1%) and was obtained by counting stained cells in five different fields at 100 times magnification. Each point was done in triplicate. Values represent means obtained from 6 experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ vs BSA 0.1%.

In the cell counting assay, the NK_2-receptor selective agonist [β-Ala^8]-NKA(4-10) and the NK_3-receptor selective agonist [MePhe^7]-NKB exerted some stimulant activity which, however was not concentration-related and did not exceed 20% increase over controls (Figure 3).

The stimulant activity of the NK_1-selective receptor agonist was confirmed in the [^{3}H]-thymidine incorporation assay (Figure 4). Even in this test, [β-Ala^4, Sar^9, Met$(O_2)^{11}$]-SP(4-11) stimulated fibroblasts DNA synthesis and proliferation, with a significant effect at 10^{-10} M ($33 \pm 1.4\%$ labelled nuclei). At 10^{-12} M neither SP nor NKA had a significant effect in this assay (Figure 2). Neither [MePhe^7]-NKB nor [β-Ala^8]-NKA(4-10) stimulated [^{3}H]-thymidine incorporation by human fibroblasts, up to 10^{-6} M (Figure 4).

Effect of thiorphan on the response to substance P and neurokinin A

Data in Figure 5 show that in the presence of thiorphan (THR) (10^{-5} M) the proliferative response to 10^{-12} M SP was markedly enhanced as compared to control. By contrast, no

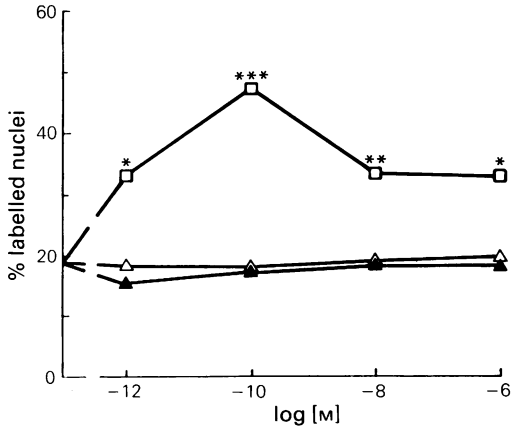

Figure 4 [^{3}H]-thymidine uptake by human fibroblasts in presence of selective NK_1- (□), NK_2- (△) and NK_3- (▲) receptor agonists. Data are expressed as percentage of labelled nuclei over a mean of 300 cells counted at 100 times magnification in 5 fields per well. Values represent means from 4 experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ vs BSA 0.1%, the coefficient of variation does not exceed 10%.

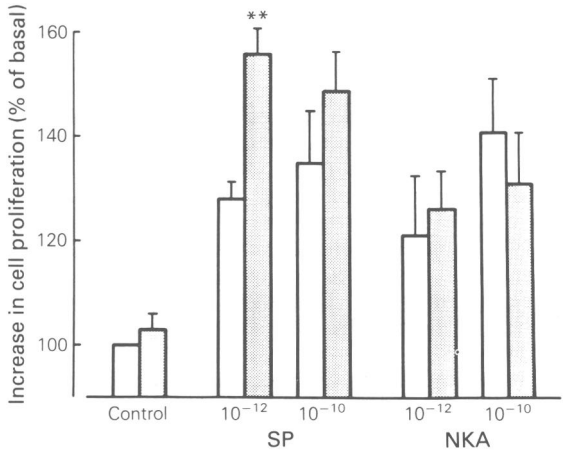

Figure 5 Effect of thiorphan (THR) on proliferative response to substance P (SP) and neurokinin A (NKA) of human fibroblasts. Open columns represent cellular proliferation in presence of 0.1% BSA, SP and NKA, stippled columns represent the effect of THR addition (10^{-5} M). Proliferation is expressed as the percentage increase in cell number compared to serum-free conditions (BSA 0.1%) and was obtained by counting stained cells in five different fields at 100 times magnification. Each point was done in triplicate. Values represent means of 5 experiments. $**P < 0.05$ vs SP alone.

further enhancement was observed for the response to 10^{-10} M SP nor to 10^{-12} or 10^{-10} M NKA (Figure 5).

Discussion

In this study we confirm (Nilsson *et al.*, 1985) that natural TKs such as SP or NKA exert a potent proliferative action on human skin fibroblasts. Both peptides have been shown to be synthesized in the body primary sensory neurones from which they are transported to peripheral endings. Accordingly, sensory nerves containing TKs have been shown to be present in mammalian skin, at which level their release is thought to exert a trophic function and to take place during neurogenic inflammation (Lembeck, 1988; Maggi & Meli, 1988; Holzer, 1988).

In this context, proliferation of skin fibroblasts is presumably relevant for the wound healing process (Nilsson *et al.*, 1985; Maggi *et al.*, 1987a; Kjartansson *et al.*, 1987). Present findings indicate that NK_1-tachykinin receptors are the main if not the only mediators of this response. In the cell counting assay, both NK_2- and NK_3-agonist displayed some activity, although markedly less potent and effective than that observed with [β-Ala^4, Sar^9, Met$(O_2)^{11}$]-SP(4-11), a potent and selective NK_1-agonist. The weak activity of the NK_2- and NK_3-receptor agonists was not observed in the [^{3}H]-thymidine incorporation assay, in which the potent stimulant action on fibroblast proliferation of the NK_1-selective agonist was confirmed.

It is interesting to note that, when using natural TKs, NKA appeared slightly more potent and/or effective than SP, particularly in the cell proliferation assay. At first sight this might suggest some involvement of NK_2-sites, an hypothesis not confirmed by experiments with [β-Ala^8]-NKA(4-10). This latter peptide is a potent and selective agonist at NK_2-sites in different bioassays, such as the rat isolated vas deferens (Rovero *et al.*, 1989), guinea-pig isolated gallbladder (Maggi *et al.*, 1989a), human isolated ileal longitudinal muscle (Maggi *et al.*, 1989c) or hamster isolated trachea (Maggi *et al.*, 1989b). In these bioassays [β-Ala^8]-NKA(4-10) has been shown to be as potent as NKA or even more effective than the natural decapeptide (rat vas deferens), this latter behaviour being ascribable to metabolic resistance of the synthetic peptide toward certain tissue enzymes which otherwise degrade TKs (Patacchini *et al.*, 1989). [β-Ala^8]-NKA(4-10) was distinctly

less active than NKA, if not completely inactive ([^{3}H]-thymidine incorporation) in stimulating human skin fibroblast proliferation. Therefore, the present findings do not support the idea that NK_2-sites might be involved in this response. When considering the strong activity of the NK_1-selective agonist, one could hypothesize, on theoretical grounds, that SP should have been more potent than NKA, a pattern consistently observed in various bioassays involving NK_1-receptors (Maggi *et al.*, 1987b; Regoli *et al.*, 1988). By contrast, we found NKA slightly more active than SP and, even more important, the NK_1-selective agonist was distinctly more active than natural TKs. This suggests that full expression of the activity of natural TKs was somehow prevented by local metabolism.

Recent evidence from several groups indicates that certain peptidases such as endopeptidase 24.11 or angiotensin converting enzyme (ACE), restrict the biological activity of TKs in the mammalian periphery (Sekizawa *et al.*, 1987a,b; Devillier *et al.*, 1988; Shore *et al.*, 1988; Patacchini *et al.*, 1989; Maggi *et al.*, 1989a).

Here we found that 10^{-5} M thiorphan (THR), a well known inhibitor of endopeptidase 24.11, markedly enhanced the activity of SP but not of NKA. Further studies are needed to assess the identity of the enzyme system which attenuated the response to SP in our experimental conditions. Irrespective of this, experiments with thiorphan indicate that the biological activity of SP in our assay is underestimated because of peptide degradation. Thus the greater activity of NKA as compared to SP in stimulating fibroblast proliferation is only apparent and this finding is in keeping with the conclusion that NK_1-receptors are the main, if not the only mediators of the proliferative response of human skin fibroblasts to TKs.

We thank Prof. A. Giotti and Dr P. Geppetti for helpful discussion in writing this manuscript. [β-Ala^4, Sar^9, Met$(O_2)^{11}$]-SP(4-11) and [MePhe^7]-NKB were kindly provided by Dr D. Regoli, Department of Physiology and Pharmacology, University of Sherbrooke, Canada. [β-Ala^8]-NKA(4-10) was synthesized by Dr P. Rovero, Chemistry Department, A. Menarini Pharmaceuticals, Florence, Italy. This work was supported by grants from MPI 40% and 60%.

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Inhibitory effects of MK-886 on arachidonic acid metabolism in human phagocytes

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1 We have investigated the inhibitory activity of compound MK-886 (formerly L-663,536), an indole derivative, on 5-lipoxygenase product synthesis in various human phagocytes stimulated with either the ionophore A23187, in the presence and absence of exogenous arachidonic acid, or platelet-activating factor (PAF). The lipoxygenase products were analysed by reversed-phase h.p.l.c.

2 MK-886 inhibited the formation of 5-hydroxy-eicosatetraenoic acid (5-HETE), leukotriene B_4 (LTB_4), its Ω-oxidation products and 6-*trans*-isomers with an IC_{50} value of 10–14 nM in A23187-stimulated neutrophils. In the same system, nordihydroguaiaretic acid (NDGA), AA-861 and L-655,240 showed IC_{50} values of 250–510, 110–420 nM and 1.7–3.9 μM, respectively.

3 MK-886 inhibited 5-lipoxygenase product synthesis in A23187-stimulated blood eosinophils and monocytes, and in neutrophils primed with granulocyte-macrophage colony-stimulating factor and stimulated with PAF with IC_{50} values of 1–13 nM.

4 The inhibitory activity of MK-886 was not reversed by addition of 10 μM arachidonic acid to A23187-stimulated neutrophils.

5 Compound MK-886 had no effect on 15-lipoxygenase product synthesis in blood eosinophils and neutrophils up to a concentration of 1 μM.

6 At 100 nM compound MK-886 had no significant effects on calcium ion mobilization, superoxide anion production and actin polymerization in neutrophils.

7 In conclusion, MK-886 is a very potent and specific inhibitor of both LTB_4 and LTC_4 synthesis in various types of human phagocytes.

Introduction

Leukotrienes constitute a group of metabolites of arachidonic acid with potent biological activities. The discovery some years ago that the peptido-leukotrienes (leukotriene C_4 (LTC_4), D_4 and E_4) account for the biological activity in various preparations of slow reacting substances of anaphylaxis (SRS-A) (Samuelsson, 1983; Samuelsson *et al.*, 1987) has raised a strong interest in these compounds as potential mediators of inflammation and allergy. There is compelling evidence that leukotrienes are produced at sites of inflammation and allergic reactions (Borgeat *et al.*, 1985) and the use of synthetic leukotrienes has established that the peptido-leukotrienes are potent agents that increase vascular permeability (Bray *et al.*, 1981) and constrict respiratory smooth muscle in man (Dahlen *et al.*, 1980). Furthermore, LTB_4 is a potent chemotactic stimulus for polymorphonuclear leucocytes (PMNLs) and other phagocytes (Ford-Hutchinson *et al.*, 1980; Naccache *et al.*, 1989). Leukotrienes are formed from arachidonic acid through the action of the enzyme 5-lipoxygenase, and their biosynthesis has been demonstrated in various cell types involved in inflammation and immune responses, i.e. PMNLs, monocytes/macrophages and mast cells (Poubelle *et al.*, 1986). The synthesis of leukotrienes is triggered by inflammatory stimuli such as the chemotactic peptide C5a or formyl-methionyl-leucyl-phenylalanine (FMLP), platelet-activating factor (PAF), the phagocytosis of particles, or by antigen stimulation of sensitized cells (Poubelle *et al.*, 1986). Finally, there is evidence that leukotrienes are formed during the course of several inflammatory and allergic diseases in man (Ford-Hutchinson, 1987).

Over the past years major efforts have been dedicated to the development of specific inhibitors of leukotriene synthesis. We have studied the *in vitro* inhibitory properties of a novel inhibitor of leukotriene synthesis, compound MK-886, formerly named L-663,536 (Gillard *et al.*, 1989), in a variety of human cells producing leukotrienes. Compound MK-886 is (3-[3-(4-chlorobenzyl)-3-t-butyl-thio-5-isopropylindol-2-yl]2,2-dimethylpropanoic acid) developed by Merck Frosst (Montréal, Qc, Canada); it was recently shown to have potent anti-inflammatory properties *in vivo* (Gillard *et al.*, 1989). We have compared the activity of MK-886 with that of L-665,240 (3-[1-(4-chlorobenzyl)-5-fluoro-3-methyl-indol-2-yl]2,2-dimethyl-propanoic acid), a compound having weak 5-lipoxygenase product inhibitory properties and strong thromboxane A_2 antagonistic activity (Hall *et al.*, 1987) and from which compound MK-886 was derived. The inhibitory activities of MK-886 and L-665,240 were also compared to that of two antioxidant-type lipoxygenase inhibitors nordihydroguaiaretic acid (NDGA) and AA-861 (Cashman, 1985; Mita & Shida, 1986).

Methods

Cell isolation procedures

Human peripheral blood was obtained from healthy volunteers by venous puncture and collected in heparin or sodium citrate. PMNLs were isolated following red cell sedimentation by Dextran T-500, centrifugation over Ficoll-Paque (Pharmacia, Montréal) cushions and NH_4Cl lysis of the remaining red blood cells as described previously (Nadeau *et al.*, 1984). The mononuclear leucocytes were collected on top of the Ficoll-Paque cushions and the monocytes were further purified by elutriation using a type JE-6 rotor and a J-6M centrifuge (Beckman) as described previously (Poubelle *et al.*, 1987). The purity of the monocyte preparations was greater than 70% (70–85% monocytes and 15–30% lymphocytes) as determined by size distribution by flow cytometry. Eosinophil-rich PMNL populations were prepared from peripheral blood of hypereosinophilic patients suffering from asthma and obtained as described above for normal PMNL suspensions.

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Eosinophils accounted for 50–60% of the PMNLs in the suspensions used. The patients (2 women, 70 and 76 year old and one man, 42 year old) were using bronchodilators (theophylline and salbutamol) and corticosteroids during the course of the study.

Alveolar eosinophils were obtained by bronchoalveolar lavage performed on one asthmatic patient (68 year old man) using sterile saline as described previously (Laviolette *et al.*, 1986). The bronchoalveolar lavage fluid cells consisted of eosinophils (58%), macrophages (29%), and lymphocytes (12%). Eosinophils were purified with Ficoll-Paque cushions as described above for PMNLs. The final suspension contained ~95% eosinophils.

Cell incubations

The different cell populations were resuspended in Hank's balanced salt solution (HBSS) containing 10 mM HEPES (pH 7.4), 2 mM $CaCl_2$ and 0.5 mM $MgCl_2$. According to the cell type, incubations were performed at cell concentrations of 0.5 to 5 million cells ml^{-1} as indicated in the figure and table legends. In studies in which A23187 was used as a stimulus, the cells were preincubated in the presence of the different drugs for 10 min at 37°C before the addition of ionophore A23187 at a final concentration of 2 μM. After a further 5 min at 37°C, the reaction was stopped with 0.5 volume of an ice-cold acetonitrile:methanol (1:1) mixture.

In studies with platelet-activating factor (PAF) as stimulus, PMNLs were preincubated for 50 min at room temperature with 200 pM granulocyte-macrophage colony-stimulating factor (GM-CSF), and then for an additional 10 min at 37°C with the different drugs (and GM-CSF). PAF was then added at a final concentration of 100 nM for a further 15 min incubation period and the reaction stopped as described above.

The effect of MK-886 on PMNL 15-lipoxygenase was determined by measuring the production of 15-hydroxy-eicosatetraenoic acid (15-HETE) following a 10 min incubation of the cells with 50 μM arachidonic acid.

Reversed-phase h.p.l.c. analysis of arachidonic acid metabolites

Metabolites of arachidonic acid were analysed directly from the aqueous-organic phase of the denatured incubation media after removal of cellular debris by centrifugation (Borgeat & Picard, 1988). The levels of metabolites (LTB_4, LTC_4, 5-HETE, 15-HETE, 6-*trans*-isomers of LTB_4 and 20-hydroxy-LTB_4 (20-OH-LTB_4), 20-carboxy-LTB_4 (20-COOH-LTB_4)) were determined by reversed-phase h.p.l.c. by use of an octadecylsilyl silica cartridge (Resolve C18, 5 μm particles, 5 × 100 mm, Waters Ass.) and gradients of methanol and acetonitrile. The metabolites were detected by monitoring u.v. absorption with two Beckman model 160 u.v. detectors (280 and 229 nm) in series. The levels of specific metabolites were calculated from calibrated standards (LTB_4, LTC_4, 20-OH-LTB_4) after correction for recoveries with the internal standards (prostaglandin B_2, 19-OH-prostaglandin B_2). The detection limits were ~1 ng for the leukotrienes and ~5 ng for the hydroxy-eicosatetraenoic acids.

Cellular toxicity

The toxicity of the different drugs were determined from the cellular release of lactate dehydrogenase (LDH). The cells were preincubated with the different drugs for 10 min at 37°C and stimulated as described above. LDH activity was measured in the supernatant by the Sigma LDH kit (Sigma Chemicals, St. Louis, MO) following centrifugation of the cell suspension. Maximal release of LDH was obtained by disrupting cells with 0.1% Triton X-100.

Measurement of intracellular calcium

Intracellular free calcium was monitored by the fluorescent probe fura-2 as described by Grynkievicz *et al.* (1985) and Faucher & Naccache (1987). Briefly, neutrophil suspensions (1×10^7 cells ml^{-1}) were incubated with 1 mM fura-2/acetoxymethyl ester for 30 min at 37°C. The cells were then washed free of the extracellular probe, resuspended at 5×10^6 cells ml^{-1} and allowed to re-equilibrate for 10 min at 37°C. They were then transferred to the thermostatted (37°C) cuvette compartment of the fluorimeter and the fluorescence monitored (excitation and emission wavelengths, 340 and 510 nm respectively). The internal calcium concentrations were calculated as described in Tsien *et al.* (1982).

Measurement of superoxide production

Superoxide production was monitored as the superoxide dismutase-sensitive reduction of cytochrome C, by a slight modification of the method described by Metcalf *et al.* (1986). Briefly, neutrophil suspensions (1×10^6 cells ml^{-1}) were incubated under the desired experimental conditions in the presence of 130 μM cytochrome C for 5 min at 37°C. The reactions were stopped by transferring the tubes to an ice-cold bath and adding superoxide dismutase (final concentration, 62.5 μg ml^{-1}), followed by centrifugation. The optical density of the supernatants was read at 550 nm and the amount of superoxide produced was calculated using an extinction coefficient of 21000.

Measurement of actin polymerization

Actin polymerization was estimated by a modification of the 7-nitrobenz-2-oxa-1,3-diazolyl-(NBD) phallacidin staining method originally developed by Howard & Oresajo (1985). Briefly, 10^6 cells were resuspended in 0.8 ml HBSS and stimulated with the desired agonists. The cells were fixed by addition of formalin (3.7% final concentration) followed by a 15 min incubation at room temperature. The cells were then centrifuged (12,000 *g*, 15 s) and the pellet resuspended in 175 μl of HBSS and 25 μl of a solution containing NBD phallacidin (final concentration 8.25×10^{-7} M) and lysophosphatidylcholine (final concentration 40 μg ml^{-1}) was added. The cells were allowed to stand for 10 min at room temperature before being washed free of unbound NBD phallacidin and resuspended in 0.9 ml HBSS. The fluorescence of the stained cell suspension was then measured (excitation and emission wavelengths 465 and 535 nm, respectively). The data are expressed as ratios of the fluorescence intensities of the control and treated (or stimulated) cell suspensions.

Drugs and chemicals

Ionophore A23187, FMLP, PAF, superoxide dismutase, cytochrome C, lysophosphatidyl-choline, phorbol 12-myristate 13-acetate and NDGA were obtained from Sigma Chemicals (St. Louis, MO). L-655,240 and MK-886 (L-663,536) (see Figure 1) were provided by Dr R. Young from Merck Frosst (Montréal, Qc, Canada). AA-861 (2-(12-hydroxydodeca-5,10-diynyl)-3,5,6-tri-methyl-1,4-benzoquinone) was provided by Dr Y. Oka from Takeda Chemical Industry (Osaka, Japan). Fura-2/acetoxymethyl ester and NBD-phallacidin were purchased from Molecular Probes (Eugene, OR, U.S.A.). Recombinant human GM-CSF was a generous gift of the Genetic Institute and Sandoz Laboratories. (Cambridge, MA, U.S.A.). H.p.l.c. solvents (HPLC grade) were obtained from Anachemia (Montréal, Qc, Canada). Stock solution of drugs, PAF and A23187 were kept at −20°C in dimethyl sulphoxide at a concentration of 10^{-2} M. Arachidonic acid (Nu Check Prep. Inc., Elysian, MN, U.S.A.) was purified by silicic acid column chromatography before use and kept at −20°C in hexane; before use, hexane was evaporated and arachidonic acid was dis-

solved in dimethyl sulphoxide. Small aliquots of 200 nM GM-CSF solutions were kept at −70°C in phosphate buffered saline containing 0.1 mg ml^{-1} bovine serum albumin.

Results

Inhibition of leukotriene synthesis by MK-886 in A23187-stimulated PMNLs

The capacity of MK-886 to inhibit the formation of 5-lipoxygenase products was compared to that of the structurally related drug L-655,240 (Figure 1) and to that of NDGA and AA-861, two compounds frequently used to inhibit lipoxygenase enzymes in A23187-stimulated human PMNLs.

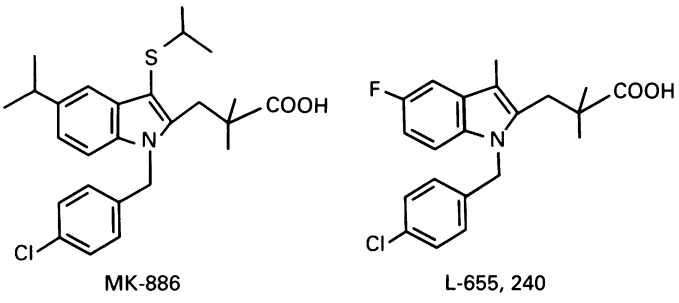

Figure 1 Structures of L-655,240 and of MK-886 (L-663,536).

Table 1 IC_{50} values (nM) of L-655,240, NDGA, AA-861 and MK-886 for inhibition of 5-lipoxygenase in A23187-stimulated human PMNLs

5-Lipoxygenase product	*L-655,240*	*NDGA*	*AA-861*	*MK-886*
20-COOH-LTB_4	3900 ± 1900	510 ± 50	420 ± 230 (n = 2)	14 ± 5
20-OH-LTB_4	3100 ± 900	510 ± 50	270 ± 120	14 ± 4
LTB_4	2500 ± 400	460 ± 110	240 ± 130	13 ± 4
6-*Trans*-LTB_4*	1700 ± 650	250 ± 70	110 ± 70	10 ± 5
5-HETE	2900 ± 1100	430 ± 130	170 ± 140	13 ± 4

Unless indicated in parentheses, IC_{50} values are means ± s.d. from 4 experimens each being carried out in triplicate.
The amounts of metabolites (ng/10^6 cells) in the controls (no drug) were: 20-COOH-LTB_4, 2.1 ± 0.9; 20-OH-LTB_4, 7.1 ± 2.3; LTB_4, 19.8 ± 4.0; 6-*trans*-LTB_4*, 11.9 ± 4.9; 5-HETE, 27.2 ± 11.1.
Cells were incubated at a density of 2 × 10^6 ml^{-1}.
* Represents the 2 isomers 6-*trans*-LTB_4 and 6-*trans*-12-epi-LTB_4.

Figure 2a shows that the four compounds produced a concentration-dependent inhibition of the synthesis of LTB_4. Figure 2b and c shows that the inhibition curves observed for 5-HETE, trans-LTB_4 and the Ω-oxidation products of LTB_4, i.e. 20-OH- and 20-COOH-LTB_4, with compound MK-886 paralleled that of LTB_4. Among the four compounds tested, MK-886 with an IC_{50} of 10–14 nM was clearly the most potent inhibitor; Table 1 summarizes their IC_{50} values. In another series of experiments we investigated the effect of exogenous arachidonic acid on the inhibitory effects of MK-886 and NDGA on leukotriene synthesis in A23187-stimulated PMNLs. Table 2 shows that the addition of 10 μM arachidonic acid did not significantly alter the IC_{50} values obtained

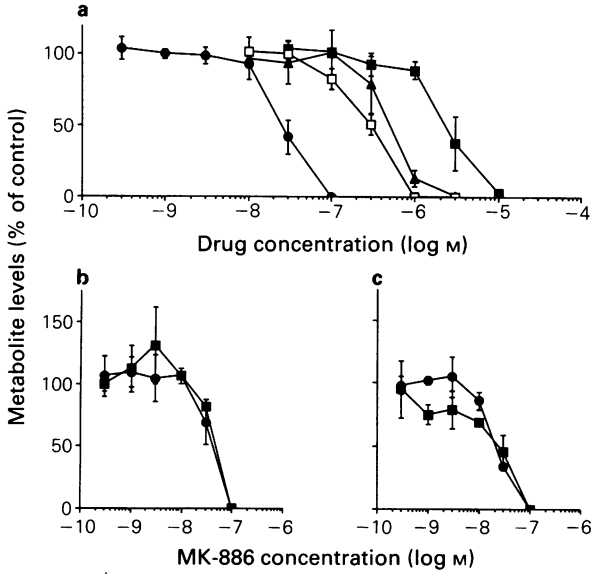

Figure 2 Inhibition of 5-lipoxygenase product synthesis in human polymorphonuclear leukocytes (PMNLs) by MK-886, nordihydroguaiaretic (NDGA), AA-861 and L-655,240. Incubations were performed at a cell density of 2 × 10^6 ml^{-1}. The data shown are mean values from a single experiment (representative of 4) carried out in triplicate, and within experiment replication error (± s.d.) is indicated by bars. (a) The effects of MK-886 (●), NDGA (▲), AA-861 (□) and L-655,240 (■) on leukotriene B_4 (LTB_4) synthesis. LTB_4 production in the controls (no drug) was 23.4 ± 1.2 ng per 10^6 cells. (b) The effects of compound MK-886 on the synthesis of 20-COOH-LTB_4 (■) and 20-OH-LTB_4 (●), while (c) shows the effects of MK-886 on 5-hydroxy-eicosatetraenoic acid (5-HETE, ■) and the 6-*trans* isomers of LTB_4 (●) (6-*trans*-LTB_4 + 6-*trans*-12-epi-LTB_4); the production of these 4 metabolites in the controls (no drug) was 1.8 ± 0.3, 3.3 ± 0.9, 26.7 ± 13.8 and 7.1 ± 0.7 ng per 10^6 cells, respectively.

Table 2 Effect of exogenous arachidonic acid (AA) on the IC_{50} values (nM) of NDGA and MK-886 for inhibition of 5-lipoxygenase in A23187-stimulated PMNLs

5-Lipoxygenase product	*NDGA*	*NDGA + AA*	*MK-886*	*MK-886 + AA*
20-COOH-LTB_4	475 (450, 500)	400 (550, 250)	11 (15, 7)	4 (5, 3)
20-OH-LTB_4	475 (450, 500)	400 (550, 250)	11 (15, 7)	5 (6.5, 3)
LTB_4	375 (450, 300)	550 (550, 550)	10 (15, 5)	4 (6, 2.5)
6-*Trans*-LTB_4*	195 (200, 190)	350 (300, 400)	5 (7, 3.8)	2 (4, 0.9)
5-HETE	350 (450, 250)	550 (550, 550)	11 (15, 6.5)	4 (2, 6.5)

The IC_{50} values are means from 2 experiments (individual values between parentheses) each being carried out in triplicate.
The amounts of metabolites (ng per 10^6 cells) formed in the controls (no drug) in the absence of exogenous AA were: 20-COOH-LTB_4, 1.4 ± 0.3; 20-OH-LTB_4, 6.5 ± 0.9; LTB_4, 17.1 ± 3.4; 6-*trans*-LTB_4*, 9.3 ± 3.0; 5-HETE, 24.8 ± 12.5.
The amounts of metabolites formed in the controls (no drug) in the presence of exogenous AA were: 20-COOH-LTB_4, 1.1 ± 0.0; 20-OH-LTB_4, 3.6 ± 1.5; LTB_4, 14.3 ± 7.6; 6-*trans*-LTB_4*, 20.3 ± 18.0; 5-HETE, 51.9 ± 45.2.
Cells were incubated at a density of 2 × 10^6 ml^{-1}.
* See Table 1 legend.

with either inhibitor for the various 5-lipoxygenase products measured. MK-886 at a concentration of 10^{-6} M had no effect on 15-HETE production in neutrophils (data not shown). The toxicity of these compounds was evaluated by the LDH-release assay and none of them (at concentrations up to 10 times their IC_{50} values) caused greater release of LDH into the medium than did A23187 alone (data not shown).

Table 3 IC_{50} values (nM) of NDGA and MK-886 on leukotriene synthesis in GM-CSF primed and PAF-stimulated human PMNLs

5-Lipoxygenase product	*NDGA*	*MK-886*
20-COOH-LTB_4	150 ± 130	8 ± 1
20-OH-LTB_4	150 ± 130	6 ± 1
LTB_4	130 ± 120	6 ± 1
6-*Trans*-LTB_4*	150 ± 120	9 ± 8

The IC_{50} values are means ± s.d. from 3 experiments being carried out in triplicate.
In the absence of inhibitor the total amount of 5-lipoxygenase metabolites formed was 5.7 ± 3.1 ng per 10^6 cells.
5-HETE formation was not detectable.
Cells were incubated at a density of 5×10^6 ml^{-1}.
* See Table 1 legend.

Table 4 IC_{50} values (nM) of NDGA and MK-886 for inhibition of 5-lipoxygenase in A23187-stimulated eosinophil-rich PMNL suspensions and monocyte suspensions

5-Lipoxygenase	*PMNLs*[a]		*Monocytes*	
product	*NDGA*	*MK-886*	*NDGA*	*MK-886*
20-COOH-LTB_4	560 ± 170	13 ± 2	ND	ND
20-OH-LTB_4	580 ± 120	10 ± 4	ND	ND
LTB_4	480 ± 90	10 ± 4	230 ± 30 (n = 3)	3 ± 1
6-*Trans*-LTB_4*	300 ± 50	7 ± 1	140 ± 30 (n = 3)	1 ± 0
5-HETE	410 ± 50	10 ± 3	150 ± 0	3 ± 1
LTC_4	480 ± 130 (n = 3)	5 ± 1 (n = 3)	220 ± 0 (n = 3)	3 ± 1

Unless indicated in parentheses, IC_{50} values are means ± s.d. from 4 experiments each being carried out in triplicate.
The amounts of metabolites formed (ng per 10^6 cells) in the controls (no drug) were: 20-COOH-LTB_4 and 20-OH-LTB_4, 1.8 ± 0.8 and 6.2 ± 2.5 (in PMNLs). The amount of metabolites formed in PMNLs and monocytes (respectively) were: LTB_4 (10.9 ± 3.8; 11.8 ± 3.4), 6-*trans*-LTB_4* (13.1 ± 1.2; 11.5 ± 1.0), LTC_4 (20.8 ± 5.4; 7.6 ± 3.8), 5-HETE (23.3 ± 9.3; 35.7 ± 8.0).
Monocytes and PMNLs were incubated at densities of 1×10^6 ml^{-1} and 2×10^6 ml^{-1} respectively.
[a] PMNL suspensions consisted of 50–60% eosinophils. ND, not detectable.
* See Table 1 legend.

Inhibition of leukotriene synthesis by MK-886 in GM-CSF-primed and PAF-stimulated PMNLs

The inhibitory activity of MK-886 on leukotriene synthesis was also tested in PMNLs primed with the haematopoietic growth factor GM-CSF and stimulated with PAF for 15 min. Under these conditions, the metabolites accumulating in the PMNL suspensions are the 20-OH- and 20-COOH-LTB_4, while LTB_4 is present in much smaller amounts and 5-HETE is usually undetectable (McColl *et al.*, 1989). Table 3 shows that under such conditions of cell stimulation by natural agonists, MK-866 had an IC_{50} value of 6–9 nM for inhibition of the various 5-lipoxygenase products detected. In the same experiments the IC_{50} values observed for NDGA were 15–25 fold higher.

Inhibition of leukotriene synthesis by MK-886 in A23187-stimulated eosinophils and monocytes

Both eosinophils and monocytes are important sources of 5-lipoxygenase products but the profile of metabolites generated by these cells is different from that of neutrophils. Eosinophils produce LTC_4 (Weller *et al.*, 1983), while monocytes produce both LTC_4 and LTB_4 and do not metabolize LTB_4 (Goldyne *et al.*, 1984).

Table 4 indicates that MK-886 inhibited synthesis of 5-lipoxygenase products induced by A23187 in blood eosinophils (IC_{50} value of 5–13 nM) as well as in monocytes (IC_{50} value of 1–3 nM), as observed for 5-HETE, LTB_4, 20-OH- and 20-COOH-LTB_4, 6-*trans*-LTB_4 isomers and LTC_4. In the same experiments NDGA showed much weaker inhibitory activity. Interestingly, MK-886 did not inhibit the formation of the 15-lipoxygenase product 15-HETE in eosinophils (data not shown). In the single suspension of purified eosinophils tested (alveolar eosinophils from an asthmatic patient), MK-886 inhibited 5-lipoxygenase product synthesis (LTC_4 and 6-*trans*- LTB_4 isomers) with an IC_{50} value of 3 nM (data not shown).

Effects of MK-886 on calcium mobilization, superoxide anion production and actin polymerization in PMNLs

MK-866 was without significant effect on the mobilization of calcium induced by either of the three chemotactic factors tested, on the stimulation of the polymerization of actin induced by FMLP and LTB_4 or on the production of superoxide anions stimulated by FMLP or phorbol 12-myristate 13-acetate (PMA) (Table 5).

Table 5 Effect of MK-886 on PMNL responses

	Calcium mobilization[1]		*Superoxide production*[2]		*Actin polymerization*[3]	
Stimulus	*Control*	*MK-886*	*Control*	*MK-886*	*Control*	*MK-886*
FMLP	399 ± 68 (n = 4)	361 ± 64 (n = 4)	7.4 ± 1.2 (n = 5)	5.9 ± 1.5 (n = 5)	1.46 ± 0.19 (n = 3)	1.81 ± 0.10 (n = 3)
LTB_4	393 ± 113 (n = 4)	357 ± 128 (n = 4)	ND	ND	1.40 ± 0.11 (n = 3)	1.67 ± 0.21 (n = 3)
PAF	627 ± 65 (n = 3)	555 ± 111 (n = 3)	ND	ND	ND	ND
PMA	ND	ND	5.2 ± 2.2 (n = 5)	5.5 ± 2.3 (n = 5)	ND	ND

[1] Increase in cytosolic free calcium (nM) induced by 10 nM of each stimulus. The cytosolic-free calcium concentration was 116 ± 9 nM in unstimulated cells both in absence and presence of the inhibitor.
[2] Amount of superoxide anions produced (nmol per 10^6 cells) 5 min after addition of each stimulus (10 nM). The basal release of superoxide was not altered by the drug.
[3] Ratio of the NBD-phallacidin fluorescence of cells stimulated in the presence or the absence of MK-886 to unstimulated cells. Cells were stimulated with formyl-methionyl-leucyl-phenylalanine (FMLP, 100 nM) for 30 s or with LTB_4 (100 nM) for 10 s.
The compound did not alter the NBD-phallacidin fluorescence in unstimulated cells.
Where indicated, cells were incubated with MK-886 (100 nM) for 1 min before stimulation.
Results are means ± s.d. from (n) separate experiments. ND, not determined. PMA = phorbol 12-myristate 13-acetate.

Discussion

Compound MK-886 (3-[3-(4-chlorobenzyl)-3-t-butyl-thio-5-isopropyl indol-2-yl]2,2-dimethylpropanoic acid) (Figure 1) is a potent inhibitor of leukotriene synthesis. In a recent study Gillard *et al.* (1989) have shown by radioimmunoassay procedures, that MK-886 inhibits LTB_4 formation in A23187-stimulated human and rat PMNLs as well as in whole blood, but is virtually inactive against platelet 12-lipoxygenase and cyclo-oxygenase. In the present study, we have characterized the inhibitory properties of MK-886 on 5-lipoxygenase product synthesis in human isolated blood neutrophils, eosinophil-rich blood PMNLs, as well as in one preparation of alveolar eosinophils and blood monocytes under various stimulating conditions. We also determined the activity of MK-886 on 15-lipoxygenase activity in neutrophils and eosinophils. The lipoxygenase products were measured by an h.p.l.c. procedure that enables the measurements of all 5- and 15-lipoxygenase products of interest and therefore enables the assessment of the effects of the drug on the various enzymes involved in their synthesis.

The results of our studies on A23187-stimulated neutrophils were in excellent agreement with those of Gillard *et al.* (1989) who obtained an IC_{50} value of 2.5 nM (for LTB_4) for MK-886. The small difference in the IC_{50} value presented herein (13 nM, Table 1) is probably explained by the difference in the number of cells used in these experiments (2×10^6 cells vs 5×10^5 cells ml^{-1}), since we and others (Gillard *et al.*, 1989) have observed that the IC_{50} values were related to the amount of cells used in the incubations. The IC_{50} values found for AA-861 and NDGA were also very close to those previously obtained for these compounds (Cashman, 1985; Salari *et al.*, 1984; Mita & Shida, 1986). Thus the results of our experiments with A23187-stimulated neutrophils emphasized the high potency of MK-886 as an inhibitor of leukotriene synthesis compared with AA-861, NDGA and L-655,240 (Figure 2a; Table 1). These data also indicate that MK-886 inhibited leukotriene synthesis at an early step in the biosynthetic pathway, i.e. at the level of the 5-lipoxygenase or above, since not only the synthesis of LTB_4 and its metabolites were inhibited but also the synthesis of 5-HETE and the 6-*trans*-LTB_4 isomers, the products of the non-enzymic hydrolysis of LTA_4 (Borgeat & Samuelsson, 1979). Furthermore, the results shown in Table 2 indicate that MK-886 (and NDGA) do not act through inhibition of release of endogenous substrate, since exogenous arachidonic acid did not restore normal levels of 5-lipoxygenase product synthesis.

Although the ionophore A23187 is well known and widely used as a potent stimulant of leukotriene synthesis, it is in fact a non-specific activator of arachidonic acid metabolism in many cell types. Thus it was of interest to investigate the effect of MK-886 on leukotriene synthesis induced by a physiological agonist acting through activation of a cell membrane receptor. PAF has previously been shown to induce leukotriene synthesis in PMNLs (Borgeat *et al.*, 1988) and furthermore, we recently observed that the stimulant effect of PAF on leukotriene synthesis was strongly enhanced after priming of the PMNLs with the haematopoietic growth factor GM-CSF (McColl *et al.*, 1989). Table 3 shows that under these conditions of cell activation compound MK-886 inhibited leukotriene synthesis with an IC_{50} comparable to that observed in A23187-stimulated PMNLs (Table 2).

Another goal of the present study was to assess the effect of MK-886 on leukotriene synthesis in human phagocytes other than neutrophils. It is well known that blood monocytes produce both LTB_4 and LTC_4 (Goldyne *et al.*, 1984), while eosinophils produce exclusively LTC_4 (Weller *et al.*, 1983; Shaw *et al.*, 1984). While monocytes are important cells in the inflammatory process, eosinophils are important for defence against parasites and are found in increased numbers in blood and bronchoalveolar lavages of allergic patients (Frigas & Gleich, 1986). The present data have shown that various types of human phagocytes which are likely to produce leukotrienes in different inflammatory or allergic conditions show similar sensitivity to inhibition of leukotriene synthesis by MK-886 (Tables 1 and 4).

Finally, other studies were performed to assess further the specificity of compound MK-886 as a leukotriene synthesis inhibitor. Gillard *et al.* (1989) have previously shown that MK-886 does not inhibit the synthesis of 12-lipoxygenase or cyclo-oxygenase products, and in the present study we found that synthesis of the 15-lipoxygenase product 15-HETE is not affected by MK-886 either in neutrophils or eosinophils (data not shown). Furthermore, the present study emphasized the selectivity of the action of MK-886. Thus, we examined its potential to interfere with several aspects of excitation-response coupling in human neutrophils initiated by chemotactic factors such as FMLP, PAF and LTB_4. The parameters tested included the mobilization of calcium (Sha'afi & Naccache, 1981), the polymerization of actin (a prerequisite of the motile functions of neutrophils, Sha'afi & Molski, 1988), and the production of superoxide anions (Rossi, 1986; Lambeth, 1988). At a concentration that completely inhibited leukotriene synthesis (100 nM), MK-886 did not significantly alter any of these responses (Table 5).

The results shown in Figure 2 and Table 2 suggest that MK-886 acts at the level of, or prior to, the 5-lipoxygenase and not at the level of arachidonic acid release as briefly discussed above. Gillard *et al.* (1989) and Rouzer & Kargman (1989) have proposed that the mechanism of action of MK-886 differs from that of other dioxygenase inhibitors such as AA-861 and NDGA which are believed to act through redox mechanisms. Indeed, these investigators have shown that compound MK-886 does not inhibit the 5-lipoxygenase in a cell-free system but only acts in intact cells, probably by preventing the translocation of the 5-lipoxygenase from the cytosol to the membrane, a process linked to the activation of the enzyme (Rouzer & Kargman, 1988; 1989).

In conclusion, MK-886 is, to our knowledge, the most potent inhibitor of leukotriene synthesis *in vitro* presently available. It inhibits the synthesis of all 5-lipoxygenase products and is active in neutrophils, eosinophils and monocytes activated with either the ionophore A23187 or with physiological stimuli. Compound MK-886 appears to be a highly specific 5-lipoxygenase inhibitor. It does not alter other biochemical events important in neutrophil functions, and has already been shown to inhibit leukotriene synthesis *in vivo* in animal models. It is thus likely that it will be a valuable tool for the assessment of the role of 5-lipoxygenase products in health and disease.

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Native and oxidized low-density lipoproteins have different inhibitory effects on endothelium-derived relaxing factor in the rabbit aorta

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1 The effect of native low-density lipoproteins (LDL) and oxidized LDL (OXLDL) on the relaxations to endothelium-derived relaxing factor (EDRF) in isolated, intact aortic rings of the rabbit were investigated.

2 Native LDL induced a concentration-dependent reversible inhibition of the relaxations elicited by acetylcholine (ACh) or A23187, in rings pre-contracted by noradrenaline (NA), adrenaline (Ad) and 5-hydroxytryptamine (5-HT), but not phenylephrine (PE), which was not influenced by indomethacin.

3 The inhibition was surmountable in the rings pre-contracted with NA and Ad and only partially in those pre-contracted with 5-HT.

4 OXLDL induced an inhibition of the relaxations elicited by ACh and A23187 which was independent of the contractile agonist. The extent of inhibition and its reversibility varied with the LDL from individual donors, but was unaffected by indomethacin.

5 Native and oxidized LDL inhibited relaxations evoked by exogenous nitric oxide (NO) to the same extent. Higher concentrations of NO overcame the inhibition. The inhibition was independent of the contractile agonist and the preparation of LDL from individual donors.

6 Only OXLDL inhibited reversibly relaxations evoked by glyceryl trinitrate (GTN) and the inhibition was independent of the LDL preparation from individual donors.

7 This study demonstrates that native and OXLDL influence the response to EDRF in isolated aorta. We suggest that these lipoproteins may contribute to the attenuation of responses to EDRF found in isolated arteries from hypercholesterolaemic and atherosclerotic animals.

Introduction

Furchgott & Zawadzki, (1980) were the first to demonstrate that endothelium-dependent relaxation in the rabbit aorta is accounted for by the release of endothelium-derived relaxing factor (EDRF), which is now known to be nitric oxide (Moncada *et al.*, 1988). The attenuation of this response in arteries from cholesterol-fed animals (Verbeuren *et al.*, 1986; Shimokawa & Vanhoutte, 1989) and from human atherosclerotic arteries (Förstermann *et al.*, 1988) has led to the suggestion that a dysfunction in EDRF release is important in atherosclerosis. We have linked this observation to low-density lipoproteins (LDL) which at high plasma concentrations are known risk factors for coronary heart disease. In support of this claim, we have shown in our laboratory that LDL inhibit, in an irreversible manner, relaxations mediated by EDRF in rabbit aorta precontracted by noradrenaline or 5-hydroxytryptamine (Andrews *et al.*, 1987). Furthermore, LDL-modified by oxidation, which are known to be present in the intima of atherosclerotic arteries and thought to be the atherogenic form of LDL (Ylä-Herttuala *et al.*, 1989), behaved in a similar way to that of native LDL (Dunn *et al.*, 1988). In preliminary publications we have found that the extent of inhibition and its reversibility depends on several factors, including the degree of oxidation and the nature of the LDL of individual donors (Jacobs *et al.*, 1989; Plane *et al.*, 1989). Here, we describe in more detail the effects of LDL on EDRF-mediated relaxations and compare them to those of LDL modified by oxidation.

Methods

Preparation of lipoproteins

Native low-density lipoproteins Fresh plasma was obtained from apparently healthy human volunteers. Native LDL (density, 1.019–1.063 g ml^{-1}) was prepared separately from the plasma of each donor by discontinuous density gradient ultracentrifugation in a Kontron vertical rotor (TFT 70.38) with a Centrikon T 2080 ultracentrifuge. To remove contaminating plasma proteins, LDL was recentrifuged for 10 h in a conventional rotor at 190,000 *g* (r = 6.75 cm) in buffer with density adjusted to 1.063 g ml^{-1} (Andrews *et al.*, 1987). Buffers contained 0.3 mM EDTA to prevent oxidation. The purified LDL was finally dialysed for 24 h against three changes of 21 of modified Tyrode buffer (composition (M): NaCl 0.13, $NaHCO_3$ 0.012, NaH_2PO_4 0.0042, KCl 0.0027) containing 0.3 mM EDTA. LDL prepared by this procedure had no significant oxidation as measured by lipid peroxidation or fluorescence spectroscopy (excitation 350 nm, emission 420 nm), a method which correlates well with the production of thiobarbituric acid reactive substances as shown previously by Dunn *et al.* (1988).

Oxidized low-density lipoproteins The oxidative modification of LDL was performed by incubation of freshly prepared LDL with 5 μM Cu^{2+} for 24 h at 18°C, followed by extensive dialysis against Tyrode buffer. Under these conditions, the LDL of individual donors was oxidized to a similar extent as determined by fluorescence spectroscopy. Cu^{2+}-oxidized LDL have been shown to have similar properties to cell-modified LDL (Steinbrecher *et al.*, 1984).

Measurement of endothelium-dependent relaxation

The descending thoracic aortae were removed from 6 month-old New Zealand White rabbits and trimmed free of fat and adhering connective tissue. Two mm wide, transverse rings were cut and paired tissues were mounted under a resting tension of 2 g, in oxygenated Krebs-bicarbonate buffer containing 0.3 mM EDTA at 37°C, for isometric force measurements (Andrews *et al.*, 1987). Tissues were equilibrated for 90 min and pre-contracted with noradrenaline (NA), adrenaline (Ad), 5-hydroxytryptamine (5-HT), phenylephrine (PE) or

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prostaglandin $F_{2\alpha}$ to give 75% of the maximal contraction obtained with NA. They were then relaxed by graded doses of acetylcholine (ACh), A23187, nitric oxide solution (NO) or glyceryl trinitrate (GTN). After washout and equilibration, the tissues were preincubated with native LDL or oxidized LDL (OXLDL, made from the same native LDL preparation) at concentrations in the range of 0.5–2 mg protein ml^{-1} or Tyrode buffer (control) for 0–60 min and then the contraction/relaxation cycle was repeated. Control tissues were used to correct for time-dependent changes in the responses. In some experiments, LDL was added to the pre-contracted rings. Finally, after removal of the lipoproteins by washing, the EDRF responses of the tissues were again assessed to determine the reversibility of the effects. After the experiment, the integrity of the endothelium was assessed by silver staining (Poole *et al.*, 1958).

Statistical analysis

Comparisons were made by use of Student's *t* test for unpaired samples where $P < 0.05$ was considered significant.

Drugs and chemicals

NA, Ad and PE were from Sigma, U.K. 5-HT and all other chemicals were from B.D.H. (U.K.).

Results

Native LDL reversibly inhibits responses to EDRF

Figure 1a shows a typical trace of the endothelium-dependent relaxation evoked by graded doses of ACh in an aortic ring pre-contracted with NA, as described by Furchgott & Zawadzki (1980). These relaxations could be repeated in the presence of Tyrode buffer and after further washout, without significant change in the sensitivity to ACh. LDL (2 mg protein ml^{-1}), caused a reduction in the relaxations evoked by ACh, compared to the initial relaxations. which were overcome at higher concentrations of ACh (Figure 1b). This decrease in sensitivity to ACh was reversed after washout. The threshold concentration for inhibition was about 0·5 mg protein ml^{-1} and rapidly increased to a maximum at 2 mg protein ml^{-1} (results not shown). Experiments were therefore routinely done at this latter concentration.

Similar inhibitory effects by LDL of endothelium-dependent relaxations elicited by ACh were found when rings were pre-contracted with Ad (not shown). In addition, pre-contraction with 5-HT (0.3 μM), showed a significant decrease in maximal relaxation in the presence of LDL (Figure 2). LDL, on the other hand, had no influence on relaxations elicited by ACh in rings pre-contracted with phenylephrine (Figure 1c) or prostaglandin $F_{2\alpha}$ (not shown), whereas if a subthreshold concentration of 5-HT (10 nM) which did not enhance contraction, was also present, an inhibition similar to that shown in Figure 2 was found. The lipoproteins themselves had no contractile effects in the absence or presence of a pre-contraction.

Prolonged pre-incubation with LDL for up to 1 h did not intensify the inhibition of relaxation, and the results were similar even if LDL was added to the pre-contracted ring immediately before the addition of ACh (not shown). The onset of the effect was therefore very rapid. Fifty μM indomethacin, 20 units ml^{-1} of superoxide dismutase or 10 μM ascorbic acid had no effect on the inhibition of relaxation. No significant differences were detected in the inhibition of relaxation by LDL obtained from the plasma of different donors. The inhibition by LDL of relaxations elicited by the Ca^{2+} ionophore, A23187 which act via a receptor-independent mechanism, was not significantly different (Figure 3a,b), showing that interference with receptor binding of the relaxant agent is not implicated in the inhibition. Furthermore, no visible loss of endothelium was found in the rings treated with LDL, as determined by silver staining.

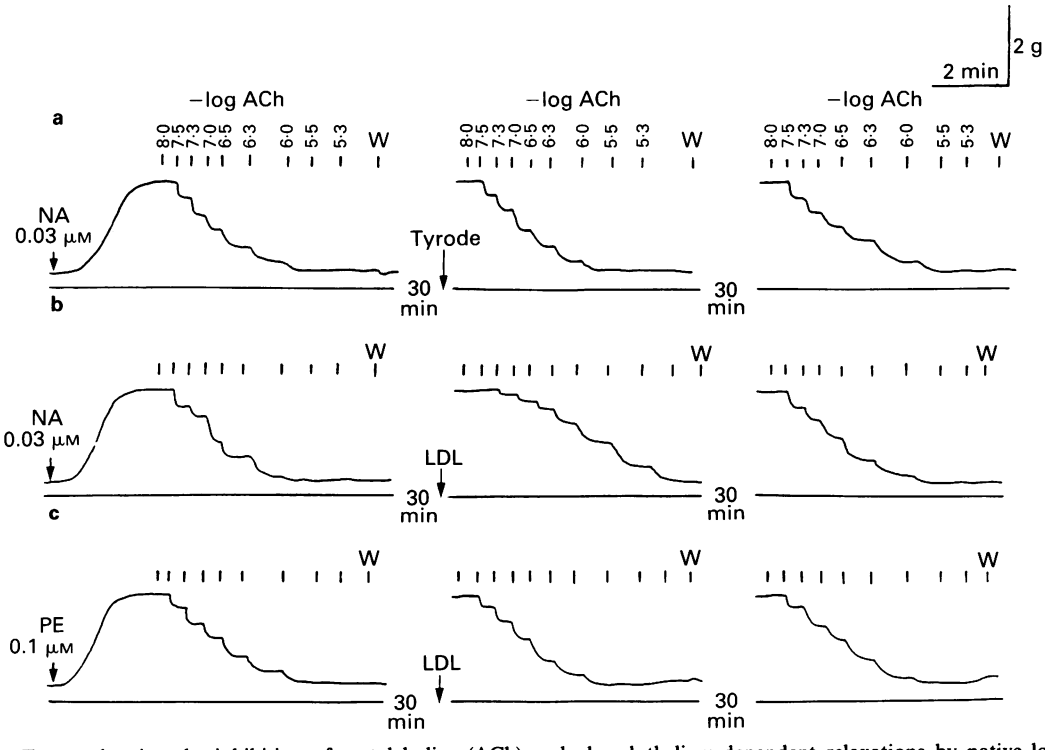

Figure 1 Traces showing the inhibition of acetylcholine (ACh)-evoked endothelium-dependent relaxations by native low-density lipoproteins (LDL) in intact aortic rings of the rabbit. Tissues were precontracted with 0.03 μM noradrenaline (NA) (a and b) or 0.1 μM phenylephrine (PE) (c), and relaxed with cumulative doses of ACh (0.01–1 μM). The cycle of contraction-relaxation was repeated in the presence of (a) Tyrode buffer (control), (b and c) LDL (2 mg protein ml^{-1}) and then after removal of the LDL by washing. Addition of ACh is shown by — and cumulative concentrations are shown in −log unit.

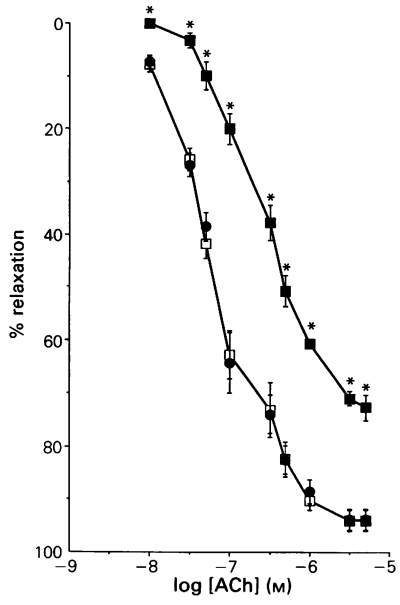

Figure 2 Dose-response curves showing the effect of native low-density lipoproteins (LDL) on endothelium-dependent relaxations evoked by acetylcholine (ACh) in rings pre-contracted with 0.3 μM 5-hydroxytryptamine (5-HT). Experiments were performed as in Figure 1. Curves were constructed from responses in tissues before and after treatment with native LDL. Relaxations are expressed as the % relaxation of the initial maximum contraction. Each point is the mean of 5 preparations of native LDL from 5 different donors. Vertical bars indicate s.e.mean. Comparisons were made by Student's t test for unpaired samples, * $P < 0.05$. (●) Control; (■) + LDL; (□) after washout.

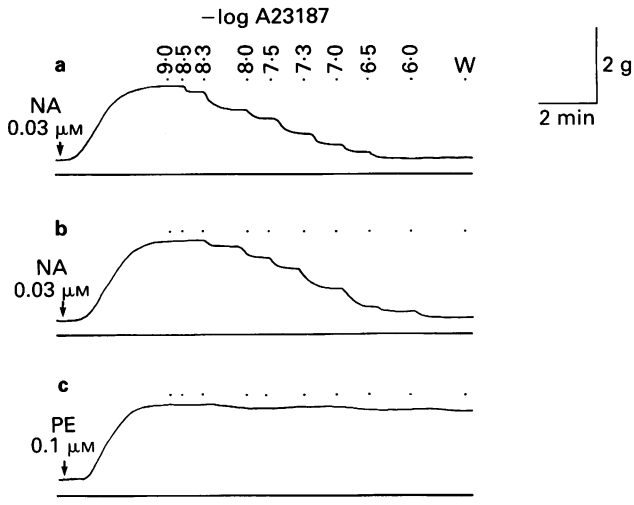

Figure 3 Traces showing the inhibition of Ca^{2+} ionophore, A23187-evoked endothelium-dependent relaxations by low-density lipoproteins (LDL) and oxidized LDL (OXLDL). Tissues: (a) control, (b) treated with LDL, (c) treated with OXLDL. The experiment was performed as in Figure 1 except that in (c) rings were pre-incubated with OXLDL (2 mg protein ml^{-1}) for 30 min before pre-contraction with noradrenaline (NA) or phenylephrine (PE).

Inhibition of relaxation by OXLDL

Distinctive differences were found between the effect of LDL and oxidized LDL on endothelium-dependent relaxation. It was discovered that to achieve maximum inhibition with OXLDL, it was necessary to pre-incubate the rings with the lipoprotein at 2 mg protein ml^{-1} for 30 min. In contrast to native LDL, inhibition occurred when PE as well as NA and 5-HT were used to pre-contract the rings. In addition, the inhibitory behaviour of OXLDL was found to fall into three major categories which were reproducible on different occasions of preparation from particular donors and were unaffected by 50 μM indomethacin.

Combined data from experiments using OXLDL prepared from 4 donors are shown in Figure 4a. As demonstrated by the dose-response curves, maximal relaxations evoked by ACh were inhibited by 80–90% in the presence of OXLDL and the inhibition persisted after washout. In contrast, OXLDL from 3 other donors, inhibited the response to ACh and partially reduced the maximal relaxation (Figure 4b). These effects were reversed after washout. Inhibition by OXLDL from 3 further donors, on the other hand, resembled in some respects that of native LDL and was reversible (cf. Figures 2 and 4c). However, a 30 min pre-incubation was necessary to produce the inhibition. Relaxations elicited by A23187 were also inhibited by OXLDL from these different donors in a similar

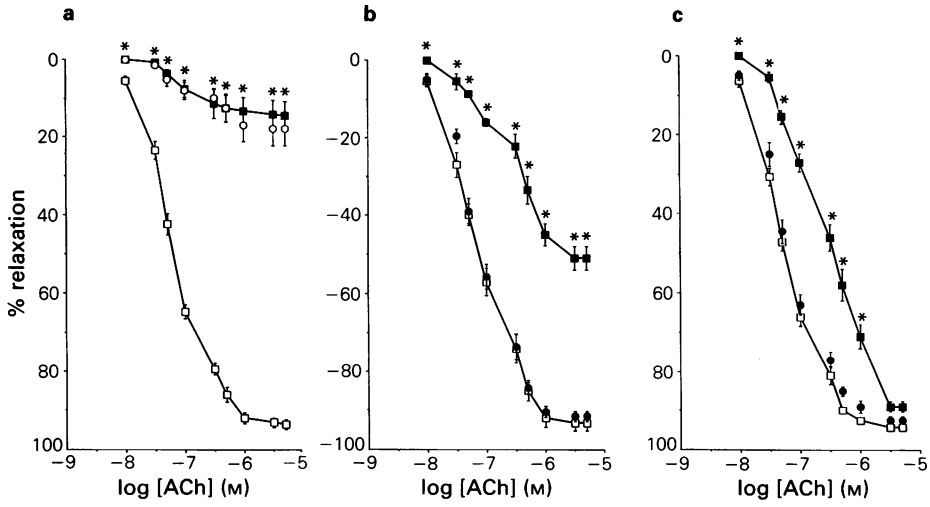

Figure 4 Dose-response curves showing the effect of oxidized low-density lipoproteins (OXLDL) on endothelium-dependent relaxations evoked by acetylcholine (ACh). Tissues were pre-contracted with 0.1 μM phenylephrine (PE). Experiments were performed as in Figure 1 except that tissues were pre-incubated for 30 min with OXLDL before contraction. Curves are constructed from the initial relaxations and those after treatment OXLDL (2 mg protein ml^{-1}). OXLDL was prepared from the plasma of donor: (a) TH, $n = 4$, TB, $n = 4$, RR, $n = 4$, GW, $n = 3$; (b) MT, $n = 4$; GD, $n = 4$; RB, $n = 3$; (c) HP $n = 3$; FP $n = 3$; MJ, $n = 4$. Relaxations are expressed as the % relaxation of the initial maximum contraction. Each point is the mean of 10–15 experiments with preparations of OXLDL; vertical bars indicate s.e.mean. Significant differences were found between donors in groups (a–c) but not within the groups. (□) Control; (■) + OXLDL; (●) after washout. * $P < 0.05$, significantly different from control.

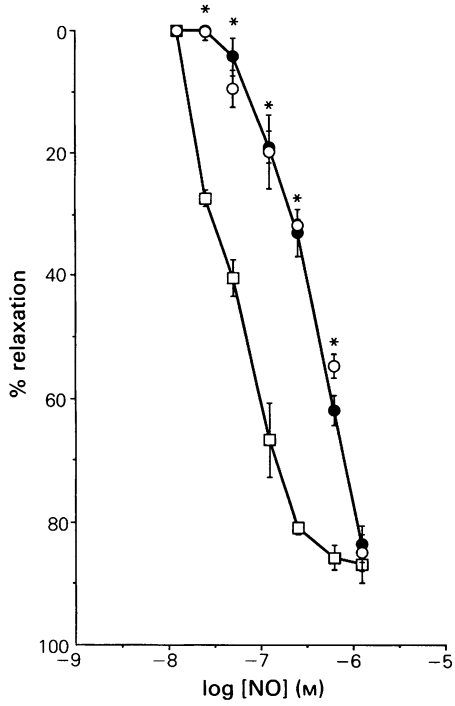

Figure 5 Dose-response curves showing the effects of lipoproteins on endothelium-independent relaxation by nitric oxide. Rings denuted of endothelium were pre-contracted with phenylephrine (PE) and relaxed by graded doses of nitric oxide (NO), the relaxation cycle was repeated in the presence of Tyrode buffer, native low-density lipoproteins (LDL) or oxidized LDL (OXLDL) (without pre-incubation) and after washout. Each point is the mean of 4 experiments with different preparations of lipoproteins from different donors; vertical bars indicate s.e.mean. (□ Control; (○)+ LDL; (●)+ OXLDL. * $P < 0.05$, significantly different from control.

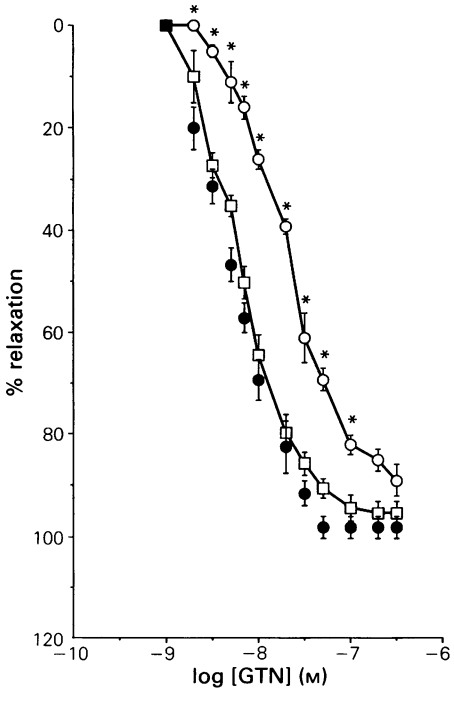

Figure 6 Dose response curves showing the effects of lipoproteins on endothelium-independent relaxation by glyceryl trinitrate. Rings pre-contracted with 0.1 μM phenylephrine (PE) and relaxed by graded doses of glyceryl trinitrate (GTN), the relaxation cycle was repeated in the presence of Tyrode buffer, native low-density lipoproteins (LDL) or oxidized LDL (OXLDL) (without pre-incubation). Each point is the mean of 4 experiments with different preparations of lipoproteins from different donors; vertical bars show s.e.mean. (□) Control; (○) + OXLDL; (●) + LDL.

fashion to those of ACh. This is illustrated by the effect of one of the most potent LDL preparations (donor: TH; Figure 3a,c). No visible loss of endothelial cells was found after treatment with any of the preparations of OXLDL.

LDL and OXLDL inhibit relaxations by nitric oxide

Nitric oxide solutions evoked dose-dependent relaxations in endothelium-denuded aortic rings which were inhibited by both native LDL and OXLDL. The inhibitions by the two forms of LDL were not significantly different as shown by the dose-response curves in Figure 5 and were reversible after washout. Furthermore, the inhibitions were independent of the agonist used to contract the tissues and the preparation of LDL from the plasma of different donors. Pre-incubation of the tissues for up to 1 h with the lipoproteins did not alter the inhibitions.

The effect of LDL and OXLDL on relaxations evoked by glyceryl trinitrate

Figure 6 shows the dose-response curves obtained from the data of 4 experiments in which rings pre-contracted with PE and relaxed by the addition of cumulative concentrations of glyceryl trinitrate (GTN) were used. No significant shift in the curve was found when relaxations were performed in the presence of native LDL. When similar experiments were performed in the presence of OXLDL, a small but significant rightward shift in the dose-response curve was found compared with the control (Figure 6). This inhibition was reversed by removal of the lipoproteins. The inhibition by OXLDL was independent of the donor, the presence or absence of endothelium and the agonist used to pre-contract the tissues.

Discussion

This study has revealed that native LDL at comparable concentrations to those found in hypercholesterolaemic plasma of human patients (2–4 mg LDL protein ml^{-1}) and OXLDL inhibit endothelium-dependent relaxation elicited by receptor activation or by a receptor-independent pathway. The inhibitory effects of native LDL and OXLDL in other respects appear to be different and operate by distinctive mechanisms.

The reversibility and almost immediate effects of native LDL make it unlikely that the inhibition involves the endocytosis of the lipoproteins (Brown & Goldstein, 1979; Baker *et al.*, 1984) or their transcytosis into the subendothelial space (Simionescu, 1989), processes which are both time-dependent. We have found that the inhibition of ACh-evoked relaxations by both native or oxidised LDL takes place if non-atherosclerotic aortic rings from 2 month-old Watanabe hereditary hyperlipidaemic rabbits are used instead of those from normal rabbits (Bruckdorfer *et al.*, 1988 and unpublished results). Since endothelial cells of this strain of rabbits lack the functional high affinity receptors implicated in the endocytosis and degradation of native LDL (Baker *et al.*, 1984), it is most unlikely that such receptors are involved in the inhibition of endothelium-dependent relaxation. Furthermore, the high affinity receptors would be fully saturated at concentrations of LDL (100 $\mu g\,ml^{-1}$) lower than the threshold concentration for inhibition (0.5 $mg\,ml^{-1}$).

Several other mechanisms which may explain the effects of native LDL are also excluded. Inhibition is not mediated by cyclo-oxygenase products or by the generation of superoxide. Ascorbate, an inhibitor of NA oxidation does not prevent the effect of LDL. Furthermore, the rapidity of the inhibition rules out oxidation of LDL, which requires long exposures to transitional metal ions or endothelial cells. A simple binding of ACh or A23187 by LDL is unlikely because of the lack of inhibition with PE as a contractile agonist. The failure of native LDL to inhibit relaxations elicited by glyceryl trinitrate, a drug which is thought to release NO intracellularly

in the vascular smooth muscle, also rules out an action on soluble guanylate cyclase. An inhibition of the relaxations elicited by EDRF and not GTN by various inhibitors has similarly been found in bioassay experiments (Moncada *et al.*, 1986). Nevertheless, LDL inhibit relaxations elicited by NO which is added directly into the organ bath. This suggests that LDL are able to react with or sequestrate NO and this might be the mechanism by which the responses mediated by endogenous NO are inhibited. This mechanism is clearly not sufficient since it does not occur in rings pre-contracted with PE alone, although there was inhibition when subthreshold concentrations of 5-HT (1 nM) which do not enhance contraction, were also present. Further investigations are required to clarify whether at this low concentration, 5-HT receptors are implicated in the inhibition.

The modification of LDL during oxidation causes significant changes in the composition and structure of LDL. The changes include lipid peroxidation, the formation of conjugates between fragments of fatty acids and apolipoprotein B and the conversion of phosphatidylcholine to lysophosphatidylcholine (Steinbrecher *et al.*, 1984). As a result, the uptake of the modified lipoproteins by cells is no longer by receptor-mediated endocytosis, but by the so-called scavenger receptors; found on macrophages (Steinbrecher *et al.*, 1984) and also present on endothelial cells (Baker *et al.*, 1984). Subtle differences in these oxidation products may result from the variable composition of polyunsaturated fatty acids and antioxidants in the initial LDL from individual donors, as shown by Esterbauer *et al.* (1989). Thus the changes that occur on oxidation may explain many of the differences between native and OXLDL, including the variation in the inhibitory properties of the OXLDL from individual donors. Different constituents in OXLDL may account for the rapid inhibition of the relaxations evoked by NO and GTN compared with the time-dependent and variable inhibition of endothelium-dependent relaxation. Furthermore, the effect of many preparations of OXLDL on relaxations mediated by endogenous NO were more potent than those on the former relaxants, suggesting that the interaction of OXLDL with NO or soluble guanylate cyclase is unlikely to be the major mechanism. In addition, transcytosis of OXLDL (Simionescu, 1988) or uptake via the scavenger receptors may be required before these modified lipoproteins can influence events within the endothelial cells or on the abluminal side of the endothelium. Recently, it was shown by Jialal & Chait (1989) that OXLDL or a lipid extract from the lipoproteins inhibit cholesterol esterification in cultured endothelial cells, independently of the receptor for native LDL or the scavenger receptor, and it was suggested that this might result from the translocation of a lipid component across the cell membrane. A similar time-dependent translocation of constituents of OXLDL into the endothelial cells or subendothelial space may be an alternative mechanism to account for the inhibition of endothelium-dependent relaxation.

Our observations on the different degrees of inhibition produced by OXLDL correlate with the various degrees of attenuation of EDRF responses obtained in isolated arterial preparations from cholesterol-fed animals (Verbeuren *et al.*, 1986; Harrison *et al.*, 1987; Shimokawa & Vanhoutte, 1989) and atherosclerotic human arteries (Forstermann *et al.*, 1988). An attenuation of responses of glyceryl trinitrate was also found in severely diseased arteries in some of these investigations. An exhaustive study in coronary arteries from hypercholesterolaemic pigs (Shimokawa & Vanhoutte, 1989), without signs of atherosclerosis, showed a moderate impairment in the responses to selective relaxants, whereas where atherosclerosis was present inhibition was more severe with all relaxants and EDRF release was inhibited (Shimokawa & Vanhoutte, 1989). In atherosclerotic arteries from monkeys (Harrison *et al.*, 1987), a surmountable inhibition of responses to A23187 were observed, not unlike that found with native LDL and some preparations of OXLDL.

In summary, we suggest that isolated non-atherosclerotic arteries of animals at the very early stages of hypercholesterolaemia which are exposed mainly to native LDL, might be expected to show only modest impairment of EDRF responses. An enhancement in platelet aggregation is predicted from the interaction of LDL with NO. OXLDL, on the other hand, represents the result of long-term hypercholesterolaemia, by which time oxidatively modified LDL will have accumulated in the intima and given rise to the continuum of inhibition of EDRF responses found in isolated atherosclerotic arteries.

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Electrophysiological and mechanical effects of calcitonin gene-related peptide on guinea-pig atria

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1 The effects of calcitonin gene-related peptide (CGRP) on mechanical and electrophysiological responses were studied in the guinea-pig atrial muscle preparations and in single cells.

2 CGRP ($>10^{-9}$ M) enhanced the twitch contraction in a concentration-dependent manner in electrically driven left atria and increased heart rate in spontaneously beating right atria. The positive inotropic and chronotropic effects of CGRP were not inhibited by propranolol but were attenuated by reduction of the calcium concentration in the bathing medium.

3 In single left atrial cells, CGRP slightly hyperpolarized the resting potential but did not affect the other action potential parameters significantly.

4 Under whole-cell voltage-clamp conditions, CGRP increased the calcium inward current. The peptide also increased the steady inward current elicited by hyperpolarization and the late outward current by depolarization.

5 These results suggest that CGRP may produce the positive inotropic and presumably chronotropic effects by increasing calcium inward current. CGRP also increases the potassium permeability. Such effects on ionic currents may not produce any apparent change in the action potential conformation, due to their opposite directional actions and relatively weak potencies.

Introduction

Calcitonin gene-related peptide (CGRP) is a 37 amino acid peptide, which has been encoded by the same gene as calcitonin and is generated in neural cells by an alternative tissue specific splicing of the mRNA (Amara *et al.*, 1982; Craig *et al.*, 1982; Rosenfeld *et al.*, 1983). CGRP-immunoreactivity is found in heart particularly around the sinoatrial and atrioventricular node, as well as in periadventitial nerves in association with blood vessels (Manson *et al.*, 1984; Wisenfeld-Hallin *et al.*, 1984; Mulderry *et al.*, 1985; Gennari & Fischer, 1985).

CGRP shows potent positive chronotropic and inotropic effects on the atrial muscles of rats and guinea-pigs (Franco-Cereceda & Lundberg, 1985; Marshall *et al.*, 1986; Saito *et al.*, 1987). Since CGRP enhances adenylate cyclase activity resulting in an elevation of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) content, it is suggested that the positive inotropic effect of CGRP may be attributable to an increase in calcium influx (Ishikawa *et al.*, 1988). However, there is no direct evidence for the effect of CGRP on ionic currents. In the present study, we examined the electrophysiological and mechanical effects of CGRP.

Methods

Male guinea-pigs weighing 250–350 g were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, i.p.), exsanguinated from the carotid arteries and the hearts were quickly isolated. Atria were dissected and mounted vertically in 20 ml organ baths containing modified Tyrode solution of the following composition (in mM); NaCl 136.9, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.0, $NaHCO_3$ 23.8, NaH_2PO_4 0.21 and glucose 5.6, gassed with a mixture of 95% O_2 and 5% CO_2 at 30°C. Square wave pulses of 1 ms duration and suprathreshold voltage were applied at a frequency of 1 Hz in left atria. In right atria, spontaneous beating was recorded. Contractile force was measured isometrically by a force displacement transducer (T7-30-240, Toyo Boldwin, Japan) and recorded on a strip chart recorder (RJG-4128, Nihon Kohden, Japan). The preparations were equilibrated at a resting tension of 1 g for 90 min before experiments started.

In some experiments, the calcium concentration in the bathing medium was reduced to a half (0.9 mM) or a quarter (0.45 mM) of the normal calcium concentration.

Single atrial myocytes from guinea-pig hearts were prepared as described in a previous paper (Ohmura *et al.*, 1990). A patch clamp amplifier (CEZ-2100, Nihon Kohden) was used to study action potentials and membrane currents (for details see Nishio *et al.*, 1988). Patch electrodes were filled with a solution of following composition (in mM): potassium aspartate 100, KCl 5, $MgCl_2$ 5, HEPES (hydroxyethylpiperazinyl-ethane-sulphonic acid) 5, EGTA (ethylene-glycol-*bis*(β-aminoethyl-ether)N,N,N′,N′-tetraacetic acid) 2.5, adenosine 5′-triphosphate (ATP) 5 and phosphocreatine 5. The pH was adjusted to 7.3 with KOH. The solution for superfusing the cells was as follows (in mM): NaCl 136.9, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, glucose 10 and HEPES 5. The pH was adjusted to 7.3 with NaOH.

Calcitonin gene-related peptide (CGRP, human) was supplied by Peptide Institute Inc. (Osaka, Japan) and dissolved in saline solution containing 2% gelatin.

Data and analysis

Experimental values are given as mean ± s.e.mean. The effects of drugs were considered significant if $P < 0.05$ by Student's t test for unpaired data.

Results

Effects of CGRP on mechanical responses in atrial preparations

CGRP produced a positive inotropic response in electrically driven left atria (1 Hz) and a positive chronotropic response in spontaneously beating right atria. Figure 1 shows the concentration-response curves to CGRP, in which CGRP was applied cumulatively and the maximum response at each concentration was plotted. The responses were concentration-dependent; the EC_{50} values being (9.7 ± 1.5) × 10^{-9} M for the

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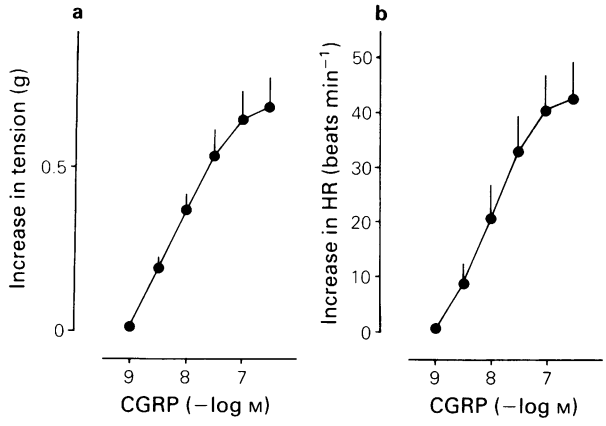

Figure 1 Effects of calcitonin gene-related peptide (CGRP) on the twitch contraction of left atria (a) and on the heart rate (HR) of right atria (b). Left atria were driven at a frequency of 1 Hz. Net increases in contractile force and heart rate were plotted against the concentration of CGRP. Each value indicates the mean with s.e.mean indicated by vertical lines (n = 5–6).

positive inotropic effect and $(1.3 \pm 0.3) \times 10^{-8}$ M for the positive chronotropic effect, respectively. The maximal inotropic response of CGRP corresponded to 81.5% of that of isoprenaline in left atria (mean of 5 experiments). The effects of CGRP were not affected by propranolol (10^{-6} M) or atropine (10^{-6} M). However, the positive inotropic and chronotropic responses were markedly attenuated in low calcium medium. Figure 2 shows the effects of 10^{-7} M CGRP at various calcium concentrations. Reduction of calcium concentration from 1.8 mM to 0.45 mM significantly attenuated the responses to CGRP.

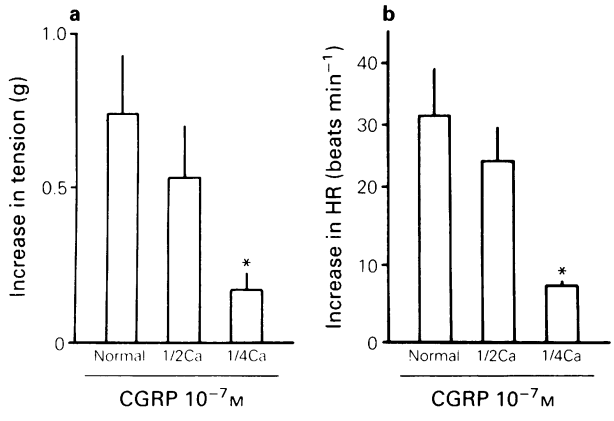

Figure 2 Influence of extracellular calcium concentration on the positive inotropic effect in left atria (a) and the positive chronotropic effect in right atria (b) of calcitonin gene-related peptide (CGRP, 10^{-7} M). Left atria were driven at a frequency of 1 Hz. Net increases in contractile force and heart rate were plotted against the concentration of CGRP. Each value indicates the mean with s.e.mean indicated by vertical lines (n = 5–6).

Effects on resting and action potentials in single atrial cells

Single atrial cells were stimulated at a frequency of 1 Hz and the membrane potentials were recorded before and after perfusion of 10^{-7} M CGRP. No apparent change in the membrane potential parameters was observed before and after 10^{-7} M CGRP, except that the resting potential was slightly hyperpolarized in all 4 cells tested (Figure 3 and Table 1).

Effects of CGRP on ionic currents in single atrial cells

Whole-cell voltage clamp experiments were carried out on single left atrial cells of guinea-pig. The membrane potential was held at the resting potential (−85 mV in Figure 4) and the depolarizing or hyperpolarizing pulses of 300 ms duration to various potentials were delivered every 5 s. Figure 4a shows representative current recordings. When the membrane was

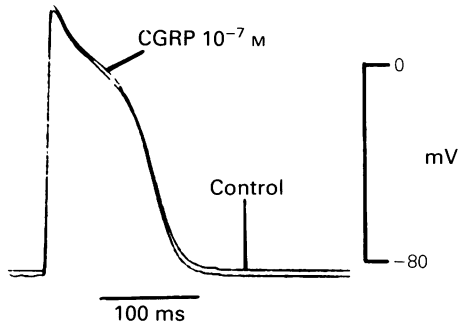

Figure 3 Effects of calcitonin gene-related peptide (CGRP) on the action potentials of a single atrial cell. The cell was driven at a frequency of 1 Hz. Action potentials before (control) and 5 min after the treatment of 10^{-7} M CGRP are superimposed.

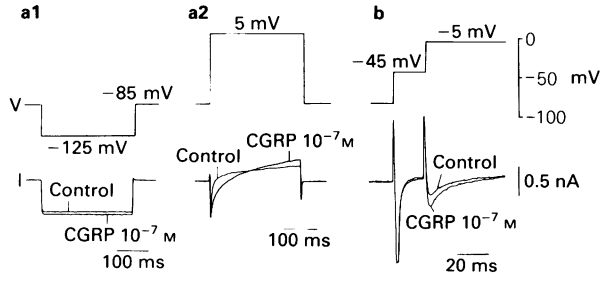

Figure 4 Effects of calcitonin gene-related peptide (CGRP 10^{-7} M) on ionic currents in a single atrial cell. Upper traces indicate the voltage commands. (a1) Effect on the inward current induced by a hyperpolarization from a resting potential (−85 mV) to −125 mV. (a2) Effects on the inward and outward currents induced by a depolarization to 5 mV. (b) Effects on inward calcium current, which was elicited by double pulse protocol (see text for further explanation).

Table 1 Effects of calcitonin gene-related peptide (CGRP) on resting and action potentials of guinea-pig atrial myocytes

	RP	*APA*	*APD*$_{30}$	*APD*$_{50}$	*APD*$_{90}$
Control	−80.3 ± 1.0	106.0 ± 1.8	63.8 ± 8.0	89.3 ± 10.5	135.0 ± 12.4
CGRP10^{-7}M	−82.3 ± 0.8 (2.0 ± 0.2*)	108.5 ± 3.5	62.5 ± 9.2	85.6 ± 10.3	127.5 ± 7.8

RP, resting potential; APA, action potential amplitude; APD_{30}, APD_{50} and APD_{90}; action potential duration at 30%, 50% and 90% repolarization. Each value is mean ± s.e.mean of 4 experiments.
* All four cells hyperpolarized after CGRP perfusion; the net value of hyperpolarization is shown.

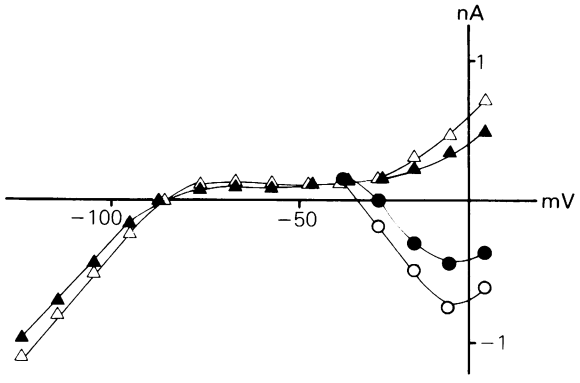

Figure 5 Current-voltage relationships for the inward calcium current (circles) and the current measured at the end of 300 ms clamp pulses (triangles); closed symbols: before 10^{-7} M calcitonin gene-related peptide (CGRP), open symbols: after 10^{-7} M CGRP. The membrane was held at −85 mV.

hyperpolarized to −125 mV (Figure 4a1), a steady state current flowed during the application of the pulse, which was significantly increased by 10^{-7} M CGRP (18 ± 9% increase in 3 cells tested). On the other hand, a depolarizing pulse to +5 mV produced a transient inward current followed by an outward current. CGRP (10^{-7} M) apparently slowed down the decaying phase of inward current and increased the outward current at the end of depolarizing pulse (Figure 4a2). Such responses were not affected by propranolol 10^{-6} M.

In order to observe the effect on calcium current clearly, we used a double pulse protocol, in which the membrane was depolarized to −45 mV for 20 ms before application of test pulses. Under such conditions, sodium channels were inactivated during the prepulse and calcium current was seen in the early phase of the test pulse. CGRP (10^{-7} M) significantly increased the calcium current (Figure 4b).

Figure 5 shows the current-voltage relationships in which the calcium currents (circles) were elicited with the use of double pulse protocol (see Figure 4b) and the late currents (triangles) were measured at the end of 300 ms single pulses (see Figure 4a). CGRP increased the inward calcium current and the steady state current induced by hyperpolarizing pulses. The outward currents elicited by depolarization to less negative potentials than −35 mV were also increased by CGRP.

Discussion

CGRP produced positive inotropic and chronotropic effects in the guinea-pig atria with a potency comparable to isoprenaline. Since the responses were markedly attenuated by reducing the calcium concentration in the bathing medium from 1.8 mM to 0.45 mM, it was likely that the positive inotropic and chronotropic effects of CGRP were dependent on the extracellular calcium concentration, probably on the calcium influx. When we examined the electrophysiological parameters in single atrial cells, no apparent change in the action potential parameters was observed, except a slight but consistent hyperpolarization of the membrane potential in each cell after CGRP application. Such lack of significant change by CGRP in action potential parameters has also been found in the multicellular preparations of guinea-pig atria (Ishikawa *et al.*, 1988).

Since the atrial action potential is constructed under the sophisticated balance of ionic currents (Beeler & Reuter, 1977; Trautwein & McDonald, 1978), we examined the effects of CGRP on the individual ionic currents by using the voltage-clamp method. Three effects were observed: (1) an increase in inward calcium current, (2) an increase in inward current which was elicited by the application of hyperpolarizing pulses from the resting potential and (3) an increase in the late outward current upon depolarization to the membrane potential over −40 mV. However, the extent of such effects was relatively small. Furthermore, the directions of the first effect and the second and third effects mentioned above were opposite in terms of their contribution to the action potential duration, resulting in an apparent lack of change in the action potential.

The effects of CGRP on cardiac ionic currents is reminiscent of the responses to β-adrenoceptor agonists (Reuter, 1974; Kass & Wiegers, 1974). Like isoprenaline, CGRP has been recently reported to increase cyclic AMP content in the guinea-pig atria (Ishikawa *et al.*, 1988). Since CGRP responses were not inhibited by propranolol, it is likely that the mechanisms subsequent to the receptor are common to the responses to CGRP and β adrenoceptor agonists.

The guinea-pig heart is innervated by various peptide-containing nerves. CGRP-immunoreactivity has been found in the sensory nerves. Since sensory nerves can act peripherally (Muramatsu, 1987), CGRP may be released from the sensory nerves and then produce positive inotropic and chronotropic effects (Franco-Cereceda & Lundberg, 1985; Saito *et al.*, 1987). In contrast, somatostatin is distributed in the parasympathetic nerves (Franco-Cereceda *et al.*, 1986). Recently, we have found that somatostatin inhibits the inward calcium current and then produces negative inotropic responses in the guinea-pig atria (Ohmura *et al.*, 1990). This evidence, together with the present results, suggests that the calcium channels of atrial cells may be reciprocally regulated by both peptides (CGRP and somatostatin), in addition to the classical adrenergic and cholinergic control.

Note added in proof

After this work was submitted, a paper by Ono, Delay, Nakajima Irisawa and Giles was published in Nature (**340**, 721–724, 1989). Their results are similar to ours in demonstrating that CGRP increases calcium current in frog atrial myocytes.

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Reactivity and sensitivity of mesenteric vascular beds and aortic rings of spontaneously hypertensive rats to endothelin: effects of calcium entry blockers

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1 The vasoconstrictor effects of endothelin-1 were studied in perfused mesenteric vascular beds (MVB) and aortic rings of 14–16 week-old spontaneously hypertensive rats (SHR) and age-matched Wistar Kyoto rats (WKY).

2 Reactivity to endothelin-1 was increased in MVBs of SHR, as indicated by the maximum perfusion pressure obtained (264 ± 8 and 141 ± 9 mmHg respectively) ($P < 0.001$), whereas sensitivity was not significantly different between the two strains (EC_{50} 171 ± 21 and 102 ± 19, respectively).

3 In aortic rings, in constrast, reactivity to endothelin-1 was reduced in SHR as compared to WKY, whereas sensitivity was similar (EC_{50} 0.78 ± 0.08 and 0.87 ± 0.09 nM).

4 As with endothelin-1, reactivity to noradrenaline and potassium chloride was increased in MVBs, but not in aortic rings of SHR. Endothelin-1 was 30 times more potent than noradrenaline in MVBs of SHR, and 15 times more potent than noradrenaline in aortic rings.

5 In both strains, nifedipine and nitrendipine almost completely blocked potassium-induced contractions in MVB and aortic rings, respectively, whereas contractions induced by endothelin-1 or noradrenaline were only partially inhibited.

6 It is concluded that calcium influx via the voltage-operated calcium channel is only partially responsible for the vasoconstrictor action of endothelin-1 in MVBs and aortic rings of SHR and WKY rats. The increased reactivity of the MVB of SHR to endothelin-1 at this stage of the hypertensive process is most likely to be the result of a change in vascular structure rather than due to a primary hypertensive mechanism.

Introduction

Since the original demonstration of the obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine (Furchgott & Zawadzki, 1980), the vascular endothelium has been recognized as playing an important role in vascular homeostasis (Huttner & Gabbiani, 1983; Gryglewski *et al.*, 1988; Luescher, 1988). It has also been shown that bovine cultured aortic and pulmonary endothelial cells release vasoconstrictor peptide(s), in addition to relaxant agents, into culture medium (Hickey *et al.*, 1985; Gillespie *et al.*, 1986). Yanagisawa *et al.* (1988) isolated a peptide, which they termed endothelin, from the supernatant of porcine cultured endothelial cells and determined its amino acid sequence. Porcine endothelin (endothelin-1) has been characterized as a very potent vasoconstrictor in a variety of vessels of different species including man (Yanagisawa *et al.*, 1988). *In vitro*, the vasoconstriction induced by endothelin-1 is long-lasting, difficult to wash out (Yanagisawa *et al.*, 1988; Tomobe *et al.*, 1988) and strongly dependent on extracellular calcium.

The observation that nicardipine, a dihydropyridine-type calcium entry blocker, attenuated the vasoconstriction induced by this peptide, led to the suggestion that endothelin may be an endogenous agonist of the dihydropyridine-sensitive calcium channels (Yanagisawa *et al.*, 1988). However, subsequent studies have shown the existence of specific endothelin binding sites, independent of the nicardipine binding sites (Hirata *et al.*, 1988), indicating that the site of action of endothelin may be different from that originally proposed by Yanagisawa *et al.* (1988).

The role of endothelin in the induction and/or maintenance of high blood pressure is not well understood (Yanagisawa & Masaki, 1989). Increased sensitivity to endothelin-1 has been shown to occur in renal arteries of 12 week-old spontaneously hypertensive rats (SHR) (Tomobe *et al.*, 1988), suggesting that endothelin could contribute to the maintenance of high blood pressure in this animal model. However, other authors did not find any augmented sensitivity to endothelin-1 in the same vessel of SHR (Auch-Schwelk & Vanhoutte, 1989).

The objectives of the present study were: (1) to investigate the reactivity and sensitivity to endothelin-1 in two different vessels, namely the isolated perfused mesenteric vascular bed (MVB) and rings from the thoracic aorta, taken from spontaneously hypertensive and age-matched Wistar Kyoto rats (WKY); (2) to study the effects of nifedipine and nitrendipine, two classical blockers of voltage-operated calcium channels on endothelin-1-induced vasoconstriction. For comparison, vasoconstriction was also induced with noradrenaline and potassium chloride.

Methods

All experiments were performed on 14–16 week-old male spontaneously hypertensive rats and age-matched Wistar Kyoto rats, supplied by IFFA CREDO, L'Arbresle, France. Arterial blood pressure was measured by the tail-cuff method. The mean body weights of the SHR and WKY rats were 317 ± 3.2 g ($n = 49$) and 320 ± 2.4 g ($n = 52$), respectively. The animals were anaesthetized with ether and exsanguinated by cutting both the carotid arteries. The preparation of the mesenteric vascular beds and aortic rings was performed as described below.

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Isolated perfused mesenteric vascular beds

MVBs were prepared according to a modification of the method of McGregor (1965). A cannula (PP 50) was inserted into the superior mesenteric artery at its junction with the aorta, just rostral to the left renal artery. The caecal part of the superior mesenteric artery was tied, and the MVB dissected free from the intestine. The MVBs were mounted on a perfusion system, and perfused at a constant rate of 5 ml min^{-1} with a peristaltic pump (Ismatec MP 13 GJ-4) with physiological salt solution (PSS) of the following composition (mM): NaCl 136, KCl 2.5, $NaHCO_3$ 11.9, $CaCl_2$ 1.36, $MgCl_2$ 0.5, NaH_2PO_4 0.42. The solution was aerated with 95% O_2, 5% CO_2 to give a pH of 7.4, and maintained at room temperature (22°C). Perfusion pressure, which under constant flow conditions is proportional to vascular resistance, was measured by a Gould P25 1D pressure transducer and recorded continuously (Hellige recorder).

Experimental protocol The MVBs were left for approximately 2.5 h to stabilize before the experiments were begun. To examine the effect of nifedipine on the pressor actions of KCl, noradrenaline (NA) and endothelin-1, dose-response curves were constructed in the presence of nifedipine (3, 30, 300 nM); in the control an appropriate dilution of solvent was used. Nifedipine was infused at a rate of 0.1 ml min^{-1} with a peristaltic pump (Ismatec MP 25 GJ-4) 30 min before the first dose-response curve was determined. Dose-response curves were then recorded in the following order: KCl, NA and endothelin-1; a 5 min interval was allowed between consecutive curves. KCl and NA were added as a 0.5 ml bolus injection directly into the perfusion system by a peristaltic pump (Ismatec JPN-12) at intervals of 2.5 and 5 min, respectively. Endothelin-1 was added as a 0.1 ml bolus with a syringe, at 5 min intervals. Only one MVB per concentration was used.

Thoracic aortic rings

The thoracic aorta of SHR and WKY were removed and cleaned of all loosely adherent connective tissue. Four rings from each aorta, about 2.5 mm wide, were cut close to the aortic arch. The rings were suspended under a tension of 1.5 g between two parallel hooks in a 20 ml organ bath containing a modified Krebs-Henseleit solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.52, $NaHCO_3$ 24.8, KH_2PO_4 1.2, glucose 10; at 37°C, gassed with 95% O_2 and 5% CO_2. Each preparation was allowed to equilibrate for at least one hour. Isometric responses were measured with a force transducer (K30, Hugo Sachs Electronics, Freiburg, F.R.G.) coupled to a tissue bath data acquisition system (Buxco Electronics, Inc., Sharon, CT, U.S.A.).

Experimental protocol After 30 min, the tension on the rings was readjusted to 1.5 g and after a further 30 min equilibration period, the experiments were begun. To examine the effects of nitrendipine on the contractions induced by endothelin-1, noradrenaline and potassium chloride, dose-response curves were constructed in the presence of nitrendipine 10, 100, 1000 nM; in the control an appropriate dilution of solvent was used. The rings were incubated for 30 min with nitrendipine or solvent before addition of the agonists.

Drugs and solutions

The following drugs were used: porcine endothelin (endothelin-1), nifedipine and nitrendipine (CIBA-GEIGY, Switzerland), potassium chloride (Merck, F.R.G.), noradrenaline hydrochloride (Fluka, Switzerland). Endothelin-1 was dissolved in a bicarbonate buffer solution (pH 7.4) and diluted in physiological salt solution (PSS). Albumin 1 mg ml^{-1} (Fluka, Switzerland) was added to the solutions to prevent adsorption of endothelin-1 onto the glassware. Noradrenaline was dissolved in distilled water and further diluted in PSS and 0.1 mM ascorbic acid. Stock solutions of nitrendipine (50% DMSO) and nifedipine (50% ethanol) were further diluted in PSS and protected from light.

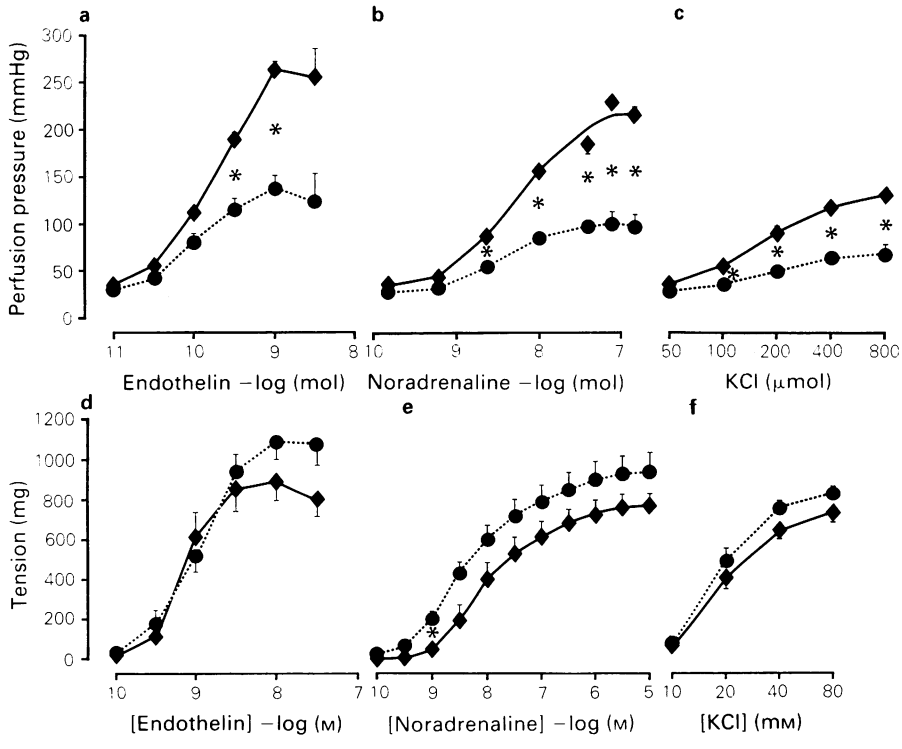

Figure 1 Vasoconstrictor effect of (a,d) endothelin-1, (b,e) noradrenaline, and (c,f) potassium chloride (KCl) in mesenteric vascular beds (a–c) and aortic rings (d–f) of spontaneously hypertensive rats (◆) and Wistar Kyoto rats (●). Results are the means of 6–12 preparations; vertical lines indicate s.e.mean. Significant differences ($P < 0.05$) are indicated by the asterisks.

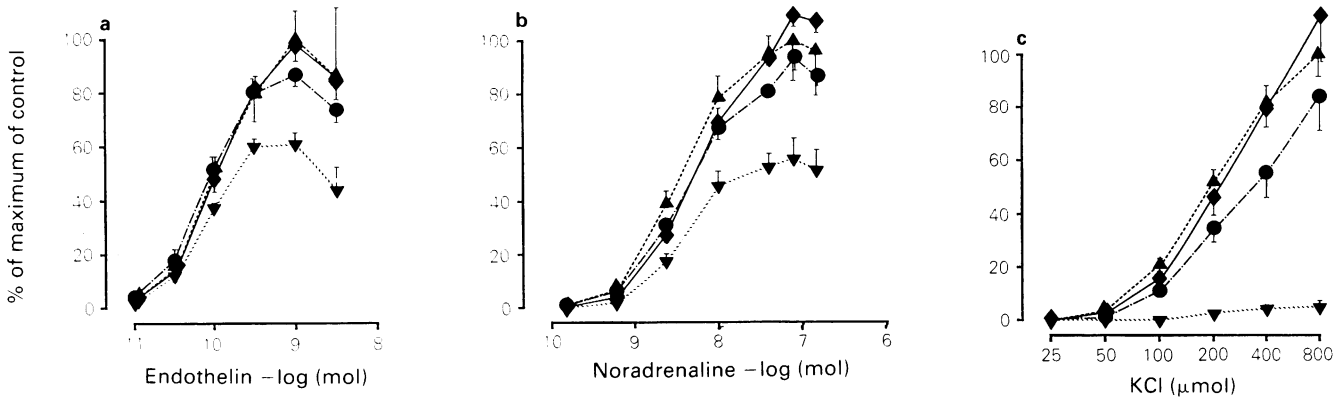

Figure 2 Inhibitory effects of nifedipine (◆ 3; ● 30; ▼ 300 nM) on the vasoconstrictor action of (a) endothelin-1, (b) noradrenaline and (c) potassium chloride (KCl) in mesenteric vascular beds of Wistar Kyoto rats. Results are expressed as percentage of the maximum vasoconstrictor response obtained with each agent in the preparations treated with appropriate concentrations of the solvent (control, ▲). Values are means of 10–12 preparations; vertical lines indicate s.e.mean.

Data presentation and analysis.

All values represent means ± s.e.mean. Data were analysed by Student's unpaired *t* test or one-way ANOVA, followed by Bonferroni's procedure (Wallestein *et al.*, 1980), as appropriate. Differences were considered statistically significant when $P < 0.05$.

Results

Effects of endothelin-1, noradrenaline and potassium on mesenteric arteries and aortic rings

In SHR the initial systolic blood pressures were significantly higher than those of the WKY rats: 199 ± 3.6 ($n = 49$) vs 150 ± 2.2 mmHg ($n = 52$); $P < 0.001$. After the stabilization period of 2.5 h, baseline perfusion pressure in MVB of SHR was significantly higher than in MVB of WKY (33 ± 0.3 mmHg and 27 ± 0.4 mmHg, respectively, $P < 0.05$). Endothelin-1 produced a concentration-dependent increase in perfusion pressure (i.e. vasoconstriction) in the MVBs of both strains (Figure 1a). MVBs of SHR were, however, more reactive to endothelin-1 than MVBs of WKY rats. The maximum increase in perfusion pressure obtained was 264 ± 8 mmHg in MVBs of SHR and 141 ± 9 mmHg in the MVBs of WKY rats. However, sensitivity to endothelin-1 was not significantly different between the two strains. In fact, the EC_{50} values were 171 ± 21 pmol for SHR and 102 ± 19 pmol for WKY. A similar trend was observed after noradrenaline and potassium chloride (Figure 1b,c). In both strains the maximum perfusion pressure obtained after noradrenaline and potassium were significantly ($P < 0.05$) lower than that induced by endothelin (Figure 1a,b,c). In SHR endothelin was 30 times more potent than noradrenaline.

In contrast to the mesenteric vascular beds, in aortic rings of SHR and WKY rats endothelin-induced contractions were similar. EC_{50}s were 0.78 ± 0.08 and 0.87 ± 0.09 nM, respectively (Figure 1d). In rings of SHR rats maximum developed tension was less than in WKY (Figure 1d). There was no significant difference in developed tension after noradrenaline and potassium chloride in rings of either strain (Figure 1e,f).

Effects of nifedipine on endothelin-induced vasoconstriction in the mesenteric vascular beds

In the MVBs of both strains, nifedipine at concentrations of 30 and 300 nM inhibited potassium-induced vasoconstriction. The concentration of 300 nM almost completely blocked the effects of potassium (Figures 2c and 3c). The same concentration of nifedipine only partially inhibited endothelin- and noradrenaline-induced vasoconstriction in a non-competitive manner (Figures 2 and 3, a and b). In MVB of SHR nifedipine was more effective than in WKY in inhibiting endothelin- and noradrenaline-induced vasoconstriction. However, only the values for 30 nM nifedipine were significantly different; $P < 0.05$.

Effects of nitrendipine on endothelin-induced contractions in thoracic aortic rings

Similar to the MVBs, in aortic rings of both strains, potassium-induced contractions were inhibited after 10, 100 and 1000 nM nitrendipine, and the latter concentration exerted an almost complete inhibition (Figures 4c and 5c). However,

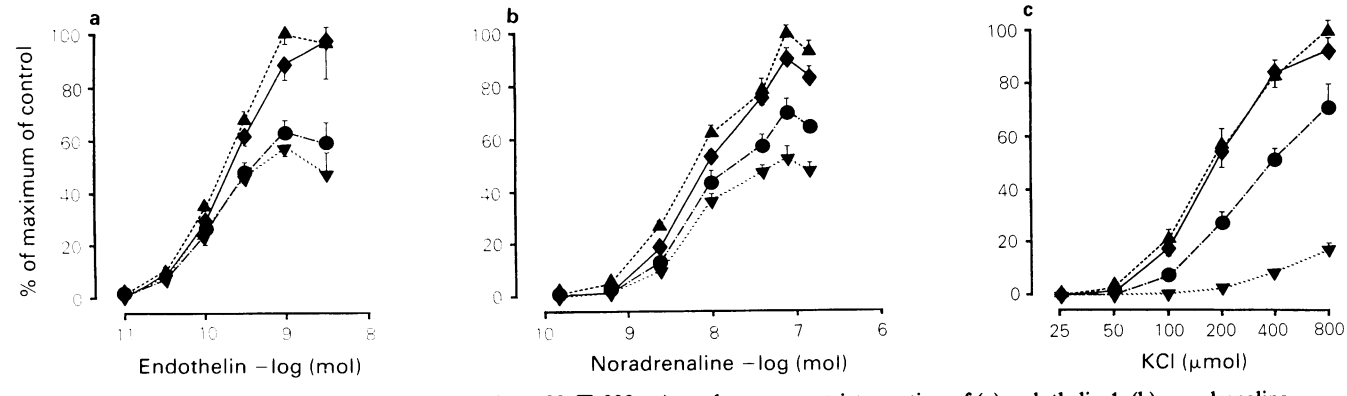

Figure 3 Inhibitory effects of nifedipine (◆ 3, ● 30, ▼ 300 nM) on the vasoconstrictor action of (a) endothelin-1, (b) noradrenaline and (c) potassium chloride (KCl) in mesenteric vascular beds of spontaneously hypertensive rats. Results are expressed as percentage of the maximum vasoconstrictor response obtained with each agent in the preparations treated with appropriate concentrations of the solvent (control, ▲). Values are means of 10–12 preparations; vertical lines indicate s.e.mean.

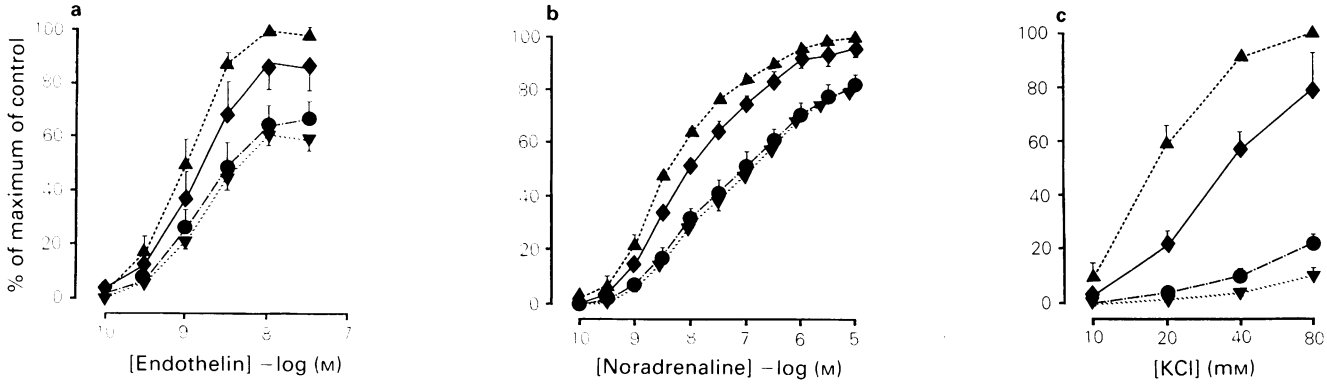

Figure 4 Inhibitory effects of nitrendipine (◆ 10, ● 100, ▼ 1000 nM) on contractions induced by (a) endothelin-1, (b) noradrenaline and (c) potassium chloride (KCl) in aortic rings of Wistar Kyoto rats. Results are expressed as percentage of the maximum vasoconstrictor response obtained with each agent in the preparations treated with appropriate concentrations of the solvent (control, ▲). Values are means of 4–6 preparations; vertical lines indicate s.e.mean.

endothelin- and noradrenaline-induced contractions were only partially inhibited by nitrendipine in the same concentration range (Figures 4 and 5, a and b).

Discussion

The present experiments demonstrate that the reactivity to endothelin-1 of perfused mesenteric vascular beds, but not aortic rings is greater in spontaneously hypertensive than in Wistar Kyoto rats. However, sensitivity to endothelin-1 is similar in the two strains and both types of vessel. Since an increase in sensitivity would indicate a role of endothelin-1 in the genesis of high blood pressure, these data suggest that endothelin-1 is not a primary hypertensive mechanism at this stage of hypertension in SHR. Our findings also show that calcium influx via 1,4-dihydropyridine-sensitive calcium channels is only partly responsible for the vasoconstrictive action of endothelin-1. The same is true of noradrenaline, but not of potassium chloride.

The greater reactivity of mesenteric vascular beds of SHR to endothelin-1 is most likely the result of structural changes (i.e. increases in wall-to-lumen ratio) and is not selective for endothelin-1, since it was also found with the other two vasoconstrictors used, i.e. potassium chloride and noradrenaline. Increased reactivity to noradrenaline in perfused vascular preparations of SHR has also been obtained by other authors (see Triggle & Laher, 1985, for review). Folkow (1978) has suggested that increased vascular resistance and exaggerated vascular reactivity in hypertension are attributable to thickening of the vessel walls. They demonstrated that the vasoconstrictor response of the perfused hindquarters of SHR, or of rats with renal hypertension, closely corresponds to that of a theoretical model based solely on circumferential shortening of the media and its encroachment on the lumen. According to Folkow (1978), the characteristic features of this response are (1) elevated resistance at maximal dilatation, (2) unchanged threshold, (3) supranormal maximal response, and (4) proportionally steeper vasoconstrictor response. All these criteria are met by the data presented here for the perfused mesenteric vascular beds of SHR. In fact, at the time of maximal dilatation, perfusion pressure in the vascular beds of SHR was significantly higher than that of WKY rats. Since, under conditions of constant flow, perfusion pressure is proportional to vascular resistance, these data indicate an elevated vascular resistance in the mesenteric vascular beds of the SHR. The differences in the perfusion pressure values in the experiments on mesenteric beds described here are very close to those obtained for perfused hindquarters of SHR (Folkow, 1978) and for mesenteric arteries (Triggle & Laher, 1985). In our study, baseline perfusion pressures at maximal dilatation were 33 mmHg in SHR and 27 mmHg in WKY rats. According to Folkow's model (1978), this difference in resistance corresponds to an increase in the thickness of the media of SHR vessels by about 30% as compared to WKY vessels. This means that the influence of structural adaptations can be taken into account to explain the abnormalities obtained in perfused vascular preparations from hypertensive animals.

However, in contrast to the mesenteric beds, aortic rings of SHR showed a decreased reactivity to both of these vasoconstrictors by comparison with those of WKY rats. Other investigators have also noted a lower reactivity of aortic rings to noradrenaline and potassium (Spector *et al.*, 1969). Similar to

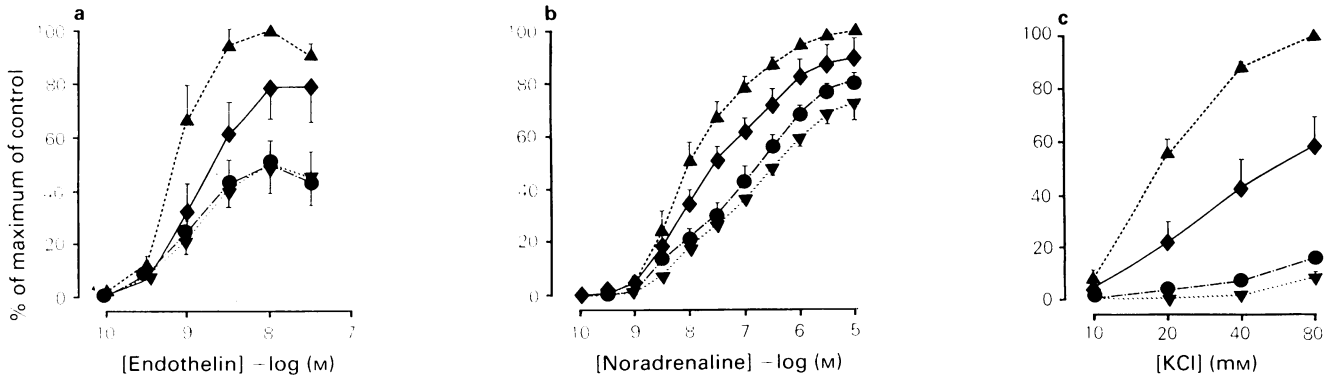

Figure 5 Inhibitory effects of nitrendipine (◆ 10, ● 100, ▼ 1000 nM) on contractions induced by (a) endothelin-1, (b) noradrenaline and (c) potassium chloride (KCl) in aortic rings of spontaneously hypertensive rats. Results are expressed as percentage of the maximum vasoconstrictor response obtained with each agent in the preparations treated with appropriate concentrations of the solvent (control, ▲). Values are means of 4–6 preparations; vertical lines indicate s.e.mean.

the results presented here, Auch-Schwelk & Vanhoutte (1989) found a reduced contraction to endothelin-1, by a similar extent, in rat aorta and renal arteries from SHR, as compared to those of WKY. The absence of increased sensitivity to endothelin-1 in the aortic preparations, where geometric factors play a minor role (Webb & Bohr, 1981), indicates that there is no alteration in smooth muscle function in response to endothelin-1 at this stage of hypertension in SHR. However, increased sensitivity of renal arteries of SHR to endothelin-1 has been demonstrated (Tomobe *et al.*, 1988). These authors came to a different conclusion, namely that endothelin-1 contributes to the maintenance of high blood pressure in SHR. The reasons for this discrepancy are at present unclear. Further studies, especially in blood vessels of prehypertensive SHR, are needed to clarify the exact role of endothelin-1 in hypertension.

Originally, endothelin-1 was represented as 'an endogenous agonist of the dihydropyridine-sensitive Ca^{2+} channels' (Yaganisawa *et al.*, 1988). Several lines of experimental evidence, however, indicate that the substance has no affinity for dihydropyridine binding sites (Hirata *et al.*, 1988; Van Renterghem *et al.*, 1988). The first specific endothelin-1 binding sites were detected in vascular smooth muscle. Endothelin-1 and [^{3}H]-nitrendipine do not displace each other from their respective binding sites (Hirata *et al.*, 1988).

The finding that high concentrations of nifedipine, or nitrendipine, only partially inhibited endothelin-induced vasoconstriction indicates that the calcium needed for this effect is not primarily derived from the extracellular medium via voltage-operated calcium channels. The dependency of endothelin-1 contractions on extracellular calcium (Yanagisawa *et al.*, 1988) has also been observed in experiments with other vasoconstrictors, such as noradrenaline (Karaki, 1987). Thus the absence of contractions after removal of extracellular calcium, or after inhibition by calcium-entry blockers, is not necessarily indicative of a direct effect of a vasoconstrictor on the voltage-dependent calcium channels, particularly with regard to the coronary arteries, originally used by Yanagisawa *et al.* (1988). In fact, the activation of contraction of the coronaries is more dependent on extracellular calcium than that of other arteries (Van Breemen & Siegel, 1980; Sato *et al.*, 1982). In addition, coronary and cerebral vessels are particularly susceptible to the effects of calcium-entry blockers (Nakayama *et al.*, 1983). More recent studies have indicated that endothelin-1, at a concentration of 10 nM, is capable of contracting porcine coronary arteries independently of the presence of extracellular calcium (Kodama *et al.*, 1989). The inhibitory action of calcium-entry blockers on endothelin-induced contractions is quantitatively very similar to their effects on contractions due to other vasoconstrictors, such as noradrenaline (Godfraind, 1985). Thus the effects of endothelin-1 resemble those of an activator of receptor-operated calcium channels (Godfraind, 1985). According to Godfraind (1985), activation of receptor-operated channels is typically only incompletely inhibited by calcium-entry blockers.

Recently, it has been shown that endothelin-1 stimulates the metabolism of inositol phosphates, leading to mobilization of intracellular free calcium stores (Resink *et al.*, 1988 Van Renterghem *et al.*, 1988; Marsden *et al.*, 1989). In addition, it has been shown that by transiently activating calcium-sensitive K-channels, endothelin-1 initially provokes hyperpolarization of the membrane, followed by sustained depolarization due to the opening of a non-specific cation channel permeable to Ca^{2+} and Mg^{2+}. It is this depolarization that then activates L-type Ca^{2+}-channels (Van Renterghem *et al.*, 1988). Hence, the effect of endothelin-1 on L-type Ca^{2+}-channels is only a part of its total effect, and, only an indirect one. This mode of action, which is very likely shared by other vasoconstrictors, is supported by the finding that calcium-channel blockers only partially inhibited endothelin-induced vasoconstriction. As suggested by Van Renterghem *et al.* (1988), the remaining component of endothelin-induced contraction in the presence of calcium-entry blockers is probably due to influx of calcium via non-selective cation channels and to calcium released from intracellular stores.

In conclusion, reactivity to endothelin-induced vasoconstriction was found to be greater in isolated perfused mesenteric vascular beds, but not aortic rings, of SHR than in those of WKY rats. Similar patterns of activity were observed with the other vasoconstrictor agents used, noradrenaline and potassium chloride. These data indicate that in SHR with fully developed hypertension the augmented reactivity to endothelin-1 is due to a structural rather than a functional change. They do not rule out the involvement of endothelin-1 in some primary hypertensive mechanism operating at a prehypertensive age.

