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Impairment of nitrergic-mediated relaxation of rat isolated duodenum by experimental diabetes

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1 Diabetes mellitus is associated with changes in gastrointestinal motility. The effects of experimental diabetes, induced by streptozotocin administration to rats 3-4 weeks previously, on the nitric oxide (NO)-mediated (nitrergic) relaxation of the duodenum have now been investigated.

2 The non-adrenergic, non-cholinergic (NANC) relaxation of the isolated duodenum induced by nicotine (0.3-10 μM) or the nicotinic agonist, 1,1-dimethyl-4-phenylpiperazinium (DMPP; 10 μM) was inhibited by the NO synthase inhibitor, N^G-nitro-L-arginine (3-100 μM).

3 This nitrergic relaxation induced by nicotine or DMPP of the duodenum from diabetic rats was substantially smaller than that of the tissue from control rats.

4 By contrast, the relaxation of the duodenum from diabetic rats to the NO donor, nitroprusside (0.3-10 μM) was similar to that of control tissue, whereas the relaxation to ATP (0.1-3 μM) was enhanced to a small but significant degree.

5 Incubation of duodenal tissue from control rats at 4°C for 72 h, which leads to neuronal disruption, significantly attenuated the relaxation to nicotine or DMPP whereas the relaxation induced by nitroprusside or ATP was not affected. Comparable cold-storage did not affect the endothelium-dependent relaxation of rat aortic rings induced by acetylcholine (0.01-2 μM).

6 The calcium-dependent NO synthase activity in duodenal tissue, determined by the conversion of radiolabelled L-arginine to citrulline, was significantly reduced in cold-stored tissue and in tissue obtained from diabetic rats.

7 These findings in the rat duodenum indicate that a reduction in intestinal NO synthase activity is associated with an impairment of the NANC relaxation. A defect in the intestinal nitrergic innervation could thus contribute to the motility dysfunction observed in diabetes.

Keywords: Nitric oxide; nicotine; neuronally-induced relaxation; non-adrenergic, non-cholinergic; nitrergic system; intestinal motility; diabetes; cold storage; NO synthase; duodenum; intestinal smooth muscle

Introduction

Nitric oxide (NO) is synthesized from L-arginine by calcium-dependent constitutive NO synthase in endothelial cells and neuronal tissue (Moncada *et al.*, 1991). Based on the use of inhibitors of NO synthase (Rees *et al.*, 1990; Moore *et al.*, 1990) such as N^G-monomethyl L-arginine (L-NMMA) or N^G-nitroarginine (L-NOARG), NO has been proposed as a mediator of the non-adrenergic, non-cholinergic (NANC) relaxation of the intestinal smooth muscle, including that of rat or guinea-pig stomach and duodenum and the canine ileo-cecal juction and duodenum (Li & Rand, 1990; Bult *et al.*, 1990; Toda *et al.*, 1990; Desai *et al.*, 1991; Irie *et al.*, 1991). In addition, NO synthase has been located in the myenteric plexus and other neuronal structures of the rat gastro-intestinal tract (Bredt *et al.*, 1990; Belai *et al.*, 1992), although the contribution of the nitrergic neuronal enzyme to the overall NO synthase activity in the gastrointestinal tissues is not yet fully clear (Whittle *et al.*, 1992; Grider *et al.*, 1992; Sanders & Ward, 1992; Tepperman *et al.*, 1993).

Abnormalities of gastrointestinal motility are common in diabetes mellitus (Chesta *et al.*, 1990; Iber *et al.*, 1993; Yang *et al.*, 1984) and have been associated with degenerative changes in the peripheral nervous system. Alterations in the myogenic actions of different agonists in the intestinal smooth muscle from animal models with experimental diabetes have been found (Mathison & Davison, 1988; Lucas & Sardar, 1991). Furthermore, morphological and histological abnormalities occur in the intestinal myenteric neurones in the diabetic state, with alterations in the adrenergic, cholinergic, 5-hydroxytryptaminergic and peptidergic systems (Lincoln *et al.*, 1984; Ballmann & Conlon, 1985; Belai *et al.*, 1985; Bailey *et al.*, 1986; Nowak *et al.*, 1986; Belai & Burnstock, 1987; Lucas & Sardar, 1991).

It is also possible that the gut motility disorders of diabetics are associated with dysfunction in the nitrergic innervation in the gastro-intestinal tract. Thus, in the present study, the effects of the experimental diabetes induced by pretreatment with streptozotocin 3 weeks prior to study, have been evaluated on the nitrergic-mediated responses induced by nicotine in the rat isolated duodenum (Irie *et al.*, 1991). These responses of duodenal tissue from diabetic rats have also been compared to those resulting from denervation of the duodenum induced by cold storage treatment of the freshly removed control tissue (Holman & Hughes, 1965; Hattori *et al.*, 1972). In addition, the NO synthase activity in duodenal tissue from control and diabetic rats has been compared to that in cold stored tissues.

Preliminary accounts of this work have been presented in abstract form (Whittle *et al.*, 1993; Martinez-Cuesta *et al.*, 1994).

Methods

Tissue preparation and isometric tension recording

Segments (2.5 cm) of proximal duodenum were removed from male Wistar rats (250-300 g) after exsanguination. Two duodenal segments from each rat were rinsed and were set up in organ baths containing 25 ml of modified Krebs solution (composition in mM; NaCl 118, KCl 4.8, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 1.25, $NaHCO_3$ 24 and glucose 11). The

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solution was maintained at 32°C and aerated with a mixture of 95% O_2 and 5% CO_2. Mechanical activity of the duodenal segments, under a resting tension of 1 g, was measured isometrically with a Grass FT-03 transducer connected to a Lectromed MT 8P coupler and Rikadenki R-64 recorder.

In some experiments the duodenal tissues were obtained from diabetic rats. Rats were starved in grid-bottomed cages for 24 h, and diabetes was induced by a single intraperitoneal injection of streptozotocin (70 mg kg^{-1}), administered 3–4 weeks before the study. Age-matched control animals received vehicle alone. The blood glucose levels were measured by a reflectance photometer, and rats were characterized as diabetic by a blood glucose level >12.0 mmol l^{-1}.

For cold-storage treatment, the duodenal tissues removed from control rats were stored in Krebs solution at 4°C for 72 h without additional oxygenation. On the day of experiment, the tissues were slowly prewarmed to 32°C and then mounted in an organ bath and treated identically to the fresh preparations from control rats.

The effects of cold-storage (at 4°C for 72 h) on the response to acetylcholine of endothelium-intact rings (5 × 2 mm) from thoracic aorta of control rats were also evaluated. In these studies, the freshly removed or cold-stored aortic tissue was suspended in Krebs solution containing indomethacin (5 μM) under an initial tension of 2 g which was reduced to 1 g after a 60 min equilibration period.

Experimental protocols

After an equilibration period of 1 h, the preparations were exposed 2–3 times for 3 min periods followed by washing, at 30 min intervals, to a single concentration of nicotine (0.3–10 μM) or 1,1-dimethyl-4-phenylpiperazinium (DMPP, 1–10 μM) until a reproducible response was obtained. To characterize the nature of this relaxation, tissues were incubated for 15 min with atropine (1 μM), phentolamine (1 μM), propranolol (1 μM), guanethidine (6 μM) or hexamethonium (100 μM), prior to the exposure to nicotine. Separate tissues were incubated with N^G-nitro-L-arginine (L-NOARG, 3–100 μM), 15 min prior to nicotine (10 μM) or DMPP (10 μM) and in further studies L-arginine (1 mM) or its enantiomer D-arginine (1 mM) was added to the organ bath 5 min prior to L-NOARG (30 μM).

The responses to nicotine (0.3, 1 and 3 μM), DMPP (10 μM), the NO-donor nitroprusside (0.3–10 μM) or adenosine 5′-triphosphate (ATP, 0.1, 1 and 3 μM) were also investigated in duodenal segments obtained from control and diabetic rats.

The effect of cold-storage (72 h at 4°C) on the response of the duodenal tissues to L-NOARG (30 μM), nicotine (3 μM), DMPP (10 μM), ATP (1 μM) and nitroprusside (10 μM) was also investigated. In further studies, control and cold-stored (72 h at 4°C) aortic rings were precontracted with phenylephrine (0.25 μM) prior to evaluation of the endothelium-dependent relaxation induced by the cumulative addition of acetylcholine (0.01–2 μM).

Nitric oxide synthase activity

In the duodenal tissues obtained from control or diabetic rats or after cold-storage treatment, NO synthase activity was measured as the conversion of L-[^{14}C]-arginine monohydrochloride to [^{14}C]-citrulline as described before (Tepperman *et al.*, 1993). Intestinal tissue was homogenized in buffer containing DL-dithiothreitol (1 mM), leupeptin (10 μg ml^{-1}), soybean trypsin inhibitor (10 μg ml^{-1}) and aprotonin (2 μg ml^{-1}). Following centrifugation (10 000 *g*, 20 min, 4°C), the supernatant was added to buffer (pH 7.4) containing (final concentration) L-valine (6 mM); NADPH (100 μM); $MgCl_2$ (1 mM) and $CaCl_2$ (200 μM), L-arginine (20 μM) and L-[^{14}C]-arginine monohydrochloride (0.271 μCi, 11.8 GBq $nmol^{-1}$) and incubated for 10 min at 37°C. The reaction was terminated by the addition of Dowex-AG50W.

Product formation that was abolished by *in vitro* incubation with L-NOARG (300 μM) was taken as an index of NO synthase activity and calculated from the total of added substrate, as the formation of citrulline, nmol min^{-1} g^{-1} of tissue. The activity of NO synthase in the intestinal tissue was further characterized *in vitro* by incubation with EGTA (1 mM) to determine the dependence of the enzymic activity on calcium, the calcium-dependent activity being taken as constitutive NO synthase.

Drugs

Acetylcholine chloride, L-arginine hydrochloride, D-arginine hydrochloride, atropine sulphate, adenosine 5′-triphosphate (ATP), 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), guanethidine monosulphate hexamethonium bromide, nicotine hydrogen tartrate, sodium nitroprusside, N^G-nitro-L-arginine (L-NOARG), phentolamine hydrochloride, phenylephrine hydrochloride, propranolol hydrochloride and streptozotocin were obtained from Sigma Chemical Company (Poole, Dorset, U.K.). These compounds were dissolved in isotonic saline, except streptozotocin which was dissolved in 0.01 M citrate buffer at pH 4.5. L-[U-^{14}C]-arginine monohydrochloride was obtained from Amersham International (U.K.). All other reagents were from the Sigma Chemical Company.

Statistical analysis

The data were expressed as mean ± s.e.mean where (n) indicates the numbers of rats. Student's t test for paired and unpaired observations was used as appropriate, where P values of less than 0.05 were considered to be significant.

Results

Characterization of the response to nicotine

Nicotine (0.3–10 μM) or DMPP (1–10 μM) induced a rapid and concentration-dependent relaxation of the duodenal tissues which was sustained for 1 or 2 min. The near-maximal relaxation induced by nicotine (3 μM; −447 ± 63 mg tension, $n = 12$) was not significantly modified by the pretreatment of the duodenal tissues with atropine (1 μM) nor by incubation with phentolamine (1 μM) in combination with 1 μM propranolol or by guanethidine (6 μM) ($n = 5$ for each; data not shown) but was significantly reduced by 100 μM hexamethonium (to −60 ± 36 mg tension, $n = 8$, $P < 0.01$).

Effect of N^G-nitro-L-arginine on response to nicotine and DMPP

The relaxation of the duodenal segments induced by submaximal doses of nicotine (1 and 3 μM) was abolished by L-NOARG (100 μM; $n = 5$ for each, $P < 0.001$; data not shown). The maximal relaxation induced by nicotine (10 μM) was antagonized in a concentration-dependent manner by incubation with L-NOARG (3, 10 and 100 μM), as was the relaxation induced by DMPP (10 μM) (Figure 1). The incubation of the duodenal tissues for 15 min with L-NOARG (30 μM) significantly ($P < 0.01$) reduced the relaxation induced by nicotine (10 μM) from −562 ± 30 mg to −244 ± 95 mg tension ($n = 8$). Pretreatment of the tissues with L-arginine (1 mM) significantly ($P < 0.01$) attenuated the inhibitory effect of L-NOARG (30 μM), the response to nicotine (10 μM) being reversed to −518 ± 51 mg tension ($n = 8$), whereas D-arginine (1 mM) had no such effect (−142 ± 87 mg tension, $n = 6$).

Effect of diabetes on responses to nicotine and DMPP

The duodenal tissues from diabetic rats showed a substantial reduction in the relaxation induced by nicotine (0.3, 1 and

3 μM), being inhibited by 98 ± 2%, 83 ± 10% and 80 ± 10% ($n = 7$, 10 and 9; $P < 0.001$) for each nicotine concentration respectively compared with the control. The relaxation to DMPP (10 μM) was likewise abolished in tissue from diabetic rats (Figure 2).

In contrast to the responses to nicotine or DMPP, the relaxation to ATP (0.1, 1 and 3 μM) was significantly ($P < 0.001$) increased in the tissue from diabetic rats compared with the control tissues, over the range of concentrations used (Figure 3a).

Nitroprusside (0.3–10 μM) likewise induced a concentration-dependent relaxation of duodenal tissue from control rats. In the tissue from diabetic rats, the relaxation to nitroprusside (0.3–10 μM) was not significantly different from that in the control tissue (Figure 3b).

Effect of cold-storage on responses to nicotine and DMPP

In duodenal tissue following cold-storage treatment, the relaxation to nicotine (3 μM) or DMPP (10 μM) was abolished (Figure 4). By contrast, the relaxation induced by ATP (1 μM) or nitroprusside (10 μM) in the cold-stored tissues was not significantly different from that determined in the control tissue (Figure 4).

The endothelium-dependent relaxation induced by acetylcholine (0.01–2 μM) of the aortic ring precontracted with phenylephrine (0.25 μM) was not significantly different from the relaxation obtained in cold-stored aortic tissue (4°C for 72 h) over the concentration-range studied. Thus, acetyl-

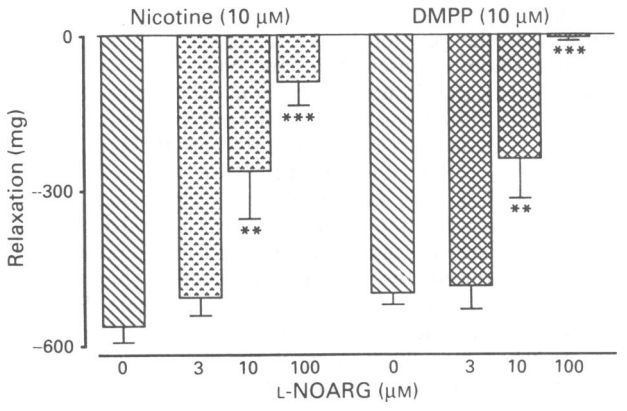

Figure 1 Effect of N^G-nitro-L-arginine (L-NOARG; 3–100 μM) on the relaxation induced by nicotine (10 μM; stippled column) or 1,1-dimethyl-4-phenylpiperazinium (DMPP; 10 μM; cross hatched column) of the isolated duodenum from control rats. The results, shown as the relaxation of the tissue (mg), are the means ± s.e.mean of at least 7 studies for each group. The significant differences from the respective nicotine or DMPP control responses (hatched columns) are shown as $^{**}P < 0.01$ and $^{***}P < 0.001$.

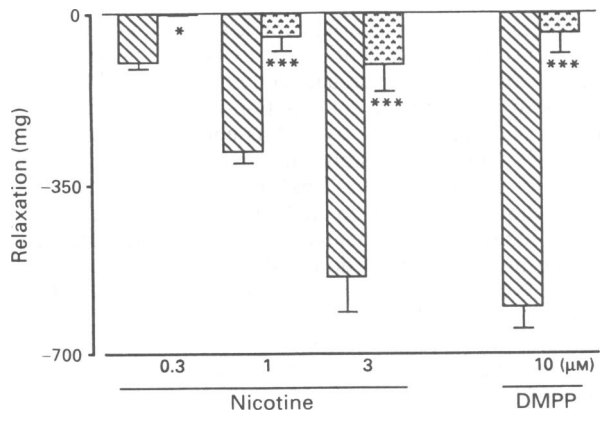

Figure 2 Relaxation induced by nicotine (0.3–3 μM) on 1,1-dimethyl-4-phenylpiperazinium (DMPP, 10 μM) in the isolated duodenum from control rats (hatched columns), or from streptozotocin-induced diabetic rats (stippled columns). The results, shown as the relaxation of the tissue (mg), are the mean ± s.e.mean of at least 8 studies for each group. The significant difference from the control tissues are shown as $^{*}P < 0.05$, $^{***}P < 0.001$.

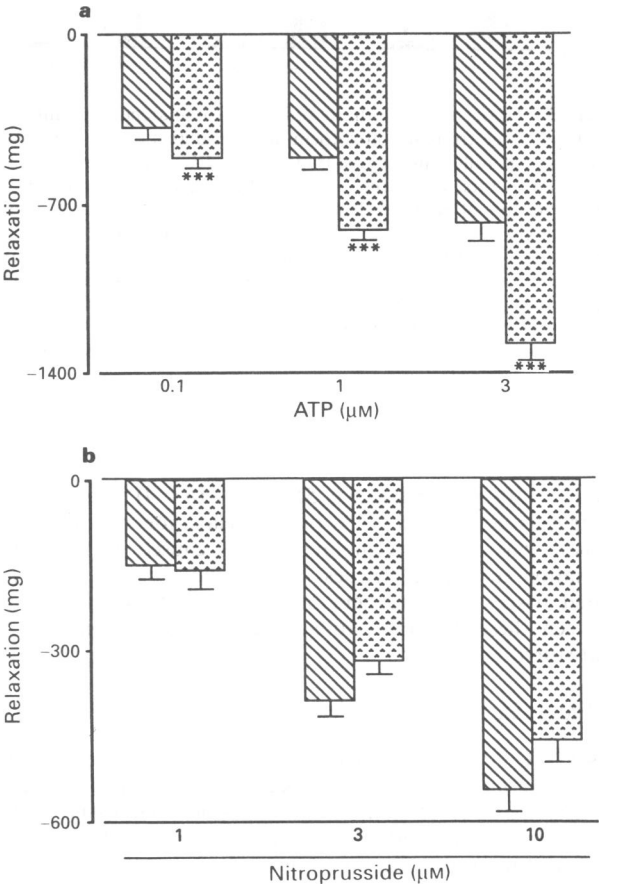

Figure 3 Relaxation induced by (a) adenosine 5-triphosphate (ATP, 0.1–3 μM) or (b) nitroprusside (0.3–10 μM) in the isolated duodenum from control (hatched columns) and streptozotocin-induced diabetic (stippled columns) rats. The results, shown as the relaxation of the tissue (mg), are the mean ± s.e.mean of at least 13 studies for each group, where *** shows significant differences ($P < 0.001$) from the control tissues.

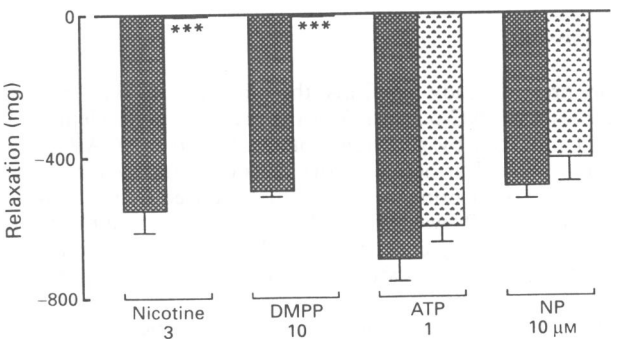

Figure 4 Effect of cold-storage (72 h, 4°C; stippled columns) of the rat isolated duodenum tissues on the relaxation induced by nicotine (3 μM), 1-1-dimethyl-4-phenylpiperazinium (DMPP, 10 μM), adenosine 5-triphosphate (ATP, 1 μM) or nitroprusside (NP, 10 μM). The results, shown as the relaxation of the tissue (mg), are the mean ± s.e.mean of at least 8 studies for each group, where *** shows the significant differences ($P < 0.001$) from the control tissues (cross hatched columns).

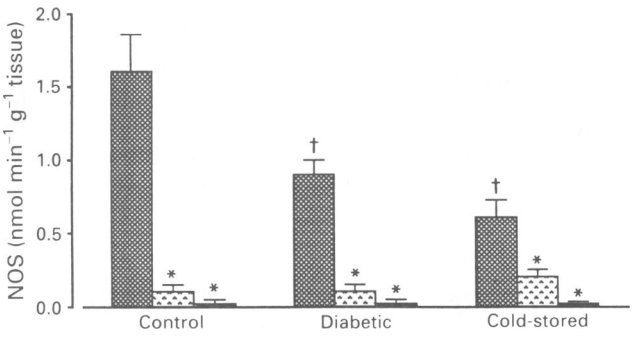

Figure 5 Nitric oxide synthase activity (cross-hatched columns) in duodenal tissue from streptozotocin-induced diabetic rats, from control rats and following cold-storage (72 h, 4°C) treatment of tissue from control rats. Constitutive NO synthase activity, determined as the conversion of radiolabelled L-arginine to citrulline (nmol min^{-1} g^{-1} tissue), was characterized by its inhibition *in vitro* by N^G-nitro-L-arginine (L-NOARG, 100 μM, stippled columns) or EGTA (1 mM, filled columns). NO synthase activity is expressed as the mean ± s.e.mean of at least 5 experiments, where a significant decrease in NO synthase activity from the control is given as $\dagger P < 0.001$, and significant inhibition by incubation with EGTA or L-NOARG is shown by $*P < 0.001$.

choline (0.08 μM) induced a 51 ± 13% ($n = 4$) relaxation in control tissues and 38 ± 6% ($n = 4$) relaxation in cold-stored tissues, whereas acetylcholine (1 μM) caused a 83 ± 10% relaxation in control tissues ($n = 4$) and 62 ± 6% in cold-stored tissues ($n = 4$), there being no significant differences between these responses to acetylcholine with control and cold-stored tissue.

Nitric oxide synthase activity

Basal NO synthase activity, that was abolished by the incubation *in vitro* with L-NOARG (300 μM), was detected in the supernatants of homogenates of duodenal tissues. This activity in the supernatants was abolished by incubation with EGTA (1 mM) as shown in Figure 5.

In duodenal tissues from diabetic rats or from cold-stored treatment, the basal NO synthase activity was significantly reduced compared with that observed in tissues freshly removed from control rats (by 43 ± 6% and by 64 ± 5% respectively; $P < 0.05$). As with control tissue, incubation of the supernatants of these tissues with L-NOARG (100 μM) near-maximally inhibited, and EGTA (1 mM) abolished, such activity (Figure 5).

Discussion

The present study confirms the observation that nicotine induces a NANC-relaxation of the rat isolated duodenum, an effect inhibited by ganglionic blockade and L-NOARG (Irie *et al.*, 1991). Furthermore, the relaxation induced by maximal doses of nicotine, as well as that induced by the nicotinic agonist, DMPP, was effectively inhibited by L-NOARG, an action reversed by concurrent incubation with L-arginine but not D-arginine, indicating the response to be essentially nitrergic in nature.

In duodenal tissue from rats made diabetic by treatment with streptozotocin, 3–4 weeks previously, however, the relaxation induced by nicotine or DMPP was substantially reduced. The effect was not a consequence of failure of the contractile elements of the duodenal smooth muscle from diabetic rats since the relaxation to ATP was not reduced. Indeed, as found with L-NOARG treatment of control tissue, the relaxation to ATP in diabetic tissue was significantly enhanced, perhaps reflecting the modulation of this response by endogenous NO. Moreover, the inhibition of the nicotine-induced relaxation from diabetic rats was not a result of diminished reactivity of the smooth muscle to NO, since relaxation induced by the NO-donor, nitroprusside was not significantly affected. This observation contrasts with recent findings on the mechanisms underlying the attenuated NANC-relaxation in the anococcygeus muscle of diabetic rats, where reduced responses to nitroprusside were observed (Way & Reid, 1994). The present findings therefore suggest an impairment in the pre-junctional neuronal synthesis or release of NO in the duodenum of diabetic rats.

In the vasculature, functional defects have been identified in the endothelial cells from diabetic rats. Thus, an attenuation of endothelium-dependent relaxation to acetylcholine has been noted in aortic and mesenteric tissue *in vitro* (Oyama *et al.*, 1986; Meraji *et al.*, 1987; Taylor *et al.*, 1992) and intestinal arterioles *in vivo* (Lash & Bohlen, 1991), as well as the vasodilator response to calcitonin gene-related peptide in the skin (Lawrence & Brain, 1992) of diabetic rats. These findings implicate a deficiency in the mechanisms underlying the endothelium-dependent NO-mediated vascular responses. However, the reduced vascular responses *in vitro* in diabetes have also been attributed to the local production of oxygen radicals limiting the activity of endogenous NO (Pieper *et al.*, 1992; Diederich *et al.*, 1994). Such observations on the endothelium, along with the current findings on neuronal mechanisms indicate a complex influence of diabetes on the physiological modulation of vascular and intestinal smooth muscle tone by NO. It is therefore of relevance that the failure of erection in diabetes (Saenz de Tejada *et al.*, 1989) has been associated with impairment of both NO-mediated neurogenic and the endothelium-mediated relaxation of penile smooth muscle, as observed *in vitro* (Azadozi & Saenz de Tejada, 1992; Rajfer *et al.*, 1992).

As with diabetes and incubation with L-NOARG, cold-storage of duodenal tissue at 4°C for 72 h suppressed the relaxation induced by nicotine or DMPP, but did not attenuate the relaxation to ATP or nitroprusside. Similarly, the relaxation of the guinea-pig ileum to nicotine or DMPP was abolished by cold storage of the tissue (Hattori *et al.*, 1972), as was the motor sympathetic response of the mouse anococcygeus muscle (Gibson *et al.*, 1992). The present observations are therefore likely to reflect the disruption of NO-containing neurones, rather than direct actions of cold storage on NO synthase. Indeed, a similar duration of cold-storage of aortic tissue did not impair the acetylcholine-induced relaxation, as observed previously over this time-period (Torok *et al.*, 1993).

Cold storage of duodenal tissue caused a significant reduction of NO synthase activity, determined as the conversion of radiolabelled L-arginine to the NO co-product, citrulline. This suggests a neuronal origin for a substantial component of the constitutive NO synthase activity in homogenates of duodenal tissue, the residual activity after cold storage presumably reflecting the contribution of other mucosal cells including endothelial cells, as well as epithelial cells (Tepperman *et al.*, 1993) or possibly intestinal smooth muscle cells (Grider *et al.*, 1992). Duodenal tissue from diabetic rats likewise showed a reduction in NO synthase activity, which thus correlates with the attenuation of the nitrergic relaxation following nicotinic stimulation and suggests a common link, involving NO, between the denervating effects of cold-storage and the actions of diabetes. The extensive neuronal destruction that follows cold-storage is not seen in the intestinal tissue from diabetic rats, although some damage to neuronal elements has been observed (Lincoln *et al.*, 1984; Belai & Burnstock, 1987). Whether the change in NO synthase activity in duodenal tissue could reflect down-regulation of neuronal NO synthase in the diabetic condition rather than neuronal damage, however, awaits detailed biochemical, molecular and histochemical evaluation.

These findings thus suggest that failure of the nitrergic modulation of intestinal tone, as demonstrated in the rat

isolated duodenum, may contribute to the pathogenesis of the gastrointestinal disturbances that are associated with diabetes.

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Role of endothelin-1 and the ET_A receptor in the maintenance of deoxycorticosterone acetate-salt-induced hypertension

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1 To search for a possible role for endothelin-1 (ET-1) in deoxycorticosterone acetate (DOCA)-salt-induced hypertension, we examined changes in concentration of ET-1 in vascular and renal tissue in DOCA-salt hypertensive rats and evaluated the antihypertensive effect of the ET_A receptor antagonist, FR139317.

2 There was an increase in aortic immunoreactive-ET (IR-ET) concentrations in association with hypertension-induced treatment. There were no significant changes in ET-1 levels in the kidney with DOCA-salt treatment.

3 In DOCA-salt hypertensive rats, a significant correlation ($r = 0.83$, $P < 0.01$) was found between aortic IR-ET concentrations and systolic blood pressure.

4 High-performance liquid chromatography analysis of the aortic extract from DOCA-salt rats revealed one major component corresponding to the elution position of synthetic ET-1.

5 The intravenous bolus injection of FR139317 (10 mg kg^{-1}) produced a slight decrease in blood pressure in the control rats and in the DOCA-salt hypertensive rat, FR139317 had a more pronounced hypotensive effect.

6 We propose that ET-1 production in vascular tissues is increased in DOCA-salt hypertensive rats. In addition, our study indicates the pathophysiological importance of increased endogenous ET-1 in the maintenance of DOCA-salt-induced hypertension, through interaction of the peptide with ET_A receptors.

Keywords: Endothelin-1 (ET-1); endothelin receptor; ET_A antagonist; deoxycorticosterone acetate-salt hypertension

Introduction

Endothelin-1 (ET-1) is the most potent endogenous vasoconstrictor substance so far identified (Yanagisawa *et al.*, 1988). This peptide possesses a wide variety of biological actions (Rubanyi & Botelho, 1991) and may play a role in the various cardiovascular disorders such as cerebral vasospasm after subarachnoid haemorrhage (Matsumura *et al.*, 1991), acute renal failure (Kon & Badr 1991), heart failure (Margulies *et al.*, 1990), atherosclerosis (Lerman *et al.*, 1991) and hypertension (Vanhoutte, 1993; Lüscher *et al.*, 1993). Circulating ET-1 concentrations are increased during hypertension (Kohno *et al.*, 1991; Widimsky *et al.*, 1991). However, circulating ET-1 levels do not reflect the local production of the peptide. Indeed, the change in ET-1 content of tissues occurs without change in circulating ET-1 levels (Hughes *et al.*, 1992; Larivière *et al.*, 1993).

One study found that there was an increased vascular content of ET-1, with no change in circulating ET-1 concentrations in deoxycorticosterone acetate (DOCA)-salt hypertensive rats (Larivière *et al.*, 1993). It has also been reported that renal ET-1 production is altered in spontaneous hypertensive rats (Kitamura *et al.*, 1989; Hughes *et al.*, 1992). Thus, these changes in local ET-1 production might participate in the development and/or the maintenance of hypertension in these models. However, the functional significance of increased or decreased local ET-1 levels in these hypertensive models remains obscure.

Several ET receptor antagonists have been developed and may be useful for evaluating physiological or pathophysiological roles of endogenous ET-1 and its receptor subtypes (Ihara *et al.*, 1992; Clozel *et al.*, 1993; Sogabe *et al.*, 1993). FR139317 ((R)2-[(R)-2-[(S)-2-[[1-hexahydro-1H-azepinyl-1H-indoyl)]propionyl]amino-3-(2-pyridyl)propionic acid) is an ET_A receptor antagonist which inhibits ET-1-induced vasoconstrictor effects *in vitro* and *in vivo* (Sogabe *et al.*, 1993). To explore the contribution of ET-1 in DOCA-salt-induced hypertension, we examined changes in ET-1 concentrations of vascular and renal tissues in DOCA-salt hypertensive rats and evaluated the antihypertensive effect of FR139317.

Methods

DOCA-salt treatment and blood pressure measurement

Male Sprague-Dawley rats, weighing 160–180 g were anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, i.p.) and the right kidney was removed via a right flank incision. After a 1-week postsurgical recovery period, the rats were treated twice weekly with deoxycorticosterone acetate (DOCA) suspended in corn oil, which was administered subcutaneously (15 mg kg^{-1}) and 1% NaCl added to their tap water for drinking. Control rats were uninephrecotomized but not given DOCA or salt. Systolic blood pressure was monitored with a tail cuff and a pneumatic pulse transducer. The rats were exsanguinated 1 or 4 weeks after treatment and concentrations of ET-1 were measured.

Tissue extraction and ET-1 measurement

ET-1 was extracted from the kidney, according to the method of Fujita *et al.* (1994). Briefly, kidneys were weighed and homogenized for 60 s in 8 vol ice-cold organic solution

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(chloroform/methanol, 2:1, including 1 mM N-ethylmaleimide). The homogenates were left overnight at 4°C, then 0.4 vol distilled water was added to the homogenates. In case of extraction from the aorta, thoracic aortae (4 cm) were removed from animals, rapidly cleaned of fat and adherent connective tissue, weighed and homogenized for 60 s in 4 ml ice-cold organic solution, as described above. The homogenates were left overnight at 4°C, then 0.4 ml of distilled water was added to the homogenate. Those aortic or renal homogenates were then centrifuged at 3000 r.p.m. for 30 min and the supernatant was stored. Aliquots of the supernatant were diluted 1/10 with a 0.09% trifluoroacetic acid (TFA) solution and applied to Sep-Pak C18 cartridges. The sample was eluted with 3 ml of 63.6% acetonitrile and 0.1% TFA. Eluates were dried in a centrifugal concentrator and the dried residue was reconstituted in assay buffer for radioimmunoassay (RIA). The clear solution was subjected to RIA. Recoveries of ET-1 from aorta and renal tissues in our extraction procedures were approximately 80%.

RIA for ET-1 was carried out as described elsewhere (Matsumura *et al.*, 1990b). The limit of detection of ET-1 in this assay was 3 pg/tube. ET-1 antiserum (a generous gift from Dr Marvin R. Brown, Department of Medicine, University of California, San Diego, U.S.A.) did not cross-react with big ET-1, as described (Hexum *et al.*, 1990).

Reverse-phase high-performance liquid chromatography

After application of the tissue sample to a Sep-Pak C18 cartridge, the dried residue was dissolved in 0.5 ml of 0.02% TFA instead of radioimmunoassay buffer and a 0.4 ml portion was then applied to a Capcell-Pak C18-SG300 column (4.6 × 250 mm, Shiseido, Tokyo, Japan), using a high-performance liquid chromatography (h.p.l.c.) system (model 600E, Waters Chromatography Division). Elution was performed with 0.02% TFA in water (solvent A) and 0.02% TFA in acetonitrile (solvent B). The gradient was linear from 0% to 35% v/v solvent B for 15 min, followed by isocratic elution at 35% v/v solvent B for 15 min and a linear gradient from 35% to 63% v/v solvent B for 15 min. The flow rate was 0.5 ml min^{-1}. Each fraction was evaporated and assayed for immunoreactive-ET (IR-ET) by RIA.

Effect of FR139317 (ET_A receptor antagonist)

Experiments were carried out on rats treated with DOCA-salt for 4 weeks and on age-matched control rats. The animals were anaesthetized with sodium thiobutabarbitone (Inactin, 100 mg kg^{-1}, i.p.) and placed on a heated surgical tray that maintained the rectal temperature between 37° and 38°C. After tracheotomy, the right femoral vein was cannulated for bolus injection of the drug. The right femoral artery was also cannulated for blood pressure measurement with a pressure transducer. After a 90 min equilibration period, FR139317 (10 mg kg^{-1}) or vehicle was administered intravenously by slow bolus injection (2 min). The doses of FR139317 used in this study have been shown to produce complete inhibition of ET-1-induced pressor action (Sogabe *et al.*, 1993). Blood pressure was recorded continuously on a polygraph (Nihon Koden, RM 6000G, Tokyo, Japan).

Statistical analysis

All values are expressed as mean ± s.e.mean and were analyzed statistically by an unpaired t test. Aortic IR-ET levels were correlated with systolic blood pressure by linear regression analysis. $P<0.05$ was considered significant.

Drugs

FR139317 was a kind gift from Fujisawa Pharmaceutical Co. Ltd., Osaka, Japan. FR139317 was dissolved in 1 N NaOH and diluted with saline. Other chemicals were purchased from Nacalai Tesque, Inc (Kyoto, Japan).

Results

Table 1 summarizes the comparative data on groups of animals treated with DOCA-salt for 7 or 28 days and of age-matched controls. There were no differences in systolic blood pressure of the control animals and those treated with DOCA-salt for 7 days. After 28 days of DOCA-salt treatment, systolic blood pressure was significantly elevated, compared with control animals (116 vs 187 mmHg). The increase in body weight of DOCA-salt rats was smaller than that in control rats. A significant difference between control and DOCA-salt rats with respect to aortic weight was seen at 28 days.

Figure 1a shows the change in aortic IR-ET concentrations in control and DOCA-salt rats. After 7 days of DOCA-salt treatment, a slight increase in aortic IR-ET content was observed compared with age-matched control rats. However, this increase was not statistically significant. After 28 days of DOCA-salt treatment, aortic IR-ET content was significantly higher than in the aged-matched control (2.23 ± 0.37 and 0.93 ± 0.10 ng g^{-1} aortic tissue). On the other hand, no significant changes occurred in renal IR-ET contents of the two experimental groups at 7 and 28 days (control rats; 0.20 ± 0.04 and 0.14 ± 0.01 ng g^{-1} tissue at 7 and 28 days, respectively vs DOCA-salt rats; 0.17 ± 0.02 and 0.13 ± 0.01 ng g^{-1} tissue at 7 and 28 days, respectively, Figure 1b). There was a positive correlation between systolic blood pressure and aortic IR-ET content in the DOCA-salt animals (Figure 2).

The dilution curve of aortic extract clearly revealed a parallel displacement with the standard curve (Figure 3a). To

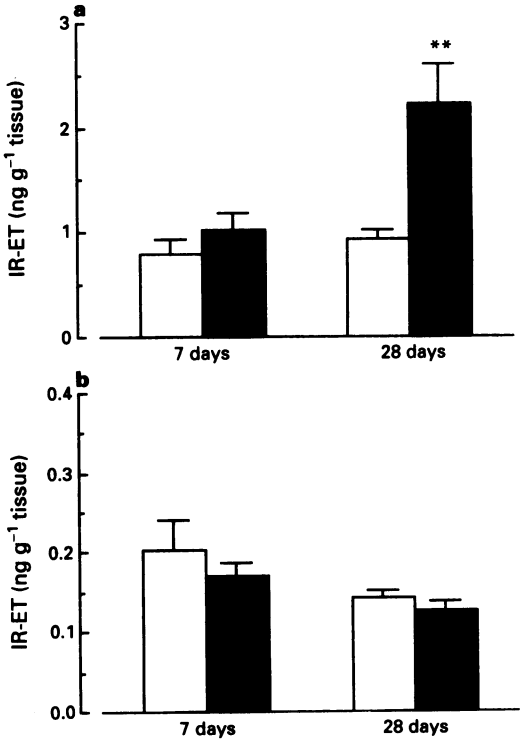

Figure 1 (a) Aortic immunoreactive endothelin (IR-ET) concentrations in rats 7 and 28 days after treatment with deoxycorticosterone (DOCA)-salt (solid columns; $n = 5-11$) and age-matched controls (open columns; $n = 4-14$). (b) Renal IR-ET levels in DOCA-salt ($n = 5-6$) and age-matched controls ($n = 4-6$). Values are mean ± s.e.mean. $^{**}P<0.01$ compared with values of age-matched controls.

Table 1 Effects of deoxycorticosterone acetate (DOCA)-salt treatment of the rat

	Treatment group	n	*SBP* (mmHg)	*Body weight* (g)	*Aorta weight* (mg)
7 days	Control	4	115 ± 5	268 ± 7	40.5 ± 4.9
	DOCA/salt	5	113 ± 6	257 ± 1	41.2 ± 5.2
28 days	Control	14	116 ± 3	361 ± 6	42.4 ± 1.2
	DOCA/salt	11	187 ± 5**	322 ± 11**	52.7 ± 1.9**

n indicates the number of the rats in each group. Values are means ± s.e.mean. $^{**}P<0.01$, compared with values of control rats.

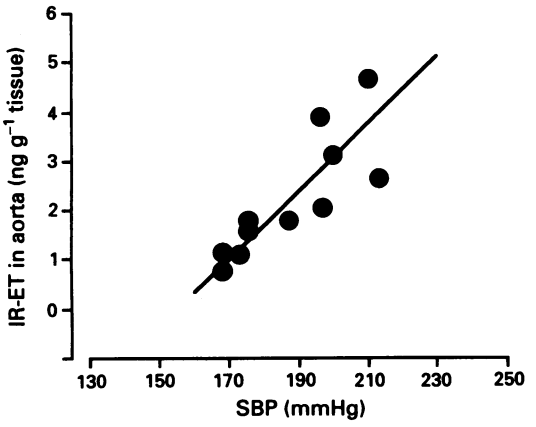

Figure 2 Correlation between concentration of aortic immunoreactive-endothelin (IR-ET) and systolic blood pressure (SBP) in rats 28 days after treatment with deoxycorticosterone acetate (DOCA)-salt. $r=0.83$; $P<0.01$.

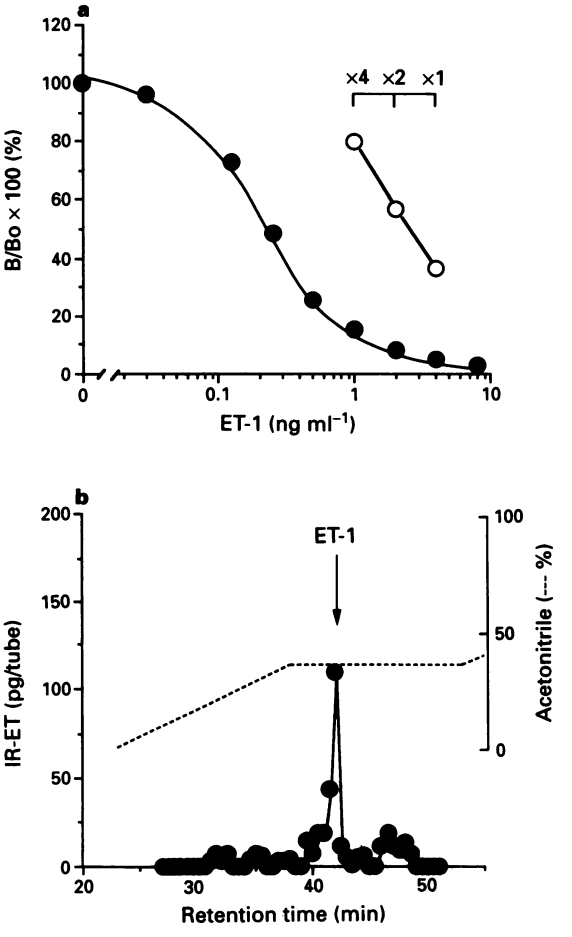

Figure 3 Characterization of immunoreactive-endothelin (IR-ET) in aortic extracts of deoxycorticosterone acetate (DOCA)-salt hypertensive rats. (a) A typical standard curve of endothelin-1 (ET-1) (●) and a dilution curve of the aortic extract (○). (b) Reversed-phase h.p.l.c. profile of IR-ET in aortic extracts from DOCA-salt hypertensive rats. The arrow indicates the elution of synthetic ET-1.

characterize further the IR-ET in aorta of DOCA-salt treated animals, we examined the elution profile of pooled aortic extract on reverse-phase h.p.l.c. coupled with RIA. As shown in Figure 3b, the elution profile revealed one major IR-ET component corresponding to the elution position of synthetic ET-1.

The time course of changes in mean arterial blood pressure (MAP) in control and DOCA-salt treatment after intravenous administration of FR139317 are shown in Figure 4. The average values for MAP after anaesthesia in DOCA-salt rats were significantly higher ($P<0.01$) than in control rats ($n=9$; 146 ± 6 mmHg vs $n=12$; 111 ± 4 mmHg). In control rats, MAP was slightly (by about 10–15 mmHg) decreased after intravenous injection of 10 mg kg^{-1} FR139317 compared with vehicle treatment. A significant decrease in MAP was observed 30–60 min after the injection. The MAP of DOCA-salts rats was markedly reduced by FR139317, at the same dose. Significant hypotensive effects were obtained at 15 min and lasted more than 90 min. Maximum responses (about 35 mmHg decrease from basal values) were observed 45–60 min after injection of the antagonist.

Discussion

The results of the present study clearly demonstrated increased vascular endothelin-1 concentrations in DOCA-salt hypertensive rat. The ET_A receptor antagonist, FR139317, produced a significant decrease in MAP in these DOCA-salt hypertensive rats. As this hypotensive effect was greater than that seen with control rats, the specificity of the finding in DOCA-salt hypertensive rats seem clear. It is most likely that ET-1 makes an important contribution to the maintenance of DOCA-salt-induced hypertension through interaction of the peptide with the ET_A receptor.

We observed an increase aortic ET-1 concentration in association with the hypertension induced by DOCA-salt treatment. Since circulation ET-1 concentrations were not increased in DOCA-salt hypertensive rats, except for the malignant model (Suzuki *et al.*, 1990; Kohno *et al.*, 1991), it seems that the increase in aortic ET-1 is due to a local increased production of the peptide in vascular tissue. We found a significant correlation between the aortic ET-1 level and the systolic blood pressure. These results suggest that ET-1 has a role in the regulation of blood pressure. On the other hand, there were no significant changes in ET-1 levels in the kidney, with DOCA-salt treatment. However, kidney is heterogeneous organ and the homogenates include vascular tissue, tubular epithelial cells and interstitial cells etc., so that it is impossible to estimate the ET-1 content of the renal vasculature from the whole kidney ET-1 level. Therefore, our results do not exclude the possibility that intrarenal vascular ET-1 concentration is altered.

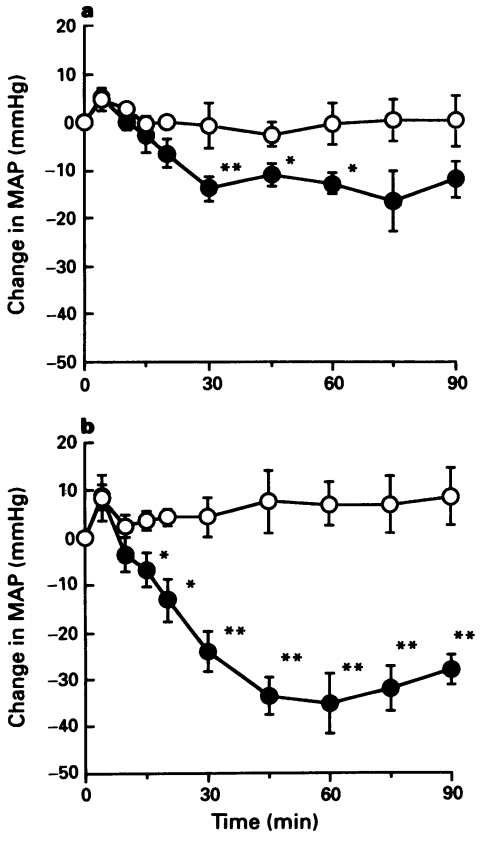

Figure 4 The effects of FR139317 (●; $n = 5-6$) or vehicle (○; $n = 4-6$) on mean arterial pressure (MAP) in anaesthetized (a) control and (b) deoxycorticosterone acetate (DOCA)-salt hypertensive rats. FR139317 was administered as an i.v. bolus injection (10 mg kg^{-1}). Each point represents the mean ± s.e.mean. $^{*}P<0.05$; $^{**}P<0.01$ compared with values of vehicle treatment at the same time.

The question arises as to whether the increase in aortic ET-1 levels in DOCA-salt hypertensive rats is merely the result of hypertension. From our results, the possibility that hypertension itself can induce the vascular ET-1 production cannot be ruled out. However, Larivière *et al.* (1993) noted that vascular ET-1 was not increased in spontaneous hypertensive rats, thereby suggesting that hypertension itself does not enhance ET-1 production in vascular tissues.

There is considerable evidence that many factors, including transforming growth factor-β_1 (TGF-β_1), thrombin, and a vasoactive agent such as angiotensin II and arginine-vasopressin (AVP) can stimulate the expression of prepro ET-1 mRNA in cultured endothelial cells (Kurihara *et al.*, 1989; Imai *et al.*, 1992; Umekawa *et al.*, 1994). It has been reported that AVP plays an important role in DOCA-salt hypertensive rats (Crofton *et al.*, 1979). Mohring *et al.* (1977) observed that plasma concentrations of AVP were elevated in this model of hypertension. Thus, AVP may be one possible candidate for the factor that enhances ET-1 production in the vascular wall. In a study by Sarzani *et al.* (1989), aortic TGF-β_1 mRNA levels were increased in DOCA-salt rats; this growth factor may also be responsible for the increased aortic ET-1 concentrations.