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Inositol phospholipid hydrolysis in human brain; adenosine inhibition of the response to histamine

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1 Inositol phospholipid hydrolysis was examined in human cerebral cortex slices by a [^{3}H]-inositol prelabelling assay.

2 Enhancement of [^{3}H]-inositol phosphates accumulation was observed in the presence of carbachol, noradrenaline, histamine, 5-hydroxytryptamine (5-HT) and depolarizing concentrations of KCl.

3 Despite having no effect alone, adenosine (and its analogue 2-chloroadenosine) selectively inhibited the direct response to histamine.

4 The inhibition due to adenosine was antagonized by theophylline, but not by 8-cyclopropyltheophylline.

Introduction

It is becoming increasingly apparent that the effects of neurotransmitters and hormones on second messenger formation in the central nervous system can be influenced, positively and negatively, by the simultaneous activation of other neurotransmitter receptor systems (Hill & Kendall, 1989). These neuromodulations show a remarkable degree of species specificity; for instance, we have previously shown that histamine-stimulated inositol phospholipid hydrolysis is enhanced in the presence of adenosine and some adenosine analogues in guinea-pig cerebral cortex (Hill & Kendall, 1987), while the response is inhibited in mouse cerebral cortex (Kendall & Hill, 1988). It is thus impossible to make predictions concerning the occurrence of such interactions in human brain. We have therefore examined the effects of adenosine on histamine-stimulated inositol phospholipid hydrolysis in slices of human cerebral cortex taken during neurosurgical procedures.

A preliminary account of some of these data has been communicated to the British Pharmacological Society (Kendall & Firth, 1989).

Methods

Tissue preparation

Pieces of cerebral cortex were taken during neurosurgical operations which involved corticectomy. The indications for the procedures varied, including sub-cortical tumour removal or biopsy and cerebral decompression, and tissue from a variety of cortical regions was included in the study. In all cases the tissue would normally have been discarded. The mean age of the donors was 50.2 years and the range was 23–77 years. There were approximately equal numbers of men and women.

Immediately following removal, the tissue was placed in ice-cold Krebs-Hensleit buffer of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 1.3, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11.7, equilibrated with 95% O_2/CO_2 to pH 7.4. The tissue was taken to the laboratory (in less than 5 min) where it was cut into slices (350 μm × 350 μm × about 1 mm) with a McIlwain tissue chopper. The slices were dispersed in Krebs solution at 37°C in a glass conical flask gassed with 95% O_2/5% CO_2, stoppered and then incubated for 60 min with 3 intermediate changes of buffer. The tissue was treated as being potentially infectious and all transfer procedures, chopping and gassing were done in a microbiological safety cabinet in a designated human tissue laboratory.

Incorporation of [^{3}H]-inositol and agonist stimulation of total inositol phosphates formation

Twenty five μl aliquots of gravity packed slices were dispensed into flat-bottomed plastic insert vials containing 25 kBeq [^{3}H]-*myo*-inositol, 5 mM LiCl and Krebs buffer. The vials were gassed and shaken in a water bath at 37°C for 45 min. When appropriate, antagonists were added (10 μl), followed by agonists (10 μl) 10 min later. The final incubation volume was 300 μl. Agonist incubations were stopped, usually after 45 min, by the addition of 100 μl, 10% (w/v) perchloric acid. The samples were cooled on ice for 15 min and were then neutralized by the addition of an appropriate volume of 0.15 M KOH. After centrifugation (1000 *g* for 10 min at 4°C) 0.8 ml of the sample supernatant was taken and added to 2.25 ml of Tris buffer (50 mM, pH 7.0) before separation of total [^{3}H]-inositol phosphates ([^{3}H]-IP) by anion exchange chromatography on Dowex-1 resin in the Cl^- form. [^{3}H]-inositol was eluted from the columns with 20 ml H_2O and [^{3}H]-IP with 3 ml 1 M HCl. [^{3}H]-IP were quantified by liquid scintillation counting after addition of 10 ml scintillation cocktail.

Separation of individual [^{3}H]-inositol phosphates

In some experiments agonist incubations were stopped with 10% perchloric acid after 5 min incubations, before estimation of separate [^{3}H]-inositol phosphates by the gradient h.p.l.c. procedure described by Whitworth & Kendall (1989).

Estimation of [^{3}H]-inositol incorporation into phospholipids

After removal of the aqueous supernatant, slices were treated with 0.94 ml chloroform/methanol/conc. HCl (100/200/1) followed by 0.31 ml chloroform and 0.31 ml H_2O. Following centrifugation to separate the phases, an aliquot (200 μl) of the lower, chloroform phase was taken, evaporated overnight at room temperature and counted in scintillation fluid for estimation of [^{3}H]-inositol incorporation into phospholipids.

Data analysis

The IC_{50} for inhibition curves was determined from log probit analysis, and the inhibition constant (K_i) calculated from the

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relationship;

$$K_i = \frac{IC_{50}}{\left(1 + \frac{C}{EC_{50}}\right)}$$

where C is the concentration of histamine or adenosine and EC_{50} the concentration of histamine that produces 50% of its maximum response (for the experiment illustrated in Figure 4) or the dissociation constant for adenosine inhibition of the response to histamine (Figure 5).

Results

Total [^{3}H]-inositol phosphate accumulation

After 45 min incubations there were significantly enhanced accumulations of [^{3}H]-IP in the presence of the muscarinic agonist carbachol (10^{-3} M), noradrenaline (3×10^{-4} M), histamine (10^{-3} M), 5-hydroxytryptamine (5-HT, 3×10^{-4} M) and elevated KCl (31 mM) (Figure 1).

The incorporation of [^{3}H]-inositol into the phospholipids was somewhat variable. In the absence of agonist there was an incorporation of 4249 ± 1198 d.p.m. ($n = 10$) per 200 μl aliquot of the chloroform phase. This was increased after incubation with agonists; in the presence of histamine (10^{-3} M) by $143 \pm 10\%$ ($n = 8$) and in the presence of carbachol (10^{-3} M) by $173 \pm 16\%$ ($n = 7$) of basal levels. Basal and stimulated accumulations of [^{3}H]-IP varied from sample to sample. Unstimulated levels ranged from 300 d.p.m. to over 3,500 d.p.m., and the stimulations due to 10^{-3} M histamine from 1.5 to 5.3 fold over basal.

The increases in [^{3}H]-IP accumulation were concentration-related (Figure 2) with EC_{50} values of; carbachol 5×10^{-5} M; noradrenaline, 1×10^{-5} M and histamine 4×10^{-5} M (all $n = 2$). The responses were abolished in the presence of the Ca^{2+} chelator EGTA (2 mM) (data not shown).

Separate [^{3}H]-inositol phosphates

It would appear that the majority of [^{3}H]-IP accumulating upon stimulation is accounted for by [^{3}H]-inositol monophosphate ([^{3}H]-IP_1) (Figure 3). Although the basal levels of the higher phosphates were relatively large, following 5 min stimulation with 10^{-3} M carbachol, the increases in [^{3}H]-inositol bisphosphate ([^{3}H]-IP_2), [^{3}H]-inositol trisphosphate ([^{3}H]-IP_3) and [^{3}H]-inositol 1,3,4,5-tetrakisphosphate ([^{3}H]-IP_4) fractions were minor, in the absence of LiCl. When 5 mM LiCl was included in the incubations, the effect of carbachol was greatly enhanced with regard to [^{3}H]-IP_1; there were small additional increases in [^{3}H]-1,3,4-IP_3 and [^{3}H]-1,3,4,5-IP_4, and the [^{3}H]-1,4,5-IP_3 fraction was reduced somewhat.

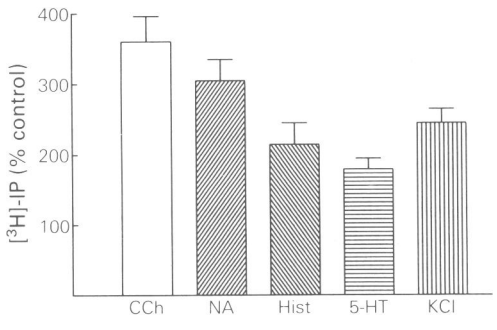

Figure 1 [^{3}H]-inositol phosphates ([^{3}H]-IP) accumulation in slices of human cerebral cortex. Each column represents the mean of three separate experiments with tissue from three different donors, each performed in triplicate; bars show s.e.mean. The accumulations are expressed as percentages of those in the absence of stimulation. CCh = carbachol 10^{-3} M; NA = noradrenaline 3×10^{-4} M; Hist = histamine 10^{-3} M; 5-HT = 5-hydroxytryptamine 3×10^{-4} M; KCl, 31 mM.

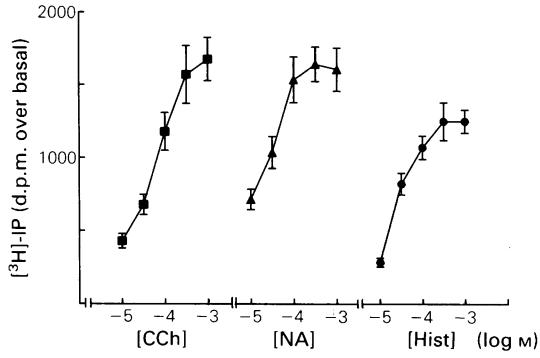

Figure 2 Dose-response relationships for [^{3}H]-inositol phosphates ([^{3}H]-IP) accumulation in the presence of carbachol (CCh), noradrenaline (NA) and histamine (Hist). Each curve represents data from a separate tissue sample, and the experiments (performed in triplicate) were repeated on another occasion. EC_{50} values were estimated by inspection. Vertical lines show s.e.mean.

Effect of adenosine on [^{3}H]-inositol phosphate accumulation

Despite having no significant effect alone, adenosine reduced, in a concentration-dependent manner, the response to histamine (Figure 4), with an IC_{50} of $4 \pm 0.7 \times 10^{-5}$ M ($n = 4$) in the presence of 10^{-3} M histamine, giving a K_i of 1.5×10^{-6} M.

This effect of adenosine was selective for the histamine response, of the three agonists examined, since the [^{3}H]-IP accumulations due to carbachol and noradrenaline were unaffected by the presence of adenosine. The mean response to 3×10^{-4} M noradrenaline was $235 \pm 22\%$ of basal in the absence and $255 \pm 74\%$ in the presence of 3×10^{-4} M adenosine ($n = 3$). The response to 10^{-3} M carbachol was $350 \pm 25\%$ of basal in the absence and $370 \pm 15\%$ in the presence of 3×10^{-4} M adenosine ($n = 3$).

The adenosine analogue 2-chloroadenosine (10^{-4} M) was as effective as the parent compound (data not shown), although no concentration-response experiments were performed.

Adenosine antagonists

The inhibitory effect of 10^{-4} M adenosine on histamine-stimulated [^{3}H]-IP accumulation was completely reversed by the non-selective adenosine receptor antagonist theophylline

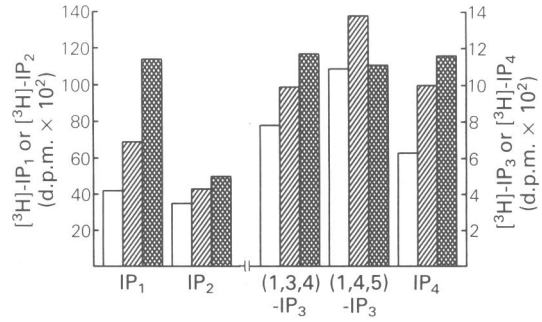

Figure 3 Separation of [^{3}H]-inositol phosphates from human brain slices in the absence of stimulation and LiCl (open columns) following 5 min stimulation with 10^{-3} M carbachol (diagonally-hatched columns) or 10^{-3} M carbachol plus 5×10^{-3} M LiCl (cross-hatched columns). The effects of LiCl alone were not determined. Twenty five μl tissue aliquots were prelabelled for 60 min with 240 kBq [^{3}H]-*myo*-inositol before agonist addition. [^{3}H]-inositol phosphates were extracted by the freon/trioctylamine method and five samples were combined for separation by gradient h.p.l.c. Abbreviations; IP_1, inositol monophosphate; IP_2, inositol bisphosphate; (1,3,4)-IP_3, inositol 1,3,4-trisphosphate; (1,4,5)-IP_3, inositol 1,4,5-trisphosphate; IP_4, inositol 1,3,4,5-tetrakisphosphate. The experiment was repeated on two further occasions with qualitatively similar results, but variations in absolute levels of radioactivity precluded their combination.

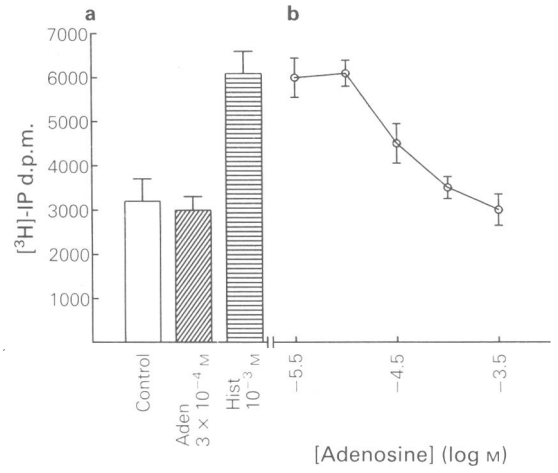

Figure 4 Inhibition of histamine-stimulated [^{3}H]-inositol phosphates ([^{3}H]-IP) accumulation by adenosine in human cerebral cortex slices. The data represent the mean of triplicate incubations with tissue from a single donor. The experiment was repeated on three further occasions. (a) The open column represents control; the diagonally-hatched column, adenosine (3×10^{-4} M) and the horizontally-hatched column, histamine (10^{-3} M). (b) The data points represent the effect of increasing concentrations of adenosine in the presence of histamine. Vertical bars show s.e.mean in (a) and (b).

(Figure 5). In two separate experiments the K_i values calculated for theophylline were 7.4×10^{-7} M and 6.0×10^{-7} M. In contrast, 8-cyclopropyltheophylline, at concentrations up to 10^{-4} M, was, in three separate experiments, unable to reverse the inhibitory effects of 3×10^{-4} M adenosine significantly.

Discussion

Previous studies from our own and other laboratories (Hollingsworth *et al.*, 1986; Hill & Kendall, 1987; Kendall & Hill, 1988) have shown that the neuromodulator, adenosine (Snyder, 1985) can selectively influence inositol phospholipid hydrolysis due to histamine in both a positive and negative fashion in the brains of experimental animals. Considering the species diversity in the effects of adenosine (enhancement in the guinea-pig, inhibition in the mouse, no apparent effect in the rat), it was not possible to make predictions concerning the human response, so that it was necessary to assess directly the prospective interaction in human brain.

Given the paucity of studies on human central nervous system tissue, it was also thought to be a useful aim to examine some general features of agonist-stimulated phosphoinositide hydrolysis, which is now accepted to be a ubiquitous intracellular signalling system (Berridge, 1987), leading to Ca^{2+} mobilization and protein kinase C activation.

The accumulations of [^{3}H]-IP in human cortical slices in the presence of LiCl (which prevents the dephosphorylation of inositol monophosphate, Berridge *et al.*, 1982) were generally comparable with those seen in rat brain (Brown *et al.*, 1984), apart from the muscarinic response which was somewhat smaller (Figure 1). The responses were apparently dependent upon extracellular Ca^{2+}, being abolished in the presence of the Ca^{2+} chelator EGTA (2 mM), as is the case in rat brain (Kendall & Nahorski, 1984).

The amount of [^{3}H]-inositol incorporated into the phospholipids was less than that observed in rat, mouse and guinea-pig brain and there was a significant enhancement of incorporation due to histamine and carbachol stimulation. Whether this is a direct effect on the incorporation process or a reflection of a reduction in the phosphoinositide pool due to stimulated breakdown, can only be speculated upon given the available data. There were relatively small increases in the accumulations of the higher phosphates following 5 min stimulations with 10^{-3} M carbachol, but whether this reflects a low rate of polyphosphoinositide metabolism or a rapid flux of label through the higher inositol phosphates would need further investigation (see Batty & Nahorski, 1989). As expected, lithium enhanced the accumulation of [^{3}H]-IP_1 and, probably, [^{3}H]-IP_2 and [^{3}H]-1,3,4-IP_3.

In rat and mouse brain slices lithium-induced decreases in [^{3}H]-1,4,5-IP_3 and [^{3}H]-1,3,4,5-IP_4 have been demonstrated (Batty & Nahorski, 1987; Whitworth & Kendall, 1988), but in the human slices there was no indication of a reduction in [^{3}H]-IP_4 accumulation, although [^{3}H]-1,4,5-IP_3 stimulation by carbachol may be reversed in the presence of 5 mM LiCl.

It is clear, however, that the response to histamine was inhibited by adenosine in human brain slices. The degree of inhibition was greater than that previously seen in mouse cerebral cortex, with complete reversal at the top of the adenosine concentration range. Also the effect was selective, in that the responses to carbachol and noradrenaline were unchanged.

There is some preliminary evidence that the adenosine receptor mediating the modulator response in human brain is different from that in the mouse. Adenosine was more potent in human cortex having a K_i of 1.5 μM compared with 16 μM in the mouse (Kendall & Hill, 1988), although this could be explained by more efficient receptor/effector coupling in the human cortex. On the other hand, the non-selective adenosine receptor antagonist theophylline (Bruns *et al.* 1986) was more potent in the human tissue, K_i versus adenosine about 700 nM compared with 12 μM in the mouse (Kendall & Hill, 1988). In addition the adenosine A_1-selective antagonist 8-cyclopropyltheophylline (H.H. Stein, personal communication) was without effect in human brain despite blocking the response to adenosine in mouse brain with a K_i of 0.9 μM. Clearly a more detailed pharmacological investigation would be needed to clarify the identity of the adenosine receptor involved.

The complicating factors involved in the interpretation of data generated from experiments with human tissue should be taken into account before the involvement of different receptors is invoked. For instance, these studies used small segments of tissue from various areas of the human cortex compared with the whole cortices employed in animal experiments. Regional differences in the responses may therefore be important and could account, in part, for some of the observed variability in basal and stimulated [^{3}H]-IP accumu-

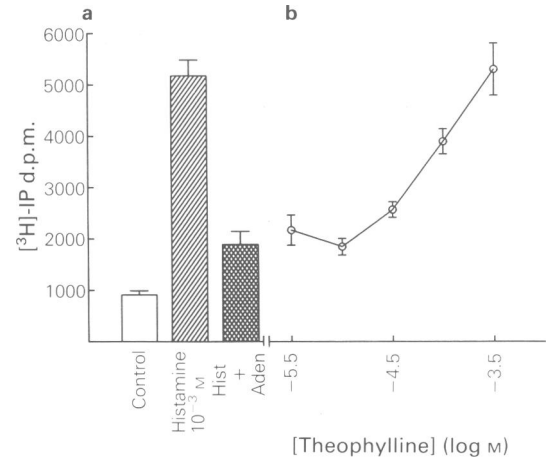

Figure 5 Reversal of the adenosine inhibition of histamine-stimulated [^{3}H]-inositol phosphates ([^{3}H]-IP) accumulation by theophylline in human cerebral cortex slices. The data represent the mean of triplicate incubations with combined tissue from two donors. The experiment was repeated on another occasion, with essentially identical results. The inhibition constant for theophylline was calculated from the relationship given under Methods. (a) The open column represents control, the diagonally-hatched column, histamine (10^{-3} M) and the cross-hatched column, histamine plus adenosine (10^{-4} M). (b) The data points represent increasing concentrations of theophylline in the presence of histamine and adenosine. In (a) and (b), vertical bars show s.e.mean.

lation. Also, the human donors were anaesthetized when their tissue was taken and, although the preincubation procedure would be expected to clear the tissue of anaesthetic, residual effects (see Miller, 1985) cannot be ignored. Further, the donors comprised a heterogeneous group in terms of sex, age, disease state and genetic make-up so that a greater degree of inter-experimental variance compared with in-bred laboratory animals would be expected.

In conclusion, agonist-stimulated inositol phospholipid hydrolysis can be demonstrated in human cerebral cortex slices by a [^{3}H]-inositol prelabelling assay. The system has many features in common with those previously demonstrated in the brains of experimental animals, including modulation of the direct response to histamine by adenosine. The determination of the pharmacological profile of the receptors involved must remain the subject of future investigations.

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Actions of second messengers synthesized by various spasmogenic agents and their relation to mechanical responses in dog tracheal smooth muscle

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1 The effects of the spasmogenic agents, carbachol (CCh), histamine, 5-hydroxytryptamine (5-HT) and 9,11-epithio-11,12-methano-thromboxane A_2 (STA_2) were investigated on smooth muscle tissues of the dog trachea.

2 CCh (10 μM) produced a larger contraction than high K (128 mM), 10 μM histamine, 5-HT or STA_2. Histamine and 5-HT produced the same amplitude of contraction as each other. In Ca-free solution containing 0.2 mM EGTA, only a phasic contraction was evoked by the above agents (except for K which induced no contraction at all).

3 In skinned muscle tissues, the maximum amplitude of contraction that could be induced by Ca (10 μM) was slightly larger than the maximum CCh-induced contraction (also at 10 μM) evoked in intact muscle tissues. Caffeine and inositol 1,4,5-trisphosphate (IP_3) both produced contraction.

4 CCh, histamine and 5-HT (10 μM) produced a sustained contraction for over 30 min and also increased phosphorylation of the 20 kD protein of myosin light chain (MLC_{20}) for over 30 min with no attenuation. Greater concentrations of the above agents caused more phosphorylation of MLC_{20}.

5 CCh (above 1 nM), histamine (above 10 nM) and 5-HT (above 100 nM) increased the amount of IP_3, in a concentration-dependent manner. Synthesis of IP_3 induced by the above agents reached its peak value within 30 s and lasted for about 3 min. The potencies for the synthesis of IP_3 were in the following order: CCh > histamine > 5-HT > STA_2.

6 Isoprenaline (10 μM) markedly enhanced but CCh (10 μM) slightly reduced the amount of cyclic AMP. 5-HT (10 μM) and STA_2 (10 μM) reduced, but histamine (10 μM) and CCh (10 μM) increased the amount of cyclic GMP.

7 Using fura 2, cytosolic Ca was measured by monitoring the ratio of the fluorescent signal excited at 340 and 380 nm wavelengths in the presence of extracellular Ca. CCh (10 μM) increased the Ca transient from 182 nM to 1.42 μM. When the CCh-induced peak Ca transient (10 μM) was normalised, 10 μM histamine, 5-HT and STA_2 showed smaller values such as 0.49, 0.53 and 0.04 times the control, respectively, and these values corresponded well with the amplitudes of contraction evoked by each of the stimulants.

8 The results can be summarized as follows: stronger spasmogenic responses occur on application of CCh than on application of 5-HT or histamine, and STA_2 may have a minor role as a spasmogenic agent. The maximum amplitudes (peaks) of contraction evoked by the above spasmogenic agents are closely related to the maximum increase in cytosolic Ca, but sustained contraction and increased phosphorylation of myosin cannot be explained by the increased amount of Ca. In the case of 5-HT and histamine, synthesized cyclic nucleotides may interact with the action of IP_3 for the regulation of contraction in a positive or negative manner, respectively.

Introduction

Airway smooth muscle tissues show both regional and species differences in their responses to various spasmogenic agents. For example, in the rabbit airway, the potassium (128 mM K)- and histamine (10 μM)-induced contractions in the trachea are much smaller than the acetylcholine (ACh, 10 μM)-induced contraction, but in the third branch of the right middle bronchial tree, these same three stimulants generate much the same amplitude of contraction and, moreover, the amplitude of the ACh-induced contraction is larger than that observed in the trachea (Fujiwara *et al.*, 1988). In the guinea-pig, dog, bovine and rabbit tracheal and bronchial tissues, histamine produces contraction by stimulation of the H_1-receptor, though H_2-receptors are also present (Chand & Eyre, 1975; Kirkpatrick, 1975; Kotlikoff *et al.*, 1987). Ito & Tajima (1982) demonstrated that in the dog trachea, isoprenaline produces hyperpolarization of the membrane, reduces the resting tone and relaxes tissues precontracted by ACh. However, in the bovine trachea, isoprenaline stimulates the β_2-adrenoceptor and increases Ca influx through activation of the receptor-operated (dihydropyridine- and pertussis toxin-sensitive) Ca channel, whereas isoprenaline reduces Ca influx following pretreatment with carbachol (CCh) (Felbel *et al.*, 1988). Furthermore, the same authors showed that CCh increases the influx of Ca and releases Ca from the sarcoplasmic reticulum and also that the channel responsible for the accelerated influx of Ca is the same as that by which isoprenaline causes Ca influx. However, in the rabbit, cat and guinea-pig trachea the maximum amplitude of K-induced contraction, i.e. when activation of the voltage-dependent dihydropyridine-sensitive Ca channel is maximum, is about 20% of the CCh-induced contraction and in the rabbit trachea, Ca-channel blockers (Ca antagonists) inhibit the tonic response but not the phasic response of the contraction.

In the dog tracheal smooth muscle cell, ACh and 5-hydroxytryptamine (5-HT) stimulate the production of myo-inositol 1,4,5-trisphosphate (IP_3) in the soluble fraction (Hashimoto *et al.*, 1985). Histamine increases the amount of adenosine 3′: 5′-cyclic monophosphate (cyclic AMP) through stimulation of the H_2-receptor (Beaven, 1978). In the bovine and guinea-pig tracheal smooth muscle tissues, histamine increases guanosine 3′: 5′-cyclic monophosphate (cyclic GMP; Duncan *et al.*, 1980; Nakagawa *et al.*, 1986). This latter action

is blocked by diphenhydramine, an H_1-antagonist (Ganellin & Parsons, 1982). It is therefore apparent that many second messengers, such as Ca, cyclic nucleotides, IP_3 and diacylglycerol may contribute to the generation of contraction induced by various stimulants.

The present experiments were intended to clarify the effects on dog tracheal smooth muscle tissues of the spasmogenic agents, CCh, histamine, 5-hydroxytryptamine (5-HT) and 9,11-epithio-11,12-methano-thromboxane A_2 (STA_2; a stable thromboxane A_2 analogue). To obtain parameters of muscle responses, measurements were made of the mechanical response, amounts of free Ca, of cyclic nucleotides (cyclic AMP and cyclic GMP) and of IP_3, and the phosphorylation of the 20 kD protein of the myosin light chain.

Methods

Materials

Adult mongrel male dogs weighing 10–15 kg were anaesthetized with intravenous pentobarbitone (10–30 mg kg^{-1}). Segments of the cervical trachea were excised and a dorsal strip of transversely running smooth muscle was separated from the cartilage. The mucosa and adventitial areolar tissues were carefully removed, under the microscope. The tracheal smooth muscle was cut into sections: (a) 0.05–0.08 mm in width and 0.3–0.4 mm in length for tension recording in intact and skinned muscle tissues and for measurements of Ca transients using fura 2, and (b) 2 mm in width and 5–7 mm in length for the phosphorylation of the 20 kD protein of the myosin light chain.

For measurements of cyclic nucleotides and IP_3, dispersed smooth muscle cells were prepared with collagenase as described by Sumimoto & Kuriyama (1986). The cell viability, as assessed by the trypan blue exclusion test, was over 85%.

Solutions

The Krebs solution contained (mM): Na^+ 137.5, K^+ 5.9, Cl^- 134.4, Mg^{2+} 1.2, Ca^{2+} 2.6, HCO_3^- 15.5, $H_2PO_4^-$ 1.2 and glucose 11.5. It was of pH 7.3 and was bubbled with 97% O_2 and 3% CO_2. High K-solution was prepared by replacing NaCl with equimolar KCl (128 mM K). Ca-free solution was prepared by substituting $MgCl_2$ for $CaCl_2$ in the Krebs solution and adding 0.2 mM EGTA. For the experiments on skinned muscle strips, the following solutions were used (mM): K-methanesulphonate (KMs) 110, $Mg(Ms)_2$ 5, Na_2ATP 5, ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) 4, piperazine-N-N′-bis-(2-ethanesulphonic acid)(PIPES) 20; the pH being adjusted to 6.8 with KOH at 25°C. Solutions of desired Ca concentration were prepared by adding appropriate amounts of Ca to the relaxing solution. The precise methods for calculating free ionic concentrations and the binding constants used have been described by Fujiwara *et al.* (1989).

Tension recordings from intact and skinned muscle strips

Mechanical responses were measured by attaching smooth muscle strips prepared from the trachea to a strain gauge (U-gauge, Shinko, Tokyo), as described previously (Fujiwara *et al.*, 1989). Tension recordings were started after repetitively generated K-induced contractions showed the same amplitude (about 2–4 h after placing the tissue in the bath) and spasmogenic agents were applied for 3 min at 10–15 min intervals. Responses were found to be reproducible with this procedure. Control responses, recorded before and after each trial, were also reproducible. Concentration-response relationships were obtained by application of various concentrations of spasmogens for 3 min at 15 min intervals.

Skinned tissues were prepared by use of saponin (40 μg ml^{-1}, for 20 min), as described previously (Itoh *et al.*, 1986). To prevent deterioration of the Ca-sensitivity of the contractile proteins, 0.1 μM calmodulin was present throughout the experiments. The tension-pCa relationship was obtained by cumulative application of solutions containing various Ca concentrations and buffered with 4 mM EGTA (Itoh *et al.*, 1986).

Measurements of phosphorylation of 20 kD protein of myosin light chain

The muscle strips were stimulated with spasmogenic agents and were quickly frozen by acetone-dry ice. The strips were then homogenised in a solution containing 5% trichloroacetic acid (TCA) and were centrifuged at 12,000 *g* for 15 min. The pellet was washed with the solution containing 2% TCA and 5 mM KH_2PO_4 and centrifuged at 12,000 *g* for 15 min at 4°C, dissolved in lysis buffer containing 0.25 M Na_2HPO_4, 0.3% sodium dodecyl sulphate (SDS) and 5% 2-mercapto-ethanol and then homogenised. Two-dimensional gel electrophoresis involving isoelectric focusing (IEF) in the first dimension and SDS electrophoresis in the second dimension, as developed by O'Farrell (1975), was used for the resolution of myosin light chain phosphorylation. The IEF gels 4% polyacrylamide (1.0 mm in diameter and 110 mm in length) containing 8.5 M urea, 2% Nonidet P-40 (NP-40), and 2% Pharmacia carrier ampholytes (1.6% for pH 4–6.5 and 0.4% for pH 3.5–10) were compared before and after application of spasmogenic agents. Lysis buffer containing 500 μg of protein was applied and focused at a constant voltage of 200 V for 2 h, 440 V for 13 h and 800 V for 1 h. After being focused, the gels were loaded onto the SDS electrophoresis unit. The SDS electrophoresis gels (140 mm in width and 1.5 mm in thickness) comprised stacking gels (50 mm in height with 4% polyacrylamide in 0.1% SDS and 0.125 M Tris-HCl at pH 6.8) and separating gels (100 mm in height with 13.5% polyacrylamide in 0.1% SDS and 0.345 M Tris-HCl at pH 8.8). The gels were run at a constant current density of 25 mA in the stacking gels and 40 mA in the separating gels. The gels were stained and fixed overnight with 0.25% Coomassie Brilliant Blue R-250, 45% ethanol, and 10% acetic acid, and then destained. The distribution of the stained protein (20 kD myosin light chain; MLC_{20}) exhibited the first and second (and sometimes third) spots from higher to lower pI values. The intensity of these spots was measured with a chromatography densitometer equipped with an automatic integrator (CS-910, Shimazu, Kyoto). The first area and the second area at around pI 5.45 were meausured to obtain the relative value of MLC_{20} phosphorylation, which is expressed as a percentage, derived from a division of the second spot area by the sum of the first and second spot areas, as described by Driska *et al.* (1981).

Measurements of cyclic nucleotides and IP_3

Cell suspensions (450 μl; 0.5 × 10^6 cells ml^{-1}) were incubated at 37°C for 30 min, and 50 μl of each agonist dissolved in Krebs solution was added. The reaction was terminated by adding 500 μl ice cold 15% (w/v) trichloroacetic acid. After homogenisation, proteins were sedimented by centrifugation at 3000 *g* for 15 min at 4°C. Supernatants were separated and extracted three times with 3 volumes of H_2O-saturated diethyl ether and then titrated to pH 7.5 with $NaHCO_3$.

[^{3}H]-IP_3, [^{125}I]-cyclic AMP and [^{125}I]-cyclic GMP assay systems (Amersham TRK. 1000, RPA. 509 and RPA. 525, respectively) were used for the measurements of IP_3, cyclic AMP and cyclic GMP, respectively. The protein concentration was determined by the method of Lowry *et al.* (1951).

Measurements of Ca concentrations with fura 2

Muscle strips (0.3–0.5 mm long and 0.1 mm diameter) were loaded for 2 h in Krebs solution containing 5 μM acetoxy-

methyl ester (fura 2-AM) dissolved in dimethyl sulphoxide (DMSO) (premixed with cremophor EL; final concentration 0.02%), at room temperature (25 ± 1°C). The loaded muscle strips were fixed at both ends with 'Scotch' double-sided tape in a 1 ml chamber, the bottom was covered with 24 × 24 mm width and 0.13–0.17 mm thickness micro cover glass (Matsunami Glass IND. Ltd., Tokyo, Japan) and immersed in Krebs solution containing 2.6 mM Ca at room temperature. The chamber was fixed on the stage of a microscope (DIAPHOTO-TMD, Nikon, Tokyo, Japan).

Experiments were performed with a fluorimeter (Spex Fluorolog-2 Spectrofluorometer, Spex Industries Inc., N.J. U.S.A.). The muscle strips were excited by light obtained from xenon high pressure lamp (1907 OFR, 450 W) with power supplied by 1970 P and the measured field was a round spot 250 μm diameter in the middle portion of thin muscle strip. The slits at the entrance and exit ports of both the excitation and emission spectrophotometers were fixed at 2 mm and the bandpass was 3–4 mm. Two alternative excitation wavelengths, 340 nm (F340) and 380 nm (F380), were used and the emission was monitored at 505 nm. Both F340 and F380 were measured continuously and the ratio of F340/F380 was calculated.

The data were acquired and analysed on a DM 3000F Spectroscopy Computer (Spex, N.J., U.S.A.) with DM 3000 software. Determinations of absolute values of free Ca were difficult because of the difficulty in eliminating autofluorescence from the signal, the amount of fura 2-AM remaining (Luckhoff, 1986; Himpens & Somlyo, 1988) and also the unknown value of the dissociation constant of the fura 2-Ca complex in smooth muscle cytoplasm (Sato *et al.*, 1988). Therefore, in the present experiments absolute calibrations of the Ca transient (Grynkiewicz *et al.*, 1985) were only performed at the end of experiments and the maximum (Fmax) and minimum (Fmin) fluorescences were obtained by use of 10 μM ionomycin in Krebs and Ca-free solutions, respectively. An assumed dissociation constant of 224 nM (Tsien *et al.*, 1985) was used for calculating the cytosolic free Ca.

Statistics

The values are expressed as the mean ± s.d. or mean ± s.e. and accompanied by the number of observations. Statistical significance was assessed by Student's *t* test and *P* values less than 0.05 were considered significant.

Results

Mechanical responses evoked by spasmogenic agents in intact and skinned muscle tissues

Figure 1a shows the mechanical effects that five spasmogenic agents (high K, CCh, histamine, 5-HT and STA_2) evoked in tracheal smooth muscle strips. The amplitude of the K-induced contraction (128 mM) was much smaller than the CCh-induced contraction (10 μM), as previously observed in the rabbit trachea (Fujiwara *et al.*, 1988). Histamine- and 5-HT (10 μM)-induced contractions were larger than the K-induced contraction but smaller than the CCh-induced contraction. The minimum concentration of STA_2 required to evoke a contraction was 1 μM, and at 10 μM the amplitude was slightly enhanced, but the amplitudes were always smaller than those evoked by other agents. In Figure 1b, the concentration-response relationships are shown for CCh, histamine, 5-HT and STA_2. The contraction evoked by 10 μM CCh was normalised as a relative tension of 1.0. The ED_{50} values for CCh, histamine and 5-HT were 0.8, 1.1 and 0.6 μM, respectively (n = 5–7 preparations). When Ca-free solution containing 0.2 mM EGTA was applied 3 min before application of the spasmogenic agents, the K-induced contraction

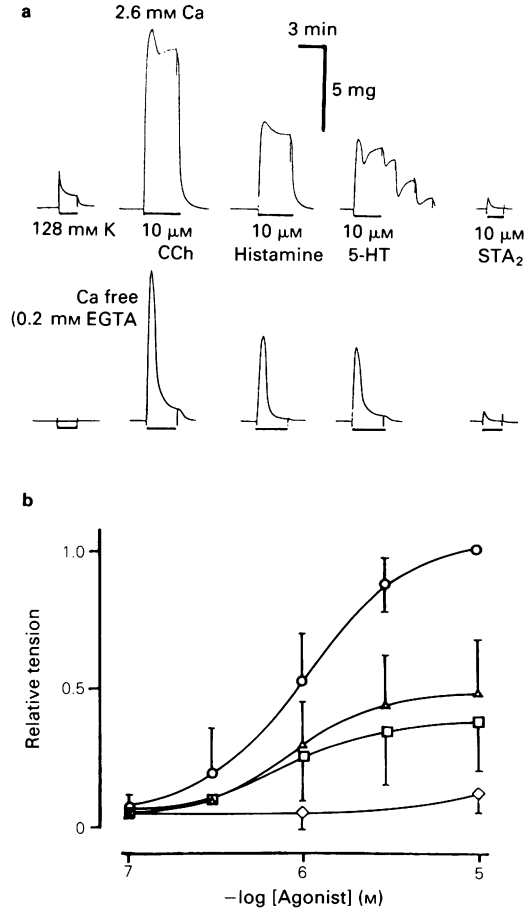

Figure 1 Contractions induced by 128 mM K, 10 μM carbachol (CCh), histamine, 5-hydroxytryptamine (5-HT) and 9,11-epithio-11,12-thromboxane A_2 (STA_2) in smooth muscle tissues in Krebs or Ca-free solution containing 0.2 mM EGTA (a) and the concentration-response relationships for the above spasmogenic agents (b). The peak amplitude of CCh-induced contraction (10 μM) was normalised as a relative tension of 1.0. Symbols with vertical bars indicate the mean value with s.d., n = 5. In (b), (○), CCh, (△) histamine, (□) 5-HT and (◇) STA_2.

and tonic responses of the CCh- histamine- and 5-HT-induced contractions ceased, but the phasic responses evoked by these agents remained almost unchanged. When 0.1 μM nifedipine was applied with the spasmogenic agents (10 μM), the tonic response was markedly inhibited but the phasic response only slightly inhibited (not shown), as previously observed in the rabbit trachea (Fujiwara *et al.*, 1988).

Figure 2 shows the contraction evoked by 10 μM CCh in an intact muscle tissue and that evoked by cumulatively applied Ca at various concentrations to skinned muscle tissue (see Methods). The minimum concentration of Ca required to evoke a contraction was 0.1 μM in relaxing solution and the maximum amplitude was obtained with 10 μM Ca. The contraction evoked by 1–3 μM Ca was of much the same amplitude as the CCh-induced contraction (10 μM). The concentration-response relationship for Ca observed in skinned muscle tissues (Figure 2b) was almost the same as that observed in the guinea-pig mesenteric artery (Itoh *et al.*, 1981). Figure 2c and d shows, respectively, the effects of IP_3 and caffeine on the Ca store site in skinned smooth muscle tissues. In skinned muscle tissue, 1 μM Ca buffered with 4 mM EGTA-containing relaxing solution was applied to accumulate Ca in the storage sites, and subsequently the tissue was rinsed with relaxing solution containing 0.1 mM EGTA with 6 mM inorganic phosphate (P_i) to relax the tissue. After the tissue had relaxed completely, that solution was replaced with 0.1 mM EGTA-containing relaxing solution for 2 min and either 20 μM IP_3 or 25 mM caffeine was applied. Used in this

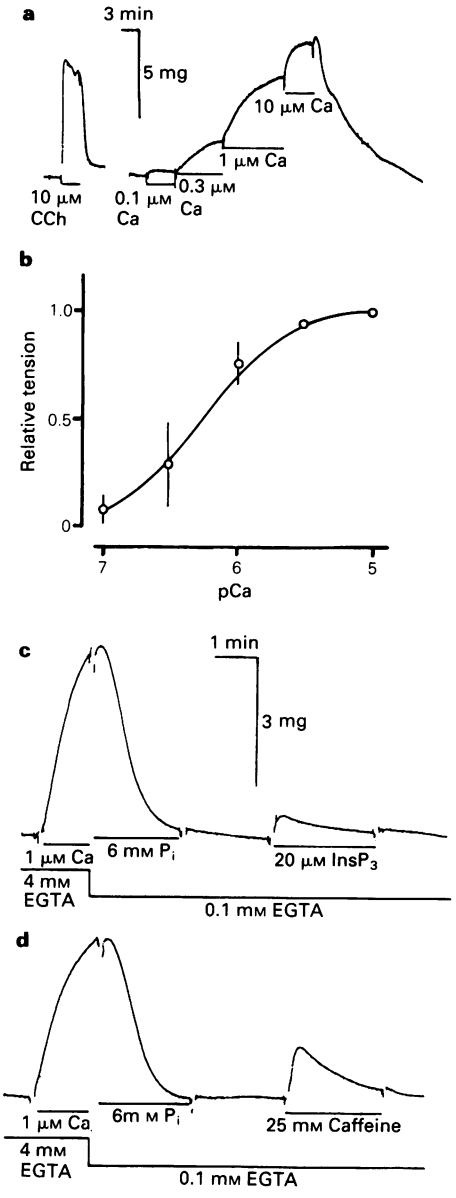

Figure 2 Effects of various concentration of Ca on skinned muscle tissues prepared by saponin treatment. (a) In intact tissues, 10 μM CCh was applied. After the CCh-induced contraction had been recorded, saponin 40 $\mu g\,ml^{-1}$ was applied for 20 min in relaxing solution in order to prepare skinned muscle. Various concentrations of Ca (0.1–10 μM) were cumulatively applied. (b) The concentration-response relationship for Ca in skinned muscle tissues. The contraction evoked by 10 μM Ca was normalised as 1.0 ($n = 5$). Symbols with vertical bars indicate mean ± s.d. ($n = 5$). (c) and (d), effects of IP_3 (c) or caffeine (d) on skinned smooth muscle tissues after accumulation of Ca in the storage site. P_i indicates inorganic phosphate added in the relaxing solution. The experimental procedures are described in the text. $InsP_3$ = inositol 1,4,5-trisphosphate.

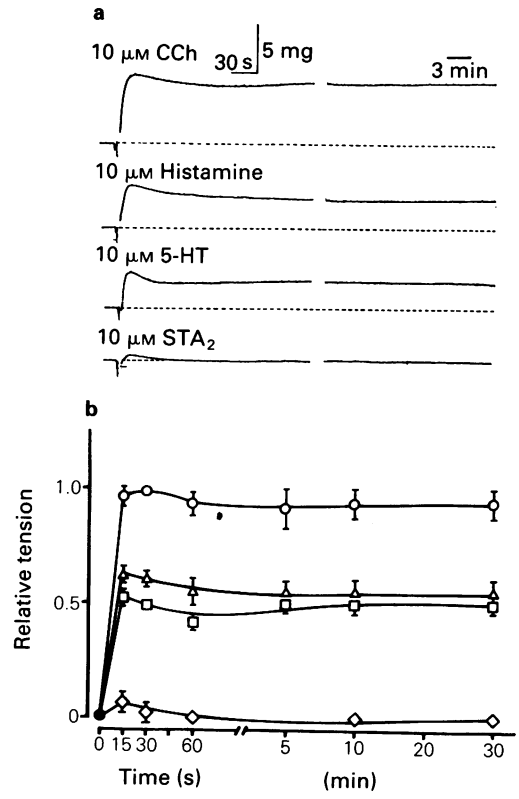

Figure 3 Mechanical responses evoked by 10 μM carbachol (CCh), histamine, 5-hydroxytryptamine (5-HT) or 9,11-epithio-11,12-thromboxane A_2 (STA_2) in smooth muscle tissues. (a) Mechanical responses evoked by the 4 different spasmogenic agents. (b) Time courses of mechanical responses evoked by the same agents. The peak amplitude of the CCh (10 μM)-induced contraction (at 30 s) was normalised as 1.0. Symbols and vertical bars indicate mean and s.d., $n = 6$. (○) CCh, (△) histamine, (□) 5-HT and (◇) STA_2.

way, both IP_3 and caffeine evoked a contraction. (Figure 2c and d).

Relationship between the contraction and phosphorylation of MLC_{20} evoked by spasmogenic agents

When spasmogenic agents, with the exception of STA_2, were applied for over 30 min, a sustained tonic contraction could be recorded (Figure 3a). In Figure 3b, the peak amplitude of the phasic response evoked by 10 μM CCh (measured at about 30 s after application) was normalised as a relative tension of 1.0. A few minutes after application of 10 μM CCh, histamine or 5-HT, the amplitude of contraction reached a steady value, which was slightly lower than the peak value, and then remained unchanged for over 30 min.

To investigate the relationship between contraction and phosphorylation of MLC_{20} under treatment with spasmogenic agents, the phosphorylation was measured by two-dimensional gel electrophoresis (see Methods). Phosphorylation of MLC_{20} induced by CCh, 5-HT and histamine increased in a concentration-dependent manner (Figure 4a–c). The amount of phosphorylation observed in the resting condition was 23.5 ± 4.6% ($n = 11$, control). The minimum

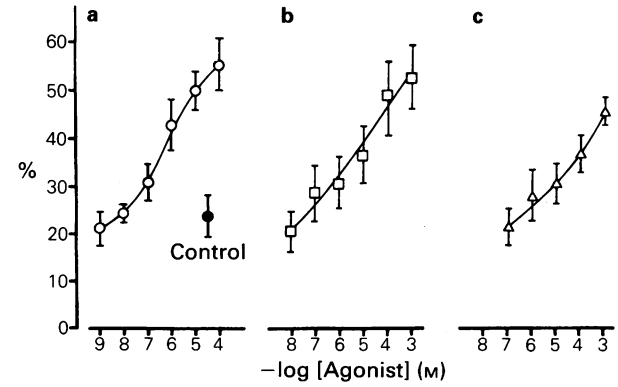

Figure 4 Effects of various concentrations of carbachol (CCh, a), 5-hydroxytryptamine (5-HT, b) or histamine (c) on phosphorylations of the 20 kD protein of myosin light chain (MLC_{20}). The measurement procedure was as described in the Methods. Control; basal phosphorylation level of MLC_{20} (see text; $n = 11$). Symbols and vertical bars indicate mean and s.d., $n = 5$–9.

concentration required to phosphorylate MLC_{20} was lower for CCh (10 nM) than for 5-HT or histamine (0.1 and 1 μM, respectively, $n = 5$–9). Even with 0.1 mM CCh or with 1 mM of 5-HT or histamine, phosphorylation did not reach the maximum value possible.

As shown in Figure 5a, when higher concentrations of CCh (0.1 mM), 5-HT (1 mM) and histamine (1 mM) were applied (i.e. the maximum concentrations of spasmogenic agents used in Figure 4), the peak increase in phosphorylation was observed within 1 min, but 30 min later, the amount of phosphorylation had fallen slightly.

Figure 5b shows the phosphorylation of MLC_{20} induced by applications of 10 μM CCh, histamine, or 5-HT for 30 min (cf. Figure 3). In Krebs solution, the phosphorylation was 23.5 ± 4.8% ($n = 11$) and 1 min after application of the spasmogenic agents, phosphorylation had increased to 49.3 ± 4.0 (CCh), to 30.7 ± 4.5 (histamine) or to 36.4 ± 6.6% (5-HT) ($n = 6$). In these experiments, attenuation of the phosphorylation of MLC_{20} in the presence of individual spasmogenic agents occurred very slowly, if at all. It was slight in the case of CCh even after 30 min, and with 5-HT and histamine, the increased phosphorylation remained unchanged (42.2 ± 4.3% for CCh, 31.6 ± 3.2% for histamine and 34.7 ± 3.2% for 5-HT, $n = 5$–6). The levels of phosphorylation induced by histamine and 5-HT (10 μM) reached almost the same value ($P > 0.05$).

Amount of cyclic nucleotides and IP_3 following application of spasmogenic agents

Figure 6 shows the amount of IP_3 synthesized following the application of various spasmogenic agents against time. Following the application of 10 μM CCh, 5-HT, histamine or STA_2, the maximum increase of IP_3 occurred after 15–30 s, and then rapidly declined to the control level within 3 min.

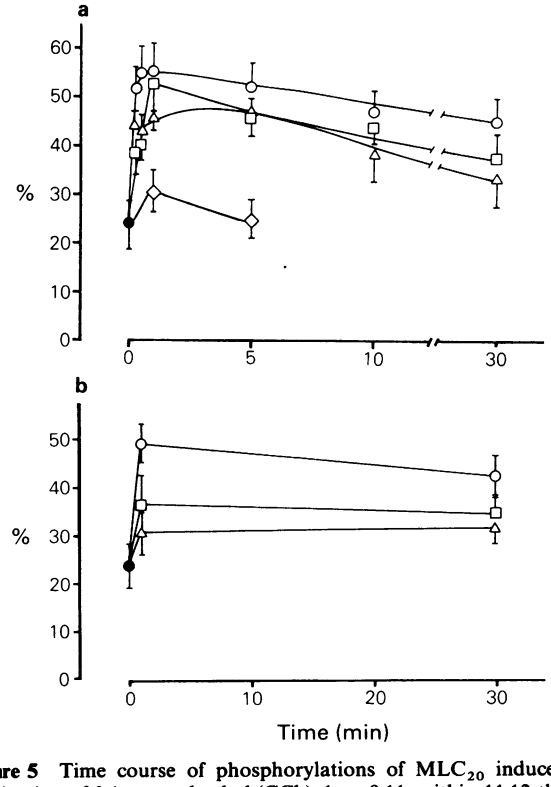

Figure 5 Time course of phosphorylations of MLC_{20} induced by application of 0.1 mM carbachol (CCh), 1 μM 9,11-epithio-11,12-thromboxane A_2 (STA_2), 1 mM 5-hydroxytryptamine (5-HT) or histamine (a) and 10 μM CCh, 5-HT, histamine or STA_2 (b). (●) Control (23.5 ± 4.8%, $n = 11$); (○) CCh, (□) 5-HT, (△) histamine and (◇) STA_2. Values are expressed as mean and vertical lines show s.d. ($n = 5$–6).

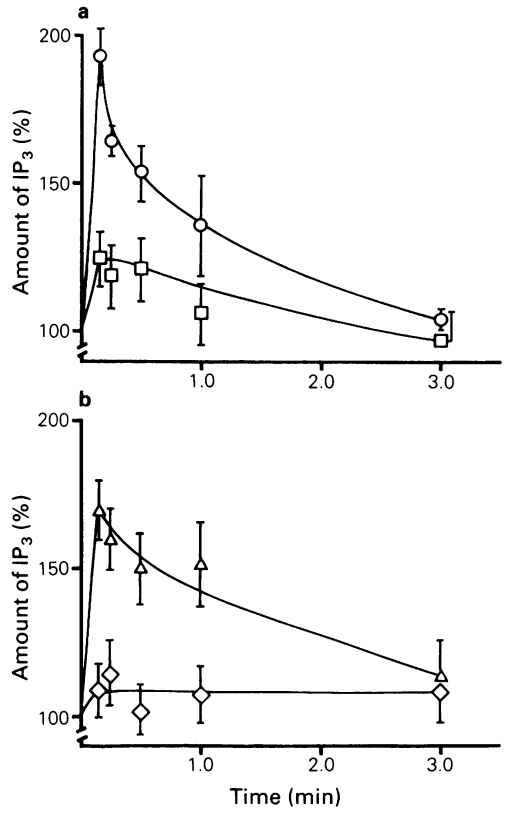

Figure 6 Time courses of synthesis of inositol, 1,4,5-trisphosphate (IP_3) induced by 10 μM carbachol (○) and 5-hydroxytryptamine (□) (a), and histamine (△) and 9,11-epithio-11,12-methano-thromboxane A_2 (◇) (b). Symbols and vertical bars indicate mean and s.d., $n = 4$–5. Concentrations of IP_3 measured before application of spasmogenic agents was normalised as 100% (3.77 ± 1.66 pmol mg^{-1} protein, $n = 18$).

The amount of IP_3 observed in the resting state (3.77 ± 1.66 pmol mg^{-1} protein, $n = 18$) was normalised as 100%. The maximum amount of IP_3 occurring on application of CCh (193 ± 9%, $n = 4$) was greater than that on application of histamine (169 ± 9%, $n = 4$) or 5-HT (125 ± 10%, $n = 5$). Following application of 10 μM STA_2, the synthesis of IP_3 was small and the difference from control was not statistically significant (114 ± 11%, $n = 5$, $P > 0.05$).

The concentration-response relationships for synthesis of IP_3 observed following applications of spasmogenic agents are shown in Figure 7. The amount of IP_3 synthesized before the application of agents was normalised as 100% (as shown in Figure 6). The relative amounts of IP_3 synthesized by spasmogenic agents was: CCh > histamine > 5-HT. The EC_{50} values for synthesis of IP_3 for CCh, histamine and 5-HT were 1 nM, 1.6 nM and 60 nM ($n = 4$–6), respectively. On application of cimetidine (100 μM), 5 min before and during application of 10 μM histamine, the increase in IP_3 induced by histamine remained unchanged, whereas, after application of mepyramine (10 μM) histamine failed to increase IP_3 (data are not shown).

The amount of cyclic AMP or cyclic GMP was also measured after application of spasmogenic agents. Isoprenaline, a substance known to produce cyclic AMP, was used for comparison with the spasmogenic agents (Figure 8a). The concentration of cyclic AMP before the application of any of the agents was normalised as a relative value of 100% (22.4 ± 2.96 pmol mg^{-1} protein, $n = 25$). Isoprenaline (10 μM) consistently increased cyclic AMP and after 3 min, the level had increased to twice control (198.7 ± 5.8%, $n = 5$). However, CCh (10 μM) within 15 s had slightly reduced the amount of cyclic AMP to 78.7 ± 10.9% ($n = 5$; $P < 0.05$), but 3 min after application the amount of cyclic AMP had recovered to 89.7 ± 6.9% of control ($n = 5$; $P > 0.05$). 5-HT (10 μM)

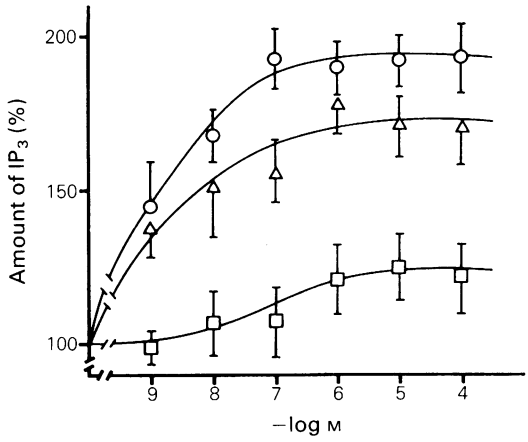

Figure 7 Effects of various concentrations (1 nM–0.1 mM) of carbachol (○), 5-hydroxytryptamine (□) or histamine (△) on synthesis of inositol 1,4,5-trisphosphate (IP_3). The maximum increase in concentration of IP_3 (15–30 s after application) is plotted. The amount of IP_3 measured before application of spasmogenic agents was normalised as 100% (3.77 ± 1.66 pmol mg^{-1} protein, $n = 18$). Symbols and vertical bars indicate mean and s.d., ($n = 4$–6).

and STA_2 (10 μM) had almost no effect on the synthesis of cyclic AMP (Figure 8a).

The effects of the various spasmogenic agents on the synthesis of cyclic GMP were also determined (Figure 8b). The amount of cyclic GMP measured before application of any spasmogenic agent was normalised as 100% (mean value 0.925 ± 0.11 pmol mg^{-1} protein, $n = 20$). Histamine (10 μM) consistently increased the amount of cyclic GMP, the maximum value being obtained 1 min after application (144.8 ± 12.8%, $n = 5$) and after 3 min it was still 123.9% of the control ($n = 5$).

This histamine-induced increase in cyclic GMP was marginally inhibited by cimetidine (100 μM; reduced to 121.0 ± 13.2% after 1 min, $n = 5$; $p > 0.05$) and significantly inhibited by mepyramine (10 μM; reduced to 116.0 ± 4.8%, after 1 min, $n = 5$; $p < 0.05$).

As shown in Figure 8b, CCh (10 μM) also increased cyclic GMP within 30 s, but after 1 min the amount of cyclic GMP had fallen by more than it had with histamine. On the other hand, 5-HT (10 μM) reduced the amount of cyclic GMP consistently and 3 min after its application, the amount was still lower than the control value (64.7 ± 4.7%, $n = 5$, $P < 0.01$). STA_2 (10 μM) had no effect on the amount of cyclic GMP.

Effects of CCh, histamine, 5-HT and STA_2 on the free concentration of Ca as measured with fura 2

The mean basal amount of Ca was calculated to be 180 nM ($n = 8$). When CCh (10 μM) was applied to the fura 2 loaded strips in Krebs solution, the Ca transient increased from the basal level of 182 nM to 1.42 μM within 10 s (see Methods), then the peak intensity gradually declined to a certain sustained level (480 nM after 3 min). Figure 9A shows the effects of 10 μM CCh, 5-HT, histamine and STA_2 on the Ca transient (F340 and F380 in Figure 9Aa and the ratio of F340/F380 in Figure 9Ab) compared to the mechanical responses induced by the various spasmogenic agents, Figure 9B shows a summary of the results obtained with the spasmogenic agents on the Ca transient ($n = 4$–8) at the peak and 2 min after their application. The peak amplitude of the CCh-induced fluorescence

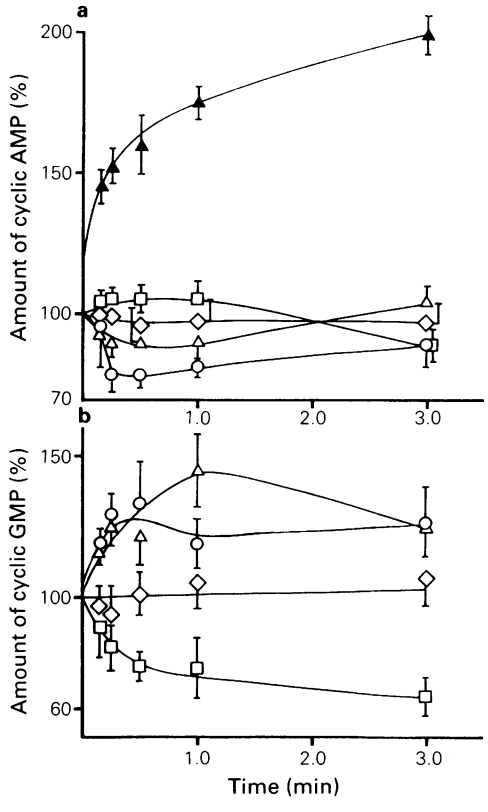

Figure 8 Effects of 10 μM carbachol (○), 5-hydroxytryptamine (□) histamine (△) or 9,11-epithio-11,12-methano-thromboxane A_2 (◇) on the synthesis of cyclic AMP (a) and cyclic GMP (b). To obtain a control synthesis of cyclic AMP, isoprenaline (10 μM) was applied (▲, a). The amounts of cyclic AMP and cyclic GMP measured before application of the drugs were normalised as 1.0 (22.4 ± 2.96 pmol mg^{-1} protein, $n = 25$ and 0.925 ± 0.11 pmol mg^{-1} protein, $n = 20$, respectively). Symbols and vertical bars indicate mean and s.d., $n = 5$.

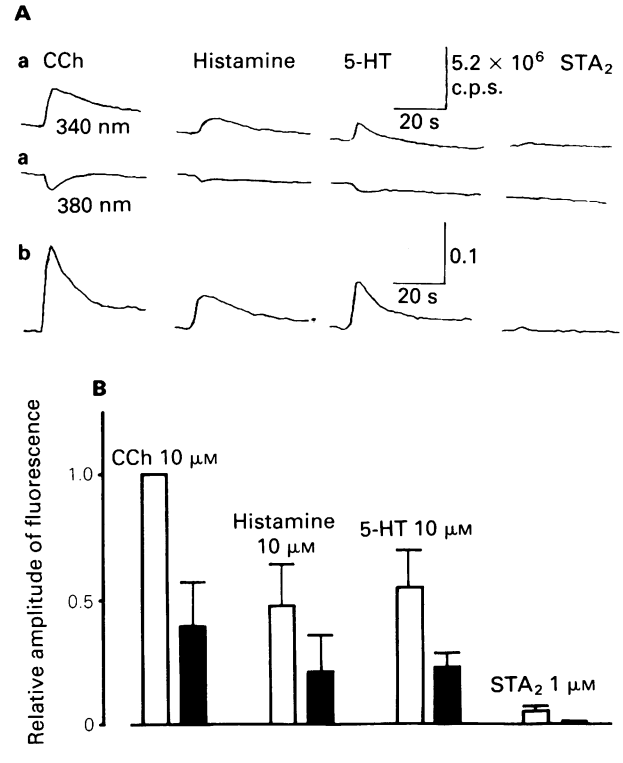

Figure 9 Ca transients measured on application of 10 μM carbachol (CCh), histamine, 5-hydroxytryptamine (5-HT) or 10 μM 9,11-epithio-11,12-methano-thromboxane A_2 (STA_2) with fura 2. (Aa and b) Measurements of fluorescence intensities at F340 and F380 and the ratio of these two intensities (F340/F380). (b) Comparison of fluorescence intensities measured in the presences of 10 μM CCh, histamine, 5-HT or STA_2 at peak (open columns) and 2 min after application (solid columns). The peak fluorescence intensity measured on application of 10 μM CCh was normalised as 1.0. Bars indicate s.d., $n = 6$.

intensity (F340/F380) was normalised as a relative value of 1.0. The intensity of fluorescence measured in the presence of 5-HT or histamine was only half that measured in the presence of CCh.

Discussion and conclusions

In the present experiments, the spasmogenic agents used on tracheal smooth muscle tissues were CCh, histamine, 5-HT and STA_2. All the agents produced contraction but to different extents. CCh produced the largest contraction, and 5-HT and histamine both produced a contraction which was half the amplitude of the CCh-induced contraction. STA_2 produced the smallest amplitude of transient contraction. Therefore, the experiments were carried out mainly with CCh, 5-HT and histamine.

The amplitude of the CCh-induced contraction (10 μM) was equivalent to the contraction evoked by 2 μM Ca, as estimated from the concentration-response relationship observed in skinned muscle tissues, and with 1.42 μM free Ca in the cytosol as estimated by fura 2, and about 50% of MLC_{20} was phosphorylated. Initial increases in the concentrations of Ca (as indicated by fura 2) are probably related to the increase in both the phasic contraction and synthesis of IP_3 (about 200%). In addition, CCh inhibited the synthesis of cyclic AMP and slightly increased the synthesis of cyclic GMP. Such concomitant changes in the cyclic nucleotides may partly modify the amplitude of contraction induced by CCh.

The minimum concentrations of the spasmogenic agents required to produce a contraction and the ED_{50} values for the mechanical responses were much larger than those required to synthesize IP_3. From the present experiments, we failed to explain these discrepancies. However, it is natural to postulate that some discrepancies may occur between the synthesis of IP_3, increased free Ca and generation of contraction. For example, the minimum concentration of Ca required to produce a contraction in skinned muscle strips was 100 nM (Figure 2), but since the intact tissue has a resting tone, much increased concentrations of the cytosolic Ca (or IP_3) might be required for the generation of a contraction.

Histamine (10 μM) and 5-HT (10 μM) produced only about half the amplitude of contraction evoked by 10 μM CCh. Histamine increased IP_3 to about 170% of the control, whereas 5-HT increased the amount of IP_3 to 125%. When the phosphorylation of MLC_{20} was compared, 5-HT and histamine produced much the same effect (30.7% vs 36.4% from 23.5%, respectively, $P < 0.05$). Thus, Ca released from the sarcoplasmic reticulum by IP_3 stimulated by histamine is less completely utilised for the phosphorylations of MLC_{20} through Ca-calmodulin-myosin light chain kinase-MLC_{20} processes than is the case with 5-HT. When the effects of synthesis of cyclic AMP or cyclic GMP were compared, the amount of cyclic AMP was not modified by either agent but the amount of cyclic GMP was increased by histamine, yet reduced by 5-HT. A histamine-induced increase in cyclic GMP has been demonstrated in bovine and guinea-pig tracheal smooth muscles (Duncan *et al.*, 1980; Ganellin & Parsons, 1982; Nakagawa *et al.*, 1986). It is known that cyclic GMP accelerates Ca extrusion by activation of Ca-ATPase at the sarcolemmal membrane (Popescu *et al.*, 1980; Suematsu *et al.*, 1984). This Ca-pump process may not occur through direct phosphorylation of Ca-ATPase (Eggermont *et al.*, 1988) but through an associated phosphorylation of phosphatidylinositol (Vrolix *et al.*, 1988). Furthermore, the amount of free Ca measured by fura 2 is reduced by application of nitrocompounds, such as nitroglycerin or nicorandil (Kobayashi *et al.*, 1985; Sumimoto *et al.*, 1987). Thus, Ca released by synthesized IP_3 may be partly pumped out in the extracellular space by cyclic GMP and, as a consequence, the amount of available free Ca bound to calmodulin may be reduced. Furthermore, cyclic GMP is known to reduce the affinity of the Ca-calmodulin complex for MLCK through phosphorylations of MLCK, and to inhibit the Ca-induced contraction in skinned muscles tissues (Itoh *et al.*, 1985; Nishikawa *et al.*, 1984). However, to clarify whether or not this inhibition of MLC_{20} by cyclic GMP plays a physiological role, further experiments are required (Kamm & Stull, 1986; 1987).

Histamine increased both IP_3 and cyclic GMP and these actions were inhibited by cimetidine and mepyramine respectively. Thus, IP_3 is synthesized through activation of the H_1-receptor. Synthesis of IP_3 requires activation of the receptor-GTP-activating protein-phospholipase C complex, but it is not clear whether synthesis of cyclic GMP requires the activation of the receptor-GTP activating protein-guanylate cyclase or direct activation of the agonist-receptor (guanylate cyclase) complex. Significantly, Chinkers *et al.* (1989) have shown that the receptor of atrial natriuretic peptide (ANP) is guanylate cyclase itself and this protein is a new class of mammalian sarcolemmal receptor which contains an extracellular ligand binding domain. Furthermore, Rapoport (1986) found that cyclic GMP synthesized by α-human ANP and nitro-compounds inhibited the synthesis of IP_3. On the other hand, Kajikuri & Kuriyama (1990) demonstrated that α-human ANP but not endothelium-derived relaxing factor inhibits the synthesis of IP_3 and this action has no causal relation to the synthesized cyclic GMP, since cyclic GMP and dibutyryl cyclic GMP had no effect on the synthesis of IP_3. It is, therefore, plausible to postulate that metabolic paths for the synthesis of second messengers may also interact with the synthesis of IP_3. During the application of 5-HT, cyclic GMP was reduced below the basal level. This response may act to preserve the increased amount of free Ca in the cytosol and, thus, produce a larger contraction than that expected from the synthesis of IP_3. However, the mechanism underlying the reduction in cyclic GMP is not yet clear.

In the present experiments, when CCh, histamine or 5-HT (10 μM) were applied the tissue produced a sustained contraction. When CCh was applied, the Ca transient measured with fura 2 was reduced to 0.34 times the peak value within 3 min (mean value of 480 nM) and the maximum synthesis of IP_3 occurred within 30 s and ceased within 3 min. Much the same effects were observed on application of 5-HT or histamine. Therefore, dissociations occurred on synthesis of IP_3 and the free Ca level during a sustained contraction. These discrepancies, may, in part, be due to increases in Ca influx during activation of receptors in intact tissues. The receptor-activated Ca influx may occur during the application of agonists. On the other hand, discrepancies also occurred between phosphorylations of the MLC_{20} and free Ca during the generation of a sustained contraction, i.e. phosphorylation remained unchanged after application of spasmogenic agents but the free Ca concentration transiently increased and then declined to a lower level. These responses are unlikely to be explained solely by a latch phenomenon, because the latch phenomenon is defined by a sustained contraction, with a reduction in the free concentration of Ca nearly to basal level, reduction in the phosphorylation of MLC_{20} and lowering of the cyclic rate of cross bridges (Gerthoffer & Murphy, 1983; Chatterjee & Murphy, 1983; Rembold & Murphy, 1986; Chatterjee & Tejada, 1986). Kamm & Stull (1986, 1987) noted that in smooth muscle tissues, the latch phenomenon was not a prerequisite for the production of a sustained contraction. The present experiments failed to explain the discrepancies observed between the sustained contraction, free Ca and phosphorylation.

In conclusion, CCh showed a stronger spasmogenic action than 5-HT, histamine or STA_2. In intact tissues, the actions of the spasmogenic agents, CCh, 5-HT, histamine and STA_2, for generation of the peak amplitude of contraction (phasic) may occur through increases in the phosphorylation of myosin. Increased cytosolic free Ca induced by synthesis of IP_3 has a causal relation to the peak amplitude and the phosphorylation, but not in a quantitative manner. The increase in the cytosolic Ca induced by IP_3 may be negatively or positively controlled by other second messengers. However, the

sustained contractions evoked by the spasmogenic agents cannot be explained by the interactions of the second messengers themselves or their metabolic paths. Thromboxane A_2 may play a minor role as a spasmogenic agent.

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Demonstration of extrapulmonary activity of angiotensin converting enzyme in intact tissue preparations

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1 The activity of angiotensin converting enzyme (ACE) has been studied on functional parameters of intact isolated preparations of extrapulmonary tissues. The conversion of angiotensin I (A I) to angiotensin II (A II) and the cleavage of bradykinin (BK) were used as indicators of ACE activity. Captopril was employed as a specific inhibitor of ACE.

2 Captopril augmented the BK-induced contractions of the rat isolated uterus, the BK- and substance P-induced contractions of the guinea-pig ileum, and the BK-induced venoconstriction in the isolated perfused ear of the rabbit. Degradation of BK by ACE was calculated to be 52% in the rat uterus and 75% in the rabbit perfused ear.

3 Captopril inhibited the A I-induced contractions of the rat isolated colon, the A I-induced vasoconstriction in the isolated perfused ear of the rabbit and the rise in blood pressure induced by i.a. injections of A I in pithed rats. Conversion of A I to A II was calculated to be 13% in the rat colon and 26% in the rabbit perfused ear.

4 From estimations of the A II activity (bioassay on the rat colon) in the effluent of the perfused ear of the rabbit after injections of A I into the arterial inflow cannula it was calculated that approximately one tenth of A I was converted to A II during a single passage through the ear (less than 15 s).

5 The present experiments suggest that the high activity of ACE in endothelium of blood vessels of extrapulmonary tissues may provide an additional (endothelium-dependent) local vasoconstrictor mechanism by the rapid formation of A II and inactivation of BK. The ACE activity in non-vascular smooth muscles, other than those of blood vessels, may also affect the physiological functions of these tissues.

Introduction

The angiotensin converting enzyme (ACE) catalyzes the conversion of angiotensin I (A I) to angiotensin II (A II) and inactivates bradykinin (BK). The antihypertensive effect of captopril has been mainly attributed to its action as an inhibitor of ACE and consequently to the prevention of the formation of A II. The inhibition of the cleavage of BK may also contribute to its antihypertensive effect (Carretero *et al.*, 1981; Lindsey *et al.*, 1987). Some of the side effects of ACE inhibitors, both beneficial and adverse, were also attributed to inhibition of BK cleavage (Wilkin *et al.*, 1980; Schölkens *et al.*, 1988). It is well established that the major site for the conversion of A I to A II by ACE is the vascular bed of the lung (Vane, 1974). By means of immunohistochemical and biochemical methods, ACE has also been demonstrated in other tissues such as kidneys, testes, and brush border membranes of the intestine (Huggins & Thampi, 1968; Roth *et al.*, 1969; Cushman & Cheung, 1971). In blood vessels the enzyme is localized in endothelial cells (Caldwell *et al.*, 1976). The physiological significance of ACE in extrapulmonary blood vessels has, however, been questioned (Ng & Vane, 1968; Oparil *et al.*, 1970; Marceau *et al.*, 1981; Whalley, 1987).

In order to gain information about a possible physiological role of extrapulmonary ACE, studies on intact tissue preparations appear to be more suitable than biochemical enzyme studies on tissue homogenates. Thus, in the present work pharmacological experiments on ACE activity in visceral smooth muscles, the isolated perfused ear of the rabbit and rat blood pressure were carried out with captopril as an inhibitor of ACE.

Methods

Smooth muscle preparations

Rat colon The ascending colon of Sprague-Dawley rats of either sex was cut longitudinally into halves and suspended in Krebs solution containing indomethacin (5.6 μM) and propranolol (5.0 μM) at 32°C as in the method of Regoli & Vane (1964). Isotonic contractions were recorded with a resting tension of 0.5 g. A I or A II was added to the organ bath at intervals of 3 min; the bath fluid was changed 1 min after the addition of the angiotensins. When the colon was used for the bioassay of A II activity in effluents from the isolated perfused ear of the rabbit, captopril (46 μM) was added to the Krebs solution.

Rat uterus The uterus of Sprague-Dawley rats was used in which oestrous had been induced by s.c. injection of diethylstilboestrol 100 μg kg^{-1}, 16 h before cervical dislocation and exsanguination. The distal parts of the uterine horns were suspended at 32°C in De Jalón solution which contained indomethacin (2.8 μM) and atropine (0.35 μM). Isotonic contractions were recorded with a resting tension of 0.5 g. BK was added to the incubation medium at 5 min intervals and left in the organ bath for 1 min.

Guinea-pig ileum Pieces of ileum (1.5 cm) were suspended in Tyrode solution at 37°C. Isotonic contractions were recorded with a resting tension of 2.0 g. BK, substance P (SP), histamine or carbachol were added at intervals of 3 min and left in contact with the ileum for 1 min. The contractile responses were expressed as a percentage of the maximum response.

Isolated perfused ear of the rabbit

Vasoconstriction Rabbits of either sex were killed by an i.v. injection of an overdose of pentobarbitone sodium and the auricular arteries of both ears were cannulated. The ears were separated from the head and perfused with Tyrode solution at 37°C under constant pressure; the initial flow rate was adjusted to 6 ml min^{-1}. Noradrenaline (NA), BK, A I or A II were injected at intervals of 45–60 min. The venous outflow was measured with an electronic drop interval recorder. In each experiment captopril (3.8 μM) was added to the perfusion medium of one ear and the other ear was used as control.

Removal of the endothelium Rabbit ears were perfused as described above. The vasoconstrictor effects of NA (50 pmol),

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A I (100 pmol) and BK (10 pmol) were determined in the presence or absence of captopril (3.8 μM). Subsequently, the endothelium was removed by a modification of the method of Spokas & Folko (1984): air was pushed through the blood vessels with a 20 ml syringe, followed by a quick short perfusion with 20 ml Tyrode solution. Both procedures were repeated 3 times. Normal perfusion was then continued for at least 30 min. The injections of NA, A I and BK were then repeated.

Bioassay on the rat colon of angiotensin II contained in the effluent of the perfused rabbit ear For this purpose the rabbit ear was perfused as described above. However, the flow rate was kept constant at 1.5 ml min^{-1} using a roller pump. Indomethacin (5.6 μM) was added to the Tyrode solution to eliminate the formation of prostaglandins which may interfere with the bioassay of A II on the rat colon. A I or A II were injected into the arterial inflow cannula. The venous effluent was collected in 1 min fractions before and during the 2 min period following the angiotensin injections. A II equivalent activity was then determined in these fractions by comparing the effects of 100–200 μl portions of the test samples with the effects of known amounts of A II on the rat colon.

Blood pressure in pithed rats

Male Sprague-Dawley rats were pretreated with hyoscine butylbromide (30 mg kg^{-1}, s.c.); 30 min later the rats were anaesthetized with ether and pithed (Shipley & Tilden, 1947); artificial respiration with a mixture of 95% O_2 and 5% CO_2 was applied and the rectal temperature was maintained at 37°C. Blood pressure was recorded in one carotid artery with a pressure transducer. The contralateral carotid artery was cannulated retrogradely and used for i.a. injections of A I and A II (in volumes of 0.1 ml). The cannula was rinsed with 0.2 ml of a 154 mM NaCl solution after each injection. Captopril (23 nmol min^{-1}, 0.05 ml min^{-1}) was infused into a jugular vein.

Physiological salt solutions

The composition of the salt solutions (in mM) was as follows: Krebs: NaCl 118.1, KCl 4.6, $CaCl_2$ 2.5, $MgSO_4$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 15.5, glucose 10.0. De Jalón: NaCl 153.9, KCl 5.6, $CaCl_2$ 0.7, $MgCl_2$ 0.1, $NaHCO_3$ 5.9, glucose 3.0. Tyrode: NaCl 136.9, KCl 2.9, $CaCl_2$ 1.8, $MgCl_2$ 1.15, NaH_2PO_4 0.4, $NaHCO_3$ 11.9, glucose 5.0. The solutions were gassed with a mixture of 95% O_2 and 5% CO_2.

Substances

The following substances were used: bradykinin, ^{5}Ile-angiotensin I, noradrenaline (Sigma, U.S.A.); ^{5}Ile-angiotensin II (Calbiochem-Behring, U.S.A.); substance P (SP) (Bachem, Switzerland); carbachol (Ebewe, Austria); histamine, diethylstilboestrol (Serva, F.R.G.); captopril (Squibb, Austria); atropine, indomethacin (Merck Sharp & Dohme, U.S.A.); (−)-propranolol (ICI, U.K.); pentobarbitone sodium (Nembutal; Ceva, F.R.G.); hyoscine butylbromide (Buscopan; Boehringer Ingelheim, F.R.G.).

Statistical analysis

The log-logit dose-response curves to BK, NA, A I and A II in the perfused ear of the rabbit, on the rat uterus and on the rat colon were calculated by the least squares method. The regression lines were tested for non-identity and non-parallelism by F tests (Zar, 1984). When the regression lines were found to be non-coincidental and parallel, the horizontal distance was calculated (Geigy, 1980) to estimate the extent of degradation or conversion. These values are given with 95% confidence limits. All other values are given as means ± s.e.mean.

When individual groups of responses to the same dose of a drug in the absence and presence of captopril were compared the Mann-Whitney U test was used (see Figures 1, 4 and 5). To compare contractions of the guinea-pig ileum in response to drugs before and after the addition of captopril to the bath fluid, multiple nonparametric comparisons with a control (Zar, 1984) were made (see Figure 3). Drug-induced vasoconstrictions in the perfused ear of the rabbit before and after removal of the endothelium were compared by the Wilcoxon matched pairs signed rank test; vasoconstriction in the absence or presence of captopril was compared by the Mann-Whitney U test (see Figure 6). For the comparison of the hypertensive effects of A I and A II in pithed rats before and during an infusion of captopril the Wilcoxon-Wilcox test was used (see Figure 8).

Results

Smooth muscle preparations

Rat colon Concentration-response curves to A I and A II were established on the rat colon (Figure 1). After testing for parallelism and difference in location ($F_{(1;69)} = 312.8$; $P < 0.001$) the horizontal distance was determined as 0.89 (0.85–0.94) log units giving an estimate for the conversion of A I to A II of 13% (12%–14%) during the contact time of 1 min. The validity of this estimate requires that A I does not possess an intrinsic contractile activity. To test this requirement captopril (46 μM) was added to the bathing solution. In the presence of captopril A I in concentrations up to 125 nM no longer elicited contractions of the colon. Only when the concentration of A I was increased to 250 nM was a contraction observed and this was significantly ($P < 0.01$) smaller than that elicited by the same concentration of A I in the absence of captopril (see Figure 1).

Rat uterus Concentration-response curves for BK were established on the rat uterus in the absence and presence of captopril (46 μM) (Figure 2). The log-logit regression lines were

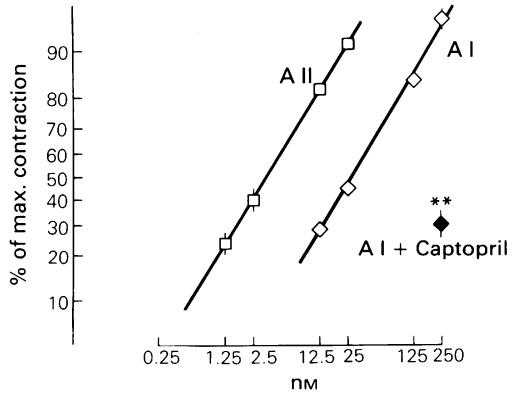

Figure 1 Rat isolated colon: concentration-response curves for angiotensin I (A I, ◇) and angiotensin II (A II, □); (◆) contraction induced by A I (250 nM) in the presence of captopril (46 μM). The log-logit regression lines were calculated by the least squares method. Values are given as means with s.e.mean (vertical lines); where no s.e.mean is given it was smaller than the symbol. $n = 5$–13. Significance of difference from value obtained in the absence of captopril: ** $P < 0.01$ (Mann-Whitney U test).

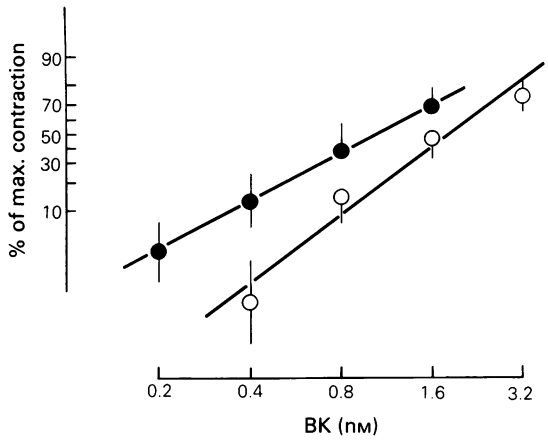

Figure 2 Rat isolated uterus: concentration-response curves to bradykinin (BK) in the absence (○) and presence (●) of captopril (46 μM). Least squares regression analysis of the log-logit regression lines showed the regression lines to be non-coincidental and no significant difference in the slopes could be detected. The difference in location was highly significant ($P < 0.01$); the values given represent mean values and vertical lines show s.e.mean; $n = 6$.

found to be parallel but with a significant difference in location ($F_{(1;45)} = 9.70$; $P < 0.01$). The horizontal distance between the regression lines of 0.32 (0.20–0.45) log units leads to an estimate of 52% (37%–65%) for the degradation of BK by ACE.

Guinea-pig ileum Test doses of BK (23 nM), SP (2.2 nM), carbachol (33 nM) or histamine (90 nM) were applied to the isolated guinea-pig ileum until reproducible contractions were obtained (Figure 3). Captopril was then added to the organ bath. Subsequent contractions induced by the test dose of BK were more than doubled ($P < 0.01$ and $P < 0.05$). For SP the increase in the response was much smaller but still significant ($P < 0.05$), following captopril. This confirms that SP is a poor substrate for ACE (Skidgel *et al.*, 1984). The effects of carbachol and histamine were not affected by captopril.

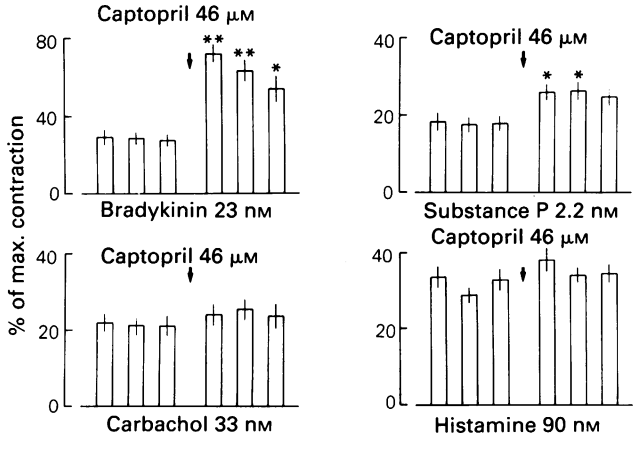

Figure 3 Guinea-pig isolated ileum: bradykinin (23 nM), substance P (2.2 nM), carbachol (33 nM) and histamine (90 nM) were added to the organ bath at regular intervals of 5 min. After a contact time of 1 min the tissue was washed by emptying and refilling the organ bath three times. When the response was constant, a single dose of captopril (46 μM) was added at the points indicated by arrows and the additions of the contracting drugs were continued as previously. Magnitude of contractions is given as % of the maximum contraction. Means with s.e.mean shown by vertical lines; $n = 6$. Significance of difference from the last contraction before the addition of captopril: * $P < 0.05$; ** $P < 0.01$ (multiple nonparametric comparisons with a control).

Isolated perfused ear of the rabbit

Vasoconstriction in response to noradrenaline, angiotensins and bradykinin Isolated rabbit ears were perfused under constant pressure and dose-response curves were established for the vasoconstrictor effects of NA, A I and A II, and BK (Figures 4 and 5) in the presence and absence of captopril (3.8 μM) in the perfusion fluid. The response to NA (Figure 4) was not affected by captopril ($F_{(2;74)} = 1.10$; $P > 0.10$).

For A II a linear dose-response relation in the absence of captopril was established (Figure 4). When captopril was present, the regression analysis showed a significant deviation from linearity ($F_{(1;24)} = 6.26$; $P < 0.05$) and no regression line was calculated. The effects of 100 pmol A II were significantly ($P < 0.05$) enhanced by captopril. The effects of higher doses of A II were not influenced by captopril. A few experiments with a dose of 50 pmol A II also suggested an augmentation by captopril (data not shown).

The dose-response curve of A I was linear and of a slope similar to that of A II, but with a significantly different location ($F_{(1;47)} = 21.33$; $P < 0.001$; Figure 4). The horizontal distance between the regression lines for A I and A II was 3.78 (2.85–5.10) log units, which suggests a conversion of 26% (20%–35%) of A I to A II during its passage through the ear. In the presence of captopril (3.8 μM) A I was completely ineffective in doses up to 1 nmol; the vasoconstriction observed in response to 2 nmol A I was significantly smaller than that observed in the absence of captopril ($P < 0.05$) (Figure 4).

The reduction of the venous outflow from the perfused ear of the rabbit induced by BK is due to venoconstriction (Goldberg *et al.*, 1976). The log-logit dose-response regression lines for BK in the absence and presence of captopril (3.8 μM) were parallel and non-coincidental ($P < 0.001$). When captopril (3.8 μM) was present in the perfusion medium, the dose-response curve to BK was shifted to the left by 0.61 (0.49–0.72) log units when compared with the curve obtained under control conditions (Figure 5). In the presence of captopril the effect of 50 pmol BK was larger than that of 200 pmol BK in the absence of captopril. It was calculated that degradation of BK by ACE in the perfused ear of the rabbit was 75% (68%–81%).

By injecting Evans blue it was found that the perfusion time of the rabbit ear under the experimental conditions used was less than 15 s.

Removal of the endothelium Using an immunofluorescence technique, Caldwell *et al.* (1976) have found that ACE is

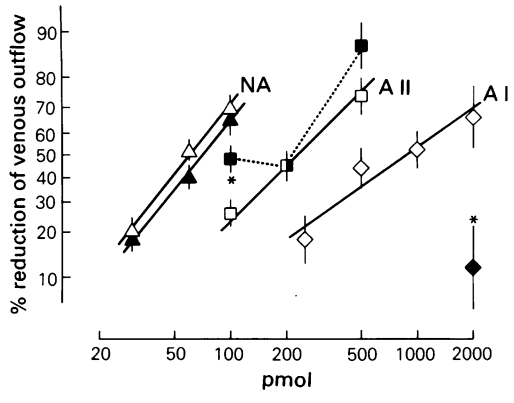

Figure 4 Isolated perfused ear of the rabbit: vasoconstriction induced by angiotensin I (A I, ◇), angiotensin II (A II, □) and noradrenaline (NA, △) in the absence (open symbols) and presence (closed symbols) of captopril (3.8 μM). The least squares method was used to calculate log-logit regression lines. In the presence of captopril no regression line was calculated for A II because of significant deviation from linearity ($P < 0.05$). Each symbol represents % reduction of venous outflow and vertical lines show s.e.mean; $n = 5$–14. Significance of difference from control values: * $P < 0.05$ (Mann-Whitney U test).

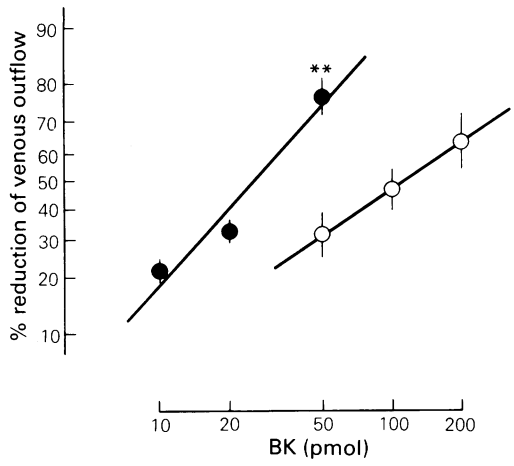

Figure 5 Isolated perfused ear of the rabbit: vasoconstriction induced by bradykinin (BK) in the absence (○) and presence (●) of captopril (3.8 μM). Log-logit regression lines were calculated by the least squares method. Significance of difference from control values: ** $P < 0.01$ (Mann-Whitney U test). Each symbol represents % reduction of venous outflow, vertical lines give s.e.mean; $n = 7$–10.

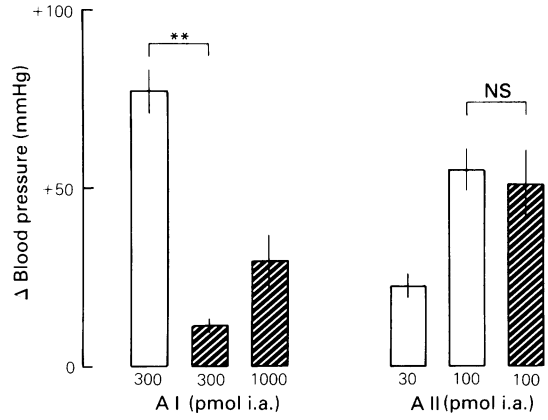

Figure 8 Rise in mean arterial blood pressure induced by i.a. injections of angiotensin I (A I) and angiotensin II (A II) in pithed rats; doses (in pmol) given below the columns, before (open columns) and during an i.v. infusion of captopril (23 nmol min^{-1}, hatched columns). Column height represents mean values, vertical lines show s.e.mean; $n = 5$. Significance of difference from control values: ** $P < 0.002$ (Wilcoxon-Wilcox test); NS, not significant.

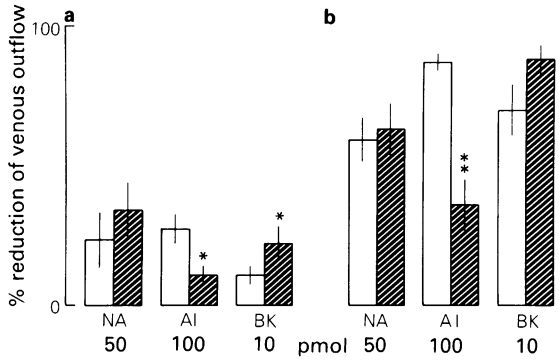

Figure 6 Isolated perfused ear of the rabbit: % reduction of venous outflow induced by i.a. injections of noradrenaline (NA), angiotensin I (A I) and bradykinin (BK), at doses given in pmol below the columns, in the absence (open columns) or presence (hatched columns) of captopril (3.8 μM). (a) Endothelium intact; (b) endothelium removed. Column heights represent mean values, vertical lines show s.e.mean; $n = 6$–8. Significance of difference from values obtained in the absence of captopril: * $P < 0.05$, ** $P < 0.01$ (Mann-Whitney U test).

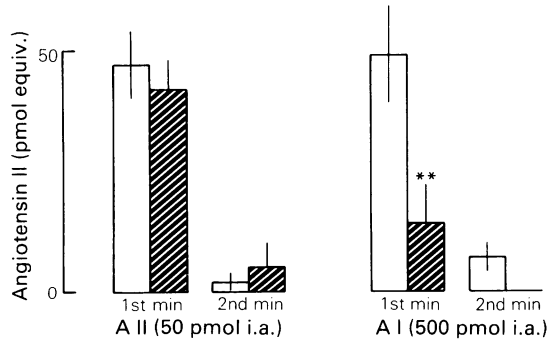

Figure 7 Bioassay on the rat colon of angiotensin II (A II) in the effluent of the perfused ear of the rabbit: 50 pmol A II or 500 pmol angiotensin I (A I) were injected i.a. into the rabbit ear which was perfused at a constant flow of 1.5 ml min^{-1}. The venous effluent was collected in 1 min fractions, from ears perfused with captopril (3.8 μM) either present (hatched columns) or absent (open columns). Captopril (46 μM) had been added to the bathing solution of the rat colon in order to prevent the conversion of any A I left in the test samples by angiotensin converting enzyme (ACE) present in the colon. Column height represents mean values, vertical lines show s.e.mean; $n = 5$–8. Significance of difference from controls: ** $P < 0.01$ (Mann-Whitney U test).

almost exclusively localized in vascular endothelial cells. Therefore, we investigated the effect of captopril on the actions of NA, A I and BK in the rabbit ear before and after removal of the endothelium. From Figure 6 it can be seen that removal of the endothelium strongly enhanced the effects of NA, AI and BK ($P < 0.01$). Before removal of the endothelium captopril reduced the effect of A I and enhanced the potency of BK ($P < 0.05$) as seen previously (compare Figures 4 and 5). After removal of the endothelium the A I-induced vasoconstriction was still significantly inhibited by captopril ($P < 0.01$).

Bioassay on the rat colon of angiotensin II in the effluent of the rabbit perfused ear The results obtained with the ACE inhibitor captopril and the methods described above provided indirect evidence for the formation of A II and for the degradation of BK in several tissue preparations. For the quantitative estimation of the amount of A II formed from A I in the isolated perfused ear of the rabbit perfusion was carried out at a constant flow of 1.5 ml min^{-1}; fractions of the effluent were collected over periods of 1 min and tested for A II activity on the rat isolated colon. Captopril was present in the bathing solution of the colon in order to prevent further conversion of any A I in the effluent by ACE present in the rat colon (compare Figure 1).

When 50 pmol A II were injected into the inflow cannula of the rabbit ear, the total amount was recovered in the effluents collected during the 1st and 2nd min after the injection (Figure 7). This excludes any significant degradation of A II during its passage through the rabbit ear. None of the samples collected before or during the 3rd min after the injections showed any activity on the rat colon. Addition of captopril to the perfusion medium of the ears did not alter the recovery of A II in the effluents (Figure 7).

When 500 pmol A I was injected into the circulation of the rabbit ear, the effluent collected during the 1st min after the injection contained 49 ± 10 pmol A II equivalent activity; 7 ± 3 pmol A II equivalent activity was found in the sample collected during the 2nd one min period after the injection (Figure 7). When captopril was present in the perfusion medium, the A II equivalent activity in the venous outflow was reduced to 14 ± 8 pmol in the 1st one min fraction; none was detectable thereafter. The amounts of A I required to elicit contractions of the rat colon (even in the presence of captopril compare Figure 1) were much larger than the amounts that could be expected to be present in the venous outflow of the perfused ear after the injection of A I. Therefore, the A II equivalent activity estimated on the rat colon

represents in all probability the amount of A II formed from A I during a single passage through the rabbit ear. It indicates a conversion of approximately one tenth.

Blood pressure in pithed rats

The pithed rat preparation was used because of its high sensitivity to pressor substances (Shipley & Tilden, 1947). Various doses of A I and A II were injected retrogradely into a carotid artery followed by a fast injection of 0.2 ml of a 154 mM NaCl solution. The peak of the rise in blood pressure induced by A I as well as by A II occurred already before the end of the saline injection. When captopril (23 nmol min^{-1}) was infused into a jugular vein starting 5 min before the next injection, the effect of A II was the same as under control conditions (Figure 8), that of A I was reduced by about 90% ($P < 0.01$). The effect elicited by 1000 pmol A I was comparable to that of 30 pmol A II in the absence of captopril. Thus, extensive conversion of A I to A II occurred in less than 12 s.

Discussion

Immunohistochemical and biochemical methods allow precise localization of ACE in tissues and the estimation of its activity in tissue homogenates. Its functional significance depends, however, on the availability of its substrates, A I or BK, which are supplied either through the blood stream or are formed in the same tissue where ACE is active. Although there is good evidence that most of the circulating A I is converted to A II by ACE in the lungs, some A I might reach the extrapulmonary circulation and may be converted to A II by ACE present in vascular endothelium in other tissues (Caldwell *et al.*, 1976). Some A I might also be converted to A II in extravascular tissues, e.g. in the brush border membranes of the intestine (Ward *et al.*, 1980). It has been demonstrated that angiotensinogen can be synthesized in extrahepatic tissues such as the aorta (Hellmann *et al.*, 1988). In addition, renin was found to be synthesized in aortic smooth muscles (Re *et al.*, 1982). Both these observations point to a possible local formation of A I. In the present study the specific ACE inhibitor captopril has been employed to gain information on the possible physiological function of ACE in extrapulmonary tissues.

In isolated smooth muscle preparations which contain brush border membranes such as the rat colon and the guinea-pig ileum (Figures 1 and 3), the conversion of A I to A II was found to occur at a fast rate. This was also the case in the rat uterus (Figure 2). Regoli & Barabé (1980), however, could not find a change in the response of the rat isolated uterus to BK after application of captopril.

During the short time required for the perfusion through the vascular bed of the rabbit isolated ear (less than 15 s), a considerable proportion of A I injected into the arterial inflow cannula was converted to A II as indicated by the degree of the ensuing vasoconstriction (Figure 4) and by the bioassay on the rat colon of A II equivalent activity in the venous effluent (Figure 7). These experiments do not allow a precise determination of the rate of conversion of A I to A II. However, the differences between the results obtained in control experiments and in experiments in which ACE was inhibited by captopril suggest a conversion rate of 10 to 25% in less than 1 min. After the removal of the endothelium from the vessels in the ear some ACE activity was still present (Figure 6). This could be due to incomplete removal of the endothelium and/or to the presence of ACE activity in deeper layers of the vessel wall as suggested previously (Saye *et al.*, 1984; Story & Ziogas, 1986; Schölkens & Tilly, 1986; Pipili *et al.*, 1989).

Observations on the blood pressure of the pithed rat (Figure 8) did not allow a distinction between the conversion of A I to A II in the pulmonary circulation and that in peripheral vessels because of the short circulation time. As the immediate rise in blood pressure induced by A I i.a. was greatly inhibited by captopril, a considerable contribution of ACE in peripheral vessels seems to be likely.

In contrast to A I, BK is formed within the tissues. Its short half-life in the blood (less than 30 s; Saameli & Eskes, 1962; McCarthy *et al.*, 1965; Ferreira & Vane, 1967) indicates a rapid inactivation by ACE not only in pulmonary but possibly also in peripheral vessels (Griesbacher *et al.*, 1989). BK causes local arterial vasodilatation, venoconstriction, plasma extravasation, prostaglandin release and nociceptor stimulation. In the present study the effects of BK on the rat uterus (Figure 2), guinea pig ileum (Figure 3) and isolated perfused ear of the rabbit (Figure 5) were augmented by captopril. This is evidence for cleavage of BK by ACE in these tissues. In the rabbit ear, the apparent inactivation of BK (75%) by ACE was more pronounced than the conversion of A I to A II (see above); this finding is consistent with the results of Stewart *et al.* (1981) who found that BK is a better substrate for ACE than A I. In contrast, Whalley & Wahl (1983), working with cat cerebral arteries, found that ACE converts A I but does not inactivate BK. It is still controversial whether BK contributes to the regulation of vascular tone but experiments with a BK antagonist have indicated that BK could have a significant effect on vasomotor tone when ACE is inhibited (Schölkens *et al.*, 1988).

The results of the present investigation suggest that the ACE activity in extrapulmonary blood vessels and smooth muscles may be of physiological significance. If, under *in vivo* conditions, A I reaches extrapulmonary vessels or is formed there locally from angiotensinogen, it would be rapidly converted to A II by endothelial ACE. The rate of conversion may be modulated by factors such as thrombocyte adhesion, inflammatory plasma extravasation or other forms of endothelial irritation. In addition to endothelial-derived relaxing factor and endothelin, the local conversion of A I to A II may, thus, contribute to the local regulation of vasomotor tone.

Note added in proof

Recently, Schalekamp *et al.* (*Br. J. Clin. Pharmacol.*, **28**, 105S–113S (1989)) reported that, in man, a major fraction of regionally produced angiotensin I appears to be formed locally, i.e. not in circulating plasma.

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Interactions between adenosine and phorbol esters or lithium at the frog neuromuscular junction

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1 Interactions between the effects of adenosine or 2-chloro-adenosine (CADO) and the effects of substances that interfere with the phosphoinositides/protein kinase C transducing system or with the adenylate cyclase transducing system, on endplate potentials (e.p.ps), were investigated. The preparation used was the innervated sartorius muscle of the frog in which twitches had been prevented with high magnesium concentrations.

2 The activator of protein kinase C, 4β-phorbol-12,13-diacetate (PDAc), reversibly increased the amplitude and the quantal content of e.p.ps and attenuated the inhibitory effects of adenosine and CADO on e.p.p. amplitude. The affinity of the adenosine receptor antagonist, 8-phenyltheophylline, was not modified by PDAc.

3 The phorbol ester 4α-phorbol-12,13-didecanoate, which does not activate protein kinase C, did not modify either e.p.p. amplitude or the inhibitory effect of adenosine on e.p.ps.

4 The inhibitor of protein kinase C, polymyxin B, reversibly decreased the amplitude and the quantal content of e.p.ps, prevented the enhancement caused by PDAc on e.p.p. amplitude, but did not modify the inhibitory effect of adenosine on e.p.ps. H-7, another inhibitor of protein kinases, also decreased e.p.p. amplitude but did not modify the effect of PDAc on the amplitude of e.p.ps.

5 Lithium chloride, which alters phosphoinositide signal transduction by inhibiting the breakdown of inositol phosphates, reversibly increased the amplitude and the quantal content of the e.p.ps. In the presence of adenosine or CADO the effect of lithium on e.p.p. amplitude was markedly attenuated.

6 The activator of adenylate cyclase, forskolin, reversibly increased the amplitude and the quantal content of the e.p.ps. MDL 12,330A, an inhibitor of adenylate cyclase, irreversibly decreased e.p.p. amplitude, an effect which was prevented by forskolin. Neither forskolin nor MDL 12,330A modified the inhibitory effect of adenosine on e.p.ps.

7 The results suggest that the phosphoinositides/protein kinase C transducing system, but not the adenylate cyclase transducing system, might be involved in the inhibitory effect of adenosine on neuromuscular transmission.

Introduction

Adenosine inhibits transmitter release at the neuromuscular junction (Ginsborg & Hirst, 1972; Ribeiro & Walker, 1975) by activating a xanthine-sensitive adenosine receptor (Ribeiro & Sebastião, 1985; Sebastião & Ribeiro, 1988a; 1989). The most frequently proposed mechanism to explain the inhibitory effect of adenosine on transmitter release involves calcium (e.g. Ribeiro & Sebastião, 1986). Receptors affecting calcium mobilization may operate through modifications of the phosphoinositides/protein kinase C transducing system (e.g. Abdel-Latif, 1986; Putney, 1987).

In the present work we investigated whether activators and inhibitors of the phosphoinositides/protein kinase C transducing system could affect the inhibitory action of adenosine on neuromuscular transmission. The ability of an activator and an inhibitor of adenylate cyclase to modify the effect of adenosine on neuromuscular transmission was also investigated because in some cells adenosine acts through modifications of adenylate cyclase activity (e.g. Daly *et al.*, 1981; Ribeiro & Sebastião, 1986), and interactions between the adenylate cyclase and the phosphoinositide/protein kinase C transducing systems might occur (see Nishizuka, 1986).

Brief accounts of some of the results have already appeared (Sebastião & Ribeiro, 1988b;c).

Methods

The experiments were carried out at room temperature (22–25°C) on innervated sartorius muscles of the frog (*Rana ridibunda*). The preparations were mounted in a Perspex chamber of 5 ml capacity through which the solutions flowed continuously at a rate of 5 ml min^{-1} via a roller pump. The bath volume was kept constant by suction. Solutions were changed by transferring the inlet tube of the pump from one flask to another. This involved a minimum of disturbance to the preparation and allowed prolonged recording from the same fibre with many solution changes. However, because of the slow rate of flow it was not possible to estimate rates of onset of the effects of the substances. The change-over times in the figures of this paper indicate the times at which the inlet tube of the pump was transferred to a new solution.

Evoked endplate potentials (e.p.ps) were recorded in the conventional way (Fatt & Katz, 1951) with intracellular electrodes filled with 3 M KCl and 10–20 MΩ resistance. The bath electrode was an Ag-AgCl pellet. The nerve was stimulated supramaximally with rectangular pulses of 20 μs duration applied once every 2 s. Evoked responses of 64 consecutive stimuli were averaged after amplification, with a Datalab DL-4000 computer. The output of the computer was coupled to a pen recorder. The usual procedure was to continue to record averages in the same solution until a stable value was obtained, i.e. until two or three averages differed by less than 2%. The nerve was stimulated at a constant rate throughout the experiments and at least 30 min to 1 h before the recording was begun. The time from the beginning to the peak of each individual e.p.p. was less than 1 ms.

The normal bathing solution (pH 7.0) contained (mM): NaCl 117, KCl 2.5, NaH_2PO_4 1, Na_2HPO_4 1, $MgCl_2$ 1.2 and $CaCl_2$ 1.8. The twitches of the muscle in response to nerve stimulation were prevented by increasing the concentration of magnesium ($MgCl_2$ 9–12.5 mM) in the bath.

Statistical analysis

Data are expressed as mean ± s.e.mean. The significance of the differences between means was calculated by Student's *t*

test. P values of 0.05 or less were considered to represent statistically significant differences.

Drugs

The following drugs were used: adenosine, 2-chloro-adenosine, forskolin, 1-(5-isoquinolinylsulphonyl)-2-methyl-piperazine, 4β-phorbol-12,13-diacetate, 4α-phorbol-12,13-didecanoate, polymyxin B sulphate (Sigma); 8-phenyltheophylline (R.B.I.). Lithium chloride (analytical grade) was from Merck. MDL 12,330A (N-(as-2-phenylcyclopentyl)azacyclo-tridecan-2-imine hydrochloride) was a gift from Merrell Dow Pharmaceuticals Inc., U.S.A., 4β-phorbol-12,13-diacetate and 4α-phorbol-12,13-didecanoate were made up, respectively, in 20 mM and 10 mM stock solutions in dimethylsulphoxide (DMSO). Forskolin was made up in a 10 mM stock solution in absolute ethanol. 1-(5-Isoquinolinylsulphonyl)-2- methylpiperazine was made up in 60 mM or 20 mM stock solutions in absolute ethanol. 8-Phenyl-theophylline was made up in a 10 mM stock solution in 80% methanol (v/v) containing 0.2 M NaOH. Dilutions of these solutions were used.

Results

Phorbol esters

Figure 1A illustrates the effect of 4β-phorbol-12,12-diacetate (PDAc, 100 nM), an activator of protein kinase C (Castagna *et al.*, 1982; see also Baraban, 1987), on the average amplitude of e.p.ps. PDAc (100 nM) reversibly increased the average amplitude of e.p.ps without modifying in a consistent manner their decay phase or the membrane resting potential of the muscle fibre. The 4α-phorbol-12,13-didecanoate (PDDec) which is inactive on protein kinase (Castagna *et al.*, 1982), applied to the same endplate in a concentration (1 μM) ten times higher than that of PDAc, had virtually no effect on e.p.ps (Figure 1B). In fifteen endplates from different preparations, PDAc (100 nM) increased e.p.p. amplitude by 24 $\pm$ 1.4% ($P < 0.05$). PDAc (100 nM) caused similar increases (22 $\pm$ 0.5%) in the quantal content of the e.p.ps (determined by the variance method, Hubbard *et al.*, 1969), without affecting in an appreciable way the quantal size. This suggests that the enhancing effect of PDAc on neuromuscular transmission is mainly presynaptic, increasing evoked transmitter release. A higher concentration (250 nM) of PDAc was also used in two experiments and it caused greater effects both on the amplitude (62 $\pm$ 4% increase) and on the quantal content (67 $\pm$ 1% increase) of the e.p.ps.

The full effect of PDAc (100–250 nM) on e.p.p. amplitude was usually seen in the first 20 to 30 min after starting perfusion and disappeared within 30 min after returning the preparation to the control bathing solution. The increase in e.p.p. amplitude caused by PDAc could not be attributed to its solvent, dimethylsulphoxide (DMSO) since the concentration of DMSO present in the PDAc solutions (0.0005–0.00125% v/v) was lower than that present in the PDDec solutions (0.01% v/v), which did not modify e.p.p. amplitude.

Figure 2 illustrates the action of PDAc (100 nM) on the concentration-response curve for the inhibitory action of adenosine (1–10 μM) on e.p.p. amplitude. In each endplate the effects of different concentrations of adenosine on the averaged amplitude of e.p.ps were tested cumulatively, first in the absence of PDAc. Adenosine was then washed out and PDAc (100 nM) was applied to the preparation until its full effect was observed. A cumulative concentration-response curve for the effect of adenosine in the presence of PDAc (100 nM) was then performed. In some experiments, the effect of adenosine after washing out PDAc was also tested and no appreciable differences were found between the effects of the same concentration of adenosine applied to the preparations before PDAc or after washing out PDAc. Adenosine (1–10 μM) caused a concentration-dependent decrease in e.p.p. amplitude both in the presence and in the absence of PDAc but, as illustrated in Figure 2, in the presence of PDAc (100 nM) the effect of aden-

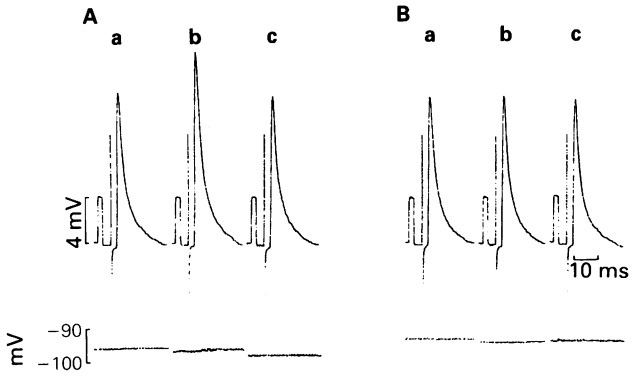

Figure 1 Effect of 4β-phorbol-12,13-diacetate (PDAc) (A) and absence of effect of 4α-phorbol-12,13-didecanoate (PDDec) (B) on the averaged amplitude of evoked endplate potentials (e.p.ps) recorded from a frog sartorius muscle fibre. Solutions contained magnesium 11 mM, which prevented muscle action potentials and twitches in response to nerve stimulation. Upper part: pen recorder traces of averaged e.p.ps; each trace is the computed average of sixty-four successive e.p.ps preceded by the stimulus artifact and by a calibration pulse of 4 mV amplitude and 2 ms duration. Lower part: membrane resting potential. (A) Responses recorded in the control bathing solution before applying PDAc (a); 20 min after starting perfusion of PDAc (100 nM) (b); 20 min after returning to the control bathing solution (c). (B) Responses recorded in the control bathing solution before applying PDDec (a); 20 min after starting the perfusion of PDDec (1 μM) (b); 10 min after returning to the control bathing solution (c). The responses in (A) and (B) were recorded from the same endplate.

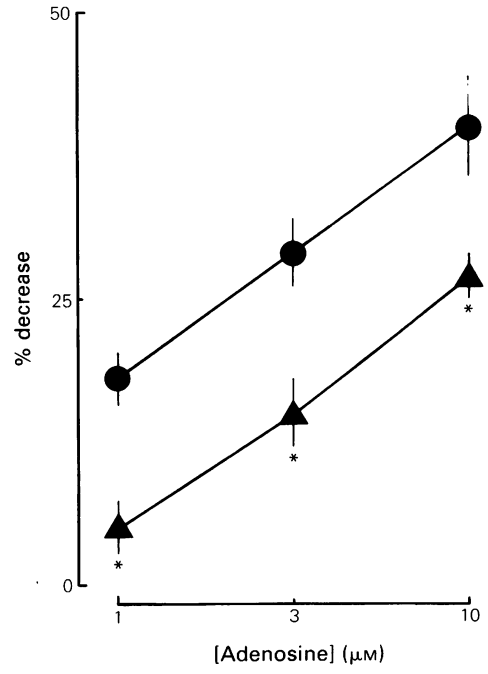

Figure 2 Action of 4β-phorbol-12,13-diacetate (PDAc) on the log concentration-response curve for the inhibitory effect of adenosine on the amplitude of evoked endplate potentials (e.p.ps). The ordinate scale shows the percentage decrease in the amplitude of e.p.ps recorded intracellularly from innervated frog sartorius muscle preparations paralysed with magnesium 9–10 mM. (●) Adenosine; (▲) adenosine + PDAc (100 nM). Zero % is the e.p.p. amplitude in the absence of adenosine (13.0 $\pm$ 3.5 mV for (●), and 15.5 $\pm$ 3.9 mV for (▲)) and 100% represents a complete inhibition of e.p.ps. The results are the average of four experiments where the effects of adenosine in the absence and in the presence of PDAc (100 nM) were compared in the same endplate. Averaged resting membrane potential: -98 ± 4 mV. * $P < 0.05$ (paired Student's t test) when compared with the effect of the same concentration of adenosine in the absence of PDAc. The vertical bars represent $\pm$ s.e.mean.

osine was significantly ($P < 0.05$) attenuated. The concentration of adenosine causing 25% decrease in e.p.p. amplitude (which corresponds to about 50% its maximal effect), calculated from the data shown in Figure 2, was increased by a factor of 3.3 ± 0.2 ($n = 4$) in the presence of PDAc (100 nM).

PDDec (1 μM), which is inactive on protein kinase C (Castagna *et al.*, 1982), did not modify in an appreciable manner the inhibitory effect of adenosine on e.p.p. amplitude. Thus, in one experiment, 1 μM and 3 μM of adenosine decreased e.p.p. amplitude by 19% and 36%, respectively, in the absence of PDDec and by 17% and 39%, respectively, in the presence of PDDec (1 μM).

As with adenosine, the inhibitory effect of 2-chloroadenosine (CADO, 0.1–3 μM) on e.p.p. amplitude was attenuated by PDAc (100 nM). The concentration of CADO that caused 25% decrease in e.p.p. amplitude was increased by a factor of 3.1 ± 0.4 ($n = 4$) in the presence of PDAc (100 nM), a value which is not statistically different ($P > 0.05$) from that observed when adenosine was used as the adenosine receptor agonist.

The finding that PDAc attenuates, to a similar extent, the inhibitory effects of adenosine and of CADO, which has low affinity for the adenosine uptake system (Jarvis *et al.*, 1985), might indicate that either the adenosine receptor or the transducing system associated with the adenosine receptor is modified in the presence of PDAc. In an attempt to discriminate between these two possibilities experiments were designed in which the ability of PDAc to modify the affinity of an adenosine receptor antagonist, 8-phenyltheophylline (8-PT), was investigated. In these experiments a concentration-response curve for the inhibitory effect of the adenosine receptor agonist, CADO, was first performed. After removing CADO from the bath, 8-PT, in a concentration (1 μM) virtually devoid of effect on e.p.p. amplitude, was applied to the preparation. After an equilibration period of at least 30 min with 8-PT, a concentration-response curve for the inhibitory effect of CADO in the presence of 8-PT (1 μM) was then obtained and the ratio between equiactive concentrations (CR) of CADO in the presence and in the absence of 8-PT was calculated at the level of 25% inhibition of e.p.p. amplitude. 8-PT and CADO were then washed out and PDAc (100 nM) was applied to the preparation until its full effect was observed. With a constant concentration of PDAc (100 nM) throughout the experiment, a second pair of concentration-response curves for the inhibitory effect of CADO in the absence and in the presence of 8-PT (1 μM) was performed and the CR value caused by 8-PT (1 μM) in the presence of PDAc (100 nM) was calculated. Figure 3 shows the results obtained in one experiment. As can be seen, 8-PT (1 μM) shifted to the right in a near parallel manner the concentration-response curve for the inhibitory effect of CADO on e.p.p. amplitude, and this shift was of similar magnitude either in the absence (Figure 3a) or in the presence (Figure 3b) of PDAc (100 nM). Similar results were obtained in two other experiments and the averaged CR, pA_2 and K_i values obtained for 8-PT in the three experiments are shown in Table 1. None of these values was modified in a statistically significant ($P > 0.05$) manner by PDAc (100 nM). The antagonism by 8-PT of the inhibitory effect of CADO on e.p.p. amplitude could not be attributed to its solvent (80% methanol with 0.2 M NaOH) since all solutions without 8-PT had the same concentration (0.01% v/v) of the solvent.

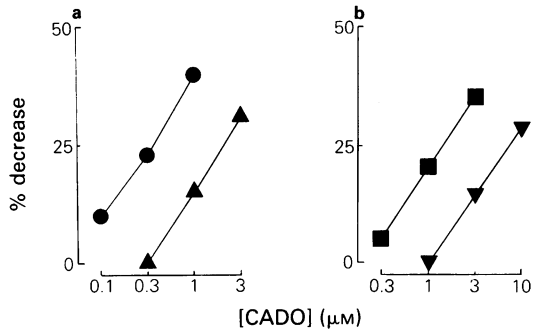

Figure 3 Comparison between the ability of 8-phenyltheophylline (8-PT) to antagonize the inhibitory effect of 2-chloroadenosine (CADO) on the amplitude of evoked endplate potentials (e.p.ps) recorded from a muscle fibre in the absence (a) and in the presence (b) of 48-phorbol-12,13-diacetate (PDAc, 100 nM). The solutions contained magnesium 12 mM. Each panel shows the log concentration-response curves for the inhibitory effect of CADO in the absence (●, ■) and in the presence (▲, ▼) of 8-PT (1 μM). The ordinates show the percentage decrease in e.p.p. amplitude caused by CADO. Zero % is the amplitude of e.p.ps before starting the perfusion of CADO (13.7 mV in (a) and 16.7 mV in (b)) and 100% represents a complete inhibition of e.p.ps. The results in (a) and (b) were obtained in the same endplate. Resting membrane potential: −87 mV.

H-7 and polymyxin B

The effect of the protein kinase C inhibitor, 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine (H-7) (see Hidaka & Hagiwara, 1987), on neuromuscular transmission was tested in one experiment. H-7 in concentrations of 20 μM and 60 μM reversibly decreased by 11% and 26%, respectively, the averaged amplitude of e.p.ps, but increased duration and prolonged the decay phase of the e.p.ps. This contrasts with the action of PDAc which did not affect the decay phase of the e.p.ps. The effects of H-7 on e.p.ps cannot be attributed to the solvent, ethanol, since all solutions without H-7 contained the same concentration (0.1% v/v) of ethanol. H-7 (20–60 μM) did not prevent the enhancement of neuromuscular transmission caused by PDAc (100 nM) which in the same experiment increased e.p.p. amplitude by 23% in the absence of H-7, and by 24% and 23% in the presence of 20 μM and 60 μM H-7, respectively. Since H-7 affected the decay phase of the e.p.ps (suggesting a postsynaptic action) and was unable to modify the effect of PDAc on neuromuscular transmission, no further experiments with H-7 were performed.

Figure 4 illustrates the effect of another inhibitor of protein kinase C, polymyxin B (Kuo *et al.*, 1983), on the averaged amplitude of e.p.ps. Polymyxin B (0.5–1 μg ml^{-1}) caused a reversible and concentration-dependent decrease in the amplitude of e.p.ps virtually without modifying the decay phase or the resting membrane potential of the muscle fibre. The averaged decrease in e.p.p. amplitude caused by 0.5 μg ml^{-1}, 1 μg ml^{-1} and 2.5 μg ml^{-1} of polymyxin B were 8 ± 1.0% ($n = 2$), 19 ± 1.4% ($n = 6$) and 31 ± 2.0% ($n = 2$) respectively. The quantal content (determined by the variance method) of

Table 1 Adenosine receptor antagonism by 8-phenyltheophylline (8-PT) in the absence (control) and in the presence of 4β-phorbol-12,13-diacetate (PDAc) at the frog neuromuscular junction

	CR	pA_2	K_i (nM)
Control	5.67 ± 0.69	6.66 ± 0.07	225 ± 37
With PDAc (100 nM)	5.28 ± 0.65	6.62 ± 0.07	245 ± 38

Concentration-ratio (CR) values were determined at the level of 25% decrease in endplate potential (e.p.p.) amplitude caused by 2-chloroadenosine in the presence of 8-PT (1 μM) and in the absence of the xanthine in the same endplate. pA_2 values were calculated by an abbreviated Schild analysis, where a slope of unity was used for log (CR − 1) on log[8-PT] (cf. Sebastião & Ribeiro, 1989). K_i values were taken as the negative antilog of the pA_2 value. The results are the mean ± s.e.mean obtained in 3 experiments. The values in the absence (control) and in the presence of PDAc (100 nM) were obtained in the same endplates. Averaged e.p.p. amplitude in the control bathing solution: 6.3 ± 3.7 mV. Averaged increase in e.p.p. amplitude caused by PDAc: 21 ± 5%. Averaged resting membrane potential: 92 ± 2 mV. Solutions contained magnesium 10–12 mM.

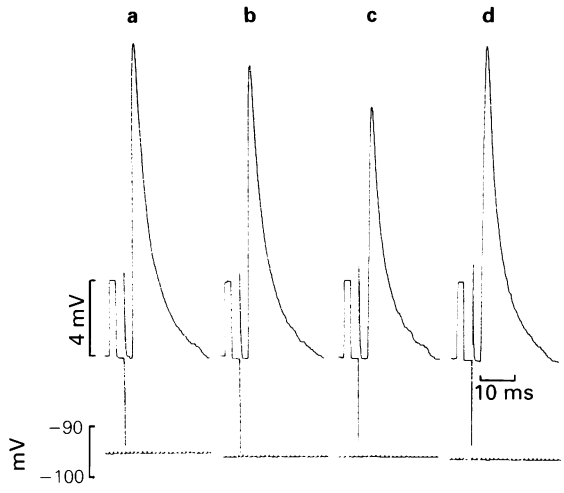

Figure 4 Effect of polymyxin B on the averaged amplitude of evoked endplate potentials (e.p.ps). Solution contained magnesium 11.5 mM. Upper part: pen recorder traces of averaged e.p.ps recorded: in the control bathing solution before applying polymyxin B (a); 15 min after starting perfusion of polymyxin B (0.5 μg ml^{-1}) (b); 25 min after starting perfusion of polymyxin B (1 μg ml^{-1}) (c); 30 min after returning to the control bathing solution (d). Calibration pulse: 4 mV amplitude and 2 ms duration. Lower part: membrane resting potential. Assuming a M_r-value of 1000 for polymyxin B (Kuo *et al.*, 1983) the concentrations of polymyxin B are 0.5 μM in (b) and 1 μM in (c). Details as in legend to Figure 1.

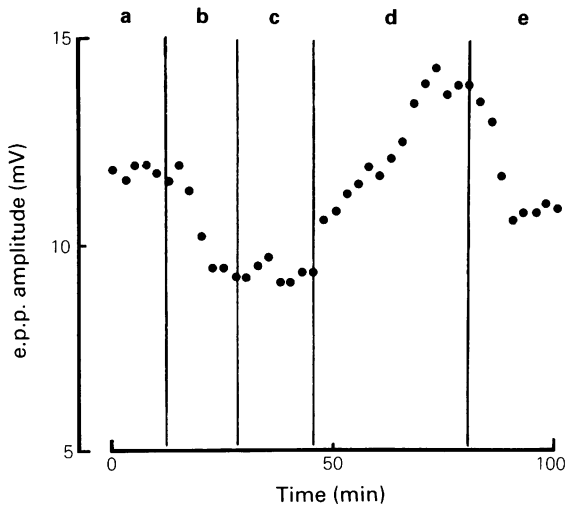

Figure 5 Blockade by polymyxin B of the enhancement caused by 4β-phorbol-12,13-diacetate (PDAc) on the amplitude of evoked endplate potentials (e.p.ps) recorded intracellularly from an innervated frog sartorius muscle preparation. The solutions contained magnesium 9 mM. The ordinate scale shows the amplitudes of the computed averages of 64 successive e.p.ps and the abscissa scale the times the averaging began. (a) and (e) Control bathing solution; (b) polymyxin B (1 μg ml^{-1}); (c) polymyxin B (1 μg ml^{-1}) + PDAc (100 nM); (d) PDAc (100 nM). Membrane resting potential: −80 mV. For the molar concentration of polymyxin B see legend to Figure 4.

the e.p.ps was decreased (20 ± 2%) by polymyxin B (1 μg ml^{-1}) without a change in the quantal size. This indicates that the main effect of polymyxin B on neuromuscular transmission is presynaptic to decrease evoked transmitter release.

The full effect of polymyxin B (0.5–2.5 μg ml^{-1}) on e.p.p. amplitude was usually seen in the first 15 to 25 min after the start of perfusion and disappeared within 30 min after returning to the control solution.

In contrast with that observed with H-7, polymyxin B (1 μg ml^{-1}) prevented the enhancement caused by PDAc (100 nM) on neuromuscular transmission. Thus, PDAc (100 nM) when applied to the preparation in the presence of polymyxin B (1 μg ml^{-1}) did not increase e.p.p. amplitude, but after removing polymyxin B from the bath the phorbol ester increased, in the usual way, the amplitude of the e.p.ps (Figure 5). In two determinations from the same endplate, PDAc increased e.p.p. amplitude by 23 ± 3% in the absence of polymyxin B and by only 4 ± 4% in the presence of polymyxin B (1 μg ml^{-1}).

The effects of adenosine (1–3 μM) on e.p.p. amplitude in the absence and in the presence of polymyxin B (1 μg ml^{-1}) were compared in six experiments. In these experiments 1 μM and 3 μM of adenosine inhibited e.p.p. amplitude by 11 ± 1.8% and 25 ± 7.8%, respectively, in the absence of polymyxin B and by 12 ± 1.5% and 27 ± 8.6%, respectively, in the presence of polymyxin B (1 μg ml^{-1}). No statistically significant ($P > 0.05$) differences were found between the effects of the same concentration of adenosine in the absence and in the presence of polymyxin B (1 μg ml^{-1}).

Lithium ions

The effect of lithium chloride, which alters phosphoinositol signal transduction by inhibiting breakdown of inositol phosphates (see e.g. Sekar & Hokin, 1986), on e.p.ps is illustrated in Figure 6. LiCl (10 mM) reversibly increased the amplitude of e.p.ps without modifying the decay phase or the resting membrane potential of the muscle fibre. The enhancement caused by LiCl (10 mM) on neuromuscular transmission could not be attributed to an increase in the osmolarity of the solutions since in the experiments using LiCl (10 mM) all solutions without LiCl contained sucrose (20 mM). The osmolarity of the solutions was measured and differed by less than 2%.

The average increase in e.p.p. amplitude caused by LiCl (10 mM) in thirteen experiments was 18 ± 1.1% ($P < 0.05$). In two experiments where the quantal content of the e.p.ps was calculated, LiCl (10 mM) increased both the quantal content and the amplitude of the e.p.ps by 15 ± 1%, without a change

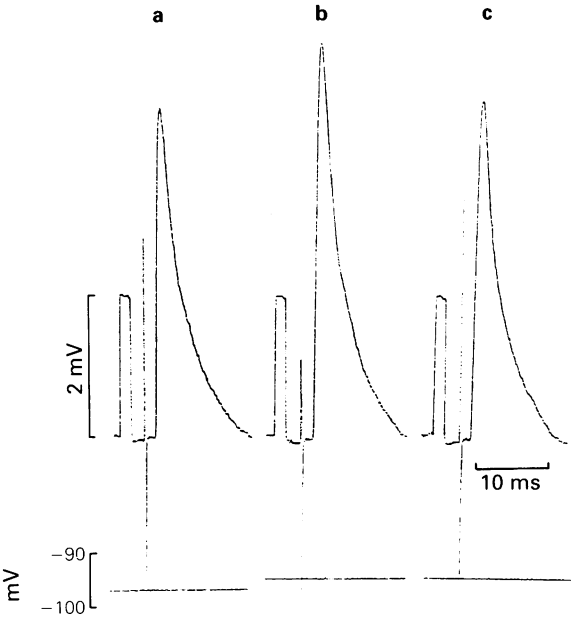

Figure 6 Effect of lithium (LiCl 10 mM) on the averaged amplitude of evoked endplate potentials (e.p.ps). Solutions contained magnesium 12 mM. Upper part: pen recorder traces of averaged e.p.ps recorded: in the control bathing solution before applying LiCl (a); 30 min after starting perfusion of LiCl (10 mM) (b); 40 min after returning to the control bathing solution (c). Calibration pulse: 2 mV amplitude and 2 ms duration. Lower part: resting membrane potential. All solutions without LiCl contained sucrose (20 mM). Details as in legend to Figure 1.

in quantal size. It appears, therefore, that the main effect of LiCl (10 mM) on neuromuscular transmission is presynaptic to increase the evoked release of the transmitter.

The full effect of LiCl (10 mM) on neuromuscular transmission was usually seen in the first 30 to 40 min after starting perfusion and disappeared within 20 to 40 min after returning the preparation to control solution.

The ability of adenosine (10 μM) to modify the enhancement caused by LiCl (10 mM) on neuromuscular transmission was tested in four experiments. The effect of LiCl (10 mM) was tested first in the absence of adenosine. LiCl (10 mM) was then washed out and adenosine (10 μM) was perfused until the full effect was observed. The effect of LiCl (10 mM) in the presence of adenosine (10 μM) was then tested. In four experiments LiCl (10 mM) increased e.p.p. amplitude by 17.5 $\pm$ 2.2% before adenosine and by 4 $\pm$ 0.7% in the presence of adenosine (10 μM) (Figure 7A). In three of these experiments the effect of LiCl (10 mM) after washout of adenosine was tested again and it was observed that LiCl increased e.p.p. amplitude by a similar amount (16.5 $\pm$ 0.5%) to that observed during the first application of the preparation. Thus, the effect of LiCl (10 mM) on e.p.p. amplitude was markedly reduced in the presence of adenosine.

In one endplate where adenosine caused typical reduction of the excitatory effect of LiCl (10 mM) on e.p.p. amplitude, (i.e. LiCl (10 mM) increased e.p.p. amplitude by 15% in the absence of adenosine and by only 4% in the presence of adenosine (10 μM)), the effect of LiCl (10 mM) was tested after the magnesium concentration in the bath was increased from 10 mM to 14 mM. This increase in the concentration of $MgCl_2$ decreased e.p.p. amplitude by 53%, which was similar to the decrease in e.p.p. amplitude caused by adenosine (10 μM) in the same endplate before increasing the concentration of $MgCl_2$. However, after reducing e.p.p. amplitude with $MgCl_2$ (14 mM), LiCl (10 mM) still caused enhancement of neuromuscular transmission and increased e.p.p. amplitude by 17%. This suggests that the ability of adenosine to prevent the excitatory effect of LiCl on neuromuscular transmission does not result only from its ability to reduce e.p.p. amplitude.

CADO also attenuated the excitatory effect of LiCl on neuromuscular transmission (Figure 7B). In three experiments LiCl (10 mM) increased e.p.p. amplitude by 17 $\pm$ 1% in the absence of CADO and by only 5 $\pm$ 1% and 10 $\pm$ 1% in the presence of 1 μM and 0.3 μM of CADO, respectively.

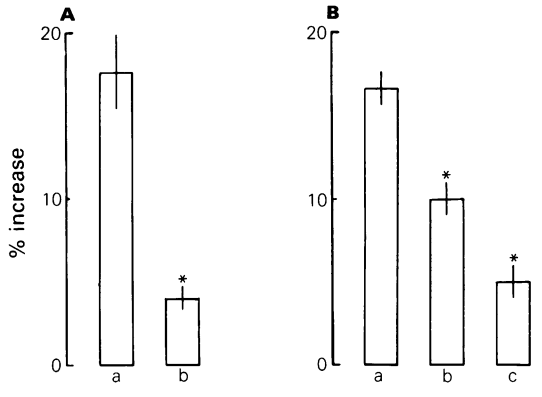

Figure 7 Comparison between the actions of adenosine (A) and 2-chloroadenosine (B) on the enhancement caused by LiCl (10 mM) on neuromuscular transmission. The ordinate scale represents the percentage increases caused by LiCl (10 mM) on the amplitude of evoked endplate potentials (e.p.ps) recorded intracellularly from innervated frog sartorius muscle preparations paralysed with magnesium 10 mM to 12.5 mM. Zero % is the amplitude of the e.p.ps before perfusing LiCl. (A) Effect of LiCl (10 mM) in the absence (a) and in the presence (b) of adenosine (10 μM). (B) Effect of LiCl (10 mM) in the absence (a) and in the presence of 0.3 μM (b) or 1 μM (c) CADO. The solutions without LiCl contained sucrose (20 mM). Each column represents pooled data from 3 to 4 experiments, and in each panel (A and B) are compared results obtained in the same experiments. The vertical bars represent $\pm$ s.e.mean. * $P < 0.05$ (Student's t test) as compared with the effect of LiCl (10 mM) in the absence of adenosine or CADO. Averaged e.p.p. amplitude in the control bathing solution: 8.7 $\pm$ 1.9 mV. Averaged decrease in e.p.p. amplitude caused by adenosine (10 μM): 50 $\pm$ 5%. Averaged decrease in e.p.p. amplitude caused by 0.3 μM and 1 μM CADO: 36 $\pm$ 1% and 47 $\pm$ 4%, respectively. Averaged resting membrane potential: -96 ± 2 mV.

Forskolin and MDL 12,330A

The effect of forskolin (2.5 μM), an activator of adenylate cyclase (Seamon *et al.*, 1981), on e.p.ps is illustrated in Figure 8. Forskolin (2.5 μM) reversibly increased the amplitude of e.p.ps and did not modify the decay phase or the resting membrane potential of the muscle fibre. In concentrations of 0.1 μM, 1 μM and 2.5 μM forskolin increased e.p.p. amplitude by 9 $\pm$ 3% ($n = 2$), 26 $\pm$ 2% ($n = 2$) and 24 $\pm$ 2% ($n = 3$), respectively. The quantal content of the e.p.ps was calculated (variance method) in two experiments using 0.1 μM or 1 μM of forskolin. In these concentrations forskolin increased the quantal content of the e.p.ps by 6% and 28%, respectively, which suggests that forskolin increased the evoked release of the transmitter.

The full effect of forskolin (0.1–2.5 μM) on e.p.p. amplitude was usually seen in the first 15 to 20 min after starting perfusion and disappeared within 30 to 40 min after returning the preparations to control solutions. The enhancement caused by forskolin on neuromuscular transmission could not be attributed to its solvent, ethanol, since in the experiments using forskolin all solutions without forskolin contained equivalent amounts (0.001–0.025% v/v) of ethanol.

To test whether forskolin modified the inhibitory effect of adenosine on e.p.p. amplitude, experiments were designed to compare in the same endplate the effect of adenosine in the absence and in the presence of forskolin. Forskolin (0.1–2.5 μM) did not cause consistent modifications of the concentration-response curve for the inhibitory effect of adenosine (1–30 μM) on e.p.p. amplitude (four experiments). The results obtained in one of these experiments are illustrated in Figure 9 which compares in the same endplate the action of forskolin (2.5 μM) with that of PDAc (100 nM) on the effect of

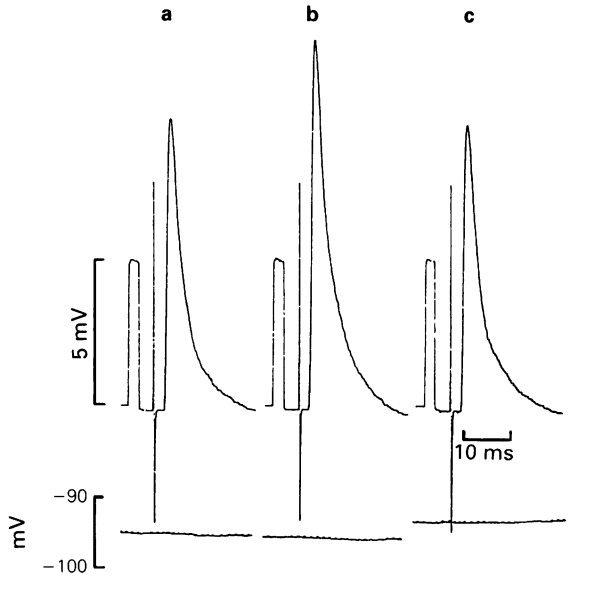

Figure 8 Effect of forskolin on the averaged amplitude of evoked endplate potentials (e.p.ps). Solutions contained magnesium 10.5 mM. Upper part: pen-recorder traces of averaged e.p.ps recorded: in the control bathing solution before applying forskolin (a); 15 min after starting perfusion with forskolin (2.5 μM) (b); 30 min after returning to the control bathing solution (c). Calibration pulse: 5 mV amplitude and 2 ms duration. Lower part: membrane resting potential. Details as in legend to Figure 1.

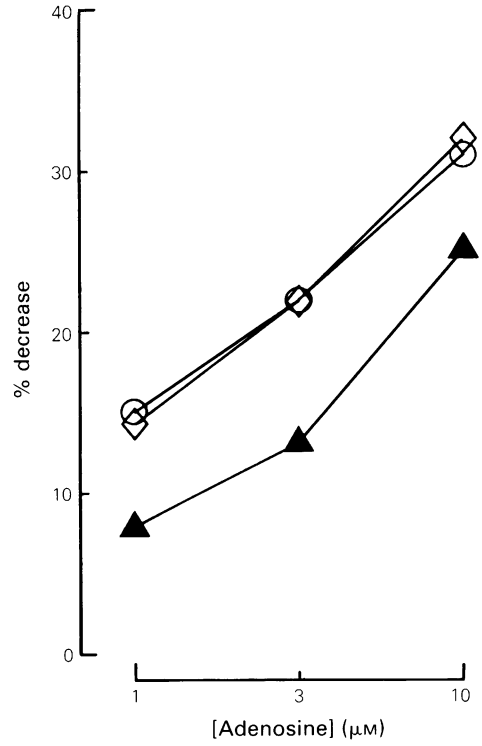

Figure 9 Comparison between the actions of forskolin and 4β-phorbol-12,13-diacetate (PDAc) on the inhibitory effect of adenosine on neuromuscular transmission. The ordinate scale represents the percentage decrease in the amplitude of evoked endplate potentials (e.p.ps) caused by adenosine alone (○), adenosine in the presence of forskolin (2.5 μM) (◇) and adenosine in the presence of PDAc (100 nM) (▲). Zero % is the e.p.p. amplitude before starting perfusion with adenosine and 100% represents a complete inhibition of e.p.ps. The results were obtained from the same endplate where forskolin (2.5 μM) and PDAc (100 nM) increased e.p.p. amplitude by 27% and 26%, respectively; e.p.p. amplitude in the control bathing solution: 9.6 mV. Resting membrane potential: −95 mV. Solutions contained magnesium 10.5 mM.

adenosine on neuromuscular transmission. Both forskolin (2.5 μM) and PDAc (100 nM) increased e.p.p. amplitude by a similar proportion (see legend to Figure 9) but forskolin, in contrast to PDAc, did not modify the effect of adenosine on e.p.p. amplitude.

The effect of the adenylate cyclase inhibitor, MDL 12,330A (formerly named RMI 12,330A) (Hunt & Evans, 1980), on e.p.p. amplitude and its ability to modify the inhibitory effect of adenosine on neuromuscular transmission was investigated in one experiment. Since MDL 12,330A (1 μM) caused an irreversible inhibition of neuromuscular transmission (cf. Silinsky & Vogel, 1986) it was left in contact with the preparation for 15 min and then removed from the bath. Twenty minutes after removing MDL 12,330A (1 μM) from the bath, the amplitude of the e.p.ps was reduced to 73% of the control value and remained at this level for the next 10 min. After the pretreatment with MDL 12,330A (1 μM) the effect of adenosine on e.p.p. amplitude was not modified. Thus, 1 μM and 3 μM of adenosine decreased e.p.p. amplitude by 35% and 49% before MDL 12,330A and by 31% and 53% after MDL 12,330A.

In another experiment, a higher concentration (10 μM) of MDL 12,330A was left in contact with the preparation for 10 min. In these conditions MDL 12,330A markedly decreased e.p.p. amplitude, which continued to decline even after the removal of MDL 12,330A from the bath. This progressive decrease in e.p.p. amplitude stopped when forskolin (2.5 μM) was applied to the preparation, but after removal of forskolin from the bath the amplitude of the e.p.ps started to decrease again until a near complete blockade of neuromuscular transmission was achieved.

Discussion

The present results show that a phorbol ester that activates protein kinase C, PDAc (see e.g. Baraban, 1987), attenuates the inhibitory effects of adenosine and of the adenosine receptor agonist CADO, on neuromuscular transmission, and that the enhancement of neuromuscular transmission caused by lithium ions, which inhibit breakdown of inositol phosphates (e.g. Sekar & Hokin, 1986), is markedly attenuated by adenosine and CADO. Neither an activator of adenylate cyclase, forskolin (Seamon *et al.*, 1981), nor an inhibitor of adenylate cyclase, MDL 12,330A (Hunt & Evans, 1980) modified the inhibitory effect of adenosine on neuromuscular transmission.

The finding that PDAc increases the amplitude and the quantal content of e.p.ps, and thus increases evoked transmitter release, conforms with the results obtained by others using phorbol esters active on protein kinase C either at the neuromuscular junction (Haimann *et al.*, 1987; Shapira *et al.*, 1987; Caratsch *et al.*, 1988) or the central nervous system (Zurgil *et al.*, 1986; Nichols *et al.*, 1987; Huang *et al.*, 1988; Fredholm & Lindgreen, 1988). The enhancement of neuromuscular transmission caused by PDAc, as well as its ability to attenuate the inhibitory effect of adenosine receptor agonists at the neuromuscular junction, might be related to its ability to activate protein kinase C, since (1) the effective concentrations of PDAc at the neuromuscular junction are within the order of magnitude of its affinity for the enzyme (Leach *et al.*, 1983), and (2) the phorbol ester inactive on protein kinase C, PDDec (Castagna *et al.*, 1982), used in a concentration more than 100 times higher than the affinity constant of its active stereoisomer (Leach *et al.*, 1983), did not modify the amplitude of the e.p.ps or the inhibitory action of adenosine on neuromuscular transmission. Since forskolin also enhanced neuromuscular transmission and did not modify the effect of adenosine, the enhancement of neuromuscular transmission *per se* cannot explain the ability of PDAc to attenuate the inhibitory effect of adenosine on e.p.p. amplitude.

The attenuation of the inhibitory effect of adenosine on neuromuscular transmission caused by PDAc could not be attributed to an activation of the adenosine uptake system, since PDAc attenuated by a similar amount both the effect of adenosine and CADO, which is not inactivated through the adenosine uptake system at the frog neuromuscular junction (Ribeiro & Sebastião, 1987). It also seems likely that protein kinase C, after being activated by PDAc affects the adenosine receptor directly, since the affinity of the adenosine receptor antagonist, 8-phenyltheophylline, was not modified in the presence of PDAc. A third possibility is that PDAc could affect some step involved in the transducing system operated by the adenosine receptor that mediates inhibition of neurotransmitter release at the neuromuscular junction. Adenosine might decrease transmitter release either by decreasing calcium entry through calcium channels (Ribeiro *et al.*, 1979; Shinozuka *et al.*, 1985; Gross *et al.*, 1989) or by decreasing the affinity of calcium for an intracellular component of the secretory apparatus (Silinsky, 1984). It is of interest to note that both activation of calcium channels (De Riemer *et al.*, 1985; Zurgil *et al.*, 1986; Huang *et al.*, 1988) and enhanced sensitivity of the stimulus-secretion coupling processes to calcium within the nerve terminal (Nichols *et al.*, 1987), have been proposed as mechanisms to explain the enhancement of neurotransmitter release during activation of protein kinase C by phorbol esters.

As in the hippocampus (Huang *et al.*, 1988), polymyxin B inhibited evoked transmitter release at the neuromuscular junction. The ability of polymyxin B to prevent the enhancement of neuromuscular transmission caused by PDAc suggests that at the concentrations used in the present work polymyxin B inhibits protein kinase C at the neuromuscular junction. These concentrations are similar to those shown to inhibit, selectively, purified protein kinase C (Kuo *et al.*, 1983). Since PDAc attenuated the inhibitory effect of adenosine on neuromuscular transmission it was expected that polymyxin B

would enhance the effect of the nucleoside. However, the inhibitory effect of adenosine on neuromuscular transmission was virtually unaffected by polymyxin B. Whether this is a consequence of polymyxin B inhibiting protein kinase C in an indirect manner, i.e. by binding to phospholipids, rather than by direct binding to the enzyme (see Hidaka & Hagiwara, 1987), cannot be answered in the present work.

The effect of H-7 on neuromuscular transmission was probably not related to its ability to inhibit protein kinase C, since it did not modify the enhancement of neuromuscular transmission caused by PDAc. Besides inhibiting protein kinase C, H-7 also inhibits the cyclic AMP-dependent and cyclic GMP-dependent protein kinases, the affinity constants for the three enzymes ranging from 3 μM to 6 μM (Hidaka *et al.*, 1984). Thus, it is possible that the observed effect of H-7 on neuromuscular transmission results from its ability to inhibit cyclic nucleotide-dependent kinases.

Lithium increased the amplitude of e.p.ps, without modifying the resting membrane potential of the muscle fibres. The quantal content and the amplitude of the e.p.ps were increased by lithium by a similar proportion which indicates that it enhances neuromuscular transmission by increasing evoked release of transmitter. This effect of lithium might result from its ability to increase the intracellular levels of inositol phosphates (IP), namely IP_3 and IP_4 (see Sekar & Hokin, 1986; Drummond & Hughes, 1987) which enhance calcium mobilization from intracellular stores and calcium entry through the plasma membrane (e.g. Putney, 1987). So, the finding that the enhancement of neuromuscular transmission caused by lithium is markedly attenuated by adenosine receptor agonists suggests that during activation of the presynaptic adenosine receptor, LiCl induces accumulation of smaller amounts of intracellular calcium. This action of adenosine receptor agonists could result from at least two possibilities: (1) upon activation of the adenosine receptor there is inhibition of calcium entry through the plasma membrane (see Ribeiro *et al.*, 1979; Shinozuka *et al.*, 1985; Gross *et al.*, 1989) and/or inhibition of calcium mobilization from intracellular stores (see Ribeiro & Dominguez, 1978); (2) activation of the adenosine receptor leads to a reduction in the formation of phosphoinositides. The possibility that adenosine prevents the effect of lithium by decreasing calcium entry in a magnesium-like manner seems unlikely since an increase in magnesium concentration in the bath did not modify the effect of lithium on neuromuscular transmission. Evidence that adenosine inhibits phosphoinositide metabolism in nerve cells has recently been produced (Petcoff & Cooper, 1987; Kendall & Hill, 1988; Rubio *et al.*, 1989). It is worth noting that the adenosine receptor involved in the inhibition of phosphoinositide metabolism (Petcoff & Cooper, 1987; Kendall & Hill, 1988) has an agonist profile more similar to the agonist profile of the adenosine receptor mediating inhibition of neurotransmitter release (Ribeiro & Sebastião, 1986), than to the agonist profiles of the adenosine receptors involved in inhibition or in stimulation of adenylate cyclase (Daly *et al.*, 1981).

Interactions between the phosphoinositides/protein kinase C transducing system and the adenylate cyclase transducing system have been proposed (see Nishizuka, 1986), namely in relation to the increase in cyclic AMP accumulation caused by adenosine analogues at the hippocampus (Nordstedt & Fredholm, 1987). In the present work the adenylate cyclase activator, forskolin (Seamon *et al.*, 1981) increased evoked transmitter release which conforms with results obtained by others (Markstein *et al.*, 1984) in the central nervous system. MDL 12,330A irreversibly decreased e.p.p. amplitude, an effect that was prevented by forskolin. This suggests that the effect of MDL 12,330A on neuromuscular transmission results from its ability to inhibit adenylate cyclase (Hunt & Evans, 1980). The finding that the inhibitory effect of adenosine on neuromuscular transmission was not modified by forskolin or MDL 12,330A suggests that adenylate cyclase is not involved in the inhibitory effect of adenosine at the neuromuscular junction. This agrees with the idea that the adenosine receptor mediating inhibition of neurotransmitter release is not coupled to adenylate cyclase (see e.g. Ribeiro & Sebastião, 1989).

In summary, the findings that an activator of protein kinase C, PDAc (but not the inactive phorbol ester, PDDec), attenuates the effect of adenosine receptor agonists on neuromuscular transmission without modifying the affinity of an adenosine receptor antagonist, taken together with the observation that adenosine receptor agonists prevent the enhancement of transmission caused by lithium ions, suggest that the phosphoinositides/protein kinase C transducing system might be involved in the inhibitory effect of adenosine on neuromuscular transmission. Whether the presynaptic adenosine receptor inhibits phospholipase C, either directly, as was suggested for the adenosine-induced inhibition of prolactin release from cultured GH_3 pituitary tumour cells (Delahunty *et al.*, 1988), or indirectly through a reduction of intracellular calcium levels, needs to be investigated.

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P_2-, but not P_1-purinoceptors mediate formation of 1,4,5-inositol trisphosphate and its metabolites via a pertussis toxin-insensitive pathway in the rat renal cortex

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1 The adenosine receptor (P_1-purinoceptor) agonists N^6-cyclopentyladenosine and N-5'-ethylcarboxamidoadenosine at concentrations up to 10 μmol l^{-1} affected neither basal, nor noradrenaline- and angiotensin II-stimulated formation of inositol-1-phosphate, inositol-1,4-bisphosphate, and inositol-1,4,5-trisphosphate in slices of rat renal cortex.

2 In contrast, adenine nucleotides (P_2-purinoceptor agonists) markedly stimulated inositol phosphate formation. The observed rank order of potency adenosine-5'-O-(2-thiodiphosphate) (EC_{50} 39 μmol l^{-1}) > adenosine-5'-O-(3-thiotriphosphate) (587) $\geq$ 5'-adenylylimidodiphosphate (App(NH)p, 899) > adenylyl-(β,γ-methylene)-diphosphonate (4,181) was consistent with the interaction of the compounds with the P_{2y}-subtype of P_2-purinoceptors. AMP and the ADP analogue (α,β-methylene)-adenosine-5'-diphosphate were ineffective. ATP and ADP ($\leq$ 10 mmol l^{-1}) did not produce a consistent increase, owing to their hydrolytic degradation in the incubation medium.

3 Whereas the inositol phosphate response to App(NH)p was linear only up to 5 min incubation, the time-dependent stimulation of noradrenaline declined at a slower rate. Following pre-exposure of the renal cortical slices to App(NH)p, renewed addition of App(NH)p caused no further enhancement in the accumulation of inositol phosphates, whilst noradrenaline was still capable of eliciting a response. This suggests that the apparent loss of responsiveness to App(NH)p is not due to substrate depletion or enzymatic inactivation, but most likely attributable to homologous desensitization of the purinoceptor.

4 Pretreatment of the animals with pertussis toxin caused a substantial reduction of functional G_i-protein, as indicated by the lack of [^{32}P]-NAD incorporation in a membrane preparation of the renal cortex. Nevertheless, the increase in inositol phosphate formation induced by noradrenaline, angiotensin II, and App(NH)p was not significantly impaired.

5 We conclude that P_{2y}-purinoceptors are present in the renal cortex; these receptors stimulate formation of inositol phosphates via a pertussis toxin-insensitive pathway and undergo homologous desensitization. On the other hand, our results suggest that renal A_1-adenosine receptors do not use stimulation of phosphoinositide breakdown as a transmembrane signalling system.

Introduction

Adenosine acts on four major structures in the kidney, namely the vasculature, renin-containing cells, nerve endings and tubular epithelium, and causes arteriolar vasoconstriction, a decrease in renin release, a decrease in noradrenaline release, and stimulation of electrogenic chloride secretion, respectively (for reviews see Spielman *et al.*, 1987; Osswald, 1988). However, the subtype of receptor and the cellular signalling mechanism mediating each particular effect are obscure at present. We have previously shown that A_1- and A_2-adenosine receptors are located on both renal glomeruli and microvessels (Freissmuth *et al.*, 1987a), and also demonstrated the presence of A_2-receptors on renal tubules (Freissmuth *et al.*, 1987b). Whereas activation of the A_2-receptor was shown to stimulate adenosine 3':5'-cyclic monophosphate (cyclic AMP) formation, no coupling to the adenylyl cyclase system was demonstrated with the A_1-receptor, which would mediate inhibition of the enzyme.

Since A_1-receptor-mediated renal vasoconstriction and suppression of renin release have been found to be associated with a rise in intracellular Ca^{2+} in rat renal slices (Churchill & Churchill, 1985; Rossi *et al.*, 1988), which might be due to increased inositol phosphate formation (Abdel-Latif, 1987), it was the aim of the present study to investigate adenosine-stimulated changes in the phosphoinositide turnover as a signal-transduction pathway alternative to adenylyl cyclase inhibition. Further evidence that constriction of renal arteries by adenosine is probably mediated via a Ca^{2+}-dependent mechanism is provided by comparison of the mode of action of adenosine and angiotensin II (Hackenthal & Taugner 1986; Imagawa *et al.*, 1986; Rossi *et al.*, 1988). Adenosine also provides a unique synergism for angiotensin II, which, in the absence of the nucleoside, is not able to constrict afferent arterioles (Hall *et al.*, 1985). Thus, adenosine may stimulate the formation of inositol phosphates in a manner similar to angiotensin II, thereby increasing the Ca^{2+} concentration in vascular smooth muscle and juxtaglomerular cells.

According to a different nomenclature, adenosine receptors are also called P_1-purinoceptors and distinguished from P_2-purinoceptors, which are preferentially activated by adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate (ADP), and their derivatives (for review see Gordon, 1986). In contrast to adenosine receptor agonists, P_2-purinoceptor agonists have been repeatedly shown to stimulate phospholipase C, the enzyme catalyzing phosphoinositide breakdown, in various cells including hepatocytes (Okajima *et al.*, 1987), Ehrlich ascites tumour cells (Dubyak, 1986), thymocytes (El-Moatassim *et al.*, 1987), endothelial cells (Pirotton *et al.*, 1987), FRTL-5 thyroid cells (Okajima *et al.*, 1989), and turkey erythrocytes (Boyer *et al.*, 1989; Cooper *et al.*, 1989). Hence, a further aim of the present study was to establish whether P_2-receptor-mediated phospholipase C activation is also demonstrable in rat renal cortex slices and, if this proves to be the case, whether this intracellular signal is triggered via a pertussis toxin-sensitive G-protein.

Methods

Assay of inositol phosphate formation

One male Sprague-Dawley rat (200–250 g) was killed for each experiment. The kidneys were removed, decapsulated and

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transferred to freshly oxygenated modified Krebs-Ringer bicarbonate medium containing (mmol l^{-1}): NaCl 115, KCl 5, Na_2HPO_4 1.2, CH_3COONa 10, $NaHCO_3$ 25, $CaCl_2$ 1 and glucose 5.5. Slices of renal cortex (0.3 × 0.3 × 0.9 mm) were prepared with a McIlwain tissue chopper, washed and then incubated with 20 μCi ml^{-1} of *myo*-[^{3}H]-inositol. The Krebs-Ringer medium was kept at 37°C and was continuously gassed with 95% O_2/5% CO_2 mixture. Following 2 h of labelling and subsequent washing, aliquots of gravity-packed slices (20–25 mg wet weight) were dispensed into vials containing incubation buffer in a final volume of 300 μl and preincubated with LiCl (10 mmol l^{-1}) for 5 min.

The reaction was started by addition of test compounds (quadruplicate determinations) and terminated by freezing in liquid nitrogen. The chosen incubation times are indicated in the individual figures and tables. Determination of inositol phosphates was carried out as described by Scholz *et al.* (1988). After addition of 1 ml chloroform/methanol/HCl (333:666:1) frozen samples were thawed and immediately homogenized with a glass potter. Following addition of 600 μl water and 800 μl chloroform the mixture was vigorously shaken for 15 min and the phases were separated by centrifugation (1,000 *g* for 10 min). An aliquot of the aqueous phase (1.7 ml) was removed, diluted with water and incubated in a shaking water bath at 45°C to expel remnants of chloroform. A volume of 5 ml was loaded onto Dowex AG 1-X8 anion exchange columns. Inositol was removed with water and glycerophosphoinositol with 5 mmol l^{-1} sodium tetraborate in 60 mmol l^{-1} sodium formate. Inositol phosphates were then sequentially eluted with increasing concentrations (0.2–1.0 mol l^{-1}) of ammonium formate in 0.1 mol l^{-1} formic acid (Berridge, 1983). Aliquots (3 ml) of the eluate were mixed with scintillation fluid (Ready Value) and the radioactivity in the inositol, inositol-1-monophosphate ($InsP_1$), inositol-1,4-bisphosphate ($InsP_2$) and inositol-1,4,5-trisphosphate fraction ($InsP_3$) was counted in a Beckman Minaxi β-counter at an efficiency of 51%. The radioactivity of the chloroform phase (0.9 ml aliquots) containing the phosphoinosites was measured *in toto*.

In order to compensate for the variability associated with handling slice suspensions, the radioactivity in each inositol phosphate fraction was normalised for each 10,000 c.p.m. appearing together in the aqueous and the chloroform phase. The amount of *myo*-[^{3}H]-inositol incorporated into inositol phosphates in the control samples was 3–5% of the total ^{3}H-activity added.

Analysis of degradation of adenine nucleotides

The amount of adenine nucleotides still present in the reaction medium after 15 min incubation with renal cortex slices was determined in a 30 μl aliquot, withdrawn before the termination of the reactions in separate experiments. Separation of adenine nucleotides by ion-exchange chromatography (Spherisorb 5-SAX h.p.l.c.-column) was performed with a linear gradient of 0–0.5 mol l^{-1} KCl in a 50 mmol l^{-1} K_2HPO_4 elution medium (pH 5.0) for 11 min at a flow rate of 2 ml min^{-1}.

Pertussis toxin treatment of rats and pertussis toxin-catalyzed [^{32}P]-NAD-ribosylation of renal cortical membranes

Pertussis toxin (160 μg kg^{-1}) was administered by the intravenous route (tail vein) 40 h before the animals were killed. Slices of renal cortex were prepared as described above. Approximately 1/5 of each slice was removed after it had been labelled with *myo*-[^{3}H]-inositol and washed, put on ice, and homogenized in a medium containing (mmol l^{-1}) Tris-HCl 20 (pH 8), EDTA 2, EGTA 1, sucrose 250 by means of an Ultra-Turrax (2× at half-maximal speed for 15 s, 1× at maximal speed for 3 s). The homogenate was sedimented at 40,000 *g* (15 min) and washed twice in sucrose-free buffer. The resulting pellet was resuspended in assay buffer and stored under liquid nitrogen at a concentration of 15 mg ml^{-1}. Pertussis toxin catalyzed ADP-ribosylation was assayed as described by Bokoch *et al.* (1983) in a 40 μl reaction mixture containing (mmol l^{-1}): Tris-HCl 100 (pH 8), EDTA 1, dithiothreitol 1, $MgCl_2$ 2.5, thymidine 10, ATP 1, GTP 0.1, [^{32}P]-NAD (specific activity 1,000 c.p.m. pmol $^{-1}$) 0.01, 10 to 50 μg of membrane protein and 2 μg pre-activated pertussis toxin. After 1 h at 30°C, the reaction was terminated by precipitation with trichloroacetic acid (15% final concentration). Trichloroacetic acid was removed by extraction with cold acetone; the samples were dissolved in Laemmli's sample buffer supplemented with 40 mmol l^{-1} dithiothreitol and subjected to SDS-PAGE (5% stacking gel, 10% running gel). Autoradiography of the dried gel was performed with Kodak XAR-5 films with one intensifying screen over 1 to 6 days at −80°C. A bovine G_i/G_o fraction, used as reference protein, was prepared according to Sternweis & Robishaw (1984).

Materials

Myo-[2′-^{3}H]-inositol (15.2 Ci mmol $^{-1}$) and [^{32}P]-nicotinamide adenine dinucleotide ([adenylate-^{32}P]-NAD, 1,000 Ci mmol $^{-1}$) were from NEN, Boston, MA; pertussis toxin was obtained as a lyophilized powder from Peninsula Labs., St. Helens, U.K., *myo*-inositol, (−)-noradrenaline-HCl, (±)-propranolol-HCl, angiotensin II (acetate salt), N^6-cyclopentyladenosine (CPA), ATP.2Na, α,β-methylen-adenosine-5′-diphosphate (AMP-CP), and 5′-adenylylimidodiphosphate, tetralithium salt (App(NH)p), from Sigma, St. Louis, MO; ADP.2Na, AMP.2Na, NAD, adenosine-5′-O-(3-thiotriphosphate), tetralithium salt (ATPγS), adenosine-5′-O-(2-thiodiphosphate), trilithium salt (ADPβS), adenylyl-(β,γ-methylene)-diphosphonate, tetralithium salt (App(CH_2)p), from Boehringer-Mannheim, FRG; theophylline and LiCl from Merck, Darmstadt, FRG; Dowex AG 1 × 8 anion exchange resin, formate form, was from Bio-Rad, Richmond, CA, U.S.A.; and Ready-Solv EP scintillation fluid from Beckman, Palo Alto, CA, U.S.A. All other chemicals were analytical grade or best grade commercially available. Phentolamine-HCl (Ciba-Geigy, Basle, Switzerland), 5′-N-ethylcarboxamidoadenosine (NECA, Byk Gulden Lomberg, Konstanz, FRG), and 8-cyclopentyl-1,3-dipropyl-xanthine (DPCPX, Gödecke, Berlin (West)) were generous gifts from the sources indicated.

Results

Accumulation of inositol phosphates in response to noradrenaline, angiotensin II and adenosine receptor agonists (Table 1)

In rat renal cortical slices the A_1-adenosine receptor agonist CPA did not affect the basal accumulation of inositol phosphates up to a concentration of 10 μmol l^{-1} during an incubation period of 15 min. Neither was there any effect when the non-selective agonist NECA (10 μmol l^{-1}) or when the original compound adenosine (5 mmol l^{-1}) was used instead (data not shown). As expected, noradrenaline and angiotensin II markedly stimulated the accumulation of inositol phosphates in renal cortex, with almost maximal effects occurring at concentrations of 5 and 10 μmol l^{-1}, respectively. In the case of noradrenaline, a further increase was observed in the $InsP_3$ fraction only at a concentration of 100 μmol l^{-1}. On the other hand, both potentiation of the noradrenaline effect by angiotensin II, as well as reversal of the noradrenaline-induced stimulation by phentolamine were observed in experiments in which the adenosine analogue CPA failed to alter pre-stimulated inositol phosphate production; enhancing or inhibitory effects of CPA were also undetectable at an earlier time point (5 min). Likewise, the inclusion of the selective A_1-adenosine receptor antagonist DPCPX (1 μmol l^{-1}) or the

Table 1 Effect of various compounds on the accumulation of inositol phosphates in rat renal cortical slices

Compound (μmol l^{-1})	C.p.m. normalized $InsP_1$	$InsP_2$	$InsP_3$
Basal values	283.5 ± 13.4	41.8 ± 2.7	15.0 ± 1.6
CPA (10)	277.0 ± 11.1	43.8 ± 2.0	15.4 ± 1.3
NA (10)	488.8 ± 27.1*	117.1 ± 10.3*	21.9 ± 2.2*
NA (10) + Phentolamine (10)	308.3 ± 16.7	47.3 ± 4.5	11.3 ± 0.5
NA (100)	441.9 ± 16.1*	94.8 ± 6.4*	36.9 ± 4.2*
NA (100) + CPA (10)	374.9 ± 29.5*	90.5 ± 18.0*	27.7 ± 3.8*
AII (5)	346.9 ± 10.9	80.5 ± 5.0*	28.8 ± 3.1*
AII (100)	393.4 ± 10.9*	70.6 ± 1.8*	23.1 ± 2.0*
NA (100) + AII (100)	696.8 ± 28.8*	239.5 ± 21.0*	64.5 ± 6.0*

The slices had been labelled with *myo*-[^{3}H]-inositol (20 μCi ml^{-1}) and washed. Incubation lasted for 15 min and was carried out at 37°C and in the presence of 10 mmol l^{-1} LiCl. In the experiments with phentolamine, the drug was added 5 min before noradrenaline (NA). Experiments where noradrenaline and N^6-cyclopentyladenosine (CPA) and noradrenaline and angiotensin II (AII), respectively, were added together were started by concomitant addition of both drugs. InsP values are normalized for each 10,000 c.p.m. of the total amount of sample radioactivity. The data are presented as means ± s.e.mean of four different experiments. *$P < 0.05$ compared with the control values (*t* test for unpaired data).

presence of the non-selective antagonist theophylline (100 μmol l^{-1}) did not affect the level of inositol phosphate accumulation, when slices were exposed concomitantly to noradrenaline and angiotensin II (data not shown).

Time- and concentration-dependent effects of adenine nucleotides on inositol phosphate accumulation

In contrast to adenosine and its analogues, adenine nucleotides markedly increased the formation of inositol phosphates. Figure 1 shows the time course of basal and agonist-induced accumulation of inositol phosphates. Under basal conditions, the turnover of $InsP_2$ and $InsP_3$ remained steady for 30 min, whereas $InsP_1$ accumulated linearly with time. In the presence of the ATP analogue App(NH)p, the formation of $InsP_2$ and $InsP_3$ was stimulated more than 3 fold by 15 min at a continuously decreasing rate; at 30 min, no further increase was observed, whereas the $InsP_1$ component accumulated further, probably due to the inhibition of $InsP_1$ degradation by LiCl. Whereas the $InsP_2$ and $InsP_3$ response to App(NH)p increased linearly only up to 5 min, the time-dependent stimulation induced by noradrenaline declined at a much slower rate.

When renal cortical slices were pre-exposed to App(NH)p for 10 min, renewed addition of App(NH)p caused no further enhancement in the accumulation of inositol phosphates (Figure 2). In contrast, noradrenaline was still capable of eliciting a response in slices pretreated with App(NH)p, suggesting that the apparent loss of responsiveness to App(NH)p was not attributable to depletion of labelled substrate. On the other hand, if the renal cortical slices were incubated only for a period of linear increment of the App(NH)p-induced effect (5 min, see Figure 2), the simultaneous presence of noradrenaline did not cause any further stimulation of inositol phosphate accumulation (data not shown), indicating that the α-adrenoceptor and the P_2-purinoceptor are coupled to a common pool of phospholipase C.

Several ATP and ADP-analogues, which act as P_2-purinoceptor agonists, were tested for stimulating activity on inositol phosphate formation in renal cortical slices. The analogues listed in Table 2, which are mostly stable to enzymatic and chemical hydrolysis (Welford *et al.*, 1986), gave essentially similar maximal responses. The ADP-analogue AMP-CP and AMP were ineffective at concentrations up to 5 mmol l^{-1} (data not shown). ATP and ADP, however, did not produce a consistent increase in concentrations up to 10 mmol l^{-1}. Their stability to enzymatic degradation was

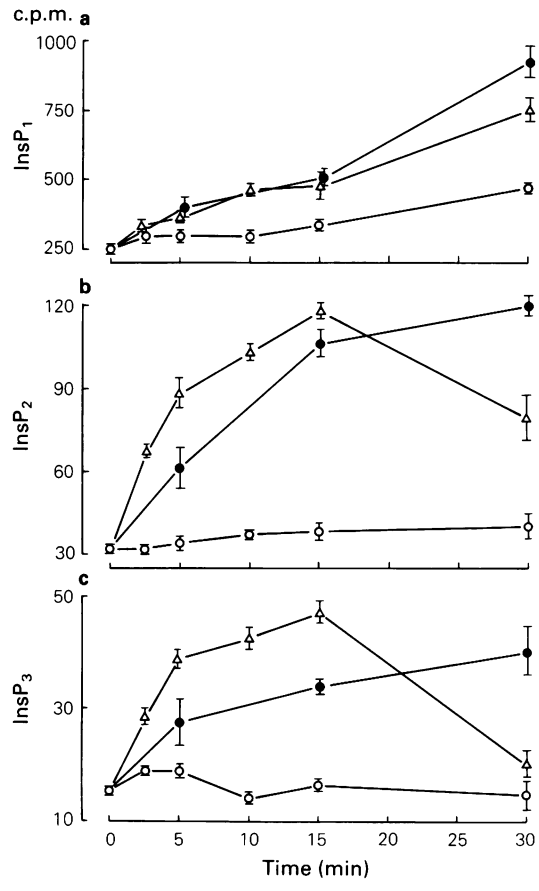

Figure 1 Time course of the accumulation of inositol phosphates (a, $InsP_1$; b, $InsP_2$; c, $InsP_3$) in response to 0.1 mmol l^{-1} noradrenaline (●) and 5 mmol l^{-1} 5'-adenylylimidodiphosphate (App(NH)p) (△) on rat renal cortical slices prelabelled with *myo*-[^{3}H]-inositol. (○) Basal accumulation. Means of 3–4 different experiments are presented; vertical lines show s.e.mean.

Table 2 Effect of P_2-purinoceptor agonists on the accumulation of inositol phosphates in rat renal cortical slices

	EC_{50} (μmol l^{-1})	% of basal value $InsP_1$	$InsP_2$	$InsP_3$
ADPβS	39	160 (142–202)	424 (237–756)	290 (227–370)
ATPγS	587	154 (104–227)	405 (266–617)	322 (237–438)
App(NH)p	899	128 (111–147)	342 (250–467)	283 (197–407)
App(CH_2)p	4,181	172 (122–241)	393 (283–545)	284 (192–419)

The maximal response is expressed as percentage increase over basal values. Prelabelled slices were incubated with each agonist at a maximally active concentration for 15 min. Data are given as geometric means and 95% confidence intervals from three different experiments. The EC_{50} values were estimated through non-linear least squares curve fitting of the experimental points to an equation describing monophasic enzyme stimulation and expressed as mean values of two separate experiments. ADPβS = adenosine-5'-O-(2-thiodiphosphate); ATPγS = adenosine-5'-O-(3-thiotriphosphate); App(NH)p = 5'-adenylylimidodiphosphate; App(CH_2)p = adenylyl-(β,γ-methylene)-diphosphonate.

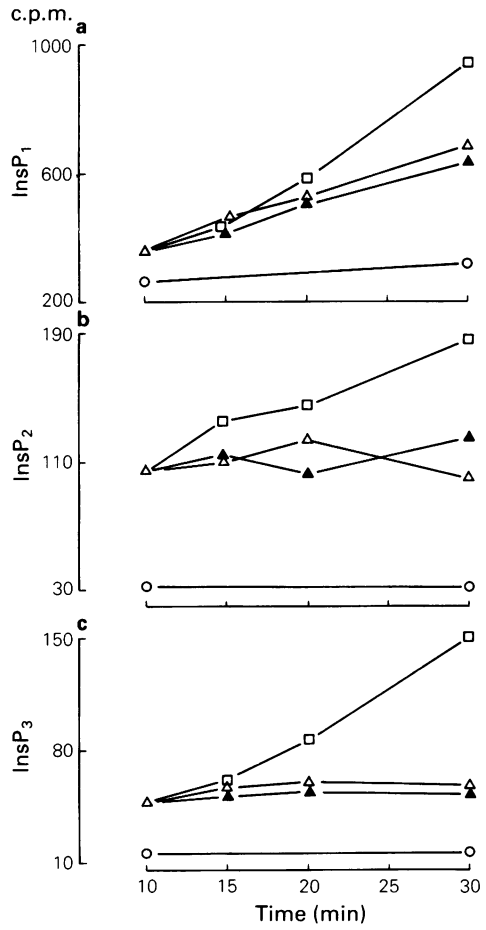

Figure 2 Rat renal cortical slices prelabelled with *myo*-[^{3}H]-inositol were preincubated with 5 mmol l^{-1} 5'-adenylylimidodiphosphate (App(NH)p) for 10 min. Following renewed addition of App(NH)p (▲) or noradrenaline (□) to the pretreated slices, inositol phosphates were measured at the indicated points of time. (○) Control without preincubation, (△) control following preincubation with App(NH)p. The data are mean values from quadruplicate determinations from one experiment. (a) $InsP_1$, (b) $InsP_2$ and (c) $InsP_3$.

therefore monitored by ion-exchange chromatography. After an incubation time of 15 min, ATP and ADP were almost completely degraded in an aliquot withdrawn from the reaction mixture; e.g., 1.5 μmol ATP added to renal cortex slices

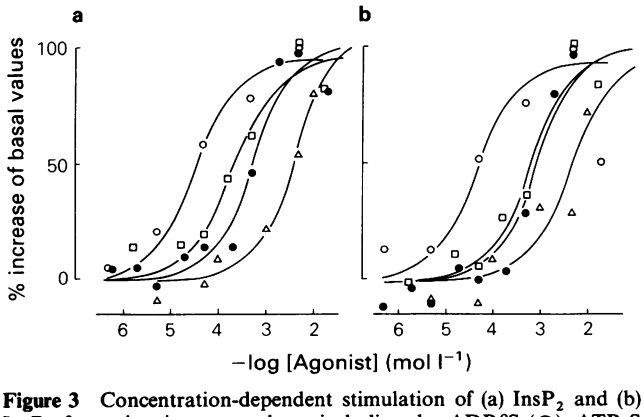

Figure 3 Concentration-dependent stimulation of (a) $InsP_2$ and (b) $InsP_3$ formation in rat renal cortical slices by ADPβS (○), ATPγS (□), App(NH)p (●) and App(CH_2)p (△). The slices, which had been pre-labelled with *myo*-[^{3}H]-inositol, were incubated with the adenine nucleotides for 15 min. The individual concentration-response curves, computed as the best fits of the values shown, are from a single experiment conducted in quadruplicate. Two further experiments gave similar results (see Table 2).

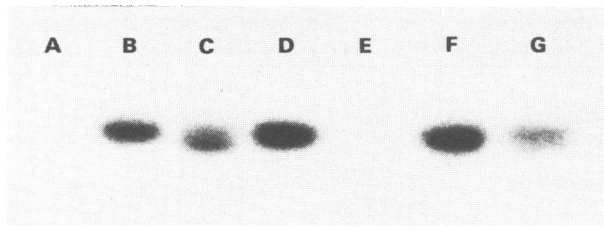

Figure 4 Pertussis toxin-catalyzed [^{32}P]-NAD labelling of rat renal cortical membranes derived from three pertussis toxin (160 μg kg^{-1} i.v.)-treated (lane A, E, G) and three control animals (lane B, D, F) receiving vehicle (sterile NaCl-solution) only. The samples containing 25 μg membrane protein were then subjected to SDS-PAGE and autoradiography. The protein in lane C represents a mixture of a purified bovine brain G-protein fraction (a mixture of G_o/G_i, but containing predominantly G_o). Only the 40 kDa region of the autoradiogram is shown. Four separate assays gave similar results.

(20–25 mg per tube) was hydrolyzed to essentially undetectable levels, with AMP forming about 80% of the original amount of ATP and ADP being present only in negligible quantities. The elution profiles of App(NH)p and ATPγS were not significantly changed after incubation with the slices.

In order to identify the P_2-purinoceptor subtype mediating the stimulation of inositol phosphate production, concentration-response curves for the effective adenine nucleotides were performed. The rank order of potency ADPβS > ATPγS ≥ App(NH)p > App(CH_2)p (Table 2, Figure 3) is consistent with the P_{2y}-purinoceptor proposed by Burnstock & Kennedy (1985).

The effects of individual adenine nucleotides were not additive. Moreover, CPA showed no detectable effect on the extent of inositol phosphate accumulation induced by App(NH)p (data not shown).

Effect of pertussis toxin-treatment on the inositol phosphate response

In order to evaluate whether a G-protein of the G_i/G_o-group is involved in the agonist-induced stimulation of inositol phosphate formation in the renal cortex, rats were treated with pertussis toxin and the inositol phosphate response was determined in the presence of noradrenaline, angiotensin II and App(NH)p. The effectiveness of the treatment was verified by quantifying the reduction of pertussis toxin-substrate in renal cortical membranes (Figure 4). In renal cortical membranes prepared from untreated control animals, the pertussis-toxin substrate migrates as a single species with a slightly lower mobility than the bovine brain G_o-standard, indicating that the predominant pertussis-toxin sensitive G-protein is a $G_{i\alpha}$-subtype (Sternweis & Robishaw, 1984). Pertussis toxin-treatment resulted in a loss of available pertussis toxin substrate in 2 out of 3 animals, which is evident from a complete lack of [^{32}P]-NAD incorporation in lane A and E. Labelling

Table 3 Effect of pertussis toxin treatment (160 μg kg^{-1} i.v.) on the agonist-induced formation of $InsP_3$ in rat renal cortical membranes

	$InsP_3$ (% of basal value)	
Agonist (μmol l^{-1})	*Control rats*	*Pertussis toxin-treated rats*
NA (100)	227 (149–345)	217 (144–327)
AII (100)	145 (101–207)	141 (107–185)
App(NH)p (5,000)	283 (197–407)	265 (190–369)

The data, presented as percentage increase over basal values, are geometric means with 95% confidence limits from pertussis toxin-treated animals, as well as from control animals receiving vehicle (sterile NaCl-solution) only ($n = 3$). NA, noradrenaline; AII, angiotensin II; App(NH)p, 5'-adenylylimidodiphosphate.

remained undetectable, even after prolonged exposure. In one treated animal, however, a faint band could still be visualized (lane G). Incorporation amounted to less than 20% of the control samples (lanes B, D, F).

In spite of the substantial reduction of functional G_i-protein, the agonist-induced increase in the accumulation of $InsP_3$ (as well as of $InsP_2$ and $InsP_1$) is not significantly impaired, much less actually reversed by pertussis toxin treatment (Table 3). Likewise, no differences were observed if the inositol phosphate response was determined in slices from the animals with complete abolition of pertussis toxin substrate and the data from the one rat with partial reduction of $G_{i\alpha}$ were evaluated separately.

Discussion

Although we have previously demonstrated on isolated renal glomeruli and microvessels that, due to GTP-dependence of agonist binding, A_1-adenosine receptors are coupled to a G-protein, the transmembraneous signalling pathway linking these receptors to their effect, e.g. vasoconstriction, inhibition of renin release, remains elusive. The results of the present study suggest that A_1-adenosine receptor activation does not affect inositol phosphate formation in the renal cortex. In particular, although we were able to demonstrate both potentiation and inhibition of noradrenaline-induced phospholipase C activation with angiotensin II and phentolamine, respectively, adenosine and its analogues had no detectable effect. Recently, A_1-receptor agonists have been shown to stimulate slightly (30%) phosphoinositide hydrolysis in a cell line of cortical collecting tubules (Arend *et al.*, 1989). Hence, identification of the A_1-receptor-activated transmembranous signalling cascade that results in vasoconstriction and inhibition of renin release would presumably require fractionation of the renal cortical tissue. Unfortunately, our attempts to measure inositol phosphate formation in a glomerular and microvessel fraction of the kidney, as described elsewhere (Freissmuth *et al.*, 1987a), were unsuccessful. Evidence is accumulating that an increase in cytosolic Ca^{2+} is responsible for the A_1-mediated effect on renal vasoconstriction and renin release, and that a pertussis toxin-sensitive G-protein intervenes between receptor occupation and an increased cellular influx of Ca^{2+} (Churchill & Churchill 1985; Rossi *et al.*, 1987; 1988).

On the other hand, the marked increase in inositol phosphate levels evoked by ATP- and ADP-analogues suggests a physiological role for adenine nucleotides in the regulation of renal function via activation of phospholipase C, which is clearly not mediated by their degradation product, adenosine. Using several types of smooth muscles, Burnstock & Kennedy (1985) have proposed that the P_2-purinoceptors, representing the site of action of adenine nucleotides, can be subdivided into an excitatory P_{2x}- and an inhibitory P_{2y}-subtype. The rank order of potency, in particular the high potency of ADPβS and the low potency of App(CH_2)p observed in the present study is similar to that recently found in the case of P_{2y}-receptor-mediated stimulation of phospholipase C in turkey erythrocyte ghosts (Boyer *et al.*, 1989). In the present experiments on renal cortical slices, the ATP- and ADP-analogues have to be used at much higher concentrations than required for activation of turkey erythrocyte-phospholipase C. A review of the literature shows that the concentrations of adenine nucleotides required to elicit a half-maximal response vary greatly (Westfall *et al.*, 1978; Burnstock *et al.*, 1985; Fedan *et al.*, 1986; Burnstock & Warland 1987; Satchell, 1988) and the low apparent affinity observed in the present study is not unprecedented in experiments performed on isolated tissues and organs. These discrepancies may, in part, be attributable to diffusion barriers for the hydrophilic agonists used, which limit their access under these conditions.

The response to adenine nucleotides was compared with the noradrenaline-induced α-adrenoceptor-mediated effect, revealing distinct differences in the time course of inositol phosphate accumulation. The data obtained in the presence of App(NH)p and noradrenaline suggest that the P_2-purinoceptor in the renal cortex undergoes homologous desensitization. This interpretation is supported by the following observations: firstly, the P_2-purinoceptors and the α-adrenoceptors appear to be coupled to a common pool of inositol phosphate generating enzymes, but noradrenaline is still capable of eliciting a response when App(NH)p has become ineffective. This argues against substrate depletion or progressive enzyme inactivation as possible explanations for the rapid loss of responsiveness. Similarly, depletion of App(NH)p can be ruled out since renewed addition of App(NH)p does not produce any stimulation in pre-exposed slices. In addition, h.p.l.c. analysis of the incubation medium did not reveal metabolism of App(NH)p to any significant degree. Rapid desensitization of P_2-purinoceptor-mediated responses has repeatedly been observed in isolated organ and tissue preparations (for review see Burnstock & Kennedy, 1985). The homologous type of desensitization observed in the present study indicates that the loss of responsiveness results from alterations at the level of the receptor, or at the level of interaction between the receptor and a putative G-protein.

Receptor-mediated stimulation of phosphoinositide hydrolysis by phospholipase C is generally believed to be controlled by a G-protein in a manner analogous to the transmembrane signalling pathways that lead to activation of adenylyl cyclase or the retinal cyclic GMP-phosphodiesterase (Freissmuth *et al.*, 1989). In many cells and tissues (e.g. liver, cardiac myocytes), activation of phospholipase C is not disrupted by pertussis toxin treatment. There are, however, several examples (e.g. granulocytes, mast cells) where pertussis-toxin catalyzed ADP-ribosylation abolishes the phospholipase C response to agonists suggesting that more than one G-protein is involved in receptor-mediated regulation of phospholipase C (Cockcroft, 1987). In this study, we demonstrate that stimulation of inositol phosphate generation by α-adrenoceptors, P_2-purinoceptors and angiotensin II receptors is not mediated by a pertussis-toxin substrate in the renal cortex. Activation of phospholipase C via P_{2y}-purinoceptors is dependent on guanine nucleotides in turkey erythrocytes (Boyer *et al.*, 1989) and binding of ADPβS to these receptors has recently been demonstrated to be heterotropically modulated by guanine nucleotide (Cooper *et al.*, 1989), thus establishing that the P_{2y}-purinoceptors belong to the family of G-protein coupled receptors. The nature of the G-protein that interacts with the P_2-purinoceptor of the renal cortex remains to be identified.

To our knowledge, the physiological role of P_2-purinoceptors in the kidney has not been investigated. However, the concept that ATP released from sympathetic nerve endings acts as a co-transmitter on postsynaptic sites is well established (Gordon, 1986). In the kidney, both the juxtaglomerular apparatus and the proximal tubules are densely innervated by adrenergic fibres (Barajas *et al.*, 1984). An increase in efferent renal sympathetic nerve activity enhances sodium and water reabsorption, which is unaffected by α_2-adrenoceptor antagonists and is only partially abolished by non-selective or α_1-selective adrenoceptor blockade (DiBona, 1985). On the other hand, the antinatriuretic effect of exogenous noradrenaline, as well as its stimulating effect on $InsP_3$ formation in rat renal slices, is fully antagonized by prazosin (Plevin *et al.*, 1988). As demonstrated in the present study, adenine nucleotides and noradrenaline are apparently coupled to the same pool of inositol phosphate generating enzymes in the renal cortex. Thus, it is attractive to speculate that ATP released as cotransmitter during renal sympathetic nerve stimulation may directly affect tubular electrolyte and water transport.

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Effects of aerosolised substance P on lung resistance in guinea-pigs: a comparison between inhibition of neutral endopeptidase and angiotensin-converting enzyme

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1 We have examined in guinea-pigs, *in vivo*, the effects of inhibition of neutral endopeptidase (NEP) and angiotensin-converting enzyme (ACE) on the airway response to aerosolised substance P (SP). We aerosolised captopril (4.6 mM, 60 breaths; 210 nmol) to inhibit ACE and acetorphan (0.3, 1 and 3 mM, 60 breaths; 9 nmol, 33 nmol and 110 nmol respectively) to inhibit NEP. We also examined the effect of the highest dose of acetorphan (110 nmol) on the response to aerosolised acetylcholine (ACh).

2 Responsiveness to SP (or ACh) was measured as the change in lung resistance (R_L) induced by nebulisation of increasing concentrations of SP (or ACh) before and after treatment with the inhibitor. PC_{200}, defined as the provocative concentration inducing an increase in R_L of 200% above baseline, was calculated for each challenge.

3 Administration of acetorphan before the second SP-challenge induced a dose-dependent decrease in PC_{200} for SP amounting to 1.8 ($\pm$0.3) log units after treatment with 11 nmol acetorphan. Treatment with vehicle before the second SP-challenge or with 3 mM acetorphan before the second ACh-challenge had no significant effect on PC_{200}.

4 Treatment with captopril (21 nmol) induced only a small, nonsignificant leftward shift of PC_{200} to SP (0.3 $\pm$ 0.2 log units).

5 We conclude that a NEP-like enzyme, but not ACE, regulates the response to aerosolised SP. We suggest that the same is true for SP released endogenously from sensory nerve endings in the airway epithelial layer.

Introduction

Substance P (SP) is a potent neuropeptide present in airway sensory nerves of several species including guinea-pigs and man, and found in close relation to the airway smooth muscle, beneath and within the epithelium, around ganglion cells and around blood vessels in the airway interstitium (Lundberg *et al.*, 1984b). SP is a potent mediator of smooth muscle contraction, mucus secretion and microvascular leakage in airways of several species (Lundberg *et al.*, 1983; 1984a; Coles *et al.*, 1984; Lötvall *et al.*, 1989), and has been implicated in the pathogenesis of asthma (Barnes, 1986) and in bronchial hyperreactivity induced by respiratory viral infections (McDonald, 1988) or toluene diisocyanate (Sheppard & Scypinski, 1988).

Enzymes which degrade SP seem to be important regulators of its actions (Schwartz *et al.*, 1985; Borson *et al.*, 1987; Stimler-Gerard, 1987; Sekizawa *et al.*, 1987; Dusser *et al.*, 1988; Shore *et al.*, 1988; Sheppard & Scypinski, 1988; Umeno *et al.*, 1989). SP is degraded *in vitro* by several peptidases including neutral endopeptidase (NEP) and, to a lesser degree, angiotensin-converting enzyme (ACE) (Skidgel *et al.*, 1984). Although the lung contains both ACE and NEP activities, they are distributed differently between airway and vascular tissues, ACE being more active in vascular and NEP in airway tissues (Johnson *et al.*, 1985). By use of immunohistochemical staining, ACE has been shown to be localized at the luminal surface of the vascular endothelium, whereas NEP has been localized within epithelial cells of the alveolar septa and within tracheal smooth muscle and epithelium (Johnson *et al.*, 1985; Sekizawa *et al.*, 1987). Thus, the relative ability of ACE-inhibitors and NEP-inhibitors to facilitate the effects of exogenous SP *in vivo* may be influenced not only by the activity of these enzymes, but also by the route of administration for SP. In the guinea-pig, inhibition of ACE by captopril and of NEP by thiorphan, shifted the dose-response curve for the bronchoconstrictor effect of intravenously-administered SP to the same extent (Shore *et al.*, 1988) in spite of the *in vitro* observation that the SP-cleaving activity of ACE is less than that of NEP (Skidgel *et al.*, 1984).

We reasoned that if different distribution in the lung of ACE- and NEP-activity explained the results of Shore *et al.* (1988), then NEP-inhibition should be much more efficient than ACE-inhibition in shifting the dose-response curve to inhaled SP, because both the higher SP-cleaving activity of NEP and its localization in airway epithelial cells would work in that direction. We therefore compared in guinea-pigs the effects of NEP-inhibition and of ACE-inhibition on changes in lung resistance (R_L) induced by aerosolised SP. We administered SP in sufficient doses to provoke bronchoconstriction before and after inhibition of the degrading enzymes, thus enabling us to calculate the shifts of the concentration-response curve for SP within the same animal. We gave the inhibitors by aerosol and used captopril to inhibit ACE and acetorphan to inhibit NEP. Acetorphan is a prodrug becoming about 1000 times more active after hydrolysation into thiorphan (Lecomte *et al.*, 1986).

Methods

Preparation

We studied male Dunkin-Hartley guinea-pigs weighing 350–600 g. The animals were fed standardized guinea-pig chow and tap water freely. On the day of study they were weighed and anaesthetized with an initial dose of 6–8 ml kg^{-1} of urethane diluted to 25% w/v in 0.9% saline, injected intraperitoneally. Additional urethane was given as required to maintain appropriate anaesthesia level. A tracheal cannula (9–11 mm in length and 2.7 mm inner diameter) was inserted into the lumen of the cervical trachea through a tracheostomy, and tied snugly with suture material. A polyethylene catheter was inserted into the left carotid artery to monitor blood pressure and heart rate with a pressure transducer. The right external jugular vein was cannulated for the administration of i.v. drugs or fluids.

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The guinea-pigs were placed in a supine position with the tracheal cannula connected to a constant volume mechanical ventilator (Harvard model 50-1718, Edenbridge, U.K.). A tidal volume of 10 ml per kg and a frequency of 60 breaths per min was used. Transpulmonary pressure was measured with a pressure transducer (Furness, model FCO 40; $\pm 1000\,mmH_2O$, Glasgow), with one side attached to a catheter inserted into the right pleural cavity and the other side attached to a catheter connected to a side port of the intratracheal cannula. The ventilatory circuit had a total volume of 20 ml. Airflow was measured with a pneumotachygraph (Mercury, model F1, Glasgow) connected to a transducer (Model FCO 40; $\pm 1\,cmH_2O$: Furness Controls Ltd, Bexhill, Sussex). Tidal volume was obtained by electronic integration of the flow signal. The aerosols were generated with an ultrasonic nebuliser (PulmoSonic, Model 2511; DeVilbiss Co, PA, U.S.A.), and were administered to the airways through a separate ventilatory system, that bypassed the pneumotachygraph. The volume of this circuit was 50 ml. The output from this system, measured at the port of the tracheal cannula with an airflow equivalent to the ventilation rate, was 40 μl per 60 breaths.

We used a 6 channel recorder (model MX6, Devices Ltd, London) for flow, transpulmonary pressure and blood pressure measurements. Expiratory lung resistance (R_L) was calculated by the method of von Neergaard & Wirz (1927). Briefly, the pressure to overcome resistance (P_r) was divided by the airflow measured at a time point 0.1 s after beginning of expiration for each calculated breath.

Protocols

The animals were divided into six groups in order to study the effect of three different concentrations of acetorphan (0.3, 1 or 3 mM), captopril (4.6 mM) or vehicle on the response to SP, and the effect of acetorphan on the response to ACh. All these treatments were given for 60 breaths. The concentrations for acetorphan were selected according to preliminary experiments. The concentration for captopril was then chosen to give approximately the same relation to the highest concentration of acetorphan, as the relation between the dose for thiorphan and captopril in an earlier study using i.v. administration of the enzyme inhibitors (Shore *et al.*, 1988). ACh-challenged animals were treated with the highest concentration of acetorphan, in order to investigate the specificity of acetorphan for SP.

All animals were intially treated with propranolol ($1\,mg\,kg^{-1}$, i.v.), to counteract inhibitory effects on the airway smooth muscle induced by catecholamines released by urethane (Spriggs, 1965). Ten minutes later the guinea-pigs were exposed to an airway challenge with aerosolised saline (0.9%), given for 20 breaths and baseline R_L measurements were made. Five minutes later, a dose-response challenge to SP or ACh was started, by increasing the concentration in the nebuliser at half log molar concentrations, starting at 3 μM for SP, and at 30 μM for ACh. For each dose, the nebuliser was filled with 3 ml of the solution. All challenge inhalations were given for 20 breaths and at 5 min intervals. Two hyperinflations with twice the tidal volume were performed between each challenge, by blocking the outflow of the ventilator. The dose-response challenges were stopped when an increase of at least 200% in R_L was seen. Subsequently the animals were allowed to recover to stable R_L values before a single dose of enzyme inhibitor, or vehicle, was given by aerosol for 60 breaths from another nebuliser.

Fifteen minutes after treatment with enzyme inhibitor, or vehicle, a second dose-response challenge, similar to the previous one, was performed. A baseline R_L value was again obtained by exposing the animals to 20 breaths of saline (0.9%). The second SP dose-response was started at 0.03 mM for animals given 11 nmol of acetorphan, at 0.3 μM when given 3.3 nmol acetorphan, and 3 μM when given 0.9 nmol. The second ACh challenge, after acetorphan 11 nmol, was started at the same dose of ACh as the first challenge. For animals given captopril 21 nmol, or vehicle, the second challenges were started at 3 μM of SP. If not significant response was seen with SP at the maximum concentration of 1 mM, a second challenge, with the same concentration in the nebuliser, but given for twice the number of breaths (40), was performed. This dose was labelled 2 mM, for calculation of PC_{200} values.

The responses were evaluated as the maximum R_L values after each challenge, and were expressed as % increase above the R_L value obtained after aerosolised saline (0.9%). PC_{200} was defined as the provocative concentration for a 200% increase of R_L over baseline. PC_{200} was determined for each dose-response challenge, by log-linear interpolation between the concentrations connecting the points where R_L reached 200% above baseline (Figure 1).

Drugs

We used the following drugs: propranolol (Inderal, ICI); acetorphan (kindly donated by Bioprojet, Paris, France); captopril (Squibb); urethane diluted to 25% w/v in 0.9% saline; SP and ACh (Sigma Chemical Company, Poole, Dorset). SP and ACh were inhaled diluted in 0.9% saline. Acetorphan was stored at 4°C in a stock solution in 5% ethanol. Further dilution was made in saline to 3 mM, which was stored at −20°C. Equivalent concentrations of diluents were stored in a similar way and were used in control experiments. Captopril was dissolved in the same solvent as acetorphan.

Data analysis

Data are reported as mean ± s.e.mean. Statistical analyses were done on log-transformed PC_{200} values. For each experiment we calculated the $\log_{10}$ shift of PC_{200} between the first and second challenge. One way analysis of variance (ANOVA) and Dunnett's test (Zar, 1974) were used to determine significant differences among groups for the shift in PC_{200} and for changes in baseline R_L. Data were analysed with a Macintosh computer (Apple Computer Inc., Cupertino, Ca, U.S.A.) using a standard statistical package (StatView 512, Abacus Concept, Inc., CA, U.S.A.).

Results

Mean baseline values after aerosolised saline (20 breaths) for the different animal groups are given in Table 1. SP aerosol caused a dose-dependent increase in R_L at concentrations of 0.1 mM and higher (Figure 1). The cut-off level for definition of the provocative concentration, 200% increase of R_L above baseline, was found on the linear part of the concentration-response curve. Treatment with acetorphan caused a dose-dependent increase in airway responsiveness to aerosolised SP. The shift in PC_{200} was significant compared to that after vehicle for all three concentrations used. The highest dose of acetorphan (110 nmol) shifted PC_{200} for SP by 1.8 (±0.26) log units to the left (Figures 2 and 3). This shift was significantly larger than that induced by 0.9 nmol ($p < 0.05$) but not significantly different from that induced by 3.3 nmol acetorphan. In contrast, treatment with aerosolised captopril (4.6 mM;

Table 1 Baseline lung resistance values ($cmH_2O\,ml^{-1} \times s^{-1}$) in the various treatment groups prior to substance P (SP) or acetylcholine (ACh) challenge

Treatment challenge	n	*First challenge*	*Second*
Vehicle + SP	5	0.28 (0.03)	0.41 (0.06)
Acetorphan 3 mM + ACh	4	0.30 (0.04)	0.50 (0.17)
Acetorphan 0.3 mM + SP	6	0.27 (0.01)	0.49 (0.06)
Acetorphan 1 mM + SP	6	0.30 (0.02)	0.77 (0.16)
Acetorphan 3 mM + SP	7	0.34 (0.03)	0.79 (0.11)
Captopril 4.6 mM + SP	6	0.23 (0.01)	0.38 (0.04)

Values are mean ± s.e.mean

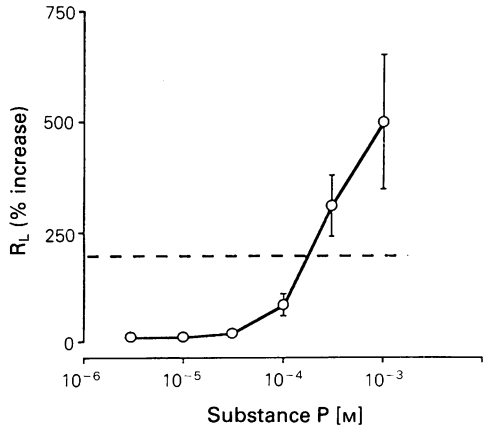

Figure 1 Mean increase in lung resistance (R_L), expressed as % above baseline, with increasing concentrations of substance P aerosol in 30 guinea-pigs ($n = 30$ up to 0.1 mM, $n = 27$ for 0.3 mM, and $n = 11$ for 1 mM), s.e.mean shown by vertical bars. We defined airway responsiveness to substance P as the provocative concentration needed to cause a 200% increase in R_L above baseline, as shown by the horizontal dotted line.

210 nmol) (Figure 2) or vehicle before the second SP challenge, or with 3 mM acetorphan before the second ACh-challenge, induced only small, nonsignificant shifts in PC_{200} (Figure 2).

The mean baseline R_L before the second dose-response challenge were in all groups, including the control group treated by vehicle, higher than before the first challenge

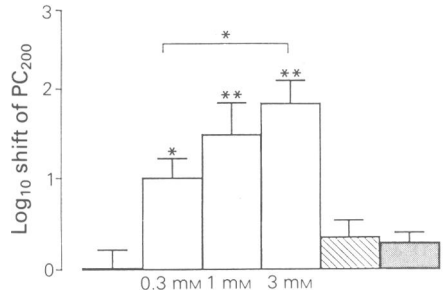

Figure 2 Leftward shift in PC_{200} (mean with s.e.mean shown by vertical bars) for substance P after treatment with vehicle (filled column), acetorphan (open columns at concentrations indicated) and 4.6 mM captopril (hatched column). The stippled column indicates the leftward shift in PC_{200} for acetylcholine after treatment with 3 mM acetorphan. Acetorphan induced a dose-dependent shift of PC_{200} for substance P, whereas captopril or vehicle had no significant effects. Acetorphan had no effect on the response to acetylcholine. (* $P < 0.05$; ** $P < 0.01$).

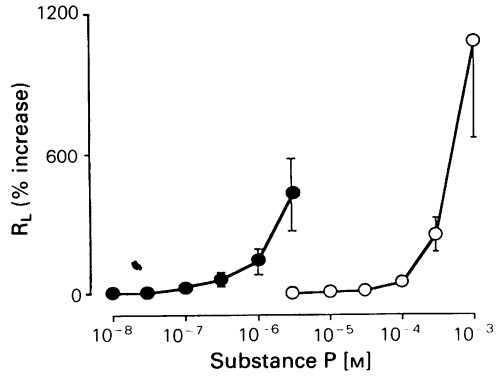

Figure 3 Mean concentration-response curves for substance P before (○) and after (●) aerosolisation of acetorphan (3 mM, 60 breaths; 110 nmol); s.e.mean shown by vertical bar. Acetorphan induced a significant leftward shift of the dose-response curve for substance P ($P < 0.01$).

(Table 1). However, this increase did not differ significantly between the groups. Baseline R_L did not correlate with the shifts in PC_{200} for SP-induced by acetorphan ($r = 0.09$).

Nebulisation of vehicle, acetorphan or captopril (60 breaths) induced a temporary increase in R_L in all groups. Compared to the increase in R_L induced by vehicle, only 2 nmol acetorphan induced significantly ($P < 0.01$) larger increases in R_L. There was a slight nonsignificant increase of the mean blood pressure in all groups 15 min after the nebulisation.

Discussion

This study shows that NEP-inhibition markedly potentiates the effect of aerosolised SP on lung resistance in guinea-pigs, whereas inhibition of ACE causes no significant potentiation. This is in contrast to studies where SP and the inhibitors were given intravenously in the same species. Inhibition of ACE was then as effective as inhibition of NEP in enhancing bronchoconstrictor responses (Shore *et al.*, 1988). Furthermore, the maximal shift of the dose-response curve for SP is clearly larger in this study using aerosol administration (1.8 log units) compared to the i.v. route (about 0.5 log units) (Shore *et al.*, 1988). The specificity of the effect of NEP-inhibition was demonstrated by its lack of effect on the response to aerosolised ACh.

Both NEP and ACE cleave SP (Skidgel *et al.*, 1984). The vascular endothelium is rich in ACE (Johnson *et al.*, 1985; Ryan *et al.*, 1985) implying SP administered i.v. is likely to be cleaved by ACE before reaching target tissues in the airways. However, the ACE-activity in airway tissue has been shown to be low compared to that of vascular tissue (Johnson *et al.*, 1985). Our data, showing no significant effect of ACE-inhibition on the response to aerosolised SP, indicates that the ACE-content in airway mucosa is low, which would explain the larger effect of NEP-inhibition on aerosolised SP compared to i.v. SP. Acetorphan and thiorphan also inhibit ACE (Schwartz *et al.*, 1981) which could contribute to the enhancement of the SP response (Skidgel *et al.*, 1984). However, this can be excluded as ACE-inhibition, since captopril had no significant effect. Furthermore, thiorphan is about 40 times less potent as an inhibitor of ACE than as an inhibitor of NEP (Schwartz *et al.*, 1981) and it does not affect ACE-activity in plasma when given by i.v. infusion (Spillantini *et al.*, 1986).

The maximal shift of PC_{200} for aerosolised SP induced by NEP-inhibition in our study agrees well with data from an earlier study by Dusser *et al.* (1988) using phosphoramidon for inhibition of NEP. They did not use sufficient SP-concentrations to elicit a bronchoconstrictor response before NEP inhibition, allowing calculation of the shift. However, if our concentration-response curve before NEP inhibition (Figure 1) is applied to their data, the calculated shift would be of the same magnitude as ours.

SP aerosol has given no bronchoconstrictor response in some earlier studies in guinea-pig (Dusser *et al.*, 1988) and in man (Joos *et al.*, 1987). However, if the SP aerosol is given in sufficiently high concentrations (about 1 mM), it induces airway narrowing in the absence of NEP inhibition (Lötvall *et al.*, 1989). The present and earlier (Dusser *et al.*, 1988) studies indicate that the probable explanation of the need for such high concentrations of SP is that NEP in airway epithelial cells constitutes a metabolic barrier to inhaled SP. When administered by the i.v. route, SP bypasses this barrier but may be metabolised by ACE in vascular tissues (Johnson *et al.*, 1985) and NEP in circulating blood (Spillantini *et al.*, 1986).

The mean baseline R_L before the second concentration-response curve had increased in all treatment groups including those treated with captopril and vehicle (Table 1). There was no significant difference between the groups in this respect, although the change in baseline R_L seemed to be more pronounced in acetorphan-treated animals, indicating a specific effect of acetorphan on R_L. This is further supported by

the observation that aerosolisation of 3 mM acetorphan, both in SP- and in ACh-challenged animals, induced a temporary increase in R_L that was significantly larger than that seen after treatment with vehicle. This indicates that aerosolisation of vehicle may induce a slight tachykinin release, the effect of which is then potentiated by acetorphan, but there is no direct evidence to support this hypothesis.

It should be pointed out that acetorphan is a prodrug that is about 1000 times more active after hydrolysation into thiorphan. Such hydrolysation has been demonstrated to take place in mouse brain membranes (Lecomte *et al.*, 1986) and in plasma (Spillantini *et al.*, 1986). Our data show that acetorphan also must be hydrolysed locally in the airways, presumably in the airway epithelium. Hydrolysation after systemic uptake would most probably provide too low concentrations to have any activity.

Our study confirms earlier studies demonstrating the key role for NEP-like enzymes in the lung for the control of the response to exogenous SP (Borson *et al.*, 1987; Stimler-Gerard, 1987; Sekizawa *et al.*, 1987; Dusser *et al.*, 1988; Shore *et al.*, 1988). It has extended these observations showing that the potentiation of the response to aerosolised SP by inhibition of NEP is indeed large, about two log units, whereas inhibition of ACE has no significant effect on the response to aerosolised SP.

This study also indicates the probable importance of NEP-like enzymes in airway epithelial damage. SP-reactive sensory nerves are present within and underneath the airway epithelium (Lundberg *et al.*, 1984b). Many airway irritants stimulate sensory nerves (Sellick & Widdicombe, 1971). It is then likely that airway epithelial damage can increase the responsiveness to various inhaled irritants via an impairment of the NEP-like activity. Support for this reasoning is given by studies showing that viral infections enhance the contractile responses to SP in guinea-pig bronchi (Saban *et al.*, 1987) and ferret trachea (Jacoby *et al.*, 1988). Furthermore, histories of infections in rats have been associated with a decreased activity of NEP and an enhanced SP-induced vascular leakage in the trachea (Borson *et al.*, 1989).

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Effects of epithelium removal on relaxation of airway smooth muscle induced by vasoactive intestinal peptide and electrical field stimulation

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1 We have studied the effect of epithelium removal on relaxation of guinea-pig isolated tracheal smooth muscle induced by vasoactive intestinal peptide (VIP) or stimulation of non-adrenergic, non-cholinergic (NANC) inhibitory nerves. Also examined were the effects of inhibitors of neutral endopeptidase (NEP) and angiotensin-converting enzyme (ACE).

2 Epithelium removal produced a 3.6 ± 0.4 fold leftward shift in the VIP concentration-response curve. The supersensitivity to VIP, following epithelium removal was abolished by phosphoramidon or thiorphan (NEP inhibitors), but unaffected by captopril (an ACE inhibitor). In intact trachea, the NEP inhibitors produced leftward shifts in the VIP curves similar to those produced by epithelium removal.

3 In contrast to responses to exogenous VIP, neurogenic NANC inhibitory responses to electrical field stimulation were affected neither by epithelial denudation nor by the peptidase inhibitors.

4 As in previous studies, epithelium removal increased tracheal sensitivity to isoprenaline. This was not altered by pretreatment with a cocktail of peptidase inhibitors. Thus, the effect of the NEP inhibitors on responses to VIP appears to be relatively specific.

5 These data indicate that exogenous VIP is a substrate for airway NEP, since inhibition of the enzyme potentiates the peptide. This is further evidence that the airway epithelium provides a source for the metabolism of mediators.

6 In guinea-pig trachea the NEP responsible for cleaving VIP may be located largely in the epithelial layer, since NEP inhibition was without effect on sensitivity to VIP in epithelium-denuded preparations. If VIP is a NANC inhibitory neurotransmitter in this tissue, its degradation endogenously does not appear to involve epithelial NEP.

Introduction

Epithelium removal modulates airway smooth muscle responsiveness to a variety of spasmogenic and relaxant agonists (see reviews by Fedan *et al.*, 1988; Goldie *et al.*, 1990), and there is evidence for an epithelium-derived inhibitory factor (EpDIF) that inhibits vascular and airway smooth muscle (Hay *et al.*, 1987; Fernandes *et al.*, 1989). In contrast, augmentation of tracheal responses to some agents may be due to loss of epithelial sites of uptake and/or enzymatic degradation for those particular agents. For example, increased sensitivity of guinea-pig trachea to the relaxant effect of isoprenaline, following epithelium removal, appears to be due solely to loss of catecholamine uptake and degradation (Farmer *et al.*, 1986). This is suggested by the observation that an inhibitor of extraneuronal uptake abolished supersensitivity to isoprenaline induced by removal of the epithelium. Moreover, epithelium removal had no effect on sensitivity to salbutamol, which is not a substrate for extraneuronal uptake (Farmer *et al.*, 1986).

Epithelium removal increases tracheal sensitivity to adenosine-induced relaxation (Farmer *et al.*, 1986; Advenier *et al.*, 1988). This, too, is due to loss of epithelial sites of adenosine degradation, as the effect is abolished by drugs which inhibit adenosine uptake and metabolism (Advenier *et al.*, 1988). The epithelium also converts exogenous arachidonic acid into relaxant products, and denudation transforms arachidonate-induced relaxation into contraction in guinea-pig trachea (Nijkamp & Folkerts, 1986; Farmer *et al.*, 1987). In addition, the increase in responsiveness to contractile effects of substance P may be due largely to loss of epithelial neutral endopeptidase (NEP; EC 3.4.24.11), which degrades this peptide (Devillier *et al.*, 1988; Frossard *et al.*, 1989).

There is much evidence to suggest that vasoactive intestinal peptide (VIP) is a non-adrenergic, non-cholinergic (NANC) inhibitory neurotransmitter in airway smooth muscle (Matsuzaki *et al.*, 1980; Sundler *et al.*, 1988; Ellis & Farmer, 1989a,b). Although the mechanisms underlying VIP degradation in the airway are unclear, it has been demonstrated that certain mast cell-derived proteases will degrade VIP (Caughey *et al.*, 1988) and that these same enzymes reverse VIP-induced tracheal relaxation *in vitro* (Franconi *et al.*, 1989). In addition, a metalloendopeptidase cleaves VIP in rat spinal tissue *in vitro* (Barbato *et al.*, 1988).

Recently, the functions of airway NEP have been the subject of much interest (Thompson & Sheppard, 1988; Djokic *et al.*, 1989; Dusser *et al.*, 1989). This enzyme is present in the plasma membrane of epithelial cells in various organs (Matsas *et al.*, 1984; Erdös & Skidgel, 1989; Ryan, 1989) and human recombinant NEP cleaves VIP into several peptide fragments (Goetzl *et al.*, 1989). The purpose of the present investigation was to assess the effects of epithelium removal on guinea-pig tracheal sensitivity to VIP and the influence of inhibitors of NEP and angiotensin-converting enzyme (ACE). We also examined the effect of epithelium removal and peptidase inhibitors on NANC inhibitory responses to electrical field stimulation (EFS). Some of these data have been presented to the British Pharmacological Society (Farmer & Togo, 1989).

Methods

Tissue preparation

Male, Dunkin-Hartley guinea-pigs (350–500 g: Hazelton, Denver, Pennsylvania) were stunned, exsanguinated and the trachea removed. This was placed in modified Krebs-Henseleit solution (composition mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, glucose 10.0). Extraneous tissue was dissected free and transverse strips, consisting of two adjacent cartilage rings, were suspended in organ chambers containing Krebs solution maintained at 37°C and

gassed with 95% O_2/5% CO_2. Alternate tracheal strips were denuded of epithelium with a cotton-tipped applicator. Tissues were equilibrated for 60 min at an initial resting tension of 1.5 g and washed with Krebs solution every 15 min.

Experimental protocol

At the end of equilibration, each preparation was exposed to an equieffective concentration of methacholine (MCh EC_{60} + epithelium 2 μM; −epithelium 1 μM; Hay *et al.*, 1986) to assess tissue viability. VIP was added to the bath in increasing concentrations cumulatively (1 nM–0.3 μM). In some experiments, VIP concentration-response curves were obtained in tissues precontracted with MCh, at the same concentrations used to assess viability. Responses to VIP are expressed as a % of the maximal relaxation to sodium nitroprusside (SNP; 30 μM) added at the end of the experiment.

Where appropriate, EFS was delivered to platinum electrodes from a Grass S-88 stimulator whose output was passed through a Stimu-Splitter II (Med-Lab Instruments, Loveland, Colorado) for signal amplification. Frequency-response curves were generated by applying stimuli (20 V, 0.2 ms, 1–20 Hz) for 30 s. All EFS experiments were conducted in the presence of atropine (1 μM) and propranolol (1 μM) to abolish cholinergic and noradrenergic responses, respectively. Both the peak magnitude of NANC relaxations, and the time taken for 50% recovery of prestimulation tone were determined. Each NANC response was expressed as a percentage of the maximum relaxation induced by 20 Hz stimulation.

The effects of phosphoramidon and DL-thiorphan, NEP inhibitors, and captopril, an ACE inhibitor (each at 10 μM) were examined after they had been added 20 min before the application of VIP or EFS. We also determined the effect of epithelium removal and the peptidase inhibitors on tracheal sensitivity to isoprenaline (0.1 nM–0.1 μM), which was also added to the bath in a cumulative manner. Each response to isoprenaline was expressed as a percentage of the maximum relaxation to this agent. All experiments with peptidase inhibitors were conducted in preparations with basal tone.

The pD_2 values for VIP or isoprenaline were determined from regression analyses of logit-transformed concentration-response curves. Responses to EFS, VIP or isoprenaline are expressed as mean ± s.e.mean. The effects of epithelium removal and peptidase inhibitors were compared with their respective controls by Student's two-tailed *t* test for paired observations. Probability values of ≤ 0.05 were considered significant.

Drugs

Methacholine Cl, atropine H_2SO_4, (±)-isoprenaline HCl, propranolol HCl, SNP, DL-thiorphan and tetrodotoxin (TTX), were obtained from the Sigma Chemical Co. (St. Louis, Missouri). Porcine VIP was purchased either from Sigma or from Bachem Inc. (Philadelphia, Pennsylvania). Phosphoramidon was obtained from Peninsula Laboratories Inc. (Belmont, California). Captopril was purchased from Squibb Pharmaceuticals Inc. (Princeton, New Jersey).

Atropine, captopril, MCh, propranolol, SNP and TTX were each dissolved in 0.9% w/v NaCl solution (saline). Isoprenaline was prepared extemporaneously, as a 10 mM solution, in saline containing 0.25% w/v ascorbic acid, and thiorphan and phosphoramidon were dissolved in dimethylsulphoxide and distilled water, respectively.

Results

Effects of epithelium removal

Epithelium removal produced an increase in potency and rate of relaxation to VIP in tracheal strips with basal or MCh-induced tone (Figure 1). In intact and denuded trachea with basal tone, the pD_2 values for VIP of 7.74 ± 0.05 and 8.25 ± 0.06 respectively, were significantly different. Epithelium removal produced a 3.6 ± 0.4 fold leftward shift in the VIP concentration-response curve ($n = 14$, Figure 2). In the presence of an EC_{60} of MCh, the VIP concentration-response curves were shifted in a dextral manner, to a similar extent in intact and denuded tissues (Figure 2). In MCh-treated trachea, the pD_2 value for VIP in intact tissues (7.02 ± 0.08, $n = 9$) was significantly lower than in denuded preparations (7.42 ± 0.10). Epithelium removal caused a 2.8 ± 0.3 fold increase in sensitivity to VIP in the presence of MCh.

The pD_2 values for VIP in intact tissues treated and not treated with atropine and propranolol were 7.82 ± 0.27 and 7.79 ± 0.21, respectively ($n = 3$). The corresponding values for

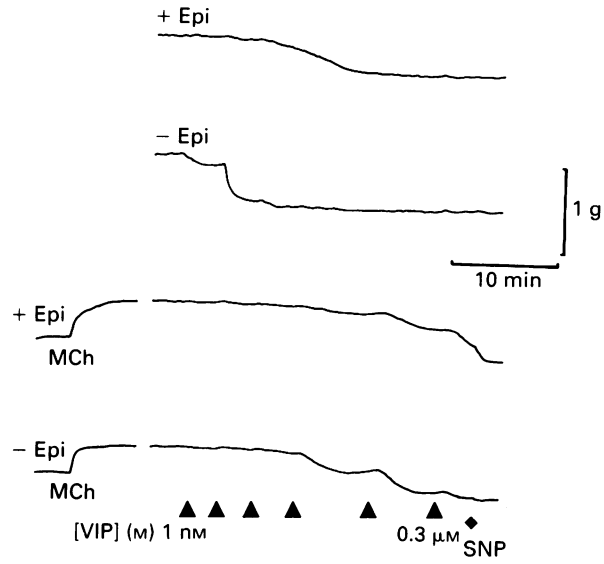

Figure 1 Relaxations of guinea-pig trachea to vasoactive intestinal peptide (VIP). The top two parts of the figure show responses in preparations with basal, spontaneous tone, and the lower two, in preparations precontracted with an EC_{60} methacholine (+ epithelium 2 μM; − epithelium 1 μM). The tissues represented by the second and fourth tracings were denuded of their epithelium. For each preparation, VIP (1 nM–0.3 μM) was added at the arrows and sodium nitroprusside (SNP, 30 μM), applied at the end of the experiment, was used to determine the maximum relaxation (i.e. zero active tension). MCh = methacholine.

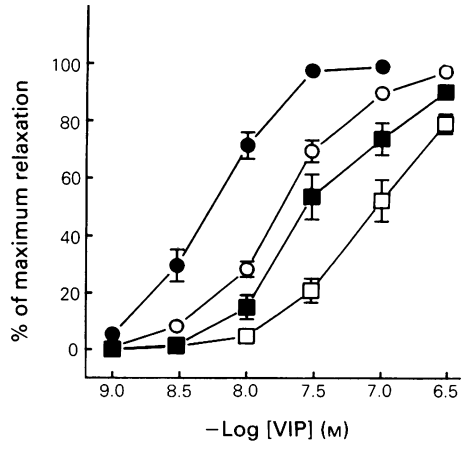

Figure 2 Concentration-response curves for vasoactive intestinal peptide (VIP), showing the effect of epithelium removal, in guinea-pig trachealis. Responses to VIP were expressed as a % of the maximum relaxation induced by sodium nitroprusside (30 μM). (○) Epithelium-intact controls with basal tone; (●) epithelium-denuded tissues with basal tone; (□) intact tissues precontracted with methacholine (MCh, 2 μM); (■) denuded tissues precontracted with MCh (1 μM). Each point represents the mean of 14 (basal tone) or 9 (precontracted) observations; vertical lines show s.e.mean.

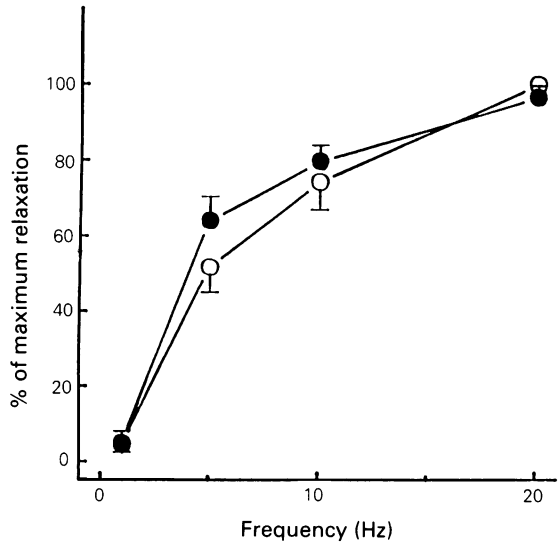

Figure 3 Frequency-dependent, non-adrenergic, non-cholinergic inhibitory responses of guinea-pig trachea to electrical field stimulation and the effect of epithelium removal. Responses were determined as a percentage of the maximum relaxation induced by stimulation at 20 Hz. Experiments were carried out in the presence of atropine and propranolol (each at 1 μM). (○) + Epithelium; (●) − epithelium. Each point represents the mean of 13 observations; vertical lines show s.e.mean.

epithelium-denuded tissues were 8.60 ± 0.41 and 8.34 ± 0.12, respectively (n = 3). Thus, VIP had similar relaxant activity in trachea treated with atropine and propranolol and, in such tissues, epithelium removal caused a similar potentiation (4.4 ± 1.3 fold, $P \leqslant 0.05$) to VIP.

In the presence of atropine (1 μM) and propranolol (1 μM), EFS induced frequency-dependent relaxations which returned slowly to baseline upon cessation of stimulation. These NANC inhibitory responses were abolished by TTX (0.1 μM) confirming their neurogenic origin. Epithelium removal did not alter the magnitude of relaxation at any frequency (Figure 3). Similarly, the maximum NANC relaxation of 658 ± 67 mg (n = 13) in intact trachea was not different from 691 ± 89 mg in denuded tissues. Epithelium removal did not influence the time taken for preparations to recover 50% of their pre-stimulation level of tone. In intact tissues at 20 Hz, for example, this time was 5.67 ± 0.42 min compared with 6.02 ± 0.82 min in denuded trachea. Thus, epithelium removal had no discernible effect on NANC responses.

Effects of peptidase inhibitors

The pD_2 values for VIP in intact tissues treated and not treated with captopril (10 μM) were 7.74 ± 0.05 and 7.64 ± 0.12 respectively. The corresponding values for epithelium-denuded tissues were 8.25 ± 0.06 and 8.10 ± 0.12 respectively. These data suggest that captopril neither modifies the action of VIP on intact trachea nor modifies the effects of epithelium removal on the action of VIP (Figure 4).

In contrast to the ACE inhibitor, both phosphoramidon and thiorphan caused leftward shifts in the VIP concentration-response curve and abolished the effect of epithelium removal (Figures 5 and 6). Phosphoramidon increased

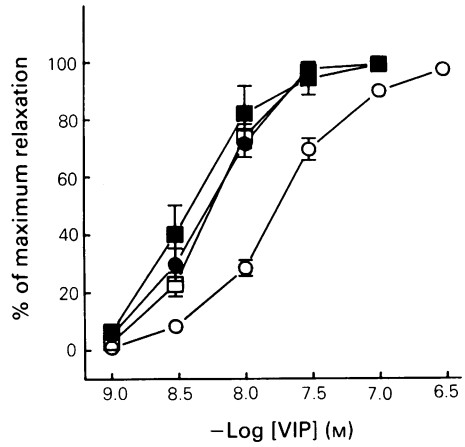

Figure 5 Concentration-response curves for vasoactive intestinal peptide (VIP), showing the effect of epithelium removal and phosphoramidon (10 μM), in guinea-pig trachealis with basal tone. Responses to VIP were expressed as a % of the maximum relaxation induced by sodium nitroprusside (30 μM). (○) Epithelium-intact controls; (●) epithelium-denuded tissues; (□) intact tissues in the presence of phosphoramidon; (■) denuded tissues in the presence of phosphoramidon. Each point represents the mean of 8 observations; vertical lines show s.e.mean.

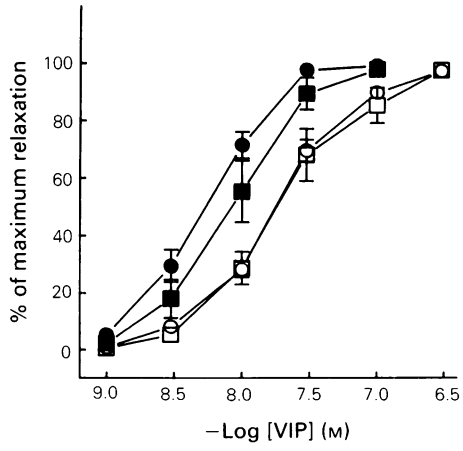

Figure 4 Concentration-response curves for vasoactive intestinal peptide (VIP), showing the effect of epithelium removal and captopril (10 μM), in guinea-pig trachealis with basal tone. Responses to VIP were expressed as a % of the maximum relaxation induced by sodium nitroprusside (30 μM). (○) Epithelium-intact controls; (●) epithelium-denuded tissues; (□) intact tissues in the presence of captopril; (■) denuded tissues in the presence of captopril. Each point represents the mean of 9 observations; vertical lines show s.e.mean.

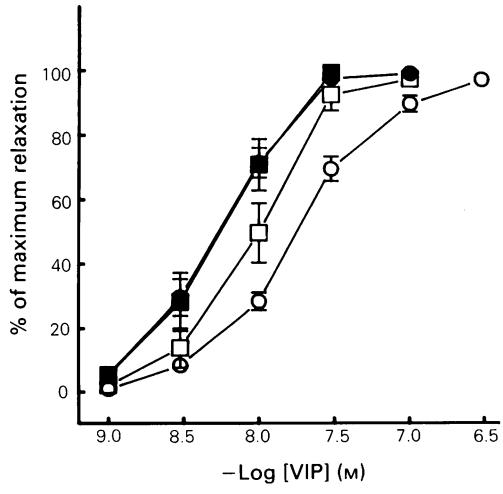

Figure 6 Concentration-response curves for vasoactive intestinal peptide (VIP), showing the effect of epithelium removal and thiorphan (10 μM), in guinea-pig trachealis with basal tone. Responses to VIP were expressed as a % of the maximum relaxation induced by sodium nitroprusside (30 μM). (○) Epithelium-intact controls; (●) epithelium-denuded tissues; (□) intact tissues in the presence of thiorphan; (■) denuded tissues in the presence of thiorphan. Each point represents the mean of 7 observations; vertical lines show s.e.mean.

Table 1 Effects of epithelium removal and peptidase inhibitors[1] on sensitivity of guinea-pig tracheal smooth muscle to the relaxant effect of isoprenaline

Treatment	pD_2 values for isoprenaline + Epithelium	− Epithelium	Mean shift
Control	8.35 ± 0.06	8.68 ± 0.14*	2.5 ± 0.6
Plus inhibitors[1]	8.38 ± 0.06	8.94 ± 0.20*	6.4 ± 3.2

[1] The cocktail of inhibitors comprised phosphoramidon and captopril, each at 10 μM. * Denotes significantly different from epithelium-intact control. Data are expressed as the mean ± s.e.mean of seven observations.

sensitivity to VIP in intact trachea only. In the presence of the NEP inhibitor, the pD_2 value in intact tissues (8.24 ± 0.06; $n = 8$), was significantly different from control and represented a leftward shift of 3.2 fold. Phosphoramidon abolished the effect of epithelium removal on sensitivity to VIP (mean shift, 1.6 ± 0.3, Figure 5). Similarly, thiorphan increased sensitivity to VIP in preparations with intact epithelium, but had no effect in denuded trachea (Figure 6). The pD_2 value for intact tissues, in the presence of thiorphan, was 8.07 ± 0.10 and this was not different from the value of 8.30 ± 0.08 in denuded trachea (shift, 1.80 ± 2.3, $n = 7$).

Phosphoramidon was without effect on the magnitude or time-course of NANC inhibitory responses to EFS, irrespective of epithelial integrity. The maximum magnitude of relaxation in intact tissues, pretreated with phosphoramidon, was 777 ± 76 mg and this was not different from 746 ± 83 mg in denuded tissues. Further, in the presence of phosphoramidon, epithelium removal had no effect on the time taken to recover 50% of initial tone (+ epithelium 6.09 ± 0.39 min; − epithelium 5.78 ± 0.55 min; 20 Hz).

Isoprenaline

Epithelium removal caused an approximately 3 fold increase in sensitivity to isoprenaline (Table 1). In the presence of the peptidase inhibitors, epithelium removal caused an apparently larger shift in the isoprenaline curve, but this was not significantly different from the shift in the absence of inhibitors.

Discussion

Removal of guinea-pig tracheal epithelium increases responsiveness of the underlying smooth muscle to tachykinin-induced contraction (Fine *et al.*, 1989; Frossard *et al.*, 1989; Tschirhart *et al.*, 1989), as well as to adenosine- (Farmer *et al.*, 1986; Advenier *et al.*, 1988) and isoprenaline-induced relaxations (Farmer *et al.*, 1986). For substance P, the effect of epithelium removal may be due partly to elimination of epithelial NEP (and thus, substance P degradation) and partly to loss of prostanoid and non-prostanoid inhibitory factors (Fine *et al.*, 1989; Frossard *et al.*, 1989). Conversely, enhancement of responses to adenosine and isoprenaline, following epithelium denudation, appears to result solely from removal of uptake and/or catabolic processes for these agents (Farmer *et al.*, 1986; Advenier *et al.*, 1988).

The present study demonstrates that epithelium removal increases tracheal sensitivity to VIP and that this potentiation is abolished by inhibitors of NEP. That the effect of the inhibitors did not influence potentiation of responses to isoprenaline suggests their action exhibits selectivity. These data provide further evidence that the airway epithelium may be an important source of metabolism for several substances. It is also suggested that increased sensitivity to VIP following epithelium removal is due to loss of NEP. Since the NEP inhibitors had no effect in epithelium-denuded tissues, the principal source of degradation of VIP by NEP probably exists in the epithelial layer. These observations concur with another study wherein it was found that substance P is degraded by guinea-pig tracheal NEP, located mainly in the epithelium (Devillier *et al.*, 1988).

VIP is the principal candidate for the NANC inhibitory neurotransmitter in the airways of guinea-pigs (Matsuzaki *et al.*, 1980; Carstairs & Barnes, 1986; Ellis & Farmer, 1989a,b,c) and cats (Ito & Takeda, 1982; Diamond *et al.*, 1988). In addition, VIP-immunoreactive nerves and VIP receptors have been localized in human airways (Dey *et al.*, 1981; Lundberg *et al.*, 1984; Carstairs & Barnes, 1986). VIP is a very potent relaxant of airway smooth muscle *in vitro* (Altiere & Diamond, 1985; Ellis & Farmer, 1989b) and inhibits bronchoconstriction in animals (Said, 1982; Diamond *et al.*, 1983). Conversely, it has proven disappointing as a bronchodilator in man (see references in Barnes, 1986; 1988), probably due to enzymatic destruction in the lungs. Indeed, one study demonstrates that inhaled VIP is ineffective as a bronchodilator, in rats, due to metabolism during its passage through the airway epithelial layer (Barrowcliffe *et al.*, 1986). This study also found that neither the serine protease inhibitor aprotinin, nor the ACE inhibitor captopril had any effect on pulmonary destruction of VIP. Further, in feline airways *in vitro* aprotinin and captopril have no effect on VIP-induced relaxation (Altiere & Diamond, 1984). Previous studies in our laboratory also showed that aprotinin had no effect on responses of guinea-pig trachea, either to exogenous VIP or to NANC inhibitory responses to EFS (Ellis & Farmer, 1989b). However, the nature of endogenous pathways for the degradation of VIP is unclear at present. The failure of captopril to potentiate airway effects of VIP in rat (Barrowcliffe *et al.*, 1986) and cat (Altiere & Diamond, 1984) is confirmed in the present study with guinea-pig trachea. Furthermore, the ability of epithelium removal to enhance sensitivity to VIP was not affected by the ACE inhibitor. Therefore, the effect of epithelium removal probably does not involve loss of ACE.

NANC inhibitory responses were not altered by removal of the epithelium, confirming previous findings in guinea-pig (Holroyde, 1986) and cat (Thompson *et al.*, 1988b) airways. That epithelium removal increased sensitivity to exogenous VIP and yet had no effect on NANC inhibitory responses, should be commented upon in view of the putative role for this peptide in neurotransmission. Furthermore, although the present study indicates that exogenous VIP is degraded by NEP located in the tracheal epithelium, inhibition of this peptidase was without noticeable effect on the NANC response. This may be interpreted as negating a transmitter role for VIP. As alluded to above, however, there is much evidence in support of VIP as a NANC transmitter. In particular, desensitization of cat or guinea-pig trachea to VIP attenuates NANC inhibitory responses to EFS (Ito & Takeda, 1982; Ellis & Farmer, 1989a). In addition, incubation of guinea-pig trachea with antiserum specific for VIP markedly reduces the NANC response (Matsuzaki *et al.*, 1980; Ellis & Farmer, 1989a). Only when specific antagonists for airway VIP receptors become available will its role in neurotransmission be confirmed. Unfortunately, known VIP antagonists are without effect, either on responses to VIP or to EFS, in guinea-pig or feline airway smooth muscle (Thompson *et al.*, 1988a; Ellis & Farmer, 1989a).

If VIP is indeed involved in NANC neurotransmission in the trachea, then the lack of effect of epithelium removal and NEP inhibition on responses to EFS also requires comment. Epithelial NEP may not be important in regulating airway levels of endogenous VIP. In fact, although myriad peptides are known to be cleaved by NEP *in vitro*, only a few (and VIP is not among them) have been shown to be cleaved *in vivo* (Erdös & Skidgel, 1989). In human airways NEP is most concentrated in the luminal membranes of epithelial cells, in addition to being present in fibroblasts (Johnson *et al.*, 1985). As mentioned earlier, human recombinant NEP cleaves VIP *in vitro* into several fragments (Goetzl *et al.*, 1989), which have little or no effect on guinea-pig trachea (Bodansky *et al.*, 1973). Nevertheless, nerve endings involved in the control of airway tone lie predominantly in the smooth muscle layer (Laitinen, 1985; Barnes, 1986; Gabella, 1987). Further, in human airways, VIP-like immunoreactive nerves are found predomi-

nantly in the smooth muscle layer and bronchial glands (Laitinen, 1985; Sundler *et al.*, 1988), whereas the epithelium does not receive VIP-like immunoreactive nerves (Laitinen, 1985). Conversely, VIP-containing nerves are present in the airway lamina propria, in close proximity to the epithelial basement membrane (Said, 1988). It has also been demonstrated that VIP receptors are widely distributed in human and guinea-pig airway tissues, including smooth muscle and the epithelium (Carstairs & Barnes, 1986). Therefore, it is conceivable that under 'normal' conditions, neuronal VIP does not reach the epithelial sites where NEP is localized, and that the ability of NEP to cleave VIP has trivial physiological significance in large airways.