Several ET receptor antagonists have been developed and these will be used to evaluate pathophysiological roles of endogenous ET-1 and may provide a new therapeutic approach for cardiovascular diseases. FR139317 is a selective ET_A receptor antagonist which inhibits ET-1-induced vasoconstrictor effects *in vitro* and *in vivo* (Sagabe *et al.*, 1993). This antagonist ameliorates cerebral vasospasm after subarachnoid haemorrhage (Nirei *et al.*, 1993). It has also been reported that FR139317 can protect against the injury in rats with extensive renal mass reduction, a model of progressive renal disease (Benigni *et al.*, 1993). These results demonstrate the efficacy of FR139317 as an ET_A receptor antagonist for investigating the pathophysiological role of ET-1. In the present study, to clarify further the functional significance of increased vascular ET-1 levels, we evaluated the antihypertensive effect of FR139317 in DOCA-salt hypertensive rats. The result clearly indicated that FR139317 produced a significant hypotensive effect in anaesthetized DOCA-salt hypertensive rats. This hypotensive effect had a slow-onset and was long lasting, a finding consistent with a study which showed that the slow reversal of the vasoconstrictor effects of ET-1 is caused by another ET_A receptor antagonist, BQ123, *in vitro* and *in vivo* (Warner *et al.*, 1994). These authors observed that it takes about 50 min for the antagonist to reverse the established pressor response to ET-1 in anaesthetized rats.

Our results obtained with anaesthetized DOCA-salt hypertensive rats agreed with the finding that another ET_A receptor antagonist, BQ123, produces a small but significant hypotensive effect in conscious DOCA-salt hypertensive rats (Bazil *et al.*, 1992). It has been reported that phosphoramidon, an endothelin converting enzyme inhibitor (Matsumura *et al.*, 1990a), produces a reduction of blood pressure in DOCA-salt hypertensive rats, by inhibiting endothelin bioconversion (Vemulapalli *et al.*, 1993). Stein *et al* (1994) reported that the orally active ET_A receptor antagonist, 5-(dimethylamino)-*N*-(3,4-dimethyl-5-isoxazolyl)-1-naphthalenesulphonamide, produced a significant decrease in blood pressure, in DOCA-salt hypertensive rats. These results show the importance of ET-1 and the ET_A receptor in the maintenance of DOCA-salt induced-hypertension.

In the present study, we observed that FR139317 produced a slight but significant decrease in MAP in control normotensive rats. This would suggest a role of ET-1 and the ET_A receptor in the maintenance of normal blood pressure. However, a previous report indicated that an acute bolus injection of FR139317 had no effect on blood pressure in conscious normotensive rats (Sogabe *et al.*, 1993). BQ123 also had no significant effect of blood pressure in conscious normotensive rats (Bazil *et al.*, 1992; Nishikibe *et al.*, 1993). One explanation for this discrepancy may relate to the experimental condition (anaesthetized rat vs conscious rat). It has been reported that the standard experimental technique such as surgery produces a significant increase in circulating ET-1 (Pollock *et al.*, 1993). Therefore, circulating ET-1 may be increased in anaesthetized rats. Other investigators reported that BQ123 produced a significant decrease in blood pressure in anaesthetized normotensive rats (Bigaud & Pelton 1992; Pollock & Opgenorth, 1993).

Our conclusions regarding the involvement of ET-1 and ET_A receptor in DOCA-salt hypertension depend on the specificity of FR139317 as an ET_A receptor antagonist. In the present study, FR139317 reduced MAP in normotensive rats as well as DOCA-salt hypertensive rats, although the reduction of MAP in the former was small, compared with that in the latter. Since any nonspecific depressor agent would be expected to have a greater hypotensive effect in animals with higher resting blood pressure, one may be doubtful of the specificity of FR139317 for DOCA-salt hypertension. However, we noted that FR139317 treatment to anaesthetized 2K-1C renal hypertensive rats produces only a moderate hypotensive effect to the same degree as that in anaesthetized normotensive rats (unpublished observation). Thus, it seems likely that the greater responses of DOCA-salt hypertensive rats are not due to a nonspecific depressor effect of the agent. In addition, the dose of FR139317 used in our study has been shown to have no effect on the initial depressor response to ET-1, which is mainly mediated by the ET_B receptor (Sogabe *et al.*, 1993). It is reasonable to consider that the antihypertensive effect of FR139317 in DOCA-salt hypertensive rat could be due to an ET_A receptor antagonism.

In conclusion, our results suggest that there is an increased vascular production of ET-1 in DOCA-salt hypertensive rats. In addition, our results indicate the pathophysiological significance of increased endogenous ET-1 in the maintenance of DOCA-salt induced hypertension, through interaction of this peptide with the ET_A receptor.

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Blockade of motion- and cisplatin-induced emesis by a 5-HT_2 receptor agonist in *Suncus murinus*

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1 The effects of (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI), a 5-hydroxytryptamine 5-HT_{2A}/5-HT_{2C} receptor agonist, on motion- and cisplatin-induced emesis were studied in *Suncus murinus*. Subcutaneous injection of DOI, 30 min prior to the emetic stimuli, dose-dependently blocked the emesis induced by motion sickness and cisplatin (20 mg kg^{-1}, i.p.) with estimated ID_{50} values of 640 and 780 μg kg^{-1}, respectively.

2 α-Methyl-5-hydroxytryptamine (α-Me-5-HT), a peripheral 5-HT_{2A}/5-HT_{2C} receptor agonist, had no effect on motion- and cisplatin-induced emesis.

3 The antiemetic effects of DOI on motion- and cisplatin-induced emesis were attenuated by pre-administration of ketanserin, a selective 5-HT_{2A} receptor antagonist.

4 The present results suggest an inhibitory role for central 5-HT_2 receptors in the emetic reflex mechanism and that a 5-HT_2 receptor agonist may be a useful tool to investigate the involvement of 5-HT receptors in the emetic reflex.

Keywords: 5-HT_2 receptor; motion sickness; cisplatin; emesis; *Suncus murinus*; (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI)

Introduction

Recently, much evidence that various subtypes of 5-hydroxytryptamine (5-HT) receptors mediate the emetic response has been obtained. Selective antagonists for the 5-HT_3 receptor inhibit emesis induced by cancer chemotherapeutic agents, such as cisplatin, in ferrets (Costall *et al.*, 1986), dogs (Smith *et al.*, 1986), cats (Lucot, 1989) and *Suncus* (Torii *et al.*, 1991a) and these antagonists have been used clinically. It has also been reported that 5-HT_{1A} receptor agonists can block emesis induced by motion and several emetic drugs in cats (Lucot & Crampton, 1989) and *Suncus* (Okada *et al.*, 1994). These results suggest that 5-HT_{1A} receptor agonists are useful as new antiemetic drugs and that a 5-HT_{1A} receptor-mediated mechanism(s) is involved in the emetic reflex.

A potential role for 5-HT_2 receptors in the emetic mechanism is suggested by experiments with LSD-25, a non-selective 5-HT_1 and 5-HT_2 receptor agonist. LSD-25 prevented apomorphine-, morphine- and hydergine-induced emesis but not emetine-, ouabain- or protoveratrine-induced emesis (Dhawan & Gupta, 1961). However, the effects of selective 5-HT_2 receptor agonists on emesis have not been reported.

Suncus murinus (a house musk shrew) is a species of insectivore, which is considered to be closer to the primates than rodents, lagomorphs and carnivores in the phylogenetic system (Colbert, 1958). We have shown previously that *Suncus* vomits in response to various emetic drugs and motion stimulus and that the animal is suitable for research on antiemetic drugs (Ueno *et al.*, 1987; 1988).

In the present study, the effects of the 5-HT_{2A}/5-HT_{2C} (formerly classified as 5-HT_2/5-HT_{1C}; Hoyer *et al.*, 1994) receptor agonist (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) (Glennon *et al.*, 1986) were investigated on motion sickness and cisplatin-induced emesis in *Suncus murinus*, to assess whether these receptors are involved in the emetic pathway.

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Methods

Animals

Experiments were performed on 3–6 month-old male *Suncus murinus* weighing 50–90 g. The animals were purchased from the Central Institute for Experimental Animals (Kanagawa, Japan) and bred in a temperature controlled room at 24 ± 1°C under artificial lighting between 08 h 00 min and 20 h 00 min at The Animal Care Institute of The University of Tokyo. They were allowed free access to pellet chow, supplied by the Central Institute for Experimental Animals, and tap water.

Experimental procedures

Emetic stimuli Experimental conditions were similar to those reported previously from our laboratory (Ueno *et al.*, 1987; 1988; Torii *et al.*, 1991a,b). Emetic stimuli employed were motion and cisplatin (20 mg kg^{-1}, i.p.). For the experiments on motion sickness, animals were selected for susceptibility to motion sickness. Motion sickness was elicited by reciprocal shaking (amplitude 40 mm, frequency 1.0 Hz, duration 5 min). Each animal was placed in a transparent cage (10W × 15L × 12H cm) fixed on a reciprocal shaker (TAITEC R-30 mini, Taiyo Scientific Industrial Co., LTD., Japan). After a 5 min acclimatisation the motion was started. Generally more than 90% of animals vomited during the motion. Then the effect of drugs was tested using the same motion. An interval longer than one week was allowed between the two motion tests to avoid habituation. The number of vomiting episodes was recorded over 5 min for motion and over 90 min for cisplatin. The latency to the first vomiting was also noted.

Drug treatment

DOI was injected subcutaneously in the shoulder region of animals 30 min prior to the emetic stimuli. Ketanserin was injected subcutaneously 15 min before the administration of DOI. α-Methyl-5-hydroxytryptamine was injected 30 min prior to the emetic stimuli.

Drugs

The following drugs were used: (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI), α-methyl-5-hydroxytryptamine maleate (α-Me-5-HT) (Research Biochemicals, Inc., Wayland, MA, U.S.A.), ketanserin tartrate (Kyowa Hakko Kogyo Co., Tojyo, Japan) and cisplatin (Sigma Chemical Co., St. Louis, MO, U.S.A.). The doses of drugs were calculated as the base. DOI, ketanserin and α-Me-5-HT were dissolved in saline. The volume of drug solution injected was adjusted to 2 ml kg^{-1} body weight except for cisplatin (10 ml kg^{-1} body weight). In the case of cisplatin, the pH of the solvent saline was adjusted to 4.0 with 0.1 N HCl. Then cisplatin was dissolved in this saline by sonication and warming to 50°C at a final concentration of 2 mg ml^{-1}. The solution was cooled to 37°C just before the administration.

Data analysis

The results are expressed as the mean ± s.e.mean. The ID_{50} value of the antiemetic effect of DOI was estimated by Brownlee's up-and-down method (Brownlee *et al.*, 1953). The dose of DOI was started from 0.5 mg kg^{-1} and decreased or increased with a fixed ratio of 2. Statistical significance between the means of the two groups was analysed by Student's *t* test (equal variances) or by Welch test (non-equal variances). A one-way analysis of variance followed by Duncan's new multiple range test was used for multiple comparison.

Results

Subcutaneous injection of DOI dose-dependently prevented the emesis induced by both motion and cisplatin in *Suncus* (Table 1). The ID_{50} values of DOI on motion- and cisplatin-induced emesis were 640 and 780 μg kg^{-1}, respectively. DOI apparently reduced the spontaneous motor activity at doses above 0.5 mg kg^{-1}. However, DOI did not cause any other behavioural change, such as head twitching or 'wet-dog' shake behaviour, in the doses used in these experiments.

Since DOI showed antiemetic effects, we tested whether or not the antiemetic effects of DOI were blocked by pretreatment with ketanserin, a selective 5-HT$_{2A}$ receptor antagonist. All animals treated only with saline vomited in response to motion or cisplatin. Administration of 2.0 mg kg^{-1} ketanserin alone 15 min prior to the saline did not significantly reduce the incidence of vomiting/tested animals in either type of emesis. Only the latency in the motion-induced emesis was significantly prolonged by ketanserin. However, the same dose of ketanserin attenuated the antiemetic effects of DOI in motion- and cisplatin-induced emesis (Table 2). In the case of motion-induced emesis, pre-administration of ketanserin prior to 1.5 mg kg^{-1} DOI increased the incidence of vomiting/tested animals to 75% from 14%. The administration of 1.5 mg kg^{-1} DOI prevented the cisplatin-induced emesis in half of the animals. At a dose of 3.0 mg kg^{-1} DOI, no animals vomited, but three out of five animals vomited when 2.0 mg kg^{-1} ketanserin was injected prior to the administration of 3.0 mg kg^{-1} DOI.

Subcutaneous injection of 2.0 mg kg^{-1} α-Me-5-HT 30 min before the emetic stimuli did not significantly change the susceptibility to motion- and cisplatin-induced emesis (Table 3). α-Me-5-HT apparently reduced the number of vomiting episodes after cisplatin. However, the effect was neither statistically significant nor dose-dependent. Other parameters were also not significantly affected. When the dose was increased to 10 mg kg^{-1}, three out of five animals in the motion and one out of five animals in the cisplatin-treated group vomited to α-Me-5-HT alone. These animals also vomited to the motion and cisplatin. Injection of α-Me-5-HT did not cause any other overt behavioural change.

Discussion

Subcutaneous injection of DOI dose-dependently prevented motion-induced emesis in *Suncus murinus*. We have shown previously that clinically-used antimotion sickness agents including antihistamines such as diphenhydramine, chlorpheniramine and dimenhydrinate were effective in preventing motion-induced emesis in *Suncus* (Ueno *et al.*, 1988; Kaji *et al.*, unpublished). The ID_{50} values of antihistamines were 10–50 mg kg^{-1}, while the ID_{50} value of DOI in this study was about 640 μg kg^{-1}. Therefore, DOI is more potent against motion-induced emesis than the antihistamines.

In the present study, the administration of DOI blocked emesis elicited by two different stimuli, motion and cisplatin. It is well-known that 5-HT$_3$ receptor antagonists selectively block the emesis induced by cancer chemotherapeutic agents (e.g., Sanger, 1990). In *Suncus*, 5-HT$_3$ receptor antagonists inhibit emesis induced by cisplatin and X-irradiation but not that induced by motion, nicotine or copper sulphate (Torii *et al.*, 1991a,b). The emetic effect of cisplatin is considered to be mediated via peripheral 5-HT$_3$ receptors because it was blocked completely by abdominal vagotomy (Mutoh *et al.*, 1992) but not by intracerebroventricular injection of a 5-HT$_3$ receptor antagonist (Torii *et al.*, unpublished observation). Although the precise mechanisms of motion sickness have not yet been clarified, it is likely that the vestibule and

Table 1 Antiemetic effect of DOI on motion- and cisplatin-induced emesis in *Suncus murinus*

Emetic stimuli	*Dose* (mg kg^{-1})	*No. of* Suncus *vomiting/tested*	*No. of vomiting episodes*	*Latency*	*ID_{50} value* (mg kg^{-1})
				(s)	
Motion	Saline	5/5	14.0 ± 1.0	47.0 ± 5.8	0.64
(1 Hz, 40 mm, 300 s)	0.25	3/3	10.7 ± 2.3	39.0 ± 10.1	
	0.5	3/5	11.0 ± 2.1	30.3 ± 7.7	
	1.0	1/4	5	69	
	2.0	0/3	–	–	
				(min)	
Cisplatin	Saline	5/5	16.0 ± 4.6	36.4 ± 5.8	0.78
(20 mg kg^{-1}, i.p.)	0.25	3/3	13.7 ± 4.4	50.3 ± 9.9	
	0.5	4/5	4.3 ± 1.4	67.3 ± 6.4	
	1.0	2/6	2, 9	78, 43	
	2.0	0/3	–	–	

Values for the number of vomiting episodes and the latency to vomit per vomiting animal are mean ± s.e.mean, but actual values are indicated when the number of vomiting animals was less than three.

Table 2 Attenuation of antiemetic effect of DOI by ketanserin in *Suncus murinus*

Emetic stimuli	*1st injection*	*2nd injection*	*No. of* Suncus *vomiting/tested*	*No. of vomiting episodes*	*Latency*
					(s)
Motion	Saline	Saline	5/5	11.4 ± 2.0	37.8 ± 5.2
	Ketanserin	Saline	5/5	5.8 ± 2.6	115.0 ± 18.7*
	Saline	DOI(1.5)	1/7	8	34
	Ketanserin	DOI(1.5)	6/8	6.5 ± 2.5	125.0 ± 36.8
					(min)
Cisplatin	Saline	Saline	5/5	9.2 ± 3.1	57.6 ± 4.5
	Ketanserin	Saline	5/5	6.4 ± 1.5	47.6 ± 6.0
	Saline	DOI (1.5)	2/4	9, 13	57, 32
	Ketanserin	DOI (1.5)	4/5	5.0 ± 1.6	66.8 ± 3.1
	Saline	DOI (3.0)	0/5	–	–
	Ketanserin	DOI (3.0)	3/5	10.3 ± 4.1	44.0 ± 4.2

Values for the number of vomiting episodes and the latency to vomit per vomiting animal are mean ± s.e.mean, but actual values are indicated when the numbver of vomiting animals was less than three. The number in parentheses indicates the dose of DOI (mg kg^{-1}). The dose of ketanserin was 2.0 mg kg^{-1}. *Significant difference from the group treated only with saline (Welch test; $P<0.05$).

Table 3 Effect of α-methyl-5-hydroxytryptamine (α-Me-5-HT) on motion- and cisplatin-induced emesis in *Suncus murinus*

Emetic stimuli	*Dose* (mg kg^{-1})	*No. of* Suncus *vomiting/tested*	*No. of vomiting episodes*	*Latency*
				(s)
Motion	Saline	5/5	13.4 ± 2.5	30.2 ± 3.2
	2.0	5/5	12.4 ± 2.2	37.2 ± 5.4
	10.0[a]	5/5	13.2 ± 2.5	39.4 ± 4.9
				(min)
Cisplatin	Saline	5/6	11.8 ± 3.5	42.6 ± 5.4
	2.0	5/5	5.4 ± 0.2	60.8 ± 9.7
	10.0[b]	5/5	7.6 ± 1.9	54.2 ± 9.2

Values for the number of vomiting episodes and the latency to vomit per vomiting animal are mean ± s.e.mean. α-Me-5-HT was injected 30 min before the emetic stimuli. [a]Three animals vomited to α-Me-5-HT alone with the latency of 3 to 10 min. [b]One animal vomited to α-Me-5-HT alone with a latency of 6 min.

semicircular canals are involved. Therefore, the two emetic stimuli are clearly distinguishable. However, DOI blocked completely the two types of emesis. DOI probably blocks common mechanism(s) necessary for the emetic reflex of *Suncus*.

The antiemetic effect of DOI may be similar to that of 5-HT$_{1A}$ receptor agonists. In cats, 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) and buspirone suppress vomiting elicited by motion, cisplatin and xylazine (Lucot & Crampton, 1989). In *Suncus*, several 5-HT$_{1A}$ receptor agonists inhibited emesis not only elicited by cisplatin but also by other stimuli (Okada *et al.*, 1994). However, it is not likely that the antiemetic effect of DOI is mediated via 5-HT$_{1A}$ receptors, since this compound has very little affinity for 5-HT$_{1A}$ receptors (Hoyer, 1988a). Furthermore, the prophylactic effect of DOI against motion- and cisplatin-induced emesis was attenuated by pre-administration of ketanserin, a selective 5-HT$_{2A}$ receptor antagonist, indicating that the antiemetic effect of DOI is mediated via a 5-HT$_{2A}$ receptor but not a 5-HT$_{1A}$ receptor. Torii *et al.* (1991a) showed that administration of ketanserin alone prevented cisplatin-induced emesis with an ID$_{50}$ value of 4.0 mg kg^{-1} in *Suncus*. However, ketanserin at doses higher than 4 mg kg^{-1} caused marked sedation in *Suncus*. In the present study, 2 mg kg^{-1} ketanserin was used, and the drug itself did not show either obvious antiemetic or sedative effects. DOI has a high affinity for 5-HT$_{2C}$ as well as 5-HT$_{2A}$ receptors (Hoyer, 1988b). Therefore, it is possible that stimulation of 5-HT$_{2C}$ receptors is also involved in the antiemetic effect of DOI.

To investigate the action of DOI, i.e. central or peripheral, the effect of another 5-HT$_{2A}$/5-HT$_{2C}$ receptor agonist, α-methyl-5-hydroxytryptamine (α-Me-5-HT) (Hoyer, 1988b; Ismaiel *et al.*, 1990), was tested on motion- and cisplatin-induced emesis. α-Me-5-HT is considered as a peripherally acting 5-HT$_{2A}$/5-HT$_{2C}$ receptor agonist (Chaouloff *et al.*, 1990; Baudrie & Chaouloff, 1992). Administration of α-Me-5-HT did not consistently change the susceptibility to motion- and cisplatin-induced emesis. Thus, this result suggests that the antiemetic effect of DOI is mediated by 5-HT$_2$ receptors in the central nervous system. The mechanism(s) of emetic reflex is probably mediated by a number of separate nuclei which include the area postrema, nucleus tractus solitarius and dorsal vagal motor nucleus. 5-HT$_{2A}$ receptors are densely located in the neocortex (lamina Va), claustrum, olfactory bulb and caudate putamen in rat brain (Appel *et al.*, 1990). DOI may act on the 5-HT$_2$ receptors of inhibitory neurones in these regions and indirectly inhibit the brain stem which mediates the emetic reflex.

Administration of DOI causes the head twitch response in mice and 'wet-dog' shake behaviour in rats. These behavioural responses are thought to be induced by the stimulation of 5-HT$_2$ receptor. In the present study, administration of DOI reduced the spontaneous motor activity, but induced neither head twitch response nor 'wet-dog' shake behaviour in *Suncus*. This may be because the doses of DOI used in this study were smaller than the reported dose of 2.5 mg kg^{-1} that induced head twitch response in mice (Darmani *et al.*, 1990).

Although the present study shows that a 5-HT$_2$ receptor agonist is a potent antiemetic, the possibility of using DOI as a clinical antiemetic is low because of the hallucinogenic effect of phenylisoplopylamine compounds including DOI (Glennon *et al.*, 1982). However, the antiemetic action of DOI indicates that 5-HT$_2$ receptor agonists may be a useful tools for investigating the further involvement of 5-HT receptors in the emetic reflex.

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Effects of β_2-adrenoceptor agonists on anti-IgE-induced contraction and smooth muscle reactivity in human airways

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1 The β_2-adrenoceptor agonists, salbutamol, salmeterol and RP 58802 relaxed basal tone of human isolated bronchial smooth muscle. Salmeterol- and RP 58802-induced relaxations persisted for more than 4 h when the medium was constantly renewed after treatment.

2 Salbutamol, salmeterol and RP 58802 reversed histamine-induced contractions in human airways (pD_2 values: 6.15 ± 0.21, 6.00 ± 0.19 and 6.56 ± 0.12, respectively).

3 Anti-IgE-induced contractions were significantly inhibited immediately after pretreatment of preparations with β_2-adrenoceptor agonists (10 μM). However, when tissues were treated with β_2-agonists and then washed for a period of 4 h, salmeterol was the only agonist which significantly inhibited the anti-IgE response.

4 Histamine response curves were shifted to the right immediately after pretreatment of tissues with the β_2-adrenoceptor agonists (10 μM; 20 min), but maximal contractions were not affected. After a 4 h washing period, the histamine curves were not significantly different from controls. Concentration-effect curves to acetylcholine (ACh) or leukotriene C_4 (LTC_4) were not significantly modified after β_2-agonist pretreatment.

5 These results suggest that β_2-adrenoceptor agonists may prevent anti-IgE-induced contraction by inhibition of mediator release rather than alterations of those mechanisms involved in airway smooth muscle contraction.