In summary, the present study indicates that increased sensitivity of guinea-pig trachea to VIP following epithelium removal, is not due to loss of epithelial diffusion barriers or epithelium-derived factors. Rather, the epithelium may contain NEP which can degrade exogenous VIP, thereby decreasing its availability to its smooth muscle receptors and limiting tissue sensitivity to the relaxant action. That epithelium removal and NEP inhibition had no effect on NANC inhibitory responses to EFS suggests that epithelial NEP may not be an important regulator of endogenous VIP. The mechanisms whereby neuronal VIP are removed and/or degraded in the airway await clarification.

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Vasopressin and the pathogenesis of chronic renal failure

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1 Partial (5/6) renal ablation was performed in Long Evans rats treated with vehicle or a vasopressin V_1-receptor antagonist, in control Long Evans rats, and in homozygous Brattleboro rats which lack endogenous vasopressin.

2 In control and vasopressin-blocked Long Evans rats, 3 weeks following partial renal ablation, systolic blood pressure was 215 ± 5 and 199 ± 9 mmHg and, urinary protein excretion was 54 ± 4 and 50 ± 3 mg day^{-1}, respectively.

3 The pressor response to exogenous vasopressin was significantly ($P < 0.05$) reduced in rats treated with the V_1-receptor antagonist (ED_{50mmHg} 5.0 ± 1.6 vs. 0.09 ± 0.01 $\mu g\,kg^{-1}$).

4 In control Long Evans and in Brattleboro rats, 3 weeks following renal ablation, systolic blood pressure was 204 ± 10 and 191 ± 7 mmHg, and urinary protein excretion was 97 ± 27 and 71 ± 5 mg day^{-1}, respectively.

5 Histological examination of the remaining kidney tissue demonstrated significant glomerular hyalinization following renal ablation but no differences between any of the groups.

6 The data indicate that neither vasopressin nor the urinary concentrating mechanism is likely to be involved in the hypertension and proteinuria associated with partial renal ablation.

Introduction

The progression of chronic renal failure appears to be mediated, in part, by glomerular hypertension (Anderson *et al.*, 1985). Ichikawa and his colleagues (Yoshida *et al.*, 1987), however, indicated that mechanisms other than glomerular hypertension, such as mesangial cell proliferation, may be important. Since vasopressin preferentially constricts the efferent arteriole of the kidney (Edwards *et al.*, 1987), causes mesangial cell growth (Ganz *et al.*, 1988), and is increased in uraemia (Benmansour *et al.*, 1982), it is considered a potential mediator in the progression of chronic renal failure. Indeed, it has been reported that increasing water intake in rats with partial renal ablation leads to an attenuated proteinuria, hypertension and renal hypertrophy (Bouby & Bankir, 1988). These authors concluded that vasopressin and/or operation of the urinary concentrating mechanism influenced the progression of the chronic renal failure. In the present study, therefore, we have investigated the effect of chronic antagonism of vasopressin V_1-receptors in rats with partial renal ablation and the effect of partial renal ablation in vasopressin-deficient homozygous Brattleboro rats.

Methods

Experimental protocols

Twenty male Long Evans rats each weighing approximately 400 g were maintained with free access to standard laboratory chow (24% protein by weight) and water. Once a week, rats were housed in metabolic cages for 48 h, and urine collected for the second 24 h period. Urine volume and urine osmolality (freezing point depression) were measured, and urinary protein excretion determined by the sulphosalicylic method (Davidsohn & Henry, 1969). At the end of the urine collection period, systolic blood pressure (SBP) was measured indirectly by tail plethysmography (Narco Biosystems). Following baseline measurements, animals underwent 5/6 renal ablation. Under sodium pentobarbitone anaesthesia (60 mg kg^{-1}, i.p.), a midline incision was made, the right kidney was removed, and approximately 2/3 of the left kidney was infarcted by ligating 2 or 3 branches of the left renal artery. At the time of surgery, an osmotic minipump (2002; Alzet) was inserted into the peritoneal space. Pumps were filled with vehicle (isotonic sterile saline; $n = 10$) with or without the vasopressin antagonist to provide a dose of 7 $\mu g\,day^{-1}$ (Hofbauer *et al.*, 1985) at a flow rate of 0.47 $\mu l\,h^{-1}$. Two weeks following surgery, the pump was replaced. Following surgery, 24 h urinary protein excretion and systolic blood pressure were determined at weekly intervals for 3 weeks. After the final urine collection, animals from each group were anaesthetized with urethane (1.5 g kg^{-1}, i.p.), and a femoral artery and vein cannulated. Blood pressure was measured with a Statham pressure transducer and a Gilson recorder, and the blood pressure changes measured after cumulative dosing with exogenous arginine-vasopressin.

In a second experiment, 5/6 renal ablation was performed in 13 male homozygous DI rats and 9 male Long Evans rats. Urinary protein excretion, SBP, urine flow, and urine osmolality were determined prior to and for 3 weeks following renal ablation.

Histology

At the end of both experiments, animals were killed and the remaining kidney removed for histological examination. This was performed in a 'blind' fashion. Kidneys were fixed by immersion in 10% buffered formalin and embedded in glycol methacrylate or paraffin. From each block, 2 μm sections were cut and stained with the periodic acid Schiff's reagent and hematoxylineosin. A subjective scoring system (from 0 to 4 as: 0, no change; 1, minimal change; 2, mild change; 3, moderate change; or 4, severe) was used to rank glomerular lesions, tubular changes (dilatation, cast formation) and interstitial inflammatory cell infiltrate. After light microscopic evaluation, it appeared that the degree of hyalinization corresponded with the other changes, and therefore, scores for glomerular hyalinization are given.

Drugs

The vasopressin antagonist, [1-(β-mercapto-β, β-cyclopentamethylene propionic acid), 1-(0-methyl tyrosine), 8-arginine] vasopressin (Manning compound; Kruszynski *et al.*, 1980; Manning & Sawyer, 1985), was prepared by chemists of

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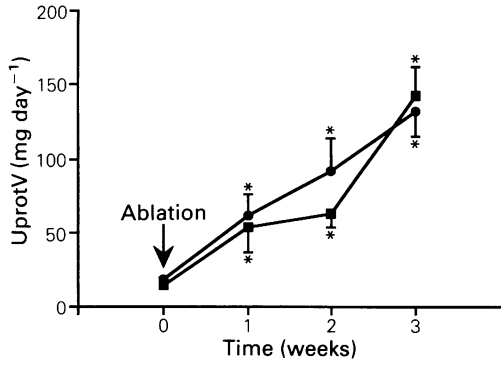

Figure 1 Effect of 5/6 renal ablation on the urinary protein excretion (UprotV) of Long Evans rats treated with vehicle (●, $n = 10$) or a vasopressin V_1-receptor antagonist (■, $n = 10$). * $P < 0.05$ vs. week 0.

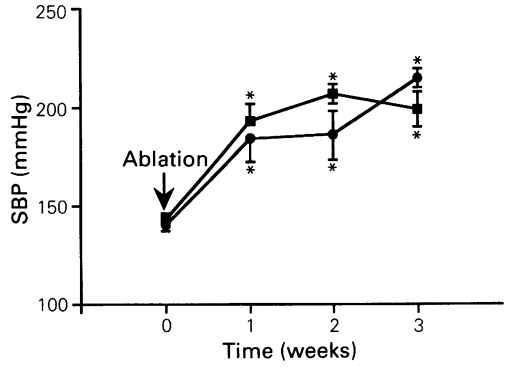

Figure 2 Effect of 5/6 renal ablation on systolic blood pressure (SBP) of Long Evans rats treated with vehicle (●, $n = 10$) or a vasopressin V_1-receptor antagonist (■, $n = 10$). * $P < 0.05$ vs. week 0.

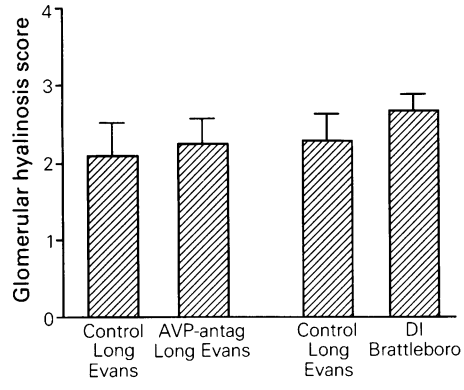

Figure 3 Glomerular hyalinosis associated with 5/6 renal ablation in vehicle ($n = 9$) and in vasopressin V_1-receptor antagonist (AVP-antag) ($n = 9$) infused Long Evans rats and in Long Evans ($n = 9$) and in Brattleboro ($n = 13$) rats.

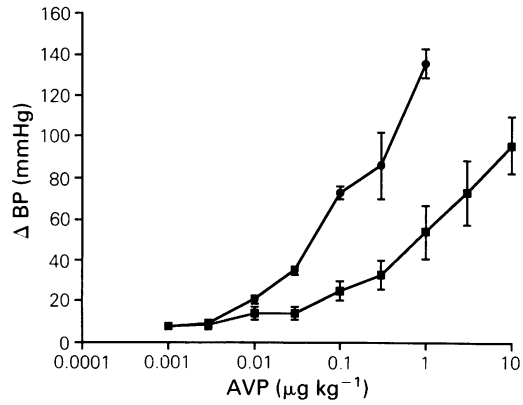

Figure 4 Effect of chronic vasopressin V_1-receptor antagonism (■, $n = 6$) or vehicle infusion (●, $n = 6$) on the pressor response (Δ BP) to exogenous vasopressin in Long Evans rats with 5/6 renal ablation.

SmithKline and French Laboratories. Arginine vasopressin (acetate salt) was purchased from Sigma Chemical Corp.

Statistical analyses

Data are presented as mean ± s.e.mean throughout, and were analyzed using an analysis of variance for repeated measures and subsequently a Dunnett's test.

Results

In the first study, partial renal ablation in Long Evans rats was associated with significant increases in urinary protein excretion (Figure 1) and systolic blood pressure (Figure 2). Treatment with a vasopressin V_1-receptor antagonist did not alter these responses (Figures 1 and 2). In both groups, urinary protein excretion was below 20 mg day^{-1} before partial renal ablation and increased to greater than 100 mg day^{-1} 3 weeks following ablation. This was associated with glomerular hyalinosis as determined histologically; however, there was no difference between groups (Figure 3). Systolic blood pressure was approximately 140 mmHg before renal ablation, and increased in both groups to approximately 200 mmHg 3 weeks later. Urine flow increased approximately 100%, and urine osmolality decreased approximately 50% in both groups of animals following renal ablation (Table 1). Body weight was reduced following renal ablation, and there was no difference between the vehicle- and the vasopressin antagonist-treated groups. Three weeks following ablation, the blood pressure response to exogenous vasopressin was significantly reduced in the vasopressin antagonist-treated animals. Thus, the cumulative dose-response curve to vasopressin in

Table 1 Body weight, urine volume and urine osmolality in Long Evans rats with partial renal ablation and treated chronically with vehicle (control; $n = 10$) or a vasopressin V_1-receptor antagonist (AVP-antag, $n = 10$)

	Time (weeks)			
	0	1	2	3
Body weight (g)				
Control	407 ± 10	369 ± 9	348 ± 13[a]	357 ± 17[a]
AVP-antag	400 ± 6	381 ± 10	377 ± 11	373 ± 11
Urine flow (ml day^{-1})				
Control	18 ± 1	40 ± 4[a]	43 ± 3[a]	54 ± 4[a]
AVP-antag	15 ± 1	37 ± 3[a]	40 ± 3[a]	50 ± 3[a]
Urine osmolality (mOsm kg^{-1} H_2O)				
Control	1963 ± 76	717 ± 45[a]	707 ± 36[a]	626 ± 31[a]
AVP-antag	2025 ± 123	788 ± 39[a]	699 ± 39[a]	655 ± 58[a]

Results are mean ± s.e.mean. [a] $P < 0.05$ vs. time 0.

urethane-anaesthetized rats was shifted significantly to the right in the vasopressin-blocked rats (Figure 4). The ED_{50} (effective dose to increase blood pressure by 50 mmHg) was significantly ($P < 0.05$) higher in the vasopressin-blocked rats than in vehicle-treated rats (5.0 ± 1.6 vs. $0.09 \pm 0.01\ \mu g\,kg^{-1}$).

In the second study, initial systolic blood pressure was slightly lower and urinary protein excretion slightly higher in the homozygous Brattleboro rats prior to renal ablation compared to the control rats (Figures 5 and 6). Following renal ablation, urinary protein excretion and systolic blood pressure increased to a similar level in both groups (Figures 5 and 6). There was, again, glomerular hyalinosis which was of the same degree as observed previously (Figure 3). Brattleboro rats had an approximately 8 fold higher urine flow and 6 fold lower urine osmolality compared to control Long Evans rats prior to 5/6 renal ablation (Table 2). Following renal ablation, urine flow increased slightly in Long Evans rats and decreased slightly in Brattleboro rats. Urine osmolality did not change significantly in the Brattleboro rats, but was significantly reduced in the Long Evans rats (Table 2) in a similar way as was observed in the previous experiment (Table 1). Body weight was significantly lower in the Brattleboro rats. Both groups lost a small amount of weight following renal ablation (Table 2).

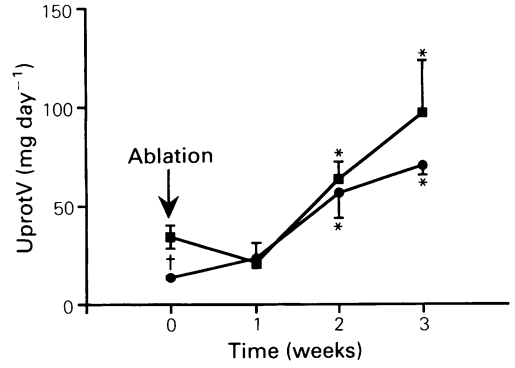

Figure 5 Urinary protein excretion (UprotV) in Long Evans (●, $n = 9$) and Brattleboro (■, $n = 13$) rats with 5/6 renal ablation. * $P < 0.05$ vs. week 0; † $P < 0.05$ vs. Long Evans rats.

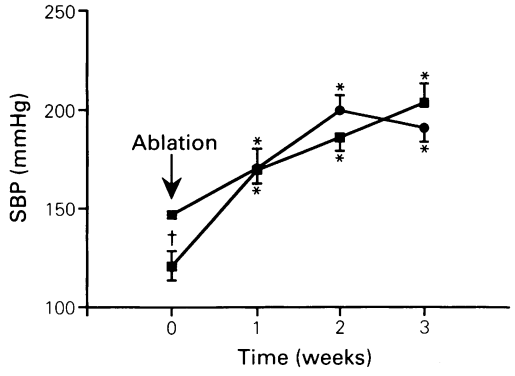

Figure 6 Systolic blood pressure (SBP) in Long Evans (●, $n = 8$) and in Brattleboro (■, $n = 13$) rats with 5/6 renal ablation. * $P < 0.05$ vs. week 0; † $P < 0.05$ vs. Long Evans rats.

Discussion

It has been shown recently (Bouby & Bankir, 1988) that feeding a liquid diet to rats provided some protection against the progression of renal disease after 5/6 renal ablation. The authors concluded that the reduced urinary concentration and/or the lower plasma vasopressin was the mechanism for the protection. Since vasopressin can cause both preferential efferent arteriolar constriction (Edwards *et al.*, 1987) and is mitogenic to mesangial cells (Ganz *et al.*, 1988), it is not unreasonable to suggest that vasopressin, by inducing glomerular hypertension and/or mesangial cell proliferation, may participate in the progression of chronic renal failure. Furthermore, plasma vasopressin levels in chronic renal failure are elevated, possibly due to decreased renal clearance (Benmansour *et al.*, 1982). The present study, however, suggests that vasopressin is unlikely to play a role in the progression of renal failure associated with 5/6 renal ablation. Thus, a vasopressin V_1-receptor antagonist, at a dose that significantly reduced the pressor responses to exogenous vasopressin, failed to reduce the hypertension, proteinuria or glomerular hyalinization observed in rats with chronic renal failure. The lack of effect of chronic vasopressin antagonism on the hypertension associated with partial renal ablation confirms the observation of Lee-Kwon *et al.* (1981) who showed that acute treatment with a V_1-receptor antagonist in rats with partial nephrectomy-salt hypertension had little effect on blood pressure. Since the effects of vasopressin on both the efferent arteriole (Edwards *et al.*, 1989) and mesangial cells (Jard *et al.*, 1987; Takeda *et al.*, 1988) appear to be mediated via a V_1-receptor, the vasopressin antagonist would be expected to provide some protection if vasopressin were involved.

The absence of a role for vasopressin in chronic renal failure was confirmed in the homozygous Brattleboro rat which lacks vasopressin. In these rats, the hypertension, proteinuria and glomerular hyalinization associated with 5/6 renal ablation was similar to that observed in the control Long Evans rats. Since Brattleboro rats cannot concentrate their urine to a great degree, these data also suggest that the urinary concentrating mechanism is not involved in the pathogenesis of chronic renal failure. The presence in the Brattleboro rats of hypertension of a similar magnitude as in the Long Evans rats also makes it unlikely that vasopressin acting through V_2-receptors is involved. This is in contrast to

Table 2 Body weight, urine volume and urine osmolality in Long Evans ($n = 9$) and in homozygous Brattleboro ($n = 13$) rats with partial renal ablation

	Time (weeks) 1	2	3	4
Body weight (g)				
Long Evans	426 ± 12	413 ± 12	392 ± 15	413 ± 10
Brattleboro	297 ± 11[b]	288 ± 11[b]	292 ± 12[b]	306 ± 14[b]
Urine flow (ml day^{-1})				
Long Evans	20 ± 2	34 ± 7	27 ± 5	37 ± 3[a]
Brattleboro	172 ± 8[b]	110 ± 7[ab]	130 ± 8[ab]	116 ± 11[ab]
Urine osmolality (mOsm kg^{-1} H_2O)				
Long Evans	1734 ± 106	887 ± 90[a]	1065 ± 137[a]	866 ± 76[a]
Brattleboro	293 ± 19[b]	328 ± 15[b]	311 ± 14[b]	305 ± 13[b]

Results are mean ± s.e.mean. [a] $P < 0.05$ vs. time 0. [b] $P < 0.05$ vs. Long Evans rats.

the rat DOCA-salt hypertension model where the antidiuretic activity of vasopressin appears to be a prerequisite for the development of hypertension (Möhring *et al.*, 1977; Crofton *et al.*, 1979; Saito *et al.*, 1980). In other experimental models of hypertension, as with partial nephrectomy-induced high blood pressure, there is little evidence for the role of vasopressin (Share, 1988).

In summary, the present study demonstrates that rats lacking vasopressin or rats undergoing chronic blockade of vasopressin V_1-receptors develop partial nephrectomy-induced hypertension, proteinuria and glomerular damage at the same rate and to the same extent as control rats. This suggests that vasopressin is unlikely to play an important role in the development of chronic renal failure.

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Pharmacological properties of FPL 63547, a novel inhibitor of angiotensin-converting enzyme

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1 FPL 63547, in its active diacid form, was a potent inhibitor of rabbit lung angiotensin converting enzyme (ACE) *in vitro* (IC_{50} 0.51 nM).

2 In conscious normotensive dogs, FPL 63547 (10–300 $\mu g\,kg^{-1}$ i.v.) produced prolonged, dose-related inhibition of plasma ACE activity and angiotensin I pressor responses, without affecting basal blood pressure, heart rate or pressor responses to angiotensin II.

3 In anaesthetized dogs, FPL 63547 diacid (3–300 $\mu g\,kg^{-1}$ i.v. cumulatively) produced dose-related increases in cardiac output accompanied by falls in total peripheral resistance indicative of vasodilatation. Mild stimulation of cardiac rate and contractility was also observed. Enalapril diacid had a similar profile.

4 FPL 63547 was a highly effective antihypertensive agent after oral administration to spontaneously hypertensive rats (SHR) pretreated with a diuretic. It lowered systolic blood pressure (SBP) on acute administration over the range 3×10^{-7}–$10^{-5}\,mol\,kg^{-1}$ p.o. ($\simeq$0.13–4.5 $mg\,kg^{-1}$ p.o.). FPL 63547 was more potent than other ACE inhibitors tested, threshold active doses for lisinopril, enalapril and captopril being 10^{-6}, 10^{-6} and $3 \times 10^{-5}\,mol\,kg^{-1}$ p.o., respectively. The antihypertensive effects of FPL 63547, unlike those of enalapril and captopril, were of long duration.

5 The antihypertensive efficacy of FPL 63547 was also observed following chronic oral administration. A dose of 0.5 $mg\,kg^{-1}\,day^{-1}$ once daily for 23 days produced a sustained reduction of SBP. By the end of the treatment period, SBP was significantly lowered both pre- and post-dose, i.e. effective 24 h control had been achieved.

6 The profile of FPL 63547 is consistent with it being a potent, selective and long-acting ACE inhibitor. As an antihypertensive agent in SHR it compared favourably with other members of this class with respect to potency and duration of action.

Introduction

The biological rationale for inhibition of angiotensin-converting enzyme (ACE: E.C.3.4.15.1) is well known (Cushman & Ondetti 1980). Agents acting through this mechanism are now established in the treatment of heart failure and hypertension, and recent appreciation of the possible pathophysiological roles of tissue renin-angiotensin systems (Dzau, 1988) suggests that further extensions of therapeutic utility are likely. Clinical demonstrations that ACE inhibitors can reduce mortality in congestive heart failure patients (CONSENSUS Trial Study Group, 1987) and improve quality of life (Croog *et al.*, 1986) add considerably to the attractiveness of this class of compound. Overall, ACE inhibitors appear destined to form one of the major drug classes in future cardiovascular therapy, possibly becoming first line treatment for certain conditions (Brunner *et al.*, 1987).

FPL 63547 (Figure 1) is the ester prodrug of a novel thiadiazoline ACE inhibitor. This paper describes some of the biological properties of FPL 63547 and its active diacid form (FPL 63547 diacid) in comparison with captopril, enalapril, enalapril diacid and lisinopril. A preliminary account of this work has been presented to the British Pharmacological Society (Carr *et al.*, 1988).

The ACE inhibitors described to date are generally considered to be similar, differing mainly in potency or duration of action. This has led to recognition (Lancet, 1988) that newer agents of this class should demonstrate biological properties which distinguish them from the first generation ACE inhibitors. In the accompanying paper (Carr *et al.*, 1990) we describe the marked preference of FPL 63547 for the biliary route of elimination, which is a novel feature of this compound.

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Methods

Angiotensin-converting enzyme inhibition in vitro

Inhibition of rabbit lung ACE was assessed by a method based on that described by Cushman & Cheung (1971). Enzyme activity was determined by measuring release of radiolabelled hippurate from the synthetic enzyme substrate hippuryl-histidyl-L-leucine (HHL).

Rabbit lung ACE was purified by lisinopril-Sepharose CL-4B affinity chromatography, as described by Bull *et al.* (1985) with minor modifications. This scaled-down version, suitable for 35 g wet weight of lung tissue, used 0.5% Nonidet P-40 (NP-40) in 50 mM potassium phosphate, 300 mM NaCl, 20 μM $ZnCl_2$, pH 8.3 for solubilisation of enzyme, and washing the column (volume 10 ml) to remove unbound protein (five column volumes at 15 $ml\,h^{-1}$). NP-40 was then

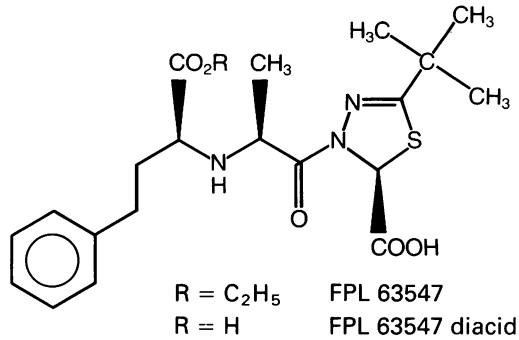

Figure 1 Structures of FPL 63547 and its active diacid.

omitted and the column was washed with a further five volumes of buffer. ACE was eluted with 100 μM captopril in buffer, pooled and dialysed against 50 mM potassium phosphate, 100 μM ethylenediaminetetraacetic acid (EDTA) pH 8.3 (seven changes of 60 volumes), followed by dialysis against 50 mM potassium phosphate pH 8.3 (two changes of 60 volumes). The enzyme was divided into aliquots and stored at -70°C.

[^{14}C]-HHL was used as substrate (2 mM, containing 10 nCi [^{14}C]-HHL per tube). ACE was incubated with the ACE inhibitor/substrate mixture (final volume 0.25 ml) for 30 min at 37°C in 100 mM potassium phosphate, 300 mM NaCl, 20 μM $ZnCl_2$ at pH 8.3. Reactions were terminated by adding 0.25 ml 1 M HCl and [^{14}C]-hippurate extracted into 1.5 ml ethyl acetate and counted. The enzyme concentration was adjusted so that cleavage of the substrate did not exceed 10% in uninhibited controls.

Angiotensin-converting enzyme inhibition in vivo

Adult male Beagle dogs (10–17 kg) underwent aseptic surgery under general anaesthesia (pentobarbitone 30 mg kg^{-1} i.v.) to implant catheters into the femoral artery to record blood pressure and into the femoral vein for drug adminsistration. Catheters were exteriorised in the nape of the neck and terminated on two-way titanium valves attached to skin buttons. Titanium electrodes were implanted subcutaneously in the neck and on the rump to record ECG.

Experiments were initiated 2–3 weeks following the operation at a time when the animals were fully recovered and had been trained to sit quietly in harnesses. Systolic (SBP) and diastolic (DBP) blood pressures were measured and mean blood pressure (MBP) derived from the pressure transducer signal. This signal also triggered a ratemeter to give a record of heart rate (HR). All variables, including ECG, were displayed on a Lectromed recorder. On each experimental day, dogs received a single i.v. dose of FPL 63547 (10–300 μg kg^{-1}) or vehicle (sterile citric acid–phosphate buffer). SBP, DBP, HR and ECG were measured before drug administration and at various times in the 24 h period afterwards. Plasma ACE activity, measured by a spectrophotometric method (Holmquist *et al.*, 1979), was determined in arterial blood samples taken at -0.5, $+1$, $+6$ and $+24$ h. Angiotensin I (150 ng kg^{-1} i.v.) and angiotensin II (150 ng kg^{-1} i.v.) were administered sequentially at -0.5, $+0.5$, $+1$, $+4$, $+5$, $+23$, $+24$ and $+25$ h approximately and the pressor responses produced measured as changes in MBP. Drug-induced effects on all variables were compared for statistical significance with the corresponding changes produced by vehicle in the same dogs ($P < 0.05$, paired t test).

Haemodynamic effects in anaesthetized dogs

Haemodynamic profiles of FPL 63547 diacid and enalapril diacid were compared in anaesthetized adult male Beagles (10–15 kg). The methodology has been described in detail previously (Humphries & O'Connor, 1988). Briefly, anaesthesia was induced with thiopentone and maintained with chloralose. The animals were artificially ventilated and surgically prepared to record mean blood pressure (BP), mean cardiac output (CO) using an electromagnetic flow probe positioned around the ascending aorta, and cardiac contractility (dP/dt P^{-1}) derived from measurement of left ventricular pressure. Total peripheral resistance (TPR) was obtained from continuous division of BP by CO and heart rate (HR) was triggered from the left ventricular pressure signal.

FPL 63547 diacid (1–300 μg kg^{-1} i.v. $n = 4$), enalapril diacid (1–300 μg kg^{-1} i.v. $n = 4$) or vehicle (saline) was administered as a series of cumulative i.v. bolus doses. Each dog received vehicle, followed by either FPL 63547 diacid cumulatively or enalapril diacid cumulatively. Percentage changes from predose levels for each variable were calculated and analysed statistically to test for differences between drug and vehicle treatments ($P < 0.05$, unpaired t test).

Antihypertensive effects in spontaneously hypertensive rats

Conscious adult male spontaneously hypertensive rats (SHR), weight range 300–500 g, underwent a training period to accustom them to experimental procedures before use. Only rats with pretreatment systolic blood pressures > 180 mmHg and which responded normally to a standard antihypertensive agent (nifedipine 10^{-5} mol kg^{-1} p.o.) were included in the study.

Systolic blood pressure (SBP) was recorded indirectly by the tail-cuff technique. This involved restraining the animals and placing them in an incubator (32 $\pm$ 2°C) for 20 min to establish a satisfactory arterial tail pulse. SBP was measured by automatic inflation of a sphygmomanometer cuff (Narco Bio Systems) around the proximal portion of the tail. Each SBP value was the mean of at least three readings. Oral pretreatment with hydrochlorothiazide (HCTZ) was used in all experiments to increase the dependence of blood pressure on the renin-angiotensin system, thus producing a model which is more sensitive to the antihypertensive effects of ACE inhibitors (Natoff *et al.*, 1985).

Acute dosing studies The antihypertensive properties of FPL 63547 have been compared with those of other ACE inhibitors after acute oral dosing. For each experiment, rats were allocated into two groups of six with approximately equal basal mean SBP. HCTZ (2×10^{-5} mol kg^{-1} p.o.) was administered at -0.5 h. One group of animals received a single oral dose of ACE inhibitor at time zero in PEG 300 (1 ml kg^{-1}) and the other group received vehicle alone. Compounds tested in this fashion were FPL 63547 (10^{-7}–10^{-5} mol kg^{-1} p.o.), captopril (10^{-5}–10^{-4} mol kg^{-1} p.o.), lisinopril (3×10^{-7}–10^{-5} mol kg^{-1} p.o.) and enalapril (3×10^{-7}–10^{-5} mol kg^{-1} p.o.). To provide a composite picture of drug-induced changes in SBP over the 24 h period after dosing, each dose of compound was tested twice, on separate occasions. Initially in a group where SBP readings were taken 1 h before (-1 h) and then at 1, 3 and 5 h after dosing (morning-dosed), and secondly when readings were taken at -1, 15, 18, 21 and 24 h (evening-dosed). Percentage changes in SBP at each time interval were calculated relative to the pre-dose (-1 h) reading. Post-drug treatment SBP values were compared against pre-dose readings and time-matched control group readings and were considered to have changed significantly only if $P < 0.05$ (unpaired t test) for both comparisons.

Chronic dosing study Three groups of SHR were used to investigate antihypertensive properties after chronic oral dosing. One received FPL 63547 treatment, one received enalapril for comparison and the third functioned as a control (vehicle). The rats were monitored and dosed daily over a continuous seven week period divided up as follows. A 14 day lead-in period (Days -14 to -1) during which the animals received HCTZ (5 mg kg^{-1} p.o.) and vehicle (PEG 300) each day. A 23 day treatment period during which the animals received HCTZ and the substance under test daily, i.e. either FPL 63547 0.5 mg kg^{-1} day^{-1} p.o., enalapril 5 mg kg^{-1} day^{-1} p.o. or PEG 300 1 ml kg^{-1} day^{-1} p.o. Finally, a 13 day recovery period where the treatment was as for the lead-in period. SBP was measured 1 h pre-dose (-1 h) and 5 h post-dose ($+5$ h) at 2–3 day intervals throughout the study. Time-matched SBP values were compared between drug-treated and control groups to determine statistical significance ($P < 0.05$, unpaired t test).

Materials

FPL 63547, enalapril and their diacids were synthesized in the Department of Medicinal Chemistry, Fisons plc; lisinopril was a gift from Merck and captopril from Squibb; HCTZ, HHL, NP-40, EDTA were obtained from Sigma; Sepharose CL-4B from Pharmacia; [^{14}C]-HHL from DuPont; thiopentone from May and Baker and α-chloralose from Koch Light.

Results

Angiotensin-converting enzyme inhibition in vitro

Results from this study are shown in Table 1. FPL 63547 diacid was a highly potent inhibitor of rabbit lung ACE, active at subnanomolar concentrations. On the basis of mean IC_{50} values it was slightly more potent than lisinopril and enalapril and approximately four times more potent than captopril. The data for FPL 63547 indicate that the ester prodrug has no significant intrinsic ACE inhibitory properties.

Angiotensin-converting enzyme inhibition in vivo

FPL 63547 was a potent inhibitor of plasma ACE and angiotensin I pressor responses in the conscious dog after i.v. bolus dosing. These inhibitory effects were rapid in onset and long-lasting. They are summarized in Figure 2 which shows data generated 1 h after dosing, approximately the time of peak inhibition. At this time point FPL 63547 (10–100 $\mu g\,kg^{-1}$) produced dose-related inhibition of plasma ACE, reaching a peak of 97 ± 1%. Angiotensin I pressor responses were also inhibited by FPL 63547 in a dose-related fashion over the range 10–300 $\mu g\,kg^{-1}$, but were a less sensitive index. Mean ID_{50} values calculated with individual animal data were 12.3 ± 1.3 $\mu g\,kg^{-1}$ against plasma ACE and 60 ± 28 $\mu g\,kg^{-1}$ against angiotensin I pressor responses. Even at the highest dose used in the study (300 $\mu g\,kg^{-1}$) FPL 63547 did not affect pressor responses to angiotensin II (Figure 2). The time course of inhibition of plasma ACE by FPL 63547 is shown in Figure 3. Peak inhibitory effect was achieved at +1 h for all doses and declined slowly thereafter, still achieving statistical significance 24 h after dosing for each of the three highest doses tested.

Table 2 shows a comparison of haemodynamic variables in FPL 63547-treated and vehicle-treated dogs 1 h after dosing, the time point approximating to peak ACE inhibitory effect. No statistically significant changes in SBP, DBP, HR or ECG were observed throughout the dose range used (10–300 $\mu g\,kg^{-1}$ i.v.).

Haemodynamic effects in anaesthetized dogs

The effects of cumulative i.v. administration of FPL 63547 diacid on the haemodynamics of the anaesthetized dog are illustrated in Figure 4. Vehicle (not shown) produced negligible changes. A dose of 1 $\mu g\,kg^{-1}$ of FPL 63547 diacid

Table 1 Inhibition of rabbit lung angiotensin-converting enzyme *in vitro*

	IC_{50} (nM)	95% CL	n
FPL 63547 diacid	0.51	0.26–1.02	5
Lisinopril	0.63	0.42–0.95	6
Enalapril diacid	0.95	0.70–1.30	6
Captopril	2.27	1.46–3.54	6
FPL 63547	>100	—	2

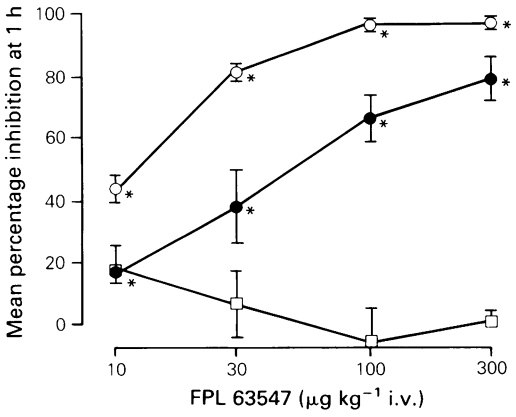

Figure 2 Effects of a series of bolus i.v. doses of FPL 63547 on plasma angiotensin-converting enzyme (ACE) activity (○), angiotensin I pressor responses (●) and angiotensin II pressor responses (□) in conscious dogs. Results are from drug-treated animals 1 h after dosing expressed as percentage inhibition (mean with vertical lines indicating s.e., $n = 4$) when compared with pre-dose (−0.5 h) readings. *$P < 0.05$ compared with corresponding changes produced by vehicle.

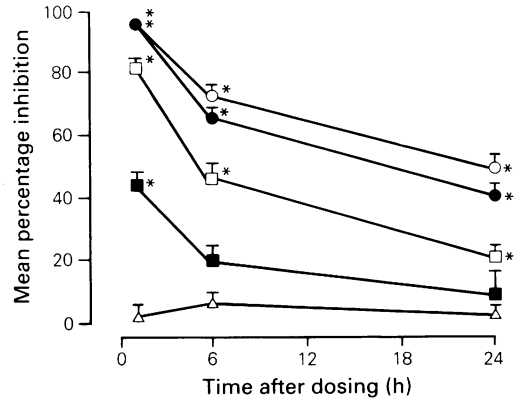

Figure 3 Time course of inhibition of plasma angiotensin converting-enzyme (ACE) activity in conscious dogs by various i.v. doses of FPL 63547. Results shown are percentage inhibition compared with pre-dose (−0.5 h) readings (mean with vertical lines indicating s.e., $n = 4$). FPL 63547 10 (■), 30 (□), 100 (●) and 300 (○) $\mu g\,kg^{-1}$ i.v., vehicle (△). *$P < 0.05$, versus vehicle group.

Table 2 Effects of FPL 63547 on systolic (SBP) and diastolic (DBP) blood pressures and heart rate (HR) of conscious dogs ($n = 4$)

	SBP (mmHg)	DBP (mmHg)	HR (bpm)
Vehicle	169 ± 12	88 ± 14	75 ± 5
FPL 63547:			
10 $\mu g\,kg^{-1}$ i.v.	171 ± 11	88 ± 6	90 ± 8
30 $\mu g\,kg^{-1}$ i.v.	159 ± 11	83 ± 10	79 ± 7
100 $\mu g\,kg^{-1}$ i.v.	155 ± 2	79 ± 2	68 ± 6
300 $\mu g\,kg^{-1}$ i.v.	171 ± 11	87 ± 4	81 ± 11

All readings taken 1 h following dosing (+1 h).

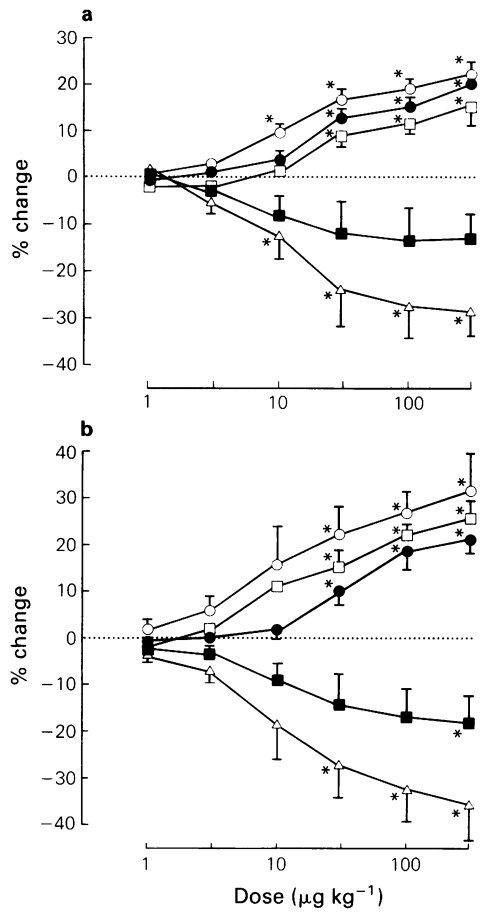

Figure 4 Haemodynamic effects of (a) FPL 63547 diacid and (b) enalapril diacid in anaesthetized dogs (1–300 $\mu g\,kg^{-1}$ i.v.). Results are expressed as a percentage change from pre-dose (mean with vertical lines indicating s.e., $n = 4$). Cardiac output (○), $dP/dt\ P^{-1}$ (●), heart rate (□), mean blood pressure (■) and total peripheral resistance (△). $^*P < 0.05$ versus vehicle.

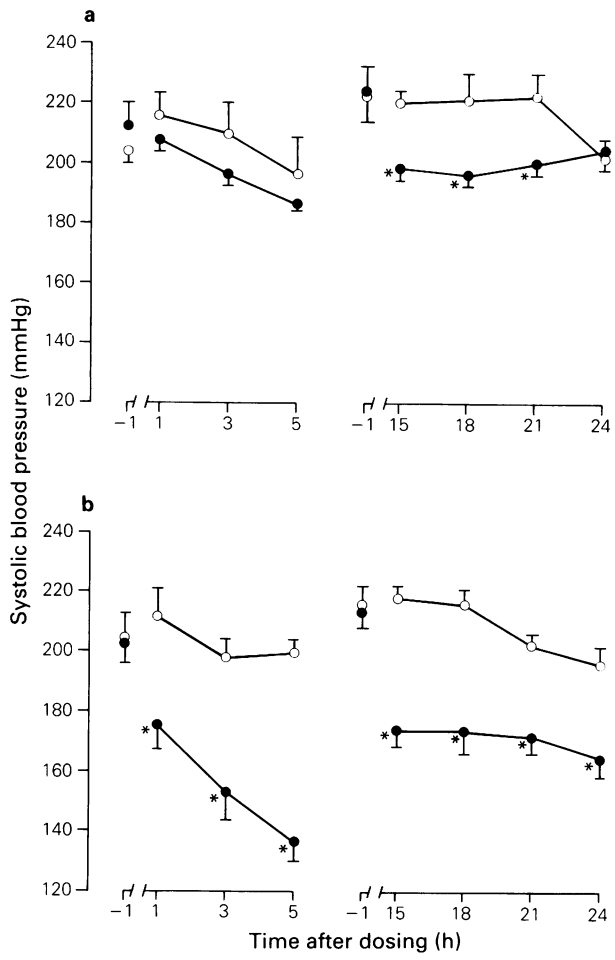

Figure 5 Antihypertensive effects of FPL 63547 (a) $3 \times 10^{-7}\,mol\,kg^{-1}$ p.o., (b) $10^{-5}\,mol\,kg^{-1}$ p.o., in diuretic-pretreated spontaneously hypertensive rats. Two panels shown on each graph represent results from morning-dosed (−1–5 h) and evening-dosed (−1–24 h) experiments respectively, drug-treated groups (●), vehicle-treated groups (○) (mean with vertical lines indicating s.e., $n = 5$–6). $^*P < 0.05$ versus both pre-dose and vehicle group readings.

appeared to be sub-therapeutic. At 3–300 $\mu g\,kg^{-1}$ it produced dose-related increases in CO (maximum +22%) accompanied by falls in TPR (maximum −29%), the latter indicative of vasodilatation. These changes were statistically significant (versus vehicle) for all doses above 3 $\mu g\,kg^{-1}$. Mild dose-related increases in HR and $dP/dt\ P^{-1}$ were observed over the same dose range. FPL 63547 diacid also produced small falls in BP which did not reach statistical significance. No alterations in ECG waveform or heart rhythm were noted at any dose.

In a separate group of animals, enalapril diacid (1–300 $\mu g\,kg^{-1}$ i.v.) exhibited a qualitatively similar profile of haemodynamic changes which tended to be slightly more marked than those observed in the FPL 63547 diacid-treated group (Figure 4).

Antihypertensive effects in spontaneously hypertensive rats

On acute oral dosing, FPL 63547 produced significant falls in SBP in HCTZ-pretreated SHR over the dose range 3×10^{-7}–$10^{-5}\,mol\,kg^{-1}$ (≃0.13–4.5 $mg\,kg^{-1}$). Antihypertensive effects of the minimum-active and maximum doses are shown in full in Figure 5. Each graph is a composite of morning-dosed and evening-dose experiments, providing the complete time course of drug- and vehicle-induced changes in SBP. A dose of $10^{-5}\,mol\,kg^{-1}$ produced marked antihypertensive effects (SBP − 32% at 5 h) which persisted throughout the 24 h period following dosing. Long duration of action was a feature of FPL 63547 in this model. At all active doses antihypertensive effects were regularly observed in the evening-dosed animals, i.e. between 15–21 h after dosing.

Comparison of the acute antihypertensive properties of FPL 63547 with those of enalapril, captopril and lisinopril is summarised in Table 3. FPL 63547 was the only agent to reduce SBP significantly at $3 \times 10^{-7}\,mol\,kg^{-1}$ p.o., the potency order being FPL 63547 > lisinopril = enalapril ≫ captopril. Lisinopril, like FPL 63547, was long-acting, whereas enalapril and captopril were relatively short-acting, even at high doses.

Figure 6 shows the effects of chronic oral dosing with FPL 63547 (0.5 $mg\,kg^{-1}\,day^{-1}$ for 23 days). During the lead-in period pre- and post-dose SBP in the two groups were stable and well-matched. Dosing with FPL 63547 produced a marked, consistent (≃20%) and statistically significant fall in SBP 5 h post-dose throughout the study. A trend towards lowering of pre-dose (−1 h) SBP was also evident which became statistically significant from Day 11 onwards and for the remainder of the dosing period. By Day 21 of treatment pre- and post-dose SBP had fallen to similar levels (≃180 mmHg). SBP readings in vehicle-treated animals remained stable at ≃220 mmHg throughout the study. On termination of dosing, SBP in the drug-treated group recovered progressively towards, but did not exceed, control levels.

Chronic dosing with enalapril at a higher dose (5 $mg\,kg^{-1}\,day^{-1}$ for 23 days) produced similar changes (data

Table 3 Antihypertensive effects of FPL 63547 in conscious diuretic-pretreated SHR—comparison with other angiotensin-converting enzyme inhibitors

Dose (mol kg^{-1} p.o.)	*Percentage fall in systolic pressure* 1 h	3 h	5 h	15 h	18 h	21 h	24 h
FPL 63547							
10^{-5}	13%	25%	32%	19%	19%	20%	26%
3×10^{-6}	NS	11%	15%	13%	10%	20%	NS
10^{-6}	NS	10%	19%	21%	NS	16%	NS
3×10^{-7}	NS	NS	NS	12%	13%	11%	NS
10^{-7}	NS	NS	NS	NS	NS	NS	NS
Enalapril							
10^{-5}	NS	17%	26%	NS	NS	NS	NS
3×10^{-6}	NS	20%	22%	–	–	–	–
10^{-6}	NS	10%	NS	–	–	–	–
3×10^{-7}	NS	NS	NS	–	–	–	–
Captopril							
10^{-4}	NS	19%	21%	NS	NS	NS	NS
3×10^{-5}	NS	14%	NS	–	–	–	–
10^{-5}	NS	NS	NS	–	–	–	–
Lisinopril							
10^{-5}	NS	28%	24%	21%	19%	17%	18%
3×10^{-6}	NS	NS	NS	NS	17%	18%	NS
10^{-6}	NS	NS	25%	NS	NS	16%	17%
3×10^{-7}	NS	NS	NS	NS	NS	NS	NS

Percentage changes calculated relative to pre-dose reading (−1 h). Values shown (n = 5–6) were significant ($P < 0.05$ unpaired t test) against both pre-dose reading and time-matched control group reading; NS = not significant.

not shown), suggesting that enalapril was approximately 10 times less potent than FPL 63547. Post-dose SBP was lowered consistently throughout the treatment period to a similar level to that achieved in the group treated with FPL 63547. Falls in pre-dose SBP did occur but were less marked, e.g. Day 21, SBP 196 mmHg, and statistically significant on Days 11, 16 and 21 only.

Discussion

In its active diacid form, FPL 63547 proved to be a highly potent inhibitor of rabbit lung ACE *in vitro*. Comparisons with lisinopril, enalapril diacid and captopril presented above, together with unpublished observations on cilazapril and ramipril diacids in the same test system (IC_{50} 0.45, 0.59 nM respectively), illustrate that FPL 63547 diacid is at least equivalent in potency to the most active first and second generation ACE inhibitors. With such a potent inhibitor, the IC_{50} value is at best only an approximation of affinity, and is probably an underestimate. Based on its similar potency to lisinopril we would predict that FPL 63547 diacid would be lisinopril-like in its kinetic properties, i.e. a 'tight binding' inhibitor with a slow rate of dissociation from the enzyme (Bull *et al.*, 1985). If so, this may contribute to the long duration of action of FPL 63547 *in vivo*.

The ester prodrug FPL 63547 possessed no significant ACE inhibitory activity *in vitro*. Efficacy of this form *in vivo* was demonstrated by its ability to inhibit plasma ACE activity and angiotensin I pressor responses after i.v. administration to conscious dogs. Inhibition was evident within 10 min of dosing and peaked at approximately one hour. This relatively rapid onset suggests ready de-esterification to the active diacid, probably by the combined action of plasma and liver esterases. Persistence of plasma ACE inhibition 24 h after a single i.v. bolus dose of FPL 63547 is a clear demonstration of its intrinsically long duration of action. Of the two indicators measured, plasma ACE was five fold more sensitive than angiotensin I pressor responses to inhibition by FPL 63547. Other investigators have made similar observations (Shaller *et al.*, 1985), and suggested that 80–90% inhibition of plasma ACE was necessary to obtain significant inhibition of pressor responses to angiotensin I. Presumably *in vivo*, there is considerable spare enzymic capacity which has to be inhibited before conversion of i.v. angiotensin I is impaired, i.e. some reduction in rate of angiotensin II formation may occur without the total product concentration being significantly affected. Another possible explanation for the differential sensitivity is that plasma ACE may not be solely responsible for conversion of i.v. angiotensin I to angiotensin II in this situation, pulmonary vascular tissue ACE might also be expected to be involved. Pressor responses to angiotensin II were unaffected by FPL 63547 at doses > 200× the ID_{50} for inhibition of plasma ACE.

In chloralose-anaesthetized dogs, FPL 63547 diacid produced falls in TPR accompanied by increases in CO and mild

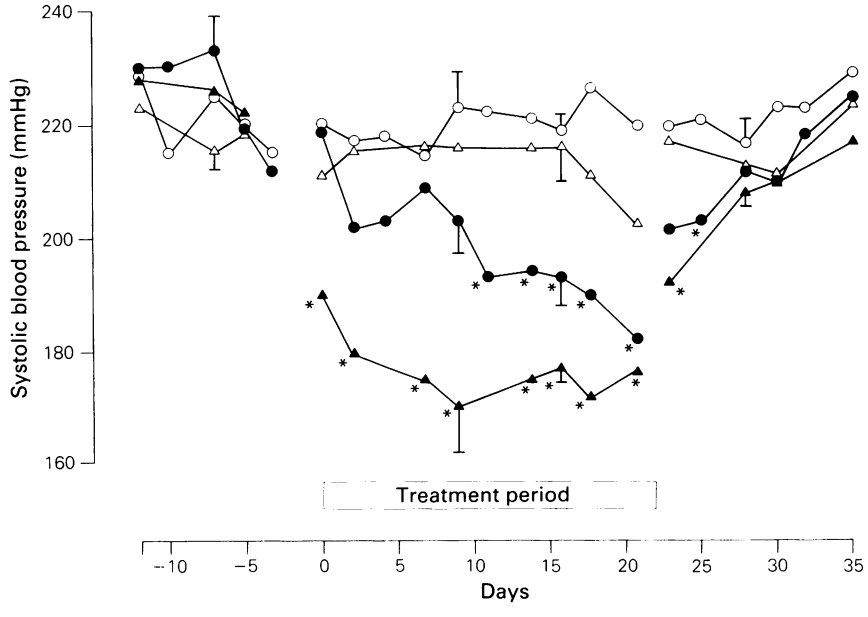

Figure 6 Antihypertensive effects of FPL 63547 in diuretic-pretreated spontaneously hypertensive rats after chronic oral dosing. Open symbols—vehicle group, solid symbols—FPL 63547 treated group (0.5 mg kg^{-1} day^{-1}, between Day 0–Day 22). Systolic blood pressure was measured both pre-dose (−1 h, ○, ●) and post-dose (+5 h, △, ▲). Values are means and vertical lines indicate s.e., n = 5–6. *$P < 0.05$ versus vehicle group.

stimulation of cardiac rate and contractility, over a wide dose-range. This profile is consistent with vasodilatation. We have observed qualitatively similar effects in the same model with the calcium channel blocker arteriolar dilator, nifedipine (Humphries & O'Connor, 1988). The haemodynamic properties of FPL 63547 diacid in the anaesthetized dog would appear to be adequately explained by ACE inhibition alone since enalapril gave an essentially similar profile. Further, the dose range over which vasodilatation was observed corresponds well with the dose-response relationships for inhibition of plasma ACE and angiotensin I pressor responses in the conscious dog. The observed vasodilatation may be due to a lowering of endogenous angiotensin II levels with subsequent lessening of its direct or indirect (i.e. mediated by facilitation of sympathetic nerve activity) vasoconstrictor properties, and/or elevation of bradykinin and prostaglandin levels. In conscious dogs, FPL 63547 produced no haemodynamic changes (SBP, DBP, HR) even at doses which caused maximal inhibition of plasma ACE. The latter finding is not uncommon for ACE inhibitors; spirapril, for example, was similarly ineffective in conscious normotensive dogs (Baum *et al.*, 1987), and probably reflects a relatively low dependence of BP on the renin-angiotensin system in this model.

In the anaesthetized dog both FPL 63547 and enalapril diacids produced mild but significant cardiac stimulation which was presumably reflex in origin. This deserves comment since ACE inhibitors as a class are considered to differ from other types of vasodilator by not causing reflex tachycardia, although the exact mechanism is unclear (Imai *et al.*, 1982), and may relate to the choice of chloralose as anaesthetic in our study. Unlike pentobarbitone, chloralose will preserve or even exaggerate the baroreceptor reflex (Brown & Hilton, 1956) and we have previously demonstrated that chloralose-anaesthetized dogs will respond with reflex tachycardia to a vasodilator (dipropyldopamine) (O'Connor *et al.*, 1982) which, under other circumstances, will lower heart rate by sympatho-inhibition. FPL 63547 and enalapril diacids consistently increased CO over a wide dose range. This contrasts with the fall in this variable obtained after administration of cilazapril to anaesthetized dogs (Holck *et al.*, 1986). This difference is probably model-dependent and may again relate to choice of anaesthetic or to the level of activation of the renin-angiotensin system. For example, Hof and colleagues (1987) have shown that spirapril lowers CO and HR in salt-depleted anaesthetized rabbits but has opposite effects in salt replete animals.

FPL 63547 was a very effective antihypertensive agent after acute oral dosing to diuretic-pretreated SHR. On the basis of these studies, it shows high potency and, unlike captopril and enalapril, a duration of action compatible with once-daily treatment of hypertension. Since FPL 63547 diacid, enalapril diacid and lisinopril had similar *in vitro* ACE inhibitory potency and the former two were comparable as vasodilators after i.v. administration, other factors may have contributed to the antihypertensive effectiveness of FPL 63547 in the SHR. Oral bioavailability of the compound is high (B Mead—unpublished observation). In addition, FPL 63547 is a very potent inhibitor of local tissue ACE, producing significant effects at doses as low as $3\,\mu g\,kg^{-1}$ p.o. in the rat (Carr *et al.*, 1989). In this respect it was between 3 and 20 fold more potent than enalapril, depending on the tissue in question. Previous investigators have demonstrated the importance of inhibition of tissue rather than plasma ACE in determining the magnitude and duration of blood pressure reduction with these compounds (Unger *et al.*, 1984; 1985).

The antihypertensive efficacy of FPL 63547 was retained on chronic once-daily dosing. No tolerance was observed as judged from post-dose SBP readings and by the end of the study effective 24 h control of this variable had been achieved. As with other ACE inhibitors, but unlike certain other antihypertensives (Parker & Atkinson, 1982), treatment withdrawal did not result in any rebound hypertensive effect. Chronic dosing with enalapril produce a similar lowering of SBP although a 10 fold higher dose was required. The gradual decline observed in the pre-dose SBP may simply reflect the long plasma half-life of FPL 63547. Alternatively, it may indicate that the mechanisms responsible for chronic reduction in blood pressure can differ from those involved in the acute antihypertensive response (Lever, 1986). Tissue ACE inhibition becomes particularly important during chronic dosing (Unger *et al.*, 1984). Furthermore, ACE inhibitor-induced reversal of adaptive structural changes, i.e. vascular or cardiac hypertrophy (Antonaccio *et al.*, 1979; Freslon & Guidicelli 1983) may influence the chronic antihypertensive response, although it is doubtful that such factors could exert a significant effect within the time-scale of our study.

In summary, FPL 63547, a novel thiadiazoline, is a potent and long-acting ACE inhibitor which by virtue of vasodilator and antihypertensive properties should prove useful in the treatment of chronic heart failure and hypertension. The accompanying paper (Carr *et al.*, 1990) describes the unusual elimination profile of FPL 63547, a feature which distinguishes it from other members of this class of drugs.

We wish to thank Merck and Squibb for their gifts of lisinopril and captopril, respectively.

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Preferential biliary elimination of FPL 63547, a novel inhibitor of angiotensin-converting enzyme, in the rat

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1 The route of elimination of FPL 63547, a novel inhibitor of angiotensin-converting enzyme (ACE), has been investigated in the anaesthetized rat. Comparisons have been made with other ACE inhibitors.

2 Bile and urine samples were collected over a 5 hour period following a single i.v. dose of ACE inhibitor (2 μmol kg^{-1}). Samples were bioassayed for ACE inhibitory activity using affinity-purified rabbit lung ACE and the amounts of the active form of inhibitor present in each sample were calculated by comparison with a standard curve.

3 FPL 63547 was rapidly and extensively excreted as the diacid in the bile but appeared in the urine in negligible amounts. The bile:urine ratio was 21.4:1 indicating a marked preference for the biliary route. A similar elimination profile was observed when the compound was dosed in its active form (FPL 63547 diacid), 87.9% of which was found in the bile over the 5 h collection period, with a bile: urine ratio of 14.6:1.

4 The marked preference of FPL 63547 for biliary elimination was not shared by the other ACE inhibitors tested in this study. Lisinopril demonstrated the opposite pattern, being excreted almost exclusively by the kidney (bile:urine ratio 0.06:1). Enalapril was eliminated in approximately equal amounts in bile and urine (ratio 0.7:1) while spirapril diacid showed a slight preference for the bile (ratio 2.6:1).

5 The physical chemical properties of FPL 63547 diacid may be responsible for its unusual preference for biliary elimination. In particular, the amphipathic character and strong acid functionality of the compound are thought to favour transport into the bile.

6 Elimination by the biliary route will be preferred in patients whose renal function is impaired as a result of disease or age. In such patients the elimination of renally-excreted ACE inhibitors is known to be compromised, resulting in compound accumulation and the need for closer monitoring. Therefore, the elimination profile of FPL 63547, if confirmed in man, may prove to be clinically advantageous.

Introduction

Inhibitors of angiotensin-converting enzyme (ACE) are proving to be very effective in the treatment of cardiovascular disease, particularly heart failure and hypertension. A number of compounds of this class are already approved (e.g. captopril, enalapril, and lisinopril) or in the later stages of development (e.g. ramipril, cilazapril). However, to date, those ACE inhibitors for which pharmacokinetic data are available in man use the kidney as the primary organ of excretion. Since impairment of renal function is a relatively common accompaniment of chronic heart failure and hypertension, the route of elimination of therapeutic agents is a significant consideration in the clinical management of these diseases. There have been a number of studies (e.g. Kelly *et al.*, 1986; van Schaik *et al.*, 1987; Shionoiri *et al.*, 1987) showing that plasma levels of ACE inhibitors in current clinical use are elevated in patients with renal impairment, necessitating closer monitoring and possible dose reduction. Thus, renal excretion of ACE inhibitors is potentially disadvantageous.

FPL 63547, a novel thiadiazoline, is a potent and long acting ACE inhibitor with antihypertensive properties in spontaneously hypertensive rats (Carr *et al.*, 1990). Mackaness (1985) first suggested that ACE inhibitors may be developed which are suited to biliary transport, thus reducing the clinical problems associated with their use in patients with impaired kidney function. In the light of this, the route of elimination of FPL 63547 has been examined in the anaesthesized rat, a preliminary account of which has been presented to the British Pharmacological Society (Carr *et al.*, 1988).

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Route of elimination has been investigated by measuring levels of compound in the bile and urine by bioassay of the samples for ACE inhibitory activity. The technique therefore detects ACE inhibitors in the form in which they are biological active. Since FPL 63547 is a mono-ester prodrug, active only after de-esterification, it has been administered in both its mono-ester and active diacid forms. Route of elimination comparisons have been made with lisinopril, enalapril (mono-ester) and spirapril (diacid).

Methods

Surgery and protocol

Male Sprague-Dawley rats (250 g) were anaesthetized with pentobarbitone, 54 mg kg^{-1} i.p. The trachea was exposed and catheterised. The jugular vein was catheterised and pentobarbitone infused at a rate of 250 μg kg^{-1} min^{-1} to maintain anaesthesia. The abdomen was opened to reveal the liver and bile duct. An incision was made in the bile duct and a 0.6 mm cannula inserted and tied in place close to the point of insertion. Once adequate bile flow was achieved, pre-dose control samples were collected. After the penis has been ligated, the bladder was exposed and sampled, prior to dosing, by insertion of a fine needle and withdrawal of the urinary contents into a syringe. The bladder was kept moist with saline swabs.

The ACE inhibitor (2 μmol kg^{-1} in 10% ethanol:saline) was administered via the femoral vein by a fine needle. Over the following 5 h bile was collected continuously (sample tube changed at hourly intervals) and urine was sampled as

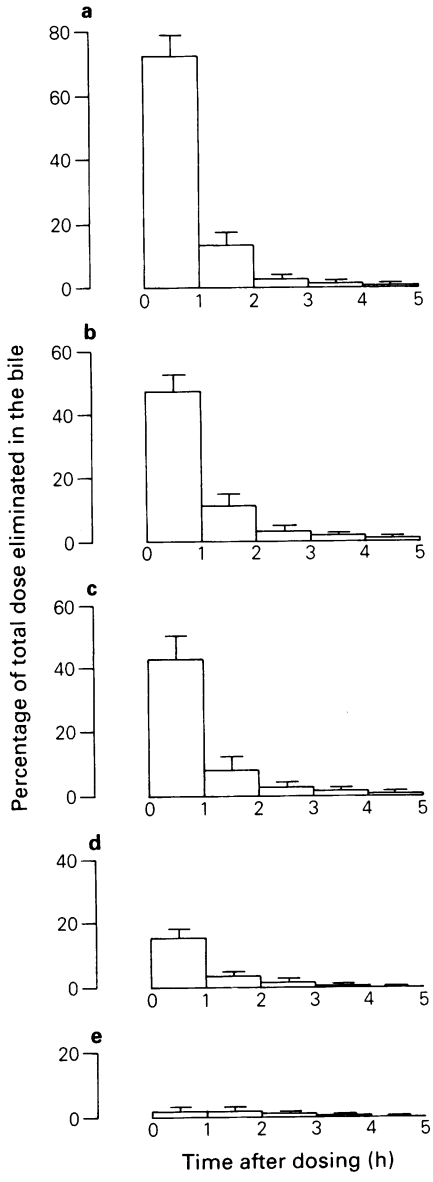

Figure 1 Time-dependence of biliary elimination of (a) FPL 63547 diacid, (b) FPL 63547 diacid (dosed as mono-ester), (c) spirapril diacid, (d) enalapril diacid (dosed as mono-ester), (e) lisinopril, in the anaesthetized rat. Bile samples were taken at hourly intervals after a single i.v. dose of angiotensin-converting enzyme inhibitor ($2\,\mu mol\,kg^{-1}$) and the amount of active form of compound present in each sample was determined by bioassay and expressed as a percentage of total dose. Results shown are means with bars indicating s.e., $n = 5$.

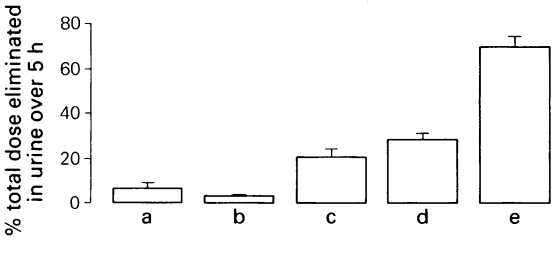

Figure 2 Total urinary elimination of (a) FPL 63547 diacid, (b) FPL 63547 diacid (dosed as mono-ester), (c) spirapril diacid, (d) enalapril diacid (dosed as mono-ester), (e) lisinopril, in anaesthetized rats over the 5 h collection period following dosing with angiotensin-converting enzyme inhibitor ($2\,\mu mol\,kg^{-1}$ i.v.). The amount of active form of compound present in the urine sample was determined by bioassay and expressed as a percentage of total dose. Results shown are means with bars indicating s.e., $n = 5$.

Table 1 Comparison of route of elimination profiles for angiotensin-converting enzyme (ACE) inhibitors in the anaesthetized rat

Active ACE inhibitor	*% of total dose eliminated* Bile	Urine	*Bile:urine ratio*
*FPL 63547 diacid	60.9 ± 6.8	2.8 ± 0.3	21.7:1
FPL 63547 diacid	87.9 ± 1.9	6.0 ± 2.0	14.6:1
Spirapril diacid	52.4 ± 11.6	20.2 ± 3.4	2.6:1
†Enalapril diacid	19.6 ± 3.1	28.0 ± 3.0	0.7:1
Lisinopril	4.1 ± 1.5	64.3 ± 5.5	0.06:1

*, † Dosed as the mono-ester (*FPL 63547, †enalapril). Values shown are means ± s.e. for five animals. Statistical analysis of the individual animal bile:urine ratios shows that the FPL 63547 diacid groups differed significantly from spirapril diacid, enalapril diacid and lisinopril groups ($P < 0.01$, Wilcoxon rank test) but not from each other.

described above. After the volumes had been noted, the samples were frozen (−20°C) before analysis.

Measurement of angiotensin-converting enzyme inhibitor levels in bile and urine

A bioassay method was employed which involved the determination of ACE inhibitory activity in bile and urine samples by use of a rabbit lung ACE assay. Enzyme activity was evaluated with a method based on that described by Cushman & Cheung (1971) which involved measurement of radiolabelled hippurate release from the synthetic enzyme substrate, hippuryl-histidyl-L-leucine (HHL). Levels of enzyme inhibitor present in samples were therefore quantified according to the degree of reduction in hippurate released in the assay.

Preparation of enzyme Rabbit lung ACE was affinity purified on lisinopril-Sepharose according to the method of Bull *et al.* (1985), modified as described in the accompanying paper (Carr *et al.*, 1990).

Preparation of substrate HHL (42.9 mg base) was suspended in 150 mM phosphate, 600 mM sodium chloride, 20 μM zinc chloride, pH 8.3 (buffer A). Phosphate buffer (50 mM, 20 μM zinc chloride, pH 8.3) containing potassium hydroxide (100 mM) was added dropwise and stirred vigorously until the substrate had dissolved (approx. 30 min). The solution was then adjusted to pH 8.3 with potassium dihydrogen phosphate (50 mM, 20 μM zinc chloride) and made up to 10 ml with phosphate buffer (50 mM, 20 μM zinc chloride, pH 8.3) to give a final concentration of 10 mM (stored at 4°C). ^{14}C-labelled HHL ($10\,\mu Ci\,ml^{-1}$) was diluted 1:25 with this stock solution prior to use in the ACE assay.

Assay of angiotensin-converting enzyme inhibition Standards and samples were diluted in buffer A. The level of dilution varied depending on the type of sample and was determined in preliminary experiments. A standard curve was assayed with each experiment and duplicates of all samples were analysed. Aliquots of sample or blank (100 μl) were pre-incubated (shaken) with 100 μl enzyme solution (from a working stock activity of $4.29\,mu\,ml^{-1}$) for five minutes at 37°C in a water bath. The reaction was started by the addition of substrate (50 μl, final concentration 2 mM) and after 30 min stopped by addition of hydrochloric acid (1 M, 0.25 ml). Radiolabelled hippurate was extracted with ethyl acetate (1.5 ml) and the sample centrifuged for 10 min at 3000 r.p.m. at 4°C (Damon Centra-7R bench top centrifuge). A 1 ml aliquot of the ethyl acetate layer was counted in Optiphase (3 ml) on a scintillation counter (C-14 programme, four minutes, Packard Tri-Carb 460).

By constructing a standard curve of inhibition of ACE-mediated hydrolysis (^{14}C-count versus log inhibitory concentration) the concentration of ACE inhibitor in the 'unknown' samples was determined.

Materials

FPL 63547 (2,3-dihydro-3-[N-(1S)-1-ethoxycarbonyl-3-phenyl-propyl)-L-alanyl]-5-(1,1-dimethyl ethyl)-1,3,4-thiadiazole-2-(S)-carboxylic acid), enalapril and their active diacids were synthesized in the Department of Medicinal Chemistry, Fisons plc; lisinopril was a gift from Merck; captopril a gift from Squibb and spirapril diacid a gift from Schering. BSA, HHL and affinity column materials (Sepharose CL-4B, 1,4 butanediol diglycidyl ether, sodium borohydride) were purchased from Sigma. Zinc chloride was obtained from JMC Ltd, and [^{14}C]-HHL from NEN Research Products. All other materials used were supplied by Fisons.

Results

Total recovery of compound over the 5 h period following i.v. administration to the anaesthetized rat, derived from individual animal data by combining the total amounts eliminated in bile and urine, was as follows: FPL 63547 diacid (93.9 ± 2.4%), spirapril diacid (72.6 ± 12.4%), lisinopril (68.4 ± 5.6%), FPL 63547 (63.7 ± 7.0%) and enalapril (47.6 ± 5.5%). Possible explanations for these variations in recovery are discussed later.

Compound content in the bile was determined hourly, whereas data from urine samples were pooled over the total 5 h period because hourly samples of a viable size could not always be obtained.

The time-dependence of biliary excretion is shown for each compound in turn in Figure 1. FPL 63547 diacid (Figure 1a) was extensively and rapidly excreted in the bile with 72.4 ± 6.4% of total dose eliminated by this route in the first hour after dosing, with the rate of elimination declining rapidly thereafter. When dosed in its mono-ester form (FPL 63547) the elimination of FPL 63547 diacid in the bile showed a qualitatively similar profile (Figure 1b). There was significant biliary excretion of spirapril diacid (Figure 1c) and, to a lesser extent, enalapril diacid (dosed as the mono-ester, Figure 1d). In each case, the maximum levels of compound were found in the first samples after dosing. The biliary elimination of lisinopril was very slight (Figure 1e), 1.2 ± 0.3% and 1.3 ± 0.5% in the first and second hours after dosing respectively, declining thereafter.

Figure 2 shows the total elimination of each compound in the urine over the 5 h sampling period. Lisinopril demonstrated extensive elimination by this route with 64.3 ± 5.5% of total dose recovered from the urine. Enalapril diacid and spirapril diacid were also present in the urine in significant amounts. By contrast, the urinary excretion of FPL 63547 diacid was at a very low level irrespective of whether diacid (6.0 ± 2.0%) or mono-ester (2.8 ± 0.3%) was administered.

Table 1 shows a summary of the biliary and urinary excretion data and the calculated bile:urine ratio which describes the overall elimination profile of each compound. FPL 63547 diacid showed a strong preference for the biliary route with similarly high bile:urine ratios achieved after dosing of diacid and mono-ester. Spirapril diacid showed some preference for the bile, while enalapril was excreted approximately equally in the bile and in the urine. Lisinopril markedly favoured the renal route of elimination. On statistical analysis of the individual animal bile:urine ratios the FPL 63547 diacid groups differed significantly from the spirapril diacid, enalapril diacid and lisinopril groups ($P < 0.01$, Wilcoxon rank test) but not from each other.

Discussion

This examination of the relative use of biliary and renal routes of elimination by different ACE inhibitors in the anaesthetized rat has uncovered a wide spectrum of elimination profiles. The two extremes are represented by FPL 63547, on the one hand, whose active form is excreted almost exclusively in the bile and by lisinopril, on the other, which strongly favours the renal route. Spirapril diacid and enalapril had intermediate elimination properties.

Total recovery levels approximated to 70%, but varied between compounds, with the recoveries of FPL 63547 diacid and enalapril being most and least complete, respectively. There are a number of possible explanations for incomplete recovery. Bile and urine samples were taken for 5 h following dosing but all the compounds tested are long-acting probably as a result of tight binding to ACE, and pharmacological and pharmacokinetic evidence supports their continued presence in the body beyond 5 h. For example, in conscious dogs the inhibition of plasma ACE produced by a single i.v. dose of FPL 63547 peaks at 0.5–1 h and is still evident 24 h after certain doses (Carr *et al.*, 1990). In all probability a proportion of each compound is taken up into tissues sites from which it is released and eliminated at a very slow rate. There are reasons to believe that the elimination profiles determined over the 5 h study period are representative. Peak biliary elimination occurred early in this period, declining steeply thereafter and, where data are available (not shown), a similar decay was evident in the urinary elimination. Longer study periods and correspondingly greater compound recovery would not therefore be expected to have influenced the profiles.

The bioassay technique used to measure compound levels in the bile and urine in this study does differ in some significant respects from other methods for evaluating elimination, e.g. detection of radiolabelled compound. In this situation it can be argued that bioassay is the more appropriate method, because it measures elimination of the biologically relevant active form only rather than total compound. Correspondingly, bioassay will not detect elimination of either inactive prodrug or inactive metabolites and non-detection of these inactive species could, theoretically, have contributed to less than complete compound recovery. There is little evidence that the ACE inhibitors used in this study undergo significant further metabolism from the diacid form, although a minor hydrolytic product of enalapril diacid has been described (Drummer & Kourtis, 1987). Some elimination of FPL 63547 mono-ester may explain the difference in recovery observed when FPL 63547 was dosed in its mono-ester and diacid forms. However, even if this occurred, it is noteworthy that the elimination profile was not influenced by the form in which the compound was dosed, favouring the bile strongly in both cases. Studies in which the route of elimination of ^{14}C-labelled FPL 63547 was measured in conscious rats have essentially confirmed the results presented here (B. Mead—unpublished observation).

The elimination of lisinopril, enalapril and spirapril diacid as determined in this study agrees well with published data on these compounds. In man, lisinopril has been shown to be eliminated primarily in the urine (Ulm *et al.*, 1982). Tocco *et al.* (1982) have shown that i.v. enalapril undergoes both urinary and biliary excretion in rat and dog with a preference for the renal route. Oral administration to man has produced similar results (Ulm *et al.*, 1982). Captopril was also investigated in our model and appeared to be eliminated predominantly by the kidney in accordance with other studies using labelled compound (Kripalani *et al.*, 1980). The data have not been included in this paper because recovery of active thiol was very poor, suggesting rapid autoxidation to inactive disulphide. This instability in biological fluids has been noted previously (Kripalani *et al.*, 1980). Spirapril was the first biliary-selective ACE inhibitor to be described quantitatively with a bile:urine ratio of ≃3:1 obtained after i.v. adminis-

tration to rats (Leitz *et al.*, 1987). Our results with spirapril diacid confirm this and also demonstrate that FPL 63547 is substantially more selective than spirapril for the biliary route in this species. Unlike spirapril diacid which also appeared in the urine in significant quantities, the renal excretion of FPL 63547 diacid was so low as to be considered negligible.

Compounds pass from the blood into the bile for elimination by means of specialised transport processes linking liver parenchymal cells and the bile canaliculi. In general, the extent to which any given compound undergoes biliary elimination is influenced primarily by its physical chemical characteristics, particularly molecular weight, polarity and other special structural features (Smith, 1973). Recently, Ondetti (1988) has attempted to relate the physical chemical properties of a series of captopril and enalapril analogues to their routes of elimination, and formed the conclusion that increased molecular weight and lipophilicity are probably responsible for preferential biliary elimination. Contrary to the conclusion of Ondetti (1988), the results of this study suggest that molecular weight is not a key determinant of the extent of biliary elimination. FPL 63547 diacid (mol.wt. 421) and lisinopril (mol.wt. 405) are of similar molecular weights but widely different elimination properties. In addition FPL 63547 diacid was considerably more biliary-selective than spirapril diacid (mol.wt. 438) despite having a lower molecular weight.

According to Smith (1973) the presence of a highly polar group in the molecule is a requirement for extensive biliary excretion. The 'carboxy-terminus' carboxyl group of FPL 63547 diacid is highly ionised (pKa 1.79, unpublished observation) and therefore comfortably fulfils this criterion. Furthermore, the strong acidity of this group distinguishes FPL 63547 diacid from the other compounds tested. However, since enalapril diacid, lisinopril and spirapril diacid each show a pKa which falls within the range 3–4 (unpublished observation) described as being compatible with biliary elimination (Smith 1973), it is by no means certain that the strong acid functionality of FPL 63547 diacid, alone, is responsible for its biliary selectivity. FPL 63547 is relatively lipophilic (unpublished observation) and, although Smith (1973) has expressed the view that there appears to be no simple relationship between lipid solubility and biliary elimination, this factor may be significant in the context of our study. One striking characteristic of many compounds excreted extensively in the bile is their amphipathic character, i.e. the presence of both strongly polar and essentially non-polar groups within their molecular structure (Smith, 1973; Gregus and Klassen, 1987). In this respect they resemble the bile salts, e.g. taurocholic acid. The balance between polar and non-polar aspects may be critical for interaction with the carrier molecules responsible for transport into the bile. FPL 63547 can be considered to have amphipathic characteristics by virtue of its highly ionised carboxylic acid residue and the hydrophobic tertiary butyl substitution. Perhaps this, rather than any single property, explains its preference for biliary elimination.

The extent to which compounds are excreted in the bile can vary very significantly with species (Smith, 1973). Although the reasons for this variation are not fully understood, it is thought that the molecular weight threshold for biliary transport alters, being lowest in the rat ($\simeq$350) and highest in the rabbit ($\simeq$475). FPL 63547 undergoes extensive biliary excretion (measured as faecal content after i.v. administration) in conscious dogs (B. Mead—unpublished observation) as well as in the rat. As the molecular weight threshold for biliary elimination in man is thought to resemble rabbit more than rat or dog these data may not be the best guide. However, information on biliary elimination of drugs in man is very scarce and since factors other than molecular weight appear to be the predominant influence responsible for the biliary selectivity of FPL 63547 in this rat study, historical concepts of species variation may not be applicable. Studies of the route of elimination of FPL 63547 in man will clarify this issue.

Renal dysfunction of varying severity, resulting from cardiovascular disease and/or advanced age (Reid, 1987), is a fairly common finding in the types of patients for whom ACE inhibitors are likely to be prescribed. In renal dysfunction, the clearance of renally-excreted ACE inhibitors is compromised, resulting in undesirable elevation of plasma levels (Kelly *et al.*, 1986; van Schaik *et al.*, 1987), supra-optimal inhibition of ACE and increased potential for adverse reactions. Hence the need for careful monitoring when these compounds are used and, usually, some reduction in dosage or dose interval in patients whose renal function is impaired. The development of biliary-eliminated ACE inhibitors would provide the clinician with a new treatment option which, by avoiding the problem of compound accumulation, may be of value in the management of such patients.

One potentially important consideration when dealing with compounds which are excreted in the bile is the possibility of enterohepatic recirculation. This reabsorption of compounds from the intestine subsequent to biliary excretion provides an additional clinical variable, usually with undesirable consequences. The phenomenon can result in significant elevation in plasma levels, thus prolonging pharmacological (e.g. digitoxin) or toxic (e.g. indomethacin) effects (Gregus & Klaassen, 1987). FPL 63547 will not be subject to enterohepatic recycling since the diacid form in which it is eliminated, although biologically active, would not be absorbed from the gut. Only the mono-ester prodrug form possesses significant oral bioavailability.

In summary, in a rat model the novel ACE inhibitor, FPL 63547, was extensively and preferentially eliminated by the biliary route. In this respect it differed from other compounds of this class tested and may be unique amongst ACE inhibitors in the level of its selectivity for the biliary route. This mode of elimination, if confirmed in man, is likely to be clinically advantageous. FPL 63547 has accordingly been selected for further development for the treatment of hypertension and heart failure.

We wish to thank Brian Mead for permission to quote route of elimination studies using labelled FPL 63547, Dave Payling and Carol Manners for physical chemical data and Merck, Squibb and Schering for their gifts of lisinopril, captopril and spirapril diacid, respectively.

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Time-dependent effects of theophylline on myocardial reactive hyperaemias in the anaesthetized dog

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1 The effects of a loading dose of theophylline (5 mg kg^{-1} i.v.) on the hyperaemias resulting from short-term (15 and 30 s) interruptions in coronary blood flow and intracoronary adenosine were studied at given intervals over a 2 h period in the anaesthetized dog.

2 These hyperaemic responses were affected differently by theophylline and each effect was time-dependent. The reactive hyperaemic response progressively decreased after drug delivery, reaching 46% of control at 2 h. In contrast, after a maximal attenuation to 23% of control 5 min after theophylline, the hyperaemia resulting from intracoronary adenosine progressively increased over the same period, reaching 64% of control 2 h after the loading dose.

3 Two-compartment model results based on plasma theophylline measurements and the time course of theophylline accumulation in pericardial infusates, suggested that complete drug distribution throughout the heart may require at least 20 min following a single intravenous dose.

4 If it is assumed that theophylline blocks coronary vascular adenosine receptors, these pharmacokinetics are consistent with the time-dependent pattern of response attenuation we observed for the adenosine-induced hyperaemias, but they cannot entirely explain the pattern of response attenuation observed for the occlusion-induced hyperaemias. The continued increase in attenuation of this response after complete drug distribution suggests an additional pharmacodynamic action of theophylline.

5 We conclude that a single therapeutic dose of theophylline results in distinct time-dependent pharmacological effects with respect to the ability of the coronary vasculature to dilate in response to temporary interruptions in oxygen supply and in response to exogenously administered adenosine. These effects deserve consideration in both experimental studies in which adenosine antagonists are used to assess adenosine action *in vivo*, and in clinical practice where theophylline pharmacotherapy for pulmonary disorders is commonplace.

Introduction

Following a coronary occlusion, blood flow increases dramatically above pre-occlusion levels and only gradually returns to control (Olsson, 1975). The mechanism responsible for mediating this reactive hyperaemic response has yet to be defined. Vasodilator metabolites, released locally from the oxygen-deprived myocardial cells, are likely candidates (Olsson, 1975). The results of many studies support a role for the purine nucleoside adenosine in causing this hyperaemia, released by myocardial cells in response to the decrease in oxygen supply (Rubio *et al.*, 1969; Olsson *et al.*, 1978; Kroll *et al.*, 1980; Saito *et al.*, 1985). However, controversy still surrounds this hypothesis because of studies finding that the adenosine antagonist theophylline (Fredholm, 1980; Snyder *et al.*, 1981; Rall, 1982) has little attenuative effect on this hyperaemia, while near maximal attenuations of hyperaemias resulting from exogenously-administered adenosine are concomitantly observed (Bittar & Pauly, 1971; Eikens & Wilcken, 1973; Giles & Wilcken, 1977; Radford *et al.*, 1984). In these latter studies, drug effects were recorded within minutes following theophylline administration. None of these studies looked at potential time-dependent effects of theophylline on these hyperaemic responses, even though exogenously delivered drugs are expected to exhibit such effects as a function of their pharmacokinetics (Nagashima *et al.*, 1969; Gibaldi *et al.*, 1971; Levy & Gibaldi, 1972; Gillette, 1973).

In the present study, both the reactive hyperaemic response to brief interruptions of coronary flow and the hyperaemic response to intracoronary adenosine were measured for 2 h following a single intravenous dose of theophylline to determine if the effects of this drug on these hyperaemias are time-dependent. The concentration of theophylline in plasma and pericardial infusates was also determined over this time; pharmacokinetic analyses of these data were utilized to determine the site(s) of theophylline action responsible for the observed pharmacological effects.

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Methods

Surgical preparation

Eighteen adult mongrel dogs of either sex (20–30 kg) were used for these studies, randomly divided into 2 groups. The dogs were anaesthetized with sodium pentobarbitone (30 mg kg^{-1} i.v.), and supplemented as needed to maintain loss of eyelid reflex. Positive pressure ventilation (Harvard Apparatus, Model 607) was established following endotracheal intubation. A femoral arterial catheter was advanced to the level of the thoracic aorta to measure arterial blood pressure continuously (Gould Brush 200), and to obtain blood samples for gas/pH analysis (Corning 158 pH/Blood Gas Analyzer) and plasma theophylline quantification. Adjustments in tidal volume or respiratory rate were used to maintain blood gases and pH within the following range: P_{O_2} = 90–150 mmHg; P_{CO_2} = 25–40 mmHg; pH = 7.35–7.45. Anaesthetic, theophylline and saline were infused via a femoral venous catheter. A small thoracotomy in the fifth right intercostal space was performed to guide a catheter (Sones 7 french) manually into the coronary sinus, via the right jugular vein, to measure coronary sinus blood oxygen content. A thoractomy in the fourth left intercostal space allowed access to the subclavian artery and left aspect of the heart and pericardium.

Heparin was administered (750 units kg^{-1}) and the circumflex branch of the left main coronary artery was perfused via an extracorporeal circuit with blood originating from the cannulated left common carotid artery. The distal end of the

circuit was composed of a metal guide cannula (o.d. = 5 mm) that was inserted into the left subclavian artery and advanced to the left coronary ostium; an inner polyethylene catheter (o.d. = 3 mm, with 4 mm o.d. flared tip) was then introduced through the ostium approximately 0.5–1.0 cm to wedge in the circumflex coronary artery. Wedge was verified by distal coronary pressures of less than 25 mmHg during occlusion of coronary flow. The pressure drop across the circuit was 10 mmHg. The pericardial sac remained intact during this procedure.

The extracorporeal shunt incorporated an electromagnetic flow probe (Biotronex, 3.5 mm) to measure circumflex coronary blood flow (Gould Brush 200). The circuit included a bypass segment to allow zero flow settings without coronary flow interruption. The flow probe was calibrated with blood, before the circuit was completed, by measuring the volume of flow at several different stages of circuit resistance. Through a side port in the circuit, pulsatile and mean coronary perfusion pressures were monitored (Gould Brush 200), and samples for coronary arterial oxygen content were obtained. For calculation of myocardial oxygen consumption, circumflex coronary arterial and coronary sinus blood samples were measured for oxygen content (Lex-O_2-Con; Lexington Instruments) after anaerobic collection into pre-chilled heparinized glass syringes. Because Silastic tubing comprised the circuit, coronary occlusions (direct clamping of the circuit tubing) and intracoronary infusion of drugs (tuberculin syringe needle inserted through tubing wall) were easily achieved. After the experiment, the animals were killed with an intravenous cardioplegic solution, and circumflex coronary flow was standardized per gram of perfused left ventricle by delivering a solution of trypan blue dye through the extracorporeal circuit, at the appropriate coronary perfusion pressure, and excising and weighing the stained left ventricular myocardium (61.2 ± 2.8 g; $n = 17$).

Experimental protocol

Group 1 Fourteen animals were utilized for the hyperaemia studies, randomly divided into two subgroups. In subgroup 1A ($n = 9$), changes in both the reactive hyperaemic response to short-term occlusions of coronary flow and the hyperaemic response to exogenous adenosine administration were measured over a 2 h period following a single intravenous dose of theophylline as follows: a control period was established during which several responses to 15 s and 30 s occlusions were obtained. The control responses to several intracoronary adenosine injections (250 ng ml^{-1}; volume adjusted for each animal over 0.1–0.2 ml) were also recorded. In all animals, a two fold minimum increase in coronary blood flow resulted from the 15 and 30 s occlusions under control conditions, and a hyperaemia of comparable peak magnitude was achieved with the bolus intracoronary adenosine dose. Repeated intracoronary injections of adenosine did not affect the intervening reactive hyperaemic responses. After the control period, theophylline (5 mg kg^{-1} in 20 ml of warm saline) was administered intravenously over a 30 s period. Identical occlusions and intracoronary adenosine infusions were then performed in duplicate 5, 10, 30, 60, 90 and 120 min after theophylline. In 5 of the subgroup 1A dogs, myocardial oxygen consumption was measured. This hyperaemia protocol was also followed with the animals in subgroup 1B ($n = 5$), wherein saline vehicle was administered instead of theophylline. In one of these animals, the responses to intracoronary adenosine were not obtained.

Group 2 Four dogs were used to obtain samples from plasma and pericardial infusates for pharmacokinetic analyses. The concentration of theophylline in 3 ml arterial plasma samples was measured at 2, 3.5, 5, 7.5, 10, 30, 60, 90 and 120 min after intravenous theophylline delivery (5 mg kg^{-1}). To determine if intramyocardial pharmacokinetics differed significantly from whole-body kinetics, pericardial infusate theophylline sampling was performed. The method is based on the assumption that the solute concentration in the pericardial infusate represents an index of the solute concentration in the myocardial interstitial fluid, and its merits as such have been previously discussed at length (Knabb *et al.*, 1983). To obtain these samples, a Silastic catheter (3 mm i.d.) was inserted through a small puncture hole in the pericardium and sutured in place to form a fluid-tight seal. Then, 25 ml of an iso-osmotic Krebs-Henseleit solution (pH = 7.4, 37°C, equilibrated with 95% O_2/5% CO_2, of the following composition (mM): NaCl 121.4, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 21.9, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 11.1) was infused into the pericardial space 5 min before theophylline administration. Infusion of this volume of buffer did not alter cardiac dynamics or coronary haemodynamics. Successive 1 ml samples were then withdrawn from the pericardial infusate at the following times after drug administration: 5, 10, 15, 30, 45, 60, 75, 90 and 120 min (fresh Krebs-Henseleit solution was not added back to the infusate with each successive sample).

Response quantification

All hyperaemic responses were quantified by computerized planimetry of the area under each hyperaemic curve (control blood flow before and after occlusion served as the horizontal baseline – see Olsson, 1975 for further details), because both duration and peak flow were typically affected by theophylline. The mean results at each test time were then normalized to control values. One-way analyses of variance were used to determine statistically significant differences between hyperaemic responses (non-parametric Kruskal-Wallis test) and statistically significant differences between pericardial infusate theophylline concentrations (Duncan's multiple range test). All results are expressed as mean ± s.e., and differences were considered significant at the 5% confidence level.

Theophylline quantification

Measurement of plasma and pericardial infusate theophylline concentrations by high performance liquid chromatography (h.p.l.c.) required the following preparatory steps. For plasma theophylline samples, centrifugation at 2500 *g* for 10 min yielded a 1 ml plasma fraction that was then added to 35% perchloric acid (4:1), vortexed, and centrifuged again (2500 *g*; 10 min). The resulting supernatant fraction was neutralized with KOH and filtered (Millipore, 0.22 μ) for removal of precipitated salts. Pericardial infusate samples were evaporated under forced air, reconstituted in 200 μl distilled water, and filtered (Gelman 0.2 μ). A 5 μ-ODS-C_{18} analytical column (Altex Ultrasphere), 1 cm-5 μ-OD-GU-RP_{18} guard column (Brownlee Labs), LKB Model 2150 h.p.l.c. pump, and LKB Model 2152 HPLC Controller were used for isocratic elution of theophylline from the above samples. We utilized a previously unpublished isocratic method for theophylline quantification: 10 mM KH_2PO_4 buffer with 10% methanol (pH = 5.0) was used at a flow of 1.4 ml min^{-1} and absorbance was monitored at 285 nm (Waters Assoc., Model 440 Absorbance Detector). The isolated theophylline peaks in the samples were identified and quantified by external standardization. Recoveries of theophylline (10 nM) added to plasma or buffer aliquots subjected to perchloric acid treatment were consistently greater than 95%.

Pharmacokinetic calculations and modelling

A biexponential least-squares regression fit with standard error confidence intervals determined by computer (Johnson & Frasier, 1985) for the plasma theophylline data as a function of time (t) in min yielded the following equation: $y = 95.5e^{-0.1649t} + 42.9e^{-0.0056t}$. These data were used to define the two compartment open model (Riegelman *et al.*, 1968; Gibaldi & McNamara, 1978). The fraction of the loading dose present in each compartment during the 2 h experimental period was then calculated (Gibaldi *et al.*, 1969;

Gillette, 1973; Greenblatt & Koch-Weser, 1975) and correlated with the observed pharmacological effects in a log dose-response manner (Levy, 1966; Levy *et al.*, 1969).

Results

In the 18 animals utilized for these studies, circumflex coronary blood flow ranged from 42 to 130 ml min^{-1} 100 g^{-1} (mean = 73 ± 9 ml min^{-1} 100 g^{-1}) and mean arterial blood pressure was 108 ± 8 mm Hg. In the 5 dogs in which myocardial oxygen consumption was measured (5.4 ± 0.5 ml min^{-1} 100 g^{-1}), flow was 51 ± 7 ml min^{-1} 100 g^{-1} and (A − V) O_2 content = 11.0 ± 1.1 ml 100 ml^{-1}). Myocardial oxygen consumption was not affected by theophylline administration except for a general increase observed 5 min after drug delivery due to the positive inotropic effect of the drug. An experiment was discarded if flow or arterial pressure varied more than 10% during the experimental period, with the exception of the first few minutes directly following theophylline delivery when increases in these variables also occurred due to the positive inotropism. Generally, these changes lasted less than 5 min, but in some animals these variables were still not back to control levels at the 5 min measurement time; nevertheless, the hyperaemic responses recorded at this time were included in our quantitative analyses.

The peak hyperaemic flow resulting from the 30 s occlusion did not differ from that resulting from the 15 s occlusion (but the duration of the former hyperaemia was greater), which is typical for this response in the dog (Olsson, 1975). Both occlusion-induced hyperaemic responses were attenuated similarly by theophylline (Group 1A), with reduction of both response duration and peak flow (see Figure 1; Table 1). Hence, the magnitudes of the two reactive hyperaemic responses at each test time were averaged for presentation in Figure 2 (with statistically significant differences noted). The effect of theophylline on this response was time-dependent. Five minutes after theophylline, the hyperaemia was reduced only to 80% of control. However, thereafter the response attenuation progressively increased, falling to 46% of control at 2 h post-theophylline. In the absence of theophylline (Group 1B), the reactive hyperaemic responses were not significantly different from control (Table 1).

A time-dependent effect of theophylline was also observed for the dilator responses to intracoronary adenosine (Group 1A), as shown in Figure 1. This hyperaemic response typically lasted 25–45 s under control conditions. Theophylline reduced both the magnitude and duration of the hyperaemic event. In contrast to the reactive hyperaemic response, the most significant hyperaemic reduction (to 23% of control) occurred directly after theophylline administration (see Figure 2, with statistically significant differences noted). Thereafter, the extent of attenuation was progressively less pronounced; 2 h after drug delivery, this hyperaemic response had returned to 64% of control. In the absence of theophylline (Group 1B), hyperaemias from exogenous adenosine were not attenuated over time (Table 1).

Figure 3 illustrates the plasma theophylline concentrations measured at various times after drug delivery (Group 2); from these data, the pharmacokinetic values in Table 2 were obtained. Note that the steady state plasma theophylline concentration resulting from the single intravenous dose was 3–9 $\mu g\,ml^{-1}$, a concentration which falls within the therapeutic range for man (Mitenko & Ogilvie, 1972; Hendeles & Weinberger, 1983; Rall, 1985). The central compartment of the model (predicted volume of distribution = 20% of body weight) was assumed to be that which received the administered drug and the compartment from which first-order drug elimination occurred (half-time of 124 min). The peripheral or tissue compartment (predicted volume of distribution = 36% of body weight) was assumed to be that into which theophylline distributed via first order kinetics (half-time of 4.1 min).

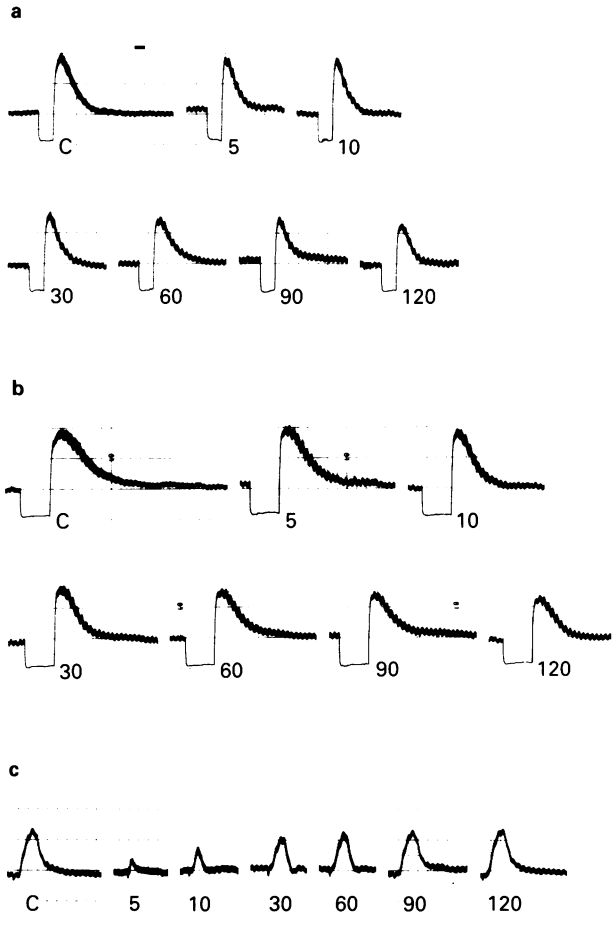

Figure 1 Circumflex coronary blood flow records from a typical experiment in which theophylline (5 mg kg^{-1} i.v.) was given, and its effects on the hyperaemic responses resulting from 15 s (a) and 30 s (b) coronary occlusions, and intracoronary administration of adenosine (c), were measured 5, 10, 30, 60, 90 and 120 min after theophylline delivery. The first response in each panel is control response (C). Horizontal bar = 10 s.

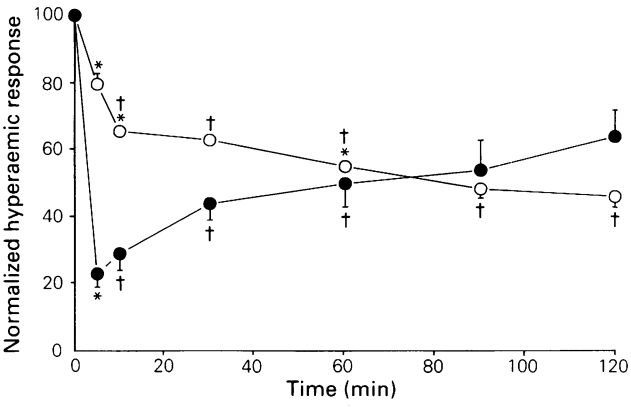

Figure 2 Effect of theophylline on the hyperaemias resulting from coronary occlusions and intracoronary adenosine. (○) The mean of the reactive hyperaemic responses resulting from 15 and 30 s occlusions of circumflex coronary blood flow recorded 5, 10, 30, 60, 90 and 120 min after a bolus dose of theophylline (5 mg kg^{-1} i.v.) in 9 animals (Group 1A). Values shown have been normalized to the control responses that preceded theophylline administration. (●) The normalized hyperaemic responses to intracoronary adenosine recorded at the same times indicated above following theophylline in Group 1A. * and † indicate that the attenuation at that time was significantly different (ANOVA; $P < 0.05$; Kruskal-Wallis) from the attenuation recorded at the previous test time, and the attenuation recorded two test times earlier, respectively. Note: some error bars fall within the symbol itself.

Table 1 Areas (mm^2) of hyperaemias resulting from 15 and 30 s coronary occlusions and intracoronary adenosine in 14 animals of Group 1(A + B)

		Time (min) *after theophylline*					
Group 1A	*Control*	*5*	*10*	*30*	*60*	*90*	*120*
15 s Occlusion							
Dog No.							
1	245	165	150	140	115	90	—
2	285	245	200	190	185	175	160
3	260	—	155	160	—	150	100
4	265	180	165	155	125	—	110
5	230	—	155	125	—	85	100
6	270	190	165	155	130	105	—
7	270	250	205	195	165	160	145
8	240	175	140	135	130	90	80
9	260	—	150	140	160	135	—
mean ± s.e.	258 ± 6	201 ± 15	165 ± 8	155 ± 8	145 ± 10	124 ± 13	116 ± 13
30 s Occlusion							
Dog No.							
1	425	325	300	295	285	265	—
2	485	395	340	335	320	285	300
3	495	—	400	375	—	260	260
4	450	360	320	255	190	—	180
5	430	—	285	290	—	170	210
6	495	400	295	320	245	—	—
7	485	400	285	305	240	215	195
8	420	335	245	250	190	165	140
9	420	—	285	280	255	200	—
mean ± s.e.	456 ± 11	369 ± 14	306 ± 15	301 ± 13	246 ± 18	223 ± 18	214 ± 23
Adenosine							
Dog No.							
1	180	20	10	35	45	35	—
2	220	70	95	125	150	165	185
3	215	—	70	100	—	105	105
4	225	30	20	50	60	—	75
5	170	—	80	110	—	125	130
6	165	55	45	75	95	—	—
7	195	35	65	90	120	115	125
8	200	60	85	115	125	120	130
9	190	—	40	75	90	80	—
mean ± s.e.	196 ± 7	45 ± 8	57 ± 10	86 ± 10	98 ± 14	106 ± 15	125 ± 15
		Time (min) *after vehicle*					
Group 1B	*Control*	*5*	*10*	*30*	*60*	*90*	*120*
15 s Occlusion							
Dog No.							
1	260	—	295	275	265	245	235
2	230	—	220	235	215	220	230
3	235	—	220	240	230	215	210
4	260	—	250	255	210	245	—
5	245	—	280	260	235	270	270
mean ± s.e.	246 ± 6	—	253 ± 15	253 ± 7	231 ± 10	239 ± 10	236 ± 11
30 s Occlusion							
Dog No.							
1	465	—	490	495	480	475	475
2	420	—	405	415	415	365	375
3	415	—	410	425	420	375	380
4	450	—	470	485	460	400	—
5	425	—	420	420	420	450	460
mean ± s.e.	435 ± 10	—	439 ± 17	448 ± 17	439 ± 13	413 ± 21	422 ± 26
Adenosine							
Dog No.							
1	—	—	—	—	—	—	—
2	195	190	195	205	200	185	220
3	190	190	180	200	200	185	205
4	220	225	220	240	245	220	—
5	210	200	200	205	210	195	175
mean ± s.e.	204 ± 7	201 ± 8	199 ± 8	213 ± 9	214 ± 11	196 ± 8	200 ± 13

Group 1A received theophylline ($5\,mg\,kg^{-1}$) i.v., $n = 9$.
Group 1B received saline vehicle i.v.; $n = 5$.

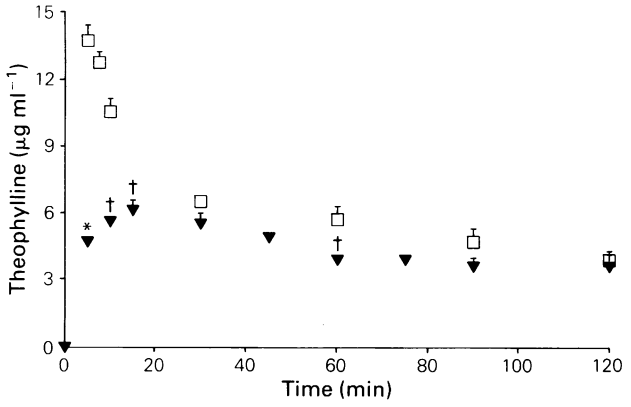

Figure 3 Theophylline concentrations in plasma and pericardial infusates (Group 2). (□) Plasma theophylline concentrations measured at the times indicated following a bolus dose (5 mg kg^{-1} i.v.). A biexponential fit of the data yielded half-times for the first distributive phase and second elimination phase of 4.1 and 124 min, respectively. (▼) Pericardial infusate theophylline concentrations measured in aliquots collected from a 25 ml infusate introduced into the pericardial space 5 min preceding theophylline (5 mg kg^{-1} i.v.). * and † indicate statistically significant differences (ANOVA; $P < 0.05$; Duncan's Multiple Range) as explained in legend of Figure 2. Note: some error bars fall within the symbol itself.

Twenty-one minutes (five half-times) were required for the drug to distribute throughout the central and peripheral body compartments and achieve a steady state (i.e. the drug concentration in the peripheral tissue compartment increased for 21 min). Total volume of distribution was slightly less than (93%) total body water. The accumulation of theophylline in the pericardial infusate buffer (Group 2) is also shown in Figure 3. The infusate theophylline concentration increased for at least 15 min after drug delivery before reaching a steady state with the drug in the plasma compartment.

Figure 4a and b shows the relationship between the two observed pharmacological effects and the log of the relative theophylline concentrations in the central and tissue compartments, resepectively. In the central compartment (Figure 4a), the fall in theophylline concentrations with time correlated positively with its decreasing effect with time on the exogenous adenosine response. In contrast, this progressive decrease in central compartment theophylline concentrations showed no correlation with the progressive increase in reactive hyperaemic attenuation. In the tissue compartment (Figure 4b), the initial rise (0 to 30 min) in theophylline concentrations correlated positively with the increase in the attenuation of the reactive hyperaemic response. However, this correlation broke down between 30–120 min, as the effect of the drug on the reactive hyperaemia continued to increase in the face of decreasing theophylline concentrations in this compartment.

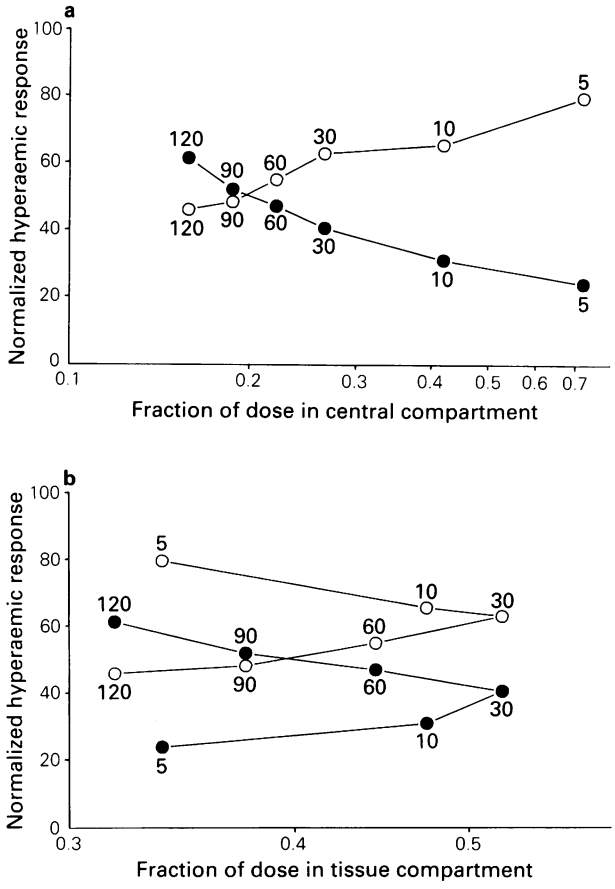

Figure 4 The log dose-response relationship between the fraction of administered theophylline present in the central compartment (a) and the peripheral tissue compartment (b) of the model 5, 10, 30, 60, 90, and 120 min after theophylline delivery and the two observed effects of the drug (attenuation of hyperaemias). (○) Reactive hyperaemia response. (●) Exogenous adenosine hyperaemia response. The numbers next to each symbol represent the time (min) when the original plasma measurements were obtained. Central compartment theophylline concentrations decreased continuously following drug administration, whereas tissue compartment drug concentrations increased for 21 min following theophylline delivery, and then decreased thereafter, in a steady state with plasma theophylline.

Discussion

The present study has shown that a single therapeutic dose of theophylline affected two different coronary hyperaemic responses in a time-dependent manner. The extent of attenuation of reactive hyperaemias resulting from brief interruptions in coronary flow increased progressively for two hours following a single dose of theophylline. However, over the same time period, the hyperaemias resulting from intracoronary adenosine were severely attenuated immediately after drug administration, and the extent of attenuation decreased progressively thereafter. These results suggest two distinct sites for theophylline action, which are likely to be two topologically distinct adenosine receptor pools. Our pharmacokinetic analyses also suggest that adenosine receptor antagonism (Fredholm, 1980; Snyder *et al.*, 1981; Rall, 1982) is not the only pharmacodynamic action of therapeutic concentrations of this drug.

If the pharmacological effects of therapeutic concentrations of theophylline are due only to blockade of adenosine receptors (Fredholm, 1980; Snyder *et al.*, 1981; Rall, 1982), then our results implicate two topologically distinct adenosine receptor pools, one of which mediates the hyperaemic response to coronary occlusion and the other of which mediates the hyperaemic response to exogenous adenosine. For many years, vascular adenosine receptors were assumed to be located on the abluminal surface of the smooth muscle cells in

Table 2 Pharmacokinetic variables describing the two compartment model

Dose (mg kg^{-1})	Q_o (mg)	V_A (ml kg^{-1})	V_B (ml kg^{-1})	V_d (ml kg^{-1})	α (min^{-1})	β (min^{-1})	k_{12} (min^{-1})	k_{21} (min^{-1})	k_{el} (min^{-1})
5	125	200	360	560	0.1694	0.0056	0.1019	0.0563	0.0169

close proximity to interstitial adenosine produced by myocardial cells (Olsson et al., 1976; Schrader et al., 1977). However, recent evidence supports the existence of an endothelial adenosine receptor pool that may mediate the vasodilator response to intracoronary adenosine (Frank & Bevan, 1983; Gordon & Martin, 1983; Nees et al., 1985; Rubanyi & Vanhoutte, 1985). We contend that the time course of the two observed pharmacological effects can be explained, in part, by time-dependent changes in drug concentrations surrounding each of these receptor pools, as dictated by the pharmacokinetic behaviour of the drug. This hypothesis is developed below in more detail for each response.

If the hyperaemia induced by intracoronary adenosine is mediated via the pool of endothelial receptors, then the resultant hyperaemic response that these receptors initiate should be affected by the relative concentrations of intravascular agonist and antagonist. Immediately after theophylline delivery, intravascular concentrations of the drug are so much greater than those resulting from the bolus injection of exogenous adenosine that vasodilatation is nearly completely blocked, as we, and others (Bittar & Pauly, 1971; Eikens & Wilcken, 1973; Giles & Wilcken, 1977; Radford et al., 1984), observed at this time. However, in the present study the period of observation was extended to include the time during which theophylline progressively loses its competitive advantage for these receptors because of a progressive decrease in its intravascular concentration due to tissue distribution and hepatic metabolism. At these later times, when the plasma concentration of theophylline had decreased nearly an order of magnitude, significant hyperaemias resulted from the same adenosine challenge. The direct correlation between the extent of exogenous adenosine hyperaemic attenuation and the plasma/central compartment theophylline concentrations (Figure 4a) reflects this dependency clearly.

If interstitial adenosine released from myocardial cells mediates the reactive hyperaemic response via abluminally-located adenosine receptors, then the magnitude of the effect of theophylline on this response at a given time would depend on the extent of interstitial theophylline distribution at that time. Based on our pharmacokinetic analyses of plasma and pericardial theophylline concentrations, we contend that the progressive increase in attenuation of the reactive hyperaemic response, observed over the initial 15–30 min following drug administration reflects a progressive increase in myocardial interstitial theophylline concentrations. A similar delay in theophylline distribution has been observed in rabbits (Hess et al., 1968) and man (Mitenko & Ogilvie, 1972; Levy & Koysooko, 1975). Even in rabbit isolated hearts, wherein capillary permeability is generally increased due to the hypo-osmotic effects of buffer perfusion (Chambers & Zweifach, 1947), 30 min were required after intracoronary theophylline ($100\,\mu g\,ml^{-1}$) before a tissue steady state was achieved (Belleman & Scholz, 1974). The observation that a 30 min intracoronary infusion of theophylline was necessary before blockade of the reactive hyperaemic response in guinea-pig isolated perfused hearts occurred (Wiedmeier, 1978) also supports our results. Thus, the controversial studies that assessed the effects of theophylline on coronary hyperaemias directly after drug administration (Bittar & Pauly, 1971; Eikens & Wilcken, 1973; Giles & Wilcken, 1977; Radford et al., 1984) now appear ill-designed, and call into question the conclusions advanced in these studies.

The continued increase in attenuation of the reactive hyperaemic response that occurred in the face of decreasing theophylline concentrations in the plasma, pericardial infusate, and tissue compartment of the model (Figure 4b) implies an additional mechanism of action of theophylline. Indeed, such an effect(s) could have been realized throughout the 2 h experimental period in conjunction with adenosine receptor antagonism, and could have contributed to the response attenuation observed during the first 30 min. How the pharmacological effect of this drug can continue to increase after complete drug distribution and a steady state of drug elimination had been achieved is not known at this time. In any event, there are data in the literature that suggest other potential sites of theophylline action. For example, methylxanthines can inhibit the synthesis of prostaglandins (Horrobin et al., 1977), which have been implicated as mediators of the reactive hyperaemic response (Alexander et al., 1975; Hintze & Kaley, 1977; Schror, 1981). Other possible targets include effects on uptake and/or release or catecholamines, calcium transport and binding, and accumulation of cyclic GMP (Rall, 1985). Besides adenosine receptor blockade, theophylline could also interfere with an adenosine-induced vasodilator response by reducing the rate of adenosine formation via inhibition of 5'-nucleotidase, as found for concentrations less than $50\,\mu M$ ($9\,\mu g\,ml^{-1}$) and $100\,\mu M$ ($18\,\mu g\,ml^{-1}$) in heart sarcolemma (Heyliger et al., 1981) and kidney (Fredholm et al., 1978), respectively. Finally, increases in 3-methylxanthine, a pharmacologically active hepatic biotransformation metabolite of theophylline (Williams et al., 1978; Rall, 1985), could account for the continued attenuation of the reactive hyperaemic response. Unfortunately, we do not have experimental evidence to confirm or refute these possibilities.

In summary, we found that theophylline antagonizes in a time-dependent manner both occlusion-induced and adenosine-induced coronary hyperaemias. We propose that these effects result, in part, from the differential blockade of two topologically distinct adenosine receptor pools; the extent of antagonism of each pool is dependent on the pharmacokinetic behaviour of the drug. Because some of these pharmacological effects increase in magnitude after the pharmacokinetics of the drug indicate that its distribution is complete, additional pharmacodynamic actions of the drug, independent of adenosine receptor blockade, are implicated. Although our intention was not to test the extent of participation of adenosine in the reactive hyperaemic response, given the wide availability of more powerful and selective adenosine antagonists, our results suggest that adenosine is a key metabolic participant in the mediation of this hyperaemia. The clinical implications of our general finding, that therapeutic levels of theophylline clearly compromise the ability of the coronary vascular bed to dilate following brief interruptions in oxygen supply, warrant further investigation of this widely used drug.

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Cardiovascular responses to verapamil and nifedipine in hypoventilated and hyperventilated rats

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1 The influence of hypoventilation or hyperventilation on blood pressure and pulse rate responses to verapamil and nifedipine was studied in chloralose-anaesthetized rats.

2 Artificial ventilation with room air at a fixed volume of $10\,ml\,kg^{-1}$ successfully induced combinations of hypoxaemia, hypercarbia and acidosis at a ventilator rate of 37 strokes min^{-1} and of hyperoxaemia, hypocarbia and alkalosis at 160 strokes min^{-1}.

3 Hypoventilation caused significant decreases in both the blood pressure and pulse rate, whereas hyperventilation produced significant increases in these parameters.

4 In the controls, intravenous injections of graded doses of either verapamil or nifedipine caused dose-dependent decreases in mean blood pressure. The effects on pulse rate were not marked.

5 The hypotensive effects of verapamil were significantly more intense in hyperventilated rats, whereas those of nifedipine were significantly less pronounced in hypoventilated animals. The hypoventilated rats exhibited a significant dose-dependent decrease in pulse rate in response to verapamil administration.

6 It is concluded that cardiovascular responses to verapamil, nifedipine and probably other calcium antagonists are altered in the presence of blood gas abnormalities.

Introduction

It is well established that changes in pH or gas content of blood or of the fluid that bathes isolated tissues affect the functions of cardiovascular tissues. Gaskel (1952) found that perfusion of the caudal half of the body of a frog with a solution of lactic acid or acetic acid resulted in significant dilatation of the arterial vessels. Bayliss (1901) showed that the rate of blood flow in the perfused frog extremities was significantly increased when the perfusate was saturated with carbon dioxide. Ng *et al.* (1967) demonstrated a consistent reduction in left ventricular systolic pressure when coronary blood pH fell and $PaCO_2$ rose, and an increase when pH rose and $PaCO_2$ fell.

Responses of tissues and organs to certain drugs may also be altered in the presence of blood gas abnormalities. Detar & Bohr (1968) showed that oxygen tension was an important determinant of the contractile force developed by the isolated helical strips of the rabbit aorta in response to adrenaline. Grant *et al.* (1985) showed that the α_1- and α_2-adrenoceptor-mediated pressor responses of pithed rats were differentially affected by changes in blood gases. Dai & Wong (1985) found that in urethane-anaesthetized rats, the effects of adrenaline on blood pressure, and of adrenaline or acetylcholine on pulse rate, were significantly reduced during hypoventilation which induced hypoxaemia, hypercapnia and acidosis. MacLean & Hiley (1988) also revealed that changes in artificial respiratory volume and the attendant blood gas and pH changes caused variations in cardiovascular responses to phenylephrine in the pithed rat.

Calcium antagonists have been advocated for the management of clinical conditions such as cerebral and cardiac ischaemia in which blood gas abnormalities are invariably associated (Steen *et al.*, 1983; 1984; Milde *et al.*, 1986). However, the possibility that the cardiovascular responses to these drugs may be altered either quantitatively or qualitatively in the presence of blood gas changes should be considered. The present study, therefore, examines the blood pressure and pulse rate responses to i.v. injected verapamil or nifedipine in rats subjected to artificial hypoventilation or hyperventilation.

Methods

Animals

Male Sprague-Dawley rats, weighing 300–350 g, were used. They were housed in an air-conditioned room where temperature was maintained at $22 \pm 1°C$ and relative humidity at 60–70%. The animals were allowed free access to a standard rat pellet diet (Ralston Purina Co., U.S.A.) and tap water.

Measurement of cardiovascular parameters

The rats were anaesthetized with inhalation of diethyl ether (Merck) followed by i.v. injection of chloralose (BDH) $60\,mg\,kg^{-1}$ through the cannulated left jugular vein. The trachea was cannulated for artificial ventilation, as was the right common carotid artery for measuring arterial pressure and pulse rate, with a Statham P23ID pressure transducer and a biotachometer coupler (Narco Bio-Systems), respectively. These parameters were displayed on a physiograph (Narco Bio-Systems). The animals were kept warm with a heating lamp throughout the whole experimental period.

Normal ventilation, hypoventilation or hyperventilation

After the cannulation procedures, the rats were subjected to artificial ventilation with room air, using a respirator (Palmer, U.K.) with a fixed volume of $10\,ml\,kg^{-1}$. In order to facilitate respiratory control of animals, alcuronium chloride (Alloferine, Roche) $150\,\mu g\,kg^{-1}$ was injected i.v. immediately before the start of artificial ventilation. The rats were randomly divided into 3 groups. They were subjected to either normal ventilation, hypoventilation or hyperventilation which was induced by adjusting the ventilator rate from 80 to 37 or 160 per min, respectively. To verify the effects of varying the ventilator rate, the arterial blood gases of the animals were measured before and at 5 min after the commencement of artificial ventilation, by a blood gas analyser (AVL Gas Check 938, Switzerland). Blood samples (0.1 ml) were collected from the cannulated carotid artery.

Responses to verapamil or nifedipine

The observations on the blood pressure and pulse rate responses to i.v. injected verapamil (Knoll AG. Ludwigshafen) or nifedipine (Bayer) during normal ventilation, hypoventilation or hyperventilation were carried out in separate groups of rats which were not used for blood gas analysis. Five min after artificial ventilation, graded doses of verapamil 20, 40, 80, 160 and 320 $\mu g\,kg^{-1}$, or nifedipine 7.8, 15.6, 31.3, 62.5 and 125 $\mu g\,kg^{-1}$, at a fixed volume of 1 $ml\,kg^{-1}$ were given i.v. at 5 min intervals. The time for i.v. injection was fixed at 10 s intervals.

Drugs were prepared freshly before use. Verapamil was dissolved in 0.9% w/v NaCl solution (saline), and nifedipine in a specially prepared vehicle which was made up of ethanol 19.2%, polyethylene glycol 400 14.4% and distilled water 66.4%. Special care was taken in handling these drugs, especially nifedipine, to avoid their degradation from exposure to light.

Before the i.v. injection of verapamil or nifedipine was started, equivalent volumes of vehicles were administered as controls by the same route and at the same injection speed, and their effects on blood pressure and pulse rate measured. Preliminary tests showed that i.v. injection of the nifedipine vehicle produced marked, but transient, effects on blood pressure and pulse rate. These vehicle-induced changes were unpredictable on the first injection (1 $ml\,kg^{-1}$), but became stable after the third consecutive dose. Therefore, in the nifedipine tests, the rats received 3 consecutive doses of vehicle i.v. at 5 min intervals before nifedipine injection; the effects of the last dose of vehicle on blood pressure and pulse rate were subtracted from the responses elicited by the subsequent increasing doses of nifedipine so as to obtain the net effects of the drug.

Statistical analysis

The peak values of systolic and diastolic blood pressure and of pulse rate after drug or vehicle injections were recorded. Calculated mean blood pressure responses and pulse rate values were compared with the corresponding values immediately before administration of drug or vehicle. The data obtained from the hypoventilated or hyperventilated groups were compared with those of the normally ventilated group for each drug on a point to point basis by Student's t test.

Results

Effects of artificial ventilation on blood gases

Table 1 shows the effects of artificial ventilation at various rates on arterial blood gases. The values of Po_2, Pco_2 and pH of the arterial blood samples obtained before artificial ventilation (control condition, with the rats breathing room air spontaneously) and at 5 min after normal ventilation at 80 strokes min^{-1} were essentially similar. When compared with these two groups, the animals subjected to hypoventilation at 37 strokes min^{-1} had a significant increase in Pco_2 and decreases in both Po_2 and pH. In contrast, the hyperventilated rats exhibited significantly greater Po_2 and pH values, and a significantly lower Pco_2.

Effects of artificial ventilation on blood pressure and pulse rate

The effects of the various rates of artificial ventilation on blood pressure and pulse rate are shown in Figure 1. In the control group, there were only slight changes in either blood pressure or pulse rate following artificial ventilation at 80 strokes min^{-1}. When the rats were hypoventilated at 37 strokes min^{-1} or hyperventilated at 160 strokes min^{-1}, there were immediate decreases or increases, respectively, in both the blood pressure and pulse rate. The changes reached their peaks by 1 min and then started to recover. In the hypoventilated group, they did not return to normal levels. In animals subjected to hyperventilation, both parameters continued to increase above normal level and reached a plateau by 4–5 min. When compared with the control group, the decreases in blood pressure and pulse rate of the hypoventilated rats and the increases in these parameters of the hyperventilated group were statistically significant at 5 min.

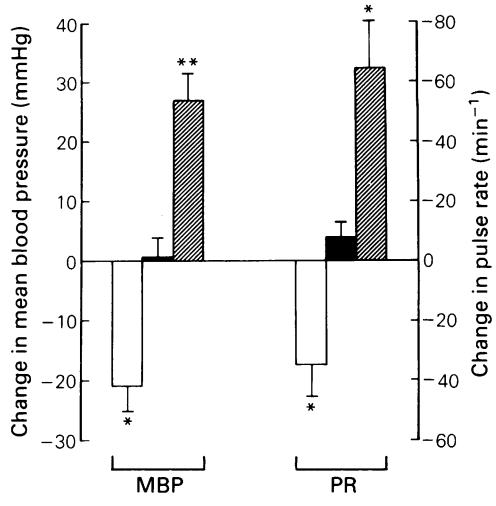

Figure 1 Blood pressure and pulse rate responses to artificial ventilation at fixed volume (10 $ml\,kg^{-1}$) and various ventilation rates. MBP = mean blood pressure; PR = pulse rate. Solid columns, control (normal ventilation, 80 strokes min^{-1}); open columns, hypoventilation (37 strokes min^{-1}); hatched columns, hyperventilation (160 strokes min^{-1}). $n = 10$ for each group. The values plotted are the means and bars show s.e.mean. * $P < 0.01$, ** $P < 0.001$ when compared with the corresponding values of the control group.

Responses to verapamil

Injections of verapamil i.v. produced a dose-dependent reduction in mean blood pressure in all groups (Figure 2). However, the changes were markedly more intense in hyperventilated rats. When compared with the control group, sig-

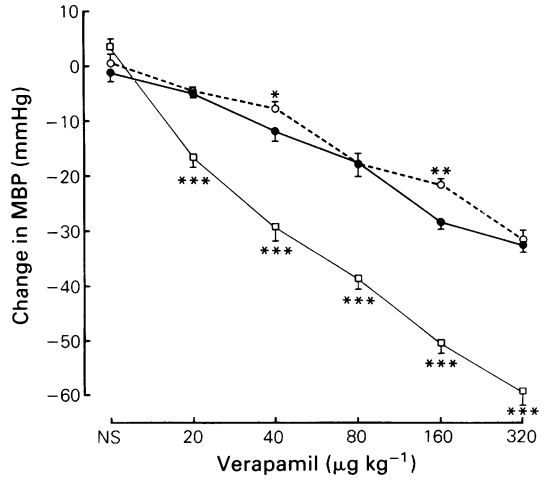

Figure 2 Mean blood pressure (MBP) responses to graded doses of verapamil in control (normally ventilated) (●); hypoventilated (○); and hyperventilated (□) rats. $n = 8$ for each group. The values plotted are the means and vertical lines show s.e.mean. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ when compared with the corresponding control values.

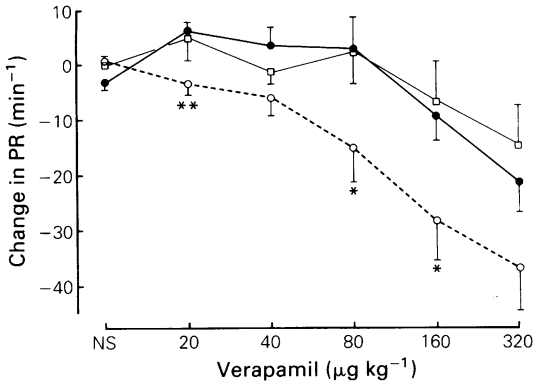

Figure 3 Pulse rate (PR) responses to graded doses of verapamil in control (normally ventilated) (●); hypoventilated (○) and hyperventilated (□) rats. $n = 8$ for each group. The values plotted are the means and vertical lines show s.e.mean. * $P < 0.05$; ** $P < 0.001$ when compared with the corresponding control values.

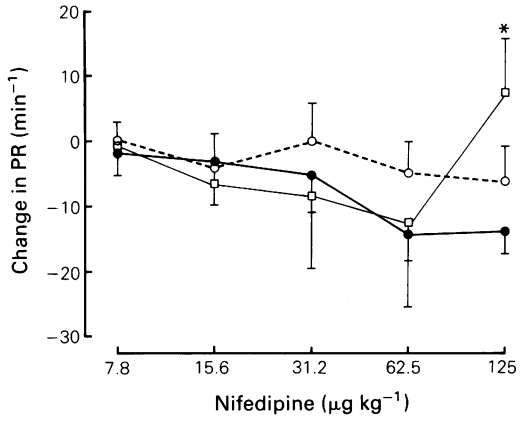

Figure 5 Pulse rate (PR) responses to graded doses of nifedipine in control (normally ventilated) (●); hypoventilated (○); and hyperventilated (□) rats. $n = 8$ for each group. The values plotted are the means and vertical line show s.e.mean. * $P < 0.05$ when compared with the corresponding control values.

nificantly greater falls were observed at all doses. In contrast, the hypotensive responses of the hypoventilated animals to verapamil appeared to be less intense than those of the control group, with statistically significant differences at doses of 40 and 160 $\mu g\,kg^{-1}$.

In both the control and hyperventilated groups, i.v. injections of verapamil, in the dose range used, caused little change in the pulse rate (Figure 3). On the other hand, these doses of verapamil produced a dose-dependent decrease in pulse rate in hypoventilated rats. These changes in pulse rate were significantly different from those of the control group at doses of 20, 80 and 160 $\mu g\,kg^{-1}$.

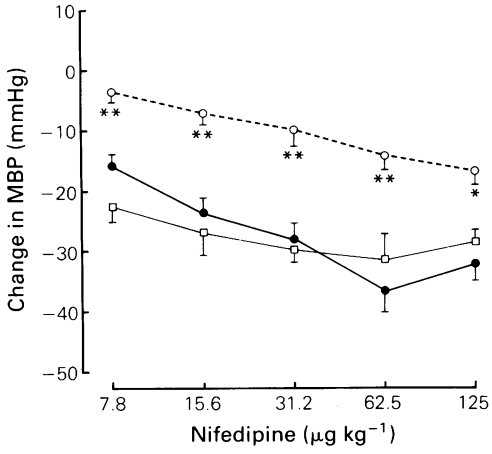

Figure 4 Mean blood pressure (MBP) responses to graded doses of nifedipine in control (normally ventilated) (●); hypoventilated (○); and hyperventilated (□) rats. $n = 8$ for each group. The values plotted are the means and vertical lines show s.e.mean. * $P < 0.01$, ** $P < 0.001$ when compared with the corresponding control values.

Responses to nifedipine

There were dose-dependent decreases in mean blood pressure in all groups in response to i.v. injections of nifedipine (Figure 4). However, the reactions of the hypoventilated rats were less intense than those of the control. They exhibited a significantly reduced blood pressure decrease at all doses of nifedipine tested. The blood pressure responses of the hyperventilated animals to nifedipine were essentially similar to those of the control at all doses tested.

Intravenous injections of nifedipine, in the dose range used, induced only a slight decrease in pulse rate. The changes in all the groups were essentially similar, except for the hyperventilated group where the highest dose (125 $\mu g\,kg^{-1}$) of the drug induced a significant increase in pulse rate (Figure 5).

Discussion

As shown in Table 1, the values of arterial blood gases of rats subjected to artificial ventilation with room air at a stroke volume of 10 ml kg^{-1} and at 80 strokes min^{-1} were essentially similar to those of the animals which were allowed to breathe spontaneously. The blood pressure and pulse rate changes at the start of artificial ventilation were also minimal (Figure 1). Therefore, it is reasonable to consider artificial ventilation with room air at 10 ml kg^{-1} and at 80 strokes min^{-1} as normal ventilation and to use this group of rats as controls in the present study. This study also showed that, by artificial ventilation with room air at a stroke volume of 10 ml kg^{-1}, a combination of hypoxaemia, hypercarbia and acidaemia or of hyperoxaemia, hypocarbia and alkalaemia could be induced by changing the ventilator rate to 37 or 160 strokes min^{-1}, respectively. The technique of varying ventilator rate was,

Table 1 The effects of various rates of artificial ventilation on arterial blood gases

Experimental condition	*Number of rats*	*Arterial blood gases* Po_2 (mmHg)	Pco_2 (mmHg)	*pH*
Control	30	93.77 ± 2.25	33.66 ± 1.30	7.48 ± 0.01
Normal ventilation	10	94.13 ± 2.33	32.78 ± 1.63	7.49 ± 0.01
Hypoventilation	10	78.00 ± 2.91*†	50.09 ± 2.27*†	7.37 ± 0.00*†
Hyperventilation	10	119.91 ± 2.52*†	23.05 ± 1.41*†	7.63 ± 0.02*†

The values are the means ± s.e.mean.
* $P < 0.001$ when compared with the corresponding values in the control period when artificial ventilation had not yet started.
† $P < 0.001$ when compared with the corresponding values in the normal ventilation group.

therefore, employed in subsequent experiments for inducing blood gas abnormalities.

Hyperventilation or hypoventilation was found to cause significant increases or decreases, respectively, in the blood pressure and pulse rate. This phenomenon is not at variance with the effects of blood gases, pH or ventilator stroke volumes on cardiovascular parameters found by others (Nahas & Cavert, 1957, Grant *et al.*, 1985; Phillips *et al.*, 1985; MacLean & Hiley, 1988). The changes in pulse rate may result from a reflex increase or decrease in activities of the vagus nerve, the pulmonary stretch mechanoreceptors or cardio-inhibitory centre, or a combination of several of these factors (Daly & Scott, 1958; Evans & Evans, 1968). It has been shown that acidosis or hypercapnia causes arteriolar dilatation while mild alkalosis causes constriction (Guyton, 1981). These factors could explain the observed blood pressure decrease or increase in the hypoventilated or hyperventilated groups, respectively. Although the mechanical effect of artificial respiration may theoretically affect venous return to the heart and subsequently blood pressure, Maloney & Handford (1954) and Smith (1965) have demonstrated that with competent cardiopulmonary systems, experimental animals tolerate artificial ventilation without major alterations in blood pressure or cardiac output. It can, therefore, be reasonably assumed that the blood pressure and pulse rate changes seen in the various experimental groups in the present study are secondary to blood gas or pH changes, but not to the mechanical effect of artificial ventilation.

Numerous studies have shown that alterations of pH or the partial pressure of O_2 or CO_2 in blood or physiological solution can change the cardiovascular dynamics (Korner & White, 1966; Ng *et al.*, 1967; Krasney & Koehler, 1977), impair the synthesis and release of neurotransmitters (Gibson & Peterson, 1982) and modify the reactions of some organs or tissues to certain drugs (Detar & Bohr, 1968; Bowman & McGrath, 1982; Dai, 1982; Ebeigbe, 1982; Dai & Wong, 1985). Working on the newborn lamb, Phillips *et al.* (1985) found that nifedipine reduced only systemic, but not pulmonary pressure during normoxia. In the presence of hypoxia, however, nifedipine decreased both systemic and pulmonary pressures. These findings led to the suggestion that cardiovascular responses to nifedipine and probably also to other calcium channel blockers, may be affected by the presence of blood gas abnormalities. This is indeed confirmed by the results of the present study. It was found that verapamil caused a significantly greater fall in blood pressure in hyperventilated rats, whereas nifedipine produced significantly smaller decreases in blood pressure in hypoventilated animals. This indicates that the sensitivity of the animals to the hypotensive effects of calcium channel blockers was enhanced or depressed by hyperventilation or hypoventilation, respectively.

Several possibilities can be considered in an attempt to explain the mechanism of these findings. Dissociation of these drugs in blood and tissues, and consequently their bioavailabilities, may be influenced by the presence of abnormal blood or tissue pH. Verapamil is known to have a pKa value of 8.75 (Hasegawa *et al.*, 1984) and nifedipine is claimed to behave as a neutral molecule (information supplied by Bayer). It is unlikely, therefore, that acidaemia or alkalaemia could significantly affect the ionisation and bioavailability of these drugs.

Force generation in cardiovascular smooth muscle is dependent on the movement of external calcium into the intracellular space, a process that initiates the further release of sarcoplasmic reticulum-stored calcium into the cytosol (Fabiato & Fabiato 1979) and the binding of the cytosolic calcium to the contractile proteins, thereby eliciting a complex process that leads to contraction. Systolic cytosolic calcium concentration ($[Ca^{2+}]_i$) is usually far from saturating the contractile proteins (Fabiato, 1981), the physiological and pharmacological controls of contractility can, therefore, be exerted by changing the size of the $[Ca^{2+}]_i$ transients. An increase in $[Ca^{2+}]_i$ transients during acidosis has been demonstrated (Allen & Orchard, 1983). It is, therefore, possible that the increased or decreased sensitivities to verapamil or nifedipine during hyperventilation or hypoventilation, respectively, as observed in the present study, may be due to up- or down-regulation of one or more of these steps leading to cellular contraction.

Another possibility is the influence of adrenergic activity. Hypercarbia and acidosis stimulate the release of adrenal catecholamines (Morris & Miller, 1962; Nahas *et al.*, 1967) and this may attenuate the sensitivity of the cardiovascular system to the hypotensive effect of calcium channel blockers. Furthermore, several workers have suggested that the hypotensive effects of the calcium antagonists may be partly attributed to the inhibition of adrenergic vasoconstriction (De Mey & Vanhoutte, 1981; Van Meel *et al.*, 1981; Cavero *et al.*, 1983). As adrenoceptor activation can be influenced by acid-base imbalance (McGrath *et al.*, 1982), it is reasonable to consider that the hypotensive effects of calcium channel blockers may be modified through this mechanism. However, results of the present investigation do not permit any speculation; further work is required.

Nifedipine exerts minimal effect on the pulse rate-determining conducting tissues of the heart, while verapamil has greater actions on these tissues (Rowland *et al.*, 1979). It is not surprising, therefore, that in the present study dose-dependent decreases in pulse rate were observed in response to verapamil, but the changes induced by nifedipine were minimal. However, the hypoventilated rats exhibited significantly greater decreases in pulse rate in response to high doses of verapamil. This may be due to an increased sensitivity of the heart to verapamil during acidosis as described by Smith & Briscoe (1985), or an impairment of the compensatory cardiovascular reflex in hypoxia as suggested by Dai & Wong (1985) and Dai *et al.* (1986). Nevertheless, further work is required to throw more light on the possible mechanisms described.

The current study also shows that the cardiovascular responses to verapamil and nifedipine were altered by blood gas abnormalities in a dissimilar manner. Hyperventilation significantly enhanced the hypotensive effects of verapamil, but not nifedipine. In contrast, hypoventilation alleviated the hypotensive effects of nifedipine but produced significantly greater decreases in pulse rates in response to increasing doses of verapamil. It is unclear at this stage whether blood gas abnormalities influence all calcium channel blockers in the same way or whether they specifically affect only certain compounds. Further studies are required.

In conclusion, the results of the present study show that the blood pressure and pulse rate responses to verapamil and nifedipine are altered in the presence of blood gas abnormalities. The mechanisms are not clear. It is suggested that in clinical conditions involving blood gas changes, the possibility of altered response patterns to calcium channel blockers may be worth investigating.

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Differences in regional vascular sensitivity to endothelin-1 between spontaneously hypertensive and normotensive Wistar-Kyoto rats

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1 The systemic and regional haemodynamic effects of porcine endothelin-1 (endothelin) have been measured in anaesthetized spontaneously hypertensive (SH) rats rendered areflexic by ganglion blockade; comparisons were made with age-matched Wistar-Kyoto (WKY) control animals.

2 In both SH and WKY rats endothelin (0.1–1 nmol kg^{-1} i.v.) elicited an initial, short-lived (< 2 min), fall in blood pressure which was associated with substantial increases in hindquarter and carotid vascular conductances. Both the blood pressure falls and the peripheral vasodilator responses were greater in SH than in WKY rats.

3 The initial depressor effects of endothelin were followed by marked and sustained increases in blood pressure associated with constriction in carotid, hindquarter, renal and mesenteric vascular beds. Vasoconstrictor responses were quantitatively similar in the two rat strains.

4 Pretreatment with indomethacin (5 mg kg^{-1} i.p. or i.v.) did not alter the responses to endothelin, 1 nmol kg^{-1}, in SH rats.

5 The regional haemodynamic effects of intravenously administered acetylcholine (0.01–1 $\mu g\,kg^{-1}$), nitroprusside (0.3–10 $\mu g\,kg^{-1}$) and angiotensin II (0.01–0.1 $\mu g\,kg^{-1}$) were similar in SH and WKY rats.

6 Endothelin (10^{-10}–3×10^{-8} M) contracted aortic rings from both SH rats and WKY control animals. Removal of the endothelium enhanced significantly the sensitivity of tissues from both WKY and SH rats to endothelin; the increase in sensitivity was greater in tissues from SH than WKY rats.

7 The results demonstrate qualitative similarities in the complex haemodynamic effects of endothelin in SH rats and WKY control animals. However, the SH rats display substantially greater vasodilator responses to endothelin than WKY. Eicosanoid generation is not the mechanism of the vasodilator action of endothelin in SH rats under the conditions of our experiments.

Introduction

Porcine endothelin-1 (endothelin) elicits complex cardiovascular effects in a number of species (Lippton *et al.*, 1988; Wright & Fozard, 1988; Yanagisawa *et al.*, 1988; Cocks *et al.*, 1989; Gardiner *et al.*, 1989a; Given *et al.*, 1989; Han *et al.*, 1989; Kitayoshi *et al.*, 1989; López-Farré *et al.*, 1989; Minkes & Kadowitz, 1989; Minkes *et al.*, 1989; Tsuchiya *et al.*, 1989; Winquist *et al.*, 1989a). In anaesthetized, ganglion-blocked, spontaneously hypertensive (SH) rats, for example, both vasoconstriction and vasodilatation are seen in vascular beds such as the hindquarter or carotid, whereas other regions, including renal and mesenteric, respond to endothelin solely with vasoconstriction (Wright & Fozard, 1988). The complexity of action of endothelin, its high potency and generally long duration of action has prompted speculation of a role for this peptide in the aetiology and/or maintenance of hypertension (Tomobe *et al.*, 1988; Yanagisawa *et al.*, 1988; Le Monnier de Couville *et al.*, 1989; Yanagisawa & Masaki, 1989).

As a preliminary step towards understanding the putative role of endothelin in experimental hypertension, we have compared the regional haemodynamic effects of endothelin in SH rats and normotensive Wistar-Kyoto (WKY) control animals. *In vitro* experiments were included in an effort to clarify the mechanism of the substantially greater regional vasodilator responses evident with endothelin in the SH rat.

A preliminary account of these findings has been presented to the British Pharmacological Society (Wright & Fozard, 1989).

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In vivo *experiments: animals and operations*

Male SH rats (333 ± 7 g, $n = 16$) and male WKY rats (380 ± 6 g, $n = 16$), both strains about 20 weeks old, were used in this study. Rats were anaesthetized with Inactin 120 mg kg^{-1} i.p. A tracheotomy was performed and catheters inserted into the right carotid artery for mean arterial pressure (MAP) measurement and in the jugular vein for drug administration. Pulsed Doppler flowprobes (Haywood *et al.*, 1981) were placed around the left carotid artery and, through a midline abdominal incision, around the left renal artery, the superior mesenteric artery and the lower abdominal aorta just above the iliac bifurcation (equated to hindquarter blood flow). The incisions were closed and parameters allowed to stabilize over one hour. Checks were made at regular intervals to validate the flow recordings by making abrupt range shifts during peak vasoconstrictor or vasodilator responses; the procedure did not result in any indication of spurious Doppler shift signals.

In vivo *experiments:*

Following the stabilization period, the rats were ganglion-blocked with mecamylamine (0.25 mg kg^{-1} infused over 15 min). Acetylcholine (0.01–1 $\mu g\,kg^{-1}$), nitroprusside (0.3–10 $\mu g\,kg^{-1}$) and angiotensin II (0.01–0.1 $\mu g\,kg^{-1}$) were then given sequentially by i.v. bolus in 0.1 ml volume. Following these agonists, i.v. bolus injections of endothelin 0.1, 0.3 and 1 nmol kg^{-1} were administered with recovery periods of 20 min between the first two doses and 80 min after the highest dose.

In vitro *experiments*

Thoracic aortae were removed from male SH rats or WKY control animals of similar weights to those used in the *in vivo* studies. Ring segments (3–4 mm long) were cut and the endothelium removed from alternate sections by gentle passage of a wooden spill through the lumen. Paired adjacent rings, with and without endothelium, were set up in Krebs solution, bubbled with carbogen, at 37°C for isometric recording of tension; a resting tension of 1 g was applied. After an equilibration period of 1 h, during which the tissues were repeatedly washed and the baseline tension readjusted to 1 g, contraction of the tissues was elicited with a supra-maximal concentration of phenylephrine (10^{-5} M). After washing to baseline and readjustment of baseline tension, a second response to phenylephrine was established. Thirty minutes after again washing to baseline, a cumulative concentration-response curve to endothelin (10^{-10}–3×10^{-8} M) was established. Twenty minutes after addition of the final concentration of endothelin, and without washing the tissue, a cumulative concentration-response curve to acetylcholine (10^{-8}–10^{-5} M) was established. The protocol is illustrated in the upper part of Figure 6. Responses to endothelin and acetylcholine were expressed in terms of the maximum response to the second response to phenylephrine.

Drugs

Porcine endothelin-1 (Peptide Institute Inc., Osaka, Japan) was dissolved in acetic acid (10 mM) to 10^{-2} M and stored in aliquots at −20°C. Other drugs were freshly prepared each day and included, acetylcholine bromide (Sigma), angiotensin II amide (Hypertensin, Ciba-Geigy), mecamylamine hydrochloride (Merck Sharp and Dohme), phenylephrine hydrochloride (Serva) and sodium nitroprusside (Sigma). Drug doses *in vivo* were prepared as their salts and in 0.9% w/v NaCl solution. *In vitro* concentrations refer to the base forms of the drugs.

Statistics

Cardiovascular variables were compared between SH and WKY rats by unpaired *t* test. Average s.e.mean within animals for a cardiovascular variable was calculated from two-way analysis of variance as (error mean square/number of animals)$^{0.5}$ after the sums of squares between animals and between times had been subtracted from the total sums of squares for each endothelin dose. This error bar (±1 average s.e.mean) is located on the average dose-response line for each variable in Figures 2–5 (Wright *et al.*, 1987). Data from *in vitro* experiments were analysed statistically by paired *t* test. In all instances *P* values less than 0.05 were taken as significant.

Results

Resting circulatory variables

Resting circulatory variables before and during ganglion blockade in anaesthetized SH and WKY rats are shown in Table 1. Autonomic blockade resulted in falls in mean arterial pressure (MAP) in both rat strains. However, the mean value from SH rats was still about 25% higher than that of WKY controls. Also, vascular conductances in both mesenteric and hindquarter beds were approximately 40% less in SH rats compared with WKY rats following mecamylamine. Renal and carotid vascular conductances were not significantly different between groups (Table 1).

Effects of vehicle

Each dose of endothelin was injected in a constant volume of 0.1 ml. This volume of vehicle produced small (<5 mmHg), brief (<20 s) increases in blood pressure often associated with brief (<20 s) falls in heart rate of up to 30 beats min^{-1}; the latter were also seen regularly following each dose of endothelin (Figures 1–4). Peripheral flow changes induced by 0.1 ml vehicle were small and short-lived.

Effects of endothelin

Changes in blood pressure The initial effects of endothelin (0.1–1 nmol kg^{-1}) were significant falls in blood pressure in both SH and WKY rats, illustrated for the SH rat in Figure 1; the mean changes (mmHg) with each dose in SH and WKY rats respectively were: 0.1 nmol kg^{-1}, −20 ± 3 and −19 ± 2; 0.3 nmol kg^{-1}, −24 ± 2 and −18 ± 2; and 1 nmol kg^{-1}, −33 ± 5 and −24 ± 2 (Figures 2–4). These initial falls in MAP were significantly larger in SH than in WKY rats at the two highest doses.

With the highest endothelin dose, 1 nmol kg^{-1}, the initial depressor effect was followed by an increase in blood pressure which reached a maximum about 5 min after bolus administration (Figure 4). This increase in blood pressure was significantly larger in WKY compared to SH rats; mean changes were 34.9 ± 3.3 and 25.2 ± 3.7 mmHg in WKY and SH rats, respectively. Correspondingly there was no significant difference between absolute values at that time, 122.4 ± 4.3 and 132.3 ± 6.0 mmHg in WKY and SH rats respectively. This secondary pressor effect of endothelin was very long-lasting in both rat strains with values returning to resting baseline only after approximately 60 min.

Changes in regional vascular conductances Concomitant with the initial depressor effect elicited by endothelin were large increases in both hindquarter and carotid vascular conductances (Figure 1). This vasodilatation was dose-related in SH

Table 1 Circulatory variables before and after ganglion blockade in SH and WKY rats

	WKY (n = 16)		*SH rats* (n = 16)	
Variable	*Before*	*After*	*Before*	*After*
MAP (mmHg)	99 ± 2	85 ± 2	136 ± 6*	108 ± 5*
Heart rate (beats min^{-1})	296 ± 6	296 ± 9	313 ± 6*	292 ± 8
Renal Q (kHz)	2.4 ± 0.3	2.5 ± 0.4	2.1 ± 0.3	2.4 ± 0.4
Mesenteric Q (kHz)	4.1 ± 0.6	3.3 ± 0.4	2.6 ± 0.3*	2.7 ± 0.3
Carotid Q (kHz)	0.5 ± 0.1	0.5 ± 0.1	0.3 ± 0.04	0.4 ± 0.05
Hindquarter Q (kHz)	2.3 ± 0.2	2.4 ± 0.2	1.2 ± 0.1*	1.4 ± 0.1*
RVC (units[a])	2.59 ± 0.32	3.01 ± 0.48	1.57 ± 0.19*	2.38 ± 0.39
MVC (units)	4.08 ± 0.55	3.99 ± 0.44	1.96 ± 0.22*	2.46 ± 0.30*
CVC (units)	0.48 ± 0.08	0.60 ± 0.11	0.26 ± 0.04*	0.44 ± 0.07
HVC (units)	2.38 ± 0.18	2.81 ± 0.21	0.91 ± 0.12*	1.31 ± 0.13*

Values are means ± s.e.mean. Ganglion blockade was produced by the administration of mecamylamine 0.25 mg kg^{-1} i.v.

[a] VC units are 100 [kHz mmHg^{-1}].

* Significantly different from corresponding WKY value, $P < 0.05$ unpaired *t* test. Q = mean blood flow; RVC, MVC, CVC and HVC correspond to renal, mesenteric, carotid and hindquarter vascular conductance, respectively.

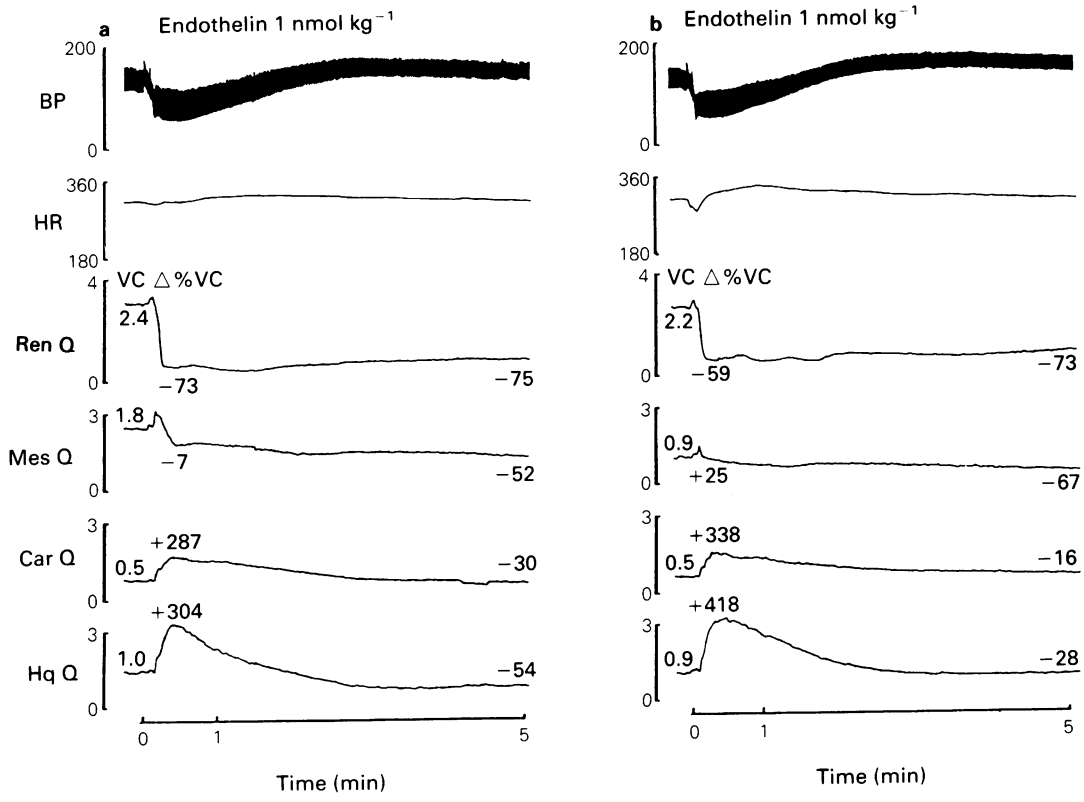

Figure 1 Representative chart records of circulatory changes following administration of endothelin 1 nmol kg^{-1} (i.v. bolus) to anaesthetized, ganglion-blocked SH rats. (a) Vehicle-treated SH rat; (b) SH rat treated with indomethacin 5 mg kg^{-1} (i.p.) 60 min before injection of endothelin. Variables recorded were: blood pressure (BP, mmHg); heart rate (HR, beats min^{-1}); mean renal blood flow (Ren Q, kHz); mean mesenteric blood flow (Mes Q, kHz); mean carotid blood flow (Car Q, kHz) and mean hindquarter blood flow (Hq Q, kHz). Indicated on the records are baseline values for regional vascular conductances (VC, units) calculated as 100 [Q/BP], and Δ% changes in VC at selected times after endothelin administration.

but not in WKY rats at the 0.1 and 0.3 nmol kg^{-1} doses. The increase in hindquarter vascular conductance in both rat strains was similar with 0.1 nmol kg^{-1} endothelin. However, high doses resulted in 30–40% greater increases in hindquarter conductance in SH compared to WKY rats (Figures 2–4). In the carotid vascular bed, conductance increases in SH rats were significantly greater by 200–300% than those in WKY rats at all three endothelin doses. Within 5 min of endothelin administration at 1 nmol kg^{-1}, hindquarter and carotid vascular responses reverted to marked vasoconstriction in both SH and WKY rats corresponding temporally with the secondary increase in blood pressure. The average changes in conductance at 5 min in SH and WKY rats respectively were -37.1 ± 6.5 and -25.4 ± 10.7% in the carotid bed, and -38.0 ± 5.2 and -44.7 ± 4.1% in the hindquarter vascular bed.

In the renal vascular bed there was no significant change in conductance following 0.1 nmol kg^{-1} endothelin in either rat

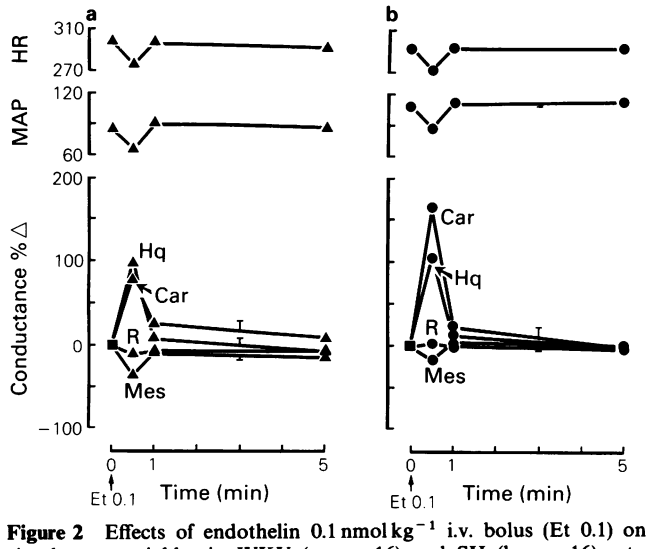

Figure 2 Effects of endothelin 0.1 nmol kg^{-1} i.v. bolus (Et 0.1) on circulatory variables in WKY (a, $n = 16$) and SH (b, $n = 16$) rats. Variables are heart rate (HR, beats min^{-1}), mean arterial blood pressure (MAP, mmHg), and % change in vascular conductance in hindquarter (Hq), carotid (Car), renal (R) and mesenteric (Mes) beds. The abscissa scale represents time (min) after injection of endothelin. Error bars on lines are average s.e.mean (see Methods).

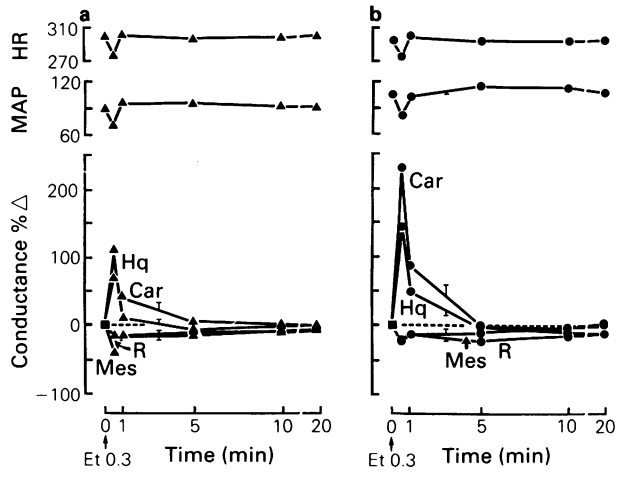

Figure 3 Effects of endothelin 0.3 nmol kg^{-1} bolus (Et 0.3) on circulatory variables in WKY (a, $n = 16$) and SH (b, $n = 16$) rats. For key to variables see legend to Figure 2.

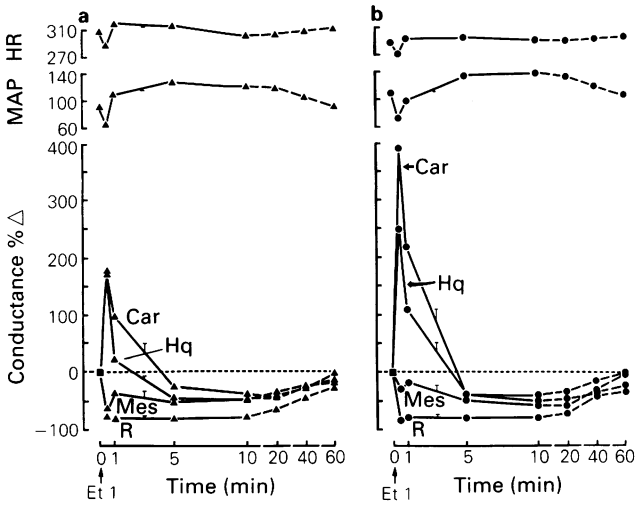

Figure 4 Effects of endothelin 1 nmol kg^{-1} i.v. bolus (Et 1) on circulatory variables in WKY (a, $n = 16$) and SH (b, $n = 16$) rats. For key to variables see legend to Figure 2.

strain. With 0.3 nmol kg^{-1} of the peptide, renal vascular conductance fell within 30 s by 19.5 ± 6.7 and 15.0 ± 9.0% in SH and WKY rats, respectively. The highest dose of endothelin, 1 nmol kg^{-1}, resulted in initial falls in renal vascular conductance of 82.3 ± 4.6 and 75.9 ± 4.7% in SH and WKY rats, respectively. This renal vasoconstriction was maintained for more than 20 min in both rat strains, with conductance values gradually returning to resting baseline over 60–80 min (Figure 4).

Mesenteric vascular conductance fell significantly with endothelin 0.1–1 nmol kg^{-1} in both SH and WKY rats. Mean values in SH and WKY rats respectively were: 0.1 nmol kg^{-1}, −16.9 ± 9.5 and −35.8 ± 5.8; 0.3 nmol kg^{-1}, −24.1 ± 10.0 and −41.5 ± 7.4; and 1 nmol kg^{-1}, −26.1 ± 16.7 and −65.9 ± 6.8%. The vasoconstriction at the latter dose was significantly greater in the WKY rats. Similar to the renal vascular response, the mesenteric vasoconstriction was of long

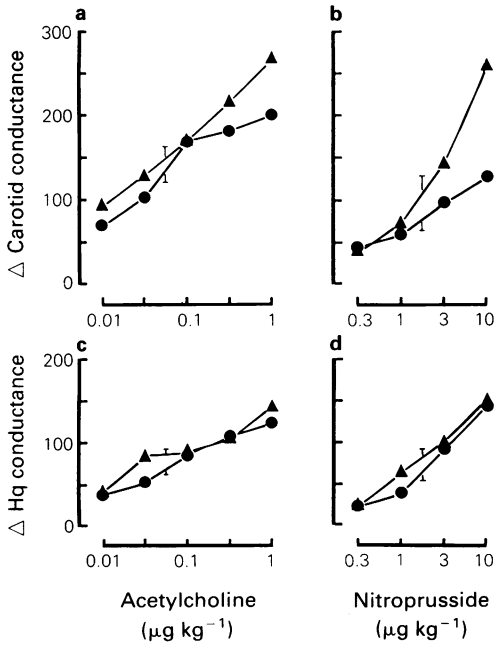

Figure 5 Effects of acetylcholine (a,c) and nitroprusside (b,d) i.v. bolus injections on carotid (a,b) and hindquarter (Hq) (c,d) vascular conductances in SH (▲, $n = 8$) and WKY rats (●, $n = 8$). Error bars are average s.e.mean (see Methods).

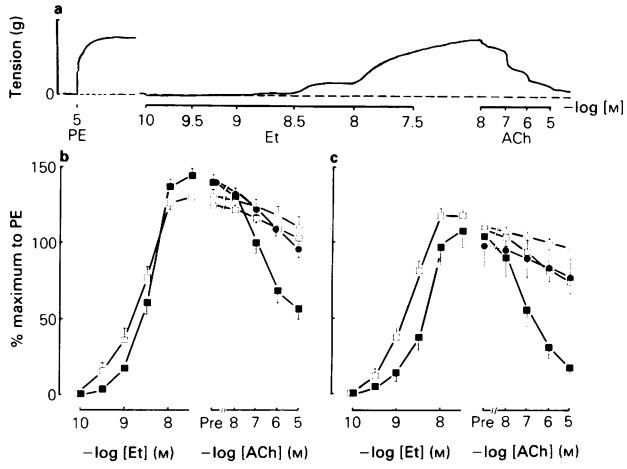

Figure 6 Contractile effects of endothelin (Et) on aortae taken from SH rats and WKY control animals; effects of endothelial removal. (a) Sample tracing (data from SH rat) to illustrate the experimental design. A cumulative dose-response curve to endothelin was established and the results expressed in terms of a supramaximal response to a prior injection of phenylephrine (PE). Twenty minutes after addition of the highest dose of endothelin (3 × 10^{-8} M), a cumulative dose-response curve to acetylcholine (ACh) or the vehicle for ACh (saline; not illustrated) was established. (b and c) Mean data from a series of such experiments comparing tissues from WKY (b) and SH (c) rats. (■) Intact aortae; (□) aortae denuded of endothelium; (●) and (○) indicate effects of the vehicle for acetylcholine (saline). $n = 10$ for endothelin concentration-response curves; 6 for acetylcholine concentration-response curves and 4 for effects of saline. The mean responses (mg; in each case, $n = 10$) to phenylephrine, 10^{-5} M, were: WKY, intact aorta 2826 ± 175; WKY, aorta denuded of endothelium 2924 ± 163; SHR, intact aorta 2084 ± 147; SHR, aorta denuded of endothelium 2333 ± 169.

duration and returned to pre-endothelin values only after approximately 60 min (Figure 4).

Effects of acetylcholine and sodium nitroprusside

Bolus injections of acetylcholine (0.01–1 μg kg^{-1}, endothelium-dependent vasodilator) or nitroprusside (0.3–10 μg kg^{-1}, endothelium-independent vasodilator) revealed no marked differences between SH and WKY rats with respect to the vasodilatation in the hindquarter bed (Figure 5). Similarly, in the carotid vascular bed there was no significant difference between rat strains in the acetylcholine-induced dilatation. However, with nitroprusside, the highest dose elicited a greater increase in carotid vascular conductance in SH than in WKY rats. The effects of both dilator agents on blood pressure were similar showing no difference between rat groups. Acetylcholine 1 μg kg^{-1} caused changes of −33.4 ± 2.9 and −26.0 ± 3.6 mmHg, and nitroprusside 10 μg kg^{-1} elicited falls of −34.6 ± 2.8 and −30.7 ± 2.1 mmHg in SH and WKY rats respectively.

Effects of angiotensin II

Angiotensin II (0.01–0.1 μg kg^{-1}) administration resulted in greater increases in blood pressure in SH compared with WKY rats. Average increases in SH and WKY rats respectively were: 0.01 μg kg^{-1}, 21 ± 2 and 13 ± 1; 0.03 μg kg^{-1}, 34 ± 3 and 20 ± 2; and 0.1 μg kg^{-1}, 53 ± 4 and 36 ± 3 mmHg. However, there were no significant differences between rat strains in the decreases in renal and mesenteric vascular conductances. For instance, decreases in renal vascular conductance with angiotensin II injections ranged from −20 ± 4 with the lowest dose to −79 ± 7% with the highest dose in SH corresponding with values in WKY rats of −27 ± 5 and −73 ± 8%.

Effects of indomethacin

In a number of additional SH rats, prepared identically to the main group, indomethacin 5 mg kg^{-1} was administered either i.p. 60 min before endothelin injection ($n = 5$), or as an i.v. bolus 10 min before endothelin 1 nmol kg^{-1} ($n = 1$). Neither route of indomethacin administration appeared to have any effect on the biphasic response to endothelin (1 nmol kg^{-1}) in these animals (Figure 1).

Sensitivity of aortae with and without endothelium to endothelin: comparison between SH rats and WKY controls

Endothelin, 10^{-10}–3×10^{-8} M, induced slowly developing and sustained contraction of aortic rings with or without intact endothelium from both SH rats and WKY control animals (Figure 6). Removal of the endothelium from rings taken from WKY rats enhanced to a small extent, but significantly ($P < 0.05$), the sensitivity to endothelin, as indicated by pEC_{50} (M) values of 8.47 ± 0.05 and 8.71 ± 0.10 (in both cases $n = 10$) for the rings with and without endothelium, respectively (Figure 6b). Rings from SH rats with intact endothelium were also significantly ($P < 0.005$) less responsive to endothelin than denuded tissues, as indicated by pEC_{50} (M) values of 8.42 ± 0.07 and 8.82 ± 0.09 respectively (in both cases $n = 10$; Figure 6c).

Acetylcholine did not relax tissues contracted with endothelin in the absence of endothelium; in intact tissues, acetylcholine relaxed tissues from both SH rats and WKY controls (Figure 6b and c). The pIC_{50} concentrations (M; calculated taking the response to 10^{-5} M acetylcholine as maximum) were not significantly different (7.16 ± 0.08 and 7.03 ± 0.17 respectively; in each case, $n = 6$) although the maximum inhibition was somewhat greater in the tissues from SH than those from WKY rats (Figure 6b and c).

Discussion

The present results emphasize the complexity of the cardiovascular effects of endothelin which is increasingly being documented in a number of species (see Introduction). However, unlike the majority of studies published to date, the present results were obtained in animals rendered areflexic by administration of the ganglion blocking agent, mecamylamine. This allows interpretation of the effects of endothelin uncomplicated by intact autonomic cardiovascular reflexes. This is particularly important when comparing cardiovascular responses of SH rats with other rat strains because of the alterations in reflex buffering capacity well known to occur in hypertension (see, e.g. Wright *et al.*, 1987).

Under our experimental conditions, the effects of endothelin were qualitatively similar in SH and WKY rats. The initial short-lived falls in blood pressure were associated with similarly short-lived vasodilator responses in both carotid and hindquarter vascular beds and it seems likely that the latter are the basis of the former. The poor correlation between blood pressure fall and the conductance changes in the carotid and hindquarters vascular beds almost certainly reflects, to a large extent, the complexity of action of endothelin and, in particular, the differential contribution of the vasoconstrictor component at the different dose levels. However, since information on conductance changes is available for only four vascular beds, precise explanation of the poor correlation is not possible. The lack of any associated tachycardia (clearly seen in SH rats with normal reflexes—Winquist *et al.*, 1989a) testifies to the adequacy of ganglion blockade and rules out the possibility that the vasodilatation results from modification of on-going autonomic tone and, in particular, the prejunctional inhibition of sympathetic neurotransmitter release (Wiklund *et al.*, 1988).

The sustained rise in blood pressure which superseded the initial fall was associated with vasoconstriction, particularly in the renal and mesenteric vasculature but also, once the vasodilatation had waned, in the carotid and hindquarters beds. The intense, long-lasting renal vasoconstriction at the higher doses is particularly noteworthy. The same phenomenon has been observed in several species (Cocks *et al.*, 1989; Minkes & Kadowitz, 1989) and *in vitro* (Firth *et al.*, 1988; Cairns *et al.*, 1988) and is associated with a marked decline in renal function (Lopez-Farré *et al.*, 1989). On this basis endothelin has been implicated as a mediator in the pathogenesis of acute renal failure (Firth *et al.*, 1988).

Despite qualitative similarities, the effects of endothelin differed quantitatively in SH compared to WKY rats. In particular, both the initial falls in MAP and the associated vasodilator responses of the hindquarter and carotid vascular beds were greater in SH than WKY rats. Although the differences may in part reflect differences in baseline values between the two strains (MAP was significantly higher, hindquarter conductance was significantly lower in SH compared to WKY rats—Table 1), this seems unlikely to be the sole explanation for our observations. Thus, the greatest differences were seen in the carotid vascular bed where baseline values did not differ significantly between the two strains. Moreover, neither the vasodilator responses to an endothelium-dependent vasodilator, acetylcholine, nor those to the directly acting agent, sodium nitroprusside, differed markedly in SH compared to WKY rats (Figure 5). Thus the vasodilator component of the cardiovascular response to endothelin appears to be increased selectively in the spontaneously hypertensive state.

The mechanism of the vasodilator response to endothelin *in vivo* has not been established, but it seems unlikely to reflect a direct action on vascular smooth muscle. Thus, no direct relaxation of arterial segments from a variety of species has ever been demonstrated (Cocks *et al.*, 1989; Eglen *et al.*, 1989; Saito *et al.*, 1989). On the other hand, endothelin has been shown to dilate the rat perfused mesenteric vascular bed when tone was induced by methoxamine (Warner *et al.*, 1989a,b; Randall *et al.*, 1989) and to release the vasodilator eicosanoid, prostacyclin, from rat perfused lungs *in vitro* (de Nucci *et al.*, 1988). In the perfused mesenteric vascular bed, vasodilatation could be inhibited by removal of the endothelium, methylene blue or haemoglobin indicating indirect effects of endothelin mediated by the release of endothelium-derived releasing factor(s), such as EDRF (Warner *et al.*, 1989a,b; Randall *et al.*, 1989). However, other experiments have failed to confirm the vasodilator effects of endothelin in the rat isolated perfused mesenteric arterial bed (Eglen *et al.*, 1989; Tabuchi *et al.*, 1989), an observation which would be consistent with the present data showing endothelin to induce exclusively vasoconstrictor responses in the mesenteric vascular bed *in vivo* (Figures 1–4). Moreover, Gardiner *et al.* (1989b) were not able to block endothelin-induced vasodilatation in conscious normotensive rats by administration of N^G-monomethyl-L-arginine (L-NMMA) at a dose adequate to increase peripheral vascular resistance by suppressing the generation of a major component of EDRF, endothelium-derived nitric oxide (EDNO) (Gardiner *et al.*, 1989c; see also Whittle *et al.*, 1989). Finally, the present data showing the cardiovascular response to endothelin in the SH rat to be unaffected by pretreatment with indomethacin (Figure 1) render it unlikely that prostacyclin is playing a major role in the vasodilator response *in vivo*, at least under the conditions of the present experiment.

Despite this, but in confirmation of the data of Eglen *et al.* (1989) and Godfraind *et al.* (1989), the endothelium does modulate the vasoconstrictor response to endothelin in rat isolated aorta (Figure 6). Intriguingly, in our experiments a somewhat greater increase in vasoconstrictor sensitivity due to endothelium removal was seen in tissues from SH rats (Figure 6), which could conceptually be used to support a greater role for endothelium-mediated vasodilator responses as the basis of the differential effects seen in SH and WKY rats *in vivo*. However, the *in vitro* effect most likely reflects not

the release by endothelin of EDRF but physiological antagonism of endothelin by a basal EDRF-mediated vasodilator tone, since similar effects are manifested against a variety of other vasoconstrictor agents (Martin *et al.*, 1986; Alosachie & Godfraind, 1988).

In seeking alternative explanations for the mechanism of the vasodilator action of endothelin, one obvious possibility is the release of an EDRF other than EDNO (see e.g. Taylor & Weston, 1989). A further possibility is the release of atrial natriuretic peptide (ANP). Such a release has been demonstrated both from cultured neonatal rat atrial cardiocytes (Fukuda *et al*, 1988) and adult rat atria *in vitro*, where release was greater in tissues taken from SH rats than WKY controls (Winquist *et al.*, 1989b). However, unlike endothelin, the fall in blood pressure induced by the acute administration of ANP in the rat is not generally associated with a decrease in systemic vascular resistance (Lappe *et al.*, 1985; Waeber *et al.*, 1986; Gardiner *et al.*, 1990). Clearly the precise mechanism of the vasodilator action of endothelin awaits further elucidation.

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Ryanodine facilitates calcium-dependent release of transmitter at mouse neuromuscular junctions

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1 Quantal release of transmitter was measured intracellularly at mouse neuromuscular junctions in the presence and absence of ryanodine (Rnd).

2 Rnd at concentrations up to 1 μM did not significantly alter the frequency of miniature endplate potentials (m.e.p.ps) in the presence or absence of Ca^{2+}, suggesting that Rnd is unlikely to alter the internal concentration of Ca^{2+} ($[Ca^{2+}]_i$) at rest.

3 In a high-K^+ (10 mM) bathing solution, Rnd further potentiated the facilitatory effect of Ca^{2+} on the frequency (F, s^{-1}) of m.e.p.ps. Rnd shifted the relationship between ln(F) and $\ln[Ca^{2+}]_o$ to lower concentrations.

4 In a high-Mg^{2+} bathing solution, Rnd did not affect the frequency of m.e.p.ps at any value of $[Ca^{2+}]_o$. However, Rnd slightly but significantly increased the quantal content (m) of e.p.ps. It shifted the relationship between ln(m) and $\ln[Ca^{2+}]_o$ to lower concentrations. These results suggest that Rnd potentiates the quantal release of transmitter after depolarization of the membrane or nerve impulse, in keeping with the cooperativity of Ca^{2+} at the active site.

5 A series of two closely spaced nerve impulses produced a facilitation of transmitter release, as judged by the quantal content (m2) of the second response in relation to that of the first one (m1), m2/m1. Rnd did not change the ratio m2/m1. Thus Rnd is unlikely to affect the rapid phase of the sequestration of Ca^{2+} inside the nerve terminal.

6 High levels of K^+ (5 mM) and caffeine (2 mM) potentiated both modes of transmitter release, in a manner dependent on $[Ca^{2+}]_o$. Caffeine did not potentiate facilitation of transmitter release.

7 These results indicate that Rnd facilitates the quantal release of transmitter presumably via an increase in $[Ca^{2+}]_i$ by a manner different from that of high-K^+ or caffeine. The results suggest that Rnd probably affects calcium turnover in neuronal cells.

Introduction

Ryanodine (Rnd) is an alkaloid extracted from the South American plant *Ryania speciosa* (Jenden & Fairhurst, 1969). It is widely accepted that Rnd specifically affects the calcium release mechanism of sarcoplasmic reticulum (SR) in a variety of muscles (Sutko & Willerson, 1980; Sutko *et al.*, 1985; Ito *et al.*, 1986; Su, 1987). Biochemical or electrophysiological studies revealed that this agent locks the Ca^{2+} release channel of SR in an open state (Fleischer *et al.*, 1985; Meissner, 1986; Rousseau *et al.*, 1987), although it at a high concentration might inhibit the opening of the channel under some conditions (Meissner, 1986; Lattanzio *et al.*, 1987). Several groups reported that the action of Rnd was accelerated by the existence of certain levels of Ca^{2+} outside SR and was attenuated by Mg^{2+} (Meissner, 1986; Lattanzio *et al.*, 1987; Hisayama & Takayanagi, 1988).

As proposed in the 'calcium hypothesis' (Katz, 1969), the release of transmitter at the motor nerve terminal is mediated by Ca^{2+} in nerve ending cytoplasm (Katz & Miledi, 1965). A growing body of evidence indicates that the nerve terminal has some sites to store Ca^{2+} (Blaustein *et al.*, 1980; Brinley, 1980). Those sites are predominantly endoplasmic reticulum (ER) and the mitochondria. Although the functional role of the ER is supposed to be similar to that of the SR in muscle cells in some respects (Blaustein *et al.*, 1980; Brinley, 1980), the mechanism by which it regulates intracellular calcium is not so clear as that of the SR. In particular clarification is required as to whether calcium handling by ER modifies the transmitter release from nerves. Recently, Rnd has been demonstrated to inhibit caffeine-induced $[Ca^{2+}]_i$ transients in sympathetic neurones (Thayer *et al.*, 1988). If it has some action on functions of ER in the motor nerve terminals, this agent could be a useful tool for a study of the regulatory mechanism of ER on transmitter release. Thus, we examined the effect of Rnd on the rate of quantal release of transmitter at mouse neuromuscular junctions. The results show that Rnd facilitates the release of transmitter quanta in a manner that depends on $[Ca^{2+}]_o$ and depolarization of the nerve terminals.

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Methods

Experiments were performed on preparations of isolated left hemidiaphragm from male *ddY* mice of 9 to 12 weeks of age. The preparation was pinned to a silicone resin which lined the bottom of a plastic chamber of about 30 ml capacity, and was soaked in Krebs-Ringer solution. The solution was constantly recirculated by means of an O_2 lift system. The circulating solution had the following composition (mM): NaCl 136, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, $NaHCO_3$ 15 and glucose 11. To depolarize the presynaptic endings (Liley, 1956), the preparation was soaked in Krebs-Ringer solution in which 10 mM NaCl was replaced by 10 mM KCl (10 mM K^+). The concentration of Ca^{2+} in this solution varied from nominally 0 (no addition of $CaCl_2$ and EGTA [ethylene glycol-bis(β-aminoethyl ether N,N,N',N'-tetraacetic acid]) to 0.5 millimolar. The preparation was equilibrated in the 10 mM K^+ solution for at least 30 min before addition of any agent. A bathing solution which contained 5 mM Mg^{2+} was also prepared, containing 0.4–0.8 mM in Ca^{2+}, for measurements of endplate potentials (e.p.ps) as well as miniature endplate potentials (m.e.p.ps). A high-Mg^{2+} bathing solution containing hypertonic 5 mM KCl (5 mM K^+) or 2 mM caffeine was also prepared. The bathing solutions were bubbled with a mixture of 95% O_2 and 5%

CO_2 and maintained at pH 7.3 and at 36°C. The temperature of the bath was monitored by a thermister (Shibaura Electric Co, Model MGA-II) and held constant by means of an external water jacket and a thermoregulatory device (Taiyo, Thermominder Mini 80) during each experiment.

Intracellular recordings were made with glass microcapillary electrodes, filled with 3 M KCl, with resistance of 5 to 8 megohms. The electrode was inserted into fibers near endplate regions. The signals were led through a high-impedance, unity-gain preamplifier (Nihon Kohden, Tokyo, MEZ-8201), displayed on an oscilloscope (Nihon Kohden, VC-10) and stored on an FM instrumentation tape recorder (Nihon Kohden, RMG-5204).

M.e.p.ps of 0.1 mV or larger amplitide were counted by a computer (Nihon Kohden, DAB-1100). M.e.p.ps were recorded for successive periods of 1 min after exposure to a given solution; from these data the mean frequency of m.e.p.ps was calculated. E.p.ps were evoked by stimulation of the phrenic nerve with 128 or 256 square pulses of 0.1 ms duration and supramaximal voltage of 1 Hz through a suction electrode in a high-Mg^{2+} bathing solution with low concentrations of Ca^{2+} (0.4 to 0.8 mM). In other experiments, the nerve trunk was electrically conditioned to change neuronal reactivity. A series of two closely spaced nerve stimuli with an interval of 4 ms between them were given 128 or 256 times at intervals of 1.5 s in a 5 mM Mg^{2+} bathing solution that contained 0.6 mM Ca^{2+}, after measurement of m.e.p.ps for about 1 min. This trial resulted in e.p.ps of the first and second responses. All the measurements in the presence of Rnd were initiated 10 min after its addition and terminated within 40 min thereafter to avoid dislocation of the electrode from the endplate region due to Rnd-induced contracture.

The quantal content (m) was estimated by the method of failures from

$$m = \log_e(N/N_0),$$

where N = number of trials and N_0 = number of failures (Crawford, 1974). Student's t tests were used for statistical analyses and a probability of less than 0.05 was deemed statistically significant.

Ryanodine was purchased from S. B. Penick Lyndhurst, NJ, U.S.A. (Lot No. 704RWP-1). All other chemicals were of analytical grade.

Results

Effect on the frequency of m.e.p.ps

M.e.p.ps were measured randomly at 5 endplates in each of 8 preparations in a standard bathing solution or in a Ca^{2+}-free bathing solution (Figure 1). The mean frequency (s^{-1}) of m.e.p.ps was slightly reduced in the Ca^{2+}-free solution. Rnd at concentrations up to 1 μM did not significantly affect the frequency of m.e.p.ps in the presence or absence of $[Ca^{2+}]_o$ within the first 10 to 50 min of the exposure period. When a preparation was exposed to Rnd for more than 60 min, a gradual increase in muscle tonus was frequently observed. This contracture made it impossible to maintain continuous recordings of the potential changes at an endplate by dislodging the electrode. Thus, measurements of the potential changes were discontinued within 50 min of the start of exposure to Rnd.

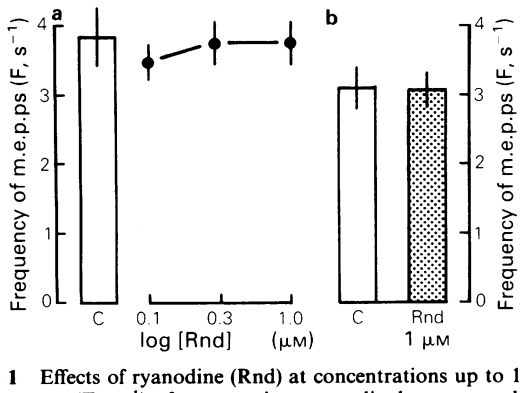

Figure 1 Effects of ryanodine (Rnd) at concentrations up to 1 μM on the frequency (F, s^{-1}) of m.e.p.ps in mouse diaphragm muscles in a standard bathing solution (a) and in a Ca^{2+}-free solution (b). Vertical bars show s.e.mean of results from 40 endplates in 8 preparations (5 endplates each). C: control. Rnd: ryanodine.

Depolarization potentiates the effect of $[Ca^{2+}]_o$ on the spontaneous release of transmitter quanta. The effect of Rnd was examined on the frequency of m.e.p.ps in a 10 mM K^+ bathing solution that contained various levels of $[Ca^{2+}]_o$, nominally 0, 0.125, 0.25 and 0.5 mM. During experiments in the presence of Rnd we measured m.e.p.ps at several endplates from 10 to 50 min after the start of exposure to this agent, then calculated the frequency of m.e.p.ps. The $[Ca^{2+}]_o$-dependent increase in the frequency of m.e.p.ps in 10 mM K^+ bathing solution was calculated by subtracting the mean value of the frequency of m.e.p.ps measured in Ca^{2+}-free, 10 mM K^+ solution from each value measured in the presence of $[Ca^{2+}]_o$, because m.e.p.ps were detectable in the absence of $[Ca^{2+}]_o$. In experiments which were performed in the presence of Rnd, the mean frequency of m.e.p.ps in the presence of Rnd and in the absence of $[Ca^{2+}]_o$ was used to correct for m.e.p.ps that occurred in the presence of Rnd with increasing $[Ca^{2+}]_o$. The frequency of m.e.p.ps increased with the increase in $[Ca^{2+}]_o$ in a nonlinear fashion (Figure 2a). Such a facilitating effect of $[Ca^{2+}]_o$ was further potentiated by Rnd. Rnd significantly elevated the mean value of the frequency (F, s^{-1}) at every $[Ca^{2+}]_o$. To determine whether Rnd exerts its effect on the spontaneous release of transmitter without changing the cooperativity of Ca^{2+}, as does sodium salicylate (Nishimura *et al.*, 1989), the effect of Rnd on the relationship between ln(F) and ln($[Ca^{2+}]_o$) was examined. Figure 2b shows the effect of Rnd on the curves that relate ln(F) to ln($[Ca^{2+}]_o$). In the absence of Rnd, the relationship between ln(F) and ln($[Ca^{2+}]_o$) was linear. The only effect of Rnd was to shift the curve to the left (towards lower values of ln($[Ca^{2+}]_o$), suggesting that Rnd facilitates the release of transmitter without changing the cooperativity of Ca^{2+}.

Effect on the quantal content of e.p.ps

E.p.ps were evoked in a Mg^{2+} (5 mM) bathing solution that contained low levels of $[Ca^{2+}]_o$ (0.4 to 0.8 mM), with or without Rnd. Measurements of e.p.ps in the presence of Rnd

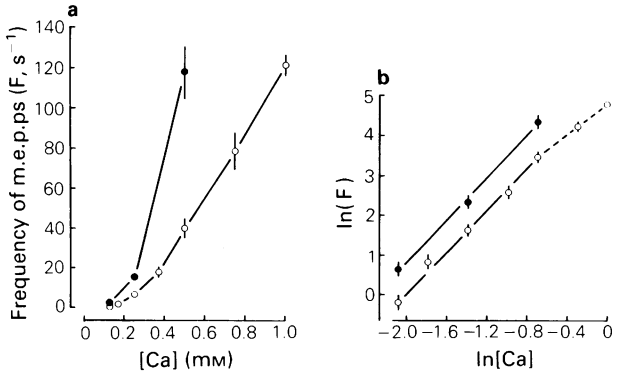

Figure 2 (a) Effect of 1 μM ryanodine (Rnd) on the frequency (F, s^{-1}) of m.e.p.ps in mouse diaphragm preparations in high-K^+ (10 mM) bathing solutions that contained various concentrations of Ca^{2+}. Vertical bars show s.e.mean, where possible, of observations from 40 endplates in 8 muscles (5 endplates in each muscle). (b) Natural logarithmic plot of the data used to generate (a). Ordinate scale: natural logarithmic values of the frequency of m.e.p.ps, ln(F). Abscissa scale: natural logarithmic values of $[Ca^{2+}]_o$ (mM), ln($[Ca^{2+}]_o$. (●): Rnd; (○): control.

were initiated 10 min after the addition of Rnd to solutions and were continued for the subsequent 40 min at several endplates. M.e.p.ps were measured at every endplate before e.p.ps were evoked in the high-Mg^{2+} bathing solution (Figure 3). The mean frequency of m.e.p.ps was slightly increased with an elevation of $[Ca^{2+}]_o$ in the presence and in the absence of Rnd. Rnd did not significantly affect the frequency of m.e.p.ps in this high-Mg^{2+} bathing solution, an observation similar to that made in the standard bathing solution (Figure 1).

The quantal content of e.p.ps was estimated by the method of failures (see Methods) (Figure 4a). The evoked release increased with an elevation of $[Ca^{2+}]_o$ in a nonlinear fashion. Rnd further and significantly ($P < 0.05$) increased the quantal content (m) at all values of $[Ca^{2+}]_o$ tested. Values for m were converted to ln(m). The value of ln(m) was plotted against $\ln([Ca^{2+}]_o)$ (Cooke *et al.*, 1973) in the presence and absence of Rnd (Figure 4b). The relationship between ln(m) and $\ln([Ca^{2+}]_o)$ was linear in the presence and in the absence of Rnd. The curve in the presence of Rnd was slightly but significantly ($P < 0.05$) shifted to lower $[Ca^{2+}]_o$, as was observed in the case of the frequency of m.e.p.ps.

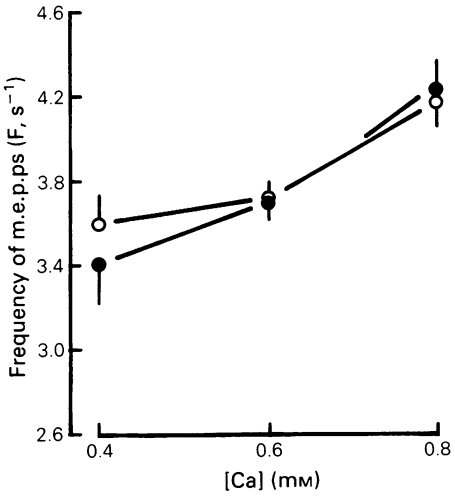

Figure 3 Effect of ryanodine (Rnd) 1 μM on the frequency (F, s^{-1}) of m.e.p.ps in mouse diaphragm preparations in high-Mg^{2+} (5 mM) bathing solutions that contained various levels of $[Ca^{2+}]_o$. (○): control; (●) ryanodine. Vertical bars show s.e.mean, where possible, of determinations from 273 to 545 endplates in 35 to 65 preparations. The resting rate of quantal release of transmitter did not change in the presence of Rnd.

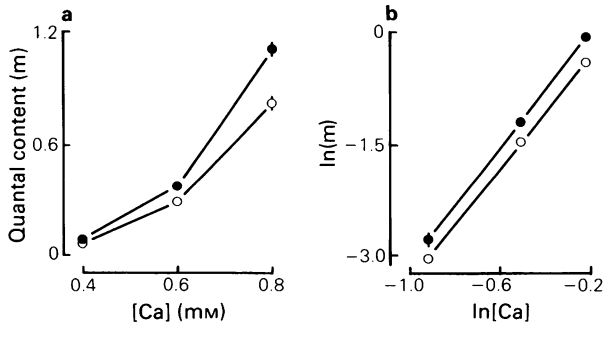

Figure 4 (a) Effect of ryanodine (Rnd) 1 μM on quantal content (m) of e.p.ps in mouse diaphragm muscles in high-Mg^{2+} (5 mM) bathing solutions that contained various levels of $[Ca^{2+}]_o$. Vertical bars show s.e.mean of results from 273 to 545 endplates in 35 to 65 muscles, where possible. (b) Natural logarithmic plot of the data used to generate (a). (●) Rnd; (○) control. Ordinate scale: natural logarithmic values of quantal content (m) of e.p.ps, ln(m). Abscissa scale: natural logarithmic values of $[Ca^{2+}]_o$ (mM), $\ln[Ca^{2+}]_o$.

Comparison of the effects of ryanodine with the effects of high K^+ and caffeine

The effects of 5 mM K^+, 2 mM caffeine and Rnd on spontaneous and evoked releases of transmitter quanta were compared in high Mg^{2+} (5 mM) bathing solution containing 0.6 mM Ca^{2+} (Figure 5). As mentioned above, Rnd did not significantly alter the frequency of m.e.p.ps at resting membrane potentials but significantly ($P < 0.05$) increased quantal content of e.p.ps. Both high K^+ and caffeine caused significant ($P < 0.05$) increases in both the frequency of m.e.p.ps and the quantal content of e.p.ps. Caffeine caused greater effects on m.e.p.ps than on e.p.ps while high K^+ showed the opposite relationship. Caffeine and high K^+ had little effect on the frequency of m.e.p.ps in a Ca^{2+}-free bathing solution.

Effect on facilitation of transmitter release

Facilitation of transmitter release was elicited by a series of two closely spaced stimuli with an interval of 4 ms on the nerve trunk. Such paired stimulation was repeated 128 or 256 times, with intervals of 1.5 s, at each endplate. Quantal contents of the first and second e.p.ps were designated as m1 and m2. Thus, the facilitation was expressed as m2/m1 (Figure 6). Measurements in the presence of Rnd or caffeine were initiated 10 min after their addition and continued for 40 min. The

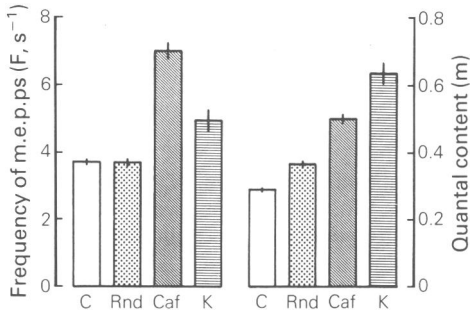

Figure 5 Comparison of effects of ryanodine (Rnd), caffeine (Caf) and high K^+ on quantal release of transmitter at mouse neuromuscular junctions in a bathing solution that contained 0.6 mM Ca^{2+} and 5 mM Mg^{2+}. Vertical bars show s.e.mean of observations from 177 to 927 endplates in 10 to 40 muscles. C: control. Rnd: 1 μM ryanodine. Caf: 2 mM caffeine. K: 5 mM K^+.

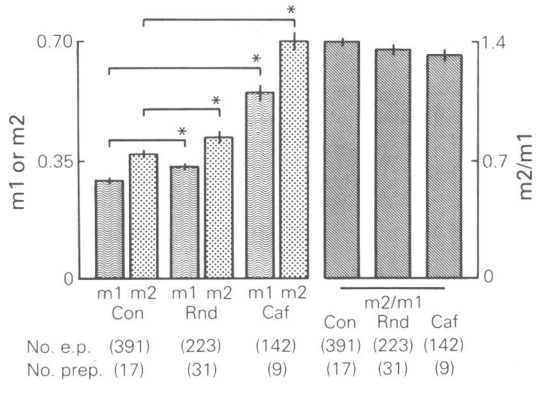

Figure 6 Effects of ryanodine (Rnd) and caffeine (Caf) on facilitation of transmitter release at mouse neuromuscular junctions in a bathing solution that contained 0.6 mM Ca^{2+} and 5 mM Mg^{2+}; Con: control. The phrenic nerve was stimulated by paired pulses with a 4 ms interval. These stimuli produced quantal contents of the first (m1) and second (m2) e.p.ps. The facilitation is shown as the ratio of m2/m1. No. e.p., numbers of endplates tested. No. prep., numbers of preparations used. Vertical bars show s.e.mean. *$P < 0.05$.

mean value of m2 was always larger than that of m1 in each case, causing the mean value of m2/m1 to be larger than 1. Both 1 μM Rnd and 2 mM caffeine significantly ($P < 0.05$) increased the value of m1 as indicated above. Since their stimulatory effects also appeared on the second e.p.ps (m2), the ratio m2/m1 was never observed to increase in the presence of either agent.

Discussion

$[Ca^{2+}]_i$ plays a central role in the process of release of transmitter quanta (Augustine *et al.*, 1987). Present experiments indicated that Rnd facilitates the quantal release of transmitter only when the nerve terminal is depolarized and $[Ca^{2+}]_o$ is greater than 0.125 mM. Such effects appear to be achieved without any alteration in the slope of the curve for the relationship between ln(F) and ln($[Ca^{2+}]_o$) or ln(m) and ln($[Ca^{2+}]_o$). It has been suggested that the non-linear dependence of quantal release of $[Ca^{2+}]_o$ shows cooperativity of Ca^{2+} at the active site (Jenkinson, 1957; Rahamimoff, 1976). Our data suggest that Rnd probably accomplishes its effect without changing the cooperativity of Ca^{2+}, and that Rnd causes a facilitation in the release process depending upon $[Ca^{2+}]_o$. Thus, according to the calcium hypothesis (Katz, 1969), the effects of Rnd are probably due to an increase in available $[Ca^{2+}]_i$.

If an increase in $[Ca^{2+}]_i$ is actually caused by Rnd, the question arises as to the source of this Ca^{2+}. Many sources have been proposed for increases in Ca^{2+} in the nerve terminal: (1) an influx through voltage-gated channels; (2) release from internal storage site(s); (3) inhibition of mechanism(s) for sequestrating Ca^{2+} in internal structures; and (4) inhibition of some extrusion mechanism(s) (Rahamimoff, 1976). The results indicate that Rnd never stimulates quantal release of transmitter at the resting state. In the sympathetic neurones, Rnd affects intracellular Ca^{2+} turnover in the presence of caffeine (Thayer *et al.*, 1988). Thus, it is unlikely that Rnd can increase $[Ca^{2+}]_i$ at rest. Furthermore, other trials showed that Rnd, as well as caffeine, did not alter the ratio of m2/m1 when a series of two closely spaced nerve impulses was adopted. This type of facilitation has been interpreted to be caused by 'residual Ca^{2+}' which outlasts a presynaptic activation and enhances the response to a subsequent impulse (Katz & Miledi, 1968; Rahamimoff, 1968; Charlton *et al.*, 1982). Some finite period of time is required for sequestration of extrusion of Ca^{2+} which enters the nerve terminal during nerve impulse. The subsequent stimulation given when the postulated residual Ca^{2+} exists in the nerve terminal would potentiate the second response of quantal release. Consistent with this suggestion is the observation that the ratio of m2/m1 is larger than 1. If Rnd inhibits the sequestering process for internal Ca^{2+}, such an effect would cause an increase in this ratio. However, the ratio of m2/m1 was never changed in the presence of Rnd. Thus, it is unlikely at least that Rnd inhibits the sequestration of Ca^{2+} involved in this type of facilitation.

We demonstrated the dependence of the effect of Rnd on depolarization of the nerve terminal as well as on values of $[Ca^{2+}]_o$ greater than 0.125 mM. Ca channels at presynaptic terminals function to transduce changes in membrane potential into an influx of Ca^{2+} down their electrochemical gradient (Augustine *et al.*, 1987). Therefore the effect of Rnd on transmitter release seems to be associated with depolarization or a transmembrane Ca^{2+} influx. In this regard, two possibilities arise, one of which is that Rnd augments the transmembrane Ca^{2+} influx upon activation of plasmalemmal Ca^{2+} channels. However, a possibility that Rnd has a direct action on plasmalemmal Ca^{2+} channels in cardiac and vascular smooth muscles was excluded (Mitchell *et al.*, 1984; Ito *et al.*, 1986) so that Rnd is unlikely to affect at least the L-type Ca^{2+} channel. No evidence was obtained as to whether Rnd acts on N-type Ca^{2+} channels which exist in nerve membranes. This should be clarified in a future study. A second possibility is that Rnd may interfere with the calcium handling of an intracellular organelle. If ER behaves similarly to SR in releasing Ca^{2+} in response to depolarization or Ca^{2+} influx and if Rnd affects the Ca^{2+} release mechanism, the substance could alter the transmitter release from nerve terminals. Rnd was shown to lock the Ca^{2+} release channel of skeletal and cardiac SR in an open state (Fleischer *et al.*, 1985; Rousseau *et al.*, 1987; Lai *et al.*, 1988) thereby allowing Ca^{2+} to leak out from SR. The action of Rnd is dependent on the history of the opening of the Ca^{2+} release channel (Sutko *et al.*, 1985; Aoki & Ito, 1988; Iino *et al.*, 1988). These features of the action of Rnd in muscles can explain its mode of action on transmitter release observed in this study. Attenuation of the Rnd effect by high Mg^{2+} is also similar to the action of Rnd observed in muscles (Meissner, 1986; Meissner & Henderson, 1987). Therefore, it is likely that Rnd has an intracellular action, at least part of which is analogous to its action on muscle SR. Indeed Rnd has been shown to inhibit caffeine-induced $[Ca^{2+}]_i$ transients in sympathetic neurones (Thayer *et al.*, 1988).

This study has demonstrated that Rnd can affect quantal release of transmitter depending on Ca^{2+}. Further study is needed to determine if Rnd specifically acts on ER and if this agent can be used as a specific tool for a study of intracellular calcium regulatory mechanisms in the nerve terminal.

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Microionophoretic study with milacemide, a glycine precursor, on mammalian central nervous system cells

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1 The effect of milacemide, a glycine percursor known to increase γ-aminobutyric acid (GABA) and glycine content in the brain, and to have anticonvulsant properties, was tested by ionophoresis on 247 neurones situated in the cerebral cortex and in deeper structures of cats and rats anaesthetized with urethane.

2 Virtually all the neurones, either firing spontaneously or exogenously driven by the excitatory amino acids, glutamate, N-methyl-D-aspartate (NMDA), kainate and quisqualate or by acetylcholine, were reversibly depressed in a dose-dependent fashion. The same depressant effect was observed in animals pretreated with the monoamine oxidase B inhibitor (IMAO-B) deprenyl which is known to reduce milacemide metabolism into glycinamide and glycine. Intravenous administration of milacemide (10 to 100 mg kg^{-1}) also depressed the firing induced by glutamate, NMDA and acetylcholine.

3 When compared to GABA, milacemide was a weaker depressant. However, its effect could still be observed in the presence of the reversible $GABA_A$ antagonist, SR 95531, and thus milacemide is unlikely to act through GABA receptors. In addition, on cells unaffected by glycine, milacemide also had a depressant effect, and on cells inhibited by glycine, it was still capable of depressing cell firing during reversible blockade by strychnine of the glycine inhibitory action; thus milacemide is unlikely to act through glycine receptors. Simultaneous release of milacemide and GABA or of milacemide and glycine, did not show potentiation of the inhibitory amino acid action. However, the depressant effect of milacemide was additive with that of GABA and glycine.

4 No consistent depression of glutamate-induced firing was obtained by ionophoresis of glycinamide, the first metabolite of milacemide.

5 It is concluded that milacemide by itself is a depressant agent and that its depressant effect does not necessarily require its metabolism into glycine, or its stimulator effect on the production of GABA.

Introduction

Milacemide, 2-n-pentylaminoacetamide, is active against convulsions in various acute and chronic animal models of epilepsy; it has also been shown to improve epileptic patients without sedative effects (Roba *et al.*, 1986).

In early studies, milacemide appeared particularly effective against convulsions induced by bicuculline (Van Dorsser *et al.*, 1983). This prompted the suggestion that it could interfere with the metabolism of γ-aminobutyric acid (GABA), the dysfunction of which has been suspected in some types of epilepsy (Meldrum, 1975). Biochemical investigations revealed that acute or chronic administration of milacemide to rats, at a dose of 25 to 100 mg kg^{-1}, increases the GABA content in the brain, particularly in the substantia nigra (Janssens de Varebeke *et al.*, 1983). The substantia nigra is known to be a site of anticonvulsant activity mediated by GABA (Iadarola & Gale, 1982; Bonhaus *et al.*, 1986).

Milacemide is transformed, through a monoamine oxidase B inhibitor (IMAO-B)-sensitive process, into glycinamide and glycine (Janssens de Varebeke *et al.*, 1988). Following its administration, the glycine content of the brain is significantly increased (Christophe *et al.*, 1983). The action of milacemide is believed to be indirectly due, at least in part, to its degradation into glycine (Hunter *et al.*, 1986; Roba *et al.*, 1986; Janssens de Varebeke *et al.*, 1988; Youdim *et al.*, 1988). However, milacemide does not appear very potent against convulsions induced by strychnine (Van Dorsser *et al.*, 1983; Roba *et al.*, 1986), which is a known glycine antagonist (Curtis & Johnston, 1974). It has been suggested (Janssens de Varebeke *et al.*, 1988) that glycine produced from milacemide preferentially influences the strychnine-insensitive glycine system, recently described by Johnson & Ascher (1987), which modulates N-methyl-D-aspartic (NMDA) acid-induced currents. However, such a hypothesis, if compatible with the enhancement of cognitive processes shown to occur in the course of milacemide treatment (Saletu *et al.*, 1986; Janssens de Varebeke *et al.*, 1988), would not account for the antiepileptic effect of milacemide.

The above studies thus suggest that anticonvulsant properties of milacemide are related to the increased concentrations of inhibitory amino acids which it induces in the nervous tissue, with emphasis on the GABA system. However, recent electrophysiological studies have pointed out that antiepileptic agents, supposed to act on the GABA inhibitory system, exert direct effects on nerve cells (Slater & Johnston, 1978; Schmutz *et al.*, 1979; Aickin *et al.*, 1981; Harrison & Simmonds, 1982; Carlen *et al.*, 1983; McLean & MacDonald, 1983; Morre *et al.*, 1984; Heinemann *et al.*, 1985; Willow *et al.*, 1985; Meldrum & Chapman, 1986; VanDongen *et al.*, 1986; Willow, 1986). These direct effects are of major importance, because they could indeed account for some of the anticonvulsant properties of these drugs.

Can milacemide and/or its metabolite glycinamide also affect nerve cell excitability directly?

Brief accounts of the results in this paper have been presented at meetings of the European and American Neuroscience Societies (Godfraind, 1986a,b; 1988).

Animal preparation

Experiments were performed on 38 adult animals breathing spontaneously: 16 cats were anaesthetized with 1.5 g kg^{-1} urethane administered through a catheter inserted into the

femoral vein under local anaesthesia; 22 rats were anaesthetized with the same drug injected intraperitoneally. When needed, supplementary doses of anaesthetic were given. After tracheotomy, animals were fixed in a stereotaxic frame and subjected to routine care (Godfraind, 1978).

Recording

Extracellular unit recordings were performed through the central barrel of five-barrelled pipettes filled with a 2 M NaCl solution, from cerebral cortex (most often in cats), or from deep structures e.g. hippocampus and thalamus (most often in rats). No attempt was made to characterize the neurones. The spikes were converted to standard pulses and fed into an integrator (Gould integrator amplifier; recycling time: 1 s) the output of which was connected to a small computer (CED 1401) and to a chart-recorder, in order to follow the firing frequency. Occasionally, spikes were also recorded on magnetic tape for analysis and photographic display.

Ionophoresis

The outer barrels of the five-barrelled pipettes were filled with the following substances: glutamate, 1 M, pH 7.4 (Fluka); N-methyl-D-aspartic acid (NMDA), 20 mM in 165 mM NaCl, pH 8 (Cambridge Research Chemicals); DL-2-amino-5-phosphono-valeric acid (APV), 20 mM in 165 mM NaCl, pH 8 (Sigma); kainic acid, 20 mM in 165 mM, pH 8 (Cambridge Research Chemicals); quisqualic acid, 20 mM in 165 mM NaCl, pH 8 (Cambridge Research Chemicals); acetylcholine, 1 M, pH 4.4 (BDH); GABA, 0.5 or 1 M, pH 4.3 (Fluka); the $GABA_A$ antagonist SR 95531 (2-(carboxy-3'-amino-6-parametoxy-phenylpyridazinium bromide)), 5 mM in 165 mM NaCl, pH 3.8 (Clin-Midy, Sanofi); glycine HCl, 500 mM, pH 4 (Aldrich Chemie); strychnine sulphate, 10 mM in 165 mM NaCl, pH 5.2 (Vel); NaCl, 165 mM, pH 3.6; glycinamide, 1 M, pH 3.6 (Janssen Chimica), and milacemide, 1 M, pH 4.3 for the first batch, and pH 4.4 for the second batch (Continental Pharma Laboratories). Retaining currents ranged from 10 to 20 nA. All drugs, except glutamate, glutamate agonists and APV, were delivered with positive currents. Cells were allowed to recover between successive and regular applications of the drugs. When comparing the effect of milacemide on the excitatory effects of the three glutamate agonists, these agonists were applied successively in a sequence determined at random for a given cell.

Intravenous administration of milacemide

In 6 experiments on the cat, milacemide was injected intravenously at doses ranging from 10 to 100 mg kg^{-1}. Milacemide was dissolved in 2 or 3 ml of 0.9% NaCl solution and slowly injected over 2 or 3 min. In these animals, the effect of milacemide applied by ionophoresis was also examined before and after the intravenous injection of milacemide.

Analysis

For each substance, the results are based on observations made on at least 3 different animals, except when the effects of APV and milacemide were compared, 2 animals were used. The test responses, obtained during application of the various drugs made with different ionophoretic currents, were measured and expressed as a percentage of the control responses. The values thus obtained were plotted against ionophoretic currents. A microcomputer programme (Linefit of Barlow, 1983), using the least squares method, calculated the regression lines corresponding to these X-Y values, and produced the resulting slope values.

Statistics

The median and interquartile range of the slopes, obtained during the applications of milacemide, glycinamide, GABA, APV or acetylcholine, were determined. The Mann-Whitney U test was used to compare the slope values corresponding to the responses obtained during the applications of the three pairs of drugs: GABA-milacemide, milacemide-glycinamide and GABA-glycinamide (NP-Scat and U test (Siegel, 1956; Barlow, 1983)). Data based on glutamate, glutamate agonists and acetylcholine-induced responses obtained during the release of milacemide or APV, were submitted to an analysis of variance (T & ANOVAR, Barlow, 1983). For comparing the potency of milacemide, GABA or glycine in the absence and presence of the antagonists of GABA and glycine, the responses obtained with the appropriate excitants in the control and test situations were paired and submitted to a Wilcoxon matched-pairs signed-ranks test (Conover, 1980; Barlow, 1983). The Wilcoxon test was also used to compare the effect obtained during release of milacemide and GABA or glycine to those obtained during the simultaneous release of milacemide and GABA or glycine.

Results

Two hundred and forty seven neurones were obtained, 93 from 22 rats, and the remaining 154 from 16 cats.

Effect of milacemide on the spontaneous activity

Ionophoretic applications of milacemide induced a reversible reduction of the spontaneous firing, on 9 neurones in 5 experiments. This effect was dose-dependent. At doses of 30 to 60 nA of milacemide, the firing rate slowed down within some 60 s after the beginning of the release. At the end of application, the firing returned gradually to its previous level within about 40 s.

Effect of milacemide on the responses evoked by glutamate and its agonists

In the course of 38 experiments, milacemide was applied by ionophoresis on 190 neurones driven by glutamate or glutamate agonists.

Effect of milacemide on glutamate-evoked responses and comparison with the effect of glycinamide For virtually all the cells (150 cells, 26 experiments), the glutamate-evoked responses were depressed, sometimes up to complete suppression, with ionophoretic currents as low as 5 nA. Of course, the depressant effect and the recovery occurred within a time course depending on the amount of ionophoretic current used (Figures 1, 3 and 5).

The depressant effect of milacemide was clearly dose-dependent (Figures 1, 2 and 3). The potency of milacemide was estimated using the median of the slope values of the regression lines (dose-response) for 24 neurones. The corresponding ionophoretic current needed to produce 50% inhibition was thus determined to be 36 nA.

The depressant effect of milacemide was not due to a direct current effect: indeed, the depression often outlasted the milacemide application (Figure 1), and, on several cells, a positive current of the same intensity applied through a NaCl-filled barrel never mimicked the milacemide effect (Figure 5). In addition, the action potential amplitude was not affected during milacemide release (Figure 2).

The milacemide effect was compared to that of its first metabolite, glycinamide. Out of 22 cells examined in 3 experiments, 9 were either little affected or slightly excited following ionophoresis of glycinamide (Figure 1). The firing of the thirteen other cells was depressed during glycinamide ejection. However, except in one case, the depressant effect of glycina-

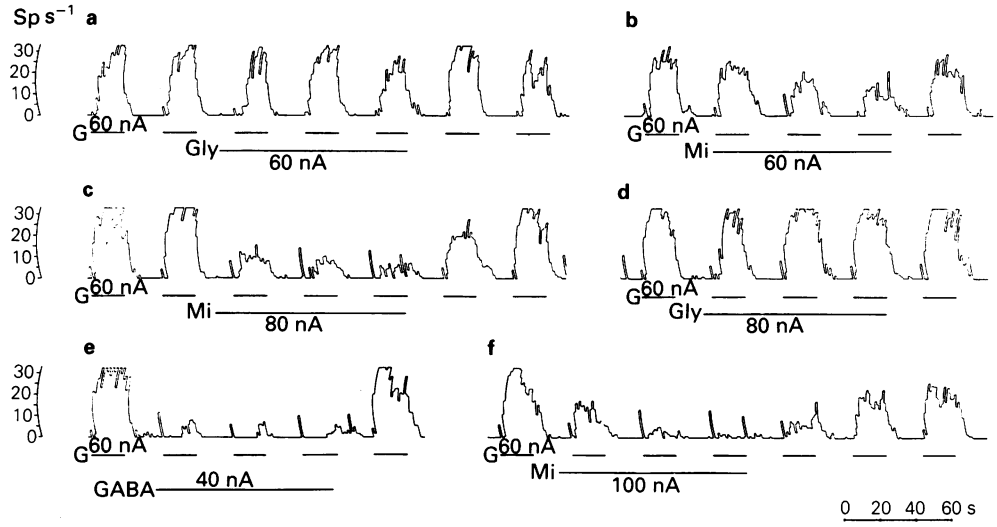

Figure 1 A comparison of the effects of milacemide, GABA and glycinamide on glutamate-induced discharges. The glutamate-firing (G) was reduced respectively during 60, 80 and 100 nA milacemide (Mi) applications, to 47%, 23% and 16% of the control (mean of three consecutive responses; b, c and f). During glycinamide (Gly) application with 60 and 80 nA, the glutamate-firing was little affected: firing rate reached 85% and 100% of the control (mean of three responses; a and d). GABA was the most potent depressant substance: 40 nA reduce the firing to 16% of the control (e). Between the upper and middle traces, and the middle and lower traces, no interruption occurred; upper and lower traces were interrupted respectively for 152 and 120 s. In the middle trace, the two halves are quasi continuous traces. In this and subsequent rate-meter records: dark lines below the records indicate the time of drug application; the ordinate scales: spikes per s (Sp s^{-1}); the abscissa scales: time in s.

mide was smaller than that of milacemide. The slope values of the dose-response curves clearly indicated that glycinamide was a poor depressant agent. It was calculated by extrapolation that an ionophoretic current of about 190 nA should be applied to reach 50% inhibition. The Mann-Whitney U test applied to the slope values of the pairs GABA-glycinamide and milacemide-glycinamide, showed that the populations were significantly different ($1 \times 10^{-6} < P < 1 \times 10^{-7}$ for GABA-glycinamide, and $1 \times 10^{-5} < P < 1 \times 10^{-6}$ for milacemide-glycinamide; one-tail test).

Effect of milacemide on NMDA-, kainate- and quisqualate-evoked responses To test whether or not milacemide acts preferentially on one of the glutamate receptor subtypes, it was ejected during regular cycles composed of four responses induced by successive applications of the glutamate agonists. The order of amino acid presentation was determined at random for each cell, and only the three last responses of a given sequence were retained for statistics.

These studies were performed on two groups of five rats, control animals and animals pretreated with deprenyl (to

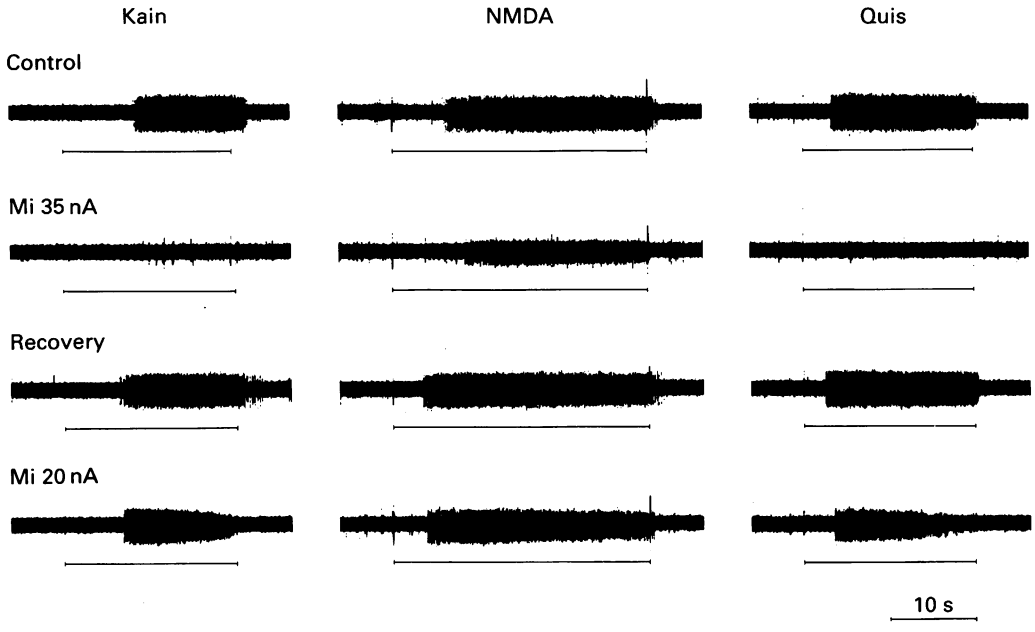

Figure 2 Effect of milacemide on glutamate agonists. A cell was fired by successive applications of 10 nA kainate (Kain), 91 nA N-methyl-D-aspartic acid (NMDA) and 2 nA quisqualate (Quis) during, respectively, control (upper horizontal row), milacemide release (second and lowest rows), and recovery (third row). During the 35 nA milacemide application, responses induced by the glutamate agonists are clearly depressed, and much less affected by 20 nA milacemide.

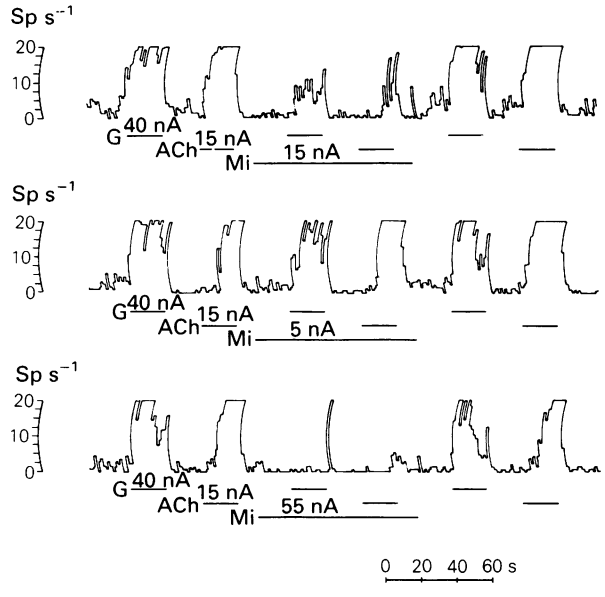

Figure 3 Effect of milacemide on acetylcholine-induced firing. A thalamic cell was fired by alternate applications of 40 nA glutamate (G) and 15 nA acetylcholine (ACh). Milacemide applied with 15, 5 and 55 nA (respectively upper, middle and lower row), depressed the firing induced by both substances in a dose-dependent manner. Upper and middle traces are continuous recordings; middle and lower traces were interrupted for about 6 min.

reduce milacemide catabolism). Deprenyl was injected intraperitoneally at a dose of 1 mg kg^{-1} late in the afternoon preceding the experiment, some 19 h before recording. In the first group, dose-response curves were based on data obtained from 9 out of 12 cells. The responses to all glutamate agonists were depressed (Figure 2). The slope values corresponding to the dose-response curves of the three agonists were submitted to an analysis of variance (T & ANOVAR from Barlow, 1983). The F test value was below 1, which indicates that milacemide was about equally potent in depressing the NMDA, kainate and quisqualate responses. In the second series of experiments, data were obtained from 15 cells but only 11 were suitable for analysis. Variance analysis of the slope values yielded a F value below 1. Thus, whether or not the milacemide degradation was impaired, milacemide appeared to depress the responses to the glutamate agonists similarly.

For 10 cells (2 rats), APV, a specific NMDA antagonist, was found to be more powerful than milacemide in depressing NMDA responses. Variance analysis of the slope values obtained for APV and milacemide, made on 6 cells out of these 10, yielded an F value above 15 ($P = 0.01$), confirming that APV was more potent than milacemide.

Effect of milacemide on acetylcholine-induced firing

Milacemide was also tested on 30 cholinoceptive cells in the cerebral cortex and deeper structures (hippocampus, thalamus) of 8 animals (6 cats and 2 rats). Milacemide did reversibly depress the acetylcholine-induced excitations, whether these were of the prolonged, muscarinic type, or of the shorter duration, so-called mixed nicotinic-muscarinic responses (Figure 3). This effect was also dose-dependent. Further, this depressant effect was clearly non-specific, in that it affected both glutamatergic and cholinergic responses (Figure 3), in all neurones. Data collected from 11 cells in 4 experiments, enabled dose-response lines to be drawn. Variance analysis of the slope values produced an F value below 1, i.e. the two populations, glutamate and acetylcholine, could not be statistically differentiated, thus showing significantly that the effect of milacemide was non-specific. On two other cells, NMDA and acetylcholine responses were compared: in contrast to the effect of APV, milacemide depressed both responses.

Comparison of the milacemide- and GABA- or glycine-evoked depressant effects

GABA GABA was clearly more potent than milacemide in depressing cell firing, as observed on 68 cells (Figures 1 and 4). Data collected from 24 neurones on which both GABA and milacemide were applied alternately, were used to draw dose-responses regression lines. The medians of the slope values were used to calculate the amount of ionophoretic current needed to produce 50% inhibition with milacemide and GABA, 36 nA and 10 nA respectively. A Mann-Whitney U test applied to these slope values, showed that the GABA and milacemide populations were significantly different ($1 \times 10^{-6} < P < 1 \times 10^{-7}$; one-tail test). To check for a possible interference between the two substances, the effect of milacemide was tested on the inhibition (sub-maximal or near-threshold) evoked by GABA, on 39 neurones driven by regular pulses, or by continuous release of glutamate. The effect of GABA was never enhanced nor prolonged during or just after the application of milacemide. However, the glutamate-evoked responses were significantly more depressed by the simultaneous release of GABA and milacemide than by GABA alone (Wilcoxon test: $0.021 < P < 0.022$; $n = 5$; one-tail test); this indicates that the effects of GABA and milacemide are additive.

We further examined whether milacemide could still depress glutamate-evoked discharges while GABA inhibition was specifically suppressed by applying the very potent $GABA_A$-antagonist, SR 95531, (Michaud *et al.*, 1986; Wermuth & Bizière, 1986). Sequences such as that shown in Figure 4, are easy to obtain provided that the dose of SR 95531 is minimal, to avoid the occurrence of paroxysmal discharges against which milacemide is ineffective. When SR 95531 was ejected with currents sufficient to reversibly abolish (6 cells) or significantly reduce (3 cells) the depressant effects of GABA, it had no significant effect on the depressant action of milacemide (same 9 neurones in 4 experiments; Wilcoxon test).

Glycine On 29 cells in 6 experiments, observations made with glycine and milacemide paralleled those obtained for GABA and milacemide, though with some differences. In the cerebral cortex, glycine was without effect on 7 cells and had only a weak effect on another 10 neurones; this was, however, less marked than that of milacemide. On the other cells situated in deep nuclei, a few nA glycine did suffice to bring complete silence. These cases were the most favourable to perform the tests with strychnine.

On cells unaffected by glycine, milacemide nevertheless proved to be an active depressant agent. To check for a possible interference between glycine and milacemide, a 'window' test was performed in 4 cells. At near-threshold dose, milacemide did progressively induce a slight depression, followed by slow recovery. During continuous release of glycine, a similar depression was obtained by a re-application of milacemide.

In cells where both glycine and milacemide depressed the firing rate, an additive effect was also observed when both drugs were released simultaneously (3 cells).

When strychnine was applied with currents sufficient to reversibly abolish (9 cells) or significantly reduce (2 cells) the glycine inhibition, it did not alter significantly the depressant effect of milacemide (11 cells, 4 experiments; Wilcoxon test).

Effect of intravenous injections of milacemide

Milacemide was injected intravenously in 6 cats at doses ranging from 10 to 100 mg kg^{-1}. Some animals received more

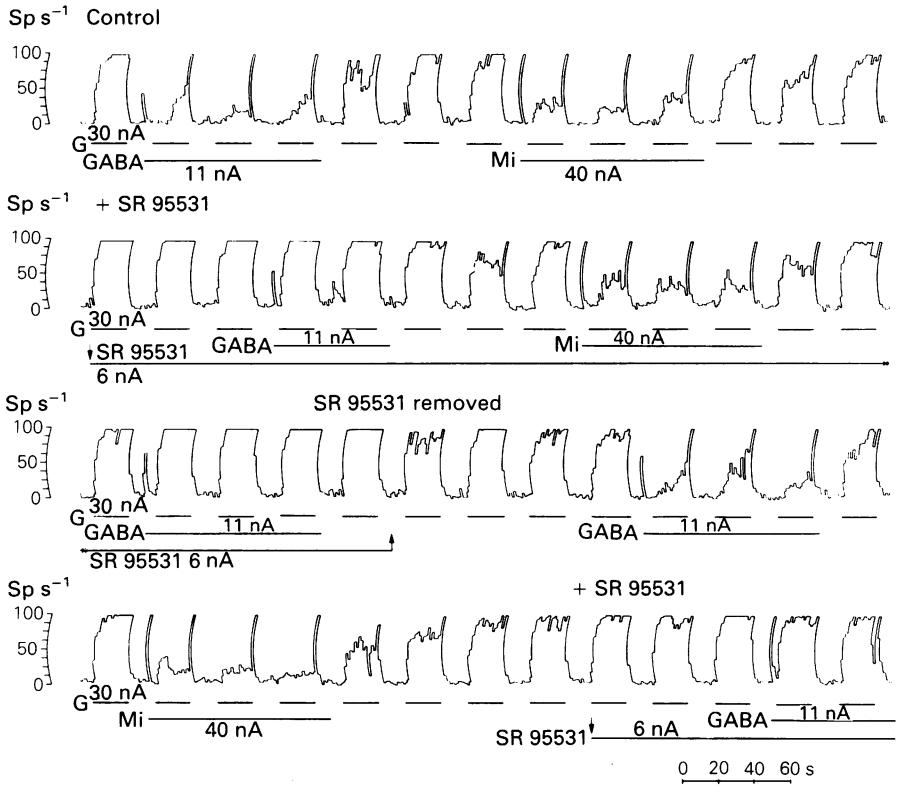

Figure 4 Effect of milacemide in the presence of SR 95531, a $GABA_A$-antagonist. In the upper row, 11 nA GABA and 40 nA milacemide depressed the glutamate firing respectively to 32% and 41% of the control. In the ejection of the $GABA_A$-antagonist (downwards arrow SR 95531 at the beginning of second row), the inhibitory effect of GABA was completely abolished (second and third row), while milacemide still depressed the firing. The GABA-induced inhibition recovered rapidly after SR 95531 release (third row). Lower trace right, SR 95531 was reapplied, and the sequence replayed. Note the excitatory effect of SR 95531. The four traces were continuous records.

than one injection, and the time elapsed between two injections varied from 30 min to several hours. Hence the cumulative dose could eventually exceed 100 mg kg^{-1}. In four experiments, control intravenous injections of saline did not alter the amplitude of the glutamate-evoked responses (Figure 5).

About 10 min after the intravenous injection of a small dose of milacemide (10 to 30 mg kg^{-1}), the amplitude of the

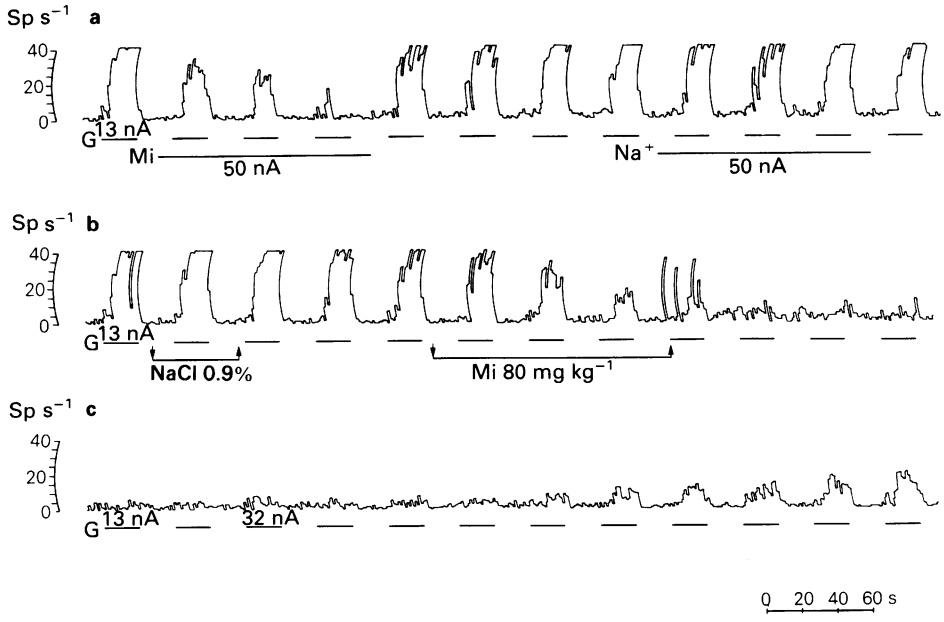

Figure 5 Effect of milacemide injected intravenously. (a) Ionophoretic ejection of 50 nA milacemide reduced progressively the glutamate-induced firing to almost complete silence. Na^+ current application failed to mimic the effect of milacemide. (b) the firing rate was not affected following intravenous injection of saline, whereas, intravenous injection of milacemide at a dose of 80 mg kg^{-1}, was quickly followed by a complete disappearance of the glutamate-response. (c) After increasing the glutamate ionophoretic current, the cell was slightly reactivated. The traces are continuous records.

glutamate-evoked responses was usually reduced by 10 to 50% (4 cells). Such an effect could be overcome by increasing the ionophoretic current of glutamate but ionophoretic application of milacemide was still able to depress the remaining glutamate responses. This suggests that the sites acted upon by milacemide were not saturated.

The depressant effect of milacemide was more clear-cut after injection of larger doses ($80\,mg\,kg^{-1}$) (first injection for this preparation) (Figure 5b). The discharges induced by glutamate, NMDA or acetylcholine were then totally suppressed for several minutes and recovered progressively after about 9–10 min. It should be mentioned that at the end of intravenous injections of such large doses of milacemide, a clear and transistory change in the respiratory rhythm could be observed. As previous investigations had not described any effect of milacemide on the cardiovascular system (Roba *et al.*, 1986), blood pressure and heart rate were not monitored during these experiments.

Discussion

It has been shown here that milacemide, applied by ionophoresis or by the intravenous route, reversibly depresses neuronal excitation, i.e. spontaneous activity, glutamate-, glutamate agonists- and acetylcholine-evoked firing, without interfering with the inhibitory mechanisms of GABA and glycine. These observations were made on unidentified cells recorded in the cerebral cortex and in deep structures from two different animal species; this covers a wide range of neuronal types, and thus the milacemide effects described here are unlikely to be linked either to a particular cell type, to a particular neuronal microcircuit, or to a defined neurotransmitter system. Since glycinamide is much less effective than milacemide in reducing cell firing, it is proposed that milacemide is the active agent. This proposition is further strengthened by the experiments made with animals pretreated with deprenyl, which reduces milacemide metabolism into glycinamide and glycine (Janssens de Varebeke *et al.*, 1988): the firing was as depressed in untreated as in treated animals.

In addition, just at the end of an intravenous injection of a high dose of milacemide ($80\,mg\,kg^{-1}$) in cats, a transient modification of the respiratory rhythm was noticed. Excitatory amino acids have been shown to play a role in respiration, and therefore it should not be surprising to find that an intravenous injection of milacemide alters the respiratory rhythm as do other specific and unspecific glutamate antagonists (McCrimmon *et al.*, 1986; Foutz *et al.*, 1988). The amounts of milacemide used here were low compared to those used in other studies (Albertson *et al.*, 1984; Youdim *et al.*, 1988).

The effect of milacemide on the three main classes of glutamate receptors (Watkins & Olverman, 1987) was also investigated: the NMDA-, kainate- and quisqualate-induced responses were all equally significantly depressed by milacemide, in control as well as in deprenyl-treated animals. A comparison of milacemide with APV, a specific NMDA antagonist (Davies *et al.*, 1981), on NMDA responses, showed that APV was significantly more potent as a depressant agent than milacemide. Milacemide appeared to be rather non-specific: it depressed the glutamatergic responses as well as those induced by acetylcholine, whether of the muscarinic type (cortical neurones) or of the mixed nicotinic-muscarinic type (deep cells).

The non-specific depressant effect of milacemide on excitation induced through a variety of receptors, together with the observation that $100\,\mu M$ milacemide does not influence directly ionic channels in patch-clamp experiments (Johnson, personal communication), and does not affect membrane input resistance (our unpublished data), all suggest that it could be acting via an intracellular biochemical mechanism.

Milacemide is significantly weaker than GABA in depressing cell firing. It does not affect the GABA inhibitory action. However, both drugs have additive effects. Results with the $GABA_A$-antagonist SR 95531, showing that the milacemide-induced depression was still present when the GABA inhibitory action was abolished, indicate that milacemide is unlikely to be acting through the GABA system. Binding studies did not reveal any interaction between milacemide and GABA receptors (Roba *et al.*, 1986).

Although milacemide is a precursor of glycine and is able to induce a widespread and significant increase of glycine in the brain (Christophe *et al.*, 1983), its depressant action is unlikely to be mediated by the glycine system. Its depressant effect persists in the presence of strychnine and in deprenyl-treated animals. In addition, milacemide is an active depressant agent on neurones unaffected by glycine. Glycine plays only a minor role in the inhibitory mechanisms observed in the cerebral cortex (Kelly & Krnjevic, 1969; Bernardi *et al.*, 1979). The 'window type' experiments conducted on the cells unaffected by glycine, showing that milacemide still depressed the firing during continuous glycine release, suggest that glycine and milacemide do not directly interact or share common sites of action. Also, binding studies did not reveal an interaction with glycine (Roba *et al.*, 1986), and milacemide is not very effective against strychnine-induced convulsions (Van Dorsser *et al.*, 1983). In addition, patch-clamp experiments made with milacemide do not show an interaction with glycine (Johnson, personal communication).

It was observed, on glycine-sensitive cells, that simultaneous release of glycine and milacemide does produce a more intense depression than that evoked by separate applications of the drugs. GABA and glycine share a common ionic mechanism to induce inhibition (Curtis & Johnston, 1974; Krnjevic, 1974). However, such a mechanism of action is unlikely to be the one through which milacemide is acting: intracellular recording in granule cells ($n = 3$) of the hippocampus maintained *in vitro*, showed that milacemide, at a concentration up to 1 mM in the bath, induced no change in membrane potential or in membrane input resistance (our unpublished data).

In conclusion, these experiments have shown that milacemide has a direct depressant effect on cell firing whether spontaneous or exogenously driven, and indicate that its site of action is likely to be postsynaptic. Without excluding the biochemical hypothesis (based mainly on increased levels of GABA and glycine), the present results call for further experiments to be done in order to identify the precise physiological mechanisms by which milacemide alters cell excitability.

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The effects of cholecystokinin octapeptide on human isolated alimentary muscle

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1 We studied cholecystokinin octapeptide (CCK-OP) for its motor effects and sites of action on human isolated muscle from stomach, small intestine and colon.

2 CCK-OP induced a concentration-dependent contraction of all the longitudinal muscles and of circular muscle from the stomach and large intestine. The peptide acted directly on these muscles at a site not involving muscarinic receptors.

3 CCK-OP relaxed the circular muscle of the small intestine and/or reduced the contractions to acetylcholine, by stimulating intramural postganglionic inhibitory neurones.