Keywords: β_2-Adrenoceptor agonist; salbutamol; salmeterol; RP 58802; anti-IgE; human airways; relaxation; contraction

Introduction

In addition to their bronchodilator activity, β_2-adrenoceptor agonists have been reported to inhibit mediator release from inflammatory cells. Previous studies have shown that the immunological release of histamine from human chopped lung (Assem & Schild, 1969; Orange *et al.*, 1971; Butchers *et al.*, 1979) or from isolated lung mast cells (Church & Hiroi, 1987) can be reduced by prior treatment with β_2-agonists. Furthermore, the production of leukotrienes induced by an allergenic challenge of sensitized lung parenchymal tissue was prevented by treatment with β_2-agonists (Butchers *et al.*, 1991). A number of studies have demonstrated that antigen- and anti-IgE-induced contractions are dependent on the release of several spasmogenic factors (Schild *et al.*, 1951; Adams & Lichtenstein, 1979; Dahlén *et al.*, 1983). A considerable number of studies have been performed to evaluate the bronchodilator actions of β_2-agonists on basal or induced tone (Cerrina *et al.*, 1986; De Jongste *et al.*, 1987; Whicker *et al.*, 1988); however, few experiments have been performed to assess the inhibitory actions of β_2-agonists against spasmogenic agents. Furthermore, a limited number of reports have been published to evaluate the inhibitory action of β_2-agonists against antigen- or anti-IgE-induced contraction of airways (Buckner *et al.*, 1979).

The aim of the present study was to determine whether human bronchial smooth muscle responses to spasmogens such as histamine, acetylcholine (ACh) and leukotriene C_4 (LTC_4) are altered by β_2-agonist pretreatment. In addition, an evaluation of the inhibitory effects of the β_2-adrenoceptor agonists, salbutamol, salmeterol (Ullman & Svedmyr, 1988) and RP 58802 (Underwood *et al.*, 1992) on the functional response of human bronchial tissues to an anti-IgE challenge was carried out.

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Methods

Bronchial tissues

Human bronchial tissues were obtained from patients who had undergone surgery for lung carcinoma. No subject had a history of asthma and baseline lung function and IgE titres were not assessed in these patients prior to surgery. Subsequent to pulmonary lobe resection, macroscopically normal bronchial tissues were dissected free of surrounding parenchyma, cut as rings and placed in Tyrode solution at 4°C. The composition of the Tyrode solution was (mM): NaCl 139.2, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 0.49, $NaHCO_3$ 11.9, NaH_2/PO_4 0.4 and glucose 5.5; pH 7.4. Tissues were used within 18 h after dissection.

Experimental procedure

Bronchial preparations from 3 to 5 mm in internal diameter were set up in 10 ml organ baths under initial loads of 2-3 g. The tissues were maintained at 37°C in Tyrode solution gassed with 5% CO_2 in O_2. Changes in force were recorded by isometric force transducers (Narco F-60) and Linseis physiographs. Tissues were equilibrated (90 min), during which period Tyrode solution was replaced every 10 min. Preparations were then contracted with histamine (50 μM) and washed until basal tone was re-established.

Salbutamol, salmeterol and RP 58802 cumulative concentration-effect curves were constructed on precontracted tissues (histamine, 50 μM) by adding incremental concentrations of relaxant agonist every 15 min until no further relaxation was achieved. In another series of experiments, the contracted preparations (histamine, 50 μM) were relaxed with a single dose of agonist (10 μM).

After addition of the β_2-agonists (10 μM; 20 min) to preparations at resting tone, contractions to either histamine, ACh, LTC_4 or anti-IgE were produced. In another series of

experiments β_2-agonists were added to the preparations at resting tone (20 min) and the tissues were washed for a period of 4 h before either anti-IgE or histamine curves were performed.

Drugs

The following drugs were used: histamine dihydrochloride and acetylcholine chloride (Sigma Chemical Co., St. Louis, MO, U.S.A.), leukotriene C_4 (Bayer U.K. Ltd, Research Dept., Stoke Poges, England), ascorbic acid (Prolabo, France). Salbutamol, salmeterol, RP 58802 (α[[(1H-benzimidazolyl-1)-3 methyl-1 propyl]amino-methyl] hydroxy-4 methoxy-3-benzene methanol dichlorhydrate, dihydrate) were synthesized at Rhône-Poulenc-Rorer and were kindly provided by Dr I. Cavero (Rhône-Poulenc-Rorer, Alfortville, France). The anti-human IgE (sheep anti-(human IgE) serum; Nordic Immunological Laboratories, Tilburg, Netherlands) was directed against the Fc fraction of the IgE (i.e. the constant domains on the ε chain).

Histamine, ACh and salbutamol were dissolved in Tyrode's solution. Salmeterol, RP 58802 and LTC_4 were dissolved in dimethylsulphoxide (DMSO). Subsequent dilutions were made in Tyrode solution. The final dilution of DMSO was less than 1/10 000. This dilution did not affect either the resting tone or the maximal response to histamine. All β_2-agonist solutions were prepared just prior to use and contained ascorbic acid (0.1 mM).

Data analysis

Histamine precontractions, effects of β_2-adrenoceptor agonists on basal tone and anti-IgE responses are expressed in grams (g). Maximal relaxations (E_{max}) to salbutamol, salmeterol and RP 58802 were expressed as a percentage reversal of the histamine response. ACh, histamine and LTC_4 concentration-effect curves are presented in figures as percentage of agonist maximal contraction. The E_{max} for these agonists are given in tables as percentage of initial histamine precontraction. Agonist potency was assessed by determination of the EC_{50} values, which were defined as the concentration of agonist which gave 50% of the maximal response. EC_{50} values were transformed to pD_2 values which are the negative logarithm of the EC_{50} values. Data are expressed as means ± s.e.mean of (n) experiments, where (n) represents the number of human lung samples. Statistical evaluation was performed using Student's t test for paired or unpaired variates where appropriate and a P value of less than 0.05 was considered to be significant.

Results

β_2-Adrenoceptor agonist-induced relaxation

The β_2-adrenoceptor agonists (10 μM) all caused relaxation of basal tone (Figure 1). After a 4 h washing period, salbutamol-treated preparations returned to their control basal tone; in contrast, the baseline for salmeterol and RP 58802 at 4 h remained significantly lower than in control tissues (Figure 1). The relaxant effects of β_2-agonists on human isolated bronchial muscle ring preparations contracted with histamine (50 μM) are shown in Figure 2. Maximal responses obtained in the concentration-effect curves and potencies (pD_2 values) for these agonists are given in Table 1. Salmeterol produced a partial relaxation equivalent to approximately 50% reversal of histamine-induced tone. When the tissues were contracted with histamine (50 μM) and then relaxed by the addition of a single concentration (10 μM) of salbutamol, salmeterol or RP 58802, the relaxations obtained under these conditions were 75%, 49% or 80% reversal of the induced tone, respectively. The time necessary to attain these values was markedly different for each agonist (salbutamol, 26.4 ± 10.5 min; salmeterol, 72.0 ± 13.2 min and RP 58802, 16.8 ± 5.0 min; $n = 3$).

Effects of β_2-agonists on contractile responses

The immediate effects of β_2-agonists (10 μM; 20 min) on the anti-IgE-induced contraction and following a 4 h washing period are presented in Figure 3. β_2-Agonist treatment significantly inhibited the anti-IgE-induced contraction. Salmeterol was the only relaxant agonist which exerted a prolonged inhibitory action on the anti-IgE-induced contraction.

The maximal contraction to histamine (E_{max}) obtained from concentration-effect curves in controls was 5.49 ± 1.09 g and represented 127 ± 10% of the initial histamine (50 μM) response. These values were not significantly different from the histamine response (E_{max}) obtained in curves performed after the 4 h washing period (123 ± 12% of histamine precontraction). Immediately after incubation with salbutamol, salmeterol or RP 58802, histamine curves were shifted to the

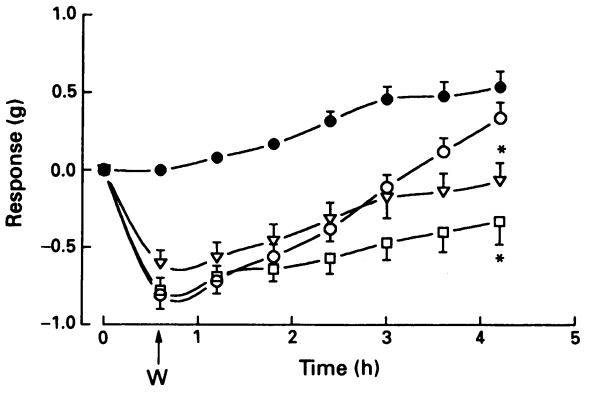

Figure 1 Human airway muscle tone in preparations incubated with either Tyrode solution (control; ●) or Tyrode solution containing 10 μM salbutamol (○), salmeterol (▽) or RP58802 (□) followed by a 4 h washing period with Tyrode solution. The arrow indicates the beginning of the washing period (W). Values are means ± s.e.mean obtained from 18 lung samples. *Significantly different from data obtained in control tissues. ($P < 0.05$, Student's t test).

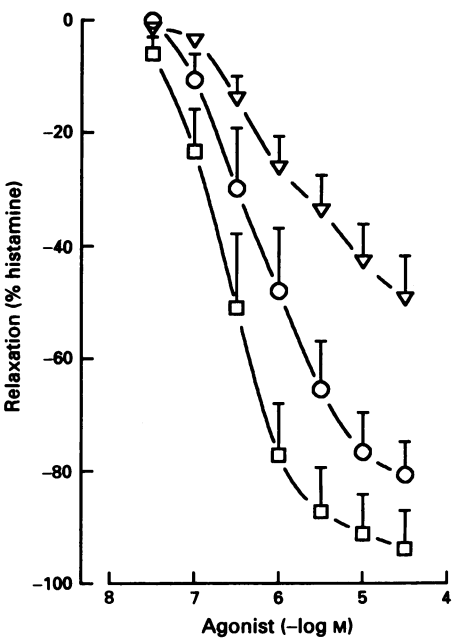

Figure 2 Relaxant effects of salbutamol (○), salmeterol (▽) and RP 58802 (□) in human airways contracted with histamine (50 μM). Relaxant responses are expressed as percentage reversal of histamine-induced tone. Values are means ± s.e.mean obtained from 5 lung samples.

right without alteration in maximal response. However, after the washing period (4 h), the response and potency of histamine were not significantly different from data derived from control preparations (Figure 4, Table 2). Neither the E_{max} for ACh and LTC_4 nor the pD_2 values were modified after treatment with β_2-agonists (Figure 5, Table 3).

Discussion

Salbutamol, salmeterol and RP 58802 relaxed basal tone and reversed histamine-induced contraction of human airways. Immediately following β_2-adrenoceptor agonist pretreatments, anti-IgE-induced contractions were significantly blocked, an effect also observed with salmeterol at 4 h. After β_2-agonist pretreatment, histamine curves were shifted to the right. This displacement was small and not observed after the 4 h washing period. In contrast, incubation with β_2-agonists did not significantly alter ACh- and LTC_4-induced responses. These data suggest that while β_2-agonists blocked IgE-mediated contractions, they did not modify human airway reactivity to several contractile agonists.

Human bronchial preparations *in vitro* relax when treated with antihistamines, leukotriene antagonists, TP-receptor antagonists or muscarinic antagonists (Norel *et al.*, 1991; Gorenne *et al.*, 1994; Ellis & Undem, 1994). These results suggest that resting tone in bronchial preparations, which is stable under control conditions during the experimental protocols, is maintained by the endogenous release of mediators. Therefore, the relaxation observed on basal tone with the β_2-agonists may be the consequence of both a direct relaxant effect on smooth muscle and inhibition of spontaneous mediator release. Salbutamol and salmeterol were equipotent, while RP 58802 was slightly more potent in reversing

Table 1 β_2-Adrenoceptor agonist relaxation on histamine-induced tone in human airways

	Histamine (g)	E_{max} (%)	*pD_2 values*
Salbutamol	2.87 ± 0.32	81 ± 6	6.15 ± 0.21
Salmeterol	2.70 ± 0.72	49 ± 8	6.00 ± 0.19
RP 58802	2.14 ± 0.27	94 ± 7	6.56 ± 0.12

Bronchial preparations were contracted with histamine (50 μM) and relaxed with cumulative concentrations of β_2-adrenoceptor agonists. Histamine precontractions are expressed in g. Maximal relaxations to β_2-adrenoceptor agonists (E_{max}) are presented as percentage reversal of histamine contraction. Values are means ± s.e.mean obtained from 5 lung samples.

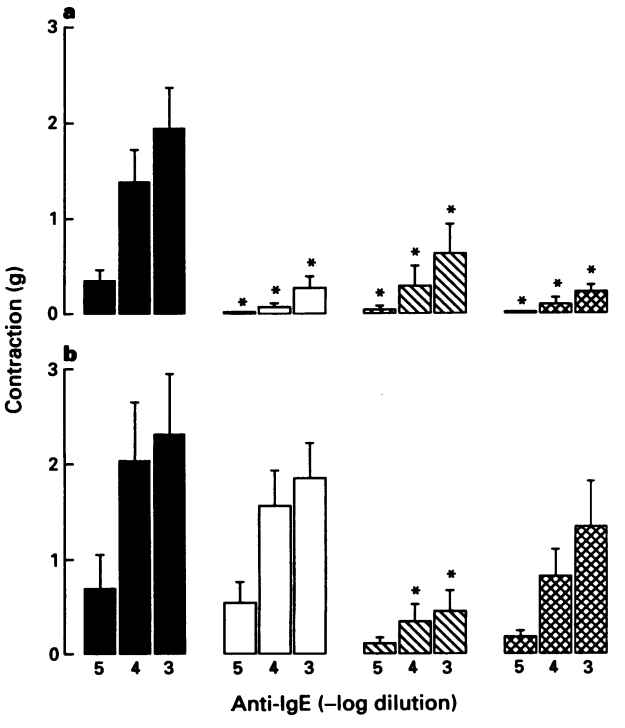

Figure 3 Inhibitory effects of β_2-adrenoceptor agonists on the response to cumulative dilutions of anti-IgE in human airways. (a) Anti-IgE-induced contractions were obtained after incubation with Tyrode solution (control; solid columns) or Tyrode solution containing salbutamol (open columns), salmeterol (hatched columns) or RP 58802 (cross-hatched columns) (10 μM; 20 min). (b) Anti-IgE-induced contractions were obtained after β_2-agonists treatment followed by a 4 h washing period. Results are expressed in g. Values are means ± s.e.mean obtained from 4–6 lung samples. *$P<0.05$.

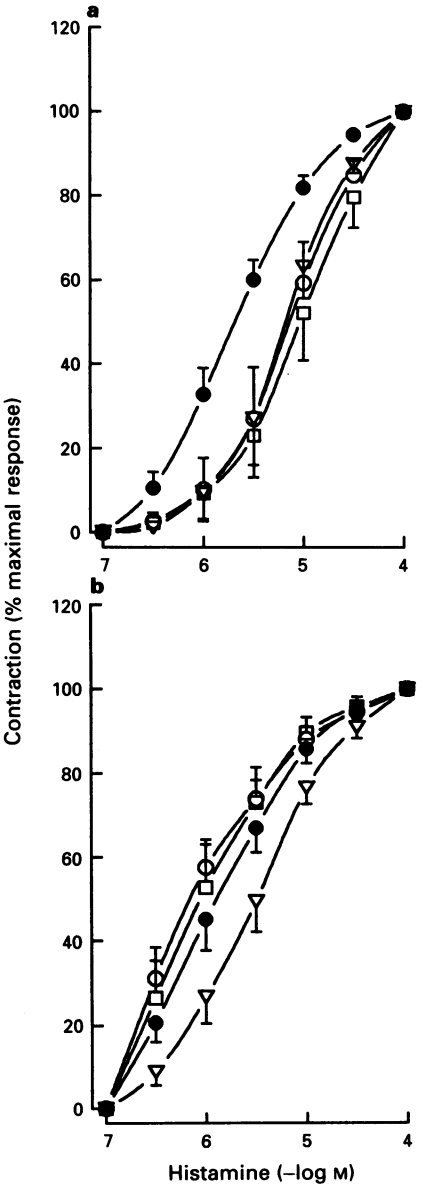

Figure 4 Effects of β_2-adrenoceptor agonists on histamine concentration-effect curves in human airways. (a) Curves were obtained after incubation (20 min) with Tyrode solution (●, control) or Tyrode solution containing 10 μM salbutamol (○), salmeterol (▽) or RP 58802 (□). (b) Histamine curves obtained after the β_2-agonists treatment followed by a 4 h washing period. Results are expressed as percentage of agonist maximal response. Values are means ± s.e.mean obtained from 3–4 lung samples.

Table 2 Effects of β_2-adrenoceptor agonists on histamine concentration-effect curves in human airways

	Immediate		*4 h*	
	E_{max} (%)	pD_2 *values*	E_{max} (%)	pD_2 *values*
Control	127 ± 10	5.70 ± 0.11	123 ± 12	5.91 ± 0.14
Salbutamol	142 ± 29	5.16 ± 0.18*	107 ± 16	6.13 ± 0.12
Salmeterol	114 ± 15	5.20 ± 0.16*	115 ± 7	5.49 ± 0.12
RP 58802	104 ± 10	5.05 ± 0.20*	158 ± 4	6.08 ± 0.18

Bronchial preparations were initially precontracted with a single dose of histamine (50 μM) and washed until basal tone was re-established. After preincubation with Tyrode solution or Tyrode solution containing the β_2-adrenoceptor agonist (10 μM; 20 min), histamine concentration-effect curves were performed either immediately after treatment (Immediate) or after treatment followed by a 4 h washing period. Maximal contractions (E_{max}) to histamine obtained from concentration-effect curves are expressed as percentage of initial histamine precontraction. Values are means ± s.e.mean obtained from 3–4 lung samples. *Significantly different from control ($P<0.05$; Student's *t*-test).

Table 3 Effect of β_2-adrenoceptor agonists on acetylcholine (ACh) or leukotriene C_4 (LTC_4) concentration-effect curves in human airways

	ACh response		*LTC_4 response*	
	E_{max} (%)	pD_2 *values*	E_{max} (%)	pD_2 *values*
Control	134 ± 8	4.53 ± 0.25	139 ± 29	8.05 ± 0.10
Salbutamol	145 ± 10	4.07 ± 0.12	113 ± 20	7.49 ± 0.33
Salmeterol	124 ± 6	4.14 ± 0.13	150 ± 35	7.67 ± 0.27
RP 58802	154 ± 15	4.34 ± 0.32	110 ± 9	7.63 ± 0.18

Bronchial preparations were initially precontracted with histamine (50 μM) and washed until basal tone was re-established. After incubation with Tyrode solution or Tyrode solution containing the β_2-adrenoceptor agonist (10 μM; 20 min), ACh or LTC_4 cumulative concentration-effect curves were produced. Maximal contractions (E_{max}) to ACh and LTC_4 obtained from concentration-effect curves are expressed as percentage of initial histamine precontraction. Values are means ± s.e.mean obtained from 5–8 lung samples.

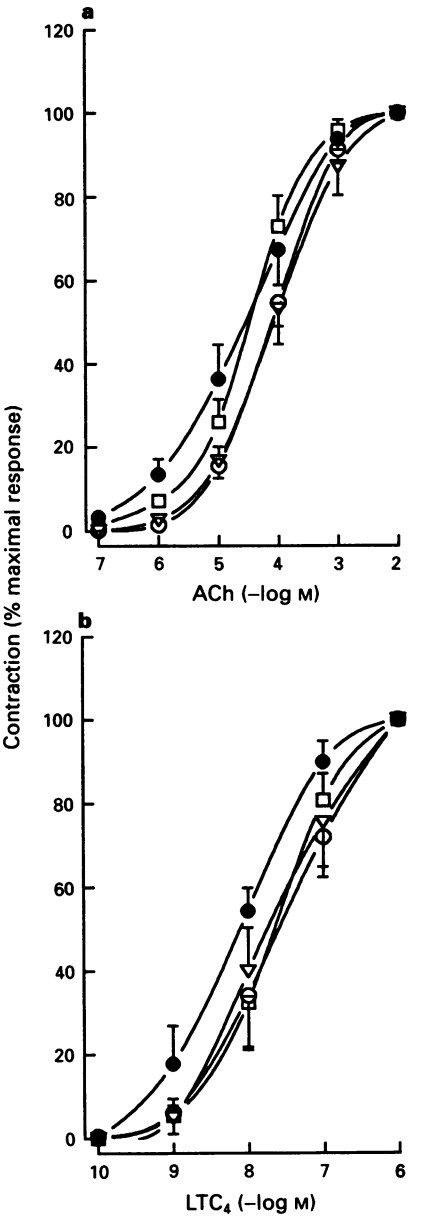

Figure 5 Effects of β_2-adrenoceptor agonists on concentration-effect curves to acetylcholine (ACh) (a) or leukotriene C_4 (LTC_4) (b) in human airways. All curves were obtained after incubation (20 min) with Tyrode solution (●, control) or Tyrode solution containing 10 μM salbutamol (○), salmeterol (▽) or RP 58802 (□). Results are expressed as percentage of agonist maximal response. Values are means ± s.e. mean obtained from 5–8 lung samples.

histamine-induced tone. The pD_2 value for salmeterol was found to be different from that previously reported (Ball *et al.*, 1991; Nials *et al.*, 1993). An inverse relationship appears to exist between the degree of agonist-induced contraction and the ability of β_2-agonists to elicit relaxation (Buckner & Saini, 1975; Torphy *et al.*, 1983; Van Amsterdam *et al.*, 1990). Hence, the variations in the pD_2 values observed with β_2-adrenoceptor agonists may, in part, be due to the contraction of smooth muscle by different concentrations of contractile agents. Whether or not differences in potency of relaxant agonist may also be related to the methods used to establish the relaxations has not adequately been explored. A previous report (Dougall *et al.*, 1991) has shown that salmeterol is a partial agonist in guinea-pig airways, and is similar in potency to sabutamol (Lindén *et al.*, 1993). Data derived from human airways (present paper) confirm this observation, since salmeterol produced approximately 50% reversal of histamine contraction.

Previous reports have shown that anti-IgE-induced contraction in human airways produced a response which ranged between 59% to 83% of an acetylcholine (100 μM; Björck & Dahlén, 1993) or methacholine (50 μM; Jones *et al.*, 1989)-induced contraction. Gorenne and coworkers (1994) have reported that the response to anti-IgE was 46% to 68% of that to acetylcholine (100 μM). In the present report the anti-IgE response was approximately 75% of that to histamine (50 μM, 2.61 ± 0.48 g; immediate stimulation) and 81% of that to histamine (50 μM, 2.32 ± 0.63 g; stimulation at 4 h). These data suggest that the magnitude of the IgE-induced contraction is comparable with a contractile agonist response and is dependent upon inflammatory mediator release (Björck & Dahlén, 1993; Gorenne *et al.*, 1994).

However, few studies in airways have been performed where β_2-agonists have been evaluated against immunological contractions. The present report shows a marked inhibition of anti-IgE-induced contractions in human airways. These results are consistent with previous reports where β_2-mimetics inhibited the release of mediators during anaphylactic challenge in either isolated mast cells (Church & Hiroi, 1987), or human lung parenchymal tissues (Assem & Schild, 1969; Butchers *et al.*, 1979; 1991; Hughes *et al.*, 1983). The inhibition of mediator release was dependent on the β_2 subtype of adrenoceptors (Assem & Schild, 1969; Butchers *et al.*, 1980). Butchers *et al.* (1991) demonstrated *in vitro* that salmeterol

had a prolonged inhibitory effect on antigen-induced mediator release from human lung. However, measurements of mediator release during anti-IgE stimulation after treatment with β_2-agonists in human bronchial preparations remain to be established. The anti-IgE-induced contractions in human airways were also blocked by salmeterol even after 4 h (present paper). Several *in vivo* studies (Ullman & Svedmyr, 1988; Twentyman *et al.*, 1990; Taylor *et al.*, 1992) demonstrated the prolonged inhibitory action of salmeterol on both the early and late phase bronchoconstriction observed in asthmatic patients after antigen inhalation.

Airway smooth muscle reactivity following β_2-adrenoceptor agonists activity was examined by Hay and coworkers (1988) who reported that salbutamol treatment, in guinea-pig trachea, caused a small shift in carbachol dose-response curves without modifying the maximal response. In human airways, Advenier *et al.* (1991) demonstrated no inhibition of either the ACh or histamine contractions following treatment with salbutamol. The results presented in this paper show that histamine-induced contractions were significantly displaced to the right while ACh response curves were not altered.