Introduction

Within the gut, cholecystokinin (CCK) has principal actions on gallbladder contraction (Ryan, 1981) and pancreatic secretion (Fried *et al.*, 1983), but it also affects gastrointestinal muscle. In isolated gut tissues from several species CCK can act directly on the smooth muscle, and indirectly through intramural postganglionic cholinergic neurones (Dockray, 1987). There are few studies on human isolated tissues. A purified CCK-pancreozymin preparation increased the spontaneous contraction amplitude of human gastric muscle *in vitro* with little effect on the resting tone (Cameron *et al.*, 1970). CCK-octapeptide (CCK-OP) often produced contractions of gastric strips by a direct action on the muscle, but did not affect circular muscle from the duodenum (Lüdtke *et al.*, 1988). CCK and CCK-OP contracted human isolated taenia coli, also by a direct action (Egberts & Johnson, 1977). The aim of our study was to investigate further the effects and sites of CCK-OP action on human isolated gastrointestinal muscle.

Methods

Specimens of human tissue, free from any macroscopically visible lesion, were obtained at operation for benign or malignant conditions, placed in Krebs solution at room temperature, and studied either immediately or after storage at 4°C for up to 24 h. The tissue was laid flat in Krebs solution and the mesentery, mucosa and submucosa were carefully cut away. Depending on the specimen size, 6–16 strips 3–4 mm wide and 20–30 mm long, were cut through the muscle coat parallel to either the circular or longitudinal muscle fibres, and set up in tissue baths (5–7 ml), containing Krebs solution maintained at 37°C and bubbled with 95% O_2:5% CO_2. The composition of the Krebs solution was as follows (mM): NaCl 121.5, $CaCl_2 \cdot 6H_2O$ 2.51, K_2HPO_4 1.18, KCl 4.7, $MgSO_4 \cdot 7H_2O$ 1.17, $NaHCO_3$ 25 and dextrose 5.55. The strips were placed under a load of 1 g and allowed to equilibrate for at least 1 h. Responses, magnified 10–15 times, were registered on pen recorders using isotonic transducers.

Each experiment started with a concentration-response curve to acetylcholine (ACh, 0.01–1 μM). Consistent ACh-induced submaximal contractions were obtained with ACh 0.01–0.2 μM that gave 40–80% of the maximal response. The contact time was always 30 s but although the cycle time was constant in each experiment it varied between experiments, usually from 7–10 min depending on the amount of spontaneous activity, the time taken for the tissue to relax after the ACh was washed out and the amount of time needed to study all of the strips used. CCK-OP was then added for 2 min followed, without washing out, by ACh for a further 30 s. This procedure allowed us to examine the effect on the response to ACh, and was particularly important in the tissues showing an inhibitory response to the peptide. Each strip was tested with repeated doses of CCK-OP, given at intervals of 1 h to avoid tachyphylaxis. Since it was therefore often not possible to generate a full CCK-OP concentration-response curve in a single muscle strip, neighbouring strips from the same specimen sometimes received different concentrations of CCK-OP. Contraction to CCK-OP after the 2 min contact time was measured in mm from the resting baseline, and expressed as a percentage of the maximal contraction to ACh obtained in the absence of CCK-OP. The results obtained from different specimens were then used to build up an average concentration-response curve to which was fitted a 3-variable logistic function, giving estimates of EC_{50}, slope at half of maximal response, and the upper asymptote (Black *et al.*, 1985).

The influences of drug antagonists were tested on consistent submaximal responses to CCK-OP, chosen on the basis of the composite concentration-response curve and giving contractions 30–70% of maximum, as demonstrated at the end of the experiment by giving a 3 fold higher CCK-OP concentration. On the two occasions when the test responses were >70% of maximum the experiments were omitted from the analysis. The contact times for the antagonists were: atropine, one cycle time (7–10 min) before the next addition of ACh; tetrodotoxin and hexamethonium, at least two cycle times before the addition of nicotine. Drug antagonist contact with the tissues before addition of CCK-OP was therefore substantially longer than these times.

The drugs used were: CCK-OP, acetylcholine hydrochloride, atropine sulphate, hexamethonium bromide, nicotine hydrogen tartrate, and tetrodotoxin (all from Sigma). They were added to the bathing fluid dissolved in 154 mM NaCl and washed out at the fixed time intervals found to be optimal in preliminary experiments.

Results

There were 177 strips of longitudinal muscle and 140 strips of circular muscle from 56 patients. All contracted with ACh 0.01–1 μM, in agreement with the findings of Bennett & Whitney (1966a,b). Many strips (174/317) showed spontaneous activity, as described in the latter papers, with amplitudes usually less than 10% of the maximum response to ACh.

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Table 1 The regions from which the muscle strips were taken, and a summary of the responses to various concentrations of cholecystokinin octapeptide (CCK-OP)

	Total studied	*Circular muscle (Contracting total)*		*Longitudinal muscle (Contracting/total)*	
Stomach	59 (12)	24/28	(8/9)	19/31	(8/10)
Jejunum	10 (1)	0/6*	(0/1)	3/4	(1/1)
Ileum	52 (12)	0/38*	(0/11)	10/14	(6/10)
Ascending colon	17 (4)	7/14	(2/4)	2/3	(1/2)
Transverse colon	33 (4)	1/18	(3/4)	14/15	(4/4)
Sigmoid colon	146 (23)	33/73	(14/19)	41/73	(17/21)

Some strips received the same concentration of CCK-OP repeatedly, others from the same specimen received different amounts, and others received a range of concentrations that produced partial or full sigmoidal log concentration-response curves. The numbers of strips studied are shown first, with the numbers of specimens given in parentheses.
* Relaxation or inhibition of the contraction to ACh.

When the spontaneous activity was greater than 30% of the maximum ACh-induced contraction, the strips were rejected (8 strips from two different specimens). Although the spontaneous activity varied among strips from different specimens, neighbouring strips from the same specimen showed a similar pattern. During the experiment the size of the spontaneous activity showed a tendency to reduce in 147/174 strips; the remainder showed little or no change.

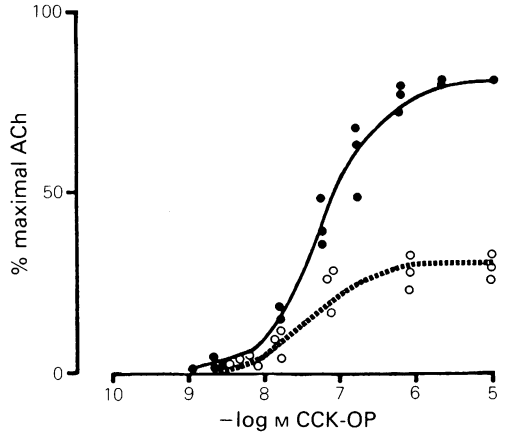

Figure 1 Derived concentration-response curve of cholecystokinin octapeptide (CCK-OP) using responses from 3 specimens of human transverse colon circular muscle. Cumulative addition (○) gave a smaller maximum response compared with non-cumulative (●) administration.

CCK-OP produced a contraction or no response in strips from all regions except the circular muscle from the small intestine which was inhibited by the peptide (Table 1). Not all strips from the same specimen responded. Most of the gastric strips responded (73% of circular and longitudinal muscle strips; 84% of specimens), compared with 50% of strips (80% of specimens) from the colon (Table 1). Storage at 4°C overnight (maximum 24 h) did not appear to affect the tissue responses, as shown previously with this and various other agonists. We did not analyse the effect of storage quantitatively, but the sensitivity to ACh and the response to CCK-OP in strips from two specimens were similar when used fresh or after overnight storage. We have obtained similar results with CCK-OP on four other specimens used immediately and after storage (unpublished observations). Results from fresh and stored tissues were therefore pooled.

Compared with the response to ACh, the contraction to CCK-OP was smaller, developed more slowly and was much more sustained. Repeated contractions were consistent, provided that the strip was not exposed to CCK-OP for more than 2.5 min or with a cycle time of less than 1 h. Preliminary experiments showed that longer contact times or shorter intervals between doses caused tachyphylaxis which was irreversible and complete for at least 12 h. Figure 1 shows the comparison between non-cumulative and cumulative administration of CCK-OP to strips of transverse colon circular

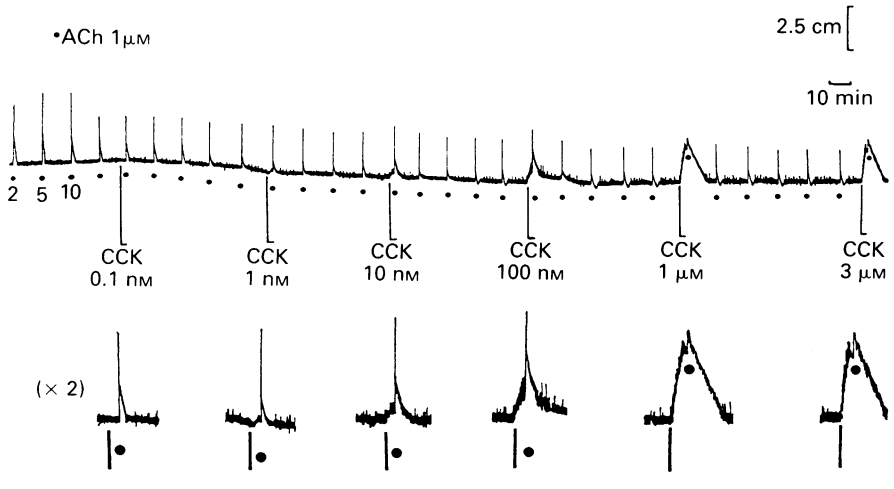

Figure 2 Representative tracing of the response to increasing concentrations of cholecystokinin octapeptide (CCK) applied to a gastric circular muscle strip. The interval between applications was 1 h, interspersed with submaximal contractions to ACh (1 μM, except at the start of the trace where 2, 5 and 10 μM were given).

muscle. Figure 2 shows concentration-dependent contractions of gastric circular muscle that began after at least 20 s, and gradually increased to a maximum at about 1 min. After washout, the tissue recovered its resting tone over about 20 min, mainly regardless of the size of the CCK-induced contraction.

Figure 3 shows the composite concentration-response curve obtained from different concentrations in different strips of taeniae from the sigmoid colon. Similar results, summarised in Table 2, were obtained with tissues from all other regions except small bowel circular muscle which was inhibited. In both layers from all the different regions of the gut the threshold for contraction was similar (range 2–20 nM CCK-OP), and the maximum contraction (about 80% of the maximum to ACh) was usually produced by 1 μM CCK-OP. In 85/164 strips contracted by CCK-OP, the response to ACh added in the presence of the peptide increased by a mean of 18% (range 5–39%). In one circular muscle strip from a sigmoid colon and one longitudinal muscle strip from the only specimen of jejunum, only the response to the subsequent addition of ACh increased. The other strips showed little or no change in the contraction to ACh added in the presence of CCK-OP.

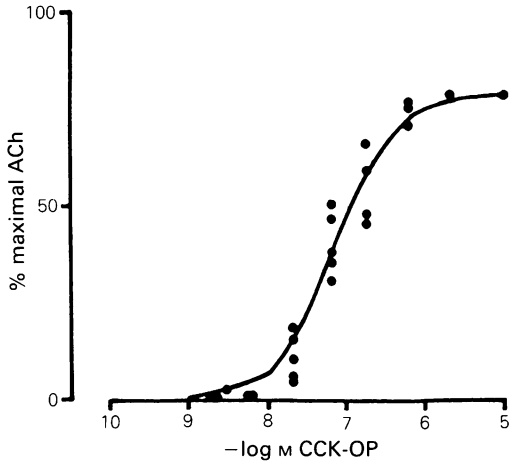

Figure 3 The derived concentration-response curve of cholecystokinin octapeptide (CCK-OP) in human sigmoid colon longitudinal muscle (*taenia coli*). Responses are expressed as percent maximal contractions induced by acetylcholine (ACh).

Atropine 500 nM always completely blocked the contraction to ACh, but there was no significant change with CCK-OP (mean contraction height ± s.e.mean 95.5 ± 5.3% (n = 12) of that in the absence of atropine: stomach, 2 longitudinal and 1 circular strip; jejunum, 1 longitudinal strip; ileum, 2 longitudinal strips; large bowel, 3 longitudinal and 3 circular strips). Tetrodotoxin 50 nM always completely blocked the response to nicotine, but had no effect on CCK-OP (mean contraction height 99.9 ± 3.4%; n = 12) of that in the absence of tetrodotoxin: stomach, 2 longitudinal and 2 circular strips; jejunum, 1 longitudinal strip; ileum, 2 longitudinal strips; large bowel, 1 ascending colon circular strip, 2 sigmoid taeniae and 2 circular strips). An experiment illustrating the influence of atropine and tetrodotoxin in one longitudinal muscle strip from the sigmoid colon is shown in Figure 4.

In contrast, none of the 27 ileal or 6 jejunal circular muscle strips (from 12 and 1 patients respectively) contracted to CCK-OP (0.02–2 μM); instead, inhibitory responses were obtained in 16/33 (11 ileal and 5 jejunal) strips. Figure 5 shows concentration-dependent relaxations and a reduction of the superimposed ACh-induced contraction in one ileal strip; this inhibitory effect was unchanged by atropine 500 nM, but was blocked by tetrodotoxin 50 nM. In another experiment CCK-OP (0.15–0.18 nM) relaxed each of the three strips from the same specimen and in two other experiments the peptide

Table 2 EC_{50} values for contractions to cholecystokinin octapeptide (CCK-OP) in human tissues (given as −log EC_{50}, mean value ± s.e.mean)

	Circular muscle	*Longitudinal muscle*
Stomach	6.71 ± 0.07 (5)	6.43 ± 0.06 (5)
Small intestine	—*	7.55 ± 0.10 (5)
Ascending colon	6.95 ± 0.80 (2)	7.60 (1)
Transverse colon	7.15 ± 0.06 (3)	7.00 ± 0.06 (4)
Sigmoid colon	7.10 ± 0.07 (6)	7.15 ± 0.07 (6)

The numbers of specimens are given in parentheses.
* Relaxation or inhibition of the contraction to ACh (one jejunal and four ileal specimens).

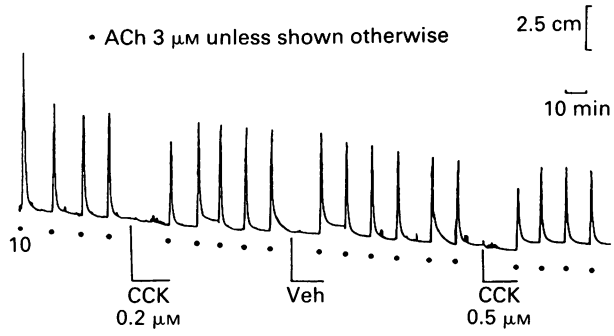

Figure 5 A concentration-dependent inhibition by cholecystokinin octapeptide (CCK) in human ileal circular muscle. Veh = vehicle.

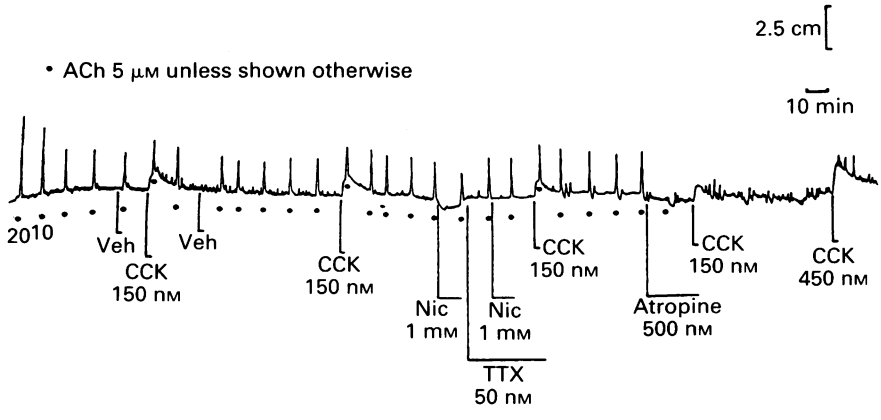

Figure 4 Representative tracing showing no effect of tetrodotoxin (TTX) and atropine on the contractile response induced by cholecystokinin octapeptide (CCK) in a longitudinal strip from the sigmoid colon. Veh = vehicle; Nic = nicotine.

reduced the contraction to ACh without altering the muscle tone. The reduction of the jejunal ACh-induced contraction was blocked by tetrodotoxin 50 nM but not by hexamethonium 20 μM ($n = 2$; Figure 6).

Discussion

The aim of the present study was to investigate the effects and sites of CCK-OP action in human isolated gastrointestinal muscle. CCK-OP has similar activity to CCK on human gut muscle (Egberts & Johnson, 1977) and is present in the human bloodstream (Walsh *et al.*, 1982). We studied both the circular and the longitudinal muscle because various substances affect these two layers differently (e.g. prostaglandin E_2, Bennett *et al.*, 1968; vasoactive intestinal peptide, Bennett *et al.*, 1984).

In agreement with the studies of Lüdtke *et al.* (1988) on human gastric strips, those regions of the gut that contracted to CCK-OP did so with nanomolar concentrations. Human gut *in vivo* is sensitive to CCK-OP at the normally circulating picomolar amounts (Kellow *et al.*, 1987). Lower sensitivity *in vitro* may be due to the difficulty of diffusion from the bath fluid into muscle strips, compared with diffusion from the blood, and to the absence of other factors that might augment motility *in vivo*.

Since the contractile effect of CCK-OP was not inhibited by atropine or tetrodotoxin, in agreement with Lüdtke *et al.* (1988), we conclude that the peptide stimulates human isolated alimentary muscles by a direct action at sites not involving muscarinic receptors.

The relaxation and/or inhibition of ACh-induced contraction in the circular muscle from the small bowel contrasts with the rest of the alimentary tract. Lüdtke *et al.* (1988) studied only duodenal circular muscle, apart from the stomach, and obtained no duodenal excitatory responses to CCK-OP; it seems that they did not look for inhibition by determining the effect on ACh-induced contraction. In our experiments the inhibition by CCK-OP in jejunal and ileal circular muscle was blocked by tetrodotoxin but not hexamethonium, suggesting an action on postganglionic inhibitory neurones.

Both direct muscle and neurally mediated effects of CCK have been described in isolated gut from laboratory animals. A direct contractile effect has been shown in canine antral muscle (Morgan *et al.*, 1978) and guinea-pig stomach (Gerner & Haffner, 1977). Evidence for a neurally mediated effect of CCK in the longitudinal muscle of guinea-pig ileum is that ACh is released (Vizi *et al.*, 1972; 1973), and that without the myenteric plexus the tissue responded to ACh but not to CCK (Hutchinson & Dockray, 1980; 1981). These authors considered that release of substance P may account for the atropine-resistant component of the CCK-induced contraction in this tissue. Grider & Makhlouf (1987) confirmed that CCK contracts guinea-pig ileum longitudinal and circular muscles both directly and by activation of cholinergic pathways, and they showed different sensitivities of the muscle cells and neurones. With regard to inhibitory effects, CCK activates receptors on nonadrenergic-noncholinergic neurones in dog stomach (Schmalz *et al.*, 1983; Schmalz & Szurszewski, 1983), cat sphincter of Oddi (Behar & Biancani, 1980) and lower oesophageal sphincter (Rattan & Goyal, 1983). It is therefore clear that species, regional, and muscle layer differences exist in the gastrointestinal responses to CCK. There also seem to be differences in the types of CCK receptors in human and guinea-pig intestine (unpublished).

Consistent with our *in vitro* findings, CCK *in vivo* inhibited human small bowel motility (Osnes, 1975) but increased colonic motor activity (Dinoso *et al.*, 1973). Thus, perhaps the pattern of motility affected by CCK released in response to a meal tends to reduce the propulsion in the small bowel, so increasing the time for digestion and absorption of the food, while the increased colonic motility stimulates defaecation (the 'gastrocolic reflex'). However, it may not be valid to extrapolate from *in vitro* to *in vivo* activity (Bennett, 1968). Nevertheless, our results are consistent with a possible regulatory role for CCK in human gastrointestinal motility.

We thank Mr John Rennie, other surgeons, and the pathologists of King's College Hospital for providing the specimens, the colleagues who helped with data analysis, and The British Council for the grant to M D'A.

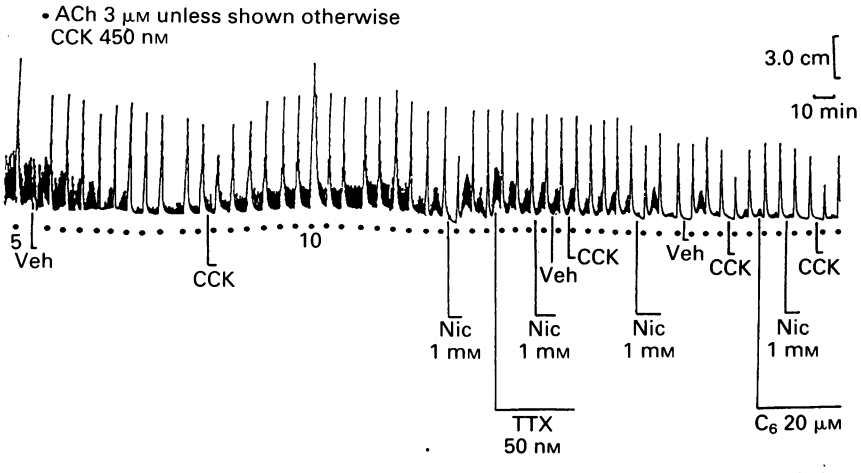

Figure 6 Block by tetrodotoxin (TTX), but not hexamethonium (C6), of the inhibitory response to cholecystokinin octapeptide (CCK-OP) in human jejunal circular muscle. Veh = vehicle; Nic = nicotine.

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Modulation of cholinergic neurotransmission in guinea-pig airways by opioids

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1 Opioid receptors have been localised on sensory fibres in the vagus nerve and opioids have previously been shown to inhibit non-adrenergic, non-cholinergic (NANC) neurotransmission in guinea-pig bronchi *in vitro* and *in vivo*. We have now investigated whether an inhibitory effect could be demonstrated on cholinergic neurotransmission.

2 Electrical field stimulation (EFS) (8 Hz, 0.5 ms, 40 V for 20 s) produced only a rapid, cholinergic response in the upper trachea but in the lower trachea and main bronchi a cholinergic response which was atropine-sensitive and a longer lasting NANC contraction that was atropine-insensitive was demonstrated. This slow contraction could be blocked by tetrodotoxin and capsaicin pretreatment.

3 [D-Ala^2, NMePhe^4, Gly-ol^5]enkephalin (DAMGO), a selective μ-opioid receptor agonist, inhibited the cholinergic response to EFS at 8 Hz in a dose-dependent manner in main bronchi (IC_{50} = 113 nM with a maximal inhibition of 35.7 ± 5.6% 10 μM, n = 5). In the lower trachea, DAMGO inhibited the cholinergic response to a similar extent (inhibition of 35.8 ± 3.5% at 10 μM, n = 5). However, DAMGO had no effect on the contractile response to exogenously applied acetylcholine in the main bronchi. By contrast, opioids had no inhibitory effect on cholinergic neurotransmission in the upper trachea. DAMGO (1 μM) inhibited the cholinergic response to EFS in a frequency-dependent manner in the main bronchi with greater inhibition at lower frequencies of stimulation.

4 The δ-opioid receptor agonist [D-Pen^2, D-Pen^5]enkephalin (DPDPE) significantly inhibited the cholinergic component of the constrictor response to EFS at 8 Hz in the bronchi but at the highest dose used (10 μM). U-50,488H, a κ-receptor agonist, had no inhibitory effect on the cholinergic constrictor component in the main bronchi (10 μM).

5 DAMGO also inhibited the NANC responses to EFS in the main bronchi in a dose-dependent manner (with an IC_{50} = 36 nM and a maximal inhibition of 63.4 ± 8.3%, at 1 μM, n = 5). DAMGO had no effect on contractile responses to exogenously applied substance P (SP). DPDPE (10 μM) was less effective in inhibition of the NANC bronchoconstriction with a maximal inhibition of 29.2 ± 4.2% (n = 7), and U-50,488H (10 μM) had no inhibitory effect.

6 After capsaicin pretreatment, which depleted sensory nerves of neuropeptides, the inhibitory effect of DAMGO (1 μM) on cholinergic constriction in main bronchi at 8 Hz was only 13.4 ± 1.9% (n = 13) compared with 32.9 ± 4.0% (n = 9) inhibition in vehicle-treated controls ($P < 0.001$).

7 Opioids may reduce the cholinergic neural responses in airways partly via an inhibitory action on excitatory NANC nerves and partly by a direct effect on cholinergic neurotransmission. The opioid receptor involved is of the μ-opioid receptor subtype.

Introduction

Opioid receptors appear to be present on capsaicin-sensitive sensory nerves (Laduron, 1984) which contain neuropeptides including substance P and neurokinin A. Opioid agonists inhibit stimulus-evoked release of substance P from the rat trigeminal nucleus *in vitro* (Jessell & Iversen, 1977).

Non-adrenergic non-cholinergic (NANC) bronchoconstriction evoked by electrical field stimulation (EFS) *in vitro* and vagal stimulation *in vivo* is due to release of tachykinins from sensory nerves in airways (Lundberg *et al.*, 1983b; Andersson & Grundstrom, 1983). Opioids inhibit the NANC constrictor response in the guinea-pig *in vivo* and in the guinea-pig bronchi *in vitro* (Frossard & Barnes, 1987; Bartho *et al.*, 1987; Belvisi *et al.*, 1988) by preventing release of tachykinins, probably via a μ-opioid receptor on airway afferent nerves.

We have now investigated whether opioids may also influence cholinergic neurotransmission in guinea-pig airways and whether this inhibition might be related to an effect on NANC bronchoconstrictor nerves.

We have also classified the receptor subtype involved *in vitro* using the more selective agonists [D-Ala^2, NMePhe^4, Gly-ol^5]enkephalin (DAMGO), [D-Pen^2, D-Pen^5]enkephalin (DPDPE) and U-50,488H (*trans*-3,4-dichloro-N-methyl-N-(2-(1-pyrrolidinyl)cyclohexyl)-benzeneacetamine) for μ, δ and κ-opioid receptors, respectively.

Methods

Male Dunkin-Hartley guinea-pigs (250–500 g) were killed by cervical dislocation. The lungs with the bronchi and trachea were removed and placed in Krebs-Henseleit (KH) solution of the following composition (mM): NaCl 118, KCl 5.9, $MgSO_4$ 1.2, $CaCl_2$ 2.5, NaH_2PO_4 1.2, $NaHCO_2$ 25.5 and glucose 5.05; it was gassed continuously with a mixture of 95% O_2 and 5% CO_2 to give pH 7.4.

After being stripped of connective tissue, the trachea was opened longitudinally by cutting through cartilage. The trachea was cut into sections upper (laryngeal) and lower (carinal) so that each segment contained 3–4 cartilaginous strips. From the proximal end of the main bronchi ring, segments 2–3 mm in length were prepared. Each segment was suspended in a 10 ml organ bath containing KH solution. Indomethacin was present throughout at a concentration of 10 μM. The solution was maintained at 37°C. The tissues were allowed to equilibrate for 1 h with frequent washing, under a resting tension of 1 g for the trachea and 0.5 g for the main bronchi, which were found to be optimal for measuring

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changes in tension. Isometric contractile responses were measured with a Grass FT.03 force-displacement transducers and recorded on a polygraph (Grass Model 7D, Grass Instruments Co., Quincy, Mass., U.S.A).

Electrical field stimulation

The effect of opioids on responses of main bronchi and trachea to EFS was studied by suspending the tissues between parallel platinum wire electrodes (approximately 1.5 cm apart). Biphasic square wave pulses were delivered for 20 s periods from a Grass S88 stimulator, with a supramaximal voltage of 40 V at source, frequencies of between 0.25–32 Hz and a pulse duration of 0.5 ms.

Stimuli were delivered every 30 min, allowing sufficient time between stimulation for each tissue to return to its resting tension. At least three consistent EFS responses were obtained to check the reproducibility of the response, and tissues giving variable results were not studied. The responses to EFS in main bronchi were stable over 4–6 h. Opioids were added 12 min before stimulation (Frossard & Barnes, 1987) in all tissues studied and only one concentration of opioid was tested in each tissue.

The contractile responses induced by EFS could be abolished by tetrodotoxin (1 μM), confirming the neural nature of the response.

Acetylcholine and substance P concentration-response relationships

The effects of DAMGO (1 μM) on cumulative concentration-response relationships to acetylcholine (ACh) (10 nM–10 μM) and to substance P (1 nM–10 μM) were studied in main bronchi. The substance P results were expressed as a percentage of the maximum contraction to carbachol (1 mM) because a maximum contraction to substance P was not determined.

Capsaicin pretreatment

Capsaicin was dissolved to a concentration of 50 mg ml^{-1} in 10% alcohol, 10% Tween 80 and 80% saline. Guinea-pigs were anaesthetized with ketamine hydrochloride (50 mg kg^{-1}, i.m.) and xylazine (0.1 mg kg^{-1}, i.m.) and pretreated 1 h before this with aminophylline (25 mg kg^{-1}, i.p.) and terbutaline (0.1 mg kg^{-1}, s.c.) to prevent bronchoconstriction. A single capsaicin injection of 50 mg kg^{-1} s.c. was then given (Lundberg *et al.*, 1983a). Animals treated in this way were killed one week after the injection. Depletion of sensory neuropeptides was confirmed at the end of each experiment by the lack of response to capsaicin (10 μM).

Drugs and solutions

Drugs and chemicals were obtained from the following sources: substance P, acetylcholine chloride, indomethacin, tetrodotoxin, Tween 80 (polyoxyethylenesorbitan monooleate), capsaicin (Sigma Chemical Co., Poole, Dorset), naloxone hydrochloride (Du Pont U.K., Stevenage, Hertfordshire), terbutaline sulphate BP (Astra Pharmaceuticals Ltd., Kings Langley, Herts), aminophylline (Antigen Ltd., Roscrea, Ireland), [D-Ala^2, NMePhe^4, Gly-ol^5]-enkephalin (DAMGO), [D-Pen^2, D-Pen^5]enkephalin (DPDPE) (Bachem Feinchemikalien AG, Bubendorf, Switzerland), *trans*-3,4-di-chloro-N-methyl-N-(2-(1-pyrrolidinyl)cyclohexyl)benzeneacetamine (U-50,488H) (a kind gift from Upjohn Company, Kalamazoo, U.S.A), ketamine hydrochloride (Ketalar) (Parke-Davis, Parke, Davis and Co., Pontypool, Gwent), xylazine (Bayer U.K. Ltd., Agrochem Division, Bury St. Edmunds, Suffolk). Aliquots of the opioid compounds were dissolved in distilled water and stored at −20°C. Indomethacin was made up in alkaline phosphate buffer (pH 7.8) of the following composition (mM): KH_2PO_4 20, Na_2HPO_4 120. Fresh drug solutions were made up daily. Drug additions did not exceed 1% of the bath volume. All concentrations refer to the final bath concentration.

Analysis of results

Contractile responses were expressed as absolute changes in tension, and then transformed to a mean response for three control stimulations obtained to EFS in each tissue. The effect of opioids on the mean responses was then expressed as a percentage inhibition. The effects of exogenous drug additions on EFS in each tissue were assessed by use of Student's *t* test for paired and unpaired data (when comparing responses of tissues from groups of animals which had undergone various pretreatments). Probability values of <0.05 were considered significant.

Results

Electrical field stimulation in trachea and main bronchi

Electrical field stimulation (EFS) of the lower trachea and main bronchi elicited a biphasic response which represented a rapid, atropine-sensitive, cholinergic contraction and a longer-lasting NANC contraction (Figure 1). This NANC contraction was abolished by capsaicin pretreatment leaving the initial cholinergic component, which was blocked by atropine (Figure 1). However, in the upper trachea EFS produced only a rapid, cholinergic response (Figure 2c).

Effect of opioid agonists on cholinergic responses to EFS in main bronchi

DAMGO (1 μM) inhibited the cholinergic response to EFS in a frequency-dependent manner (Figure 3) with greater inhibition at lower frequencies of stimulation. DAMGO (1 nM–10 μM) inhibited the cholinergic contraction produced at 8 Hz, in a concentration-dependent manner (IC_{50} = 113 nM, with a maximal inhibition of 34.1 ± 3.9% at 1 μM, n = 7) (Figure 4) and was completely reversed by naloxone at concentrations ranging from 100 nM–10 μM (Figure 2a). DAMGO (1 nM–

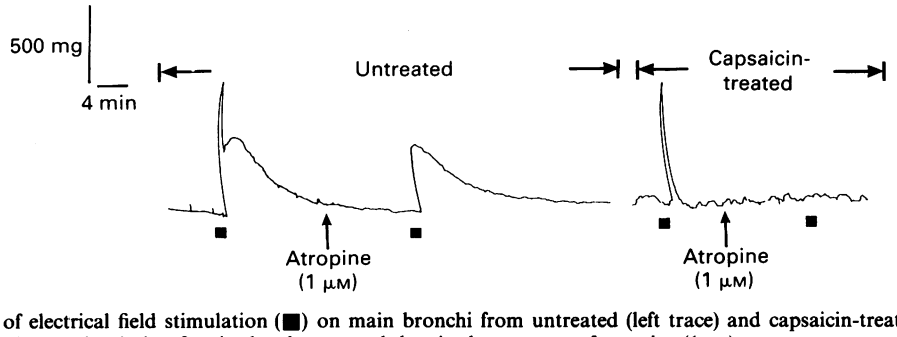

Figure 1 Effect of electrical field stimulation (■) on main bronchi from untreated (left trace) and capsaicin-treated animals (right trace). Each trace shows stimulation first in the absence and then in the presence of atropine (1 μM).

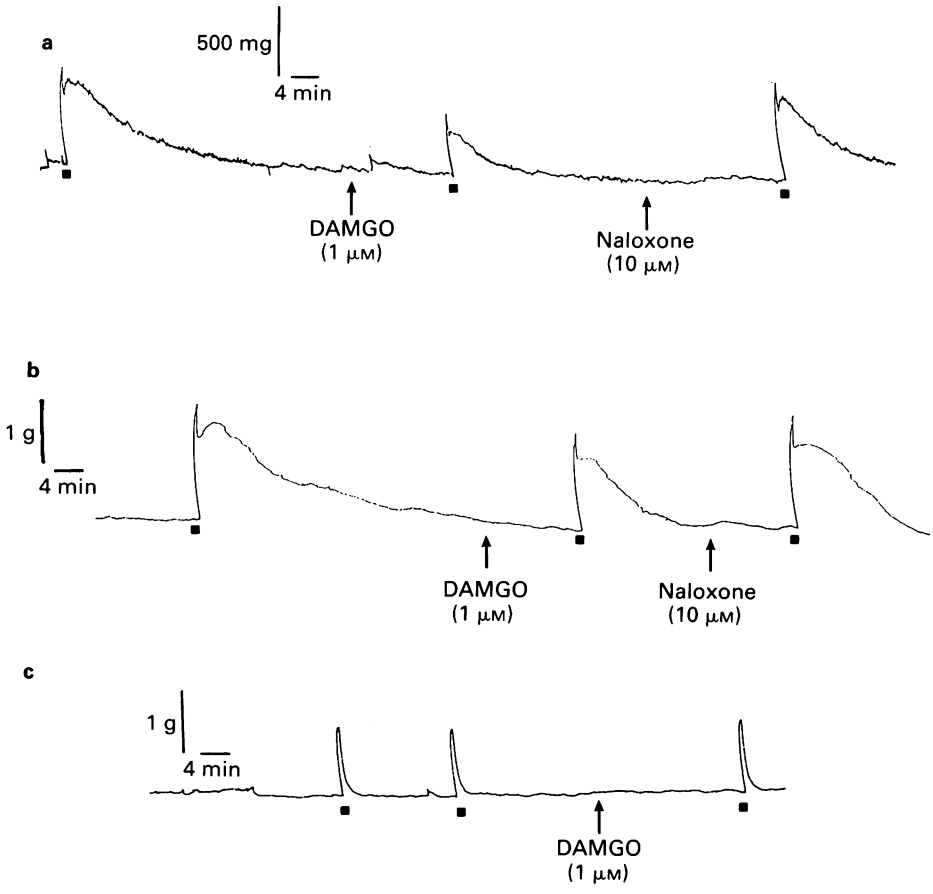

Figure 2 Effect of [D-Ala^2, NMePhe^4, Gly-ol^5]enkephalin (DAMGO) (1 μM) on electrical field stimulation (■) responses in guinea-pig airways *in vitro*, on the cholinergic and NANC components of the response. The inhibition of both responses is reversed by naloxone (10 μM). Typical traces from main bronchi (a), lower trachea (b) and upper trachea (c) are shown. Stimulation parameters: 40 V, 0.5 ms, 8 Hz for 20 s.

10 μM) had no effect on resting tone of bronchial smooth muscle (Figure 2a). In previous studies, naloxone (10 μM) has been shown to be effective in reversing the inhibitory effect of morphine without non-specific effects (Ueda *et al.*, 1985; Frossard & Barnes, 1987). Experiments were performed in which the tissues were stimulated again 32 min after the addition of DAMGO (instead of stimulating in the presence of naloxone as usual) and there was still significant inhibition of both NANC and cholinergic responses, indicating the persistence of the opioid peptides under our experimental conditions. These responses were still reversible by naloxone (10 μM) when the tissue was stimulated. Furthermore, when naloxone (10 μM) was given before the administration of DAMGO the μ-agonist had no inhibitory effect on NANC or cholinergic responses. Naloxone alone (10 μM) had no effect on the resting tone or on cholinergic or NANC responses to EFS in guinea-pig bronchi.

DAMGO (1 μM) had no effect on the contractile response to exogenously applied acetylcholine (EC_{50} = 13.4 μM before and 10.8 μM after DAMGO, n = 8) (Figure 5).

DPDPE significantly inhibited the cholinergic component of the constrictor response to EFS in the bronchi only at the highest dose used (10 μM) (n = 7) (Figure 4) whereas U-50,488H (10 μM) had no inhibitory effect on the cholinergic response to EFS in the main bronchi (n = 8) (Figure 4).

Effect of DAMGO on trachea

In the lower trachea the contractions had a NANC component; DAMGO inhibited the cholinergic neural response (Figure 2b) in a concentration-dependent manner with a maximal inhibition of 35.8 ± 3.5% at 10 μM (n = 5) (Figure 6). By contrast DAMGO had no inhibitory effect on the cholinergic response in the upper trachea (n = 8) (Figure 2c and Figure 6) which had no excitatory NANC component (Figure 2c).

Effect of opioid agonists on NANC responses to EFS in main bronchi

DAMGO inhibited the NANC component to EFS at 8 Hz in a dose-dependent manner (IC_{50} = 36 nM with a maximal inhibition of 63.4 ± 8.3% at 1 μM, n = 6) (Figure 7) which was completely reversed by naloxone (10 μM) (Figure 2a).

DAGOL (1 μM) had no effect on the contractile response to exogenous substance P (EC_{50} = 0.6 μM before and 0.6 μM after DAMGO, n = 6) (Figure 8).

DPDPE (100 nM–10 μM) inhibited the NANC constrictor response with a maximal inhibition of 29.2 ± 4.2 at 10 μM (n = 7) whereas U-50,488H (10 μM) had no inhibitory effect (n = 8) (Figure 7).

Effect of capsaicin pretreatment on the inhibitory effect of DAMGO

After capsaicin pretreatment, the excitatory NANC component in the main bronchi was abolished when compared with tissue from vehicle-treated animals (Figure 9). The inhibitory effect of DAMGO (1 μM) on the cholinergic component of the constrictor response to 8 Hz in the main bronchi was greatly reduced in capsaicin-treated animals (13.4 ± 1.9%,

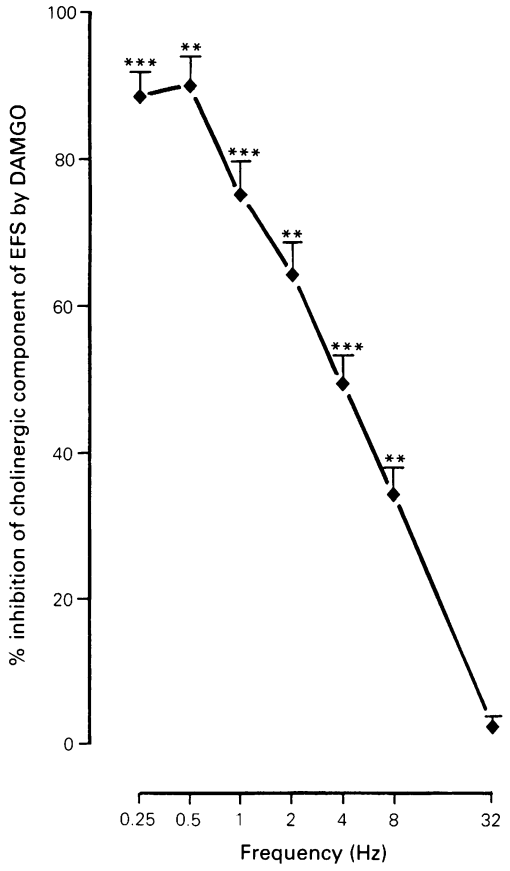

Figure 3 Frequency-dependent inhibition of the cholinergic response to electrical field stimulation (EFS, 40 V, 0.5 ms for 20 s) in main bronchi by [D-Ala^2, NMePhe^4, Gly-ol^5]enkephalin (DAMGO, 1 μM). Mean values (with s.e.mean indicated by vertical bars) of 7–10 animals are shown. Significance of inhibition: ***$P < 0.001$, **$P < 0.01$.

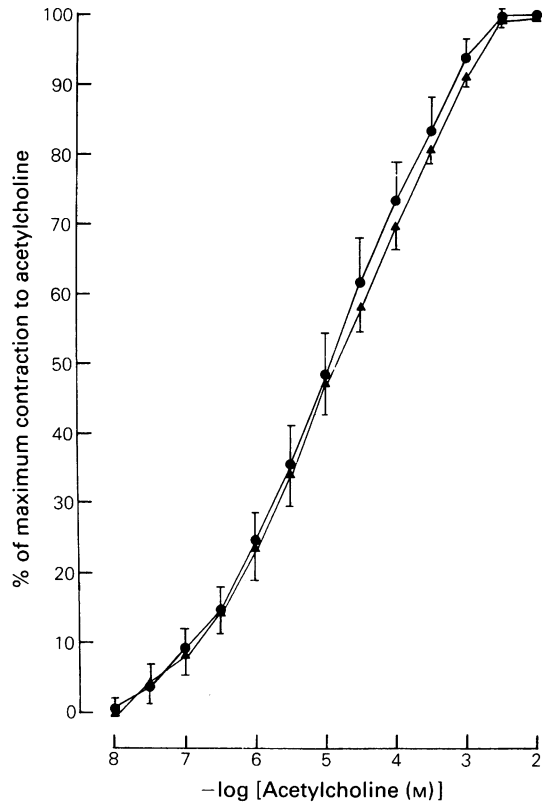

Figure 5 Concentration-response to acetylcholine (expressed as a % of the maximum contraction) in the presence (●) and absence (▲) of [D-Ala^2, NMePhe^4, Gly-ol^5]enkephalin (DAMGO, 1 μM). Points represent means, $n = 8$; vertical bars indicate s.e.means.

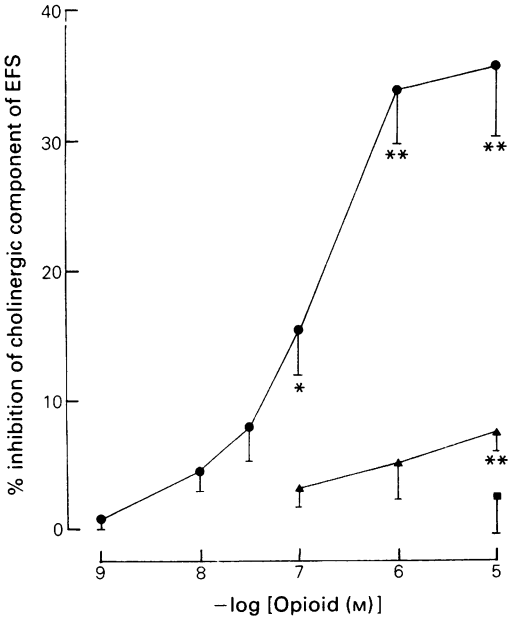

Figure 4 Concentration-dependent inhibition of cholinergic responses to electrical field stimulation (EFS, 40 V, 0.5 ms, 8 Hz for 20 s) by [D-Ala^2, NMePhe^4, Gly-ol^5]enkephalin (DAMGO, ●), [D-Pen^2, D-Pen^5]enkephalin (DPDPE, ▲) and U-50,488H (■) in main bronchi. Means of 5 animals with s.e.mean shown by vertical bars. Significance of inhibition: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

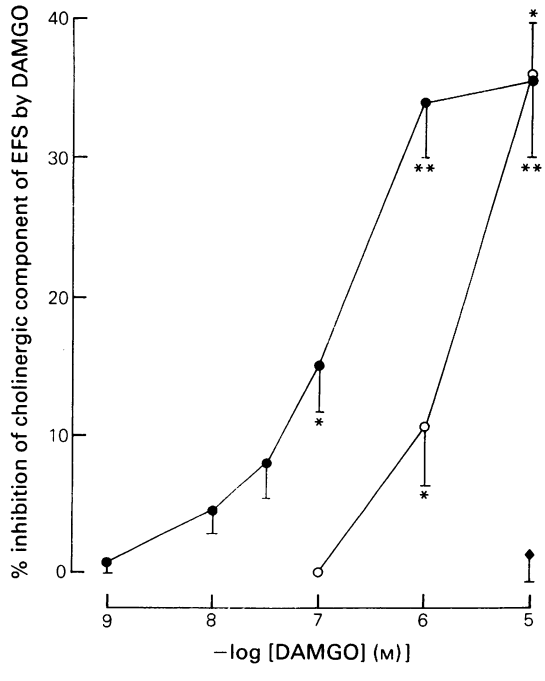

Figure 6 Inhibition of cholinergic response to electrical field stimulation (EFS, 40 V, 0.5 ms, 8 Hz for 20 s) by [D-Ala^2, NMePhe^4, Gly-ol^5]- enkephalin (DAMGO, 1 μM) in main bronchi (●), lower trachea (○) and upper trachea (◆). Means for five animals, are shown with s.e.mean indicated by vertical bars; significance of inhibition: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

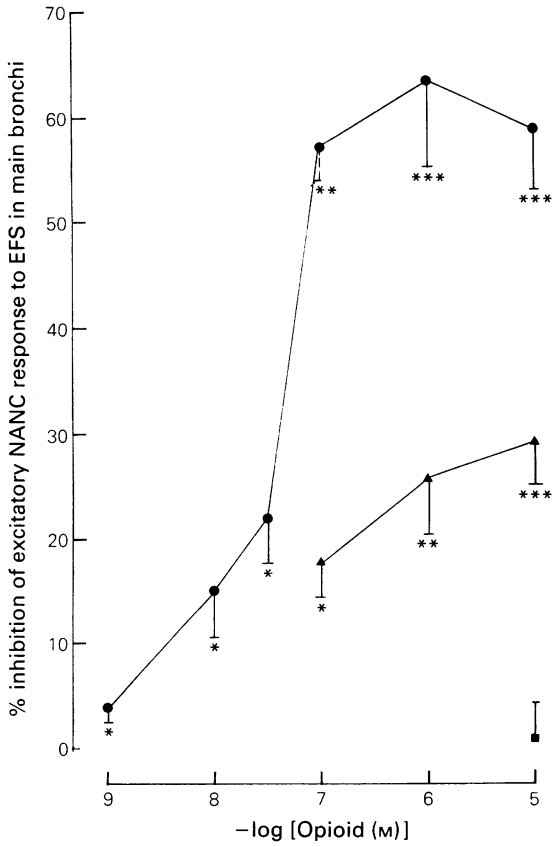

Figure 7 Inhibition of excitatory NANC response to electrical field stimulation (EFS, 40 V, 0.5 ms, 8 Hz for 20 s) by [D-Ala^2, NMePhe^4, Gly-ol^5]enkephalin (●), [D-Pen^2, D-Pen^5]enkephalin (▲), U-50,488H (■) in main bronchi. Means of 5 animals are shown with s.e.mean indicated by vertical bars; significance of inhibition: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

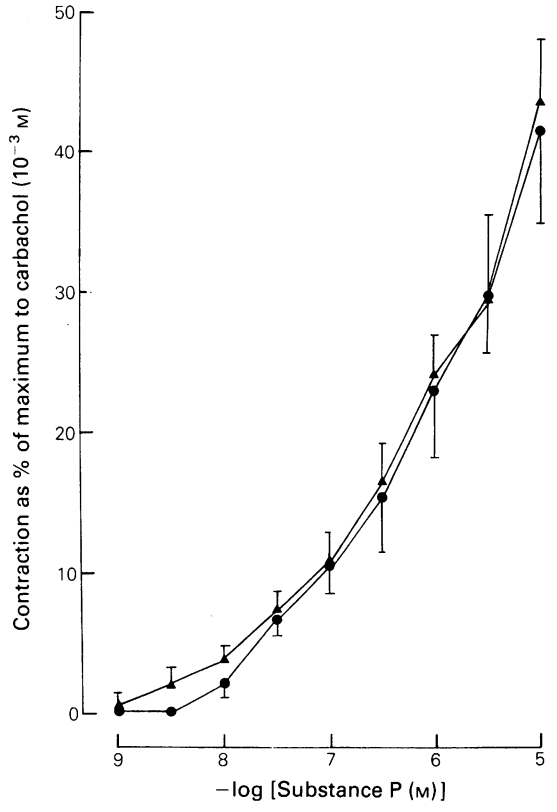

Figure 8 Concentration-response relationship to substance P (expressed as a % of the maximal contraction to carbachol 1 mM) in the presence (●) and absence (▲) of [D-Ala^2, NMePhe^4, Gly-ol^5] enkephalin (1 μM). Mean values of 6 animals are shown with s.e.mean indicated by vertical bars.

$n = 13$, compared with $34.1 \pm 3.9\%$, $n = 7$, in untreated animals and $32.9 \pm 4\%$, $n = 9$, in vehicle-treated controls; $P < 0.001$) (Figure 10).

Discussion

Our results confirm previous observations that opioids inhibit the NANC constrictor component to EFS in guinea-pig bronchi *in vitro* (Frossard & Barnes, 1987; Bartho *et al.*, 1987). DAMGO, a selective μ-opioid receptor agonist, caused a concentration-dependent inhibition of NANC bronchoconstriction, an effect that was antagonized by naloxone. A selective δ-agonist DPDPE also reduced the NANC response, although only at a high concentration (10 μM) and to a much lesser extent. A selective κ-agonist, U-50,488H, had no effect on the NANC response to EFS. This confirms that a μ-opioid receptor is involved in the modulation of NANC responses to EFS. The activity of DPDPE suggests that either δ-receptors are also involved or that DPDPE has an action on μ-receptors. The latter possibility is more likely since DPDPE is not highly selective and has been shown to interact with μ-receptors in the mouse vas deferens (Hirning *et al.*, 1985). The μ-receptor activity of DPDPE has also been demonstrated by its agonist activity in the guinea-pig myenteric-plexus in which δ-receptors are absent (Mosberg *et al.*, 1983). DAMGO had neither a direct action on bronchial smooth muscle nor any effect on substance P-induced contraction. Therefore, its

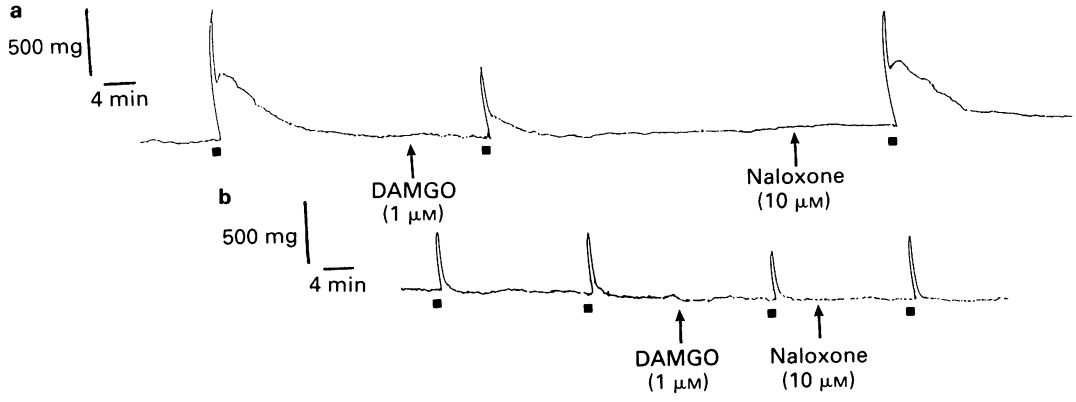

Figure 9 Tracings illustrating the inhibition of cholinergic responses to electrical field stimulation (■) (EFS, 40 V, 0.5 ms, 8 Hz for 20 s) by [D-Ala^2, NMePhe^4, Gly-ol^5]enkephalin (DAMGO, 1 μM) in (a) control (vehicle-treated) animals and (b) capsaicin-treated animals in which there was no excitatory NANC component present.

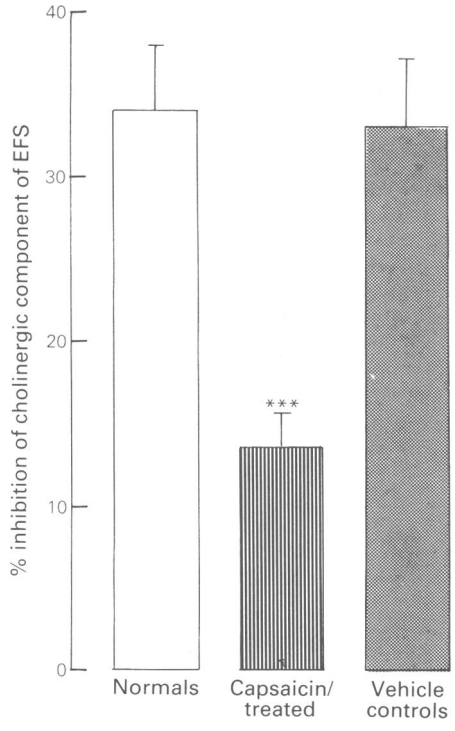

Figure 10 Histogram illustrating the degree of inhibition of responses to electrical field stimulation (EFS, 40 V, 0.5 ms, 8 Hz for 20 s) by [D-Ala^2, NMePhe^4, Gly-ol^5]enkephalin (DAMGO, 1 μM) in normal animals ($n = 7$), capsaicin-treated animals ($n = 13$) and in control animals which had been pretreated with the vehicle for capsaicin ($n = 9$). Mean values are shown, vertical bars indicate s.e.mean; significance of inhibition: ***$P < 0.001$.

effects are likely to be mediated via μ-opioid receptors on sensory nerves which modulate the release of neuropeptides. This supports *in vivo* studies which suggest that a μ-opioid receptor mediates the inhibition of excitatory NANC nerves (Belvisi *et al.*, 1988).

Our results also suggest an inhibitory effect of opioids on cholinergic neurotransmission in guinea-pig main bronchi. This is in contrast to our previous study (Frossard & Barnes, 1987) in which a higher frequency of stimulation (32 Hz) was used. Our explanation for the discrepancy between these results is that the inhibition of the cholinergic response seems to be inversely related to the frequency of stimulation. The cholinergic and NANC responses to EFS both increase with frequency but opioids are only effective at inhibiting release from cholinergic nerves at low frequencies of stimulation. These results are in accordance with the results of Russell & Simons (1985) who demonstrated that opioid-mediated inhibition of cholinergic nerves was frequency-dependent in canine airways. Previously, it has been demonstrated that morphine reduces the release of acetylcholine when the myenteric plexus longitudinal muscle preparation is electically stimulated (Cowie *et al.*, 1968) an effect which is more marked at low than at high frequencies of stimulation (Paton, 1957).

In the present study DAMGO inhibited the cholinergic response to EFS in a dose-dependent manner in main bronchi and the lower trachea, but had no effect on the contractile response to exogenous acetylcholine. This suggests a presynaptic inhibitory action of opioids on cholinergic nerves modulating the release of acetylcholine. Both the NANC response and cholinergic response were affected by DAMGO with IC_{50} values of 36 nM and 113 nM respectively.

The δ-receptor agonist, DPDPE, significantly inhibited the cholinergic component to EFS only at the highest concentration used, whereas the κ-agonist U-50,488H had no inhibitory effect. Therefore, the same subtype of opioid receptor is likely to be involved in both NANC and cholinergic nerves. Our results on inhibition of cholinergic neurotransmission are in agreement with those of Russell & Simons (1985) who demonstrated an inhibitory effect of opioids on cholinergic neural responses in canine airways although their results suggested that a δ-receptor was involved.

DAMGO had no inhibitory effect on cholinergic neurotransmission in upper tracheal segments in which an excitatory NANC response to EFS was absent. This suggests that the NANC neurotransmitter (probably a tachykinin) may facilitate cholinergic neurotransmission and that it is this facilitatory effect that is primarily inhibited by opioids. Exogenous tachykinins do indeed facilitate neurotransmission in postganglionic cholinergic nerves in this species (Hall *et al.*, 1989). Furthermore, capsaicin pretreatment, which depletes sensory neurones of SP, reduces the cholinergic response in airways to EFS *in vivo* (Martling *et al.*, 1984) and also *in vitro* (Stretton *et al.*, 1989). As expected this facilitation is more prominent in the lower trachea and bronchi than in the upper trachea. In order to establish whether opioids inhibited cholinergic neurotransmission indirectly via an effect on this facilitatory action of excitatory NANC nerves or by a direct action on the cholinergic nerves we examined the effect of DAMGO on cholinergic neurotransmission at a frequency optimal for evoking a NANC response in bronchi of capsaicinised animals. Capsaicin pretreatment completely abolished the excitatory NANC component and reduced the inhibitory effect of DAMGO by approximately 60%. Thus a component of the inhibitory effect of opioids on cholinergic neurotransmission may be mediated by an inhibition of the facilitatory action, although another component appears to be due to a direct modulation of cholinergic nerves.

In conclusion, opioids reduce the cholinergic and excitatory NANC responses to EFS in guinea-pig airways. The inhibitory action on cholinergic nerves is partly through inhibition of the facilitatory interaction of excitatory NANC nerves on cholinergic nerves.

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Role of β-adrenoceptor-adenylate cyclase system in the developmental decrease in sensitivity to isoprenaline in foetal and neonatal rat heart

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1 The inotropic and chronotropic sensitivity to noradrenaline and isoprenaline (Iso) of foetal and neonatal rat heart decreases as the heart becomes sympathetically innervated. In the present study, we have examined adenylate cyclase (AC) activation and β-adrenoceptor binding to determine whether a developmental decrease in sensitivity was demonstrable in the β-receptor-AC system of atrial and ventricular membranes from the 15 day foetus and 1 day and 7 day neonates.

2 While the maximum activation of AC by Iso increased with age, the sensitivity expressed in terms of pD_2 values decreased from the 15th foetal day to the first day after birth in the atria, and from the first day to the 7th day after birth in the ventricle.

3 In contrast, activation of AC by forskolin was almost identical at all ages both in atria and ventricle.

4 The maximum equilibrium binding of [^{3}H]-dihydroalprenolol decreased with age, the dissociation constant being about the same at all ages in both the atria and ventricle.

5 In conclusion, we have demonstrated a developmental decrease in the sensitivity of AC to Iso in myocardial membrane fractions consistent with the developmental decrease in chronotropic and inotropic sensitivity to β-adrenoceptor agonists. Although a reduction in β-adrenoceptor number partly accounts for the decrease in sensitivity, some other factors such as decreased coupling to AC may largely be responsible.

Introduction

It is known that nerves exert a trophic influence upon muscle to regulate its responsiveness to neurotransmitters and exogenously applied agonists. This phenomenon has been most extensively investigated in skeletal and smooth muscle. However, relatively little is known regarding the trophic influence of autonomic nerves on cardiac muscle. There are several reports including those from our own laboratory, which suggest that sympathectomy of the rat heart results in postjunctional supersensitivity to agonists (Nomura *et al.*, 1980; Ishii *et al.*, 1982; 1985; Goto *et al.*, 1985). In organ culture conditions, in the absence of sympathetic innervation, rat atria acquire a high sensitivity similar to that after denervation, which can be prevented by adding the neurotransmitter (noradrenaline) to the culture medium (Tanaka *et al.*, 1988a). These results support the hypothesis that sympathetic nerves exert a trophic influence upon cardiac muscle to maintain its sensitivity at a normal level.

With respect to changes in the chronotropic and inotropic sensitivity of cardiac muscle to noradrenaline (NA) and isoprenaline (Iso) during development, we have recently shown that the β-adrenoceptor sensitivity is high in the foetus followed by a ten fold decrease, which occurs in the late foetal period at the sinus node and during the first postnatal week in the ventricle (Shigenobu *et al.*, 1988; Tanaka *et al.*, 1988a,b). The time course of this decrease correlates well with the development of sympathetic innervation in each region (Shigenobu *et al.*, 1988; Tanaka *et al.*, 1988a,b), which also suggests that myocardial sensitivity is influenced by sympathetic innervation.

As for the molecular basis of the developmental decrease in sensitivity, we have suggested the contribution of some changes in the β-receptor-adenylate cyclase (AC) system based on pharmacological considerations (Shigenobu *et al.*, 1988; Tanaka *et al.*, 1988a), but no direct measurement has been made. Therefore, in the present study, we examined β-adrenoceptor binding and the activation of adenylate cyclase in foetal and neonatal rat myocardial membranes to determine whether the developmental decrease in sensitivity is demonstrable at the level of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) production and whether it is accompanied by changes in receptor number and/or affinity. We used atrial and ventricular membranes from the 15 day foetus, 1 day neonate and 7 day neonate, and compared the time course of the changes with the developmental decrease in chronotropic and inotropic sensitivity.

Methods

Pregnant Wistar strain rats were anaesthetized with ether and foetuses were removed on the 15th day of gestation (birth usually occurs on the 21st day of gestation). The atria and ventricle were obtained from these 15-day foetuses, and one day and 7-day old neonates and they were used either for AC measurements or for binding assays.

AC measurements were performed according to methods previously reported (Ishikawa *et al.*, 1988). Tissues were homogenized with a glass-Teflon homogenizer in 20 mM Tris/HCl buffer (pH 7.4) containing 0.25 M sucrose. The homogenate was centrifuged at 100,000 *g* for 1 h, and the pellet resuspended in the buffer was used for the measurements. Membrane fractions equivalent to 30–40 μg protein were incubated at 37°C for 10 min in 300 μl of the reaction mixture. The reaction mixture consisted of (mM): Tris/HCl 40 (pH 7.4), KCl 10, $MgCl_2$ 5, EGTA 1, ATP 1, theophylline 10, creatine phosphate 5, and GTP 1 μM, creatine kinase 75 μg ml^{-1} and bovine

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serum albumin 1 mg ml^{-1}. Various concentrations of Iso or forskolin were added. Reactions were terminated by boiling for 3 min. Cyclic AMP thus formed was assayed by means of a radioimmunoassay kit (Yamasa Shouyu). The increase in AC activity produced by each concentration of drug was calculated. The pD_2 values were obtained by proportional allotment between the two concentrations which produce nearly a half-maximal response, as described previously (Tanaka & Shigenobu, 1989). pD_2 is defined as the negative logarithm of the molar concentration of a drug required to produce a half maximum response. Therefore, pD_2 is an indicator of sensitivity to a drug and a higher pD_2 value reflects a higher sensitivity.

Binding assays were performed according to the methods previously described (Abe *et al.*, 1988; Koike *et al.*, 1988). The isolated atria and ventricles were minced and homogenized with a polytron homogenizer (Kinematica; setting 11, 5 s × 5 times) in 10 ml ice-cold sucrose buffer. The homogenate was first centrifuged at 3000 *g* for 10 min. The pellet was resuspended in 10 ml of sucrose buffer and recentrifuged under the same conditions. The supernatants from the two centrifugations were mixed well and further centrifuged at 10,000 *g* for 15 min. The resulting supernatant was centrifuged at 100,000 *g* for 60 min. The final pellet was resuspended in ice-cold Tris/HCl buffer (pH 7.4), and used for the assays: 200 μl of the final suspension equivalent to 15–20 μg protein was incubated with various concentrations of [^{3}H]-dihydroalprenolol ([^{3}H]-DHA) at 37°C for 30 min. The incubation was terminated by filtration through Whatman GF/F glass filters, which were rinsed twice with 3 ml ice-cold Tris/HCl buffer. The filters were dried and radioactivities were counted with a liquid scintillation counter (Aloka, LSC-700). The amount of [^{3}H]-DHA bound in the absence and presence of 10 μM propranolol was regarded as total and non-specific binding, respectively. The maximum binding capacity (B_{max}) and the equilibrium dissociation constant (K_d) were calculated from Scatchard analysis of the saturation data. Regression lines were drawn by the least square method.

Data were expressed as the mean ± s.e.mean of three independent observations each performed in duplicate. Statistical analyses were carried out by Bartlett's analysis of variance followed by Tukey's test for multiple comparisons (Grimm, 1973).

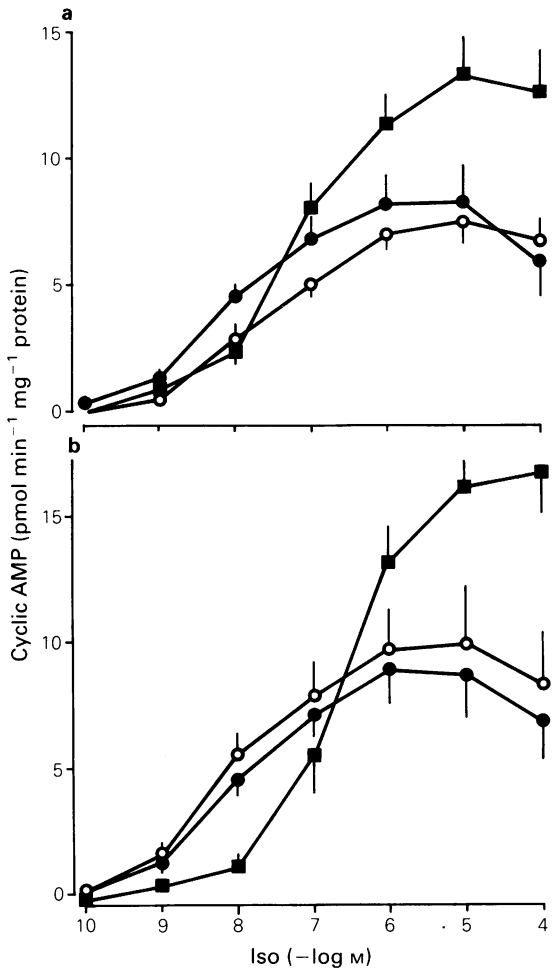

Figure 1 Dose-response curves for the effect of isoprenaline (Iso) on adenylate cyclase activity of atrial (a) and ventricular (b) membranes from the foetus (●), 1-day old neonate (○) and 7-day old neonate (■). Abscissa scale: concentration of Iso (−log M); ordinate scale: cyclic AMP formed per min per mg protein (pmol). Each point represents the mean (with s.e.mean shown by vertical bars) from 3 experiments performed in duplicate.

Results

Stimulation of adenylate cyclase by isoprenaline

Both in the atrium and in the ventricle, there were no significant changes in the basal AC activity during development (Table 1). Iso, at concentrations from 10^{-9} M to 10^{-5} M, stimulated in a concentration-dependent fashion the AC activity of both atrial and ventricular membranes of all ages. The maximum response to Iso increased during the first postnatal week both in the atrium and in the ventricle (Figure 1). However, cyclic AMP production induced by 10^{-8} M Iso (around this concentration, chronotropic or inotropic response were examined) was greater in foetal atria and foetal and 1-day old ventricle. In fact, when pD_2 values for cyclic AMP production were calculated to determine the sensitivity changes, they decreased significantly from the foetal period to

Table 1 Activation of adenylate cyclase (AC) by isoprenaline

	Atria			*Ventricle*		
	Foetus	*1-day-old*	*7-day-old*	*Foetus*	*1-day-old*	*7-day-old*
Basal activity	8.9 ± 1.2	11.8 ± 1.1	12.9 ± 1.3	9.6 ± 1.2	11.6 ± 0.9	12.8 ± 1.9
Maximum increase by Iso	8.2 ± 1.6	7.5 ± 0.7	13.4 ± 1.6*	8.6 ± 1.7	9.8 ± 2.1	16.7 ± 1.6*
Increase by 10^{-8} M Iso	4.5 ± 0.4	2.9 ± 0.5*	2.3 ± 0.4*	4.6 ± 1.4	5.2 ± 1.2	0.8 ± 0.6*
pD_2 value for Iso (AC)	8.18 ± 0.17	7.60 ± 0.18*	7.23 ± 0.15*	8.05 ± 0.19	7.93 ± 0.18	6.81 ± 0.14*
pD_2 value for Iso (mechanical)	9.48 ± 0.13	8.52 ± 0.08*	8.54 ± 0.05*	8.80 ± 0.04	8.72 ± 0.09	7.54 ± 0.06*

Activities are expressed as pmol of cyclic AMP min^{-1} mg^{-1} protein. Values are the mean ± s.e.mean from three different experiments in duplicate. pD_2 values for the mechanical (chronotropic and inotropic) responses to Iso were obtained in our previous studies (Shigenobu *et al.*, 1988).

* Indicates statistically significant difference ($P < 0.01$) from the corresponding value in the foetus.

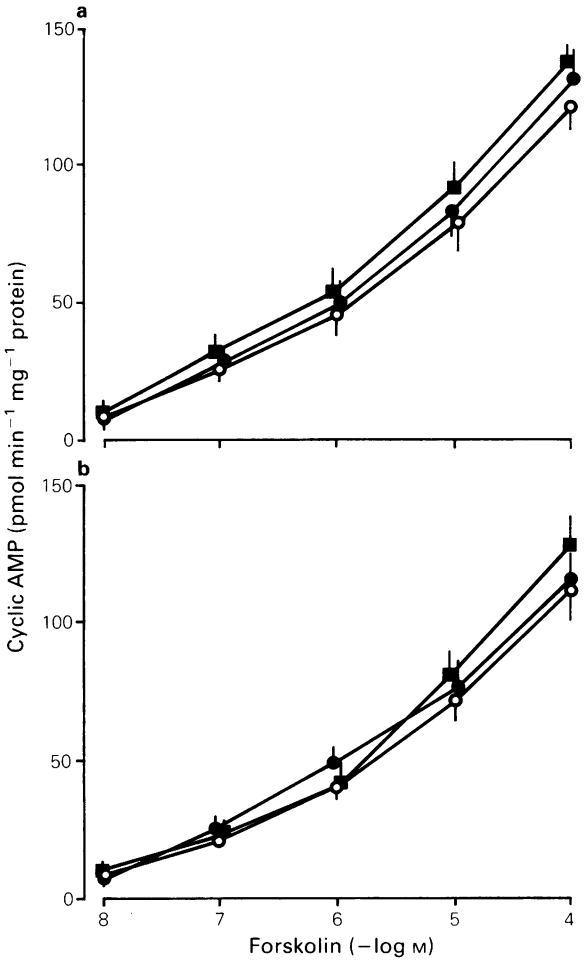

Figure 2 Dose-response curves for the effect of forskolin on adenylate cyclase activity of atrial (a) and ventricular (b) membranes from the foetus (●), 1-day old neonate (○) and 7-day old neonate (■). Abcissa scale: concentration of forskolin (−log M); ordinate scale: cyclic AMP formed per min per mg protein (pmol). Each point represents the mean (with s.e.mean shown by vertical bars) from 3 experiments performed in duplicate.

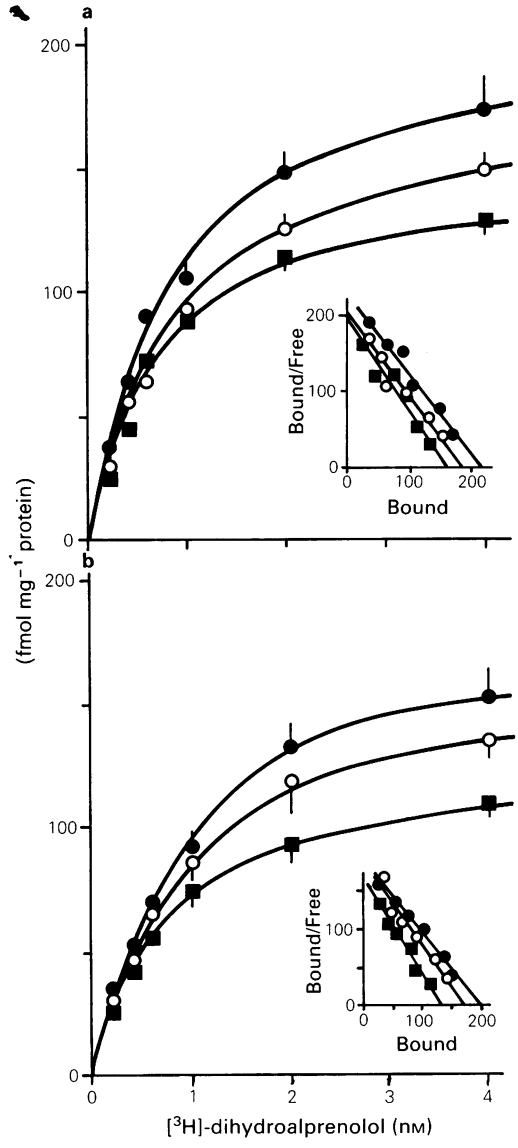

Figure 3 Saturation curves of specific [^{3}H]-dihydroalprenolol binding to atrial (a) and ventricular (b) membranes from the foetus (●), 1-day old neonate (○) and 7-day old neonate (■). Each point represents the mean (with s.e.mean shown by vertical bars) from 3 experiments performed in duplicate. Scatchard plots of the data are shown in the insets.

the first day after birth in the atrium, and from the first day to the 7th day after birth in the ventricle (Table 1). These decreases in sensitivity were paralleled by decreases in pD_2 values for the chronotropic and inotropic responses to Iso obtained in our previous study (Table 1).

Stimulation of adenylate cyclase by forskolin

Forskolin, which is known to activate directly the catalytic subunit of AC (Seamon & Daly, 1983), stimulated the AC activity in both atrial and ventricular membranes in a concentration-dependent manner and the dose-response curve was identical at all ages both in the atrium and in the ventricle (Figure 2). The increase in cyclic AMP production was much greater than that produced by Iso in all preparations.

Table 2 Summary of binding parameters

	Atria			*Ventricle*		
	Foetus	*1-day-old*	*7-day-old*	*Foetus*	*1-day-old*	*7-day-old*
B_{max} (fmol mg^{-1} protein)	217 ± 21	183 ± 11	156 ± 8*	197 ± 18	176 ± 10	127 ± 7*
K_d (nM)	0.94 ± 0.13	0.90 ± 0.08	0.79 ± 0.18	1.08 ± 0.08	0.97 ± 0.07	0.86 ± 0.12

β-Adrenoceptor number (B_{max}) and apparent dissociation constant (K_d) were determined by linear regression analysis of Scatchard plots in Figure 3. Values indicate the mean ± s.e.mean from 3 different experiments in duplicate.
* Indicates statistically significant difference ($P < 0.01$) from the corresponding value in the foetus.

[^3H]-dihydroalprenolol binding

Specific [^{3}H]-DHA binding to both atrial and ventricular membranes was saturable and of high affinity. The Scatchard plots of the data were linear in all preparations indicating the existence of a single binding site (Figure 3). The B_{max} for [^{3}H]-DHA decreased gradually with a similar developmental time course in the atrium and in the ventricle, i.e. the B_{max} value at 1 week after birth was 2/3 of that in the foetus both in the atrium and in the ventricle (Table 2). The dissociation constant was almost identical in all membranes (Table 2).

Discussion

The present study has demonstrated that a β-adrenoceptor agonist, Iso, was able to stimulate the AC system as early as the 15th day of gestation. This observation and our previous results (Shigenobu *et al.*, 1988) indicate that β-adrenoceptor agonists are able to activate AC as well as to produce chronotropic and inotropic responses as early as the 15th day of gestation, which is before the onset of sympathetic innervation (Gomez, 1958; Ishii *et al.*, 1982; Shigenobu *et al.*, 1988). It has been reported that, in the chick ventricle, Iso is able to activate AC and produce a positive inotropic effect in the 11-day old embryo while sympathetic innervation begins on the 16th embryonic day (Higgins & Pappano, 1981; Smith & Pappano, 1985). Therefore, it seems to be a general phenomenon that postjunctional cells are responsive to adrenoceptor agonists before innervation is established (Pappano 1977).

In cardiac muscle, it has been postulated that autonomic innervation may exert a trophic influence upon muscle properties (Shigenobu, 1983). With regard to the inotropic and chronotropic sensitivity (pD_2 values) of the rat myocardium during development, there seems to be general agreement that sensitivity to NA is high at foetal or neonatal stages followed by decrease with age (Mackenzie & Standen, 1980; Ishii *et al.*, 1982; Shigenobu, 1983; Shigenobu *et al.*, 1988; Tanaka *et al.*, 1988a,b). Although it has been suggested that these changes were caused by prejunctional mechanisms (Mackenzie & Standen, 1980), we have observed in the right atria and in the ventricle that sensitivity to Iso decreased in parallel to the sensitivity to NA during development (Shigenobu *et al.*, 1988; Tanaka *et al.*, 1988a,b), and concluded that the changes were mainly postjunctional in nature. This decrease in sensitivity correlates well with the increase in sympathetic innervation in each region, as determined by examining responsiveness to tyramine (Shigenobu *et al.*, 1988; Tanaka *et al.*, 1988a,b), NA content (Iversen *et al.*, 1967; Mirkin, 1972), intensity of the fluorescence of catecholamine containing fibres (De Champlain *et al.*, 1970; Lipp & Rudolph, 1972), capacity for NA uptake and retention (Iversen *et al.*, 1967) and by morphological observations (Gomez, 1958).

Under organ culture conditions, in the absence of neuronal influence, the sensitivity of foetal and neonatal atria to NA and Iso is maintained at a high level, similar to that observed in the foetal atria before innervation. Addition of the neurotransmitter (NA) to the culture medium results in normal sensitivity similar to that observed in intact atria with sympathetic innervation (Tanaka *et al.*, 1988a). Furthermore, there are several reports including those from our laboratory that sympathectomy of the rat heart results in postjunctional supersensitivity to agonists (Nomura *et al.*, 1980 Ishii *et al.*, 1982; 1985; Goto *et al.*, 1985). All these results support the hypothesis that sympathetic nerves exert trophic influences upon cardiac muscle to maintain its sensitivity at normal level.

Although the correlation between the sensitivity to agonists and sympathetic innervation has been demonstrated by many investigators, the cellular basis for these sensitivity changes has not been systematically investigated. It has been reported that sympathetic denervation of rat heart by 6-hydroxydopamine results in an increase in β-receptor number with no change in affinity (Nomura *et al.*, 1980), and that reserpine treatment induces an increase in β-adrenoceptor number (Latifpour & McNeil, 1984) as well as an increase in the maximum activation of AC by adrenaline (Cros & McNeil, 1987) in guinea-pig atrium.

We have already reported that the developmental decrease in sensitivity can be demonstrated in the presence of a phosphodiesterase inhibitor, isobutyl-methyl xanthine, and that the sensitivity to forskolin and dibutyryl cyclic AMP remains unchanged during development (Shigenobu *et al.*, 1988). These results suggest that the decrease in sensitivity is unlikely to be produced by an increase in phosphodiesterase activity or some changes in the process distal to cyclic AMP. It is possible that some change in the process leading to cyclic AMP production is the major cause of the decrease in sensitivity. In the present study, we measured the activation of AC by Iso and directly demonstrated a developmental decrease in sensitivity at the level of cyclic AMP production (Figure 1, Table 1). The decrease occurred before birth in the atria, and after birth in the ventricle, which is in good correlation with the decrease in chronotropic and inotropic sensitivity (Table 1). Therefore, the developmental decrease in chronotropic and inotropic sensitivity may be explained, at least in part, by the decrease in the sensitivity of the β-receptor-AC system.

The concentration-range of Iso that activated AC was much higher than that required to produce chronotropic and inotropic responses (Figure 1, Table 1), a phenomenon which has been documented by many investigators (Venter, 1979; Scholz, 1980; Porzig, 1982; Ishikawa *et al.*, 1988). Although the attenuation of the sensitivity of AC activity during the procedure of homogenization cannot be totally excluded (Porzig, 1982), it may be explained by considering 'spare receptors', namely, only partial activation of AC is sufficient to elicit mechanical responses (Venter, 1979). Therefore, the amount of cyclic AMP produced by threshold concentrations of Iso may be important in producing mechanical responses. Taking the above into account, the important observation made in the present study may be that there was a correlation between the increase in cyclic AMP production by 10^{-8} M Iso (which is in the concentration-range to produce chronotropic and inotropic responses) and the developmental decrease in sensitivity (Table 1).

In contrast to these changes in the responsiveness of AC to Iso, no developmental changes were observed in the responsiveness to forskolin (Figure 2). This is in agreement with our previous results which show that the inotropic and chronotropic sensitivity to forskolin remains unchanged during development (Shigenobu *et al.*, 1988; Tanaka *et al.*, 1988a). Therefore, it is most likely that the decrease in sensitivity is produced by some changes in the β-receptor and/or its coupling to AC. The degree of AC activation by forskolin was much greater than that by Iso in all preparations, which may be due to its direct action on the catalytic subunit of AC.

Both in the atrium and in the ventricle, the B_{max} of [^{3}H]-DHA binding decreased with age while the K_d values remained unchanged. This is in agreement with a previous report on ventricular membranes which showed a higher B_{max} of [^{3}H]-DHA binding in the 1-day old neonate compared to the adult (Whitsett & Beckerman, 1981). The present authors have also reported a developmental decrease in the B_{max} values for a partial β-receptor agonist [^{3}H]-befunolol in foetal and neonatal myocardium (Koike *et al.*, 1988). It is well known that the larger the number of receptors in a tissue, the larger the pD_2 value of the agonist that should be obtained (Kenakin, 1984). Therefore, the decrease in β-receptor number may partly account for the decrease in sensitivity. However, the magnitude of the decrease in receptor number seems to be rather small when compared with the tenfold decrease in sensitivity (Table 1, 2). Moreover, the time course of the developmental decrease in receptor number and that of the decrease in cyclic AMP production were not completely parallel. Therefore, it is likely that some other factors such as

decreased coupling to AC are also involved. Similar decreases in the sensitivity to Iso and changes in the β-receptor-AC system have been reported in the chick embryo ventricle before hatching (Higgins & Pappano, 1981; Smith & Pappano, 1985). As the subsensitivity was transient and was not prevented by reserpine administration, factors other than sympathetic innervation seem to be the cause of subsensitivity in the case of the chick embryo (Higgins & Pappano, 1981).

In summary, we have demonstrated a developmental decrease in the sensitivity of AC to Iso in myocardial membrane fractions, which may account for the developmental decrease in chronotropic and inotropic sensitivity to β-adrenoceptor agonists. Although a reduction in β-adrenoceptor number is partly responsible for the decrease in sensitivity, it seems most probable that some other factors such as decreased coupling to AC are also involved.

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Evidence that pinacidil may promote the opening of ATP-sensitive K^+ channels yet inhibit the opening of Ca^{2+}-activated K^+ channels in K^+-contracted canine mesenteric artery

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1 The effects of cromakalim and pinacidil on contraction and ^{86}Rb efflux were investigated in strips of canine mesenteric artery.

2 Cromakalim and pinacidil relaxed arterial strips precontracted with 20.9 mM K^+ with pD_2 values of 6.56 and 5.88, respectively.

3 High (above 10 μM) concentrations of pinacidil, but not cromakalim, relaxed arterial strips bathed by a medium containing 65.9 mM K^+, and inhibited Ca^{2+}-induced contractions in strips bathed by a medium containing 80 mM K^+. These findings suggested that pinacidil may act as an inhibitor of Ca^{2+} influx.

4 In arterial strips preloaded with ^{86}Rb, cromakalim and pinacidil increased the basal ^{86}Rb efflux.

5 When the effects of cromakalim and pinacidil on ^{86}Rb efflux were determined in arterial strips contracted with 65.9 mM K^+, both drugs increased ^{86}Rb efflux. The increase in ^{86}Rb efflux induced by pinacidil was much smaller than that induced by cromakalim. Under the same conditions, nifedipine decreased ^{86}Rb efflux.

6 After the addition of nifedipine to arterial strips contracted with 65.9 mM K^+, pinacidil produced a greater increase in ^{86}Rb efflux than in the absence of nifedipine, whereas the effects of cromakalim were the same for the two conditions. Therefore, the effects of pinacidil on ^{86}Rb efflux may be the resultant of two opposing effects: an increased ^{86}Rb efflux due to the opening of ATP-sensitive K^+ channels, and a decreased efflux due to the closing of Ca^{2+}-activated K^+ channels.

7 In causing relaxation, cromakalim was competitively antagonized by glibenclamide with a pA_2 value of 7.16. However, glibenclamide antagonism of pinacidil was not of the simple competitive type, suggesting that inhibition of Ca^{2+} influx may contribute to the relaxant action of pinacidil.

8 It may be concluded that although the ability of pinacidil to increase ^{86}Rb efflux via ATP-sensitive K^+ channel opening was similar to that of cromakalim, the inhibition of Ca^{2+} influx by pinacidil may reduce the opening of Ca^{2+}-activated K^+ channels in K^+-contracted arterial strips.

Introduction

Pinacidil is a new antihypertensive agent, which may relax vascular smooth muscle (Arrigoni-Martelli & Finucaine, 1985; Cohen, 1986; Cohen & Colbert, 1986). The mechanism of action has not yet been fully established, but it has recently been suggested that vascular relaxant responses to pinacidil are associated with the opening of smooth muscle K^+ channels (Bray *et al.*, 1987; Cook *et al.*, 1988a; Weston *et al.*, 1988; Videbaek *et al.*, 1988a). This mechanism of action was first proposed for the vasodilator effects of cromakalim on the basis that cromakalim enhanced ^{86}Rb (or ^{42}K) efflux from preloaded vascular smooth muscle preparations and hyperpolarized the cell membrane (Hamilton *et al.*, 1986; Weir & Weston, 1986; Quast, 1987; Cook *et al.*, 1988b; Quast & Baumlin, 1988). In vascular tissues, cromakalim inhibited contractions elicited by noradrenaline and low (below 30 mM) concentrations of K^+ but was ineffective against high concentrations of K^+ (Hamilton *et al.*, 1986; Weir & Weston, 1986). Recent pharmacological studies have demonstrated that glibenclamide, a blocker of adenosine 5'-triphosphate (ATP)-sensitive K^+ channels, competitively antagonizes the vascular relaxant responses to cromakalim (Winquist *et al.*, 1989; Wilson, 1989; Cavero *et al.*, 1989; Buckingham *et al.*, 1989; Quast & Cook, 1989) and inhibits the cromakalim-induced increase in ^{86}Rb efflux from vascular tissues (Quast & Cook, 1989), thus suggesting that ATP-sensitive K^+ channels are involved in the vasodilator effects of cromakalim. More recently, Standen *et al.* (1989) have demonstrated the presence of ATP-sensitive K^+ channels in arterial smooth muscle cells at the single channel level. They have also demonstrated that these channels are opened by cromakalim and inhibited by glibenclamide. These pharmacological and electrophysiological studies provide evidence that cromakalim produces vascular relaxation by opening ATP-sensitive K^+ channels in vascular smooth muscle cells.

Fewer electrophysiological and ion efflux studies using pinacidil have been performed than for cromakalim. However, in rat portal vein, spontaneous electrical activity was abolished by pinacidil and, in this tissue and in rat mesenteric resistance vessels, a pinacidil-induced increase in ^{86}Rb (or ^{42}K) efflux and a marked hyperpolarization was observed (Bray *et al.*, 1987; Cook *et al.*, 1988a; Videbaek *et al.*, 1988a,b). It has been demonstrated that pinacidil, at high (above 10 μM) concentrations, inhibits the arterial contractions produced by high (above 60 mM) concentrations of K^+ (Kaergaard Nielsen & Arrigoni-Martelli, 1981; Mikkelsen & Lederballe Pedersen, 1982; Cook *et al.*, 1988a; Videbaek *et al.*, 1988b; Hermsmeyer, 1988). Inasmuch as the response of arterial smooth muscle to high concentrations of K^+ is known to depend on transmembrane Ca^{2+} influx (Bolton, 1979), the inhibition by pinacidil of contractions induced by high concentrations of K^+ may reflect an action of this drug in inhibiting Ca^{2+} influx. Furthermore, this action of pinacidil will have a great influence on the Ca^{2+}-activated K^+ channels as has been demonstrated for other Ca^{2+} influx inhibitors (Casteels & Droogmans, 1985; Aaronson & Jones, 1985). In contrast with these observations, it was demonstrated that high concentrations (30 and 100 μM) of pinacidil had little or no effect on 80 mM K^+-induced contractions in rat aorta (Bray *et al.*, 1987;

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Weston et al., 1988). The purpose of the present study was to determine whether pinacidil interacts with both ATP-sensitive and Ca^{2+}-activated K^+ channels in strips of canine mesenteric artery. In the event of pinacidil affecting both types of K^+ channel, it was of further interest to determine the extent of any antagonism by glibenclamide of the responses to pinacidil.

Methods

Preparation of arterial strips for recording mechanical activity

Mongrel dogs of either sex weighing 7–12 kg were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, i.v.) and exsanguinated. Mesenteric artery, with an *in situ* outside diameter 0.6–0.8 mm, was excised and placed in Krebs solution of the following composition (mM): NaCl 115.0, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.2 and dextrose 10.0. Helical strips (0.8 mm in width and 7 mm in length) of mesenteric artery were prepared as described previously (Asano et al., 1987; 1988). The endothelium of the strip was removed by gently rubbing the endothelial surface with cotton pellets.

Strips were mounted vertically between hooks in water-jacketed baths containing 20 ml Krebs solution. The Krebs solution was maintained at 37°C and was gassed with a mixture of 95% O_2 and 5% CO_2. The upper end of the strip was connected to a force-displacement transducer (TB-612T, Nihon Kohden Kogyo Co., Tokyo, Japan) for isometric tension recordings. Strips were stretched passively to optical length by imposing a resting tension of 0.8 g. The optimal resting tension was determined by a length-passive tension study (Asano et al., 1987). After application of the resting tension, the strips were equilibrated for 90 min in Krebs solution with replacement of solution every 20 min. This resting tension was maintained throughout the experiments. All the experiments were conducted on phenoxybenzamine-treated strips to eliminate possible α-adrenoceptor responses to noradrenaline released by the K^+-depolarization. To this end, strips were treated with 2 μM phenoxybenzamine during the first 60 min of the 90 min equilibration period (Asano et al., 1988).

Relaxant responses to cromakalim and pinacidil

After equilibration, contractile responses of the strips to Krebs solution containing 65.9 mM K^+ (K^+ substitution for Na^+) were repeated two or three times until the responses were reproducible. To determine the relaxant responses to cromakalim, pinacidil and nifedipine, strips were contracted by the addition of 15 mM K^+ to Krebs solution (total K^+ concentration; 20.9 mM) before challenge with these drugs. In some experiments, the relaxant responses were determined in high K^+ (35.9 and 65.9 mM K^+ substitution for Na^+)-contracted strips. Cumulative concentration-response curves for the relaxant responses to these drugs were constructed. At the end of experiments, 0.1 mM papaverine was added to identify the position of the maximum relaxation. Relaxant responses to cromakalim, pinacidil and nifedipine are expressed as % of the papaverine-induced maximum relaxation.

Effects of glibenclamide on the relaxant responses to cromakalim or pinacidil were determined in strips exposed to Krebs solution containing 20.9 mM K^+. Five strips from the same animal were prepared and subjected to different treatments; test strips were treated with glibenclamide (0.03, 0.1, 0.3 or 1 μM), while control strips were treated with the vehicle for glibenclamide (ethanol). Glibenclamide was added 20 min before the K^+-induced contraction. A single concentration-response curve for the K^+ channel opening drug was determined on each strip. Each individual concentration-response curve was plotted to obtain EC_{50} values for cromakalim or pinacidil. The concentration-ratio, i.e., EC_{50} for cromakalim or pinacidil in the presence of glibenclamide divided by the EC_{50} in the control was obtained at varying concentrations of glibenclamide. Then the data were subjected to a Schild plot analysis according to the method of Arunlakshana & Schild (1959) and a pA_2 value for glibenclamide and a slope of the line were determined from the regression analysis.

Effects of cromakalim and pinacidil on Ca^{2+}-induced contraction in K^+-depolarized strips

Concentration-response curves for Ca^{2+} in K^+-depolarized strips were constructed as described previously (Asano et al., 1987). In these experiments, strips were washed several times over 90 min with (nominally) Ca^{2+}-free, K^+-depolarizing solutions (80 mM K^+ substitution for Na^+) and a cumulative concentration-response curve for Ca^{2+} was then constructed. Four strips from the same animal were prepared; one strip was treated with 10 μM cromakalim, one with 30 μM pinacidil, one with 10 nM nifedipine, while the remaining strip was used as a control.

Drug effects on ^{86}Rb efflux

Arterial strips were mounted vertically on stainless-steel rods and allowed to equilibrate in the Krebs solution for 90 min. The strips were then incubated for an additional 3 h in Krebs solution to which 14–20 μCi ml^{-1} ^{86}Rb had been added. Each strip was then dipped three times (a total of 15 s) into non-radioactive Krebs solution to remove excess radioactivity, and transferred to a temperature-controlled superfusion chamber similar to that described by Su & Bevan (1970). The upper end of the strip was connected to the force-displacement transducer as in the tension experiments. A resting tension of 2.4 g was applied because larger strips were used. The strips were superfused at a rate of 1 ml min^{-1} with Krebs solution at 37°C. Strips were superfused for the next 30 min before application of the test drugs. The superfusate was sampled by use of a collection period of 2 min and counted for radioactivity in an Aloka autowell gammer counter. The radioactivity remaining in the strip at the end of an efflux sequence was determined by dissolving the strip in 0.5 ml of 1 N nitric acid and the volume of the sample was adjusted to 2.0 ml. The rate constant of ^{86}Rb efflux was then calculated as the radioactivity released from the strip per min at time t divided by the radioactivity remaining in the strip at that time (Imaizumi & Watanabe, 1981; Bolton & Clapp, 1984; Quast, 1987).

Drug effects on ^{86}Rb efflux were generally calculated as the peak value of efflux rate constant obtained in the presence of the drug divided by the basal value of the efflux rate constant averaged over 8–10 min before drug application. However, effects of cromakalim and pinacidil faded during application (see Figure 4) and the area under the curve (AUC) of the efflux rate constant *vs.* time plot was chosen as a better measure of the drug effect. AUCs were calculated from an increase in the efflux rate constant during the 10 min application time (Quast, 1987). In experiments involving either 20.9 or 65.9 mM K^+, the K^+-rich medium itself increased ^{86}Rb efflux. In the efflux experiments, the ^{86}Rb efflux rate constant averaged over 8–10 min before drug application was used as a baseline for the drug effect in strips exposed to Krebs solution containing 5.9 and 20.9 mM K^+. When 65.9 mM K^+ was used, the efflux rate constant rose rapidly and then began to decline (Figure 6). Accordingly, the rate constant just before drug application was used as a baseline in strips exposed to Krebs solution containing 65.9 mM K^+.

Statistical analysis

When assessing the EC_{50} value, responses to drugs were calculated as % of the maximum response obtained with each drug. The EC_{50} value was obtained from a plot of % response

vs. log concentration of the drug and expressed as a negative log (pD_2 value).

Unless specified, results shown in the text, table and figures are expressed as the mean value ± s.e.mean (n = number of preparations). Statistical analysis of the data was performed using Student's t test for paired or unpaired data, or by completely randomized design, one-way analysis of variance followed by Newman-Keuls test for a significant F ratio ($P < 0.05$), depending on which test was statistically appropriate. Two groups of data were considered to be significantly different when $P < 0.05$.

Drugs and chemicals

Drugs used were cromakalim (Beecham Pharmaceuticals Research Division), pinacidil (Lilly Research Laboratories), nifedipine (Bayer Yakuhin Ltd.), phenoxybenzamine hydrochloride (Nakarai Chemicals), glibenclamide (Sigma Chemical Co.) and papaverine hydrochloride (Wako Pure Chemical Industries). ^{86}RbCl (specific activity initially 1.5–2.9 mCi mg^{-1}) was purchased from Amersham International.

Cromakalim and pinacidil were dissolved in 60% ethanol and 0.01 N HCl, respectively to make a stock solution of 10 mM with further dilution in distilled water before use. Stock solutions of nifedipine (1 mM) and phenoxybenzamine (1 mM) were prepared in 50% ethanol with further dilution in distilled water before use. Glibenclamide was dissolved in 50% ethanol to make a stock solution of 1 mM with further dilution in the same solvent before use. Aqueous stock solutions were prepared for other drugs. Concentrations of drugs are expressed as final molar concentrations in the tissue bath.

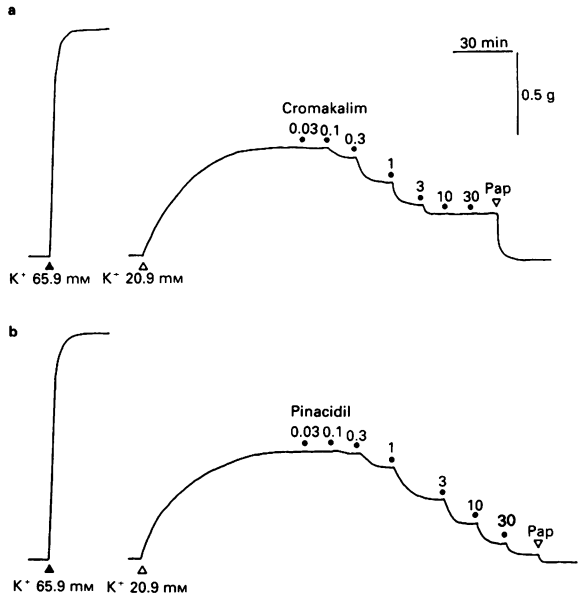

Figure 1 Canine mesenteric artery: suppression of K^+-induced contraction by K^+ channel opening drugs. Note, in (a) and (b), the initial contractile response to K^+ (65.9 mM). Following washout, contraction was subsequently induced by Krebs solution containing 20.9 mM K^+. Note that cromakalim (a) and pinacidil (b) each produced concentration-dependent suppression of the contraction. The concentrations of cromakalim and pinacidil are expressed as μM. Note also the final identification of the position of full relaxation by use of papaverine 0.1 mM (Pap).

Results

Relaxant responses to cromakalim, pinacidil and nifedipine

Relaxant responses to cromakalim and pinacidil were compared in arterial strips exposed to Krebs solution containing 20.9 mM K^+ (Figure 1). The magnitude of the K^+-induced contraction was 48.0 ± 3.6% (n = 32) of the maximum contraction induced by 65.9 mM K^+. The addition of cromakalim or pinacidil in concentrations ranging from 30 nM to 30 μM caused a concentration-dependent relaxation in these strips (Figure 1). The maximum relaxant response to 10 μM cromakalim (59.6 ± 3.8% of the papaverine-induced maximum relaxation) was significantly ($P < 0.001$) smaller than the response to 30 μM pinacidil (91.3 ± 2.9%) (Figures 1 and 2). The pD_2 value for cromakalim (6.56 ± 0.05) was significantly ($P < 0.001$) greater than that for pinacidil (5.88 ± 0.09).

The relaxant responses to cromakalim and pinacidil were also determined in arterial strips exposed to Krebs solution containing 35.9 or 65.9 mM K^+ (Figure 2). In the presence of these high concentrations of K^+, relaxant responses to cromakalim and pinacidil were decreased. When the strips were contracted with 65.9 mM K^+, cromakalim did not cause relaxation (Figure 2a). However, under the same conditions, pinacidil at relatively high (above 10 μM) concentrations produced a relaxation (Figure 2b).

Nifedipine, a Ca^{2+} influx inhibitor, also caused a concentration-dependent relaxation in strips exposed to Krebs solution containing either 20.9 or 65.9 mM K^+. The effect of nifedipine seemed independent of the K^+ concentration (Figure 2c).

Effects on Ca^{2+}-induced contractions

Ca^{2+}-induced contractions in K^+-depolarized strips were not altered by 10 μM cromakalim (Figure 3). On the other hand, 30 μM pinacidil produced a rightward displacement of the concentration-response curve for Ca^{2+} (Figure 3). Nifedipine also antagonized Ca^{2+} (Figure 3).

^{86}Rb efflux studies

Exposure of arterial strips to 10 μM cromakalim increased the ^{86}Rb efflux rate constant from the basal efflux of $2.32 \pm 0.22 \times 10^{-3}$ per min (measured between the 20th and 30th min of efflux) to a peak value of $3.66 \pm 0.12 \times 10^{-3}$ per min (Figure 4). The AUC of the increase in the efflux rate constant during the 10 min application time was $5.6 \pm 1.4 \times 10^{-3}$. Pinacidil (30 μM) also increased ^{86}Rb efflux (Figure 4). On the other hand, nifedipine (100 nM) did not change ^{86}Rb efflux (Figure 4). The data presented in Table 1 indicate the effects of cromakalim and pinacidil on ^{86}Rb efflux as measured in the presence of various concentrations of K^+.

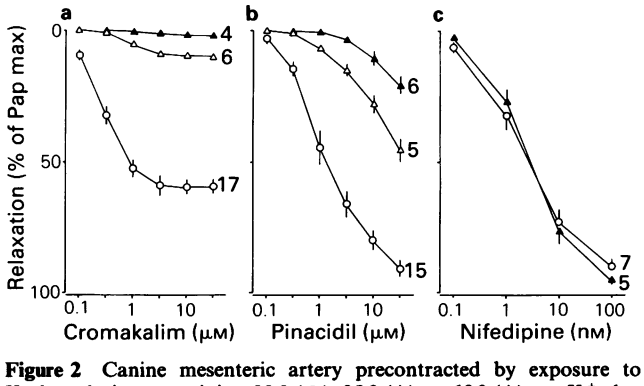

Figure 2 Canine mesenteric artery precontracted by exposure to Krebs solution containing 20.9 (○), 35.9 (△) or 65.9 (▲) mM K^+: log concentration-response curves for the relaxant actions of cromakalim (a), pinacidil (b) and nifedipine (c). Relaxation induced by 0.1 mM papaverine (Pap) was taken as 100%. Data points are means of values from the number of strips indicated beside each curve. Vertical lines represent s.e.mean.

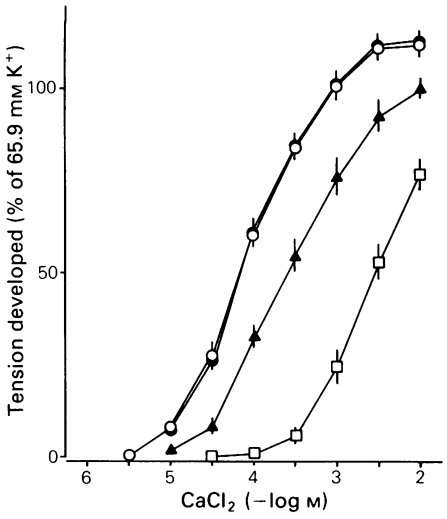

Figure 3 Effects of cromakalim, pinacidil and nifedipine on Ca^{2+}-induced contractions in K^+-depolarized strips of canine mesenteric artery. Cromakalim (10 μM) (●), pinacidil (30 μM) (▲) or nifedipine (10 nM) (□) was added 20 min before the concentration-response curve for Ca^{2+} was constructed. The control concentration-response curve (○) was constructed in the absence of any drugs. Contractile responses to Ca^{2+} are expressed as % of the contraction induced by 65.9 mM K^+ which had been evoked in Ca^{2+}-containing Krebs solution. Each point is the mean, and vertical lines represent the s.e.mean of 5 determinations.

Table 1 Effects of cromakalim and pinacidil on ^{86}Rb efflux from resting and K^+-contracted strips of canine mesenteric artery preloaded with ^{86}Rb

Condition[a]	*Concentration of drug*	*Drug effect on ^{86}Rb efflux rate constant (AUC)*[b] *Cromakalim*	*Pinacidil*
	μM	$\times 10^{-3}$	$\times 10^{-3}$
K^+ 5.9 mM	3	6.2 ± 1.5 (5)	ND
	10	5.6 ± 1.4 (6)	1.5 ± 0.5* (4)
	30	6.1 ± 1.8 (4)	6.3 ± 1.4 (6)
K^+ 20.9 mM	3	5.0 ± 2.5 (4)	ND
	10	9.4 ± 3.0 (6)	2.1 ± 0.1* (4)
	30	8.9 ± 0.9 (4)	8.8 ± 2.8 (5)
K^+ 65.9 mM	10	53.8 ± 7.6 (5)	ND
	30	ND	18.2 ± 4.3† (5)
K^+ 65.9 mM + Nif	10	53.0 ± 7.3 (5)	ND
	30	ND	32.8 ± 5.8 (4)

[a] Drug effects on ^{86}Rb efflux were determined in strips exposed to Krebs solution containing 5.9 (resting), 20.9, 65.9 mM K^+, and strips exposed to 65.9 mM K^+ plus 100 nM nifedipine (Nif).

[b] The drug effects are expressed as AUC of the increase in ^{86}Rb efflux rate constant during the 10 min application time. For details, see Methods.

Figures in parentheses indicate the number of preparations used. Data are expressed as mean ± s.e.mean. ND, not determined.

* Significantly different from the same concentration of cromakalim ($P < 0.05$).

† Significantly different from 10 μM cromakalim ($P < 0.05$).

At a K^+ concentration of 5.9 mM, pinacidil (10 and 30 μM) caused a concentration-dependent increase in ^{86}Rb efflux. In contrast, cromakalim (3–30 μM) did not exhibit a clear relationship between concentration and effect.

In order to correlate possible drug-induced alterations in ^{86}Rb efflux with mechanical effects, the effects of cromakalim and pinacidil on ^{86}Rb efflux were compared in strips exposed to Krebs solution containing 20.9 mM K^+ (Figure 5). The ^{86}Rb efflux was increased by strip exposure to 20.9 mM K^+, but the addition of 10 μM cromakalim or 30 μM pinacidil caused a further increase in ^{86}Rb efflux (Figure 5). It should be noted that the concentrations of cromakalim and pinacidil

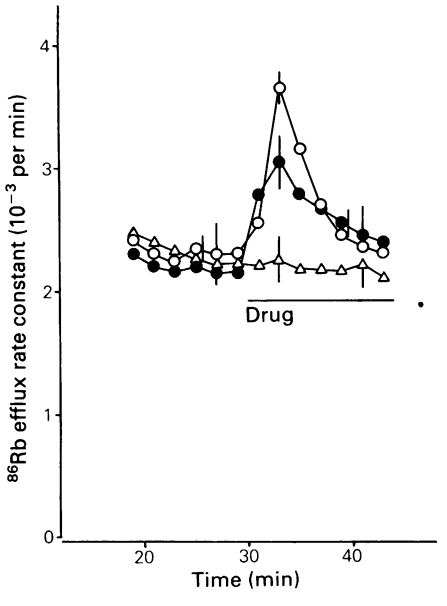

Figure 4 Effects of cromakalim, pinacidil and nifedipine on basal ^{86}Rb efflux from resting strips of canine mesenteric artery. Ordinate scale: ^{86}Rb efflux rate constant expressed as 10^{-3} per min. Abscissa scale: time (min) after start of the efflux period. Strips preloaded with ^{86}Rb were exposed to 10 μM cromakalim (○) ($n = 6$), 30 μM pinacidil (●) ($n = 6$) or 100 nM nifedipine (△) ($n = 6$) at the 30th min of the efflux period as indicated by the bar. Data points are means and s.e.means (representative values) are shown by vertical lines. The basal ^{86}Rb efflux rate constant obtained in the absence any drugs was constant between the 18th min ($2.67 \pm 0.23 \times 10^{-3}$ per min, $n = 6$) and 68th min ($2.46 \pm 0.18 \times 10^{-3}$ per min, $n = 6$) of the efflux period.

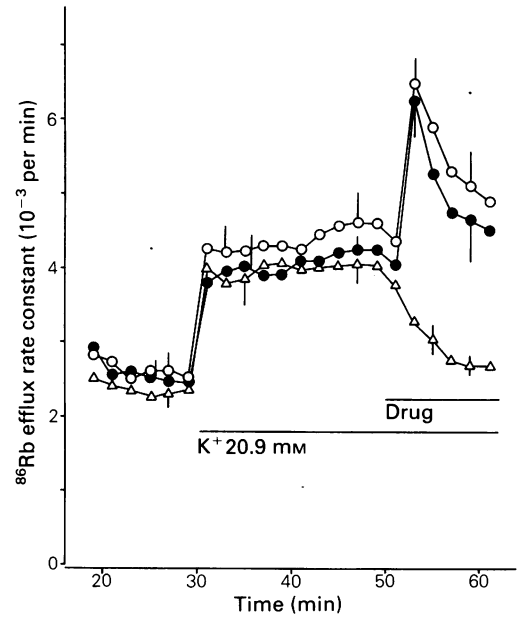

Figure 5 Effects of cromakalim, pinacidil and nifedipine on ^{86}Rb efflux from strips of canine mesenteric artery exposed to Krebs solution containing 20.9 mM K^+. Strips preloaded with ^{86}Rb were exposed to 20.9 mM K^+ at the 30th min of the efflux period and then exposed to 10 μM cromakalim (○) ($n = 6$), 30 μM pinacidil (●) ($n = 5$) or 100 nM nifedipine (△) ($n = 5$) at the 50th min. The ^{86}Rb efflux rate constant in the presence of 20.9 mM K^+ was constant between the 32nd min and 70th min of the efflux period.

required to cause a significant increase in ^{86}Rb efflux in the presence of 20.9 mM K^+ exceeded their respective EC_{50} values as regards inducing relaxation (Table 1 and Figure 2). The effect of 10 μM cromakalim in increasing ^{86}Rb efflux was significantly greater than that of 10 μM pinacidil but was comparable to the effect of 30 μM pinacidil (Figure 5 and Table 1). In contrast to cromakalim and pinacidil, nifedipine (100 nM) decreased ^{86}Rb efflux from strips exposed to Krebs solution containing 20.9 mM K^+ (Figure 5).

Further comparison of the effects of cromakalim and pinacidil on ^{86}Rb efflux was made in strips exposed to Krebs solution containing 65.9 mM K^+ (Figure 6). Exposure of strips to the K^+-rich medium greatly increased ^{86}Rb efflux. Cromakalim and pinacidil further increased ^{86}Rb efflux. However, the effect of 30 μM pinacidil was significantly smaller than that of 10 μM cromakalim (Table 1). Nifedipine again decreased ^{86}Rb efflux (Figure 6). Compared with their effects in the presence of 5.9 mM K^+, the effects of cromakalim and pinacidil on ^{86}Rb efflux in the presence of 65.9 mM K^+ were augmented (Table 1). This augmentation was significantly greater in the case of cromakalim (9.6 fold) than for pinacidil (2.9 fold). To determine whether the difference between the effects of cromakalim and pinacidil was due to the relaxant effect of pinacidil, similar experiments were performed in the presence of nifedipine. In these experiments, 100 nM nifedipine was applied before the addition of cromakalim or pinacidil to inactivate the Ca^{2+}-activated K^+ channels via the inhibition of Ca^{2+} influx (Figure 6). In the presence of nifedipine, cromakalim and pinacidil again increased ^{86}Rb efflux. The effects of cromakalim were similar in the absence and presence of nifedipine (Table 1). On the other hand, the effect of pinacidil was significantly increased in the presence of nifedipine compared with that observed in the absence of nifedipine (Table 1).

Glibenclamide antagonism of cromakalim and pinacidil in strips exposed to Krebs solution containing 20.9 mM K^+

Glibenclamide in concentrations ranging from 0.03 to 1 μM produced a rightward displacement of the concentration-response curve for cromakalim (Figure 7a) or pinacidil (Figure 7b). At the concentrations used, glibenclamide did not modify the resting tone or K^+-induced contractions. The Schild plot for antagonism by glibenclamide of cromakalim gave a regression line with a slope of 1.00 ± 0.15 (mean ± 95% CL) and a pA_2 value of 7.16 ± 0.07 (Figure 8a). In the case of glibenclamide antagonism of pinacidil, a slope of 0.85 ± 0.21 (mean ± 95% CL, not significantly different from unity) and a pA_2 value of 7.12 ± 0.08 were obtained (Figure 8b). However, the effects of glibenclamide on the concentration-response curves for pinacidil did not comprise a parallel rightward displacement (Figure 7b). The extent of rightward displacement of the concentration-response curve in the presence of 0.3 μM glibenclamide calculated at the EC_{50} (0.59 ± 0.07 log units) was significantly ($P < 0.05$) different from that calculated at the EC_{75} (0.30 ± 0.04 log units). This difference was also noted in the effect of 1 μM glibenclamide (1.04 ± 0.12 log units *vs.* 0.63 ± 0.09 log units) (Figure 7b). When these values at the EC_{75} were used the Schild plot for glibenclamide antagonism of pinacidil gave a regression line with a slope of 0.79 ± 0.25 (mean ± 95% CL, not significantly different from unity) and a pA_2 value of 6.60 ± 0.10 (Figure 8b). If the Schild plot was constructed using the values at the EC_{25}, a typical competitive antagonism by glibenclamide of pinacidil was observed, since a regression line with a slope of 1.06 ± 0.18

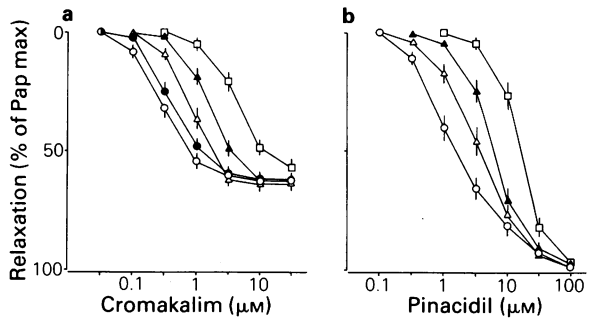

Figure 7 Effects of glibenclamide on the log concentration-response curves for cromakalim (a) and pinacidil (b) in strips of canine mesenteric artery exposed to Krebs solution containing 20.9 mM K^+. Glibenclamide in a concentration of 0.03 (●), 0.1 (△), 0.3 (▲) or 1 (□) μM was added 20 min before the K^+-induced contraction. Control concentration-response curves (○) were constructed in the presence of 0.05% ethanol, the vehicle for glibenclamide. Relaxation induced by 0.1 mM papaverine (Pap) was taken as 100%. Each point is the mean, and vertical lines represent the s.e.mean of 6 determinations.

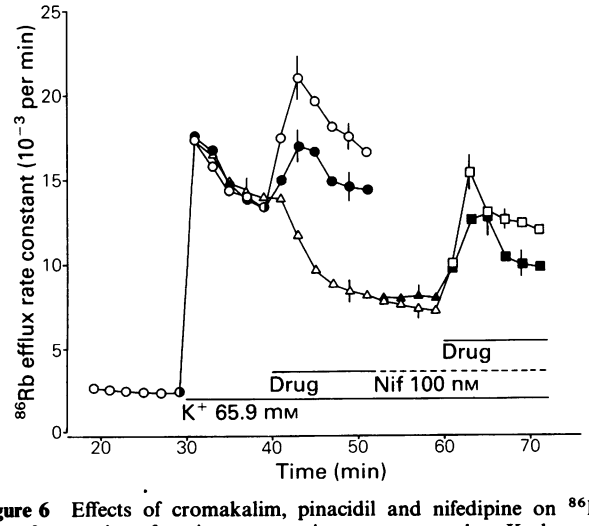

Figure 6 Effects of cromakalim, pinacidil and nifedipine on ^{86}Rb efflux from strips of canine mesenteric artery exposed to Krebs solution containing 65.9 mM K^+. Strips preloaded with ^{86}Rb were exposed to 65.9 mM K^+ at the 30th min of the efflux period and then exposed to 10 μM cromakalim (○) ($n = 5$), 30 μM pinacidil (●) ($n = 5$) or 100 nM nifedipine (△) ($n = 9$) at the 40th min. In the nifedipine (Nif) experiment, the strips were exposed to 10 μM cromakalim (□) ($n = 5$) or 30 μM pinacidil (■) ($n = 4$) at the 60th min. The ^{86}Rb efflux rate constant in the presence of 65.9 mM K^+ was constant between the 40th min and 50th min of the efflux period. The ^{86}Rb efflux rate constant in the presence of 65.9 mM K^+ plus 100 nM nifedipine was constant between the 48th min and 70th min of the efflux period.