Pretreatment of guinea-pig airway smooth muscle with high concentrations of salbutamol markedly inhibited the contractions to LTC_4 and LTD_4 (Armour *et al.*, 1982), data which were confirmed by Hay and coworkers (1988). Thus the leukotrienes which are potent airway constrictors (Dahlén *et al.*, 1983) appear to be more susceptible to inhibition by β_2-adrenoceptor agonists in guinea-pig airways. However, LTC_4-induced contractions in human isolated airways (present paper) were not modified even at β_2-adrenoceptor agonist concentrations which were 10 fold higher than reported for guinea-pig tissues. The differences observed for LTD_4 inhibition induced by the β_2-adrenoceptor agonists may be due to the species from which the airway muscle preparations are derived. The reason for this difference is not clear at present. While β_2-adrenoceptor agonists are known to relax basal and induced tone of human airways (Whicker *et al.*, 1988; present paper), the reactivity to several contractile agonists appears not to be inhibited by the β_2-agonists.

The data presented in the present paper using isolated human airways indirectly suggest that the mechanism by which β_2-adrenoceptor agonists prevent IgE-mediated contraction may be due to inhibition of endogenous mediator release, as has been previously suggested (Butchers *et al.*, 1991), rather than to alterations of smooth muscle contraction.

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4, 6-Dibromo-3-hydroxycarbazole (an analogue of caffeine-like Ca^{2+} releaser), a novel type of inhibitor of Ca^{2+}-induced Ca^{2+} release in skeletal muscle sarcoplasmic reticulum

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1 4,6-Dibromo-3-hydroxycarbazole (DBHC) was synthesized as an analogue of bromoeudistomin D (BED), a powerful Ca^{2+} releaser, and its pharmacological properties were examined.

2 In Ca^{2+} electrode experiments, DBHC (100 μM) markedly inhibited Ca^{2+} release from the heavy fraction of sarcoplasmic reticulum (HSR) induced by caffeine (1 mM) and BED (10 μM).

3 DBHC (0.1 to 100 μM) inhibited $^{45}Ca^{2+}$ release induced by Ca^{2+} from HSR in a concentration-dependent manner.

4 DBHC (100 μM) abolished $^{45}Ca^{2+}$ release induced by caffeine (1 mM) and BED (10 μM) in HSR.

5 Inhibitory effects of calcium-induced calcium release (CICR) blockers such as procaine, ruthenium red and Mg^{2+} on $^{45}Ca^{2+}$ release were clearly observed at Ca^{2+} concentrations from pCa 7 to pCa 5.5, and were decreased at Ca^{2+} concentrations higher than pCa 5.5 or lower than pCa 7. However, DBHC decreased Ca^{2+} release induced by Ca^{2+} over the wide range of extravesicular Ca^{2+} concentrations.

6 [^{3}H]-ryanodine binding to HSR was suppressed by ruthenium red, Mg^{2+} and procaine, but was not affected by DBHC up to 100 μM.

7 [^{3}H]-ryanodine binding to HSR was enhanced by caffeine and BED. DBHC antagonized the enhancement in a concentration-dependent manner.

8 9-[^{3}H]-Methyl-7-bromo-eudistomin D, an ^{3}H-labelled analogue of BED, specifically bound to HSR. Both DBHC and caffeine increased the K_D value without affecting the B_{max} value, indicating a competitive mode of inhibition.

9 These results suggest that DBHC binds to the caffeine binding site to block Ca^{2+} release from HSR. This drug is a novel type of inhibitor for the CICR channels in SR and may provide a useful tool for clarifying the Ca^{2+} releasing mechanisms in SR.

Keywords: 4,6-Dibromo-3-hydroxycarbazole; bromoeudistomin D; caffeine; skeletal muscle; sarcoplasmic reticulum; Ca^{2+}-induced Ca^{2+} release; ruthenium red; procaine; Mg^{2+}; ryanodine binding

Introduction

Ca^{2+} release from the sarcoplasmic reticulum (SR) plays a key role in excitation-contraction coupling (EC-coupling) in skeletal muscle (Ford & Podolsky, 1972; Endo, 1977; Endo *et al.*, 1981; Kirino & Shimizu, 1982; Ebashi, 1991). It is well known that ryanodine, a plant alkaloid, promotes Ca^{2+} release from skeletal and cardiac SR and interferes with the inactivation of Ca^{2+}-induced Ca^{2+} release (CICR) from SR (Meissner, 1986). The alkaloid binds with high affinity to a receptor localized in the heavy fraction of sarcaplasmic reticulum (HSR) (Fleischer *et al.*, 1985). The purified ryanodine receptor (Inui *et al.*, 1987; Imagawa *et al.*, 1987; Lai *et al.*, 1988; Wagenknecht *et al.*, 1989) is identical in morphology with the 'feet' structures that span the transverse tubule-SR junction and form caffeine-sensitive Ca^{2+} channels. It has been reported that ryanodine locks the Ca^{2+} release channels of SR in an open state and that its high affinity binding site is localized in terminal cisternae of SR (Fleischer *et al.*, 1985). These studies revealed that the ryanodine receptor is identical with CICR channels of SR (McPherson & Campbell, 1993; Sorrentino & Volpe, 1993). One of the useful approaches that may achieve a better understanding of the molecular mechanism of Ca^{2+} release is the application of specific drugs that affect the releasing mechanisms.

It has been reported that caffeine increases the Ca^{2+} sensitivity of CICR channels (Nagasaki & Kasai, 1984; Endo, 1985) and the open probability of the channels at saturating Ca^{2+} concentrations (Rousseau *et al.*, 1988). Numerous studies using skinned skeletal muscle fibres and isolated SR membrane preparations have revealed the presence of a caffeine-sensitive Ca^{2+} release pathway through CICR channels (Kim *et al.*, 1983). However, the characterization of the caffeine receptor site in Ca^{2+} release channels has not been possible because of its low affinity and the detailed molecular mechanism of Ca^{2+} release from SR remains unresolved. We have reported that bromoeudistomin D (BED), a derivative of eudistomin D isolated from the Caribbean tunicate *Eudistoma olivaceum*, induces Ca^{2+} release from the heavy fraction of SR (HSR) (Nakamura *et al.*, 1986). Our pharmacological studies indicate that BED is approximately 500 times more potent than caffeine in Ca^{2+} releasing activity. For the purpose of finding the inhibitor in order to investigate the function of CICR channels, numerous analogues of BED were synthesized (Kobayshi *et al.*, 1984; 1989; Takahashi *et al.*, unpublished observations). Here we present the first report that 4,6-dibromo-3-hydroxycarbazole (DBHC), one of these analogues, is a novel type of CICR-channel inhibitor having properties different from those of typical inhibitors such as procaine, ruthenium red and Mg^{2+}.

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Methods

Preparation of SR vesicles

HSR was prepared from skeletal muscle of male rabbits (Japanese white rabbits weighing about 2–3 kg), according to the method of Kim *et al.* (1983) in the presence of protease inhibitors: aprotinin (76.8 mM), *p*-APMSF (0.1 mM) and benzamidine (0.83 mM). Rabbit white muscle was homogenized in 5 volumes of 5 mM Tris-maleate (pH 7.0) and centrifuged at 5000 *g* for 15 min. The supernatant was centrifuged at 12 000 *g* for 30 min. The pellet was suspended in 5 mM Tris-maleate (pH 7.0) containing 90 mM KCl and centrifuged at 70 000 *g* for 40 min. The pellet (the HSR) was resuspended. These procedures were performed at 0–4°C. HSR was used within four days. The protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Ca^{2+} electrode experiments

The concentration of extravesicular Ca^{2+} in the HSR suspension was measured at 30°C with a Ca^{2+} electrode as described previously (Seino *et al.*, 1991). The Ca^{2+} electrode showed Nernstian response (slope, 27–29 mV pCa^{-1} unit) in the calibration buffer containing Ca^{2+}-EGTA between pCa decreased from 6 to 4. The assay solution (final volume, 1 ml) contained 0.05 mM $CaCl_2$, 90 mM KCl, 0.25 mM $MgCl_2$, 50 mM MOPS-Tris (pH 7.0), 1 mg ml^{-1} of HSR, 5 mM creatine phosphate, 0.13 mg ml^{-1} of creatine kinase and 0.5 mM ATP. The Ca^{2+} uptake reaction was initiated by simultaneous addition of creatine kinase and ATP.

$^{45}Ca^{2+}$ release measurement

$^{45}Ca^{2+}$ release from HSR passively preloaded with $^{45}Ca^{2+}$ was measured at 0°C according to the method of Nakamura *et al.* (1986). HSR (20 mg ml^{-1}) was preincubated in a solution containing 5 mM $^{45}CaCl_2$ at 0°C over 12 h. $^{45}Ca^{2+}$ release was started by diluting the solution 100 fold with the reaction medium containing various concentrations of Ca^{2+} or different reagents. Free Ca^{2+} concentration was controlled with Ca^{2+}-EGTA buffer. The calculation of free Ca^{2+} was accomplished by a computer programme (Fabiato *et al.*, 1979). The reaction was stopped by adding the solution containing 5 mM $LaCl_3$ and 5 mM $MgCl_2$ at an appropriate time interval. The mixture was filtered through Millipore Filters (HAWP type, 0.45 μm pore size) and washed with 10 times its volume solution containing 5 mM $LaCl_3$ and 5 mM $MgCl_2$. The amounts of $^{45}Ca^{2+}$ in HSR were measured by counting the radioactivity remaining on the filters. The reaction medium contained (mM): $CaCl_2$ 0.5, KCl 90, 4-morpholinepropanesulphonic acid (MOPS) 50 at pH 7.0.

[^{3}H]-ryanodine and 9-[^{3}H]-methyl-7-bromoeudistomin D binding assay

[^{3}H]-ryanodine binding to HSR was measured according to the method of Seino *et al.* (1991). HSR (300 μg ml^{-1}) was incubated with 10 nM [^{3}H]-ryanodine in the reaction medium at 37°C for 45 min. Bound and free [^{3}H]-ryanodine were separated by filtration through glass fibre filters (Whatman) under reduced pressure. The filters were washed five times with 2 ml of ice-cold water. Radioactivity that remained on the filter was determined by liquid scintillation counting. Nonspecific binding was determined in the presence of 10 μM unlabelled ryanodine. The reaction medium contained (mM): sucrose 300, NaCl 1000, dithiothreitol 2, $CaCl_2$ 0.2, EGTA 0.192, *p*-APMSF 0.1 and HEPES/Tris 20 at pH 7.4 and 37°C.

9-[^{3}H]-methyl-7-bromoeudistomin D ([^{3}H]-MBED) binding experiments were performed by the method of Fang *et al.* (1993). HSR (300 μg ml^{-1}) was incubated with various concentrations of [^{3}H]-MBED in the reaction medium at 0°C for 45 min. Separation of bound and free [^{3}H]-MBED was carried out as described above. Nonspecific binding was determined in the presence of 5 μM unlabelled MBED. The reaction medium contained (mM): sucrose 300, dithiothreitol 2, $CaCl_2$ 0.2, EGTA 0.192, *p*-APMSF 0.1 and HEPES/Tris 20 at pH 7.4 and 37°C.

Drugs

DBHC (Figure 1) was synthesized as follows. 3-Methoxycarbazole was synthesized by bromination of carbazole with bromine in pyridine followed by methoxylation with NaOMe/MeOH and dimethylformamide catalyzed by CuI. DBHC was synthesized by demethylation of 3-methoxycarbazole with BBr_3 in CH_2Cl_2 followed by bromination with NBS in acetic acid. ^{1}H NMR (acetone-d_6) δ10.56

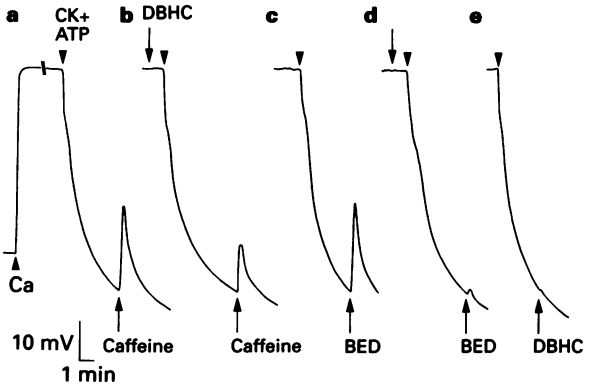

Figure 2 Effects of the preincubation of HSR with DBHC on Ca^{2+} release induced by caffeine (1 mM) and BED (10 μM). The concentrations of extravesicular Ca^{2+} were monitored at 30°C with a Ca^{2+} electrode in the assay solution containing 0.05 mM $CaCl_2$, 90 mM KCl, 0.25 mM $MgCl_2$, 50 mM MOPS-Tris (pH 7.0), 1 mg ml^{-1} of HSR, 5 mM creatine phosphate, 0.13 mg ml^{-1} of creatine kinase (CK) and 0.5 mM ATP. After addition of 0.05 mM $CaCl_2$, the reaction of Ca^{2+} uptake was started by a simultaneous addition of CK and ATP. Vertical calibration bar indicates a response for voltage change (10 mV) corresponding to 0.5 pCa unit. In (b) to (d) DBHC (100 μM) was added before addition of CK and ATP. For abbreviations in this and subsequent figures, please see text.

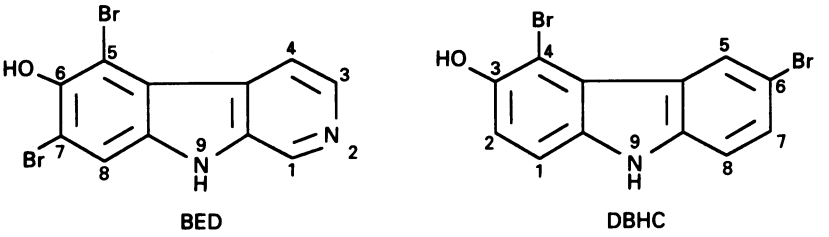

Figure 1 Chemical structure of bromoeudistomin D (BED) and 4,6-dibromo-3-hydroxycarbazole (DBHC).

(1H, br, s), 8.82 (1H, s), 7.55 (3H, m) and 7.18 (1H, d, $J = 8.8$ Hz); EIMS m/z 343, 341 and 339 (M+); Found m/z 338.8899, Calcd for $C_{12}H_7ON^{79}Br_2$: M, 338.8895. BED and MBED were synthesized as reported previously (Kobayashi et al., 1988). [^{3}H]-MBED was synthesized as described by Fang et al. (1993). $^{45}CaCl_2$ (1 Ci ml^{-1}) and [9, 21-^{3}H(N)]-ryanodine (54.7 Ci mmol^{-1}) were purchased from Du Pont New England Nuclear, Boston, MA, U.S.A. Ryanodine was purchased from S.B. Penick Company, New York, NY, U.S.A. Caffeine, procaine hydrochloride, and ruthenium red were purchased from Wako Pure Chemical Industries, Osaka, Japan. Other reagents used were of analytical grade.

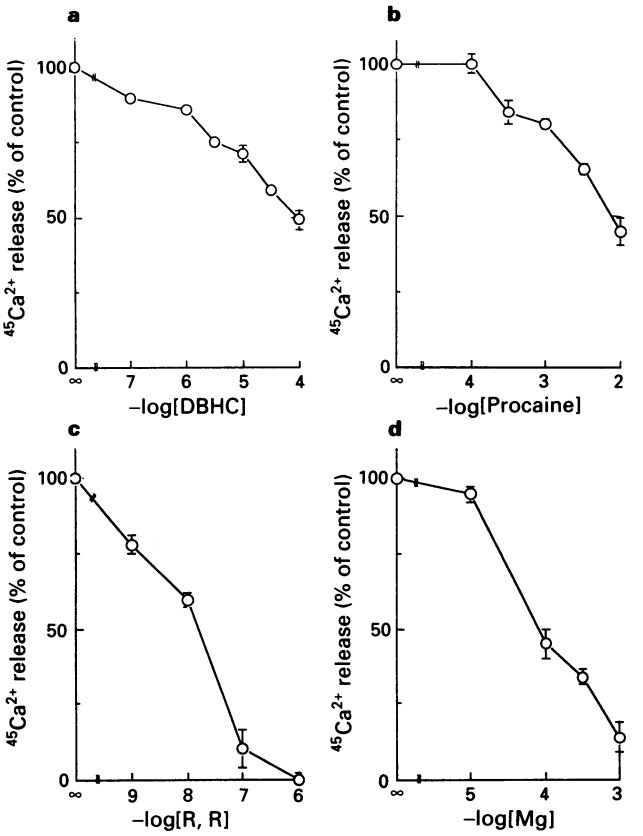

Figure 3 Effects of CICR inhibitors on $^{45}Ca^{2+}$ release induced by Ca^{2+} from skeletal muscle HSR. $^{45}Ca^{2+}$ release was measure at pCa 7.0, as described under Methods. The $^{45}Ca^{2+}$ content in HSR was measured at 0°C by the filtration method after 100 fold dilution of HSR (20 mg ml^{-1}) passively preloaded with $^{45}CaCl_2$ (5 mM) into Ca^{2+}-EGTA buffer medium. The initial $^{45}Ca^{2+}$ content of HSR was obtained by using the release medium containing 5 mM $LaCl_3$ and 5 mM $MgCl_2$. The Ca^{2+} release activity was calculated from the decrease in the $^{45}Ca^{2+}$ content in HSR vesicles during 1 min after dilution. $^{45}Ca^{2+}$ release activity was normalized against that in the absence of the inhibitors. (a) DBHC, (b) procaine, (c) ruthenium red (RR) and (d) Mg^{2+}. Data are mean ± s.e.mean (bars) values ($n = 6$).

Statistical comparison

Results of the experiments are expressed as mean ± s.e.mean. Student's t test and paired t test were used for statistical analysis of the results.

Results

Effects of DBHC on Ca^{2+} release from skeletal muscle HSR

In the course of our survey of CICR inhibitors in natural products and their derivatives, we have succeeded in finding DBHC. In electrode experiments, preincubation of DBHC

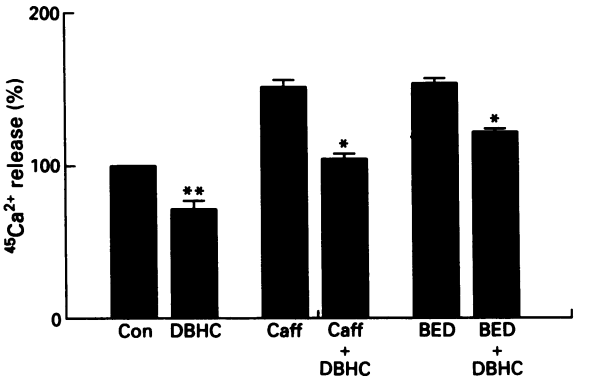

Figure 5 Effects of DBHC on $^{45}Ca^{2+}$ release induced by Ca^{2+}, caffeine (caff) and BED. $^{45}Ca^{2+}$ release was measured as described under Methods. The amount of released $^{45}Ca^{2+}$ was calculated from the decrease in the $^{45}Ca^{2+}$ content in HSR during 1 min after dilution at pCa 7.0. The concentrations of caffeine, BED and DBHC were 1 mM, 10 μM and 100 μM, respectively. Data are mean ± s.e.mean ($n = 6$). **Significantly ($P < 0.05$) different from the control values. *Significantly ($P < 0.05$) different from the values in the presence of caffeine or BED alone.

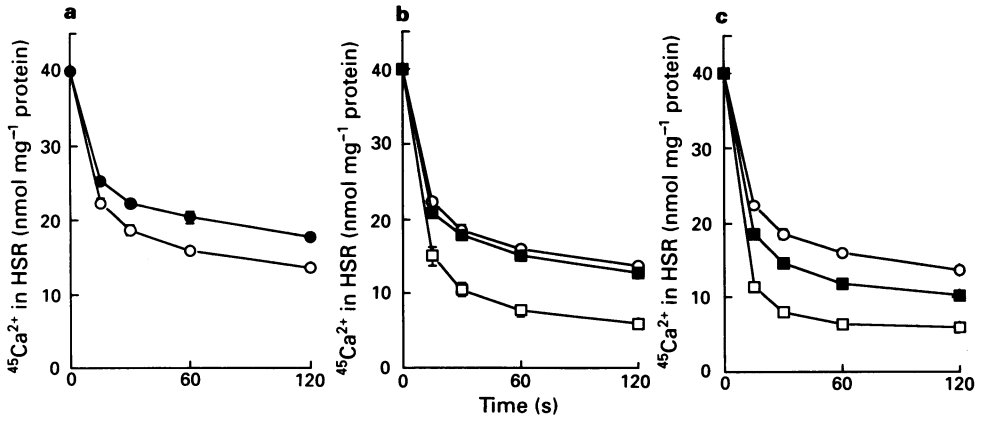

Figure 4 Effects of DBHC on the time course of the $^{45}Ca^{2+}$ release induced by Ca^{2+} (a), caffeine (b) and BED (c) from skeletal muscle HSR at pCa 7.0. The time course of the decrease in the $^{45}Ca^{2+}$ content in HSR was measured after 100 fold dilution of HSR (20 mg ml^{-1}) passively preloaded with $^{45}CaCl_2$ (5 mM) into Ca^{2+}-EGTA buffer medium. (a) Control (○), 100 μM DBHC (●); (b) control (○), 1 mM caffeine (□), 1 mM caffeine and 100 μM DBHC (■); (c) control (○), 10 μM BED (□), 10 μM BED and 100 μM DBHC (■). Data are mean ± s.e.mean values ($n = 6$).

(100 μM) with HSR resulted in the marked decrease in Ca^{2+} release induced by caffeine (1 mM) or BED (10 μM) (Figure 2a–2d). DBHC alone (100 μM) did not induce Ca^{2+} release (Figure 2e). Interestingly, DBHC was synthesized as an analogue of BED, a powerful inducer of CICR with caffeine like properties (Nakamura *et al.*, 1993). Therefore, the inhibitory effect of DBHC was investigated in comparison with the representative CICR inhibitors such as procaine, ruthenium red and Mg^{2+}. As shown in Figure 3, DBHC and these CICR inhibitors inhibited $^{45}Ca^{2+}$ release induced by Ca^{2+} from HSR in a dose-dependent manner. Their IC_{50} values were 30 μM, 3.5 mM, 15 nM and 58 μM for DBHC,

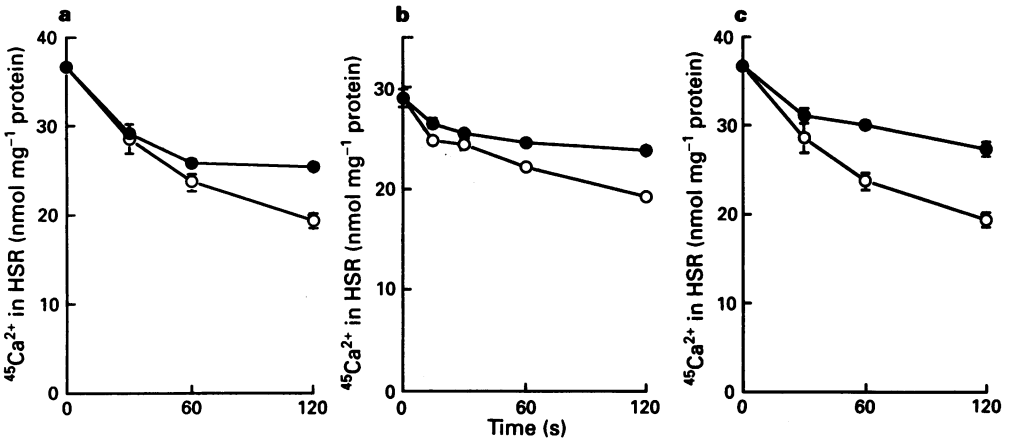

Figure 6 Effects of CICR inhibitors in the time course of the $^{45}Ca^{2+}$ release induced by Ca^{2+} from skeletal muscle HSR at pCa 7.0. The time course of the decrease in the $^{45}Ca^{2+}$ content in HSR vesicles was measured after 100 fold dilution of 20 mg ml^{-1} HSR preloaded with 5 mM $^{45}CaCl_2$ into Ca^{2+}-EGTA buffer medium: (○) control; (●) 3 mM procaine (a) or 30 nM ruthenium red (b) or 100 μM Mg^{2+} (c). Data are mean ± s.e.mean ($n = 6$).