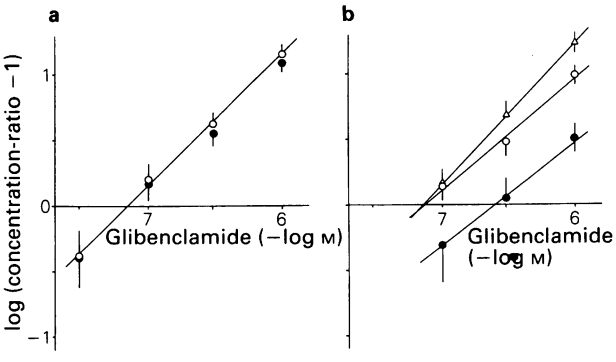

Figure 8 Schild plots for glibenclamide antagonism of cromakalim (a) or pinacidil (b) in strips of canine mesenteric artery exposed to Krebs solution containing 20.9 mM K^+. The plots were obtained by use of the concentration-ratios calculated from EC_{50} (○), EC_{75} (●) and EC_{25} (△) values of the concentration-response curves in Figure 7. The lines indicate the regression lines of best fit through 4 (a) or 3 (b) data points from 6 determinations each. Vertical lines represent the s.e.mean of calculated values of log (concentration-ratio − 1) at points corresponding to the glibenclamide concentrations used.

(mean ± 95% CL) and a pA_2 value of 7.15 ± 0.08 was obtained (Figure 8b). When the values at the EC_{75} for cromakalim were used for the Schild plot, the points on the Schild plot were in good agreement with the former points (Figure 8a).

Discussion

The experiments described in the present study evaluated the effects of two K^+ channel openers, cromakalim and pinacidil, on tension and ^{86}Rb efflux in strips of canine mesenteric artery. Arterial relaxations induced by both cromakalim and pinacidil were associated with an increase in ^{86}Rb efflux. In addition to this K^+ channel opening activity, pinacidil at high (above 10 μM) concentrations behaved like an inhibitor of Ca^{2+} influx. Cromakalim, however, showed no such action even at high concentrations. Evidence was also obtained that an action of pinacidil in inhibiting Ca^{2+} influx might affect the function of Ca^{2+}-activated K^+ channels and the antagonism by glibenclamide.

Both cromakalim and pinacidil produced arterial relaxation against the contraction induced by a low (20.9 mM) concentration of K^+ with an approximately five fold difference in their EC_{50} values. The apparent mechanism of action of cromakalim is to stimulate K^+ efflux, which in turn causes hyperpolarization and subsequent relaxation of arterial smooth muscle (Hamilton *et al.*, 1986; Weir & Weston, 1986; Quast, 1987; Cook *et al.*, 1988b; Quast & Baumlin, 1988). Under the conditions where the arterial strips were contracted with low concentrations of K^+, the equilibrium potential for K^+ (E_K) would be more negative than the actual membrane potential. On the other hand, cromakalim did not produce the relaxation in strips contracted by 65.9 mM K^+, presumably because the high K^+ reduced the E_K to a value of membrane potential less negative than that required to close voltage-dependent Ca^{2+} channels. Under these conditions, the cromakalim-induced increase in K^+ efflux would not hyperpolarize arterial smooth muscle sufficiently to inhibit transmembrane Ca^{2+} influx. Such a simple experiment easily allows Ca^{2+} influx inhibitors to be distinguished from vasodilators with K^+ channel opening activity, as demonstrated by Weston and his colleagues (Hamilton *et al.*, 1986; Bray *et al.*, 1987; Weston *et al.*, 1988; Hamilton & Weston, 1989). The present study has demonstrated that pinacidil inhibits Ca^{2+} influx as well as opening K^+ channels. This conclusion is based on the following two observations: (1) in contrast to cromakalim, pinacidil at high concentrations produced relaxation in strips contracted by 65.9 mM K^+ and (2) pinacidil inhibited the Ca^{2+}-induced contractions in strips depolarized by 80 mM K^+, whereas cromakalim did not. The maximum relaxant response to pinacidil was greater than that to cromakalim. Perhaps the difference is due to the inhibitory action of pinacidil on Ca^{2+} influx. Similar effects of pinacidil on contractions induced by high concentrations of K^+ have been demonstrated in rabbit aorta (Kaergaard Nielsen & Arrigoni-Martelli, 1981; Cook *et. al.*, 1988a), rat aorta (Mikkelsen & Lederballe Pedersen, 1982), rat mesenteric and femoral arteries (Videbaek *et al.*, 1988b), rat tail artery (Hermsmeyer, 1988) and human crural vein (Mikkelsen & Lederballe Pedersen, 1982).

^{86}Rb has been used as a marker of K^+ conductance in smooth muscles (Imaizumi & Watanabe, 1981; Bolton & Clapp, 1984; Hamilton *et al.*, 1986; Quast & Baumlin, 1988). In the resting strips, both cromakalim and pinacidil increased the basal ^{86}Rb efflux. The increase in ^{86}Rb efflux induced by 30 μM pinacidil was comparable to that induced by 10 μM cromakalim. When the effects of these concentrations of cromakalim and pinacidil were determined in strips contracted by 20.9 mM K^+, the pinacidil-induced increase in ^{86}Rb efflux was also comparable to the cromakalim-induced increase. Compared with the effect of each drug on the basal ^{86}Rb efflux from the resting strips, the effects of cromakalim and pinacidil were not increased. However, the effects of two drugs on ^{86}Rb efflux from strips contracted by 65.9 mM K^+ were significantly augmented.

The pinacidil-induced increase in ^{86}Rb efflux from strips contracted by 65.9 mM K^+ was only 34% of that of cromakalim (Table 1). This reduction in the effects of pinacidil on the K^+-contracted strips may be the net balance of two opposing effects; an increased ^{86}Rb efflux due to the opening of ATP-sensitive K^+ channels, and a decreased efflux through other K^+ channels due to the relaxation of the K^+-induced contractions. Arterial smooth muscle contraction induced by K^+ is associated with an increased ^{86}Rb efflux, presumably due to an opening of Ca^{2+}-activated K^+ channels (Bolton & Clapp, 1984; Casteels & Droogmans, 1985; Aaronson & Jones, 1985; see also this paper, Figures 5 and 6). On the other hand, the inhibition by drugs of transmembrane Ca^{2+} influx is associated with the closing of Ca^{2+}-activated K^+ channels (Casteels & Droogmans, 1985; Aaronson & Jones, 1985; see also this paper, Figures 5 and 6). In fact, pinacidil produced relaxation in strips contracted by 65.9 mM K^+, whereas cromakalim did not. Our proposal that pinacidil has two opposing effects on K^+ channel activity is supported by the following observations: (1) the relaxant response to nifedipine was associated with the decreased ^{86}Rb efflux in strips contracted by K^+ and (2) after the inactivation of Ca^{2+}-activated K^+ channels by the addition of nifedipine, pinacidil produced a greater increase in ^{86}Rb efflux than in the absence of nifedipine, whereas the effects of cromakalim were the same for the two conditions. Under conditions where the Ca^{2+}-activated K^+ channels are inactivated, ^{86}Rb efflux due to opening of ATP-sensitive K^+ channel by 30 μM pinacidil was comparable to that by 10 μM cromakalim. These observations suggest that, to determine the function of ATP-sensitive K^+ channels, it is necessary to minimize the influence of the pinacidil-induced relaxation on other K^+ channels. To this end, the elevation of K^+ to demonstrate the simultaneous measurement of the effects of K^+ channel opening drugs on tension and ^{86}Rb efflux may not be the most appropriate method of comparing their abilities to open ATP-sensitive K^+ channels.

Glibenclamide is a potent blocker of ATP-sensitive K^+ channels in pancreatic β-cells (Zunkler *et al.*, 1988) and insulin-secreting cells (Schmid-Antomarchi *et al.*, 1987). These channels have also been identified in cardiac cells (Noma, 1983; Escande *et al.*, 1988; Sanguinetti *et al.*, 1988) and in arterial smooth muscle cells (Standen *et al.*, 1989). As described in the Introduction, a large number of pharmacological studies have revealed a competitive antagonism by glibenclamide of cromakalim in vascular tissues (Winquist *et al.*, 1989; Wilson, 1989; Cavero *et al.*, 1989; Buckingham *et al.*, 1989; Quast & Cook, 1989). In the present study, glibenclamide was also demonstrated to antagonize the relaxant responses to cromakalim and pinacidil. Basically, the glibenclamide antagonism of pinacidil was not significantly different from that of cromakalim. The Schild plots for glibenclamide antagonism of cromakalin and pinacidil showed a competitive antagonism with a slope of unity and a pA_2 value of 7.16 (for cromakalim) and 7.12 (for pinacidil), respectively. These pA_2 values for glibenclamide were in good agreement with the pA_2 values for this substance reported in other studies (Wilson, 1989; Cavero *et al.*, 1989; Quast & Cook, 1989).

However, looking more closely at the effects of glibenclamide on the concentration-response curve for pinacidil, we noted that the extent of the rightward displacement of the concentration-response curve calculated at EC_{75} values was different from that calculated at EC_{50} or EC_{25} values. The Schild plot constructed from EC_{75} values for pinacidil, gave a regression line with a pA_2 value of 6.60, thus suggesting that this antagonism is not of the simple competitive type. Because pinacidil inhibits Ca^{2+} influx at high concentrations, and higher concentrations of pinacidil were necessary to obtain EC_{75} values in the presence of glibenclamide, the Ca^{2+} influx inhibitory action of pinacidil assumed progressively greater importance. Thus, it may be concluded that the ability of pinacidil to inhibit Ca^{2+} influx also contributed to the relax-

ant responses to the higher concentrations of this drug in addition to its K^+ channel opening activity.

In conclusion, the present study compared the ability of cromakalim and pinacidil to produce relaxation and to increase ^{86}Rb efflux via the opening of ATP-sensitive K^+ channels in strips of canine mesenteric artery. The ability of pinacidil to increase ^{86}Rb efflux via the opening of ATP-sensitive K^+ channels was similar to that of cromakalim. Because of the ability of pinacidil at high concentrations to inhibit Ca^{2+} influx, this similarity did not extend to the effects of these two drugs on the net ^{86}Rb efflux or to antagonism by glibenclamide.

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Hexocyclium derivatives with a high selectivity for smooth muscle muscarinic receptors

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1 The affinity of a number of derivatives of the muscarinic antagonist, hexocyclium, containing an amidine cationic head, for guinea-pig cardiac and ileal receptors was investigated.

2 All the compounds studied displayed a greater affinity for muscular than for cardiac muscarinic receptors.

3 The 5 fold ileal selectivity of hexocyclium was increased by a number of chemical substitutions. The largest discrimination between receptors (about 200 fold) was found for the formamidine derivative.

4 The selectivity displayed by the hexocyclium derivatives stemmed from a greater decrease in affinity towards cardiac as compared to ileal receptors.

Introduction

The concept that cardiac and intestinal muscarinic receptors possess different pharmacological properties was first suggested by the preferential antagonism at cardiac receptors displayed by gallamine and related neuromuscular relaxants (Riker & Wescoe, 1951; Mitchelson, 1984). Subsequently, agents endowed with the reverse selectivity, i.e. greater affinity for smooth muscle receptors were also described, namely, 4-diphenylacetoxy-*N*-methyl piperidine methiodide (4-DAMP) (Barlow *et al.*, 1976) and analogues of procyclidine (sila-procyclidine and sila-diphenidol, Mutschler & Lambrecht, 1984). The discrimination afforded by either class of molecules ranges between 10 and 30 fold.

The good correlation between pharmacological and binding results, and the data from the cloned m2 receptors demonstrate a remarkable homogeneity of the cardiac receptor population (M_2 subtype; Hammer *et al.*, 1986; Giachetti *et al.*, 1986; Maeda *et al.*, 1988). However, evidence have been provided that at least two subtypes, M_2 and M_3 (glandular type), are present on visceral smooth muscle (Giraldo *et al.*, 1987; 1988; Maeda *et al.*, 1988). At present the relative contribution of each subtype to contractile function is still unclear, hampering the design of molecules with improved selectivity for muscular tissue.

While exploring the structural requirements for M_1 receptor antagonism, we found that replacement of the tertiary amino group of pirenzepine with a guanidine moiety did not affect M_1 affinity, but considerably reduced the affinity for M_2 receptors (Cereda *et al.*, 1989). It was therefore of interest to investigate the effect of the same substitution on receptors mediating smooth muscle contractility. Hexocyclium (Figure 1) was selected as a lead molecule since: (a) the compound is endowed with clinically relevant spasmolytic activity (Martindale, 1989) and (b) its sila-derivative shows some selectivity for smooth muscle receptors (Lambrecht *et al.*, 1988).

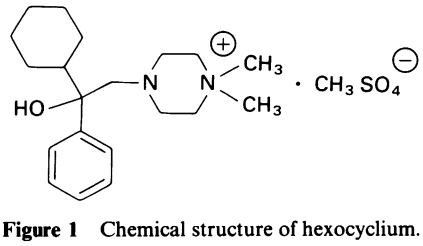

Figure 1 Chemical structure of hexocyclium.

This study describes the affinities for ileal and cardiac muscarinic receptors of derivatives of hexocyclium in which the onium head has been substituted by amidine-type groups, with the formamidine derivative, emerging as the most selective compound.

A preliminary account of these results has been given elsewhere (Donetti *et al.*, 1989).

Methods

Guinea-pig left atria

Tissues were mounted in McEwen's solution (mM: NaCl 131.6, KCl 5.6, $CaCl_2$ 2.16, $NaHCO_3$ 24.9, NaH_2PO_4 1.03, glucose 11 and sucrose 13) under 1 g tension, at 32°C, and stimulated through platinum electrodes (3 Hz, 2 ms, 100% above threshold voltage). Inotropic activity was recorded isometrically (Basile 7005 transducer, 7050 recorder).

Guinea-pig ileum

Segments of terminal ileum were suspended in Tyrode solution (mM: NaCl 137, KCl 2.68, $CaCl_2$ 1.82, $NaHCO_3$ 5.9, $MgCl_2$ 1.0, NaH_2PO_4 0.42 and glucose 5.6) at 37°C. Tension changes were recorded isotonically (Basile 7006 transducer, 7050 recorder).

Experimental procedure

In both preparations, muscarinic receptor stimulation was induced by cumulative additions of bethanechol. Antagonists were equilibrated for 60 min. Affinity measurements were estimated by either Schild analysis, fitting linear regression by least squares and verifying parallelism before calculating dose-ratios, or when only 4 replicates were performed, from the relation:

$$pA_2 = -\log([\text{Antagonist}]/(DR - 1)).$$

Compounds

Bethanechol hydrochloride was purchased from Sigma Chemical Co. (St Louis, MO, USA).

Hexocyclium (4-(2-cyclohexyl-2-hydroxy-2-phenyl ethyl)-1, 1-dimethyl piperazinium methyl sulphate) was synthesized by

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the method described in U.S. Patent 2, 907,765 (1959). The amidine derivatives (compounds 1–7, Table 1) were prepared by reacting their secondary amine precursors with ethyl formimidate, ethyl acetimidate, or cyanamide as described in the European Patent Appl. (publication number 0309424, 1989). The formamidine (1, 4–7), acetamidine (2), and guanidine derivatives (3) were obtained in satisfactory yields.

Results

Hexocyclium was a competitive antagonist of bethanechol in both guinea-pig atria and ileum, with a 5 fold greater affinity for ileal receptors ($P < 0.05$; Table 1).

The three amidine derivatives were also able to antagonize the muscarinic responses investigated in a competitive fashion. As can be seen from the affinity constants (Table 1), interaction of compounds 1–3 with muscarinic receptors was negatively affected by the substitutions. The pA_2 values at the cardiac receptor were drastically decreased (120 (compound 1) to 479 fold (compound 3)). In contrast, the decrease in ileal pA_2 values was much smaller (55 fold (compound 3) to less than 3 fold (compound 1)). This resulted in a large improvement of selectivity; from the 5 fold selectivity ratio of hexocyclium to 204 fold for compound 1 (Table 1).

Further modifications of compound 1 led to compounds with comparable M_2 affinity (compounds 4–7, Table 1). In the case of the phenyl (7) and the branched alkyl (6) derivatives, the affinity for the ileal receptors was reduced to a similar extent. None of these further chemical manipulations afforded a better selectivity than that observed for compound 1.

Discussion

The present study shows that substitution of hexocyclium quaternary nitrogen by amidine moieties leads to antagonists selective for smooth muscle receptors.

The selectivity originates from a different decremental effect exerted by the amidine substitution on the affinity for ileal and cardiac receptors. For the most favourable substitution, formamidine (compound 1), a negligible effect on ileal affinity is accompanied by a pronounced loss in cardiac affinity, resulting in the greatest discrimination (204 fold) so far reported for any smooth muscle selective antagonists. This indicates that M_2 receptors possess more stringent requirements for recognition of antagonists of this type.

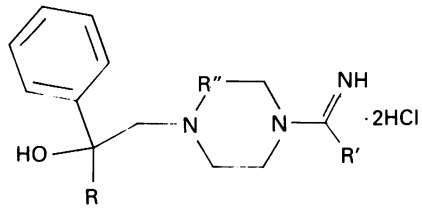
The physico-chemical features of amidines may account for the different decreases in affinity observed at the two subtypes. Owing to their pK_a values in the range 9–12 (Patai, 1975), amidine systems may be considered as structures intermediate between tertiary amines and quaternary onium compounds. They differ, however, from these latter moieties as tertiary amines and onium cationic heads possess a tetrahedral structure favouring van der Waals interactions (Belleau & Puranen, 1963), whereas amidinium cations have a high capacity to form hydrogen bonds (Sapse & Massa, 1980) and prefer the planar conformation (Mazurek *et al.*, 1983).

Further information that can be obtained from present results relates to the role of M_2 receptors in ileal contraction and is derived from inspection of affinity constants of compound 1. The concentrations employed to estimate its ileal pA_2 (10 to 100 nM) were below the K_B (575 nM) found for M_2 receptors, and indicate that this latter subtype plays a minor role in smooth muscle contraction. This supports previous similar conclusions obtained with M_2 selective compounds (Giraldo *et al.*, 1987; Roffel *et al.*, 1988; Ladinsky *et al.*, 1988).

A loss in M_2 affinity for amidine-substituted compounds is a feature found for one other type of muscarinic antagonist. Thus, for the guanyl derivative of pirenzepine, an M_1/M_2 ratio of 250 was found as a consequence of its decreased M_2 affinity (Cereda *et al.*, 1989). This suggests that amidine substituents on different classes of selective muscarinic antagonists are worth exploiting.

In conclusion, replacement of the quaternary head in the hexocyclium molecule by the amidine substituents, formamidine (1), acetamidine (2), and guanidine (3) affects affinities for ileal and cardiac receptors to a different extent, the overall result being an enhancement of smooth muscle selectivity. The greatest selectivity is found for the formamidine derivative and could not be improved by further variations of the molecular skeleton.

Table 1 General formula and affinity estimates (pA_2) for ileal and cardiac muscarinic receptors of amidine derivatives of hexocyclium



Compound	R	R′	R″	*MP* (°C)	*Ileum* ($pA_2 \pm$ s.e.)	*Atrium* ($pA_2 \pm$ s.e.)	*Selectivity ratio*
1	cyclohexyl	H	CH_2	218–220	8.56 ± 0.13 ($n = 11$) (0.96 ± 0.11)	6.25 ± 0.11 ($n = 11$) (1.30 ± 0.16)	204
2	cyclohexyl	CH_3	CH_2	201–202	7.68 ± 0.14 ($n = 9$) (1.09 ± 0.12)	6.00 ± 0.15 ($n = 15$) (1.04 ± 0.12)	48
3	cyclohexyl	NH_2	CH_2	183–185	7.27 ± 0.17 ($n = 9$) (0.97 ± 0.19)	5.65 ± 0.07 ($n = 9$) (1.22 ± 0.12)	42
4	cyclohexyl	H	CH_2CH_2	230–232	8.12 ± 0.29 ($n = 12$) (0.97 ± 0.24)	6.38 ± 0.04 ($n = 9$) (0.97 ± 0.04)	55
5	cyclopentyl	H	CH_2	>275	8.10 ± 0.11 ($n = 4$)	6.68 ± 0.15 ($n = 4$)	26
6	3-pentyl	H	CH_2	223–225	7.79 ± 0.12 ($n = 4$)	6.18 ± 0.02 ($n = 4$)	41
7	phenyl	H	CH_2	267–270	7.42 ± 0.06 ($n = 4$)	6.10 ± 0.02 ($n = 4$)	21
Hexocyclium					9.01 ± 0.28 ($n = 12$) (0.93 ± 0.19)	8.33 ± 0.27 ($n = 10$) (0.80 ± 0.16)	5

Compound 1 as monohydrochloride; for compounds 5–7 values are means ± s.e. (see methods); n = number of replicates; in parentheses slope ± s.e.

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Enhanced coronary vasoconstrictor responses to 5-hydroxytryptamine in the presence of a coronary artery stenosis in anaesthetized dogs

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1 In the anaesthetized dog, left circumflex coronary artery blood flow and external diameter were measured and the vascular responses to an injection of 5-hydroxytryptamine (5-HT, 0.02–2 $\mu g\,kg^{-1}$) assessed, before and after a screw clamp had been placed on the artery to produce a severe stenosis.

2 In the normal coronary circulation, intra-coronary (i.c.) injection of 5-HT increased coronary blood flow (CBF) but decreased the external diameter of the large coronary artery (CD), without affecting systemic mean arterial pressure or heart rate.

3 In the normal coronary circulation, the 5-HT-induced increases in CBF were unaffected by blockade of 5-HT_2-receptors with ketanserin (0.1 $mg\,kg^{-1}$ i.c.) but were significantly attenuated by blockade of 5-HT_1-like receptors with methysergide (0.1 $mg\,kg^{-1}$ i.c.).

4 In the presence of a severe stenosis, the increase in CBF produced by 5-HT was markedly attenuated and a secondary decrease in CBF was revealed. The stenosis also caused a marked enhancement of the 5-HT-induced constriction of the large artery, such that the reduction of the CD was approximately doubled at all doses of 5-HT tested. The enhanced 5-HT-induced reduction in CD was similar with placement of the stenosis either proximal or distal to the site of diameter measurement.

5 In the presence of the stenosis, blockade of 5-HT_2-receptors with ketanserin (0.1 $mg\,kg^{-1}$ i.c.) significantly attenuated the 5-HT-induced decreases in CD and CBF.

6 These results demonstrate that, in the anaesthetized dog, placement of a severe, flow-limiting stenosis enhances 5-HT-induced constriction of the large coronary arteries and reveals a reduction in coronary blood flow. These observations support suggestions that, in the presence of coronary artery disease, 5-HT could contribute to reductions in coronary blood flow leading to myocardial ischaemia.

Introduction

It has been postulated that in the presence of coronary artery disease an enhanced release of 5-hydroxytryptamine (5-HT) could contribute to coronary vasoconstriction and possibly myocardial ischaemia (Vanhoutte, 1987). 5-HT is released from aggregating platelets in sufficient amounts to cause contraction of coronary vascular smooth muscle (Cohen *et al.*, 1983a). In addition the concentration of 5-HT is elevated in dog coronary arteries at the site of a stenosis (Ashton *et al.*, 1986). Also, van den Berg *et al.* (1989) have recently shown that 5-HT is released into the coronary circulation of some patients with coronary artery disease.

However, if 5-HT is going to cause myocardial ischaemia then it must decrease coronary blood flow. 5-HT constricts large coronary arteries *in vivo* (Haddy *et al.*, 1957; Bove & Dewey, 1983; Lamping *et al.*, 1985; Chu & Cobb, 1987) and *in vitro* (Cocks & Angus, 1983; Cohen *et al.*, 1983b), but it may also produce endothelium-dependent relaxation of large coronary arteries *in vitro* (Cocks & Angus, 1983; Cohen *et al.*, 1983b) and dilate resistance vessels *in vivo* (Haddy *et al.*, 1957; Blackshear *et al.*, 1985; Meschig *et al.*, 1985). As the predominant determinant of resistance is the small rather than the large vessels the action of 5-HT in the normal coronary circulation is to increase rather than decrease blood flow.

This suggests that if 5-HT contributes to myocardial ischaemia in the presence of coronary artery disease, the vascular response must be different from that occurring in the normal vasculature. There are potentially many factors occurring in coronary artery disease which could influence the responses to vasoactive agents. One of these is stenosis of the large coronary arteries, as an atherosclerotic plaque encroaches on the lumen resulting in dilatation of the resistance vessels and the resultant loss of vasodilator reserve. As there have been no studies in which the actions of 5-HT have been examined under these conditions, the aim of this study was to compare the response to 5-HT in the normal coronary circulation with that occurring in the presence of a severe stenosis. Large coronary artery diameter and coronary blood flow were monitored, in order to assess the actions of 5-HT on both conduit and resistance vessels. A preliminary account of these findings has been presented to the Australian Physiological and Pharmacological Society (Woodman, 1989).

Methods

Greyhound dogs of either sex and weighing 25–38 kg were anaesthetized with thiopentone (25–30 $mg\,kg^{-1}$ i.v.) followed by α-chloralose (70 $mg\,kg^{-1}$ i.v., supplemented as necessary). The dogs were ventilated with room air plus additional oxygen as necessary to maintain arterial blood Po_2, Pco_2 and pH within the normal ranges (Po_2: 85–110 mmHg; Pco_2: 30–35 mmHg; pH: 7.25–7.35). Pressure in the thoracic aorta was measured by passing a catheter from the right femoral artery and connecting the catheter to a Druck pressure transducer. A continuous measurement of heart rate was obtained by a cardiotachometer coupler triggered by the ECG.

A thoracotomy was performed at the left fifth intercostal space and the pericardium was opened. A pair of 7 MHz piezoelectric transducers were sutured to opposing surfaces of the left circumflex coronary artery. External diameter of the artery was measured with an ultrasonic transit-time dimension gauge (Triton Technology Inc.). Blood flow through the artery was measured by an electromagnetic flow probe (Statham) placed on the artery. A needle tipped catheter was placed in the artery proximal to the transducers for the administration of drugs. Care was taken during the placement of the transducers to limit dissection and damage of any visible nerves.

Experimental protocol

5-HT (0.02–2 $\mu g\,kg^{-1}$) was injected as a bolus of less than 0.5 ml directly into the left circumflex coronary artery. After control responses to 5-HT had been obtained, a screw clamp was placed on the circumflex coronary artery and tightened until there was no reactive hyperaemia in response to a 10 s occlusion of the artery. This occlusion had no effect on cardiac rhythm. In ten dogs the stenosis was placed proximal to the diameter transducers, whilst in a further four dogs the stenosis was distal to the transducers. The 5-HT injections were then repeated in the presence of the stenosis. Ten of these dogs were then randomly allocated to one of two groups. In the first group the stenosis was removed and the responses to 5-HT (0.1–2 $\mu g\,kg^{-1}$ i.c.) re-examined. In the second group the stenosis was maintained and the responses to 5-HT were assessed after blockade of 5-HT_2-receptors with ketanserin (0.1 $mg\,kg^{-1}$) injected into the coronary artery every 15 min.

In a separate group of five dogs without coronary stenosis, the effect of 5-HT (0.02–2 $\mu g\,kg^{-1}$ i.c.) on coronary blood flow was assessed before and after blockade of 5-HT_2-receptors with ketanserin (0.1 $mg\,kg^{-1}$ i.c.) and combined blockade of 5-HT_1-like and 5-HT_2-receptors with methysergide (0.1 $mg\,kg^{-1}$ i.c.) plus ketanserin.

Drugs

5-Hydroxytryptamine creatinine sulphate (Sigma), ketanserin tartrate (Janssen) and methysergide maleate (Sandoz) were dissolved in distilled water with further dilutions in saline.

Statistics

Baseline values of the haemodynamic variables measured before and after placement of the stenosis were compared by Student's *t* test for paired data. Statistical comparison of the responses to 5-HT recorded after stenosis or antagonism of 5-HT receptors was achieved by a two-way analysis of variance, the two factors being the dose of 5-HT and the treatment. This test treated all doses as a group to reveal whether a treatment had any significant effect on a response. When there were 3 treatment groups, e.g. control, ketanserin and ketanserin plus methysergide, a *post hoc* comparison was performed by use of Scheffe's test (Wallenstein *et al.*, 1980), to compare each treatment group against the two others.

Results

5-Hydroxytryptamine

5-HT (0.02–2 $\mu g\,kg^{-1}$) injected into the left circumflex coronary artery caused dose-dependent increases in coronary blood flow (CBF) (Figure 1) but decreases in coronary artery diameter (CD) (Figure 2). The maximum reduction in CD was 6 ± 1% of the resting diameter or 170 ± 40 μm at the highest dose tested (Figure 2). There was no significant change in systemic arterial pressure (AP) or heart rate (HR) at any dose of 5-HT injected into the coronary artery.

Effect of ketanserin and methysergide on 5-HT-induced increases in coronary blood flow

In five dogs the effect of selective antagonists of '5-HT_1-like' and 5-HT_2-receptors on the 5-HT-induced increases in coronary blood flow were assessed. Ketanserin (0.1 $mg\,kg^{-1}$ i.c.), a selective 5-HT_2 receptor antagonist, had no significant effect on resting CBF (control = 32 ± 5 $ml\,min^{-1}$, ketanserin treated = 27 ± 5 $ml\,min^{-1}$) or on the vasodilator responses to 5-HT (Figure 3). Methysergide (0.1 $mg\,kg^{-1}$ i.c.), given after the administration of ketanserin, had no significant effect on resting CBF (methysergide-treated = 29 ± 5 $ml\,min^{-1}$). Methysergide, in the presence of ketanserin, caused a signifi-

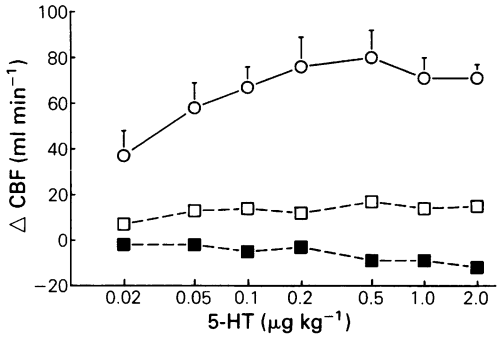

Figure 1 The effect of the intra-coronary injection of 5-hydroxytryptamine (5-HT) on left circumflex coronary artery blood flow (CBF) in the absence (○) and presence (□, ■) of a coronary artery stenosis. The values shown are the mean of 14 experiments; vertical lines indicate s.e.mean. In the normal coronary circulation 5-HT produced only increases in blood flow whereas in the presence of the stenosis the initial small increase in flow (□) was followed by a secondary vasoconstriction (■). Analysis of variance showed that the stenosis caused a significant ($P < 0.01$) attenuation of the vasodilator responses to 5-HT.

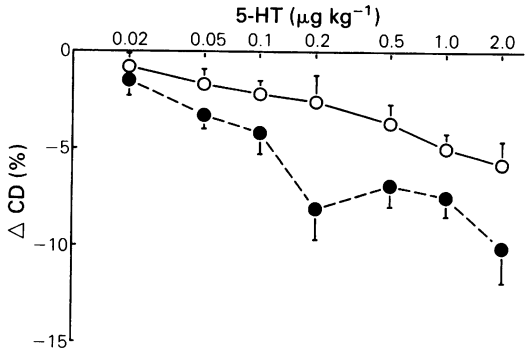

Figure 2 Percentage changes in left circumflex coronary artery diameter (CD) produced by the intra-coronary injection of 5-hydroxytryptamine (5-HT) in the absence (○) and presence (●) of a coronary artery stenosis. The values shown are the mean of 14 experiments; vertical lines indicate s.e.mean. Placement of the stenosis proximal or distal to the site of diameter measurement caused a similar enhancement of the response to 5-HT so the data were pooled for presentation in this graph. Analysis of variance showed that the stenosis caused a significant ($P < 0.01$) enhancement of the 5-HT-induced large artery constriction.

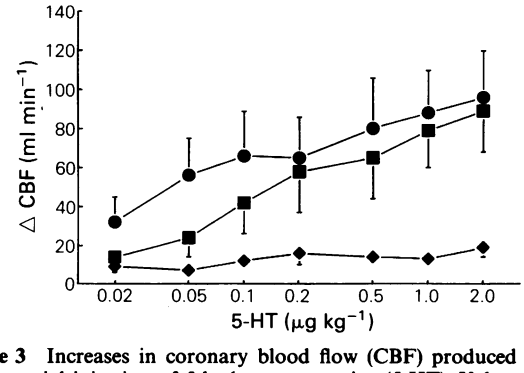

Figure 3 Increases in coronary blood flow (CBF) produced by the intra-arterial injection of 5-hydroxytryptamine (5-HT). Values shown are the mean of 5 experiments; vertical lines indicate s.e.mean. The control responses to 5-HT (●) are compared to those observed after treatment with the 5-HT_2-receptor antagonist ketanserin (0.1 $mg\,kg^{-1}$ i.c., ■) or the combined antagonims of 5-HT_2- and 5-HT_1-like receptors with ketanserin plus methysergide (0.1 $mg\,kg^{-1}$ i.c., ◆). Analysis of variance followed by Scheffe's test demonstrated that the dilator responses to 5-HT were not different in the control and ketanserin-treated groups, but that the addition of methysergide caused a significant ($P < 0.001$) attenuation of those responses.

Table 1 Baseline levels of the haemodynamic variables measured in anaesthetized dogs before and after placement of a stenosis on the left circumflex coronary artery

	Control	*Stenosis*
Mean arterial pressure (mmHg)	116 ± 4	119 ± 3
Mean external coronary artery diameter (mm)	3.1 ± 0.1	3.1 ± 0.2
Mean coronary blood flow (ml min^{-1})	63 ± 8	49 ± 7*
Heart rate (beats min^{-1})	127 ± 6	121 ± 8

Values are presented as the mean ± s.e.mean of data obtained from 14 animals. *$P < 0.01$ compared to control, Student's t test for paired comparisons.

cant attenuation of the 5-HT-induced increase in CBF (Figure 3).

Effect of coronary artery stenosis

Tightening of the screw clamp, to stenose the left circumflex coronary artery until there was no reactive hyperaemia to a ten second total occlusion of the artery, resulted in a small, but significant, reduction in CBF. The stenosis had no significant effect on CD, AP or HR (Table 1). In the presence of a stenosis the 5-HT-induced increase in CBF was markedly attenuated and, in addition, at the higher doses of 5-HT (0.5–2 μg kg^{-1}) a secondary decrease in CBF was revealed (Figure 1). The constriction of the large artery was enhanced by the stenosis at all doses of 5-HT tested. The enhancement of the large artery constriction was independent of the placement of the stenosis. On average there was a doubling of the degree of constriction observed in the presence of the stenosis at all doses tested. The degree of enhancement was similar with both proximal (2.0 ± 0.4 fold enhancement) and distal stenoses (2.5 ± 0.7 fold enhancement). The data for proximal and distal stenoses have been pooled and are illustrated in Figure 2.

Response to 5-HT after removing the stenosis

In five dogs the response to 5-HT was examined after the removal of the stenosis. After the stenosis was removed 5-HT injection caused an increase in CBF (Figure 4) of similar magnitude to the control response and there was no significant secondary decrease in flow (Figure 5). The 5-HT-induced constriction of the large artery was less than that observed in the presence of the stenosis, but of similar magnitude to the control responses (Figure 6).

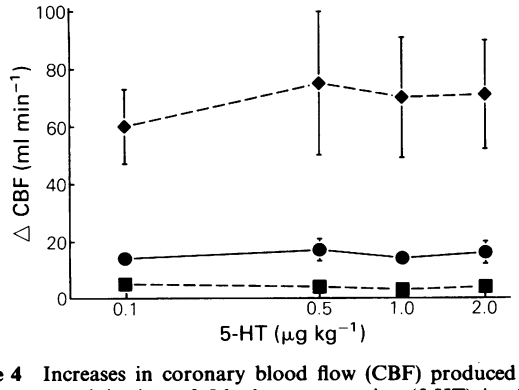

Figure 4 Increases in coronary blood flow (CBF) produced by the intra-coronary injection of 5-hydroxytryptamine (5-HT) in the presence of a stenosis (●, $n = 10$) are compared to responses to 5-HT after treatment with ketanserin (0.1 mg kg^{-1} i.c.) whilst the stenosis is maintained (■, $n = 5$) or after removing the stenosis (◆, $n = 5$). The values shown are the mean and vertical lines indicate s.e.mean. Analysis of variance showed no difference between the stenosis and the stenosis plus ketanserin-treated groups. Removal of the stenosis caused a significant ($P < 0.001$) enhancement of the 5-HT-induced dilatation.

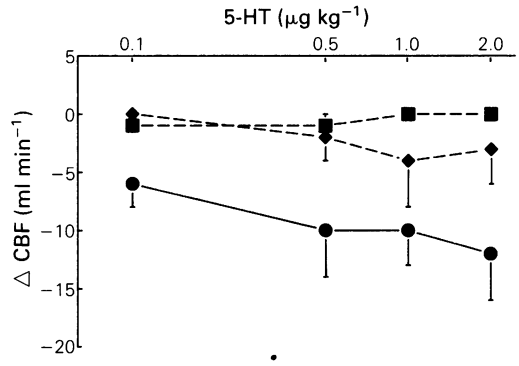

Figure 5 Decreases in coronary blood flow (CBF) produced by the intra-coronary injection of 5-hydroxytryptamine (5-HT) in the presence of a stenosis (●, $n = 10$) are compared to responses to 5-HT after treatment with ketanserin (0.1 mg kg^{-1} i.c.) whilst the stenosis is maintained (■, $n = 5$) or after removing the stenosis (◆, $n = 5$). The values shown are the mean and vertical lines indicate s.e.mean. The constrictor response to 5-HT was significantly ($P < 0.05$, analysis of variance) attenuated by both ketanserin treatment and the removal of the stenosis.

Effect of ketanserin on the response to 5-HT in the presence of the stenosis

In five dogs with stenosed coronary arteries, treatment with the 5-HT$_2$-receptor antagonist ketanserin (0.1 mg kg^{-1} i.c.) had no significant effect on the baseline levels of CD (3.1 ± 0.2 mm before ketanserin; 3.0 ± 0.1 mm after ketanserin) or CBF (30 ± 8 ml min^{-1} before ketanserin; 25 ± 6 ml min^{-1} after ketanserin). Ketanserin attenuated the constriction of the large coronary artery produced by 5-HT (Figure 6) and in addition abolished the decrease in CBF (Figure 5).

Discussion

This study has demonstrated that the injection of 5-HT into the left circumflex coronary artery of the anaesthetized dog constricts the proximal portion of that artery, but simultaneously increases blood flow in that vascular bed. As there were no accompanying changes in arterial pressure or heart rate, the increase in flow indicated that 5-HT dilates the coronary resistance vessels. The response to 5-HT was then re-examined in the presence of a coronary artery stenosis, which

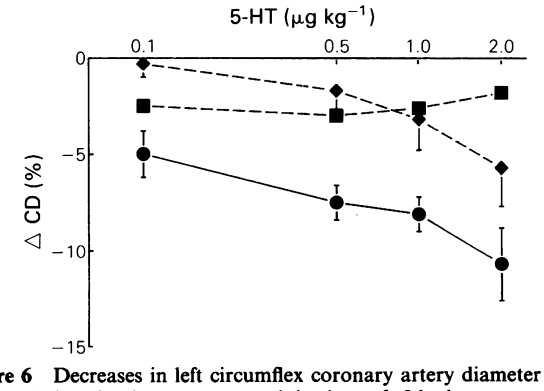

Figure 6 Decreases in left circumflex coronary artery diameter (CD) produced by the intra-coronary injection of 5-hydroxytryptamine (5-HT) in the presence of a stenosis (●, $n = 10$) are compared to responses to 5-HT after treatment with ketanserin (0.1 mg kg^{-1} i.c.) whilst the stenosis is maintained (■, $n = 5$) and after removing the stenosis (◆, $n = 5$). The values shown are the mean and vertical lines indicate s.e.mean. Analysis of variance demonstrated that both ketanserin treatment and removal of the stenosis significantly ($P < 0.01$) attenuated the 5-HT-induced contractions of the large coronary artery.

was of sufficient severity to abolish the reactive hyperaemia to a ten second occlusion of the artery indicating a loss of vasodilator reserve. In the presence of the stenosis the 5-HT-induced constriction of the large coronary artery was enhanced. The stenosis prevented the normal increase in coronary blood flow produced by 5-HT, probably due to the reduction in vasodilator reserve, as indicated by the loss of reactive hyperaemia to coronary occlusion. In addition the stenosis revealed a secondary decrease in flow.

It has been shown that 5-HT-induced constriction of large coronary arteries in the dog is enhanced by removal of the endothelium (Brum *et al.*, 1984; Lamping *et al.*, 1985). It has also been found that placement of a stenosis on a coronary artery can cause endothelial damage, although this damage was observed to be restricted to an area proximal to the stenosis (Gertz *et al.*, 1981). Observations made in the present study do not indicate a contribution of endothelial damage to the enhanced constriction to 5-HT in the presence of the stenosis, as reversal of the enhancement when the stenosis was removed suggests that there was no permanent change to the vascular tissue. Ischaemia has also been shown to alter coronary vascular reactivity (Ku, 1982; Van Benthuysen *et al.*, 1987), at least in part due to endothelial damage. However, the reversible nature of the enhancement also argues against this possibility. In addition, there were no changes in the ECG that would suggest myocardial ischaemia (data not shown).

Placement of the stenosis either proximal or distal to the site of diameter measurement was not significant in terms of the influence on the response to 5-HT. In each case the stenosis resulted in an approximate doubling of the 5-HT-induced constriction of the large coronary artery. This observation further supports the argument against the contribution of any local ischaemia or damage at the site of diameter measurement to the enhanced contractile response. The effectiveness of the distal stenosis in enhancing the contraction to 5-HT also indicates that a pressure drop across the stenosis, with a resultant passive decrease in arterial diameter, does not contribute to the observed response.

Increases in blood flow have been demonstrated to cause proximal large artery dilatation and this dilator response is endothelium-dependent (Pohl *et al.*, 1986; Inoue *et al.*, 1988). It has recently been demonstrated that when coronary blood flow is prevented from increasing, 5-HT-induced constriction of the large coronary artery in anaesthetized dogs is enhanced and this effect is abolished by removing the endothelium from the coronary artery (Lamping & Dole, 1988). This suggests that flow-induced dilatation normally opposes the direct vasoconstrictor action of 5-HT on large coronary arteries. Such a mechanism could explain the observations made in this study, that is, when the stenosis limited the increase in blood flow, the direct constrictor action of 5-HT on the large artery was not opposed by any local release of a vasodilator and the resultant reduction of the large artery diameter was enhanced. Such a mechanism would be consistent with the finding that placing the stenosis proximal or distal to the site of diameter measurement equally enhances the response to 5-HT.

Importantly, placement of the fixed stenosis also revealed a reduction in coronary blood flow in response to 5-HT. In the presence of the stenosis, the reduction in coronary artery diameter and blood flow were both attenuated by the 5-HT_2-receptor antagonist ketanserin. Antagonism of the decrease in coronary blood flow by ketanserin suggests that 5-HT may act on a population of 5-HT_2 receptors in the coronary resistance vessels. In the normal coronary circulation, the only response to 5-HT was an increase in coronary blood flow which was not affected by ketanserin alone but was significantly attenuated by methysergide plus ketanserin, indicating the involvement of 5-HT_1-like receptors as has recently been found by Ichikawa *et al.* (1989). If 5-HT_2-receptors exist in the coronary resistance vessels, these results would suggest that the population is small and any response to their activation by 5-HT would normally be masked by the greater effect on 5-HT_1-like receptors mediating vasodilatation. A second possible explanation for the decrease in flow could be that, in the presence of the stenosis, the severity of the large artery constriction was sufficient to impede flow. Though the large coronary arteries contribute less than 5% of total coronary resistance in the normal circulation (Kelley & Feigl, 1978), the presence of a stenosis may lead to a significant contribution to resistance by these vessels (MacAlpin, 1980). The enhanced constrictor response to 5-HT at the site of the stenosis could then potentially result in the observed flow reduction. It is not possible from this study to distinguish between these possible mechanisms for the 5-HT-induced decrease in coronary blood flow.

Previous studies have shown that coronary artery stenosis may lead to cyclic reductions in blood flow (Folts *et al.*, 1982; Bush *et al.*, 1984). These reductions in flow could be abolished by aspirin and 5-HT antagonists (Folts *et al.*, 1982; Bush *et al.*, 1984; Bush, 1987), suggesting that the reduction in flow was due to platelet aggregation leading to thrombi formation and transient plugging of the coronary arteries. No such cyclic flow reductions were observed in the present study, either spontaneously or after 5-HT administration. Similarly Ichikawa *et al.* (1989) have recently demonstrated that 5-HT could produce reductions in coronary blood flow in the presence of a stenosis and this did not involve platelet aggregation. Thus it appears that 5-HT could have two effects which could lead to an impairment of coronary blood flow in the presence of a stenosis, that is a vascular action, involving enhanced constriction of both large and small arteries, and/or the promotion of platelet aggregation, leading to thrombi formation and blockage of the arteries.

In summary, this study demonstrates that the 5-HT-induced constriction of large coronary arteries is enhanced by the presence of a severe, flow-limiting stenosis. Furthermore, in the presence of such a stenosis the normal effect of 5-HT to increase coronary blood flow is reversed to a decrease in flow. This flow reduction is abolished by ketanserin, indicating the involvement of 5-HT_2 receptors. These results therefore support proposals that 5-HT released from aggregating platelets in the presence of coronary artery disease could contribute to reductions in coronary blood flow, leading to myocardial ischaemia.

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Effects of indomethacin on the regional haemodynamic responses to low doses of endothelins and sarafotoxin

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1 Regional haemodynamic responses to i.v. bolus injections of low doses (4 pmol and 40 pmol) of endothelin-1, -2, -3 and sarafotoxin-S6b were assessed in conscious, Long Evans rats in the absence and presence of indomethacin.

2 Both doses of endothelin-3 and sarafotoxin-S6b caused early renal vasodilatations that were not affected by indomethacin. Endothelin-1 caused an initial renal vasodilatation only in the presence of indomethacin, indicating that this peptide produced concurrent release of cyclo-oxygenase products that caused renal vasoconstriction. Neither dose of endothelin-2 produced an increase in renal conductance.

3 The 4 pmol dose of all four peptides caused mesenteric vasoconstrictions only. With the 40 pmol dose of the peptides, none caused early mesenteric vasoconstriction except in the presence of indomethacin. Thus, in this vascular bed the primary vasoconstrictor effects of the peptides (seen with the 4 pmol dose) were offset, following the 40 pmol dose, by release of vasodilator cyclo-oxygenase products. Indomethacin alone caused significant vasoconstriction only in the mesenteric vascular bed, indicating that in this region of the circulation, vasodilator prostanoids might be involved also in the tonic control of vascular conductance.

4 All four peptides at both doses caused early hindquarters vasodilatation. However, only the initial hypotensive and hindquarters vasodilator effects of the 40 pmol dose of sarafotoxin-S6b were attenuated by indomethacin. Under these conditions the hindquarters vasodilator effects of sarafotoxin-S6b were similar to those of the other peptides, indicating that the more marked effects of sarafotoxin-S6b in the absence of indomethacin were contributed to by vasodilator cyclo-oxygenase products in the hindquarters.

Introduction

In vitro and *in vivo* data indicate that endothelin-1 (Et-1) and endothelin-3 (Et-3) can exert vasodilator effects, possibly through release of eicosanoids and/or endothelium-derived relaxing factor (De Nucci *et al.*, 1988; Warner *et al.*, 1989a,b; Rakugi *et al.*, 1989; Rae *et al.*, 1989; Thiemermann *et al.*, 1989; Lidbury *et al.*, 1989; Herman *et al.*, 1989). Although differential release of endogenous vasodilators by Et-1 and Et-3 could explain their differential pressor effects (Inoue *et al.*, 1989), pretreatment with indomethacin does not have predictable effects on the initial hypotensive effects of Et-1 in pithed or anaesthetized rats (De Nucci *et al.*, 1988; Walder *et al.*, 1989; Winquist *et al.*, 1989). However, it is feasible that indomethacin pretreatment could modify the regional haemodynamic effects of Et-1 or Et-3 without influencing the changes in systemic arterial blood pressure induced by these peptides. In the present work we investigated this possibility. In addition, we extended the experiments to include a comparison of the responses to endothelin-2 (Et-2) (Inoue *et al.*, 1989) and to sarafotoxin-S6b (S6b) (Takasaki *et al.*, 1988a; Kloog *et al.*, 1988) in the absence and presence of indomethacin, since there is a substantial structural homology between Et-1, Et-2 and Et-3 and S6b (Takasaki *et al.*, 1988a,b; Lee & Chiappinelli, 1988; Kloog *et al.*, 1988).

Methods

All experiments were carried out on male Long Evans rats 3–4 months old (380–420 g). The procedures were as described previously (Gardiner *et al.*, 1988). Under sodium methohexitone anaesthesia (60 mg kg^{-1} i.p., supplemented as necessary) pulsed Doppler probes (Haywood *et al.*, 1981) were sutured around left renal and superior mesenteric arteries and the distal abdominal aorta (to monitor hindquarters flow). At least 7 days later, animals were briefly re-anaesthetized (sodium methohexitone, 40 mg kg^{-1} i.p.) and had jugular venous catheters and a distal abdominal aortic catheter implanted. Experiments were begun the following day when animals were fully conscious, and ran over 2 days. On day 1 bolus doses (4 and 40 pmol) of Et-1, Et-2 and Et-3 and S6b were given in randomized order, but with the lower dose before the higher dose, and doses separated by at least 60 min. The following day, indomethacin was administered by primed infusion (5 mg kg^{-1} and 5 mg kg^{-1} h^{-1}) and, starting 30 min later, the bolus doses of peptides were given in the same order and with the same timing as on day 1. Mean arterial blood pressure (MBP), instantaneous heart rate (HR) and renal, mesenteric and hindquarters Doppler shift signals were recorded continuously. Percentage changes in the latter were calculated as indices of changes in regional blood flows (Haywood *et al.*, 1981), and % changes in vascular conductance were calculated from mean Doppler shift signals and MBP.

The initial hypotensive responses to the peptides were maximal about 15 s after administration of both doses. The subsequent pressor responses peaked at about 1 min with the 4 pmol dose and at about 2 min after the 40 pmol dose; these values are included in the tables. Data were subjected to two-way, non-parametric analysis of variance (Friedman's test) and Wilcoxon's ranks sum test.

Peptides and drugs

All peptides were obtained from the Peptide Institute, Osaka, Japan (through Scientific Research Associates, London) and dissolved in isotonic saline containing 1% bovine serum albumin. Administration of both doses of all peptides was in a volume of 0·1 ml; this volume of vehicle had no cardiovascular effects. No allowance was made for body weight in the peptide dose administered, since the maximum difference in injectate volumes would have been only 10 µl. Furthermore, since all animals served as their own controls, only intra-individual comparisons of peptide effects were carried out.

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Indomethacin (Merck Sharp & Dohme Ltd) was dissolved in 10 mM sodium bicarbonate; the bolus dose was given in a volume of 0.34 ml over 10 min. The continuous infusion was given at 0.3 ml h^{-1}.

Results

Table 1 summarizes the values for cardiovascular variables before peptide administration on day 1 and before and 30 min after the onset of indomethacin administration (but before peptides were injected) on day 2. There were no significant differences for any of the variables except mesenteric flow and vascular conductance.

Responses to endothelin-1

The 4 pmol dose of Et-1 caused a significant initial fall in MBP and rise in HR in the presence of indomethacin; these effects and the subsequent increase in MBP were not different from the corresponding changes in the absence of indomethacin (Table 2). In the latter condition there was an early mesenteric vasoconstriction and hindquarters vasodilatation that were not different from the responses seen in the absence of indomethacin (Table 2). The subsequent renal and mesenteric vasoconstrictions were similar also in the two conditions (Table 2).

Administration of the 40 pmol dose of Et-1 caused similar initial falls and subsequent rises in MBP and associated tachycardias and bradycardias in the absence and presence of indomethacin (Table 3). However, in the latter condition there was an initial renal vasodilatation and mesenteric vasoconstriction not seen in the absence of indomethacin (Table 3). The early hindquarters vasodilatation and the subsequent renal and mesenteric vasoconstrictions were not affected by indomethacin (Table 3).

Responses to endothelin-2

The 4 pmol dose of Et-2 did not reduce MBP significantly although there was a significant tachycardia (Table 2). The

Table 1 Cardiovascular variables in the same conscious Long Evans rats before peptide administration on day 1 and before and 30 min after the onset of indomethacin administration (i.e. before peptides were injected) on day 2

	Day 1	*Day 2*	
		Pre-indomethacin	*Post-indomethacin*
Heart rate (beats min^{-1})	319 ± 6	322 ± 8	302 ± 10
Mean blood pressure (mmHg)	106 ± 4	108 ± 3	111 ± 3
Doppler shift (kHz)			
Renal	9.8 ± 1.1	9.8 ± 0.7	9.5 ± 0.7
Mesenteric	7.6 ± 0.3	8.0 ± 0.5	6.8 ± 0.5†
Hindquarters	4.4 ± 0.4	4.2 ± 0.7	3.7 ± 0.7
Conductance (100 (kHz $mmHg^{-1}$))			
Renal	92 ± 9	91 ± 5	85 ± 6
Mesenteric	72 ± 5	74 ± 6	62 ± 5*†
Hindquarters	42 ± 4	39 ± 7	35 ± 7

Values are means ± s.e.mean (n = 8).
* $P < 0.05$ versus day 1; † $P < 0.05$ versus pre-indomethacin (Wilcoxon test).

Table 2 Cardiovascular changes following bolus injection (4 pmol) of endothelin-1, -2, or -3 or sarafotoxin-S6b in the absence or presence of indomethacin, in conscious, Long Evans rats

	Endothelin-1		*Endothelin-2*		*Endothelin-3*		*Sarafotoxin-S6b*	
	Time after injection (min)							
	0.25	*1.0*	*0.25*	*1.0*	*0.25*	*1.0*	*0.25*	*1.0*
Δ Heart rate (beats min^{-1})								
− Indomethacin	33(9)*	19(9)	31(10)*	16(7)*	35(7)*	19(8)*	36(13)*	13(11)
+ Indomethacin	29(8)*	19(12)	36(9)*	9(7)	49(5)*	33(11)*	44(7)*	7(12)
Δ Mean blood pressures (mmHg)								
− Indomethacin	−2(3)	9(2)*	−4(2)	3(1)	−7(2)*	1(1)	−4(3)	8(1)*
+ Indomethacin	−6(2)*	5(1)*	−2(3)	1(1)	−12(2)*	1(2)	−11(4)*	3(2)
Δ Renal flow (%)								
− Indomethacin	−2(3)	−13(2)*	0(1)	−7(2)*	4(2)*	−3(3)	3(2)	−11(2)*
+ Indomethacin	−1(2)	−13(3)*	1(2)	−3(2)	1(1)	−4(2)	1(2)	−8(2)*
Δ Mesenteric flow (%)								
− Indomethacin	−18(5)*	−24(2)*	−14(3)*	−20(3)*	−17(6)*	−25(5)*	−24(3)*	−31(2)*
+ Indomethacin	−19(3)*	−28(3)*	−7(3)*	−8(2)*	−24(4)*	−26(4)*	−26(5)*	−26(3)*
Δ Hindquarters flow (%)								
− Indomethacin	34(6)*	35(8)*	27(3)*	20(3)*	25(6)*	19(7)*	29(5)*	22(4)*
+ Indomethacin	36(7)*	39(12)*	24(5)*	15(7)*	52(8)*	31(9)*	40(12)*	44(20)*
Δ Renal conductance (%)								
− Indomethacin	0(7)	−20(2)*	3(3)	−9(2)*	12(4)*	−3(3)	7(4)	−17(2)*
+ Indomethacin	5(2)*	−17(4)*	2(3)	−4(2)*	13(2)*	−5(2)*	12(3)*	−10(1)*
Δ Mesenteric conductance (%)								
− Indomethacin	−16(8)	−29(1)*	−11(3)*	−22(2)*	−12(7)*	−25(6)*	−21(3)*	−36(2)*
+ Indomethacin	−15(3)*	−31(3)*	−6(3)*	−9(2)*†	−14(6)*	−27(3)*	−18(6)*	−28(3)*
Δ Hindquarters conductance (%)								
− Indomethacin	37(6)*	25(9)*	31(5)*	17(3)*	34(8)*	18(8)*	34(7)*	14(4)*
+ Indomethacin	44(9)*	34(13)*	26(7)*	14(8)*	71(9)*	30(11)*	57(18)*	40(20)*

Values are means ± s.e.mean (n = 8).
* $P < 0.05$ versus baseline (Friedman's test).
† $P < 0.05$ for corresponding values in the absence and presence of indomethacin (Wilcoxon's test).

Table 3 Cardiovascular changes following bolus injection (40 pmol) of endothelin-1, -2, or -3 or sarafotoxin-S6b in the absence or presence of indomethacin, in conscious, Long Evans rats

	Endothelin-1		*Endothelin-2*		*Endothelin-3*		*Sarafotoxin-S6b*	
	Time after injection (min)							
	0.25	*2.0*	*0.25*	*2.0*	*0.25*	*2.0*	*0.25*	*2.0*
Δ Heart rate (beats min^{-1})								
− Indomethacin	87(13)*	−44(9)*	34(15)	−24(9)*	68(13)*	−23(7)*	82(9)*	−27(11)
+ Indomethacin	62(10)*	−48(5)*	34(10)*	−39(7)*	54(13)*	−26(6)*	63(11)*	−26(19)
Δ Mean blood pressure (mmHg)								
− Indomethacin	−22(2)*	25(3)*	−9(4)	13(2)*	−13(3)*	13(2)*	−28(2)*	20(3)*
+ Indomethacin	−18(3)*	21(3)*	−7(3)	13(4)*	−15(4)*	9(2)*	−13(3)*†	14(4)*
Δ Renal flow (%)								
− Indomethacin	−18(4)*	−44(2)*	−7(4)	−29(2)*	8(2)*	−23(4)*	−13(5)	−33(4)*
+ Indomethacin	−5(4)	−46(4)*	−4(2)	−28(4)*	3(3)	−24(4)*	2(3)	−31(3)*
Δ Mesenteric flow (%)								
− Indomethacin	−19(4)*	−39(5)*	−17(7)*	−29(4)*	−10(5)	−29(8)*	−8(7)	−37(5)*
+ Indomethacin	−33(4)*	−44(3)*	−29(4)*	−33(4)*	−31(3)*	−43(4)*	−26(5)*	−42(3)*
Δ Hindquarters flow (%)								
− Indomethacin	55(7)*	−3(8)	47(9)*	10(4)*	47(5)*	1(8)	60(5)*	3(4)
+ Indomethacin	61(9)*	16(8)*	37(4)*	6(3)	37(7)*	8(4)	43(10)*	21(9)
Δ Renal conductance (%)								
− Indomethacin	4(4)	−55(2)*	3(8)	−37(3)*	24(7)*	−31(4)*	20(7)*	−43(4)*
+ Indomethacin	15(4)*†	−54(3)*	3(4)	−35(5)*	22(8)*	−30(4)*	16(4)*	−39(4)*
Δ Mesenteric conductance (%)								
− Indomethacin	3(6)	−51(4)*	−10(7)	−37(3)*	4(9)	−37(7)*	27(12)	−46(4)*
+ Indomethacin	−19(4)*†	−53(3)*	−24(4)*†	−39(6)*	−19(6)*†	−47(4)*	−15(7)†	−49(3)*
Δ Hindquarters conductance (%)								
− Indomethacin	98(11)*	−21(7)*	64(17)*	−2(4)	69(6)*	−10(7)	120(11)*	−13(5)
+ Indomethacin	96(14)*	−2(6)	48(9)*	−5(3)	61(10)*	−1(4)	64(13)*†	9(10)

Values are means ± s.e.mean ($n = 8$).
* $P < 0.05$ versus baseline (Friedman's test).
† $P < 0.05$ for corresponding values in the absence and presence of indomethacin (Wilcoxon's test).

initial hindquarters vasodilatation and mesenteric vasoconstriction and the subsequent renal vasoconstriction were not affected by indomethacin, although the later mesenteric vasoconstriction was greater in the absence than in the presence of indomethacin (Table 2).

The initial fall in MBP following the 40 pmol dose of Et-2 did not reach significance, although the subsequent rise did, but neither the MBP changes nor the associated increases and decreases in HR were affected by indomethacin (Table 3). However, in the presence of indomethacin there was an initial mesenteric vasoconstriction following Et-2 that was not seen in the absence of indomethacin (Table 2). The early hindquarters vasodilatation and later renal and mesenteric vasoconstrictions were unaffected by indomethacin (Table 2).

Responses to endothelin-3

The initial responses to the 4 pmol dose of Et-3 (falls in MBP, and mesenteric vascular conductance and rises in HR and renal and hindquarters vascular conductances) were not affected by indomethacin (Table 2). Moreover, the subsequent responses were not different in the two conditions (Table 2).

The 40 pmol dose of Et-3 caused initial hypotension and tachycardia and renal and hindquarters vasodilatations that were unaffected by indomethacin (Table 3). However, in the presence of indomethacin there was an initial mesenteric vasoconstriction in response to Et-3 that was not seen in the absence of indomethacin (Table 3). The later pressor, bradycardic and renal and mesenteric vasoconstrictor responses to Et-3 were unaffected by indomethacin (Table 3).

Responses to sarafotoxin-S6b

The initial fall in MBP following the 4 pmol dose of S6b was significant only in the presence of indomethacin, although the actual change in MBP was not different from that seen in the absence of indomethacin (Table 2). The associated regional haemodynamic changes (renal and hindquarters vasodilatation and mesenteric vasoconstriction) were not different in the absence and presence of indomethacin (Table 2), neither were the later changes (renal and mesenteric vasoconstrictions, hindquarters vasodilatation) (Table 2).

The 40 pmol dose of S6b caused an early fall in MBP that was significantly attenuated by indomethacin (Table 3). This effect was accompanied by a reduction in the hindquarters vasodilator response to S6b, and by a mesenteric vasoconstriction not seen in the absence of indomethacin (Table 3). Thereafter, the pressor effects and regional haemodynamic changes (renal and mesenteric vasoconstrictions) evoked by S6b were not affected by indomethacin (Table 3).

Discussion

The present work has shown that the initial hypotensive effects of low bolus doses of Et-1, Et-2 and Et-3 and S6b were associated with different regional haemodynamic profiles that were differentially affected by indomethacin when changes in MBP were not. The later pressor and regional constrictor effects of the endothelins and S6b were not enhanced by indomethacin. These results also indicate that while endogenous cyclo-oxygenase products could influence the initial responses to endothelins and S6b, other mechanisms must also contribute to the hypotensive and vasodilator effects of these peptides. The initial hypotensive responses to the peptides were so rapid that it is not likely they were modified by baroreflex-mediated changes in autonomic neuronal outflow to the different vascular beds investigated. Moreover, the 4 pmol bolus dose of the peptides was chosen because it had borderline effects on MBP, but clear-cut regional haemodynamic actions.

Indomethacin alone caused significant mesenteric vasoconstriction, indicating that tonic release of cyclo-oxygenase products might be important in controlling conductance in this region of the circulation. However, there was no evidence that indomethacin influenced the initial hypotension or caused a significant change in the associated haemodynamic events following the 4 pmol dose of the peptides although, after this dose of Et-1, there was an early renal vasodilatation in the

presence of indomethacin that was not seen in its absence (Table 2). Furthermore, following the 40 pmol dose of Et-1 there was a significant difference between the renal conductance changes in the absence and presence of indomethacin (Table 3), with a significant renal vasodilatation occurring in the latter condition. In rats, arachidonic acid and several prostanoids, including prostaglandin E_2 (PGE_2) and PGD_2, have renal vasoconstrictor effects (Gerber & Nies 1979; Quilley *et al.*, 1989). Therefore, the most likely explanation of the present findings is that any initial renal vasodilator effects of Et-1 were masked by concurrent release of vasoconstrictor prostanoids. This contrasts with the picture in rabbits where Et-1 stimulates the release of renal cyclo-oxygenase products that are vasodilator and act to limit the renal vasoconstrictor effects of Et-1 (Rae *et al.*, 1989).

In the present work the early renal vasodilator responses to the 40 pmol dose of Et-3 and S6b were not affected by indomethacin and were not different from the effect seen with Et-1 in the presence of indomethacin (Table 3). These findings indicate that this vasodilator mechanism does not involve cyclo-oxygenase products. It is feasible that Et-1, Et-3 and S6b release an alternative relaxing factor from endothelial cells (De Nucci *et al.*, 1988) (see below), although it was not possible to determine the extent to which autoregulatory phenomena might have contributed to the early renal vasodilatation seen following Et-1 and -3 and S6b. However, at least in the case of Et-3, this cannot be the sole explanation of the vasodilatation since the increase in renal conductance was associated with a significant increase in flow when MBP fell. Whatever the mechanism responsible for the early renal vasodilatation following Et-1 or -3 or S6b, it is noteworthy that it was not seen following the 40 pmol dose of Et-2 used in the present experiments (Table 3).

The 4 pmol dose of all four peptides caused early mesenteric vasoconstriction only, and these effects were not enhanced by indomethacin (Table 2). Hence, there was no evidence for anything other than primary constrictor responses to this dose of the peptides in this vascular bed, but administration of the 40 pmol dose of the peptides revealed a more complex picture. In the absence of indomethacin, none of the peptides caused an initial mesenteric vasoconstriction (Table 3); indeed, there was a numerical increase in mesenteric conductance following administration of S6b that just failed to reach significance (Table 3). However, in the presence of indomethacin, the 40 pmol dose of all four peptides caused an initial reduction in mesenteric vascular conductance that was significantly different from the response in the absence of indomethacin (Table 3). As mentioned above, indomethacin alone caused mesenteric vasoconstriction but, of itself, this would be expected to enhance rather than reduce vasodilator responses (Myers & Honig, 1969). Thus, it is likely that, following the 40 pmol dose, all four peptides exerted mesenteric vasoconstrictor effects that were masked by release of vasodilator cyclo-oxygenase products. These results are consistent with recently published data obtained in systems other than conscious animals (De Nucci *et al.*, 1988; Warner *et al.*, 1989a,b; Thiemermann *et al.*, 1989; Lidbury *et al.*, 1989; Herman *et al.*, 1989).

Et-1, Et-2 and Et-3 and S6b at both doses caused obvious initial hindquarters vasodilatations. With the 4 pmol dose these effects were similar and not influenced by indomethacin. However, the hypotensive effect of the 40 pmol dose of S6b (which was numerically the greatest) was reduced in the presence of indomethacin, in association with a significant attenuation of the rise in hindquarters vascular conductance (Table 3). In the presence of indomethacin, the hindquarters vasodilator effects of Et-1, Et-2 and Et-3 were unchanged and similar to the vasodilatation elicited by S6b. These results indicate that: (1) a component of the initial hypotensive and hindquarters vasodilator response to S6b was due to release of vasodilator cyclo-oxygenase products, and that this mechanism was not triggered by the same doses of the endothelins and (2) Et-1, Et-2, Et-3 and S6b can elicit marked hindquarters vasodilatation by mechanisms not sensitive to indomethacin.

Recently, Whittle *et al.* (1989) demonstrated that the hypotensive response to Et-1 in pentobarbitone-anaesthetized rats was reduced by 72% in the presence of N^G-monomethyl-L-arginine (L-NMMA), a compound that inhibits endothelial cell nitric oxide production (see Moncada *et al.*, 1989). However, we have been unable to antagonize the hypotensive and hindquarters vasodilator effects of Et-1 (40 pmol) with L-NMMA in conscious rats (Gardiner *et al.*, 1989), even with doses 5 fold higher than the highest used by Whittle *et al.* (1989). In fact, as expected (Myers & Honig, 1969), because of the hypertension and vasoconstriction caused by L-NMMA, the hypotension and hindquarters vasodilator responses to Et-1 are enhanced (Gardiner *et al.*, 1989). A similar phenomenon is observed when Et-1 is administered during infusion of arginine vasopressin, at a rate adjusted to give an increase in MBP and a decrease in hindquarters conductance the same as those seen following L-NMMA (Gardiner *et al.*, 1990). Thus, there is no evidence that endothelial cell nitric oxide production was contributing to the hypotension or hindquarters vasodilator responses to Et-1 in our present experimental protocols. However, the possible involvement of such a mechanism in the responses to Et-2, Et-3 or S6b has not been investigated. Furthermore, we can say nothing about the putative involvement of nitric oxide-mediated and/or indomethacin-sensitive processes in the responses to Et-1, Et-2, Et-3 or S6b at higher doses than those used here.

The finding that the non-significant fall in MBP elicited by Et-2 was accompanied by hindquarters vasodilatation similar to that following Et-1 or Et-3 (Tables 1 and 2) argues against the apparent difference between the effects of the peptides on MBP being simply a dose-dependent phenomenon. However, it is quite feasible that experiments conducted with higher doses of the peptides would reveal patterns of response different from those described here (for example, we have found that bolus doses of 400 pmol of Et-2 cause marked hypotension (Gardiner, Compton & Bennett, unpublished observations). In addition, with higher doses of the peptides, the patterns of interaction between vasoconstrictor and vasodilator mechanisms might vary from those observed in the current experiments.

In summary, the present work has shown that Et-1, Et-2, Et-3 and S6b have haemodynamic profiles of action that depend on the dose administered. The early renal vascular actions of the 4 and 40 pmol doses of Et-1, the initial hindquarters vasodilator effects of the 40 pmol dose of S6b, and the early actions of the 40 pmol dose of all four peptides on the mesenteric vasculature, were influenced by mechanisms inhibited by indomethacin. However, only in the case of the 40 pmol dose of S6b did indomethacin influence the initial hypotension. It is likely, therefore, that mechanisms other than the relase of vasodilator cyclo-oxygenase products are responsible for the vasodilator effects of low doses of endothelins and S6b in conscious rats. Moreover, since the later pressor and regional vasoconstrictor effects of both doses of Et-1, Et-2, Et-3 and S6b were not enhanced by indomethacin, it appears that vasodilator prostanoids do not offset the later vasoconstrictor effects of low doses of these peptides.

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Influence of plasma protein content and platelet number on the potency of PAF and its antagonist RP 59227 in rabbit platelet preparations

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1 The potency of platelet activating factor (PAF) as a pro-aggregatory agent and of RP 59227, a selective antagonist of PAF-induced platelet aggregation, was determined in several types of rabbit platelet preparations.

2 PAF produced concentration-dependent responses irrespective of whether the suspension medium for the platelets (200,000 per μl) was undiluted plasma (PRP), saline-diluted plasma (dil. PRP) or a salt solution (WP: washed platelets). The potency of PAF, expressed as pD_2, was 3 fold higher in WP than PRP or diluted PRP (dil. PRP) for which the ratio (v/v) of total plasma to saline was 1:1.5. In PRP and WP preparations, an increase in the number of platelets in the reaction medium from 200,000 to 600,000 enhanced the potency of PAF slightly (2.3 fold). Furthermore, PAF was 3 times more potent in WP than PRP when studied in preparations containing either 200,000, 400,000 or 600,000 platelets per μl.

3 RP 59227, like the reference compounds WEB 2086 and CV-6209, behaved as a competitive antagonist against PAF-evoked platelet aggregation in PRP, WP and dil. PRP (200,000 platelets per μl). Their potency was slightly greater (1.6 to 2.6 fold more) in dil. PRP than in WP, but in PRP it was 3.5 to 4.3 times lower than in WP. RP 59227 was 2.3 and 5.0 times less potent when the platelet number of the PRP suspension was increased from 200,000 to 400,000 and 600,000 per μl, respectively, whereas the potency of RP 59227 in WP was not modified by these changes in platelet number. Furthermore, CV-6209, RP 59227 and WEB 2086 were found to be 2.8 to 3.3 times more potent when studied after 10 rather than 1 min incubation time.

4 In conclusion, methodological variables such as platelet number, plasma protein content and incubation time can modify the potency not only of PAF, as an aggregatory agent, but also of its antagonists. Thus, only platelet aggregation studies carried out by using well-defined experimental conditions afford an appropriate investigational approach to establish a potency rank order for PAF-receptor antagonists. Furthermore, dil. PRP which can be easily and rapidly prepared appears to be as appropriate as WP to determine pA_2 values for PAF antagonists.

Introduction

Platelet activating factor (PAF) is an endogenous phospholipid which exerts a wide range of biological effects including platelet aggregation. This effect seems to be mediated *via* PAF receptors as suggested by the high correlation between binding and functional parameters in rabbit (Robaut *et al.*, 1987), canine (Tahraoui *et al.*, 1988) and human (Tahraoui *et al.*, 1990) platelets.

In platelets, the potency of PAF receptor antagonists is often assessed by determining the concentration of the antagonist which inhibits by 50% the aggregation response to a single concentration of PAF (IC_{50}). This method, albeit rapid and practical, has an intrinsic limitation in that it does not allow comparison of values obtained from different investigators since IC_{50} values depend on the Michaelis constant (Cheng & Prussof, 1973) and the concentration of the substrate (PAF) which varies from one laboratory to another (O'Donnell & Barnett, 1988). However, in some recent studies, pA_2 values, against PAF-induced aggregation of platelets or polymorphonuclear leukocytes, were reported for several PAF receptor antagonists, and on the basis of these results, it has been suggested that PAF may function as an agonist of more than one receptor subtype (Lambrecht & Parnham, 1986; Stewart & Dusting, 1988).

The aim of this investigation was to examine the possible influence of experimental conditions, such as number of platelets and presence of plasma protein in the suspension medium as well as incubation time, on the potency of PAF, as a platelet aggregatory agent, and of RP 59227, a novel, very potent, (+)-enantiomeric antagonist of PAF. Indeed, in a functional test using human polymorphonuclear leukocytes, RP 59227, was recently shown to be, respectively, 39, 275, 390 and 1040 fold more active than the reference compounds WEB 2086, BN 52021, kadsurenone and CV 3988, in blocking elastase release induced by PAF (Marquis *et al.*, 1989).

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Methods

The marginal ear artery of conscious male New Zealand rabbits (Hybrid Hy 2000, Achard de la Vente, Passais-la-Conception, France) weighing 2.3 to 2.8 kg, was incised and five blood samples, each of 9 ml, were collected into tubes containing 1 ml of solution. For PRP, the solution contained 3.1% (w/v) sodium citrate and for the preparation of washed platelets (WP), it contained 0.33% citric acid, 2.2% disodium citrate, 0.22% sodium dihydrogenophosphate and 0.1% dextrose. Blood was immediately centrifuged at 100 *g* for 20 min to obtain PRP and a further centrifugation at 1000 *g* for 15 min yielded the platelet-poor plasma (PPP).

Washed platelets (WP) were obtained by applying the method described by Ardlie *et al.* (1970). PRP was centrifuged at 1000 *g* for 15 min and the pellet washed firstly with a Tyrode solution containing $MgCl_2$ (2 mM) and EGTA (0.2 mM) and then with a Tyrode solution minus the EGTA. The platelets were finally suspended in an assay buffer solution (composition in mM: NaCl 140, KCl 2.7, NaH_2PO_4 0.4, $MgCl_2$ 1.0, $NaHCO_3$ 12.0, $CaCl_2$ 0.9, Tris HCl 10.0, dextrose 6.2, bovine serum albumin 0.25% and apyrase 0.2 mg ml^{-1}). PRP and WP suspensions were adjusted with PPP and the assay buffer, respectively, such that they contained 200,000,

400,000 or 600,000 platelets per μl. The diluted PRP at 200,000 cells per μl (referred to as dil.PRP) was prepared by diluting PRP with an appropriate mixture of PPP and saline which kept the ratio of total plasma to saline constant at 1:1.5 from preparation to preparation (O'Donnell & Barnett, 1988). In some experiments with 200,000 platelets per μl, different ratios of PPP to saline (1:1; 1.5:1; 2.3:1) were used.

Aggregation responses to PAF (0.12 to 16.5 nM added in 30 μl volumes) were measured by using an aggregometer (Model 470, Chrono-Log Corp., Havertown, U.S.A.). Aliquots of 340 μl platelet suspension were generally prewarmed for 10 min (1 min and 20 min prewarming times were also used in a comparative study) at 37°C in the presence (30 μl) of either solvent (control), RP 59227 (3.4–677 nM), WEB 2086 (1.6–329 nM) or CV-6209 (1.2–156 nM). The mixture was then stirred at 1000 r.p.m. for 1 min before the addition of increasing concentrations (0.12 to 16.5 nM) of PAF. Concentration-aggregatory response curves for PAF were determined in the three preparations (PRP, dil.PRP, WP) containing 200,000 platelets per μl so as to assess the influence of plasma on the studied responses. The role played by the number of platelets on responses to PAF, was determined by using PRP and WP suspensions containing 200,000, 400,000 and 600,000 platelets per μl.

Analysis of results

For each individual experiment, the log concentrations of PAF were plotted against responses, expressed as percentages of the maximum response to PAF (maximum increase in light transmittance to a supramaximal concentration of PAF) obtained in the control curve. Values of pD_2 were calculated for each PAF concentration-response curve by using a straight line regression applied to experimental values enclosed between 20 and 80% of the maximal response (E_{max} = 100%). Similarly, pA_2 values were calculated from Schild plots (results fitted with regression lines; Arunlakshana & Schild, 1959) for individual preparations in which the solvent and three concentrations of antagonists were studied. Results are reported as means ± s.e.mean for a minimum of $n = 4$ preparations for each experimental group. The significance of the difference between two mean values was assessed by use of paired or unpaired Student's t test. P values less than 0.05 were considered to be significant.

Drugs

Drugs used were platelet activating factor (1-O-octadecyl-2-O-acetyl-*sn*-glycero-3-phosphorylcholine (Bachem AG, Bubendorf, Switzerland); RP 59227 base ((+)-N-(3-benzoylphenyl)3-(3-pyridyl)-1H,3H-pyrrolo[1,2-c]-thiazolo-7-carboxamide) (Rhône-Poulenc Santé, Vitry-sur-Seine, France); WEB 2086 (3-[4-(2-chlorophenyl)-9-methyl-6H-thieno[3,2-f][1,2,4]triazolo-[4,3-a][1,4]-diazepin-2-yl]-1-(4-morpholinyl)-1-propanone (Boehringer-Ingelheim, Ingelheim-am-Rhein, F.R.G.); CV-6209 hydrochloride [2-[N-acetyl-N-(2-methoxy-3-octadecylcarbamoyloxy-propoxycarbonyl)-aminomethyl]-1-ethylpyridinium chloride) (Takeda Chemical Industries Ltd, Osaka, Japan); ADP (Diagnostica Stago, Asnières, France) and collagen (Hormon Chemie, München, R.F.A.).

PAF was initially dissolved (1 mg ml^{-1}) with ethanol (70%), then diluted by adding appropriate quantities of saline containing 0.25% bovine serum albumin (BSA: reference number A-4378, Sigma, St Louis, MO, U.S.A.). RP 59227 (3 mg) was firstly dissolved in 3 ml of PEG_{400}. Then, dilutions were made by slowly adding appropriate quantities of standard saline under continuous sonication to obtain needed transparent solutions. A stock solution of WEB 2086 (13.3 mg) was made up in saline (10 ml), stored at −4°C and diluted appropriately with saline on the day of the experiment. Solutions of other compounds were also made in saline.

Results

Studies on the potency of PAF

Influence of the suspension medium containing 200,000 platelets per μl PAF produced a concentration-dependent platelet aggregatory response in PRP, saline diluted PRP or WP (Figure 1). The potency of PAF, expressed as pD_2, was 3 fold higher in WP than in either PRP or dil.PRP for which the total plasma to saline ratio (v/v) was 1:1.5 (Table 1). However, in experiments for which the ratio of total plasma to saline was increased (1:1, 1.5:1 or 2.3:1), the potency of PAF (pD_2 = 8.64 ± 0.04, 8.59 ± 0.04 or 8.59 ± 0.03, respectively, $n = 4$) was virtually the same as that found in the dil.PRP with a ratio of 1:1.5 (pD_2 = 8.54 ± 0.02, $n = 4$).

Influence of the number of platelets in different suspension media In PRP and WP preparations, an increase in the number of platelets in the reaction medium from 200,000 to 600,000 enhanced significantly (2.3 fold) the potency of PAF. A small but significant increase (1.7 fold) in PAF potency was also found when the PRP (but not WP) platelet number was changed from 200,000 to 400,000 per μl. Furthermore, PAF was approximately 3 fold more potent in WP than in PRP when studied in preparations containing either 200,000, 400,000 or 600,000 platelets per μl (Table 1).

In PRP, the aggregatory activity of collagen but not of ADP increased by approximately 2 fold when the number of platelets was increased from 200,000 to 600,000. In WP containing 200,000 and 400,000 platelets collagen was slightly (1.7 fold), but significantly, more potent than in PRP. In dil.PRP, the potency of collagen was the same as in PRP. However, ADP was 22 times more active in dil.PRP than in PRP. ADP could not be studied in WP since it did not aggregate platelets in this preparation.

Studies on the potency of PAF receptor antagonists

In PRP, dil.PRP and WP, the PAF receptor antagonists CV-6209, RP 59227 and WEB 2086, in the concentrations tested, were all devoid of aggregatory properties. These compounds produced a concentration-dependent, dextral, parallel displacement of the control concentration-response curve to PAF without affecting its maximum.

Influence of suspension media containing 200,000 platelets per μl on the potency of RP 59227 These studies were carried in PRP, dil.PRP and WP. The potency of RP 59227, as an antagonist of PAF-induced platelet aggregation, was significantly greater in dil.PRP (pA_2 = 8.46 ± 0.05) than in WP (pA_2 = 8.16 ± 0.10). However, when the compound was

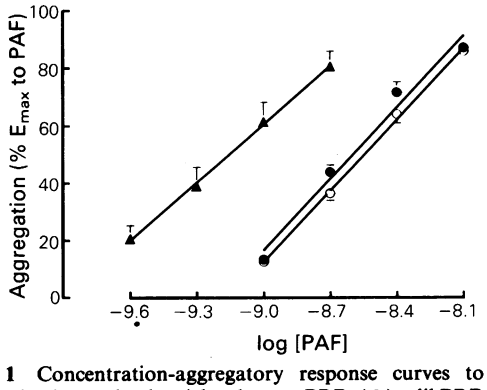

Figure 1 Concentration-aggregatory response curves to PAF in rabbit platelets: platelet-rich plasma PRP (●), dil.PRP (○) and washed platelet suspensions (▲) containing 200,000 platelets per μl. The mean maximum increase in light transmittance to a supramaximal concentration of PAF was 61.2 ± 1.5 (PRP; $n = 6$), 61.2 ± 1.5 (dil.PRP; $n = 9$) and 64.8 ± 1.3 (WP; $n = 10$)%. This value was taken as E_{max} (= 100%).

Table 1 Aggregatory potency of PAF, collagen and ADP in different platelet preparations expressed as pD_2 ($-\log EC_{50}$) values ($n = 4$ preparations for each group)

Platelet preparation	*Platelet number* (per μl)	*PAF*	pD_2 ($-\log EC_{50}$) *Collagen*	*ADP*
PRP	200,000	8.59 ± 0.02	0.23 ± 0.06	5.10 ± 0.06
	400,000	8.82 ± 0.04*	0.34 ± 0.07	5.19 ± 0.10
	600,000	8.97 ± 0.04*	0.57 ± 0.08*	5.16 ± 0.12
dil.PRP	200,000	8.54 ± 0.02	0.31 ± 0.01	6.44 ± 0.05†
WP	200,000	9.07 ± 0.07†	0.45 ± 0.04†	
	400,000	9.21 ± 0.04†	0.57 ± 0.05†	
	600,000	9.44 ± 0.04*†	0.68 ± 0.03*	

PRP, platelet-rich plasma; dil.PRP, platelets in saline-diluted plasma; WP, washed platelets.
* Value significantly ($P < 0.01$, t test) different from that obtained in the same preparation with 200,000 platelets per μl.
† Value significantly ($P < 0.05$, t test) different from that obtained in PRP with the same number of platelets.

studied in PRP ($pA_2 = 7.53 \pm 0.06$), RP 59227 was 8.5 and 4.3 fold ($P < 0.01$) less potent, respectively, than in dil.PRP and WP. Similarly WEB 2086 and CV-6209 were significantly more potent in the latter preparations than in PRP (Table 2).

Influence of the number of platelets in PRP and WP on the potency of RP 59227 In PRP, RP 59227 was 2.3 and 5.0 times ($P < 0.001$), respectively, less potent against PAF-induced aggregation when the number of platelets of the suspension was increased from 200,000 per μl to 400,000 and 600,000 per μl. However, in WP, the potency of RP 59227 was not significantly modified by these changes in platelet numbers (Figure 2).

Influence of the incubation time in dil.PRP preparations on the potency of PAF antagonists In dil.PRP, CV-6209, RP 59227 and WEB 2086 were found to be 3.2, 3.3 and 2.8 times, respectively, more potent when studied after 10 min than 1 min incubation time (Table 3). For RP 59227, prolongation of this time to 20 min did not further change the pA_2. For this reason, all the studies described above were carried out by setting the incubation time to 10 min. PAF exhibited the same potency after incubating the platelets with the solvent of the antagonists for 1 ($pD_2 = 8.63 \pm 0.03$, $n = 7$) or 10 ($pD_2 = 8.54 \pm 0.02$, $n = 4$) min. However, after a 20 min incubation, the potency of PAF was found to be significantly decreased by approximately 2 fold ($pD_2 = 8.26 \pm 0.07$, $n = 4$).

Comparison of PAF antagonists in different platelet preparations This study was carried out in PRP and WP containing 200,000 and 400,000 platelets per μl and in dil.PRP (200,000 platelets per μl). It is pertinent to note that dil.PRP with 400,000 platelets per μl could not be prepared since it would have required a PRP at 1 million platelets per μl, a

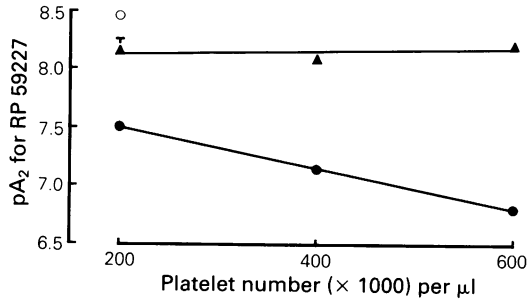

Figure 2 pA_2 values of RP 59227 against PAF-induced aggregation in PRP (●) and WP (▲) containing 200,000, 400,000 and 600,000 per μl and in dil.PRP (○) (adjusted to 200,000 pl per μl). The statistical analysis of results obtained with 200,000 and 400,000 platelet per μl is given in Table 2. The pA_2 in PRP containing 600,000 platelets per μl was significantly different ($P < 0.001$) from those obtained in preparations with 200,000 and 400,000 platelets per μl.

Table 2 Antagonism of PAF-induced aggregation in rabbit platelet rich plasma (PRP), washed platelets (WP) and diluted PRP (dil.PRP) by RP 59227, WEB 2086 and CV-6209 ($n = 4$–5 preparations per group)

Preparation (cells μl^{-1})	*RP 59227* pA_2	*Slope*	*WEB 2086* pA_2	*Slope*	*CV-6209* pA_2	*Slope*
PRP						
(200,000)	7.53 ± 0.06	0.92 ± 0.06	7.78 ± 0.05	0.84 ± 0.05	7.99 ± 0.07	0.80 ± 0.05
(400,000)	7.14 ± 0.04†	0.88 ± 0.02	7.48 ± 0.02†	0.89 ± 0.03	7.69 ± 0.07†	0.91 ± 0.08
WP						
(200,000)	8.16 ± 0.10*	0.90 ± 0.06	8.32 ± 0.04*	0.92 ± 0.03	8.60 ± 0.04*	0.75 ± 0.01
(400,000)	8.09 ± 0.06*	0.88 ± 0.04	8.45 ± 0.06*	0.96 ± 0.06	8.68 ± 0.08*	0.86 ± 0.06
dil.PRP						
(200,000)	8.46 ± 0.05*	0.86 ± 0.01	8.73 ± 0.07*	0.85 ± 0.04	8.81 ± 0.05*	0.92 ± 0.03

* Value significantly ($P < 0.01$, t test) different from that obtained in PRP containing the same number of platelets.
† Value significantly ($P < 0.05$, t test) different from that obtained in the same preparation containing 200,000 cells per μl.

Table 3 Influence of the contact time between RP 59227, WEB 2086 and CV-6209 and the platelet suspension on the potency of these compounds as antagonists of PAF-induced aggregation in dil.PRP ($n = 4$ preparations per group)

Contact time (min)	*RP 59227* pA_2	*Slope*	*WEB 2086* pA_2	*Slope*	*CV-6209* pA_2	*Slope*
1	7.95 ± 0.03	0.84 ± 0.03	8.21 ± 0.06	0.92 ± 0.05	8.36 ± 0.07	0.86 ± 0.07
10	8.46 ± 0.05*	0.86 ± 0.01	8.73 ± 0.07*	0.85 ± 0.07	8.81 ± 0.05*	0.92 ± 0.03

All three compounds were approximately 3 fold more potent after a 10 than 1 min incubation time.
* Value significantly ($P < 0.01$, t test) different from that obtained after 1 min incubation time.

value much greater than that obtainable in our laboratory (542,500 ± 7,620 platelets per μl, $n = 30$). In PRP, WP and dil.PRP with platelet numbers adjusted to 200,000 cells per μl, CV-6209 was as potent as WEB 2086 but significantly more active (2 to 3 fold) than RP 59227 (Table 2). In experiments in which the platelet number of PRP and WP was increased to 400,000 platelets per μl, the potency of CV-6209 was again not different from that of WEB 2086 but was 3.5 (PRP) and 3.9 (WP) times greater than that found for RP 59227.

Discussion

This study clearly indicates that several methodological variables can affect not only the potency of PAF as a platelet aggregating agent, but also the potency of its antagonists.

The potency of PAF in producing platelet aggregation was found to depend on the medium used to suspend the platelets. For preparations containing the same number of platelets, PAF was approximately 3 fold less potent in PRP than in WP. This may be due to the presence of plasma proteins in PRP which are known to bind lipophilic agents such as PAF and thus to lead to a reduction in the amount of PAF available for its receptor sites on platelets. This suggestion would agree with the findings of Tokumura *et al.* (1987). In the present study using PRP diluted with saline, the potency of PAF was already maximal when the protein content of the preparation was at least 40% of that present in the PRP.

The possible influence of the number of platelets on the potency of PAF was studied in PRP and WP preparations. An increase in platelets from 200,000 per μl to 600,000 per μl was accompanied by a small increase in PAF potency (2.3 fold). It is possible that the cascade of reactions leading to the PAF-induced aggregation might be facilitated by an increase in the number of platelets in the reaction medium. However, this was not due to the release of secondary mediators since this phenomenon occurred also in preparations of PRP and WP exposed to a cyclo-oxygenase inhibitor [aspirin (ASA) 1 mM] and ADP scavengers [combination of 312.5 mgl^{-1} creatine phosphate (CP) and 152.5 mgl^{-1} creatine phosphokinase (CPK)] (personal observation).

It is of interest to note that in PRP the aggregatory potency of ADP was not modified when the number of platelets in the reaction medium was increased from 200,000 to 600,000. In contrast, as already discussed for PAF, collagen activity increased slightly under these experimental conditions.

RP 59227 (Robaut *et al.*, 1988; Marquis *et al.*, 1989), WEB 2086 (Casals-Stenzel *et al.*, 1987) and CV-6209 (Terashita *et al.*, 1987) caused parallel displacements of the PAF log concentration-response curve without depressing the maximum response to PAF, in all the preparations of platelets used in this study. However, the potency of these antagonists was influenced by the medium in which platelets were suspended. In WP and dil.PRP, RP 59227 was 4.3 and 8.5 times more potent, respectively, than in PRP. It is proposed that plasma proteins present in PRP or dil.PRP could partially bind the PAF antagonists studied. These findings together with those already published by us (Tahraoui *et al.*, 1988) can help to explain, at least in part, the greater potency exhibited by many PAF antagonists in displacing [^{3}H]-PAF from its binding sites than in antagonizing PAF-induced aggregation in PRP.

It is pertinent to comment on the influence of plasma proteins in the reaction medium on the potency of PAF and of PAF antagonists. For preparations containing the same number of platelets, PAF was always approximately 3 fold more potent in WP than in PRP or dil.PRP. Similarly, RP 59227, WEB 2086 and CV-6209 were more potent in WP than PRP. However, these drugs were slightly but significantly more potent (1.6 to 2.6 fold more) in dil.PRP than WP. Thus, dil.PRP or WP would appear to be equally suitable for experiments in which pA_2 values for PAF receptor antagonists are to be determined. This finding may be of practical value since dil.PRP can be prepared more easily and rapidly than WP. It is difficult to give a satisfactory explanation for the slightly higher potency of PAF receptor antagonists in dil.PRP than WP particularly since RP 59227, WEB 2086 and CV-6209 were several times less potent in PRP than WP. It is possible that the amount of protein present in dil.PRP (60% less than in PRP) is too low to reduce significantly the biophase concentration of PAF receptor antagonists. On the contrary, for reasons as yet unknown, the amount of protein present in dil.PRP appears to enhance the potency of the PAF receptor antagonists examined in the present study.

The potency of WEB 2086 ($pA_2 = 7.31$) found by O'Donnell & Barnett (1988) is 26 fold smaller than that determined here ($pA_2 = 8.73$) using the same preparation (dil.PRP). A partial explanation for this large difference is the incubation time of the platelets with WEB 2086. This was only 1 min in the study of O'Donnell & Barnett (1988) but was 10 min for most of the experiments reported here. When we determined the pA_2 for WEB 2086 with a contact time of only 1 min, WEB 2086 was less potent ($pA_2 = 8.21$). However, despite our efforts to keep our experimental conditions as close as possible to those of O'Donnell & Barnett (1988), we still found WEB 2086 7.9 times more potent than was reported by the latter investigators. It is possible that there are other differences in experimental details which account for this discrepancy. Interestingly, the present study has provided evidence that a 1 min contact time with WEB 2086 is insufficient to achieve equilibrium between platelet PAF receptors and WEB 2086. Similarly, for RP 59227 and CV-6209, the prolongation of the incubation time from 1 min to 10 min resulted in an approximate 3 fold increase in potency.

In PRP but not in WP, the potency of RP 59227 was decreased if the number of platelets was increased from 200,000 to 400,000 or 600,000 cells per μl. This finding may indicate that plasma proteins present in PRP can facilitate the binding of RP 59227 to membrane sites either unrelated to PAF receptors or to non-functional PAF receptors. The reverse phenomenon was found for PAF which increased in potency with an increase in platelet numbers.

The slopes of the Schild plots for the PAF antagonists studied were generally less than unity. A theoretical explanation for this phenomenon may be the existence of a PAF uptake process in rabbit platelets (Kenakin, 1984). In this context, Homma *et al.* (1987) found that washed rabbit platelets are capable of internalizing PAF, a phenomenon which could lead to a decrease in the biophase concentration of PAF.

This study provides evidence that many experimental variables can influence the potency of PAF as an aggregatory agent, and the potency of PAF antagonists on rabbit platelets. Care should be given to the choice of the platelet suspension medium, especially in relation to the amount of plasma proteins used, to the number of platelets used and to the incubation time with antagonists. Furthermore, these methodological details should be clearly indicated to allow the replication of the procedure by other laboratories. If experimental conditions are not identical between laboratories, then results obtained in platelet aggregation studies may not agree.

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Contractile activity of three endothelins (ET–1, ET–2 and ET–3) on the human isolated bronchus

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1 The effects of three endothelins: (i) the classical or human/porcine endothelin (ET–1); (ii) [Trp^6, Leu^7] endothelin (ET–2) and (iii) [Thr^2, Phe^4, Thr^5, Tyr^6, Lys^7, Tyr^{14}] endothelin or rat endothelin (ET–3) were tested on the human isolated bronchus.

2 ET–1 produced a concentration-dependent contraction of the human isolated bronchus that proceeded in two different steps. The first step was observed at very low concentrations ($pD_2 = 11.01 \pm 0.17$, $n = 10$) but corresponded to a low intrinsic activity ($E_{max} = 15.6 \pm 1.8\%$ of E_{max} induced by acetylcholine (ACh) 3×10^{-3} M, $n = 10$). This effect was potentiated by Bay K 8644 10^{-7} M ($E_{max} = 26.1 \pm 2.9\%$ of ACh 3×10^{-3} M, $n = 5$, $P < 0.05$), reduced by nicardipine 10^{-6} M ($E_{max} = 6.0 \pm 2.6\%$ of ACh 3×10^{-3} M, $n = 5$, $P < 0.05$) and strongly inhibited in calcium-free medium. The second step of the action of ET–1 corresponded to a lesser potency ($pD_2 = 7.90 \pm 0.17$, $n = 9$) but a higher intrinsic activity ($E_{max} = 82.5 \pm 4.7\%$ of ACh 3×10^{-3} M). This effect was not significantly modified by nicardipine 10^{-6} M or by Bay K 8644 10^{-7} M. Neither of the two effects was modified by indomethacin 3×10^{-6} M.

3 The effects of ET–2 and ET–3 were qualitatively similar to those of ET–1 but quantitatively different; for these two steps of contracting activity and for potency and efficacy the ranking was: ET–1 > ET–2 = ET–3.

4 Thus, ET-1 appears to be the most potent of these three substances in its effect on the human isolated bronchus. Its activity seems to involve the action of voltage-dependent calcium channels at low concentrations (10^{-12} to 10^{-9} M), whereas other mechanisms are involved at higher concentrations (10^{-8} to 3×10^{-7} M).

Introduction

Endothelin (ET–1) is an endothelium-derived 21-residue peptide recently described by Yanagisawa *et al.* (1988). It possesses a very potent vasoconstrictor activity on several isolated blood vessels where its action seems to be critically dependent upon the influx of calcium ions from the extracellular space through dihydropyridine-sensitive calcium channels (Yanagisawa *et al.*, 1988). ET–1 (10^{-12}–3×10^{-7} M) also exerts a potent contractile effect on guinea-pig and human isolated bronchial tissue (Uchida *et al.*, 1988; Maggi *et al.*, 1989a; Borges *et al.*, 1989) which is partially inhibited by the dihydropyridine calcium blockers nicardipine (10^{-8} M) or nifedipine (10^{-6} M) (Uchida *et al.*, 1988; Maggi *et al.*, 1989a). On the rat trachea, Turner *et al.* (1989) observed that contractile responses to ET–1 (10^{-8}–10^{-5} M) were attenuated following incubation for 1 h in calcium-free solution and almost completely abolished following incubation in the presence of EGTA. In contrast, these responses were unaffected by treatment with nicardipine (10^{-7} M) (Turner *et al.*, 1989).

ET–1 administered *in vivo* either intravenously or by inhalation induces in the guinea-pig a marked and sustained bronchoconstriction which is suppressed by pretreatment with the cyclo-oxygenase inhibitors indomethacin or meclofenamate (Payne & Whittle, 1988; Braquet *et al.*, 1989; Lagente *et al.*, 1989; Macquin-Mavier *et al.*, 1989).

Recently, three distinct endothelin-isopeptides, produced by three separate human genes have been described by Inoue *et al.* (1989). They have been called (i) ET–1, the 'classical' human and porcine endothelin, first described by Yanagisawa *et al.* (1988), (ii) ET–2, [Trp^6, Leu^7] endothelin and (iii) ET–3, [Thr^2, Phe^4, Thr^5, Tyr^6, Lys^7, Tyr^{14}] endothelin or rat endothelin (Table 1). The three peptides induce potent vasoconstrictor activity *in vitro* and a transient depressor response followed by a sustained pressor response *in vivo*, but the quantitative profiles of their pharmacological activities are different (Inoue *et al.*, 1989; Minkes *et al.*, 1989; Rodman *et al.*, 1989).

The effects of ET–2 and ET–3 on the bronchi have not yet been tested. In the course of an extensive study of the action of endothelins on airway smooth muscle, we studied the contractile effect of these substances on the human isolated bronchus and its modifications by calcium channel blockers or openers or by reduction of calcium in the medium.

Methods

Human bronchial tissue preparation

Bronchial tissues were removed from 13 patients with lung cancer at the time of the surgical operation (12 men and 1 women; mean age, 61.3 ± 1.6 yr; 8 squamous cell carcinomas, 3 oat cell carcinomas and 2 adenocarcinomas). Just after resection, segments of human bronchi with inner diameter of 3 to 5 mm were taken as far away as possible from the malignancy. They were placed in oxygenated Krebs-Henseleit solution (composition, mM: NaCl 119, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0 and glucose 11.7) at 4°C and stored overnight at 4°C. After removal of adhering fat and connective tissue, two to eight adjacent rings from the same bronchus were prepared and suspended under an initial tension of 2.5 g in Krebs-Henseleit solution, bubbled with 95%O_2, 5%CO_2, and maintained at 37°C. Changes of tension were measured isometrically with strain gauge amplifiers and I.O.S.-Moise 2 recorder system (Celaster, Celle l'Evescault, 86600 Lusignan, France).

Protocol

Each experiment began by contraction of the bronchial strips to maximal tension with acetylcholine (ACh 3×10^{-3} M), then maximal relaxation was induced with theophylline (3×10^{-3} M). During the next 120 min, the tissues were

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Table 1 Primary structure of the endothelins used in the present study

ET-1	Cys[1]-Ser-Cys-Ser-Ser-Leu-Met-Asp-Lys-Glu[10]-Cys-Val-Tyr-Phe-Cys-His-Leu-Asp-Ile-Ile[20]-Trp
ET-2	Cys[1]-Ser-Cys-Ser-Ser-**Trp[6]**-**Leu[7]**-Asp-Lys-Glu-Cys-Val-Tyr-Phe-Cys-His-Leu-Asp-Ile-Ile[20]-Trp
ET-3	Cys-**Thr[2]**-Cys-**Phe[4]**-**Thr[5]**-**Tyr[6]**-**Lys[7]**-Asp-Lys-Glu-Cys-Val-Tyr-**Tyr[14]**-Cys-His-Leu-Asp-Ile-Ile-Trp

ET-1: Endothelin-1 (human-porcine ET); **ET-2:** Endothelin-2; **ET-3:** Endothelin-3 (rat ET).

washed every 15 min, and the resting tension was adjusted to 2 to 2.5 g, which has been found to be optimal for recording contractions in such tissues (Advenier *et al.*, 1986; Naline *et al.*, 1989). Thereafter, cumulative concentration-response curves to endothelins were constructed by applying increasing concentrations at 15 to 20 min intervals in logarithmic increments. Acetylcholine (3×10^{-3} M) was added at the end of the concentration-response curve to determine the maximal response of the preparation. Concentrations of endothelins higher than 3×10^{-7} M were not tested because of the limited availability of the peptides. In some experiments, indomethacin, Bay K 8644 or nicardipine were added to the bath 30 min before the contractile agents. In all experiments, only one concentration-response curve to endothelins was recorded in each strip.

Concentration-response curves to histamine, acetylcholine, neurokinin A and leukotriene D_4 (LTD_4) were obtained under similar conditions.

In experiments performed in Ca^{2+}-free medium, after control contractions elicited by endothelin (10^{-10} or 3×10^{-8} M), ACh (3×10^{-3} M) or KCl (3×10^{-2} M) in normal Krebs solution, the human bronchi were incubated for 45 min in Ca^{2+}-free Krebs solution, and immediately thereafter for 15 min in the same solution to which ethylenediaminetetraacetic acid (EDTA) 10^{-3} M had been added. Then the preparation was immersed again in Ca^{2+}-free Krebs solution without EDTA, and after equilibration the contractile agents were added to the bath (Godfraind *et al.*, 1968; Advenier *et al.*, 1984). The effects of the contractile agents in Ca^{2+}-free medium are expressed as a percentage of control contractions obtained in the presence of Ca^{2+}.

The data are expressed in terms of pD_2 and in mg of tension or as a percentage of the maximum tension induced by ACh (3×10^{-3} M). pD_2 values were derived from the log concentration-effect curves and defined as the negative log of the drug concentration that caused 50% of maximal effect. The maximal effect (E_{max}) was calculated as the maximal increase in tone for each endothelin or contractile agent.

Statistical evaluation of data

Statistical analysis of the results was performed with Student's *t* test for paired or unpaired data. All values in the text and tables are expressed as mean ± s.e.mean.

Drugs

Drugs used were: endothelin ET-1, ET-2 and ET-3 (Peptide Institute Inc, Osaka, Japan), acetylcholine dihydrochloride (PCH, Paris, France), histamine HCl (Sigma, St. Louis, U.S.A.), LTD_4 (Sigma, St. Louis, U.S.A.), neurokinin A (NKA, Novabiochem, 4448 Läufelfingen, Switzerland), (±)-Bay K 8644, methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate (Bayer, Wuppertal, West Germany), nicardipine HCl (Sandoz, Basel, Switzerland), indomethacin (Sigma, St. Louis, U.S.A.), phosphoramidon [N-(alpha-L-rhamnopyranosyloxyhydroxy- phosphinyl)-L-leucyl-L-trytophan] (Sigma, St. Louis, U.S.A.). All agents were dissolved in water, except nicardipine, indomethacin and (±)-Bay K 8644 which were dissolved in ethanol. As with the other drugs, these solutions were then diluted with Krebs solution.

Results

Contractile activity of ET-1

ET-1 (10^{-12}–3×10^{-7} M) induced a concentration-dependent contraction of the human isolated bronchus (Figure 1). The contraction reached a plateau 12 to 15 min after addition of the peptide to the bath, whereas under similar conditions a plateau was reached in 3–5 min with acetylcholine (ACh). Two different levels of contraction could be observed in the ET-1-induced contraction. The first one was observed for low concentrations of ET-1 (10^{-12} to 10^{-9} M) with a maximal response of 15.6 ± 1.8% of the maximal contractile activity

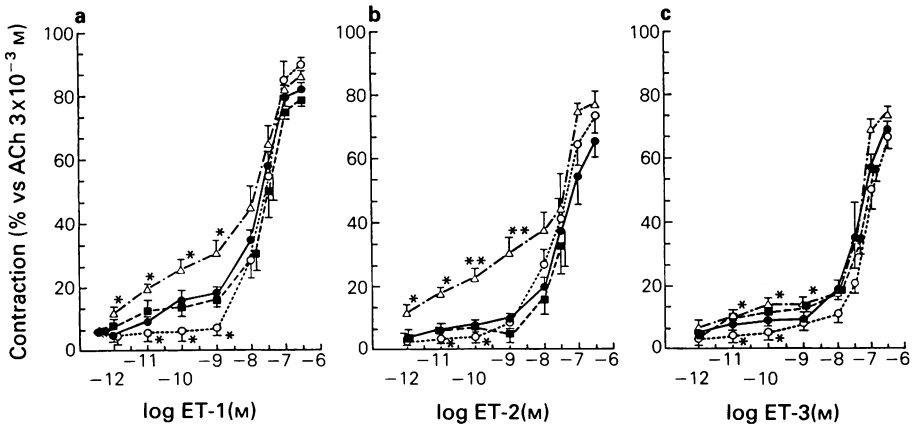

Figure 1 Response of human isolated bronchi to endothelin-1 (ET-1) (a), endothelin-2 (ET-2) (b) or endothelin-3 (ET-3) (c) in the absence (●) and in the presence of nicardipine 10^{-6} M (○), Bay K 8644 10^{-7} M (△) or indomethacin 3×10^{-6} M (■). Points represent means; vertical bars represent s.e.mean. Statistical differences are (*) $P < 0.05$ and (**) $P < 0.01$ as compared to control tissues by use of Student's *t* test for unpaired data. Number of experiments are: 7 to 10 for control concentration-response curves, 5 (ET-1) and 4 (ET-2 and ET-3) for concentration-response curves in the presence of nicardipine, Bay K 8644 or indomethacin.

Table 2 Apparent affinities (pD_2) and maximum effects (E_{max}) expressed in milligrams of tension (mg) or as percentage of the maximum effect of acetylcholine (% of ACh) measured with endothelin-1 (ET-1), histamine, acetylcholine, leukotriene D_4 (LTD_4) and neurokinin A (NKA) on human isolated bronchus

	n	pD_2	E_{max} (mg)	E_{max} (% of ACh)
ET-1 first step	10	11.01 ± 0.17*	279 ± 46*	15.6 ± 1.8*
ET-1 second step	9	7.90 ± 0.17	1276 ± 264	82.5 ± 4.7
Histamine	8	6.02 ± 0.11	1602 ± 293	92.4 ± 2.5
Acetylcholine	8	4.96 ± 0.12	1947 ± 372	100
LTD_4	4	7.67 ± 0.19	1755 ± 570	80.5 ± 6.8
NKA	6	8.48 ± 0.24	1749 ± 284	77.6 ± 6.1

Values are means ± s.e.mean. Experiments with NKA were performed in the presence of phosphoramidon 10^{-5} M.
* Assimilated to the effect observed for 10^{-10} M.

induced by ACh (3×10^{-3} M) and a pD_2 of 11.01 ± 0.17 (Table 2).

The second step of contraction was observed for higher concentrations (10^{-9} to 3×10^{-7} M) with a maximal effect of 82.5 ± 4.7% of the maximal contraction obtained with ACh 3×10^{-3} M; pD_2 value for this second step was 7.90 ± 0.17 (Table 2).

When compared with other potent constrictor agents, ET-1 appeared to be about 1,122,000 (first step) and 870 fold (second step) more potent than ACh; however, the comparison is questionable, at least as regards the first step, since the contraction observed was 7.8% with ET-1 and 50% with ACh. Under similar experimental conditions, histamine, LTD_4 and NKA were 11.5, 512 and 3311 fold respectively more potent than ACh.

When the E_{max} of the second step was examined, ET-1 appeared to have a slightly but significantly lower efficacy than ACh ($P < 0.05$), but a similar efficacy to histamine, LTD_4 and NKA (Table 2).

ET-1 responses were not modified by indomethacin (3×10^{-6} M), whatever the concentration (Table 3 and Figure 1). In contrast, the contractions induced by low concentrations of ET-1 (10^{-12} to 10^{-10} M) were suppressed by nicardipine (10^{-6} M, $P < 0.05$) or potentiated by Bay K 8644 (10^{-7} M, $P < 0.05$). But responses to ET-1 observed with high concentrations (10^{-9} to 3×10^{-7} M) were not altered by nicardipine or by Bay K 8644 (Table 3).

In calcium-free medium, the ET-1 (10^{-10} M)-induced response was markedly reduced, since the contractile response reached only 11.3 ± 7.5% of the control response in normal calcium medium (Table 4). A strong reduction was also observed in the case of 3×10^{-2} M KCl. In contrast, the ET-1 3×10^{-8} M-induced contraction was slightly reduced, reaching 56.3 ± 8.1% of the control contraction. This reduction of response was not significantly different from that observed with ACh 3×10^{-3} M (Table 4).

Contractile activity of ET-2 and ET-3

ET-2 and ET-3 elicited potent contractile activities in the human isolated bronchus (Figure 1). However, compared with ET-1, the contractile responses induced by low concentrations of ET-2 and ET-3 were lower (Figure 1). For example, at 10^{-10} M, ET-2 and ET-3 induced contractions that were 7.1 ± 0.9% and 8.0 ± 1.3% respectively of the maximal contraction induced by ACh 3×10^{-3} M, compared with the 15.6 ± 1.8% with ET-1. In concentrations higher than 10^{-9} M, the effects induced by ET-2 and ET-3 were qualitatively similar to those of ET-1. However, ET-1 was significantly ($P < 0.01$) more potent than ET-2 and ET-3 since the −log molar concentrations required to obtain 50% of the maximal contraction induced by ACh were respectively 7.77 ± 0.11 ($n = 9$), 7.23 ± 0.14 ($n = 8$) and 7.21 ± 0.08 ($n = 7$).

As was the case for ET-1, the contractile activities recorded for ET-2 and ET-3 were not altered by addition of indomethacin (3×10^{-6} M) (Figure 1 and Table 5). In contrast, in the presence of Bay K 8644 (10^{-7} M), the responses (E_{max}, %ACh) induced by small concentrations of both peptides

Table 3 Effect of indomethacin 3×10^{-6} M, nicardipine 10^{-6} M and Bay K 8644 10^{-7} M on the potency (pD_2) and maximal effect (E_{max}) of endothelin-1 (ET-1), on human isolated bronchus

	First step						*Second step*					
Pretreatment	n	pD_2	P	E_{max} (mg)	E_{max} (% of ACh)	P	n	pD_2	P	E_{max} (mg)	E_{max} (% of ACh)	P
Control	10	11.01 ± 0.17		279 ± 46	15.6 ± 1.8		9	7.90 ± 0.17		1276 ± 264	82.5 ± 4.7	
Indomethacin 3×10^{-6} M	5	10.93 ± 0.21	NS	270 ± 90	15.2 ± 3.5	NS	5	7.85 ± 0.10	NS	1182 ± 310	81.3 ± 4.5	NS
Nicardipine 10^{-6} M	5	—		64 ± 25	6.0 ± 2.6	<0.05	5	7.77 ± 0.08	NS	935 ± 160	90.3 ± 1.5	NS
Bay K 8644 10^{-7} M	5	11.35 ± 0.38	NS	445 ± 131	26.1 ± 2.9	<0.05	5	8.15 ± 0.28	NS	1035 ± 165	87.2 ± 2.8	NS

E_{max} is assimilated to the effects of ET-1 10^{-10} M (first step) and 3×10^{-7} M (second step).
Values are means ± s.e.mean.
n = number of experiments.
P = statistical difference between pretreated and control preparations (Student's t test for unpaired data).
NS = not significant.

Table 4 Residual contraction to acetylcholine (ACh), KCl, endothelin-1 (ET-1), endothelin-2 (ET-2) and endothelin-3 (ET-3) after pre-incubation of isolated human bronchi in calcium-free Krebs solution

		n	*Control contraction in normal Krebs solution* (mg)	*Contraction in calcium-free Krebs solution* (mg)	*% of control*
ACh	3×10^{-3} M	7	2224 ± 235	1500 ± 230	66.1 ± 6.4
KCl	3×10^{-3} M	4	890 ± 264	88 ± 18	13.6 ± 4.3
ET-1	10^{-10} M	4	163 ± 35	25 ± 18	11.3 ± 7.5
ET-1	3×10^{-8} M	6	1278 ± 176	691 ± 115	56.3 ± 8.1
ET-2	10^{-10} M	3	138 ± 33	6.0 ± 2.6	2.5 ± 2.5
ET-2	3×10^{-8} M	3	683 ± 298	550 ± 278	76.8 ± 13.6
ET-3	10^{-10} M	3	145 ± 15	3 ± 2	2.5 ± 2.5
ET-3	3×10^{-8} M	3	655 ± 260	525 ± 225	81.2 ± 12.5

n = number of experiments.
Values are mean ± s.e.mean.

Table 5 Effect of indomethacin 3×10^{-6} M, nicardipine 10^{-6} M and Bay K 8644 10^{-7} M on the maximal effect (E_{max}) of endothelin-2 (ET-2) (10^{-10} M) and endothelin-3 (ET-3) (10^{-10} M), on human isolated bronchus

Pretreatment	n	E_{max} (mg)	P	E_{max} (% of ACh)	P
ET-2 10^{-10} M					
Control	10	108 ± 17		7.1 ± 0.9	
Indomethacin 3×10^{-6} M	4	72.5 ± 49.0	NS	5.7 ± 3.8	NS
Nicardipine 10^{-6} M	4	55 ± 19	NS	4.5 ± 0.6	<0.05
Bay K 8644 10^{-7} M	4	365 ± 122	NS	22.8 ± 2.6	<0.01
ET-3 10^{-10} M					
Control	10	170 ± 33		8.0 ± 1.3	
Indomethacin 3×10^{-6} M	4	146 ± 32	NS	10.6 ± 1.5	NS
Nicardipine 10^{-6} M	4	75 ± 43	NS	4.7 ± 1.6	<0.05
Bay K 8644 10^{-7} M	4	285 ± 105	NS	14.3 ± 1.5	<0.05

Values are means ± s.e.mean.
n = number of experiments.
P = statistical difference between pretreated and control preparations (Student's test for unpaired data). NS = not significant.

(10^{-10} M) were markedly enhanced (Figure 1 and Table 5), so much so that the contractions observed with 10^{-10} M of either ET-1 or ET-2 were not significantly different (respectively 26.1 ± 2.9% and 22.8 ± 2.6%). In the presence of nicardipine (10^{-6} M), the responses (E_{max}, %ACh 3×10^{-3} M) induced by small concentrations (10^{-10} M) of both ET-2 and ET-3 were significantly ($P < 0.05$) inhibited (Table 5). In calcium-free medium responses to low concentration (10^{-10} M) of ET-2 and ET-3 were abolished, whereas the responses obtained with 3×10^{-8} M were 76.8 ± 13.6 and 81.2 ± 12.5% respectively of the control contraction (Table 4).