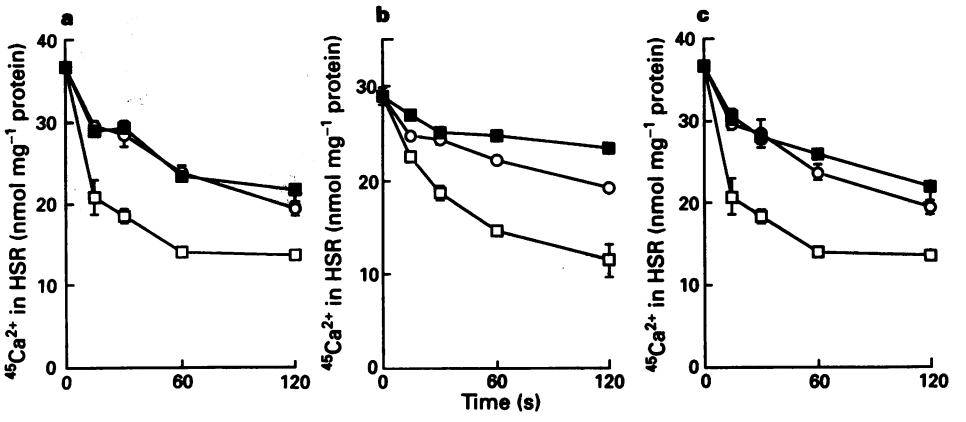

Figure 7 Effects of CICR inhibitors on the time course of the $^{45}Ca^{2+}$ release induced by caffeine from skeletal muscle HSR at pCa 7.0. Experimental protocol was similar to that described in Figure 3: (○) control; (□) 1 mM caffeine; (■) 3 mM procaine and 1 mM caffeine (a) or 30 nM ruthenium red and 1 mM caffeine (b) or 100 μM Mg^{2+} and 1 mM caffeine (c). Data are mean ± s.e.mean ($n = 3$).

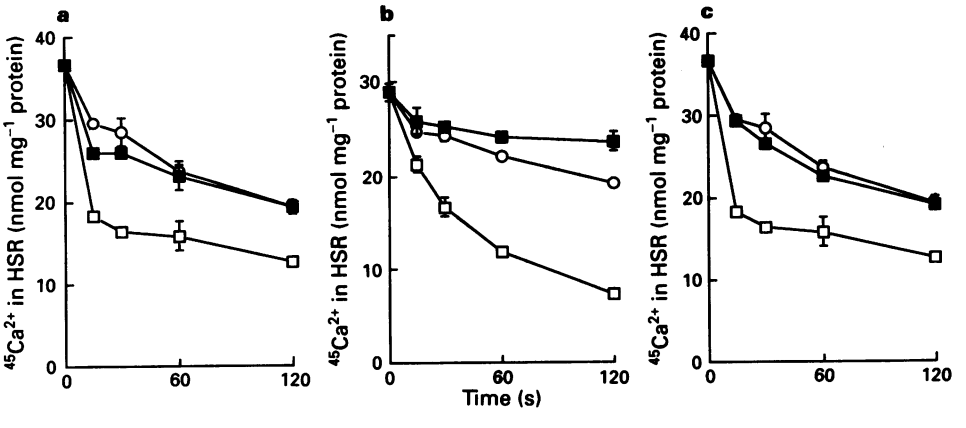

Figure 8 Effects of CICR inhibitors on the time course of the $^{45}Ca^{2+}$ release induced by BED from skeletal muscle HSR at pCa 7.0. Experimental protocol was similar to those described in Figure 3: (○) control; (□) 10 μM BED; (■) 3 mM procaine and 10 μM BED (a) or 30 nM ruthenium red and 10 μM BED (b) or 100 μM Mg^{2+} and 10 μM BED (c). Data are mean ± s.e.mean ($n = 3$).

procaine, ruthenium red and Mg^{2+}, respectively. Figure 4 depicts the time courses of inhibitory effects of DBHC on $^{45}Ca^{2+}$ release induced by Ca^{2+} (Figure 4a), caffeine (Figure 4b) or BED (Figure 4c) at the extravesicular Ca^{2+} concentration of 0.1 μM. The maximum inhibitory response to DBHC (100 μM) of $^{45}Ca^{2+}$ release induced by Ca^{2+} (0.1 μM) was obtained at 30 s and those by caffeine (1 mM) and BED (10 μM) at 15 s. Figure 5 shows that DBHC significantly inhibited $^{45}Ca^{2+}$ release induced by Ca^{2+}, caffeine and BED by approximately 25–35% at 60 s. CICR inhibitors, such as procaine (3 mM), ruthenium red (30 nM) and Mg^{2+} (100 μM) also inhibited $^{45}Ca^{2+}$ release induced by Ca^{2+} (Figure 6), caffeine (Figure 7) or BED (Figure 8).

DBHC (30 μM) maintained its inhibitory effect on $^{45}Ca^{2+}$ release induced by Ca^{2+} over the wide range from pCa 7.0 to pCa 4.0 (Figure 9a). On the other hand, procaine (3 mM), ruthenium red (30 nM) and Mg^{2+} (30 μM) powerfully suppressed Ca^{2+} release Ca^{2+} concentrations from pCa 7 to pCa 5.5, whereas the effects were decreased at Ca^{2+} concentrations higher than pCa 5.5 or lower than pCa 7 (Figure 9b–d).

Effects of CICR inhibitors on [^{3}H]-ryanodine binding to HSR

[^{3}H]-ryanodine binding to HSR was inhibited by procaine (Figure 10b), ruthenium red (Figure 10c) and Mg^{2+} (Figure 10d) in a concentration-dependent manner with IC_{50} values of 8.4 mM, 1.5 μM and 34 mM, respectively. However, DBHC had no effect on [^{3}H]-ryanodine binding to HSR up to 100 μM (Figure 10a). It is well known that [^{3}H]-ryanodine binding is dependent on Ca^{2+} concentrations. Figure 11 depicts the effects of DBHC and procaine on [^{3}H]-ryanodine binding to HSR at various Ca^{2+} concentrations. In the absence of these inhibitors, [^{3}H]-ryanodine binding increased with increase in Ca^{2+} concentrations and reached a plateau at about pCa 5. Procaine suppressed the binding over a wide range of Ca^{2+} concentrations (pCa 8–pCa 5), whereas DBHC had no inhibitory effect.

Ca^{2+} releasers such as caffeine and MBED increase the amount of [^{3}H]-ryanodine bound to CICR (Seino *et al.*, 1991). [^{3}H]-ryanodine binding increased by 21% and 41% in the presence of 2 mM caffeine and 5 μM BED, respectively (Figure 12). DBHC antagonized the enhancement of the binding in a concentration-dependent manner, suggesting that the DBHC binds to the caffeine/BED binding site in HSR.

Effects of DBHC on [^{3}H]-MBED binding to HSR

We have recently reported that 9-[^{3}H]-methyl-7-bromoeudistomin D ([^{3}H]-MBED), an ^{3}H-labelled analogue of BED shares the same binding site as that of caffeine in terminal cisternae of skeletal muscle SR (Fang *et al.*, 1993). As shown in Figure 13a, specific binding of [^{3}H]-MBED to HSR was saturable and of high affinity. Scatchard analysis showed that [^{3}H]-MBED bound to a high affinity receptor site with $K_D = 39.5$ nM and $B_{max} = 6.1$ pmol mg^{-1} (Figure 13b). Both

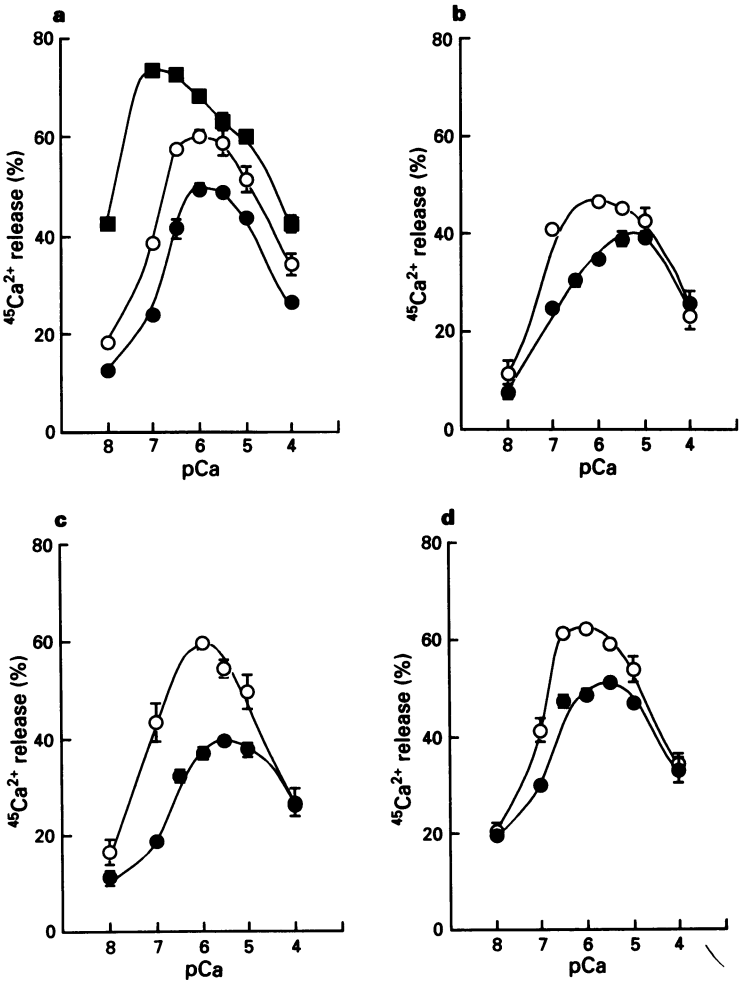

Figure 9 Inhibitory effects of CICR inhibitors on $^{45}Ca^{2+}$ release at various Ca^{2+} concentrations. $^{45}Ca^{2+}$ release at various concentrations of free Ca^{2+} was measured during 1 min after dilution. Each value was normalized against the amount of $^{45}Ca^{2+}$ in HSR at zero time. (a) Control (○), 100 μM DBHC (●), 10 μM BED (■). (b) Control (○), 3 mM procaine (●). (c) Control (○), 30 nM ruthenium red (●). (d) Control (○), 30 μM Mg^{2+} (●). Data are mean ± s.e.mean ($n = 6$).

caffeine (0.5 mM) and DBHC (50 μM) increased the K_D from 39.5 to 64.7 and 94.2 nM, respectively, without affecting the B_{max} value, indicating a competitive mode of interaction between [^{3}H]-MBED and either caffeine or DBHC. These results suggest that DBHC binds to the caffeine/BED-binding site to reduce Ca^{2+} release from HSR.

Discussion

Many compounds are known to inhibit CICR (McPherson & Campbell, 1993). Procaine, ruthenium red and Mg^{2+} are representative inhibitors of CICR. Inhibitory effects of procaine, ruthenium red and Mg^{2+} on $^{45}Ca^{2+}$ release from HSR were dependent on the extravesicular Ca^{2+} concentrations, whereas that of DBHC was not dependent on them. It has been reported that procaine binds to the site which influences the Ca^{2+} sensitivity of the Ca^{2+} regulatory site and that Mg^{2+} inhibits the Ca^{2+}-gated open state of the channels by direct competition with Ca^{2+} at the Ca^{2+}-regulatory site (Pessah *et al.*, 1987). Ruthenium red having a large positive charge (+6) has been reported to bind to the Ca^{2+}-binding site of SR (Corbalan-Garcia *et al.*, 1992), suggesting that the ruthenium red binding site is the Ca^{2+}-binding site in CICR

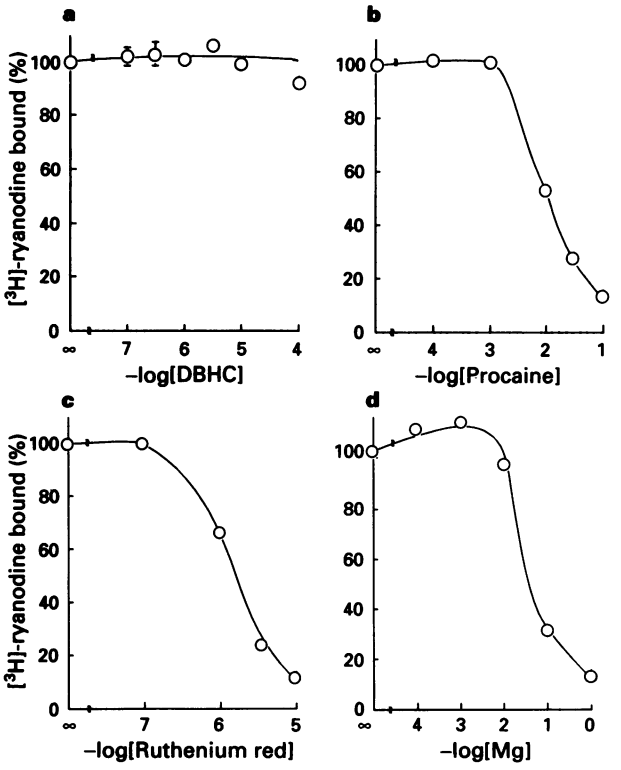

Figure 10 Effects of CICR inhibitors on [^{3}H]-ryanodine binding to skeletal muscle HSR. HSR (300 μg ml^{-1}) was incubated with 10 nM [^{3}H]-ryanodine and CICR inhibitors at 37°C for 45 min in a solution containing 0.3 M sucrose, 1 M NaCl, 10 μM $CaCl_2$, 2 mM DTT, 100 μM *p*-APMSF and 20 mM HEPES-Tris (pH 7.4). Nonspecific binding was determined in the presence of 10 μM unlabelled ryanodine. (a) DBHC, (b) procaine, (c) ruthenium red, (d) Mg^{2+}. Data are mean ± s.e.mean ($n = 4$).

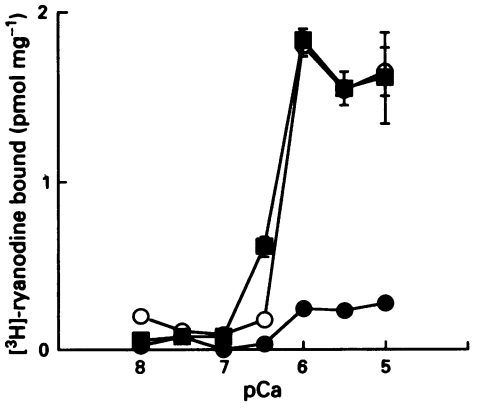

Figure 11 Effects of DBHC and procaine on [^{3}H]-ryanodine binding at various Ca^{2+} concentrations. HSR (300 μg ml^{-1}) was incubated with 10 nM [^{3}H]-ryanodine for 45 min at various Ca^{2+} concentrations in the absence (■) or presence of DBHC (100 μM) (○) or procaine (10 mM) (●). Data are mean ± s.e.mean ($n = 6$).

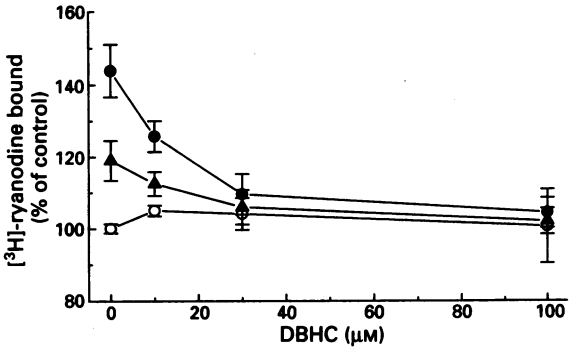

Figure 12 Effects of DBHC on the [^{3}H]-ryanodine binding to HSR enhanced by caffeine (▲) or BED (●). HSR (300 μg ml^{-1}) were incubated with 10 nM [^{3}H]-ryanodine at 37°C for 45 min in the presence or absence of either caffeine (2 mM) or BED (5 μM) in the solution containing 0.3 M sucrose, 1 M NaCl, 10 μM $CaCl_2$, 2 mM DTT, 100 μM *p*-APMSF and 20 mM HEPES-Tris (pH 7.4).

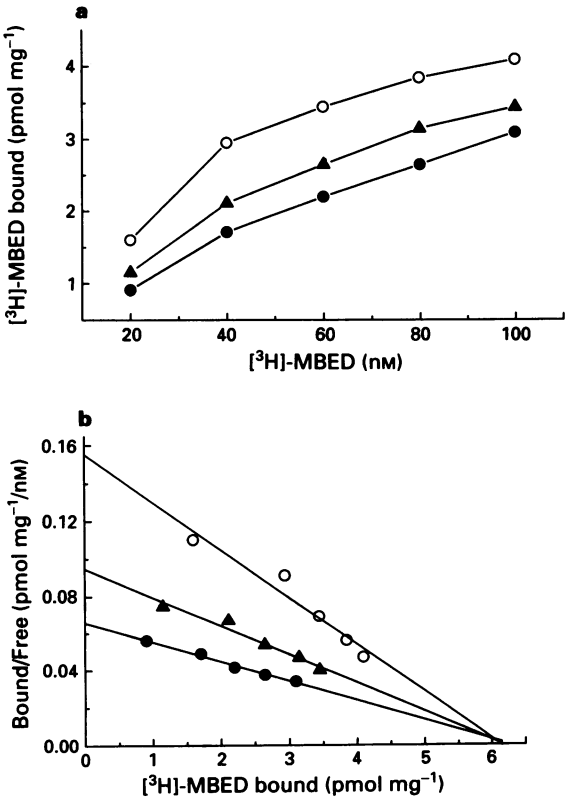

Figure 13 Effects of DBHC and caffeine on [^{3}H]-MBED binding. HSR (300 μg ml^{-1}) was incubated with increasing concentrations of [^{3}H]-MBED from 20 to 100 nM for 45 min at 0°C. (a) [^{3}H]-MBED binding was measured in the presence or absence (○) of 50 μM DBHC (●) or 0.5 mM caffeine (▲) and is plotted. (b) [^{3}H]-MBED binding in (a) is presented as a Scatchard plot.

channels of SR. These reports and our results suggest that the inhibitory effects of ruthenium red, procaine and Mg^{2+} on CICR are modified at high extravesicular Ca^{2+} concentrations because these drugs bind to the Ca^{2+} binding site or the site influenced by Ca^{2+} and that the binding site of DBHC which is thought to be the caffeine binding site is different from those of procaine, ruthenium red and Mg^{2+}. This may be the reason why the inhibitory effects of procaine, ruthenium red and Mg^{2+} on CICR channels are suppressed at high Ca^{2+} concentrations. DBHC is a novel type of CICR inhibitors having unique pharmacological properties.

The activity of the ryanodine receptor/Ca^{2+} releasing channels are modulated by various compounds. Ryanodine and Ca^{2+} have dual effects, *i.e.*, they activate the channels at low concentrations and inhibit them at high concentrations (Meissner, 1986). The binding of [^{3}H]-ryanodine to the Ca^{2+} release channels is enhanced by the compounds which activate the channels and inhibited by other agents which reduce the channel activity (Pessah *et al.*, 1987; Michalak *et al.*, 1988). Our results are in agreement with a previous observation that CICR activators such as caffeine and BED increase [^{3}H]-ryanodine binding to HSR and CICR inhibitors such as procaine, ruthenium red and Mg^{2+} decrease it (Imagawa *et al.*, 1987; Pessah *et al.*, 1987; Seino *et al.*, 1991). The inhibitory effect on [^{3}H]-ryanodine binding was strongly suppressed by Ca^{2+} (Figure 11). However, DBHC (0.1 to 100 μM) did not affect [^{3}H]-ryanodine binding. Enhancement of [^{3}H]-ryanodine binding to HSR by caffeine or BED was inhibited by DBHC, probably indicating the interference of caffeine/BED binding by DBHC.

It has been recognised that caffeine activates CICR channels of skeletal muscle SR (Endo, 1977). Caffeine potentiates Ca^{2+} sensitivity of CICR channels (Endo, 1975), increases [^{3}H]-ryanodine binding to HSR (Pessah *et al.*, 1987) and induces a contraction of the chemically skinned fibres from skeletal muscle (Nakamura *et al.*, 1986). We have found that BED is approximately 500 times more potent than caffeine in Ca^{2+}-releasing activity (Nakamura *et al.*, 1986). BED has pharmacological properties similar to those of caffeine in its action such as potentiating Ca^{2+} sensitivity (Figure 9a), increasing [^{3}H]-ryanodine binding (Figure 12) and inducing the contractions of skinned fibres (Nakamura *et al.*, 1986). Interestingly, our present studies indicate that DBHC, an analogue of BED inhibits $^{45}Ca^{2+}$ release from HSR induced Ca^{2+}, caffeine or BED and that DBHC blocks [^{3}H]-MBED binding in a competitive mode. On the basis of our observations, it is suggested that DBHC binds to the caffeine/BED binding site to block Ca^{2+} release from HSR. DBHC has been revealed to be a CICR inhibitor with novel pharmacological properties and may provide valuable information about the functions of CICR channels and excitation-contraction (E-C) coupling of skeletal muscle.

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Pharmacology of a non-selective ET_A and ET_B receptor antagonist, TAK-044 and the inhibition of myocardial infarct size in rats

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1 The aims of the present study were to characterize the pharmacological profile of a new endothelin (ET) receptor antagonist, TAK-044 and to consider whether it limits the extension of myocardial infarct size in rats.

2 Binding of [^{125}I]-ET-1 to ET receptors on rabbit ventricular and cerebellar membrane fractions was inhibited by TAK-044 with IC_{50} values of 3.8 nM and 130 nM, respectively.

3 It inhibited ET-1, ET-2 and ET-3-induced vasoconstriction of porcine isolated coronary arteries in a competitive (ET-1, ET-2) and a non-competitive (ET-3) manner.

4 In the rat *in vivo*, the ET-1-induced blood pressure changes including transient hypotension followed by sustained hypertension, were inhibited by TAK-044 (0.1–10 mg kg^{-1}, i.v.) in a dose-dependent manner.

5 Acute myocardial infarction induced by 1 h coronary occlusion followed by 24 h reperfusion in rats caused an infarct size of 60 ± 2% (n = 12) of the area-at-risk by weight.

6 Intravenous injection of TAK-044 10 min before coronary occlusion reduced the infarct size in a dose-dependent manner: 32% and 54% reductions at 1 and 3 mg kg^{-1}, respectively.

7 TAK-044 administered 10 min before or 1 h after reperfusion (1 mg kg^{-1}, i.v.) showed similar inhibitory effects: 34% and 23% reductions, respectively.

8 We conclude that TAK-044 is an ET_A/ET_B receptor antagonist which shows strong inhibitory effects on the extension of myocardial infarct size after coronary artery occlusion-reperfusion in rats.

Keywords: Endothelin receptor antagonist; TAK-044; acute myocardial infarction; porcine coronary artery contraction; rat blood pressure; ET_A and ET_B receptors

Introduction

Since the first report of endothelin (ET) and its isoforms, ET-1, ET-2 and ET-3, one of the main research issues has been to characterize their pathophysiological role in diseases. Various clinical and animal studies have addressed this (Masaki *et al.*, 1992; Haynes & Webb, 1993 for review) and have indicated that the endothelin, especially ET-1 is involved in diseases such as acute myocardial infarction (Miyauchi *et al.*, 1989; Watanabe *et al.*, 1990; 1991), acute renal failure (Tomita *et al.*, 1989; Shibouta *et al.*, 1990), subarachnoid haemorrhage (Masaoka *et al.*, 1989) and hypertension (Saitoh *et al.*, 1990). Contribution of endogenous endothelin in these conditions was justified mainly on the basis of increased plasma ET-1 levels in patients and animal disease models compared with those in normal subjects. Additional indirect evidence was provided by studies on the effects of exogenous endothelin *in vivo* and *in vitro* and on the depletion of endogenous ET-1 levels by a monoclonal antibody in animal models (Shibouta *et al.*, 1990; Watanabe *et al.*, 1991). However, since the changes in plasma endothelin contents were found to be minimal in most studies, more definitive evidence for the involvement of endothelin in pathophysiological processes, such as results of studies using specific endothelin antagonists have been awaited to clarify the issue.