Discussion

It has recently been shown that endothelin (ET-1) elicits contractile effects on airway smooth muscle *in vitro* with concentrations ranging from 10^{-12} to 3×10^{-7} M in the guinea-pig trachea (Uchida *et al.*, 1988; Maggi *et al.*, 1989a; Borges *et al.*, 1989) or from 10^{-8} to 10^{-5} M in the rat trachea (Turner *et al.*, 1989). On human airways, Uchida *et al.* (1988) also described a contractile activity for ET-1, without reporting quantitative data concerning the concentration-effect relationship. ET-1 was first considered, principally in blood vessels, to be an endogenous modulator of voltage-dependent calcium channels (Yanigasawa *et al.*, 1988), so that particular attention has been paid to the role of calcium flux in the contractile activity of ET-1. It has been demonstrated that the *in vivo* airway response to ET-1 was partially sensitive to the dihydropyridine calcium channel blockers, such as nicardipine (10^{-8} M) (Uchida *et al.*, 1988) or nifedipine (10^{-6} M) (Maggi *et al.*, 1989a). In contrast, a decrease of the contractile response was observed in calcium-free medium on the rat trachea, while no inhibitory effect of nicardipine (10^{-7} M) was observed (Turner *et al.*, 1989). Thus a relationship between transmembrane calcium flux and airway contractile activity has been established for the guinea-pig, rat and human airways.

The present results clearly show that in the human isolated bronchus the contractile activities of the three endothelins proceed in a stepwise manner. The first step is observed for concentrations of endothelin below 10^{-9} M. The maximum response observed with ET-1 is recorded for 10^{-10} M, corresponding to 15.6 ± 1.8% of the maximal tissue response obtained by addition of ACh 3×10^{-3} M at the end of the experiment, indicating a high potency but a low efficacy of the peptide. This step involves calcium flux and suggests an effect dependent upon dihydropyridine-sensitive calcium channels (L_m channels, Nayler, 1988), since the contractile effect of ET-1 is enhanced by Bay K 8644 and inhibited by nicardipine, drugs which respectively increase and decrease the voltage-dependent calcium transfer in the guinea-pig or human airways (Foster *et al.*, 1983a,b; Advenier *et al.*, 1984; 1986; Allen *et al.*, 1985). This is emphasized by the results obtained in calcium-free medium where the contractile effect of ET-1 (10^{-10} M) is markedly reduced, and to an extent similar to that of KCl (Advenier *et al.*, 1986).

The second step of the peptide airway response recorded for concentrations higher than 10^{-9} M is characterized by a higher efficacy but a lower potency than those observed for the first step. However, at these concentrations, endothelin appears to be more potent than other bronchoconstrictor agents such as histamine, ACh, NKA and LTD_4. This second step is not altered by Bay K 8644 or by nicardipine, and it is modified by the same amount as ACh in calcium-free medium, suggesting a mechanism unrelated to the dihydropyridine-dependent calcium channels. On the rat uterus, Kozuka *et al.* (1989) similarly showed dissociation between rhythmic contractions involving voltage-dependent calcium channels and slowly developing monophasic contractions insensitive to calcium channel blockers. Therefore, a direct effect of endothelin on intracellular calcium via the activation of phosphatidyl inositol hydrolysis, may be considered, as has recently been demonstrated with high concentrations (10^{-8} to 10^{-5} M) on rat or rabbit aorta (Marsden *et al.*, 1989; Huang *et al.*, 1989; Ohlstein *et al.*, 1989), in cultured A10 cells (with 10^{-7} M ET-1) (Xuan *et al.*, 1989) or in isolated canine coronary arteries (Pang *et al.*, 1989).

The cyclo-oxygenase inhibitor indomethacin (3×10^{-6} M) does not significantly modify the response to ET-1 on the isolated human bronchus. This is not concordant with the data obtained *in vivo* in the guinea-pig, where indomethacin (10 mg kg^{-1}) or meclofenamate (0.5 to 2 mg kg^{-1}) suppressed the bronchoconstriction induced by intravenous or inhaled ET-1 (Payne & Whittle, 1988; Lagente *et al.*, 1989; Macquin-Mavier *et al.*, 1989). The discrepancy may be explained by recent reports showing that the human bronchial smooth muscle may generate important quantities of prostanoids which do not modulate the contractile responses *in vitro* (Douglas & Brink, 1987; De Jongste *et al.*, 1987; Naline *et al.*, 1989).

The present data also demonstrate that ET-2 and ET-3 exert similar qualitative effects, but are quantitatively different, since they appear to be slightly less potent and/or less efficient than ET-1, if we consider the two steps of the contractile response. From a qualitative point of view, the ET-2 and ET-3 contractile activities may be divided, as with ET-1, into two steps. One is characterized by high potency and low efficacy, implicating voltage-dependent calcium channels, and the other by lower potency but higher efficacy, independent of transmembrane calcium channels.

The qualitative differences between ET-1 on the one hand and ET-2 and ET-3 on the other are similar to data obtained on the contractile responses of porcine and rat artery strips *in vitro* and on the pressor responses of anaesthetized rats or cats

in vivo (Inoue *et al.*, 1989; Rodman *et al.*, 1989; Minkes *et al.*, 1989). Indeed, ET-1 is as potent or more potent than ET-2 and more potent than ET-3. Nevertheless, in the rat, the initial transient depressor response *in vivo* was most profound with ET-3 (Inoue *et al.*, 1989); but the discrepancy between the results obtained by these authors and ours might be due to differences in animal species (rat versus guinea-pig) or in systems (cardiovascular versus respiratory). The differences we observed in the activities of the endothelins might result from differences in efficacy and/or tissue factors such as receptor density, efficiency of receptor/effector coupling and selective inactivation. They may also suggest the existence of different subtypes of endothelin receptors. The last suggestion might be supported by recent data demonstrating different high-affinity binding sites in rat lungs (Kanse *et al.*, 1989). Furthermore, it is possible to discriminate between different endothelin receptors on the guinea-pig bronchus, as demonstrated with an ET-1 analogue (Maggi *et al.*, 1989b). However, whether these endothelin receptors exist in human bronchial tissue is unknown.

To conclude, our results present evidence that the three endothelins have potent contractile activities on human isolated bronchus and that at least two mechanisms are involved in this effect. One of these, which acts when low concentrations are used, is probably dihydropyridine-sensitive calcium channel activation. Other mechanisms operate at higher endothelin concentrations, and in particular a direct effect on intracellular calcium via phosphatidyl inositol hydrolysis activation may be suggested for concentrations higher than 10^{-9} M (Marsden *et al.*, 1989; Huang *et al.*, 1989; Ohlstein *et al.*, 1989; Xuan *et al.*, 1989; Pang *et al.*, 1989).

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Investigation of the mechanism(s) of 8-OH-DPAT-mediated inhibition of plasma insulin in spontaneously hypertensive rats

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1 Effects of the prototype selective 5-HT_{1A} receptor agonist, 8-hydroxy-2-(di-n-dipropylamino)tetralin (8-OH-DPAT), were studied on the glycaemia and insulinaemia in conscious spontaneously hypertensive (SH) rats concurrently with blood pressure (BP) and heart rate (HR); underlying mechanism(s) were investigated in anaesthetized and pithed SH rats and in the perfused rat pancreas.

2 Intravenous (i.v.) injections of 8-OH-DPAT (150 $\mu g\,kg^{-1}$, i.v.) into fasted conscious but not anaesthetized SH rats increased glycaemia; glucose-stimulated (i.v. glucose tolerance test) plasma insulin levels were significantly inhibited in both cases without significant changes in glucose tolerance. Metabolic changes were associated with prominent decreases in BP and HR.

3 No inhibitory effect of 8-OH-DPAT, 150 $\mu g\,kg^{-1}$ i.v., on glucose-stimulated plasma insulin was observed in pithed SH rats; in contrast, clonidine (8 $\mu g\,kg^{-1}$ i.v.), produced marked inhibition of insulin levels in association with glucose intolerance. Neither compound decreased BP; rather, pronounced vasopressor effects were observed.

4 In the isolated perfused pancreas of the rat, 8-OH-DPAT, at 10^{-8} and 10^{-7} M, concentrations known to activate 5-HT_{1A} receptors *in vitro*, failed to modify glucose-stimulated insulin release. Inhibition ($39 \pm 7\%$) was seen only at a high concentration of 10^{-6} M.

5 The present data suggest that like the cardiovascular effects of 8-OH-DPAT, the inhibition of glucose-stimulated insulin release is mediated via the central nervous system. However, it is suggested that different mechanisms are involved in the cardiovascular actions and metabolic effects of 8-OH-DPAT in the SH rat; the latter are likely to reflect a consequence of activation of the hypothalamic-adrenal axis.

Introduction

It is now clearly established that the 5-hydroxytryptamine (5-HT) receptor family is very heterogeneous (for reviews see Fozard 1987; Peroutka, 1988). Three major classes of 5-HT receptors were identified by Bradley *et al.* (1986) and were designated 5-HT_1-like, 5-HT_2 and 5-HT_3; the 5-HT_1-like category, is now recognised to consist of at least four subtypes designated 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C} and 5-HT_{1D}. Discriminatory ligands, tissue distributions and coupling to transduction mechanisms of the various 5-HT receptor subtypes have now been well-documented (Peroutka, 1988; Hoyer, 1989).

The availability of compounds with high affinities and certain selectivities has greatly increased our understanding of the functional significance of some of the above 5-HT receptor subtypes (Hibert *et al.*, 1990). In particular, an impressive number of 5-HT_{1A} receptor-mediated functional/biological responses have been established (Dourish *et al.*, 1987; Hibert *et al.*, 1990); this remarkable progress has been possible, primarily, due to the discovery that the centrally acting 5-HT receptor agonist, 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) (Hjorth *et al.*, 1982), has high affinity and selectivity for the 5-HT_{1A} subtype of the 5-HT_1 recognition site (Middlemiss & Fozard, 1983; Gozlan *et al.*, 1983). One of the several functional responses elicited by 8-OH-DPAT is to lower blood pressure and heart rate in a number of species. The cardiovascular effects of 8-OH-DPAT have been well characterized and are mediated via activation of postsynaptic 5-HT_{1A} receptors in the central nervous system resulting in an increase in vagal tone and sympathoinhibition; in the rat, moreover, there is evidence for an indirect involvement of α_2-adrenoceptors (for review see Mir & Fozard, 1987; 1990).

More recently, it has become evident that central 5-HT_{1A} receptor activation also results in stimulation of the pituitary-adrenal axis. Thus, for example, 8-OH-DPAT has been shown to increase plasma prolactin, ACTH, corticosterone and β-endorphin levels and to decrease growth hormone levels in rats (Simonovic *et al.*, 1984; Koenig *et al.*, 1987; Gilbert *et al.*, 1988; Aulakh *et al.*, 1988). The effects of 8-OH-DPAT on endocrine/metabolic parameters are further reflected in its ability to promote hyperglycaemia and to inhibit insulinaemia in response to an intravenous glucose tolerance test in conscious Sprague-Dawley rats (Chaouloff & Jeanrenaud, 1987); on the basis of pharmacological analysis these effects were considered to be mediated via 5-HT_{1A} and α_2-adrenoceptors. Further evidence for marked inhibitory effects of 8-OH-DPAT on both the basal hyperinsulinaemia and glucose-stimulated insulin release was obtained in the conscious genetically obese (fa/fa) rat (Chaouloff & Jeanrenaud, 1988).

Despite certain similarities concerning the receptor sites involved, the hyperglycaemic and hypoinsulinaemic effects of 8-OH-DPAT are not entirely consistent with its mode of action on the autonomic nervous system i.e. sympathoinhibition and increase in vagal tone. It is generally recognised, for instance, that a physiological consequence of both an increase in vagal tone and sympathoinhibition in several species is an increase in insulinaemia associated with a decrease in glycaemia (Woods & Porte, 1974; Ahren *et al.*, 1986). It is likely, therefore, that different mechanisms are involved in the manifestation of the above endocrine/metabolic effects and the cardiovascular actions of 8-OH-DPAT. The objectives of the present study were to investigate the effects of 8-OH-DPAT on basal and glucose-stimulated (i.v. glucose tolerance test) plasma glucose and insulin levels concurrently with monitoring of blood pressure and heart rate in conscious spontaneously hypertensive (SH) rats. In addition, experiments were conducted in anaesthetized and pithed SH rats as well as isolated perfused pancreas of the rat to elucidate the mechanism(s) underlying the hypoinsulinaemic effect of 8-OH-DPAT. Some of these results was presented to the British Pharmacological Society in July, 1989 (Bouhelal *et al.*, 1989).

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Methods

Animals

Male SH rats were supplied by Tier Farm Madörin, Fullinsdorf, Switzerland, and male Wistar rats by Charles River France. All animals had free access to food and water and were maintained on a constant 12 h light cycle.

Glucose tolerance tests in conscious SH rats

Male spontaneously hypertensive (SH) rats (300–350 g) were used for these experiments. Catheters were implanted under sodium-hexobarbitone (NA-Evipan) anaesthesia (160 mg kg^{-1}, i.p.) in the abdominal aorta and femoral vein. Cannulae were passed subcutaneously and exteriorized at the back of the neck. At the end of surgery, cannulae were filled with heparinized saline to avoid blood clotting. Experiments were performed about one week after surgery.

Following an overnight fast (18–20 h) blood pressure (BP) and heart rate (HR) were recorded continuously from the arterial cannula as described in detail previously (Fozard *et al.*, 1987). After a stabilization period of about 1 h an arterial blood sample (200 μl) was taken to establish basal glucose and insulin values. Subsequently saline (1 ml kg^{-1}, i.v.) or 8-OH-DPAT (150 μg kg^{-1}, i.v.) was injected via the venous cannula and another blood sample taken 15 min later to determine the acute effects of saline or 8-OH-DPAT on resting glucose and insulin concentrations. Glucose (0.5 g kg^{-1} in 0.5 ml water) was then injected i.v. and further blood samples withdrawn after 1, 6, 12, 24, and 48 min.

Time-dependent effects of 8-OH-DPAT *per se* on basal glycaemia, insulinaemia, BP and HR were determined in a separate series of experiments. A blood sample was taken at time 0 to determine basal glucose and insulin values, subsequently saline (1 ml kg^{-1}, i.v.) or 8-OH-DPAT (150 μg kg^{-1}, i.v.) was injected and further blood samples withdrawn after 5, 15, 30 and 60 min. The arterial cannula was rinsed with heparinized saline following each blood sampling and care was taken not to dilute the sample with the residual saline in the cannula by discarding the first few drops.

Glucose tolerance tests in anaesthetized SH rats

Male SH rats fasted overnight (18–20 h) were anaesthetized with 120 mg kg^{-1} i.p. of sodium hexabarbitone (Inactin) and cannulae placed in a jugular vein and a carotid artery. An arterial blood sample (200 μl) was taken to establish basal glucose and insulin values. Subsequently saline (1 ml kg^{-1}, i.v.) or 8-OH-DPAT (150 μg kg^{-1}, i.v.) was injected and further samples withdrawn 5, 10 and 15 min later to determine the acute effects of saline or 8-OH-DPAT on resting glucose and insulin values. Glucose tolerance tests were then performed as described above for conscious SH rats. In a separate series of experiments time-dependent effects of 8-OH-DPAT (150 μg kg^{-1}, i.v.) or saline (1 ml kg^{-1}, i.v.) on basal glycaemia and insulinaemia were determined by withdrawing blood samples before injection (0 time) and 5, 10, 16, 21, 27, 39, and 63 min after injection.

Glucose tolerance tests in pithed SH rats

Non-fasted SH rats (300–350 g) were anaesthetized with sodium pentobarbitone (40 mg kg^{-1}, i.p. supplemented by s.c. injection) and pithed according to the procedure described previously (Fozard *et al.*, 1987) based on the method of Shipley & Tilden (1947). A tracheotomy was performed and rats were artificially ventilated with pure oxygen. Catheters were inserted in the right femoral vein and artery. Experiments were generally initiated between 35–45 min after pithing, to allow the preparations to stabilize. Effects of 8-OH-DPAT (150 μg kg^{-1}, i.v.) and clonidine (8 μg kg^{-1}, i.v.) on glucose tolerance were determined as described for conscious and anaesthetized animals.

Glucose-induced insulin secretion from perfused rat pancreas in vitro

Pancreases from male Wistar rats (320–380 g) anaesthetized with sodium pentobarbitone 60 mg kg^{-1} (i.p.), were isolated and perfused as described previously (Loubatieres *et al.*, 1969). Each pancreas was transferred to a plastic perfusion chamber maintained at 37.5°C, and perfused through its own arterial system with a Krebs Ringer bicarbonate solution containing (mM): NaCl 108, KH_2PO_4 1.19, KCl 4.74, $CaCl_2$ 2.74, $MgSO_4 \cdot 7H_2O$ 1.19, bovine serum albumin 2 g l^{-1} (Fraction V, Sigma Chemical Company) and glucose 1.5 g l^{-1} (8.3 mM). This glucose concentration was chosen to induce a moderate insulin secretion. The perfusion fluid was continuously bubbled with a mixture of CO_2 (5%) and O_2 (95%) in order to maintain pH around 7.35. The perfusion pressure was chosen so as to produce an output flow rate of 2.5 ml min^{-1} at the time of addition of drugs (45 min). The first sample was taken after an equilibration period of 30 min. Two additional samples were taken 10 and 15 min later in the presence of glucose (8.3 mM) alone. 8-OH-DPAT at various concentrations (10^{-8} M, 10^{-7} M or 10^{-6} M) was then added to the perfusion fluid and samples were collected every minute for 5 min then 8, 10, 15, 20 and 30 min after starting 8-OH-DPAT administration. Samples were frozen at −20°C until insulin was determined. Insulin output was obtained by multiplying the hormone concentration in the effluent (μu ml^{-1}) by the flow rate (ml min^{-1}).

Plasma glucose and insulin measurements

Blood samples were collected in Eppendorf tubes through the arterial cannula and kept at 4°C before centrifugation (8,800 *g* for 3 min). Aliquots (10 μl) of the plasma were used to determine glucose concentrations in an automatic glucose analyser (Kodak, Ektachem). The remainder of the plasma was stored at −20°C for subsequent insulin assay. Insulin was determined by radioimmunoassay as described by Hales & Randle (1963).

Drugs and solutions

(±)-8-OH-DPAT hydrogen bromide and clonidine hydrochloride (Research Biochemicals Inc.) were dissolved in 0.9% (w/v) NaCl for *in vivo* experiments. Human [^{125}I]-insulin (Amersham International) and anti-insulin antiserum raised in guinea-pigs was from Medipro (Basel, Switzerland).

Statistical analysis

Statistical analysis of data was performed by analysis of variance followed by Mann Whitney U test for unpaired data and differences were considered significant at the $P < 0.05$ level.

Results

Effects of 8-OH-DPAT on basal and glucose-induced plasma glucose and insulin levels, blood pressure and heart rate in conscious SH rats

The dose of 150 μg kg^{-1} i.v. has been previously characterized in detail for the hyperglycaemic and hypoinsulinaemic effects of 8-OH-DPAT in rats (Chaouloff & Jeanrenaud, 1987; 1988); therefore, this dose was used in the present study. Administration of 8-OH-DPAT, 150 μg kg^{-1} i.v., resulted in an increase in basal glycaemia (32 ± 6 mg dl^{-1}) which was maximal at 15 min, and was back to control values by 60 min

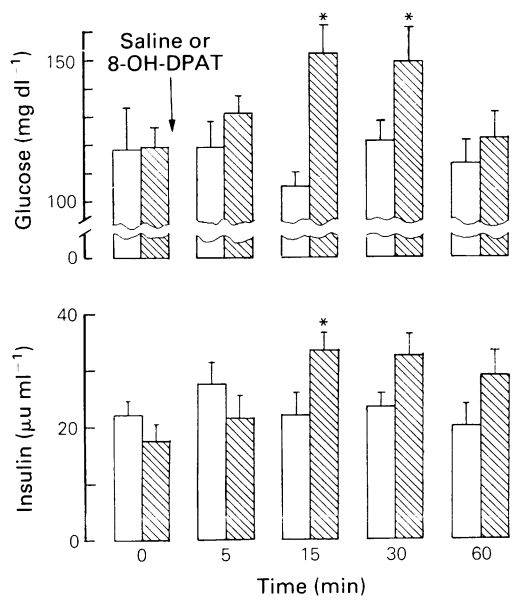

Figure 1 Effects of 8-OH-DPAT on basal plasma glucose and insulin levels in fasted conscious SH rats. Blood samples were withdrawn to estimate basal plasma glucose and insulin levels before saline or 8-OH-DPAT ($150\,\mu g\,kg^{-1}$, i.v.) administration. Further samples were taken at the times indicated in the figure. Open column: control group ($n = 6$), hatched columns: 8-OH-DPAT treated group ($n = 6$). Results are expressed as mean with s.e.mean shown by vertical bars. * $P < 0.05$, value significantly different from the corresponding control value.

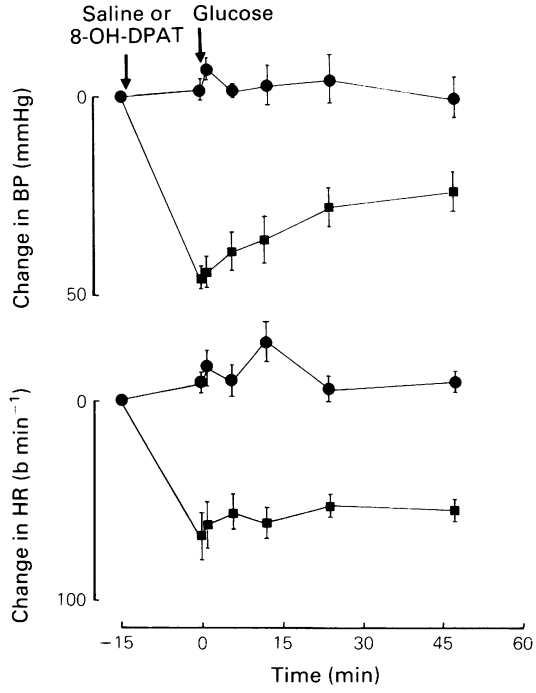

Figure 3 Effects of 8-OH-DPAT on blood pressure and heart rate during glucose tolerance tests in fasted conscious SH rats. Rats were injected with either saline or 8-OH-DPAT $150\,\mu g\,kg^{-1}$, i.v. and 15 min later subjected to an intravenous glucose tolerance test as described in the text. Changes in mean BP and HR are represented as mean with s.e.mean shown by vertical bars. (●): Control group ($n = 5$), (■): 8-OH-DPAT-treated group ($n = 8$). Resting BP and HR values (mean ± s.e.mean) respectively for control: 185 ± 10; 340 ± 13 and 8-OH-DPAT-treated group: 193 ± 5; 362 ± 10.

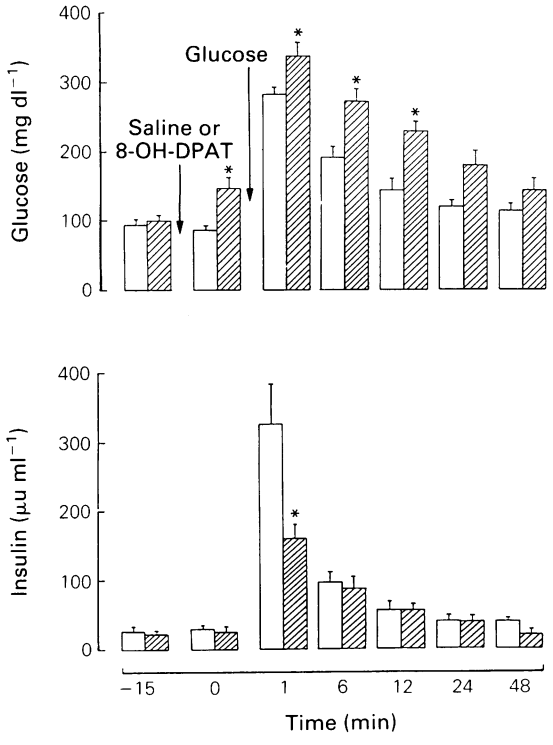

Figure 2 Effects of 8-OH-DPAT on plasma glucose and insulin in fasted conscious SH rats following a glucose load. Plasma glucose and insulin values were determined before 8-OH-DPAT ($150\,\mu g\,kg^{-1}$, i.v.) or saline injections. Acute effects of the drug were determined 15 min post injection. Glucose ($0.5\,g\,kg^{-1}$, i.v.) was then injected at time 0 and subsequent blood samples were withdrawn for glucose and insulin determinations at the times indicated. Open columns: control group ($n = 5$); hatched columns: 8-OH-DPAT-treated group ($n = 8$). Results are represented as mean with s.e.mean shown by vertical bars. * $P < 0.05$, value significantly different from the corresponding control value. For further details of the experimental protocol, see text.

(Figure 1). The increase in glycaemia was associated with a small but variable increase in basal insulinaemia (Figure 1).

In a separate series of experiments the effect of 8-OH-DPAT on an intravenous glucose tolerance test was determined. 8-OH-DPAT ($150\,\mu g\,kg^{-1}$ i.v.) increased the basal glycaemia, $100 \pm 6\,mg\,dl^{-1}$, to $146 \pm 13\,mg\,dl^{-1}$ 15 min post injection with no effect on the basal insulinaemia (Figure 2). Following the intravenous injection of glucose ($0.5\,g\,kg^{-1}$), plasma glucose levels in the saline-treated animals increased to $281 \pm 9\,mg\,dl^{-1}$ and returned to control levels within about 24 min. The increase in glycaemia was associated with a rapid increase in plasma insulin from 28 ± 5 to $326 \pm 58\,\mu u\,ml^{-1}$ (Figure 2). In contrast, the 8-OH-DPAT treated group showed a marked inhibition (49 ± 9%) in the glucose-stimulated peak insulin levels; this inhibition was not accompanied by any further increase in the glycaemia above the control levels if the increase in glycaemia caused by 8-OH-DPAT *per se* is taken into account (cf. Figures 1 and 2). Injection of 8-OH-DPAT in these animals resulted in marked and sustained decreases in BP and heart rate whereas injections of saline ($1\,ml\,kg^{-1}$, i.v.) or glucose ($0.5\,g\,kg^{-1}$, i.v.) had no significant effects on either parameter (Figure 3).

Effects of 8-OH-DPAT on basal and glucose-induced plasma glucose and insulin levels in anaesthetized SH rats

In contrast to conscious SH rats, in anaesthetized SH rats 8-OH-DPAT ($150\,\mu g\,kg^{-1}$, i.v.) had little effect on the basal plasma glucose levels; the basal levels of insulin also showed no significant change (Figure 4). The resting levels of glucose and insulin in the anaesthetized animals were similar to the levels in conscious animals.

The effects of 8-OH-DPAT on glucose-stimulated plasma insulin and glucose were determined in a separate series of experiments. Again, 8-OH-DPAT ($150\,\mu g\,kg^{-1}$ i.v.) had no effect on basal plasma glucose or insulin levels measured

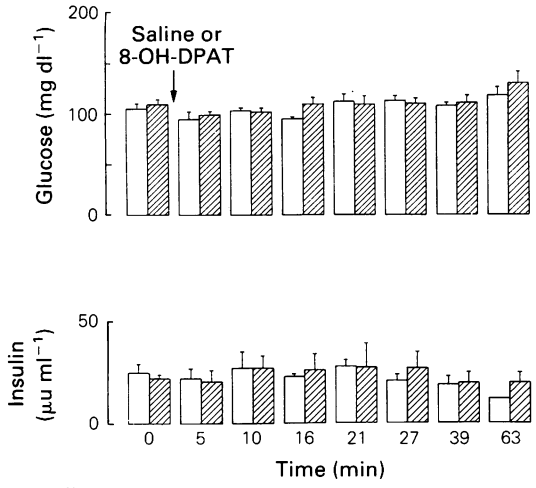

Figure 4 Effects of 8-OH-DPAT on basal plasma glucose and insulin levels in fasted anaesthetized SH rats. Blood samples were withdrawn to determine basal plasma glucose and insulin levels prior to injection of saline or 8-OH-DPAT ($150\,\mu g\,kg^{-1}$, i.v.). Further samples were taken at the times indicated in the figure. Open columns: control group ($n = 3$), hatched columns: 8-OH-DPAT-treated group ($n = 4$). Results are expressed as mean with s.e.mean shown by vertical bars. For further details of the experimental protocol, see text.

15 min post injection (Figure 5). Following a glucose load ($0.5\,g\,kg^{-1}$, i.v.), plasma glucose and insulin levels in the saline treated animals increased rapidly within 1 min to $407 \pm 18\,mg\,dl^{-1}$ and $231 \pm \mu u\,ml^{-1}$ respectively and returned to control values rather slowly as compared to the conscious animals (cf. Figures 2 and 5). 8-OH-DPAT-treated rats showed a significant reduction in the plasma insulin levels at 1 and 6 min compared to the saline-treated animals whereas

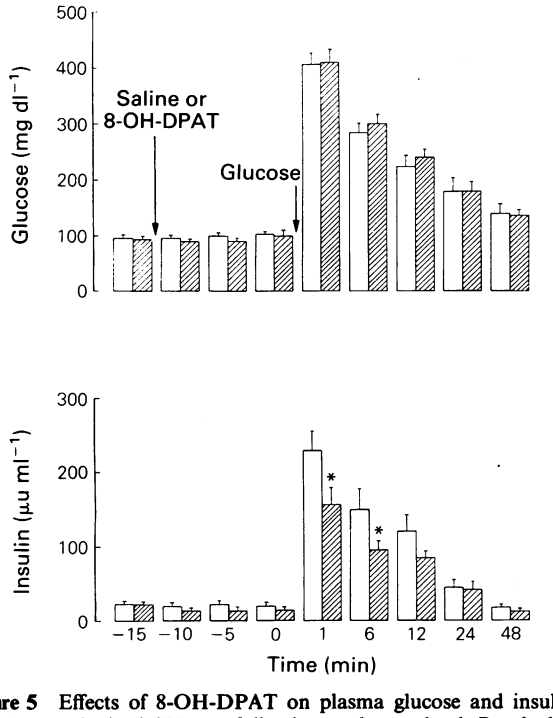

Figure 5 Effects of 8-OH-DPAT on plasma glucose and insulin in fasted anaesthetized SH rats following a glucose load. Basal plasma glucose and insulin values were determined prior to injection of saline or 8-OH-DPAT ($150\,\mu g\,kg^{-1}$, i.v.). Acute effects of the drug were determined 15 min post injection. Glucose ($0.5\,g\,kg^{-1}$, i.v.) was then injected at time 0 and subsequent blood samples withdrawn at the times indicated. Open columns: control group ($n = 5$), hatched columns: 8-OH-DPAT-treated group ($n = 6$). Results are expressed as mean with s.e.mean shown by vertical bars. * $P < 0.05$, value significantly different from the corresponding control value.

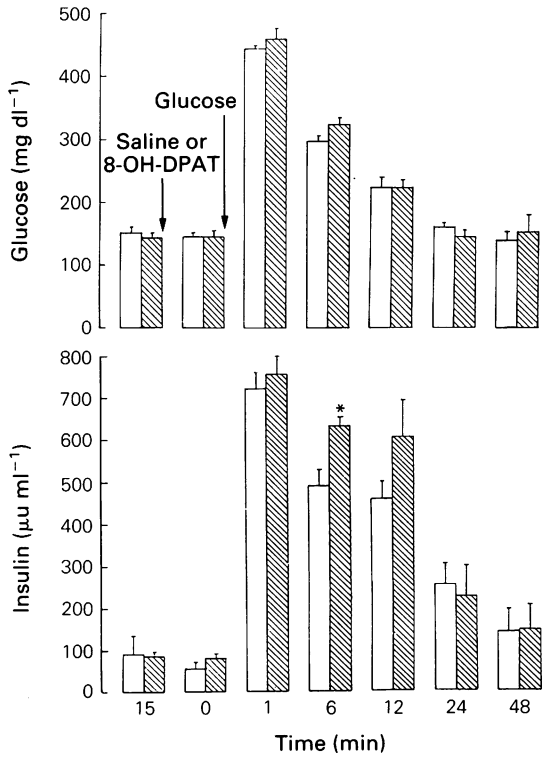

Figure 6 Effects of 8-OH-DPAT on plasma glucose and insulin levels in pithed SH rats following a glucose load. Basal plasma glucose and insulin values were determined 35 to 45 min after pithing prior to saline or 8-OH-DPAT ($150\,\mu g\,kg^{-1}$, i.v.) injections. Acute effects of 8-OH-DPAT were determined 15 min post injection. Glucose ($0.5\,g\,kg^{-1}$, i.v.) was then injected at time 0 and subsequent blood samples withdrawn at the time indicated. Open columns: control group ($n = 6$), hatched columns: 8-OH-DPAT-treated group ($n = 4$). Results are expressed as means with s.e.mean shown by vertical bar. * $P < 0.05$, value significantly different from the corresponding control value.

the glucose levels were almost identical (Figure 5). A reduction in blood pressure (resting value: 163 ± 12 mmHg; after injection of 8-OH-DPAT: 70 ± 7 mmHg) and in heart rate (resting value: 324 ± 16; 8-OH-DPAT: 254 ± 14 beats min^{-1}) was also observed with 8-OH-DPAT in the anaesthetized SH rats. However, a significant decrease in blood pressure (resting: 157 ± 18 mmHg; 12 min after saline injection: 100 ± 12 mmHg) but not heart rate (resting: 325 ± 9 beats min^{-1}; saline 306 ± 21 beats min^{-1}) was also observed in the saline-treated rats which appeared to be associated with Inactin anaesthesia.

Effects of 8-OH-DPAT and clonidine on glucose-induced plasma glucose and insulin levels in pithed SH rats

For these experiments, experimental conditions were modified as compared to the Inactin-anaesthetized SH rat model. First, Inactin was replaced by sodium pentobarbitone as the anaesthetic. Second, non-fasted animals were used instead of fasted animals. The reasons for these changes were that Inactin anaesthesia and fasting prior to pithing caused marked decreases in blood pressure (< 30–40 mmHg) and basal glycaemia (40–80 $mg\,dl^{-1}$), thus making the preparations unstable. However, it was found that under the conditions where the animals were anaesthetized with pentobarbitone and not fasted, the pithed preparations were stable throughout the duration of the experiment. Under these conditions resting blood pressure and glucose levels were respectively 63 ± 5 mmHg ($n = 6$) and $148 \pm 15\,mg\,dl^{-1}$ ($n = 6$); the basal plasma insulin in pithed animals was significantly increased compared to fasted Inactin-anaesthetized animals. Similar

Table 1 Effect of 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) and clonidine on blood pressure (mmHg) in pithed SH rats

	Baseline	*Maximal effect*	n
Control	63 ± 5	72 ± 8	6
8-OH-DPAT	69 ± 6	99 ± 12(*)	4
Clonidine	63 ± 5	166 ± 12(*)	5

Effects of saline, 8-OH-DPAT (150 $\mu g\,kg^{-1}$, i.v.) and clonidine (8 $\mu g\,kg^{-1}$, i.v.) on mean blood pressure in pithed SH rats. Data were obtained from the same experiments shown in Figures 6 and 7 and are expressed as absolute values, mean ± s.e.mean of the number of animals indicated in the table (n). * $P < 0.05$; value significantly different from the corresponding baseline value.

glucose concentrations were found in sodium pentobarbitone-anaesthetized and conscious SH rats in the non-fasting state (results not shown).

8-OH-DPAT (150 $\mu g\,kg^{-1}$, i.v.) had no significant effects on basal glucose levels or the glycaemia induced by an intravenous glucose tolerance test (Figure 6). In contrast to conscious and anaesthetized animals, no inhibition of glucose-stimulated plasma insulin levels was observed in pithed animals after 8-OH-DPAT administration; if anything a significantly higher level of plasma insulin was observed at 6 min after a glucose load in 8-OH-DPAT treated animals. 8-OH-DPAT elicited vasoconstrictor activity in pithed SH rats which lasted 7 ± 2 min and no decreases in blood pressure were observed (Table 1).

In a separate series of experiments the effects of clonidine on basal and glucose-stimulated plasma glucose and insulin concentrations were investigated. As shown in Figure 7, clonidine (8 $\mu g\,kg^{-1}$, i.v.) caused a significant reduction in basal insulinaemia (from 101 ± 14 to 26 ± 8 $\mu u\,ml^{-1}$); this inhibition was associated with a significant increase in basal glycaemia after 15 min (from 138 ± 5 to 178 ± 13 $mg\,dl^{-1}$). The decrease in insulin concentrations was also observed during the intravenous glucose tolerance test. Clonidine inhibited glucose-stimulated insulin levels by 88 ± 4% and caused impairment of the glucose tolerance (Figure 7). Like 8-OH-DPAT, clonidine also elicited vasoconstrictor activity in pithed SH rats lasting 28 ± 3 min and no decreases in blood pressure were observed (Table 1).

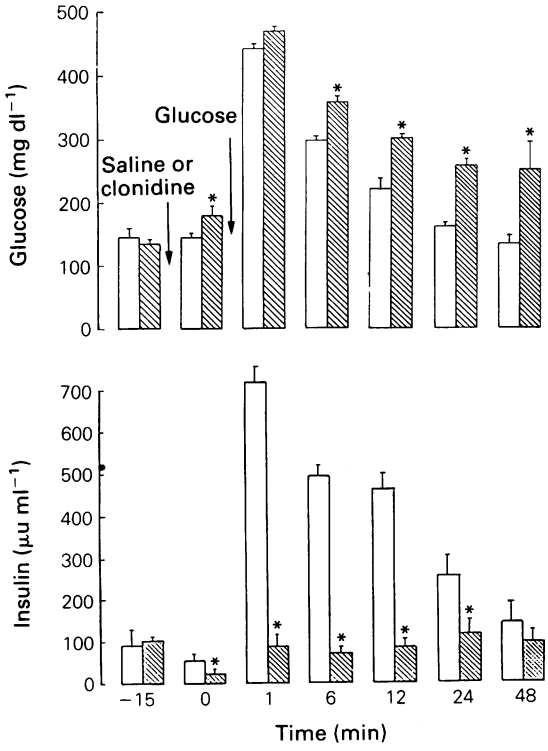

Figure 7 Effects of clonidine (8 $\mu g\,kg^{-1}$, i.v.) on plasma glucose and insulin levels in pithed SH rats following a glucose load. The experimental protocol is the same as described in the legend to Figure 6. Open columns: control group ($n = 6$), hatched columns: clonidine-treated group ($n = 5$). Results are expressed as means with s.e.mean shown by vertical bars. * $P < 0.05$, value significantly different from the corresponding control value.

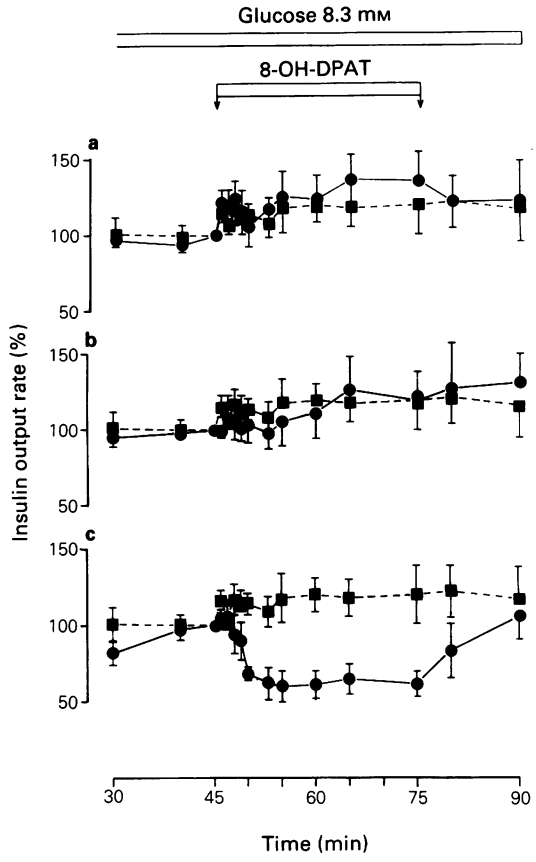

Figure 8 Effect of 8-OH-DPAT on insulin secretion from isolated perfused pancreas of the rat. The perfusion medium contained 8.3 mM (1.5 $g\,l^{-1}$) of glucose. Results are expressed in percentages in relation to the value at time 45 min taken as reference. Values are mean of 4 to 6 experiments with s.e.mean shown by vertical bars. At time 45 min, mean insulin output rates were 709 ± 134 ($n = 6$), 551 ± 98 ($n = 6$), 756 ± 76 ($n = 6$) and 673 ± 71 ($n = 4$) $\mu u\,min^{-1}$ respectively for control (■) and 8-OH-DPAT-treated tissues (●), 10^{-8} M (a), 10^{-7} M (b) and 10^{-6} M (c).

Effects of 8-OH-DPAT on glucose-induced insulin secretion from the isolated perfused pancreas

8-OH-DPAT, at two concentrations (10^{-8} and 10^{-7} M) did not modify glucose-induced insulin secretion from the isolated perfused pancreas (Figure 8); inhibition (39 ± 7%, of basal) was seen only at 10^{-6} M 8-OH-DPAT (Figure 8).

Discussion

The present results provide strong evidence that the 8-OH-DPAT-induced inhibition of glucose-stimulated plasma insulin in the SH rat is mediated via the central nervous system. This conclusion is based on two key observations. Firstly in the pithed SH rat, 8-OH-DPAT (150 $\mu g\,kg^{-1}$, i.v.)

did not elicit any inhibition of the glucose-stimulated insulinaemia, rather a small augmentation was observed. In contrast both in the conscious and anaesthetized SH rat, 8-OH-DPAT ($150\,\mu g\,kg^{-1}$, i.v.) inhibited the glucose-stimulated (i.v. glucose tolerance test) plasma insulin levels, suggesting a central mechanism of action. These effects were associated with profound decreases in blood pressure and heart rate. That the pithed rat preparation can detect agents inhibiting insulin release through peripheral mechanisms, i.e. at the level of the pancreas, is clear from the data obtained with clonidine which produced a marked and sustained inhibition of both basal and glucose-stimulated insulin levels which was associated with glucose intolerance. The hypoinsulinaemic effect of clonidine has been extensively studied *in vivo* (Angel *et al.*, 1988, Angel & Langer, 1988) and *in vitro* (Nakaki *et al.*, 1981; Hillaire-Buys *et al.*, 1985) and has been attributed to the activation of α_2-adrenoceptors present on the pancreatic β-cells. The inability of 8-OH-DPAT to inhibit insulin levels in this preparation cannot be attributed to the difference in cardiovascular actions *vis à vis* conscious and anaesthetized rats, since clonidine, like 8-OH-DPAT, showed no blood pressure lowering activity in the pithed SH rat but inhibited insulin levels.

Secondly, indirect support for the action of 8-OH-DPAT being centrally mediated comes from the inability of 8-OH-DPAT at concentrations of 10^{-8} and 10^{-7} M to inhibit glucose-stimulated insulin release from the isolated perfused pancreas of the rat. Although some inhibition of insulin release was observed at 10^{-6} M 8-OH-DPAT, this is unlikely to be due to an interaction with 5-HT_{1A} receptors. Thus, *in vitro*, functional as well as biochemical responses to 8-OH-DPAT mediated via 5-HT_{1A} receptors, such as inhibition of contraction of the transmurally stimulated guinea-pig ileum (Mir *et al.*, 1988) and inhibition of adenylate cyclase (Bockaert *et al.*, 1987) are observed in the 10^{-8}–10^{-7} M concentration range. In addition, methiotepin, a non-selective 5-HT_{1A} receptor blocker, at 10^{-7} M failed to antagonize the 8-OH-DPAT-mediated inhibition of glucose-stimulated insulin release from the isolated perfused pancreas (unpublished observation). 8-OH-DPAT has a certain affinity for α_2-adrenoceptors (pIC_{50} 6.91; Fozard *et al.*, 1987) and it is theoretically possible that at high concentrations 8-OH-DPAT can inhibit insulin release via a mechanism similar to that of clonidine (see above) at the level of pancreatic β-cells. However, 8-OH-DPAT does not exhibit α_2-adrenoceptor agonist activity in the transmurally stimulated guinea-pig ileum (Mir & Fozard, 1987) and seems to be an antagonist at these sites (Crist & Surprenant, 1987).

8-OH-DPAT did not induce hyperglycaemia in the presence of anaesthesia whereas the inhibition of evoked plasma insulin was unaffected. It appears, therefore, that anaesthesia selectively alters the mechanism(s) eliciting basal glycaemia in response to 8-OH-DPAT. An important feature of the metabolic effects of 8-OH-DPAT was that despite marked inhibition of glucose-stimulated insulin release, no worsening of the glucose tolerance was observed in either conscious or anaesthetized SH rats. In the conscious SH rat the net glycaemia induced by 8-OH-DPAT in the absence of glucose injection was similar to the difference in glycaemia between the control and 8-OH-DPAT-treated groups following the glucose injection (see for comparison Figures 1 and 2). These data suggest that despite inhibition of insulinaemia enough of the hormone is present in the circulation for adequate tissue uptake of glucose. An alternative explanation could be that 8-OH-DPAT enhances insulin sensitivity and/or metabolism of glucose via a decrease in glucagon secretion. It is interesting that fenfluramine, a 5-HT releaser, improves insulin action in patients with non-insulin dependent diabetes mellitus (NIDDM) (Pestell *et al.*, 1989), which is characterized by hyperinsulinaemia. The present data extend the results of Chaouloff & Jeanrenaud in conscious normoinsulinaemic (Chaouloff & Jeanrenaud, 1987) and genetically obese (fa/fa) rats (Chaouloff & Jeanrenaud, 1988), showing that 8-OH-DPAT increases basal glycaemia and inhibits glucose-stimulated insulin release without any adverse effects on glucose tolerance in either case. The data also support a central mechanism of action for 8-OH-DPAT. However, despite the metabolic and cardiovascular effects of 8-OH-DPAT being centrally mediated and involving pharmacologically similar receptor sites, 5-HT_{1A} and α_2-adrenoceptors (Chaouloff & Jeanrenaud, 1987; Fozard *et al.*, 1987) it is unlikely that the mechanisms of action of 8-OH-DPAT on the cardiovascular system (increase in cholinergic tone and sympathoinhibition) can account for the hypoinsulinaemic and hyperglycaemic effects of 8-OH-DPAT; an increase in vagal tone and sympathoinhibition is known generally to favour the opposite response i.e. hyperinsulinaemia and hypoglycaemia (Woods & Porte, 1974; Ahren *et al.*, 1986).

Thus, in the context of the above discrepancy it is worthwhile considering recent evidence that central 5-HT_{1A} receptor activation also results in stimulation of the pituitary-adrenal axis manifested as changes in prolactin, ACTH, corticosterone, β-endorphin and growth hormone (see Introduction). The stimulation of the endocrine axis is likely to arise from the level of the hypothalamus, since 5-HT nerve terminals make synaptic connections in the paraventricular nucleus with corticotropin releasing factor (CRF)-containing neurones in the rat hypothalamus (Liposits *et al.*, 1987), and 5-HT is reported to enhance the release of CRF (Jones *et al.*, 1976). CRF, apart from producing adrenal stimulation of corticosteroids via the pituitary axis, also directly stimulates sympatho-adrenal tone, resulting in increased plasma adrenaline, and increases blood pressure (for review see Fisher, 1989). CRF also stimulates release of somatostatin (Peterfreund & Vale, 1983). Both somatostatin (Reichlin, 1986) and catecholamines are potent inhibitors of insulin release at the level of the pancreas and therefore 8-OH-DPAT-mediated inhibition of insulin release may involve actions of somatostatin and/or catecholamines as a consequence of CRF release. In this context, 8-OH-DPAT, $62\text{-}500\,\mu g\,kg^{-1}$ i.v., has been recently shown to increase plasma adrenaline and noradrenaline levels (presumed to be of adrenal origin) associated with transient increases in blood pressure which precede the decrease in blood pressure in conscious normotensive rats (Bagdy *et al.*, 1989).

In view of the foregoing it appears likely that the hypoinsulinaemic/hyperglycaemic effects of 8-OH-DPAT reflects activation of the hypothalamic-adrenal axis and may be mediated via action(s) of CRF, somatostatin, corticosterone and catecholamines. In support of this, in preliminary experiments in adrenalectomized, conscious normotensive rats, we have found that both the hyperglycaemic and hypoinsulinaemic effects of 8-OH-DPAT, as well as the initial pressor response, are abolished whereas the hypotensive effects were unaffected (Bouhelal and Mir, unpublished data). The effects of 8-OH-DPAT on cardiovascular parameters, glycaemia and insulinaemia are being evaluated in adrenodemedullated rats to obtain further evidence for the concept that the metabolic and cardiovascular effects of 8-OH-DPAT are mediated via different mechanisms.

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ω-Conotoxin GVIA is a potent inhibitor of sympathetic neurogenic responses in rat small mesenteric arteries

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1 We have investigated the effects of the N-type calcium channel blocker, ω-conotoxin GVIA, on contractile responses to nerve stimulation, noradrenaline and KCl in rat small mesenteric arteries. In separate experiments, single and summated excitatory junctional potentials (e.j.ps) evoked by nerve stimulation were recorded with an intracellular electrode in the absence and presence of ω-conotoxin.

2 Electrical field stimulation of intramural sympathetic nerves (30 V; 0.25 ms pulse width; 3 s train length; 4–24 Hz) caused frequency-dependent contractions. Cumulative concentration-response curves for the contractions induced by noradrenaline and KCl were constructed in the same preparations. Stimulation at 0.2 Hz and 10 Hz induced respectively single e.j.ps without contractions and summated e.j.ps associated with a contractile response.

3 ω-Conotoxin (0.1 to 3 nM) inhibited markedly and in a concentration-dependent manner both the contractions and e.j.ps to electrical field stimulation. The concentration-response curves to exogenous noradrenaline and KCl remained unaffected.

4 The time-course for the effects of ω-conotoxin (0.3 to 3 nM) indicated a slow onset of action with at least one hour to achieve an equilibrium.

5 The experiments indicate that ω-conotoxin acts prejunctionally to inhibit sympathetic neurotransmission in rat small arteries presumably by inhibition of noradrenaline release. We suggest that ω-conotoxin could be a useful tool to study the control of vascular tone through the autonomic nervous system.

Introduction

The depolarization-induced release of noradrenaline from nerve terminals is triggered by a presynaptic influx of extracellular calcium ions through voltage-operated channels (Reichardt & Kelly, 1983; Smith & Augustine, 1988). Recent electrophysiological studies have shown that voltage-operated calcium channels (VOCCs) can be subdivided in L-, N- and T-type on the basis of differences in unitary barium conductance and gating properties (Nowycky *et al.*, 1985; Miller, 1987). These three types of channel also differ in their tissue distribution and pharmacological properties (Tsien *et al.*, 1988). Thus, N-type VOCCs have been demonstrated exclusively in neuronal preparations (Nowycky *et al.*, 1985; McCleskey *et al.*, 1987). Patch-clamp studies have clearly demonstrated that ω-conotoxin GVIA (ω-CTX), a 27 amino acid peptide isolated from the marine snail *Conus geographus*, is a selective and potent inhibitor of L- and N-type calcium channels in dorsal ganglion, sensory, sympathetic and hippocampal neurones of vertebrates but not in cardiac, skeletal or smooth muscle cells (McCleskey *et al.*, 1987). ω-CTX inhibited synaptsomal ^{45}Ca influx (Reynolds *et al.*, 1986) and neurotransmitter release from synaptosomes (Reynolds *et al.*, 1986), cultured neurones (Hirning *et al.*, 1988) and nerve terminals in the rat and guinea-pig vas deferens (Maggi *et al.*, 1988). In the guinea-pig vas deferens, ω-CTX abolished stimulation-evoked contractions and excitatory junctional potentials (e.j.ps) (Brock *et al.*, 1989).

Surprisingly, even though the sympathetic neuromuscular transmission in arteries is of crucial importance in the maintenance of vascular resistance, no direct information is available to date on the possible modulation by ω-CTX of sympathetic neurotransmitter release from nerve endings in the vasculature. In the present study, we used ω-CTX to explore the role of N-type calcium channels in the release of the sympathetic neurotransmitter in resistance arteries of the rat mesentery. To dissociate prejunctional from postjunctional sites of action, concentration-contractile response curves to KCl and noradrenaline were generated in the absence and presence of ω-CTX. The action of ω-CTX on e.j.ps evoked by nerve stimulation was also investigated since e.j.ps have been demonstrated to be mediated by ATP rather than noradrenaline in rat mesenteric arteries (Angus *et al.*, 1988).

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Methods

Wistar-Kyoto rats (10 to 15 weeks) were killed by CO_2 (Kraus, 1980) and small arteries from the superior mesenteric bed were carefully dissected under a Nikon 102 microscope. Mesenteric vessels were then mounted as ring preparations in an isometric myograph (J.P. Trading, Denmark) as previously described (Angus *et al.*, 1988). During dissection, mounting and the course of experiments, arteries were held in a physiological salt solution (PSS) of the following composition (in mM): NaCl 119, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.17, $NaHCO_3$ 25, $CaCl_2$ 2.5, ethylene-diaminetetracetic acid (EDTA) 0.026, glucose 5.5, bubbled with 95% O_2 plus 5% CO_2. The vessels were set up in a 15 ml bath and stretched to an internal circumference equal to 0.9 L_{100} where L_{100} is the internal circumference the vessel would have when relaxed and under a pressure of 100 mmHg (Mulvany & Halpern, 1977). Square wave field stimulation was achieved through 5 μm thick platinum electrodes contained on the mounting supports of the myograph with a low-output-resistance stimulator (Grass SD9/S88, Quincy, MA, U.S.A.). Membrane potential was measured by conventional intracellular capillary-glass microelectrodes (resistances of about 100 MΩ) filled with 0.5 mM KCl solution. The reference electrodes were similarly prepared and positioned close to the impaling electrode. The output of the force transducer amplifier was recorded on a potentiometric recorder (Model 320, W&W Scientific Instruments, Basel, Switzerland). In electrophysiological experiments, signals of force transducer and electrode amplifiers were recorded simultaneously on a potentiometric recorder and on a dual-beam storage oscilloscope (5113 Tektronix, Beaverton, OR, U.S.A.). After being mounted, the vessels were activated successively with KCl (124 mM), 10 μM

noradrenaline in KCl (124 mM) and 10 μM noradrenaline. Field stimulation (0.25 ms, 30 V) was then applied at 4–24 Hz for 3 s every minute. The field stimulation was repeated after irreversible blockade of autoinhibitory prejunctional α_2-adrenoceptors by benextramine (3 μM) treatment, added in the presence of prazosin (0.1 μM) to protect α_1-adrenoceptors. After 5 min exposure to benextramine and prazosin, the vessels were repeatedly washed with drug-free PSS to remove the reversible postjunctional α_1-adrenoceptor antagonist prazosin but leaving the prejunctional α_2-adrenoceptors irreversibly blocked with benextramine (see Angus *et al.*, 1988). Arteries were then equilibrated with desipramine (0.1 μM) and atropine (1 μM) to avoid interference from neuronal uptake or cholinergic activity.

In pilot experiments conducted to assess the optimum equilibration period for ω-conotoxin, five 3 s trains of field pulses at 24 Hz were applied at one minute intervals before and 5, 20, 40 and 60 min after addition of ω-conotoxin or its vehicle. In the definitive experiments, two 3 s trains of field pulses at 4, 8, 16 and 24 Hz were applied at one minute intervals before and one hour after incubation with vehicle (H_2O) and ω-conotoxin (0.1, 0.3, 1 or 3 nM). Only one concentration was tested in each preparation. In the same experiments concentration-response curves to noradrenaline (0.1–30 μM) or to KCl (5–70 mM) were obtained before and one hour after equilibration of the vessel with ω-CTX. In electrophysiological experiments, the tissue bath (15 ml) was perfused at 5 ml min^{-1} with oxygenated PSS. This continuous perfusion system helped to maintain the electrode impalement. The time to reach the appropriate concentration of a drug in the bath when added to the perfusate was approximately 1 min. Field pulses were applied at either 25 V, 0.25 ms, 0.2 Hz to obtain repetitive e.j.ps without measurable contraction or at 10 Hz for 500 ms to achieve e.j.p. summation and a small contraction. Thirty minutes after ω-CTX (3 nM), these two periods of field stimulation were re-applied. Before the application of ω-CTX in all experiments, tetrodotoxin (TTX, 0.1 μM) was added as a bolus to the bath to confirm that the e.j.ps and contractions to field stimulation were neurally mediated.

Drugs

The drugs and suppliers were as follows: ω-conotoxin GVIA (Peninsula Lab.), prazosin hydrochloride (Pfizer), (−)-noradrenaline bitartrate (Sigma), atropine sulphate (Sigma), desipramine hydrochloride (Sigma), benextramine tetrahydrochloride (Sigma), tetrodotoxin (Calbiochem). Solutions of noradrenaline were made fresh daily. Stock solutions of TTX and ω-CTX were stored frozen and diluted daily in distilled water and discarded. Stock solutions of other drugs were kept in the refrigerator.

Statistical analysis

Each contraction concentration-response curve to noradrenaline and KCl was fitted to a logistic equation to determine objectively the EC_{10-90}% values and the maximum contraction (Nakashima *et al.*, 1982). Comparisons of average (±1 s.e.mean) EC_{50} values and maximum responses were achieved between groups by a non-parametric Kruskall-Wallis test. Significant differences were accepted at the 5% level. For the responses to electrical field stimulation, the average s.e.mean for the responses to the entire range of frequencies within the artery was calculated from a two-way analysis of variance as (error mean square/number of arteries)$^{0.5}$ after the sums of squares between arteries and between frequencies had been subtracted from the total sums of squares. These error bars are placed on the line relating % contraction and frequency at no specific point for each concentration of conotoxin.

Results

Electrical field stimulation at 24 Hz for 3 s caused a contraction in rat mesenteric arteries (150–350 μm i.d.) of 52.2 ± 1.7% ($n = 9$) of the maximum contraction to noradrenaline (10 μM). These contractions were mediated by neural transmission since tetrodotoxin (0.1 μM) completely abolished the response after 1 min incubation (Figure 1). This effect of tetrodotoxin

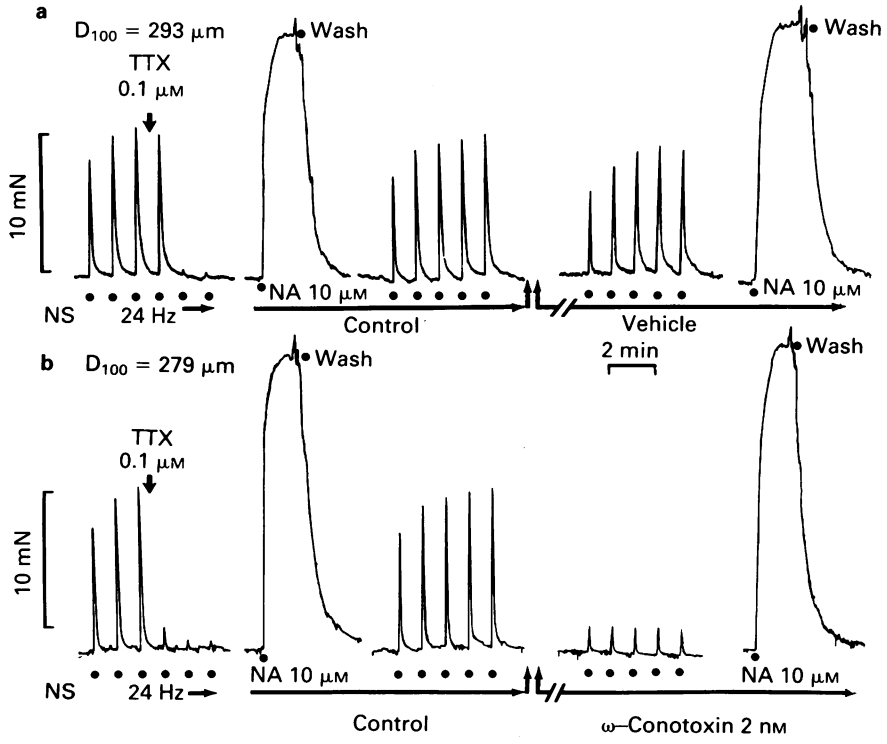

Figure 1 Representative tracings from two arteries showing the effects of ω-conotoxin (3 nM) on contractions to electrical field stimulation (30 V, 0.25 ms pulse width, 3 s train every minute, 24 Hz) and to noradrenaline (NA, 10 μM) in rat small mesenteric arteries. The effect of tetrodotoxin (TTX, 0.1 μM) is also represented. ω-Conotoxin (ω-CTX) or the corresponding vehicle was incubated with the tissue for one hour. D_{100} is the normalised internal diameter of the vessel and NS = nerve stimulation.

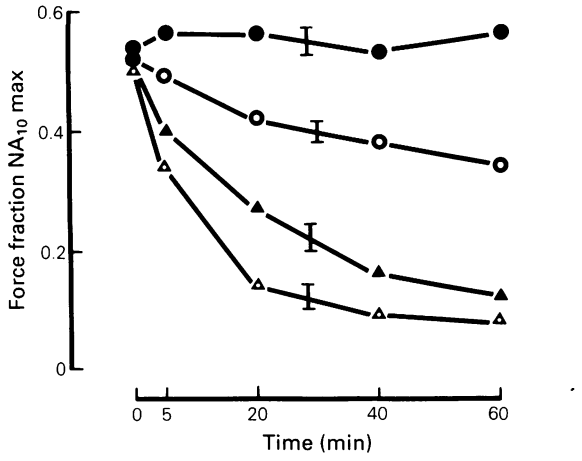

Figure 2 Time-course for the effects of ω-conotoxin on the contractions induced by electrical field stimulation (30 V, 0.25 ms pulse width, 3 s train every min, 24 Hz) in rat small mesenteric arteries. Five consecutive trains of stimulation were evoked before, 5, 20, 40 and 60 min after addition of ω-conotoxin (0.3 (○); 1 (▲); 3 (△) nM) or its vehicle (●). Error bars are ±1 average s.e.mean ($n = 4$–5) (see Methods). Normalised internal diameter, D_{100}, was 253 ± 15 μm (●), 321 ± 13 μm (○), 249 ± 22 μm (▲) and 267 ± 18 μm (△). Responses are given as fraction of wall tension response to noradrenaline (10 μM) (NA_{10} max) at start of experiment which was 2.66 ± 0.46 N m^{-1} (●), 3.42 ± 0.48 N m^{-1} (○), 2.65 ± 0.51 N m^{-1} (▲) and 2.23 ± 0.27 N m^{-1} (△).

was rapidly and completely reversed on replacing the bathing fluid with drug-free saline solution. These neurogenic contractions were inhibited by ω-CTX (3 nM) incubated for one hour, as illustrated in Figure 1b. Evidence that ω-CTX, at the most effective concentration on nerve stimulation, did not affect the postjunctional smooth muscle cell reactivity was that the peak response to noradrenaline (10 μM) was unaltered after one hour (Figure 1b). ω-Conotoxin (0.3–3 nM) was very slow in onset in reducing the peak response to 3 s electrical stimulation at 24 Hz (Figure 2). After one hour exposure to 0.3, 1 or 3 nM ω-CTX, the contraction to 24 Hz for 3 s was reduced by 38.3 ± 8.9, 76.3 ± 0.6 and 83.4 ± 1.8% of the control contraction. The neurogenic responses were reduced by ω-CTX at all frequencies of stimulation and the inhibitory effect was concentration-dependent (Figure 3). At 16 Hz, ω-CTX inhibited the amplitude of contractions by 21.1, 29.4, 76.4 and 87.4% of control values at 0.1, 0.3, 1 and 3 nM respectively. The cumulative concentration-response curves to both KCl (5–70 mM) and noradrenaline (0.1–10 μM) were unaffected by ω-CTX (Figure 4).

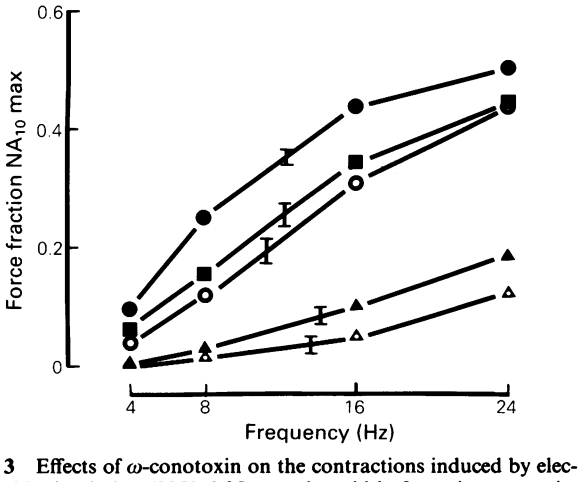

Figure 3 Effects of ω-conotoxin on the contractions induced by electrical field stimulation (30 V, 0.25 ms pulse width, 3 s train every min, 4–24 Hz) in rat small mesenteric arteries. Field stimulation at 4, 8, 16 and 24 Hz was evoked one hour after addition of ω-conotoxin (0.1 (■); 0.3 (○); 1 (▲); 3 (△) nM) or its vehicle (●). Error bars are ±1 average s.e.mean ($n = 5$–6). Normalised internal diameter, D_{100}, was 304 ± 20 μm (●), 308 ± 15 μm (■), 258 ± 39 μm (○), 296 ± 32 μm (▲) and 298 ± 22 μm (△). Responses given as fraction of wall tension response to noradrenaline (10 μM) at start of experiment which was 2.40 ± 0.38 N m^{-1} (○), 2.06 ± 0.30 N m^{-1} (■), 2.52 ± 0.29 N m^{-1} (○), 2.49 ± 0.28 N m^{-1} (▲) and 3.00 ± 0.32 N m^{-1} (△).

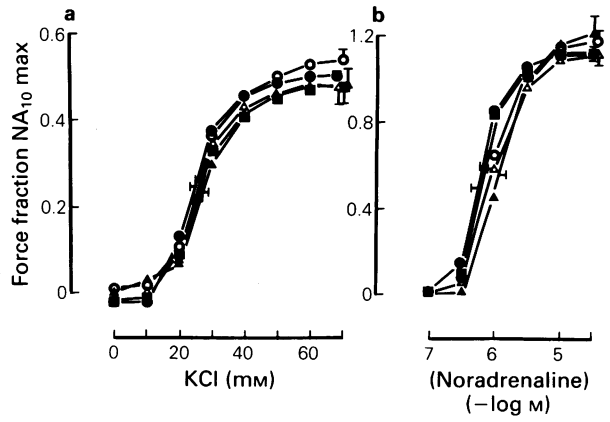

Figure 4 Effects of ω-conotoxin on the contractions induced by KCl (a) and noradrenaline (b). Cumulative concentration-response curves to noradrenaline (0.1–30 μM) and KCl (0–70 mM) were obtained one hour after addition of ω-conotoxin (0.1 (■); 0.3 (○); 1 (▲); 3 (△) nM) or its vehicle (●). Horizontal error bars are s.e.mean ($n = 5$–6) at EC_{50} values. Vessels were the same as in Figure 2. In each case EC_{50} values were not significantly different ($P > 0.05$).

Field stimulation at very low frequencies (0.2 Hz) caused e.j.ps of 5–10 mV but no contraction (Figure 5). These e.j.ps

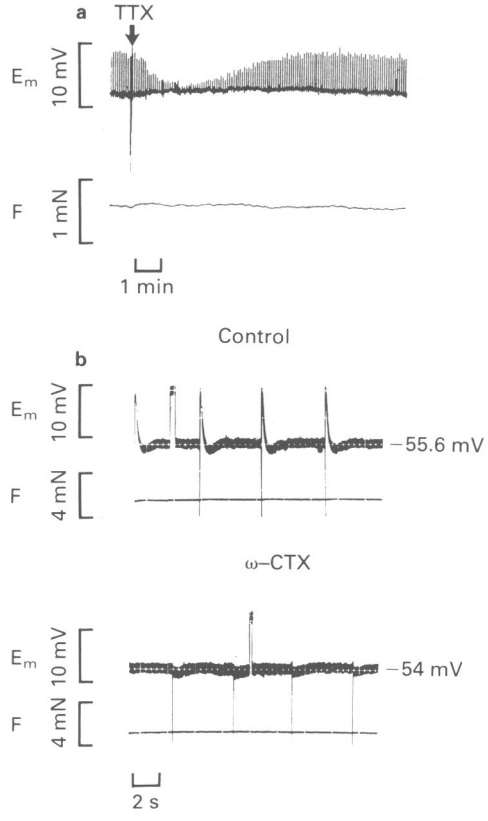

Figure 5 Simultaneous recordings of membrane potential (E_m, upper traces) and wall force (F, lower traces) of a rat small mesenteric artery before and after treatment with tetrodotoxin (a) or ω-conotoxin (b). Stimulation (25 V, 0.25 ms pulse width) was at 0.2 Hz. Tetrodotoxin (TTX) was added as a bolus to the perfused tissue (5 ml min^{-1}) so that the recovery of excitatory junctional potentials (e.j.ps) correspond to the wash-out of TTX (a). ω-Conotoxin (ω-CTX, 3 nM) was infused for 30 min (b). Resting membrane potentials indicated on the figure. The normalised internal diameter of the artery was 310 μm. Note the 10 mV calibration signal on the E_m records.

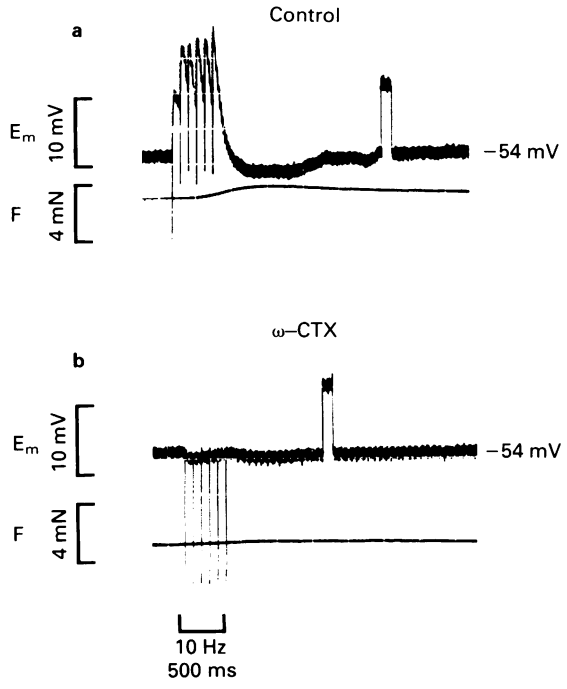

Figure 6 Simultaneous recordings of membrane potential (E_m) and wall force (F) of a rat small mesenteric artery before (a) and after treatment with ω-conotoxin (3 nM, ω-CTX) for 30 min (b). Stimulation (25 V, 0.25 ms duration, 500 ms train length) was at 10 Hz. Resting membrane potential indicated on the figure. The normalised internal diameter of the artery was 310 μm. Note the 10 mV calibration mark.

were totally abolished after 30 min equilibration with ω-CTX (3 nM).

When the frequency of 0.2 Hz was raised to 10 Hz in the absence of ω-CTX the e.j.ps summated and a small contraction resulted (Figure 6). These electrophysiological and mechanical responses were abolished by ω-CTX (Figure 6).

Discussion

The present results clearly indicate that ω-CTX is a potent inhibitor of sympathetic neurotransmission in rat small mesenteric arteries probably by interfering with noradrenaline release. We also demonstrated that ω-CTX was without effect on vascular smooth muscle cells when contractions were induced by α_1-adrenoceptor activation or by direct smooth muscle depolarization by K^+.

The small mesenteric arteries of the rat were selected as a preparation with sympathetic innervation on which pre- and post-junctional effects of ω-CTX could be functionally studied. Furthermore, arteries with an internal diameter between 150 and 350 μm are important in the control of vascular resistance (Mulvany, 1984) and the modulation of their tone by the autonomic nervous system is poorly understood. In these vessels, it has been demonstrated that the contraction to field stimulation is mediated primarily through α-adrenoceptors (Nilsson, 1984; Angus *et al.*, 1988) and it is known that the release of an adrenergic transmitter in response to electrical stimulation of nerve fibres requires the influx of calcium ions into terminals (Reichardt & Kelly, 1983; Smith & Augustine, 1988). However, the mechanism sustaining this process has not yet been established. It is thought that depolarization activates presynaptic VOCCs but these channels are relatively insensitive to classical calcium entry blockers (Beattie *et al.*, 1986; McCleskey *et al.*, 1987; Miller, 1987; Hirning *et al.*, 1988). Nifedipine, diltiazem and verapamil have been shown to inhibit the release of noradrenaline from the rabbit isolated heart and pulmonary artery, but the concentrations of calcium antagonists required were much higher than those needed to block VOCCs in smooth or cardiac muscle (Starke & Schümann, 1973; Göthert *et al.*, 1979; Zelis *et al.*, 1985). In contrast, the release of neurotransmitter from nerve terminals triggered by presynaptic VOCCs was shown to be inhibited by the 27 amino acid peptide ω-CTX in the rat isolated kidney (Mohy El-Din & Malik, 1988) and in various non-vascular peripheral tissues, such as the vas deferens (Maggi *et al.*, 1988; Brock *et al.*, 1989) and the mesenteric plexus longitudinal muscle (Lundy & Frew, 1988). In the rat isolated urinary bladder and proximal duodenum, the response to field stimulation, though TTX-sensitive, was partly resistant to ω-CTX (Maggi *et al.*, 1988). It seems therefore that the sensitivity to ω-CTX can vary according to the organ and species studied (Maggi *et al.*, 1988). Also, in the rat small mesenteric arteries the sensitivity of neurogenic contractions to ω-CTX appeared to be frequency-dependent, as previously described by Maggi *et al.* (1988) in the rat urinary bladder.

It has been proposed from electrophysiological studies that ω-CTX inhibits L- and N-type VOCCs in neurones from vertebrates (McCleskey *et al.*, 1987). The present study does not allow us to differentiate between channels but if we assume that ω-CTX blocks both the presynaptic L- and N-type VOCCs, the results indicate that L-type neuronal VOCCs are different from L-type muscular channels. It is well known that L-type calcium channels of vascular smooth muscle are inhibited by dihydropyridines (Godfraind *et al.*, 1986). In rat small mesenteric arteries, felodipine (0.1 μM) inhibited the contractile response to noradrenaline by 96.3 ± 1.3% ($n = 4$) (J.A. Angus, unpublished data). In contrast, it is clear from our results and others that vascular smooth muscle VOCCs are insensitive to ω-CTX (McCleskey *et al.*, 1987; Lundy & Frew, 1988). In support of this view is the finding that no binding interactions occur between ω-CTX and dihydropyridine-related calcium antagonists (Barhanin *et al.*, 1988; Keith *et al.*, 1989). In rat sympathetic neurones, it has been established that N-type VOCCs are dominant in the evoked release of noradrenaline (Hirning *et al.*, 1988). We suggest that in sympathetic nerve terminals of rat small arteries ω-CTX-sensitive VOCCs, probably N-type VOCCs, are present and promote the release of noradrenaline, while VOCCs located in smooth muscle cells are totally ω-CTX-insensitive. A possible inhibitory action of ω-CTX on impulse propagation in nerves has recently been ruled out in a study in the vas deferens (Brock *et al.*, 1989).

Interestingly, in the present study both the e.j.ps and corresponding contractile responses were eliminated by ω-CTX (Figure 6). In a similar preparation it was previously observed that α,β-methylene ATP (10 μM) abolished e.j.ps without affecting the accompanying contraction, whereas prazosin (0.01 μM) blocked the force response and left e.j.ps almost unaffected (Angus *et al.*, 1988). These studies suggest that e.j.ps and the concomittant contractions could be mediated by the release of ATP and noradrenaline, respectively. The finding that ω-CTX blocked both the electrical and the mechanical processes suggests that the release of noradrenaline and possibly ATP are triggered by a similar ω-CTX-sensitive influx of calcium ions.

In summary, this study provides evidence of ω-CTX-sensitive VOCCs on sympathetic nerve terminals to the rat vasculature and demonstrates that these channels control the release of noradrenaline. We propose that ω-CTX could be a useful tool to achieve pharmacological sympathectomy when the role of the autonomic nervous system in the control of vascular tone is being studied.

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Antigen induces leucopenia in non-immunised guinea-pigs injected with platelets from actively sensitized animals

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1 Ovalbumin administration to animals injected with 0.5–5 × 10^6 washed platelets μl^{-1} from actively sensitized animals induced a marked decrease (maximum of around 50% at 60 min) in the number of circulating leucocytes, whereas platelet counts were unaffected. The intensity of the decrease in leucocyte counts was dependent upon the concentration of the injected platelets.

2 No drop in leucocyte counts was measured upon antigen challenge of guinea-pigs injected with platelets from non-sensitized animals.

3 This phenomenon was unaffected by pretreatment of the recipient animals with a platelet-activating factor (PAF) antagonist WEB 2086 (3 mg kg^{-1} i.v.) but was markedly reduced (around 50% inhibition) by the anti-allergic drug nedocromil sodium (100 mg kg^{-1} i.v.). By contrast, indomethacin (5 mg kg^{-1} i.v.) caused a significant ($P < 0.01$) enhancement of the antigen-induced leucopenia, whereas the mixed cyclo-oxygenase and lipoxygenase inhibitor, BW 755C (10 mg kg^{-1} i.v.) suppressed the drop in leucocyte counts evoked by ovalbumin administration.

4 These results indicate that platelets from actively sensitized guinea-pigs transferred to normal animals still responds to the specific antigen with the activation of circulating leucocytes. This phenomenon appears to be independent of the production of PAF and of cyclo-oxygenase metabolites. By contrast, the production of the metabolites of the lipoxygenase pathway by platelets could account for the marked leucopenia observed following the immunological stimulation.

Introduction

Several lines of evidence indicate that platelets participate in inflammatory and allergic reactions. Indeed, these blood elements exhibit some of the features of other inflammatory cells such as neutrophils, eosinophils and alveolar macrophages (Page, 1988). The presence of a specific binding structure for immunoglobulin E (IgE) on their surface (Joseph *et al.*, 1986) led to the demonstration that platelets respond to immune challenges following the interaction of the antigen with the surface-bound IgE antibody. The physiopathological significance of the presence of IgE receptors has been linked to the cytocidal activity of platelets (Capron *et al.*, 1986). Platelet activation and/or accumulation within the pulmonary vasculature in several animal species (Pretolani *et al.*, 1986; Arnoux *et al.*, 1988; Lellouch-Tubiana *et al.*, 1988) and in man (Knauer *et al.*, 1981) is associated with bronchoconstriction following immunological stimulation or the administration of inflammatory mediators, including platelet-activating factor (PAF) (Page *et al.*, 1982; Lellouch-Tubiana *et al.*, 1988). Platelet activation may thus play an important role in hypersensitivity reactions. This led us to investigate whether platelets obtained from actively sensitized guinea-pigs keep the ability to recognize antibody upon their transfer to non-immunised animals and, if so, whether this recognition would be followed by relevant physiopathological events.

Sensitization procedure

Hartley guinea-pigs of either sex (400–600 g) were actively sensitized by a s.c. injection of 0.5 ml 0.9% NaCl (saline) containing 10 μg ovalbumin dispersed in 1 mg $Al(OH)_3$ (modified from Andersson & Brattsand, 1981). The injection was repeated after 2 weeks and the animals were used 7–10 days after the second injection. This sensitization procedure has been shown to produce mainly specific IgG (Pretolani *et al.*, 1988; 1989). Untreated guinea-pigs were used as controls.

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Preparations of washed guinea-pig platelets

Control or actively sensitized guinea-pigs were anaesthetized with sodium pentobarbitone (30 mg kg^{-1} i.p.) and the carotid artery (for blood collection) was cannulated. Arterial blood was collected on 1/10th its final volume of sodium citrate (3.8% w/vol) in the presence of 1 μM prostacyclin (PGI_2). Platelet-rich plasma (PRP) was prepared by centrifugation (200 *g*, 15 min) at 30°C. Platelets were washed twice by repeated centrifugations (900 *g*, 10 min) and resuspended in a Tyrode-gelatin buffer at pH 6.5, containing 0.2 mM EGTA and 1 μM PGI_2. After being centrifuged again, the platelet pellet was resuspended in saline plus 1 μM PGI_2 and counted with a Coulter counter (ZBI). The final platelet preparation was adjusted to 0.5–5 × 10^6 platelets μl^{-1} and injected i.v. in 1 ml saline to non-immunised guinea-pigs. In a separate set of experiments, after the second centrifugation, platelets were suspended and ^{111}indium oxine (20–50 μCi) added. After 5 min at room temperature, the platelets were washed once to remove the free Indium.

Examination by light microscopy of undiluted platelet preparations after staining with Toluidine blue showed that the leucocyte contamination was less than 0.02%.

The composition of the Tyrode gelatin buffer was ($g l^{-1}$): KCl 0.2, $MgCl_2 \cdot 6H_2O$ 0.198, NaCl 8, $NaHCO_3$ 1.015, glucose 1, EGTA 0.076, gelatin 2.5; pH adjusted to 6.5 with 1 N HCl.

In vivo *experiments*

The day after platelet injection guinea-pigs were anaesthetized as described above and the carotid artery (for blood collection), the jugular vein (for drug administration) and the trachea were cannulated. Animals were ventilated (Palmer miniature respiratory pump; 60 strokes min^{-1}; 10 ml kg^{-1} body weight) and spontaneous breathing was abolished with pancuronium (4 mg kg^{-1} i.v.). After a 3 h equilibration period, ovalbumin (1 mg kg^{-1} i.v.) was administered. Aliquots (200 μl) of arterial blood were collected before and following (1, 3, 6, 10, 20, 30, 60, 120 min) the antigen administration for measuring the number of circulating platelets and leucocytes. When

indicated, guinea-pigs were pretreated i.v. with either WEB 2086 (3 mg kg^{-1}), nedocromil sodium (100 mg kg^{-1}), indomethacin (5 mg kg^{-1}) or BW 755C (10 mg kg^{-1}), 5 min before the injection of ovalbumin.

In selected experiments, guinea-pigs were prepared for the recording of bronchial resistance to inflation, as previously described (Lefort & Vargaftig, 1978) and blood pressure was monitored by cannulation of a carotid artery.

The pulmonary accumulation of Indium-labelled platelets upon antigen administration was monitored continuously with a collimated 1 inch crystal scintillation probe located over the external surface of the thorax (Page *et al.*, 1982; Bureau *et al.*, 1989). The counts were measured every min for 2 h after the injection of 1 mg kg^{-1} ovalbumin. At the end of the experiments, ADP (100 μg kg^{-1}) was injected to check the sensitivity of platelets. Responses were expressed as maximal % increase in the thoracic counts (i.e., an index of intrathoracic platelet accumulation).

Reagents

Drugs used were as follows: pentobarbitone (Sodium pentobarbital, Clin Midy, Montpellier, France), pancuronium, (Pavulon, Organon, Fresnes, France), chicken ovalbumin (Miles Naperville, IL, USA); $Al(OH)_3$, gelatin (Merck, Darmstadt); PGI_2, indomethacin, EGTA, KCl, $MgCl_2 \cdot 6H_2O$, NaCl, $NaHCO_3$, glucose (Sigma, St. Louis, MO, USA); WEB 2086 (3-[4-(2-chlorophenyl)-9-methyl-6H-thieno[3,2-f][1,2,4]-triazolo[4,3-a][1,4]-diazepin-2-yl]-1-(4-morpholinyl)-1-propanone, kindly provided by Dr H. Heuer, Boerhinger Ingelheim, FRG), was dissolved in HCl 50 mM in distilled water and further diluted at the appropriate concentration in saline; nedocromil sodium (Tilade, registered trade mark of Fisons plc Pharmaceutical division) was kindly provided by Dr K. Rainey, Fisons Pharmaceuticals, Loughborough, U.K. and BW 755C (3-amino-1-[*m*-(trifluoromethyl)-phenyl]-2-pyrazoline) was a gift from The Wellcome Research Labs., Beckenham, U.K.

Statistical analysis

The results are expressed as mean ± s.e.mean of the indicated number of experiments and significance was assessed by Student's *t* test for unpaired values. $P \leq 0.05$ was considered significant.

Results

Effect of ovalbumin administration on guinea-pigs injected with platelets from non-sensitized or from actively sensitized animals

The i.v. administration of 1 mg kg^{-1} ovalbumin to non-immunised guinea-pigs injected with platelets from actively sensitized animals was followed by a marked decrease in the number of circulating leucocytes (Figure 1). This phenomenon started 10 min after the antigen injection, reached a maximum of around 50% after 30 min and lasted for at least 2 h (Figure 1). By contrast, only a minimal (less than 10%) decrease in the number of circulating platelets was observed throughout the experiment (Figure 1).

When platelets were prepared from non-sensitized guinea-pigs and transferred to recipient animals, the i.v. administration of ovalbumin was not followed by a significant drop in leucocyte counts, which was limited to 15.5 ± 3.8% ($n = 4$). Furthermore, no leucopenia was measured in animals injected with sensitized platelets kept in saline at 4°C for 48 h (not shown).

Antigen-induced leucopenia in guinea-pigs injected with increasing concentrations of platelets from actively sensitized animals

To determine the threshold number of platelets necessary to trigger leucopenia, control guinea-pigs were injected with an

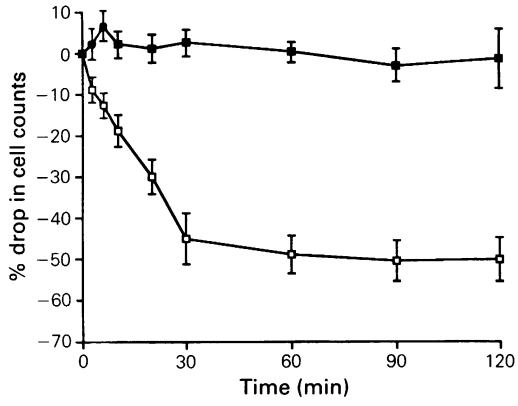

Figure 1 Time-course of the decrease in platelet (■) and leucocyte (□) counts following the i.v. administration of 1 mg kg^{-1} ovalbumin to non-immunised guinea-pigs injected the day before the experiment with platelets from actively sensitized animals. Results are expressed as % drop in peripheral cell counts and represent the mean of 6 experiments; vertical lines show s.e.mean.

increasing number of platelets (5×10^5, 1×10^6, 2×10^6, 5×10^6 μl^{-1}) and ovalbumin (1 mg kg^{-1}) was injected, as described above. As shown in Figure 2, no leucopenia was observed upon antigen challenge of guinea-pigs injected with 5×10^5 platelets μl^{-1}, whereas a transient drop in leucocyte counts was measured in those which received 1×10^6 platelets μl^{-1}). By contrast, ovalbumin administration to animals injected with 2×10^6 platelets μl^{-1} was accompanied by a marked decrease in the number of leucocytes, which reached a maximum of around 40% at 30 min. Two hours after the antigen challenge, a leucopenia of only 15% was evaluated. When the experiments were performed with the highest platelet concentration (5×10^6 μl^{-1}), a drop in leucocyte counts, which plateaued at 30 min and was irreversible, was observed.

In a selected series of experiments, blood pressure and bronchial resistance to inflation were recorded and no modifi-

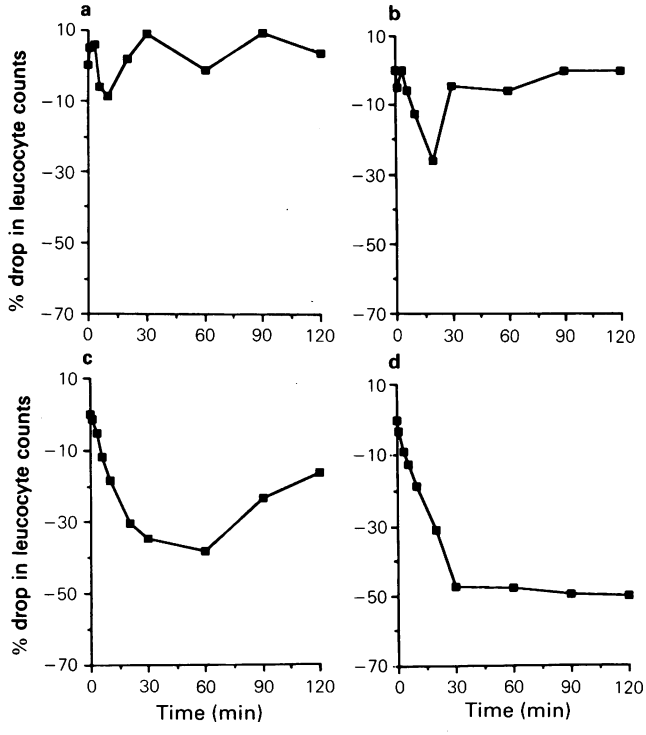

Figure 2 Decrease in blood leucocyte counts following the i.v. administration of 1 mg kg^{-1} ovalbumin to non-immunised guinea-pigs injected the day before the experiment with: (a) 5×10^5 platelets μl^{-1}, (b) 1×10^6 platelets μl^{-1}, (c) 2×10^6 platelets μl^{-1}, or (d) 5×10^6 platelets μl^{-1}. Each panel represents the results obtained in one single experiment.

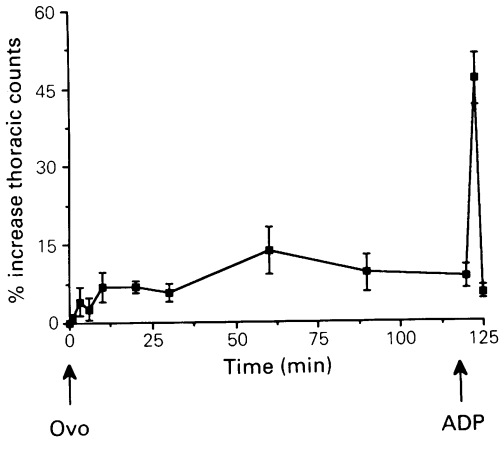

Figure 3 Time-course of ovalbumin (1 mg kg^{-1}, Ovo)- and ADP (100 μg kg^{-1})-induced lung accumulation of ^{111}indium-labelled platelets obtained from actively sensitized guinea-pigs and injected the day before the experiment to non-immunised animals. Results are expressed as % increase in the thoracic counts, calculated by comparing the values measured before the antigen challenge. Data represent the mean of 4 experiments; vertical lines show s.e.mean.

cation of these parameters was observed upon antigen administration (not shown).

Continuous monitoring of radio-labelled platelets in vivo

The administration of 1 mg kg^{-1} ovalbumin to guinea-pigs injected with ^{111}indium-labelled platelets obtained from actively sensitized animals evoked their intrathoracic accumulation, which started between 6 and 10 min after antigen challenge and reached a maximum of around 15% at 60 min (Figure 3). The i.v. injection of 100 μg kg^{-1} adenosine 5′-diphosphate (ADP) at the end of the experiments induced a marked increase (around 45%) in lung radioactivity (Figure 3), indicating the ability of the injected platelets to respond to a specific activator.

Pharmacological modulation of antigen-induced leucopenia

In a series of experiments, guinea-pigs were pretreated 5 min before the administration of ovalbumin with the platelet-activating factor (PAF) antagonist WEB 2086 (3 mg kg^{-1}). At the dose used, WEB 2086 did not modify the blood cell counts (Pretolani *et al.*, 1987). Following the antigen challenge, a drop in leucocyte counts similar to that observed in untreated animals was measured, ruling out the involvement of PAF in platelet-mediated antigen-induced leucopenia (Figure 4).

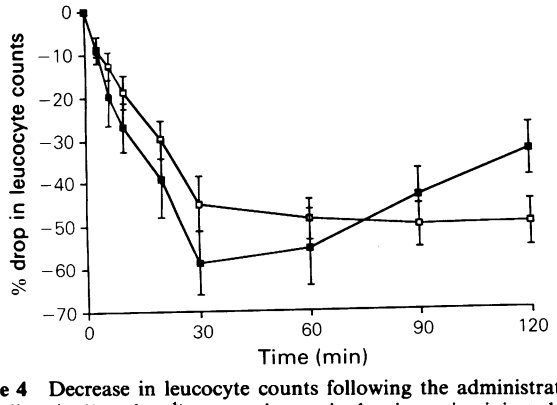

Figure 4 Decrease in leucocyte counts following the administration of ovalbumin (1 mg kg^{-1}) to non-immunised guinea-pigs injected the day before the experiment with 5 × 10^6 platelets from actively sensitized animals in the presence and absence of WEB 2086 (3 mg kg^{-1} i.v.) WEB 2086 administered 5 min before the antigen challenge. The baseline circulating leucocyte numbers in control and WEB 2086-treated guinea-pigs were 6623 ± 998 and 7121 ± 547 leucocytes μl^{-1}, respectively (difference not statistically significant). Results are expressed as mean of 4 experiments; vertical lines show s.e.mean. (□) Saline-injected guinea-pigs; (■) WEB 2086-injected guinea-pigs.

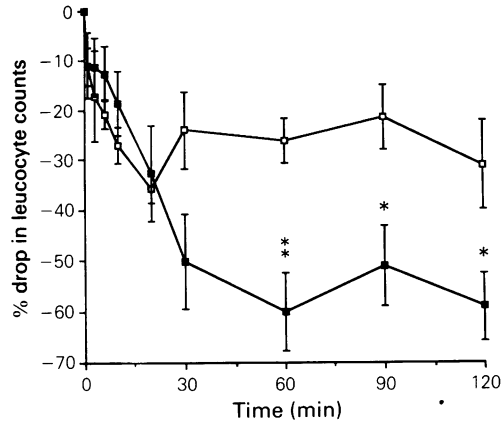

Figure 5 Decrease in leucocyte counts following the administration of ovalbumin (1 mg kg^{-1}) to non-immunised guinea-pigs injected the day before the experiment with 2 × 10^6 platelets from actively sensitized animals and the effect of indomethacin (5 mg kg^{-1} i.v., 5 min before the antigen challenge). The baseline circulating leucocyte numbers in control and indomethacin-treated guinea-pigs were 5413 ± 563 and 6064 ± 778 leucocytes μl^{-1}, respectively (difference not statistically significant). Results are expressed as mean of 4 experiments; vertical lines show s.e.mean. (□) Saline-injected guinea-pigs; (■) indomethacin-injected guinea-pigs. *P < 0.05, **P < 0.01.

The possible role of cyclo-oxygenase and lipoxygenase derivatives was further investigated. In initial experiments, indomethacin seemed to potentiate leucopenia and its possible interference was thus investigated under conditions where the drop in leucocyte counts was mild, i.e., in animals injected with 2 × 10^6 platelets μl^{-1}. The results depicted in Figure 5 show that, indeed, leucopenia following antigen challenge was significantly increased by 10 mg kg^{-1} indomethacin given i.v. 5 min before ovalbumin. In addition, leucopenia evaluated in guinea-pigs that had received platelets from actively sensitized animals was independent of the formation of cyclo-oxygenase metabolites of arachidonic acid, since platelet incubation for 15 min in the presence of 0.4 mM aspirin did not modify antigen-induced leucopenia (not shown). In contrast to the results with indomethacin, the mixed cyclo-oxygenase and lipoxygenase inhibitor BW 755C suppressed leucopenia following antigen administration (Figure 6).

Finally, 100 mg kg^{-1} nedocromil sodium injected 5 min before ovalbumin, also reduced significantly the platelet-mediated antigen-induced leucopenia (Figure 7).

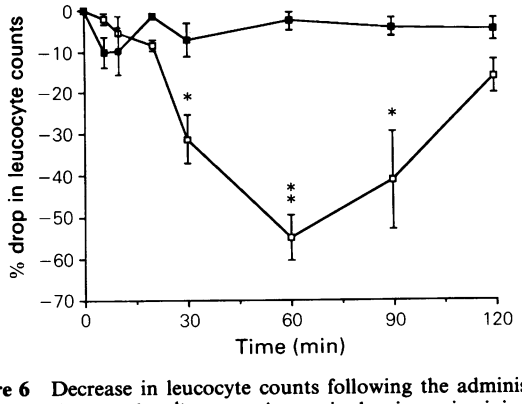

Figure 6 Decrease in leucocyte counts following the administration of ovalbumin (1 mg kg^{-1}) to non-immunised guinea-pigs injected the day before the experiment with 5 × 10^6 platelets from actively sensitized animals and the effect of BW 755C (10 mg kg^{-1} i.v., 5 min before the antigen challenge). The baseline circulating leucocyte numbers in control and BW 755C-treated guinea-pigs were 7360 ± 809 and 7840 ± 1294 leucocytes μl^{-1}, respectively (difference not statistically significant). Results are expressed as mean of 4 experiments; vertical lines show s.e.mean. (□) Saline-injected guinea-pigs; (■) BW 755C-injected guinea-pigs. *P < 0.05, **P < 0.01.

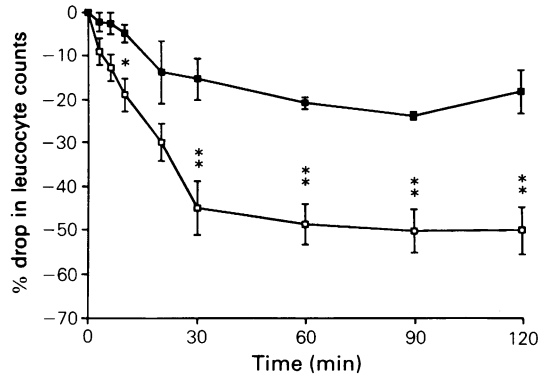

Figure 7 Decrease in leucocyte counts following the administration of ovalbumin (1 mg kg^{-1}) to non-immunised guinea-pigs injected the day before the experiment with 5×10^6 platelets from actively sensitized animals and the effect of nedocromil sodium (100 mg kg^{-1} i.v.) 5 min before the antigen challenge. The baseline circulating leucocyte numbers in control and nedocromil sodium-treated guinea-pigs were 8150 ± 838 and 7795 ± 878 leucocytes μl^{-1}, respectively (difference not statistically significant). Results are expressed as mean of 4 experiments; vertical lines show s.e.mean. (□) Saline-injected guinea-pigs; (■) nedocromil sodium-injected guinea-pigs. $^*P < 0.05$, $^{**}P < 0.01$.

Discussion

For many years platelets have been implicated only in haemostasis and thrombosis. More recently, their role in inflammation was established, particularly since it was shown that they respond to the appropriate stimuli by releasing substances which affect the pulmonary function. Thus, Joseph *et al.* (1986) and Karas *et al.* (1982) showed that platelets bear both IgE and IgG receptors, confirming their ability to respond to antigen stimulation and implicating platelets in allergic reactions.

More support for the concept of a role of platelets in immune and hypersensitivity reactions was provided by Capron *et al.* (1987), who demonstrated that platelets from asthmatic subjects as well as from *Hymenoptera* venom-sensitive patients are activated by a mechanism dependent upon IgE. Indeed, IgE-bearing platelets release cytocidal mediators and demonstrate an oxidative burst when stimulated with the specific antigen. The *in vivo* relevance of these *in vitro* observations has been established by Auriault *et al.* (1987), who obtained a significant protection against an infection induced by schistosomes after intravenous passive transfer of platelets from immunised to naïve rats.

In this study, we have demonstrated that the i.v. administration of antigen to non-sensitized guinea-pigs, which had previously been injected with platelets from actively immunised animals, induces a marked leucopenia. This indicates that, despite repeated washing, sensitized platelets keep their ability to respond to antigen challenge once transferred to a recipient non-immunised animal. The long-lasting leucopenia observed following ovalbumin administration suggests that platelets release a material which activates polymorphonuclear leucocytes. Release of this material is not secretion-dependent, since no platelet aggregation (thrombocytopenia and platelet accumulation) accompanied leucopenia. This probably rules out substances from the platelet α granules, such as platelet derived growth factor (PDGF) or platelet factor 4 (PF4). In addition, when platelets were washed in the presence of aspirin, the time-course and the intensity of leucopenia were unaffected, indicating that the antigen challenge did not trigger the activation of the cyclo-oxygenase pathway and the subsequent release of prostanoids from platelets.

Leucopenia was increased when the recipient animals were treated with indomethacin. This suggests that the inhibition of the biosynthesis of cyclo-oxygenase metabolites promotes alternative mechanisms enhancing the antigen-induced leucopenia. When non-immunised guinea-pigs were treated with the mixed cyclo-oxygenase and lipoxygenase inhibitor BW 755C, the decrease in leucocyte counts observed upon ovalbumin administration was suppressed. As the use of indomethacin had shown that prostanoids were not involved in this phenomenon, the anti-cyclo-oxygenase component does not account for the inhibition by BW 755C. Accordingly, it is likely that lipoxygenase metabolites are involved in platelet-mediated leucopenia following antigen challenge. It has been established that platelets possess a lipoxygenase which transforms the unstable metabolite 12-hydroperoxy-eicosatetraenoic acid (12-HPETE) into 12-hydroxyeicosatetraenoic acid (12-HETE), the latter being a chemotactic agent for neutrophils and eosinophils (Marcus *et al.*, 1984). In addition, the co-incubation of platelets and leucocytes in the presence of arachidonic acid induced the release of 12-HPETE from platelets which, in turn, activated the 5-lipoxygenase of leucocytes leading to the production of 5-HETE (Maclouf *et al.*, 1982).

Nedocromil sodium is the disodium salt of a pyranoquinoline dicarboxylic acid which has been shown to impair mediator release from various inflammatory cells implicated in allergic reactions (Eady, 1986). In our experimental conditions, nedocromil sodium significantly inhibited antigen-induced leucopenia in non-immunised guinea-pigs injected with platelets from actively sensitized animals. This effect might result from the interference by nedocromil sodium with the lipoxygenase metabolism, since it has previously been shown to inhibit the antigen-induced release of leukotrienes from bronchoalveolar mast cells (Wells *et al.*, 1986) and from alveolar macrophages from asthmatics (Godard, 1989). Moreover, nedocromil sodium markedly inhibited cytotoxicity against shistosomula of normal platelets incubated in the presence of IgE-rich serum from asthmatic patients and successively challenged with the specific anti-IgE (Thorel *et al.*, 1988).

In conclusion, our results demonstrate that platelets may play an important role in immediate hypersensitivity reactions, particularly leucopenia, a phenomenon not recognized earlier because of the non-involvement of classical secretion pathways. Since this platelet-leucocyte interaction can be modulated by drugs, particularly by nedocromil sodium, it may be an important target for molecules with anti-allergic activity.

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Effects of various neuropeptide Y/peptide YY fragments on electrically-evoked contractions of the rat vas deferens

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1 The effects of various neuropeptide Y (NPY) and peptide YY (PYY) fragments on electrically-evoked twitches in the rat isolated vas deferens were studied and compared with the effects of full length NPY and PYY. The aim was to identify the shortest NPY/PYY fragments that are capable of suppressing the contractions.

2 NPY (1–36) and C-terminal fragments of NPY (from 11–36 to 22–36) suppressed the electrically-evoked twitches in a concentration-dependent manner. On the whole there seemed to be a gradual lowering of the pIC_{50} values with progressive shortening of the NPY fragments (except for fragments 16–36 and 22–36 that had rather high pIC_{50} values). NPY 23–36, 24–36 and 25–36 suppressed the twitches at high concentrations (3 μM). NPY 26–36 was without effect as were C-terminal carboxy-deaminated NPY and glycine extended NPY (NPY-Gly-Lys-Arg).

3 PYY (1–36) and C-terminal fragments of PYY (from 11–36 to 23–36) suppressed the electrically-evoked twitches in a concentration-dependent manner. PYY 1–36 was more potent than any of the fragments. There was a tendency for shorter fragments to have lower pIC_{50} values. PYY 24–36 and 25–36 suppressed the twitches at high concentrations (3 μM). PYY 26–36 was without effect.

4 The findings suggest that the 12 C-terminal amino acid residues of NPY and PYY are the minimum length required to activate the Y_2-receptor.

Introduction

Neuropeptide Y (NPY) and peptide YY (PYY) are 36-amino acid peptides that belong to the pancreatic polypeptide family (Tatemoto *et al.*, 1982). NPY is widely distributed in the peripheral nervous system and is co-localized with noradrenaline (NA) in many sympathetic fibres (Lundberg *et al.*, 1982b), notably around blood vessels (Uddman *et al.*, 1985). PYY in contrast occurs mainly in endocrine cells in the gut (Lundberg *et al.*, 1982a; Böttcher *et al.*, 1984).

NPY has been attributed a variety of functional roles (for a review see Edvinsson *et al.*, 1987). It is known to cause vasoconstriction and to enhance vasoconstriction induced by exogenous and endogenous NA. These effects are mediated via receptors (tentatively referred to as Y_1-receptors) that require the whole NPY/PYY 36-amino acid molecule (Wahlestedt *et al.*, 1986). PYY and NPY are on the whole equipotent, whereas NPY 2–36 appears to be markedly less potent (Rioux *et al.*, 1986). Moreover, NPY can inhibit the electrically-stimulated release of NA from sympathetic fibres in the vas deferens (Lundberg & Stjärne, 1984) and in certain blood vessels (Dahlöf *et al.*, 1985). This effect is mediated via Y_2-receptors which recognize not only full length NPY/PYY but also C-terminal fragments of NPY and PYY (Wahlestedt *et al.*, 1986).

The aim of the present study was to identify the shortest NPY/PYY fragments that can suppress electrically-evoked twitches in the rat vas deferens.

Methods

Male Sprague-Dawley rats (200–250 g) were killed by a blow on the neck and exsanguinated. The prostatic segment of each vas deferens was cut out and placed in Krebs solution of the following composition (mM): NaCl 133, $NaHCO_3$ 16.3, KCl 4.7, $MgCl_2$ 1.0, NaH_2PO_4 1.4, $CaCl_2$ 2.5' and glucose 7.8. Segments 1.5 cm long were mounted vertically on Perspex holders in a 7 ml tissue bath maintained at 33°C. The mechanical activity was recorded isometrically by a Grass FT03 force displacement transducer and a Grass model 7 polygraph. Before the start of each experiment the preparations were allowed to equilibrate for 60 min with a tension of about 10 mN. Electrical field stimulation with square wave pulses (25 V, 1 ms and 0.15 Hz) was applied through a pair of platinum electrodes connected to a Grass S4C stimulator. As soon as a series of pulses gave identical responses, a peptide was added in a cumulative manner. Concentration-response curves were constructed in the concentration range 1 nM–3 μM. The resulting inhibition of the twitch was calculated for each concentration of peptide and expressed as a percentage. The pIC_{50} values (the negative logarithm of the concentration that leaves 50% of the twitch) were estimated by linear regression analysis of the results in the 10–90% response interval. Statistical analyses of the pIC_{50} values were performed by means of unpaired Student's t test. The results are expressed as mean values ± s.e.mean; n = number of experiments.

Drugs

NPY 1–36, 16–36, 20–36, 22–36, 26–36, desamido-NPY (NPY free acid) and PYY 1–36 were purchased from Peninsula, UK. NPY 23–36, 24–36, 25–36, and PYY 23–36, 24–36, 25–36, 26–36 were synthesized by solid-phase synthesis and purified to at least 96% by h.p.l.c. (Dr H. Franzén, Dept. of Medical Chemistry, University of Lund, Sweden). NPY 11–36, 12–36, 13–36, 14–36, 15–36; PYY 11–36, 12–36, 13–36, 14–36 and 15–36 were synthesized by solid-phase synthesis and purified to at least 96% by h.p.l.c. (W. Krzeminsky, Ferring AB, Malmö, Sweden). NPY 17–36 was a gift from Dr N. Yanaihara, Shizouka, Japan. NPY-Gly-Lys-Arg was a gift from Dr. T. Bartfai, Stockholm, Sweden. All peptides were dissolved in and diluted with 0.9% saline.

Results

NPY 1–36 and truncated NPY fragments from 11–36 to 25–36 suppressed the electrically-evoked twitches of the vas deferens; NPY 26–36 was inactive (Figure 1). With NPY 1–36 and fragments longer than 23–36 the effect was concentration-dependent (Figure 2; Table 1). NPY 1–36 was more potent than all fragments except NPY 16–36, which displayed similar

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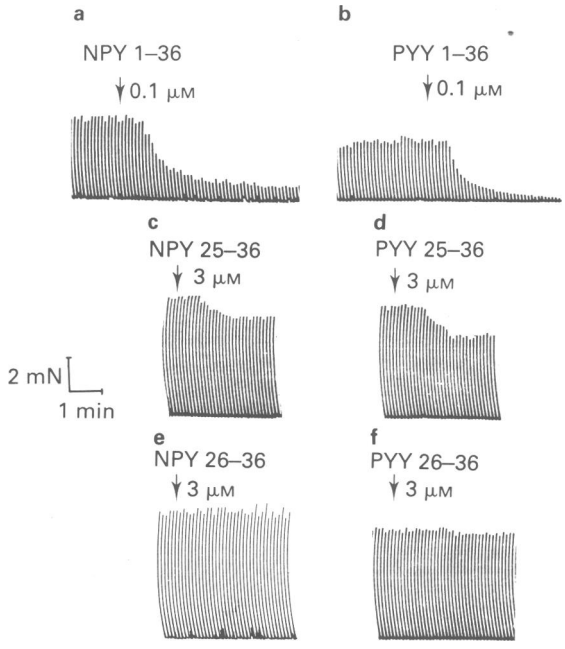

Figure 1 Tracings showing the effects of (a) neuropeptide Y (NPY) 1–36, (b) peptide YY (PYY) 1–36, (c) NPY 25–36, (d) PYY 25–36, (e) NPY 26–36 and (f) PYY 26–36 on electrically-evoked twitches in the isolated vas deferens of the rat.

potency (Table 1). However, on the whole, there seemed to be a gradual lowering of the pIC_{50} values with progressive shortening of the NPY fragments (except for fragments 16–36 and 22–36 that had unexpectedly high pIC_{50} values). High concentrations (3 μM) of NPY 23–36, 24–36 and 25–36 suppressed the twitches by 11.1 ± 3.38% ($n = 8$), 23.4 ± 5.44% ($n = 6$) and 12.2 ± 4.75% ($n = 4$), respectively. In contrast, desamido-NPY ($n = 4$) and the glycine-extended 39 amino acid peptide NPY-Gly-Lys-Arg ($n = 6$) failed to reduce the twitches even at high concentrations (3 μM). PYY 1–36 and truncated fragments of PYY from 11–36 to 25–36 suppressed the electrically-evoked twitches of the vas deferens; PYY 26–36 was inactive (Figure 1). With PYY 1–36 and fragments longer than 24–36 the effect was concentration-dependent (Figure 2; Table 1). PYY 1–36 was somewhat more potent than NPY 1–36, $P < 0.01$. There was a tendency for the shorter PYY fragments to have progressively lower pIC_{50} values, and PYY 1–36 was more potent than any of these fragments (Table 1). PYY 24–36 and PYY 25–36 (3 μM) suppressed the twitches by 19.2 ± 3.63% ($n = 6$) and 32.1 ± 3.20% ($n = 4$), respectively.

Table 1 Ability of neuropeptide Y (NPY) and peptide YY (PYY) and C-terminal fragments of NPY/PYY to suppress the electrically-evoked twitches of rat vas deferens

Peptide	*Inhibitory effect*	*pIC_{50}*	n	P
NPY 1–36	+	7.77 ± 0.09	4	—
NPY 11–36	+	7.15 ± 0.47	4	<0.05
NPY 12–36	+	6.35 ± 0.30	4	<0.001
NPY 13–36	+	6.79 ± 0.24	4	<0.001
NPY 14–36	+	6.43 ± 0.36	8	<0.001
NPY 15–36	+	6.43 ± 0.22	4	<0.001
NPY 16–36	+	7.36 ± 0.20	4	NS
NPY 17–36	+	5.78 ± 0.05	4	<0.001
NPY 20–36	+	5.85 ± 0.14	4	<0.001
NPY 22–36	+	6.22 ± 0.14	8	<0.001
NPY 23–36	(+)		8	
NPY 24–36	(+)		6	
NPY 25–36	(+)		4	
NPY 26–36	0		4	
PYY 1–36	+	8.27 ± 0.04	4	—
PYY 11–36	+	7.32 ± 0.08	4	<0.01
PYY 12–36	+	7.06 ± 0.20	6	<0.001
PYY 13–36	+	6.35 ± 0.26	4	<0.001
PYY 14–36	+	6.58 ± 0.17	4	<0.001
PYY 15–36	+	7.14 ± 0.11	6	<0.001
PYY 23–36	+	6.48 ± 0.09	4	<0.001
PYY 24–36	(+)		6	
PYY 25–36	(+)		4	
PYY 26–36	0		4	

Data shown are means ± s.e.mean.
P, the statistical significance of the difference between pIC_{50} values for NPY 1–36 or PYY 1–36 on the one hand and for the respective fragments on the other. NS = not significant. +, inhibition 100% or close to 100% can be achieved. (+), the inhibitory effect is not close to 100% even at a concentration of 3 μM and hence pIC_{50} cannot be calculated. 0, no inhibitory effect.

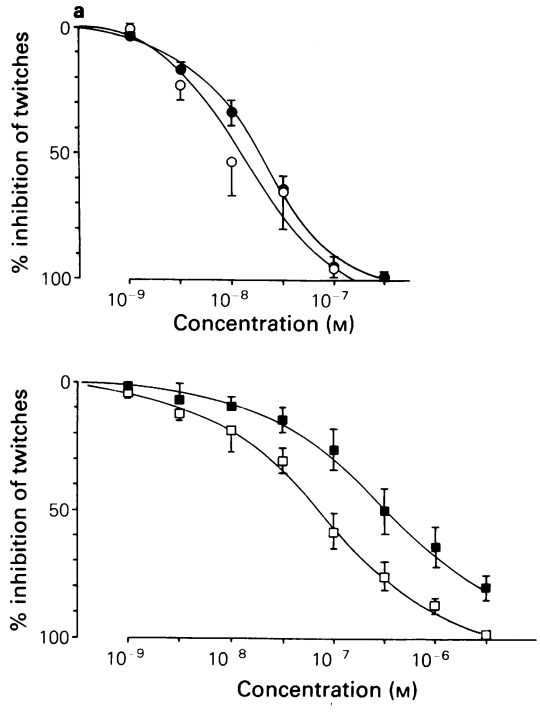

Figure 2 Concentration-response curves illustrating the suppression of electrically-evoked twitches of rat vas deferens in response to increasing concentrations of neuropeptide Y (NPY) 1–36 (●), peptide YY (PYY) 1–36 (○) (a) and of NPY 15–36 (■), PYY 15–36 (□) (b). Each point represents the mean and vertical lines indicate s.e.mean.

Discussion

NPY and PYY display significant sequence homology (Table 2). In this study, both NPY 1–36 and NPY fragments (from 11–36 to 22–36) suppressed the electrically-evoked twitches in the vas deferens in a concentration-dependent manner. This was also the case for PYY 1–36 and the PYY fragments from 11–36 to 15–36 as well as PYY 23–36. NPY 1–36 and PYY 1–36 were more potent than their respective fragments, except NPY 16–36 which displayed a potency similar to that of NPY 1–36. NPY 23–36, 24–36, 25–36 and PYY 24–36, 25–36 suppressed the twitches at high concentrations, but neither NPY 26–36 nor PYY 26–36 had any effect. This indicates that the 12 C-terminal amino acids represent the minimum length required for activating the Y_2-receptor. Interestingly, PYY 23–36 was more potent than NPY 23–36. The N-terminus of PYY 23–36 is serine. NPY 22–36 which was almost as effective as PYY 23–36 also starts with serine, suggesting that serine in this position may play an important role. The residues 15–31 of NPY and PYY contain a basic hydrophilic sequence, the so called PP-fold, and the C-terminal 32–36 pentapeptide sequence forms a hydrophobic tail (Glover *et al.*, 1985). Both these parts are highly conserved among the

Table 2 Amino acid sequences of porcine neuropeptide Y (NPY) and peptide YY (PYY)

	1 5 10 15 20 25 30 35
NPY porcine	Y-P-S-K-P-D-N-P-G-E-D-A-P-A-E-D-L-A-R-Y-Y-S-A-L-R-H-Y-I-N-L-I-T-R-Q-R-Y-NH_2
PYY porcine	Y-P-A-K-P-E-A-P-G-E-D-A-S-P-E-D-L-S-R-Y-Y-A-S-L-R-H-Y-L-N-L-V-T-R-Q-R-Y-NH_2

Identical residues are underlined.

members of the PP family (Glover *et al.*, 1985). Recently, a potent NPY agonist was synthesized by linking NPY 1–4 via epsilon-aminocapronic acid to the C-terminal peptide amide segment 25–36 (Beck *et al.*, 1989). This novel peptide excites both Y_1- and Y_2-receptors *in vivo* as well as *in vitro*.

It has been claimed that the amidated C-terminal of NPY/PYY is crucial for maintaining biological activity (Wahlestedt *et al.*, 1986). In this study, we confirmed that both desamido-NPY and NPY-Gly-Lys-Arg, the glycine-extended 39 amino acid precursor of NPY, are inactive.

In conclusion, the results of this study suggest that C-terminal fragments of NPY (from 11–36 to 22–36) and PYY (from 11–36 to 23–36) are fairly potent agonists, acting upon Y_2-receptors to suppress electrically-evoked twitches in the isolated vas deferens of the rat. Shorter fragments (NPY 23–36, 24–36, 25–36 and PYY 24–36, 25–36) are active only at very high concentrations. It appears that to be activated the Y_2-receptor requires the 12 C-terminal amino acid residues.

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