In addition, understanding of the pathophysiological roles of endothelins is complicated by the existence of ET receptor subtypes, ET_A (Arai *et al.*, 1990) and ET_B (Sakurai *et al.*, 1990). These receptor subtypes showed different sensitivities to the three endothelin isoforms so that ET-1 and ET-2 had high, almost equal, affinities for both ET_A and ET_B receptors (Sakurai *et al.*, 1990), whereas ET-3 had a higher affinity for ET_B than ET_A receptors (Arai *et al.*, 1990). Although ET_A and ET_B receptors were originally assigned to be responsible for ET-1-induced vasoconstriction and vasorelaxation, respectively (Masaki *et al.*, 1992), it has been demonstrated that both receptor subtypes were involved in ET-1-induced vasoconstriction (Sumner *et al.*, 1992; Clozel *et al.*, 1992) as well as hypertension (McMurdo *et al.*, 1993). Since increases in ET-1 levels played an important pathophysiological role in various diseases (e.g. Watanabe *et al.*, 1991), it is reasonable to speculate that both ET_A and ET_B receptors are involved in such conditions.

The recent availability of specific endothelin receptor antagonists, such as BQ-123 (Ihara *et al.*, 1992), FR139317 (Sogabe *et al.*, 1993), IRL1038 (Urade *et al.*, 1992) and Ro 46-2005 (Clozel *et al.*, 1993) and the discovery of a specific ET_B receptor agonist, sarafotoxin S6c (Williams *et al.*, 1991) made it possible to investigate some of the important physiological and pathophysiological roles of endothelins and their receptors described above. Animal studies with endothelin antagonists demonstrated the improvement of functional deterioration induced by acute renal failure (Mino *et al.*, 1992) and by subarachnoid haemorrhage (Clozel *et al.*, 1993) and suggested the involvement of ET_A receptor activation in these diseases. There is, however, little information available as to whether an ET receptor antagonist is useful for treating acute myocardial infarction. Recent animal studies using ET_A antagonists showed controversial results; BQ-123 reduced the acute myocardial infarct size in dogs

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(Grover *et al.*, 1993), whereas FR139317 did not in rabbits (McMurdo *et al.*, 1994).

The present study was, therefore, designed to address the issue whether endothelin receptor antagonists limit the infarct size in rats by use of a newly developed antagonist, TAK-044, cyclo[D-α-aspartyl-3](4-phenylpiperazin-1-yl)carbonyl]-L-alanyl-L-α-aspartyl-D-2-(2-thienyl)glycyl-L-leucyl-D-tryptophyl] disodium (Kikuchi *et al.*, 1994; Kusumoto *et al.*, 1994). First the pharmacological profile of TAK-044 was characterized and it was found to be an ET_A/ET_B receptor antagonist. Effects of TAK-044 on acute myocardial infarction in rats were, then, studied to demonstrate its limiting effects on the extension of infarct size.

Methods

Receptor binding

Cardiac and brain membrane fractions were prepared by the method described previously (Glossmann & Ferry, 1985) with minor modifications. Briefly, ventricular and cerebellar tissues were isolated from New Zealand White rabbits (2.5–3.5 kg), minced and homogenized with a Polytron homogenizer (3/4 maximum speed, 4 times for 10 s each) in 20 mM $NaHCO_3$ solution containing 0.1 mM phenylmethyl sulphonyl fluoride (PMSF). The homogenates were centrifuged at 15,000 *g* for 15 min and the resulting supernatants were centrifuged twice at 45,000 *g* for 15 min. The final pellets of ventricular and cerebellar membrane preparations were each suspended in 50 mM Tris/HCl (pH 7.4) containing 0.1 mM PMSF and stored in a deep freezer (−80°C) until required for use. The protein contents were determined by the method of Lowry *et al.* (1951). All procedures were carried out at −4°C.

Membrane fractions of ventricle (20 μg of protein) or cerebellum (5 μg of protein) were diluted with the assay solution (50 mM Tris/HCl, pH 7.4, 0.2% bovine serum albumin) to make up a final volume of 300 μl. An aliquot of each diluted membrane fraction was incubated with [^{125}I]-ET-1 (60 pM, Amersham) for 90 min at 37°C. The mixture was, then, diluted with chilled assay solution and filtered through a glass fiber filter (GF/B, Whatmann). The radioactivity on each filter was counted with a gamma counter to determine the amount of bound [^{125}I]-ET-1. The non-specific binding was determined in the presence of 0.4 μM ET-1 and the specific binding was defined as the difference between the total and non-specific binding.

Porcine coronary artery preparation

Porcine hearts were obtained from a local slaughter house, and the left anterior descending coronary arteries were isolated to make ring preparations (2-3 mm in length). They were mounted for isometric recording with a resting tension of 2 g in a 20 ml bath containing modified Krebs solution (95%O_2 5%CO_2, pH 7.4, 36°C), the composition of which in mM was: NaCl 113, KCl 4.6, $CaCl_2$ 1.2, $NaHCO_3$ 22, NaH_2PO_4 3.5, $MgCl_2$ 1.2 and dextrose 10. The experiments began after a 60 min equilibration period followed by a challenge with 60 mM KCl to obtain a standard vasoconstriction for each preparation. The contractions were measured with a transducer (FD pickup, NEC-Sanei). ET-1, ET-2 and ET-3 (Peptide Institute) was added to the bath solution in a cumulative manner to produce concentration-response curves with and without TAK-044 pretreatment (30 min before the addition of each endothelin).

Endothelium-dependency

A pair of porcine coronary arteries was prepared, one for endothelium-intact and the other for denuded preparations, the latter being prepared by removing the endothelial cells by gentle rubbing with a cotton bud. Existence of endothelial cell was examined by applying substance P (3 nM) before starting the experiments. Concentration-response curves for ET-1 were obtained using these paired preparations with and without TAK-044 pretreatment (10 nM, 30 min before the addition of ET-1).

An EC_{50} value was obtained from the concentration-response curve of each preparation, and a pA_2 value was calculated from a Schild plot of the EC_{50} values, when necessary.

Blood pressure measurements in rats

Male Wistar rats (250–350 g) were anaesthetized with 5-*sec*-butyl-5-ethyl-2-thiobarbituric acid (inactin, 120 mg kg^{-1}, i.p.). The right femoral artery and right femoral vein were cannulated with polyethylene tubes and another cannula was inserted in the bronchi to ensure respiration was maintained. After a stabilizing period of 30 min, the arterial blood pressure was measured with a transducer (Spectramed) from the femoral arterial cannula. ET-1 was injected via the femoral venous cannula two to three times with 1 h intervals between injections until stable responses in blood pressure were obtained. TAK-044 was, then, injected intravenously 10 min before the next ET-1 administration to determine its inhibitory effects.

Rat acute myocardial infarction model

Male Wistar rats (250–300 g) were anaesthetized with pentobarbitone sodium (50 mg kg^{-1}, i.p.) and the chest was opened to expose the heart. Acute myocardial infarction was induced by coronary artery occlusion for 1 h with reperfusion of the coronary blood flow for 24 h. The infarct size was determined histochemically 24 h after reperfusion as follows. Each rat was anaesthetized again 24 h after reperfusion and the heart was quickly removed. The coronary artery was ligated at the same position as the occlusion and Evans blue dye (1%w/v) was perfused through the aorta to determine the area-at-risk region. Since rat hearts showed less-developed collaterals (Tillmanns & Kübler, 1984), the area-at-risk determined by this method represents the ischaemic region during the arterial occlusion. The infarct regions were determined by incubating sliced tissues with 1% triphenyltetrazolium chloride solution (37°C) for 10 min. The undyed infarct zone was carefully cut out and weighed. The infarct size was expressed as a percentage of the area-at-risk for each heart.

All the data in the present study were expressed as means ± s.e. with the number of experiments involved. A one-way analysis of variance or Student's *t* test was performed for statistical analysis.

Chemicals

ET-1, ET-2, ET-3 and TAK-044 were dissolved in distilled water for the *in vitro* experiments and in saline for the *in vivo* experiments. TAK-044, BQ 123 (cyclo(-D-Asp-L-Pro-D-Val-L-Leu-D-Trp)) and inactin were synthesized in our chemistry division. 5-Hydroxytryptamine (5-HT), histamine, acetylcholine and substance P were dissolved in distilled water and U-46619 was in ethanol until required for use. All these agents were obtained form Sigma except for substance P (Peptide Institute).

Results

Binding of [^{125}I]-ET-1 to rabbit membrane fractions was displaced by both ET-1 and ET-3 although their inhibitory selectivities on ventricular and cerebellar preparations differed. ET-1 displaced the bound [^{125}I]-ET-1 from ventricular tissue with an IC_{50} value of 0.11 ± 0.04 nM and ET-3

showed weaker potency with an IC_{50} value of 11 ± 4 nM ($n = 4$ each, Figure 1a). In contrast, ET-1 and ET-3 showed similar displacement potencies on cerebellar membrane fractions: IC_{50} values of 0.22 ± 0.05 nM for ET-1 and 0.071 ± 0.02 nM for ET-3 ($n = 4$ each, Figure 1b).

The specific ET_A receptor antagonist, BQ-123, displaced the [^{125}I]-ET-1 bound more potently from ventricular than cerebellar preparations (Figure 1a,b). The IC_{50} value for the ventricle was 0.18 ± 0.01 μM, which was 222 times more potent than that for the cerebellum (IC_{50} of 40 ± 7 μM, $n = 4$ each). The difference in the IC_{50} values between the two preparations with TAK-044 was smaller than that with BQ-123. It displaced the bound [^{125}I]-ET-1 from the ventricular preparation with an IC_{50} value of 3.8 ± 1 nM ($n = 4$), a potency being 1/35 of ET-1 and 47 times of BQ-123 (Figure 1a). The IC_{50} value of TAK-044 for the cerebellar preparations was 130 ± 28 nM ($n = 4$, Figure 1b).

The effects of TAK-044 on ET-induced vasoconstriction were studied in porcine coronary arteries. ET-1, ET-2 and ET-3 showed strong vasoconstriction in coronary arteries. The concentration-response curve for ET-1 was similar to that for ET-2 (Figure 2a,b). Maximum responses to ET-1 and ET-2 were obtained with 0.1 μM and their amplitudes were $169 \pm 8\%$ and $176 \pm 32\%$ (%control of the 60 mM KCl-induced standard contraction, $n = 4$), respectively. EC_{50} values for the peptides were obtained from the concentration-response curves: 5.7 nM for ET-1 and 8.8 nM for ET-2. In contrast, the concentration-response curve for ET-3 showed two distinct phases (Figure 2c). Although the threshold concentration and shape of the concentration-response curve up to 10 nM ET-3 were similar to those of the other two endothelins, the curve between 10 and 300 nM was shallower than for the others.

TAK-044 shifted the concentration-response curves for these three peptides. Pretreatment with TAK-044 inhibited ET-1-induced vasoconstriction in a concentration-dependent manner from 10 nM (Figure 2a). The concentration-response curves were shifted to the right without changing the maximum response, which indicates that the inhibition was competitive: a pA_2 value was calculated to be 8.4. The potency of inhibition for ET-2-induced vasoconstriction was similar to that for ET-1. TAK-044 at 10 nM shifted the ET-2 response curve to the right without changing the maximum response (Figure 2b). In contrast, TAK-044 inhibited ET-3-induced vasoconstriction in a non-competitive manner (Figure 2c). The degree of inhibition was much greater with higher concentrations than with lower concentrations. The selective ET_A receptor antagonist, BQ-123 at 10 μM, inhibited the second phase of ET-3-induced vasoconstriction only, whereas ET-1-

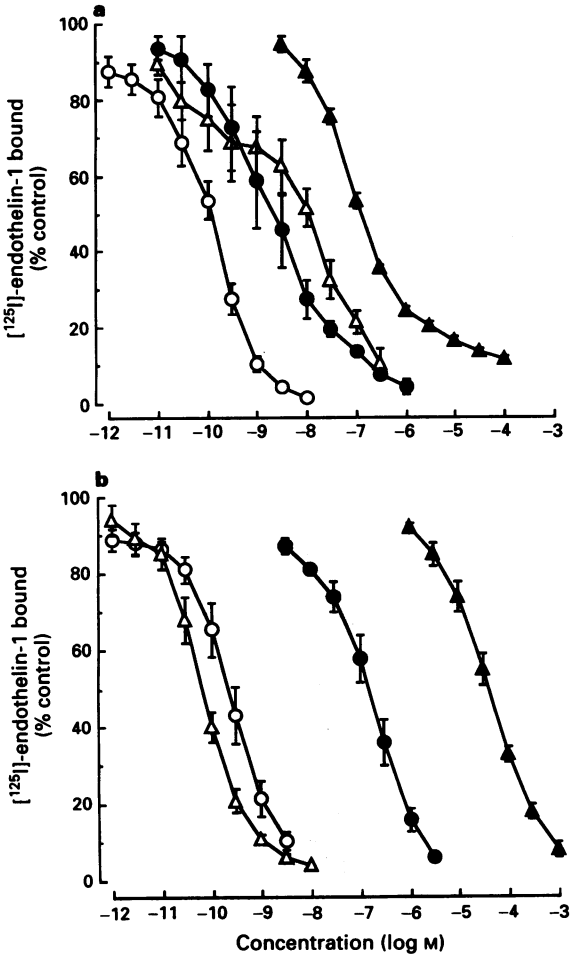

Figure 1 Binding displacement curves of [^{125}I]-endothelin-1 ([^{125}I]-ET-1) by the peptides, ET-1 (○), ET-3 (△), BQ-123 (▲) and TAK-044 (●) in rabbit ventricular (a) and in cerebellar membrane fractions (b). Each point represents mean ± s.e., $n = 4$.

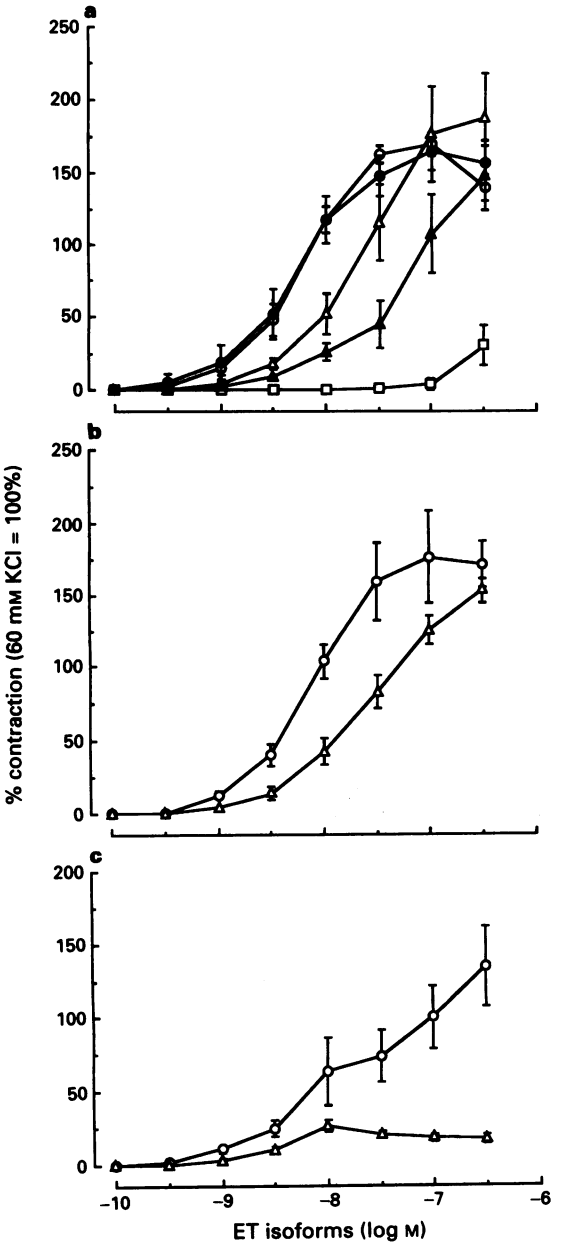

Figure 2 Concentration-response curves for endothelin-1 (ET-1) (a), ET-2 (b) and ET-3 (c) of porcine isolated coronary arteries. Each point represents mean ± s.e., $n = 4$. Control (○), and TAK-044: 1(●), 10(△), 30(▲) and 100(□) nM.

and ET-2-induced vasoconstriction was inhibited in a competitive manner (data not shown).

The specificity of inhibition by TAK-044 was studied by use of various vasoactive agents. U-46619, 5-HT, acetylcholine, histamine and KCl caused vasoconstriction in porcine coronary arteries with EC_{50} values of 11 nM, 380 nM, 540 nM, 2.6 μM and 32 mM ($n = 4$ each), respectively. Pretreatment with 10 nM TAK-044 did not alter these concentration-response curves; the respective EC_{50} values were 12 nM, 340 nM, 520 nM, 2.5 μM and 28 mM ($n = 4$ each). Next, the endothelium-dependency of the inhibitory effects of TAK-044 was studied in coronary arteries. The concentration-response curves for ET-1 with and without TAK-044 were not affected by the presence of the endothelium. The EC_{50} values ($n = 4$ each) for the endothelium-intact and denuded preparations in the absence of TAK-044 were 5.7 nM and 4.0 nM, respectively, and 21 nM for intact and 14 nM for denuded preparations after pretreatment with TAK-044 (10 nM). Finally, application of high concentrations of TAK-044 up to 100 μM induced neither relaxation nor vasoconstriction in coronary artery preparations ($n = 4$), which indicates that it had no agonist or non-selective effects.

The effects of TAK-044 on blood pressure changes induced by ET-1 were studied *in vivo*. Injection of ET-1 (0.3 nmol kg^{-1}, i.v.) evoked transient hypotension followed by sustained hypertension, which lasted for over 30 min. The maximum decrease in mean blood pressure was 13 ± 4 mmHg and the maximum increase was 24 ± 4 mmHg ($n = 9$, Figure 3). The mean blood pressure before drug injection was 104 ± 3 mmHg ($n = 9$). Pretreatment with TAK-044 (10 min before ET-1 injection) inhibited both these responses in a dose-dependent manner (Figure 3). The maximum hypertension was decreased significantly by 42% at 0.1 mg kg^{-1}, 50% at 1 mg kg^{-1} and 88% at 10 mg kg^{-1}. Similarly, the maximum hypotension was decreased significantly by 19% at 0.1 mg kg^{-1} and by over 90% at 1 and 10 mg kg^{-1}. It should be noted that injection of TAK-044 (0.01–10 mg kg^{-1}, i.v.) alone did not modify the resting blood pressure.

One of the main objectives of the present study was to determine whether TAK-044 limited the extension of myocardial infarct size in rats. Coronary artery occlusion for 1 h and reperfusion for 24 h produced myocardial infarction in rats. The infarct size measured after this occlusion and reperfusion of the coronary artery was 60 ± 2% of the area-at-risk by weight ($n = 12$, Saline control in Figure 4). Pretreatment with TAK-044 reduced the infarct size in a dose-dependent manner. Intravenous injection of 0.3 mg kg^{-1} 10 min before coronary occlusion inhibited the extension of infarct size significantly by 20% compared with the saline controls (48 ± 4% of the area-at-risk, $n = 6$, Figure 4). Reductions of 32% and 54% were obtained with 1 and 3 mg kg^{-1}, i.v., respectively: 41 ± 3% ($n = 7$) for 1 mg kg^{-1} and 28 ± 6% (% of the area-at-risk, $n = 5$) for 3 mg kg^{-1}. The area-at-risk of the saline control was 49 ± 2% of the left ventricular mass by weight and was not altered significantly by drug administration.

Another set of experiments was designed to study the effects of post-treatments with TAK-044 on the infarct size in rats. When TAK-044 was administered 50 min after coronary artery occlusion (10 min before reperfusion started), the resultant infarct size measured 24 h after reperfusion of coronary flow was reduced to 51 ± 2% ($n = 5$) and 40 ± 2% (% of the area-at-risk, $n = 12$) at 0.3 and 1 mg kg^{-1}, i.v., respectively (Figure 5). These reductions were similar to those obtained with TAK-044 pretreatment (10 min before occlusion). Similar reductions of the infarct size were also obtained with TAK-044 administered 1 h after reperfusion of coronary flow: a 16% reduction (50 ± 2% of the area-at-risk, $n = 6$) at 0.3 mg kg^{-1} and a 23% reduction (47 ± 3% of the area-at-risk, $n = 7$) at 1 mg kg^{-1} (Figure 5).

Discussion

The present study showed that TAK-044 is a specific endothelin receptor antagonist with a feature of antagonizing both ET_A and ET_B receptors on the basis of the following observations. The binding study showed that TAK-044 inhibited radiolabelled ET-1 binding to membrane fractions of rabbit ventricle and cerebellum. Since ET-1 inhibited the [^{125}I]-ET-1 binding to the ventricular preparation more than did ET-3 but both ET-1 and ET-3 showed similar inhibitory potencies on the cerebellar preparation, the major components of endothelin receptor in the ventricle appeared to be ET_A receptors and those in the cerebellum ET_B receptors (Masaki *et al.*, 1992). The binding profile of BQ-123 supported this view; it inhibited more potently in the ventricle than in the cerebellum (IC_{50} of 0.18 μM for ET_A and 40 μM for ET_B). In the experiments *in vivo*, transient hypotension (mainly ET_B stimulation) and a sustained hypertension (mainly ET_A

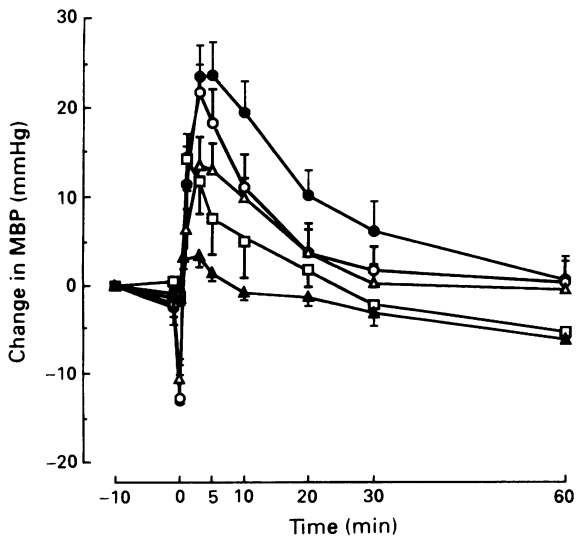

Figure 3 Effects of TAK-044 on endothelin-1 -induced blood pressure changes in rats. The data are expressed as means ± s.e., $n = 5$–9. Saline control (●), and TAK-044: 0.01 (○), 0.1(△), 1(□) and 10(▲) mg kg^{-1}. MBP: mean blood pressure.

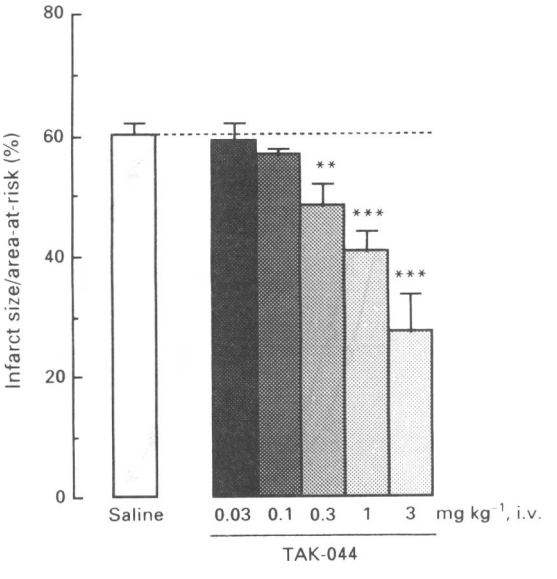

Figure 4 Effects of TAK-044 on the extension of myocardial infarct size in rats. Columns indicate mean with s.e., $n = 12$ (Saline) and $n = 5$–7 (TAK-044). $^{**}P < 0.01$, $^{***}P < 0.001$ compared with Saline.

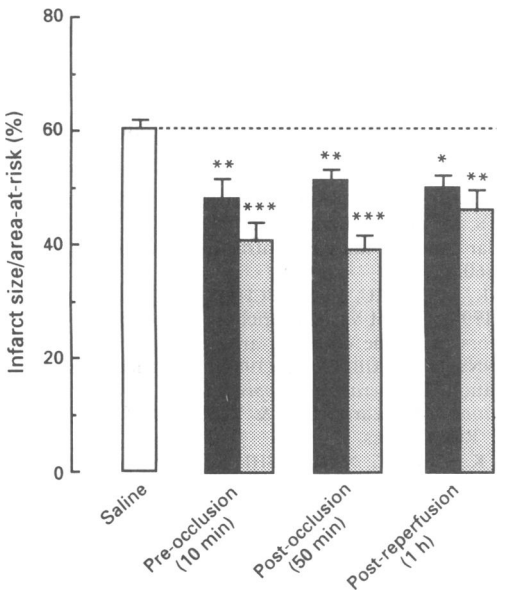

Figure 5 The effects of TAK-044 administration at different times on the myocardial infarct size in rats. The three columns on the far left-hand side are from Figure 4 as the reference (Saline and Pre-occlusion). TAK-044 0.3 (darkly stippled column) and 1 mg kg^{-1}, (lightly stippled column) i.v. was administered 50 min after coronary occlusion (10 min before reperfusion started; post-occlusion (50 min)) or 1 h after reperfusion (post-reperfusion (1 h)). Columns show mean with s.e., $n=12$ (Saline) and $n=5-12$ (TAK-044). $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with Saline.

stimulation) of rats were both inhibited by pretreatment with TAK-044, which indicates that it is an ET_A/ET_B receptor antagonist. Contractions of porcine coronary arteries evoked by various agonist, *in vitro*, were not inhibited by TAK-044, whereas those induced by ET-1, ET-2 and ET-3 were; thus TAK-044 is a selective endothelin receptor antagonist. In addition, the inhibitory effects of TAK-044 against ET-1-induced contractions of porcine coronary arteries were not endothelium-dependent.

The vasoconstriction induced by ET-3 showed two distinct phases, indicating the involvement of at least two different factors (receptors). Since ET-3 has a higher affinity for ET_B than ET_A receptors (Figure 1 and Sakurai *et al.*, 1990), the first phase of vasoconstriction (up to 10 nM in Figure 2c) is likely to be induced through stimulation of ET_B receptors and the second-phase responses are through ET_A receptor stimulation. This suggestion is supported by the results that TAK-044 inhibited ET-3-induced vasoconstriction of porcine coronary arteries in both phases (Figure 2), while only the second phase of contraction was inhibited by BQ-123. Furthermore, although ET_B receptors have been found to cause vasorelaxation and hypotension (Masaki *et al.*, 1992), ET_B receptor-induced vasoconstriction has been demonstrated in various vessels, including canine coronary arteries (Teerlink *et al.*, 1994), swine pulmonary vein (Sudjarwo *et al.*, 1993) and rat mesenteric and renal arteries (Clozal *et al.*, 1992).

We have shown the contribution of ET-1 to the extension of infarct size in the present and the previous (Watanabe *et al.*, 1991) studies using a receptor antagonist and monoclonal antibody for ET-1, respectively. However, recent studies with ET_A antagonists provided controversial results: continuous i.v. injection of BQ-123 reduced the infarct size in dogs (Grover *et al.*, 1993), while that of FR139317 showed no effects in rabbits (McMurdo *et al.*, 1994). Since monoclonal antibody for ET-1 reduced the infarct size in rats (Watanabe *et al.*, 1991), rabbits (Kusumoto *et al.*, 1993) and dogs (unpublished observations), species-dependency is not likely to be the explanation. One of the possible explanations is, thus, organ-selectivity of the endothelin antagonist: TAK-044 was demonstrated to inhibit ET-1-induced vasoconstriction of the coronary artery more than the basilar, femoral, renal or mesenteric artery (Watanabe *et al.*, 1994). Alternatively, it is possible that ET_A/ET_B receptor antagonists show stronger inhibitory effects over ET_A receptor antagonists for limiting myocardial infarct size extension. Since increases in ET-1 play an important role in it (Watanabe *et al.*, 1991; Kusumoto *et al.*, 1993) and ET-1 has been reported to activate both ET_A and ET_B receptors (Arai *et al.*, 1990; Sakurai *et al.*, 1990), it is reasonable to assume that both receptor subtypes are involved in the extension of infarct size. The existence of ET_A and ET_B receptors in smooth muscle of artery and vein (e.g. Winkles *et al.*, 1993) and in cardiac tissues (e.g. Hori *et al.*, 1992; Molenaar *et al.*, 1993) have been demonstrated to support this view. Moreover, vasoconstriction through ET_B receptors was demonstrated in coronary arteries (Teerlink *et al.*, 1994) in addition to that through ET_A receptors. It is clear that well-designed studies focused on a role of ET_A and ET_B receptors in this model should be carried out to clarify the above issue.

We have concluded that TAK-044 is a specific ET receptor antagonist with a feature of inhibiting phenomena induced by activation of both ET_A and ET_B receptors and that it limited the extension of myocardial infarct size after coronary artery occlusion and reperfusion in rats.

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The role of nitric oxide in the altered vascular reactivity of pregnancy in the rat

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1 Pregnancy is characterized by a decrease in systemic vascular resistance and a blunting of the angiotensin II (AII) pressor response. We studied the role of nitric oxide (NO) and prostanoids in these vascular changes of pregnancy in anaesthesized, ganglion blocked non-pregnant and pregnant rats.

2 Inhibition of NO synthesis with N^G-nitro-L-arginine methyl ester (L-NAME) led to an increase in mean arterial pressure (MAP) which was of a significantly greater magnitude in pregnant rats in late gestation than in non-pregnant rats, or rats in mid-gestation.

3 The pressor response to varying doses of AII was attenuated during late pregnancy, and this attenuation was partially reversed by L-NAME.

4 The pressor response to varying doses of a vasocontrictor, phenylephrine (PE), was also attenuated in late pregnancy. However, this attenuation was not reversed by L-NAME.

5 Inhibition of prostanoid biosynthesis with meclofenamate did not alter basal MAP, nor the pressor response to varying doses of AII or PE in pregnant and non-pregnant animals.

6 It is concluded that (a) increased NO synthesis occurs during late gestation and contributes both to the decrease in systemic vascular resistance, as well as the blunting of the pressor response to AII during pregnancy, and (b) prostaglandins are not important in the maintenance of basal vascular tone, or the blunting of the pressor response to AII during pregnancy.

Keywords: Pregnancy; nitric oxide; prostanoids; angiotensin II; vascular reactivity; pressor response; N^G-nitro-L-arginine methyl ester.

Introduction

Pregnancy is associated with a hyperdynamic state characterized by increased plasma volume and increased cardiac output. Systemic arterial pressure is not increased due to a decrease in systemic vascular resistance (Pan *et al.*, 1990). Concomitant with the decrease in systemic vascular resistance is a blunting of the pressor response to angiotensin II (AII) (Abdul Karim & Assali, 1961; Gant *et al.*, 1973; Pan *et al.*, 1990; Molnár & Hertelendy, 1992), plasma levels of which are increased during pregnancy (Weir *et al.*, 1975). The exact mechanism by which these vascular changes take place is not known.

Several investigators have proposed that the blunting of the AII pressor response and other vascular changes of pregnancy are due to release of vasodilator prostaglandins (Everett *et al.*, 1978; Gerber *et al.*, 1981), although this concept has been challenged by others (Conrad & Colpoys, 1986). More recently, it has been proposed that endothelium-derived relaxing factor (EDRF) (Furchgott & Zawadzki, 1980), now identified as nitric oxide (NO) (Palmer *et al.*, 1987; Ignarro *et al.*, 1987), is responsible for these vascular changes of pregnancy. Unlike PGI_2 (Conrad & Colpoys, 1986), NO has been unequivocally demonstrated to be responsible for maintaining basal vascular tone *in vivo* in all species studied (Rees *et al.*, 1989; Aisaka *et al.*, 1989; Collier & Vallance, 1989; Vargas *et al.*, 1991). NO is also released by various endothelium-dependent vasodilators (Furchgott, 1984) and probably by certain vasopressor agents such as AII (Yamaguchi & Nichimura, 1988). Findings by several investigators demonstrating increased endothelium-dependent vasodilatation of vessels obtained from pregnant animals suggest that NO may play an important role in modulating vascular tone during gestation (Weiner *et al.*, 1991).

We, therefore, decided to evaluate whether increased NO rather than prostaglandin formation in pregnant rats could account for both the fall in systemic vascular resistance as well as the blunting of the pressor response to AII observed during pregnancy. This study was performed on pregnant rats because this species offers many advantages. Some of the cardiovascular changes this species undergoes during pregnancy are similar to those in women. For example, the increase in uterine blood flow (Bruce, 1976), the decrease in mean arterial pressure (MAP) (Pan *et al.*, 1990; Molnár & Hertelendy, 1992) and attenuation of pressor responsiveness to exogenous vasoconstrictors (Pan *et al.*, 1990; Molnár & Hertenlendy, 1992) develop in the species similar to those seen in women. Rats are relatively inexpensive and their hormonal profiles during pregnancy are well defined.

Methods

Preparation of rats for blood pressure recording and drug injection

Experiments were conducted on urethane anaesthetized (1.25 g kg^{-1}, i.p.) Sprague-Dawley rats weighing 250-350 g obtained from Bantin and Kingman (Fremont, CA, U.S.A.). Rats were divided into three groups, (a) nonpregnant; (b) pregnant in mid-gestation (9-11 days pregnant); and (c) pregnant in late gestation (18-20 days pregnant). The day sperm were first seen in the vaginal lavage was considered day 1 of pregnancy. After establishment of anaesthesia, polyethelene catheters (PE50) were inserted into the right femoral and common carotid arteries for drug infusion and arterial blood pressure recordings, respectively. Blood pressure was measured by a Statham transducer and recorded on a Hewlett Packard polygraph. All drugs were dissolved in 0.9% saline and injected in a volume of 0.1 ml $100g^{-1}$ body weight. Animals were ventilated with a rodent respirator (1

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ml $100g^{-1}$, 75 strokes min^{-1}). In order to eliminate all autonomic reflexes and thereby evaluate responses at the vascular bed, animals were injected with the ganglion blocker, pentolinium (5 mg kg^{-1}, i.v.) 10 min prior to continuing the experiments. Each group contained 5–13 animals.

Role of NO in maintaining basal vascular tone during pregnancy

To assess the contribution of NO in the maintenance of basal vascular tone in pregnancy, each animal received 5 graded doses of the NO synthesis inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) injected as a bolus (0.1, 0.3, 1.0, 3.0, 30 mg kg^{-1}, i.v.). The change in mean arterial pressure (MAP) from baseline was measured after each dose when the peak rise in MAP occurred, usually after 5 min. Blood pressure was allowed to stabilize before administering each subsequent dose. In some animals, after the peak responses to L-NAME were achieved, L- or D-arginine was injected as a bolus (5 fold concentration of L-NAME) and the pressor response observed.

Role of NO in modulating the pressor response to AII and phenylephrine during pregnancy

To assess the contribution of NO in the attenuation of the vasopressor response to AII during pregnancy, animals were injected with AII before and after administration of L-NAME. Catecholamine levels, (Lederman *et al.*, 1977), in contrast to AII (Weir *et al.*, 1975) are not increased in pregnancy. We therefore also decided to evaluate whether the pressor response to the α-adrenoceptor agonist, phenylephrine (PE), during pregnancy was modulated by NO to a similar magnitude as that observed with AII. We selected non-pregnant animals and animals in late pregnancy for this protocol as initial experiments demonstrated that the blunting of the AII pressor response was observed only in animals during late pregnancy. Animals were injected with 3–4 graded doses of AII (25, 50, 100, 200 ng kg^{-1}, i.v.) and PE (0.5, 1.0, 2.0 μg kg^{-1}, i.v.) in a random fashion and changes in MAP were recorded. Following completion of these dose responses, L-NAME (45 mg kg^{-1}, i.v.) was injected as a single bolus. Fifteen minutes later the dose responses to AII and PE were then repeated in a random fashion. As the baseline tone was increased following administration of L-NAME, we decided to express the results for this protocol as percentage change from baseline values.

Role of prostanoids in modulating basal vascular tone and pressor responses to AII and PE during pregnancy

To assess the role of prostanoids in the maintenance of basal vascular tone and the blunting of the pressor response to AII during pregnancy, animals were injected with graded doses of PE and AII, in the manner described above, both before and 30 min after a single bolus injection of meclofenamate (3 mg kg^{-1}, i.v.). Changes in MAP were then recorded. This dose of meclofenamate has been shown to inhibit prostaglandin synthesis in pregnant and non-pregnant rabbits (Chaudhuri *et al.*, 1982).

Drugs

AII acetate, PE hydrochloride, urethane, pentolinium ditartrate, L-NAME hydrochloride, L-arginine, D-arginine and meclofenamate sodium were purchased from Sigma Chemical Co. (St. Louis, MO U.S.A). All drugs were dissolved in 0.9% w/v saline.

Statistical analysis

All data are expressed as mean ± s.e.mean. Differences between teatment group means were compared by two and three-way ANOVA with repeated measurements. Differences were considered significant at the $P<0.05$ level.

Results

Role of NO in maintaining basal vascular tone during pregnancy

Basal MAP following pentolinium and prior to L-NAME was 50 ± 5 mmHg in non-pregnant animals, 63 ± 3 mmHg in animals in mid gestation and 65 ± 2 mmHg in animals in late gestation. L-NAME produced a dose-dependent rise in MAP in non-pregnant animals, as well as in animals in mid and late pregnancy. However, during late pregnancy, L-NAME produced a significantly greater rise in MAP compared to that observed in either non-pregnant animals or in animals in mid pregnancy (Figure 1). In separate experiments to assess the reversibility of L-NAME by L-arginine and D-arginine,MAP following L-NAME administration in non-pregnant animals was 76 ± 4 mmHg and in pregnant animals was 157 ± 10 mmHg. Administration of D-arginine after L-NAME did not change the MAP whereas 15 min after administration of L-arginine MAP in non-pregnant and pregnant animals was reduced to 47 ± 5 mmHg and 82 ± 13 mmHg, respectively.

Role of NO in modulating the pressor response to AII and PE during pregnancy

In non-pregnant animals, basal MAP following pentolinium was 50 ± 5 mmHg before and 70 ± 9 mmHg 15 min after L-NAME. In pregnant animals in late gestation, basal MAP was 65 ± 2 mmHg before and 95 ± 6 mmHg 15 min after L-NAME. AII caused a dose-dependent rise in MAP in all three groups. However, in late pregnancy, the pressor responses to AII were attenuated at all doses (Figure 2). PE also produced dose-dependent rises in MAP which were also

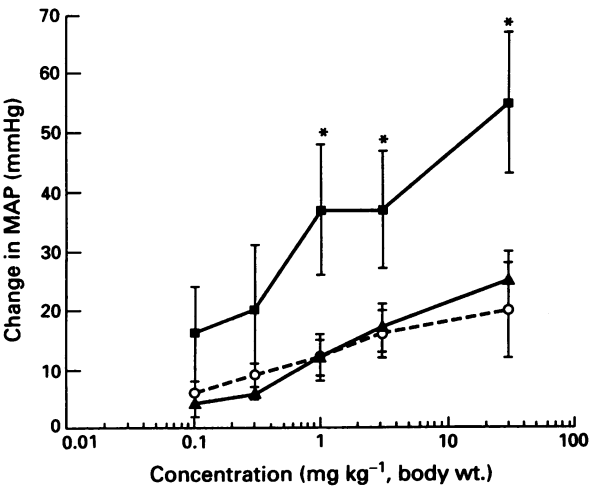

Figure 1 Pressor responses to N^G-nitro-L-arginine methyl ester (L-NAME) in anaesthetized, ganglion-blocked non-pregnant rats (▲), and pregnant rats in mid (○) and late (■) gestation. In late pregnancy, L-NAME produced a significantly greater rise in mean arterial pressure (MAP) compared to that observed in non-pregnant animals or animals in mid-gestation. MAP was measured when the peak rise in MAP occurred after each dose of L-NAME, usually after 5 min. Data are expressed as mean (±s.e.mean) rise in MAP of 9 animals in each group. $*P<0.05$ denotes significant difference from values obtained in non-pregnant animals.

attenuated in late pregnancy (Figure 2). However, the attenuation was of a lower magnitude in the PE group when compared to the AII group.

In non-pregnant animals, the pressor responses to different doses of AII were not altered by L-NAME (Figure 3). In contrast, the blunting of the pressor response to AII in late pregnancy was partially reversed by L-NAME as there was a significant potentiation of the pressor response to AII at the 25 and 50 ng kg^{-1} doses (Figure 3). The pressor response to PE was unchanged by L-NAME in non-pregnant and pregnant animals (Figure 4).

Role of prostanoids in modulating basal vascular tone and pressor responses to AII and PE during pregnancy

Inhibition of prostanoids with meclofenamate did not alter basal MAP. In non-pregnant animals basal MAP following pentolinium was 60 ± 4 mmHg before and 62 ± 4 mmHg 30 min after meclofenamate. In pregnant animals basal MAP was 64 ± 2 mmHg before and 66 ± 2 mmHg 30 min after meclofenamate. The pressor responses to AII (Figure 5) and PE (Figure 6) in non-pregnant animals were also unchanged by meclofenamate.

Discussion

The mechanisms involved in maintenance of vascular tone and altered vascular responses to pressor agents during pregnancy are not known. The role of NO in modulating these altered vascular responses has not been clearly delineated. However, there is evidence to suggest that increased NO synthesis occurs during pregnancy and that it may therefore play a role in modulating the altered vascular reactivity during pregnancy (Conrad & Vernier, 1989; Conrad *et al.*, 1993). Consistent with this concept, Conrad & Vernier (1989) and Conrad *et al.* (1993) have demonstrated an increase in endogenous NO production in pregnant rats. In addition, Weiner and colleagues (1994 a,b) have demonstrated that calcium-dependent NO synthase activity is increased in both vascular and non-vascular tissues during pregnancy.

The principle objective of this study was to elucidate whether the decrease in systemic vascular resistance, as well as the blunting of the pressor response to AII that occurs in pregnancy, can be explained on the basis of increased release of NO rather than increased release of prostanoids. This was achieved by studying the basal release of NO in non-pregnant rats and pregnant rats in mid and late gestation. We used ganglion-blocked animals because this would permit us to study the direct vascular effects of vasoactive substances, unmodified by reflex changes in vascular tone. The role of basal release of NO and prostanoids in the maintenance of vascular tone during pregnancy was studied indirectly by observing the pressor responses to L-NAME and meclofenamate. The role of NO and prostanoids in the blunting of the pressor response to AII was studied by observing the pressor responses to various doses of AII before and after the administration of L-NAME and mec-

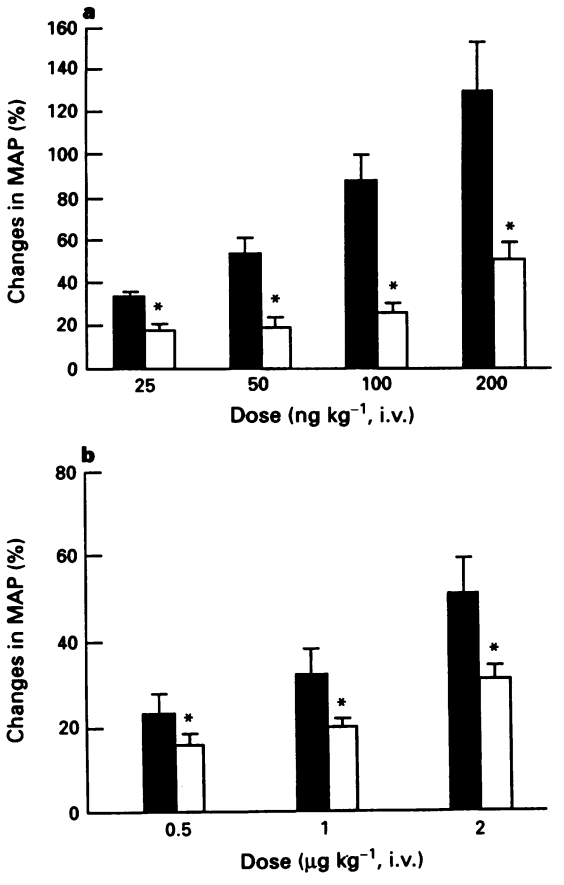

Figure 2 Pressor responses to (a) angiotensin II (AII) and (b) phenylephrine (PE) in anaesthetized, ganglion-blocked non-pregnant rats (solid columns), and pregnant rats in late gestation (open columns). A dose-dependent rise in mean arterial pressure (MAP) was seen in all groups. However, the pressor responses to both PE and AII in late pregnancy were attenuated. Data are expressed as mean (with s.e.mean) percentage changes in MAP of 7–13 animals in each group. *$P<0.05$ denotes significant difference from values obtained in non-pregnant animals.

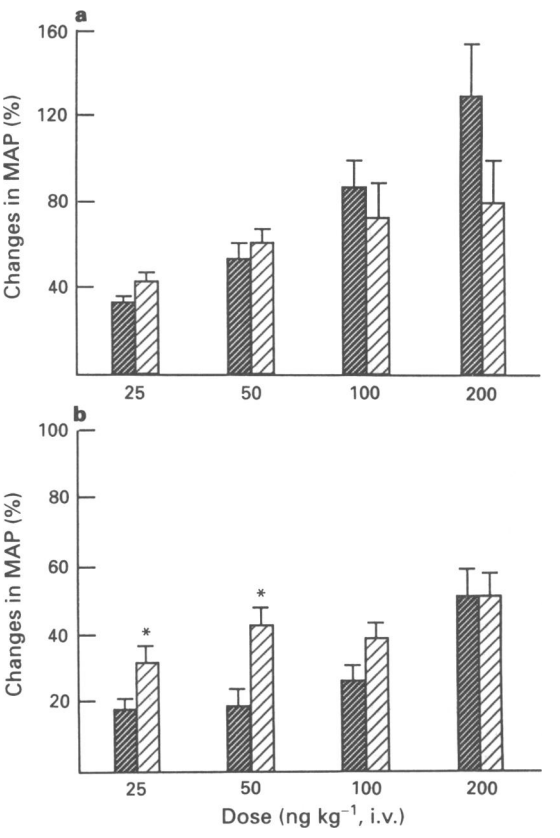

Figure 3 Pressor response to angiotensin II (AII) before (closely hatched columns) and after (widely hatched columns) N^G-nitro-L-arginine methyl ester (L-NAME) in anaesthetized, ganglion-blocked (a) non-pregnant rats and (b) pregnant rats in late gestation. In non-pregnant animals, the blunted pressor response to AII was unchanged after L-NAME administration, while in pregnant animals, the blunted pressor response to AII was partially reversed by L-NAME. Data are expressed as mean (with s.e.mean) percentage change in mean arterial pressure (MAP) of 7–11 animals in each group. *$P<0.05$ denotes significant difference from pre-L-NAME values.

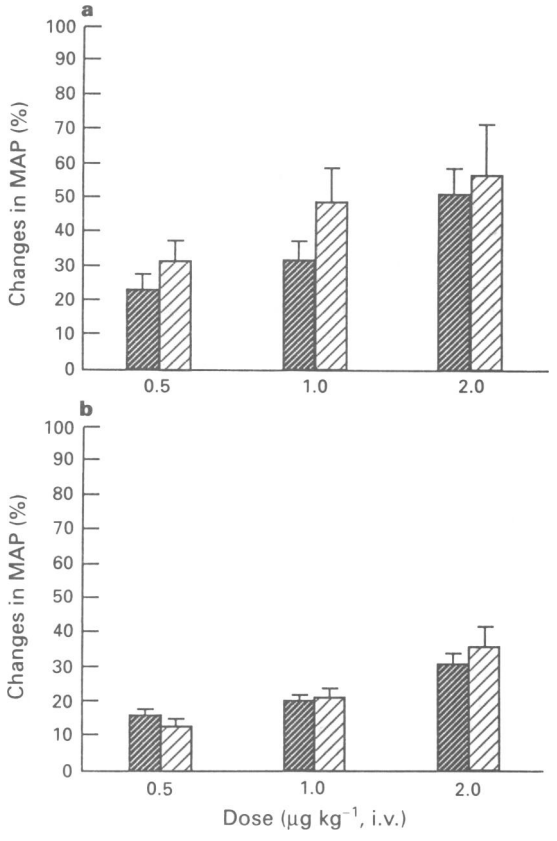

Figure 4 Pressor response to phenylephrine (PE) before (closely hatched columns) and after (widely hatched columns) N^G-nitro-L-arginine methyl ester (L-NAME) in anaesthetized, ganglion-blocked (a) non-pregnant rats and (b) pregnant rats in late gestation. The pressor response to PE was unchanged after administration of L-NAME in both pregnant and non-pregnant animals. Data are expressed as mean (with s.e.mean) percentage change in mean arterial pressure (MAP) of 8–13 animals in each group.

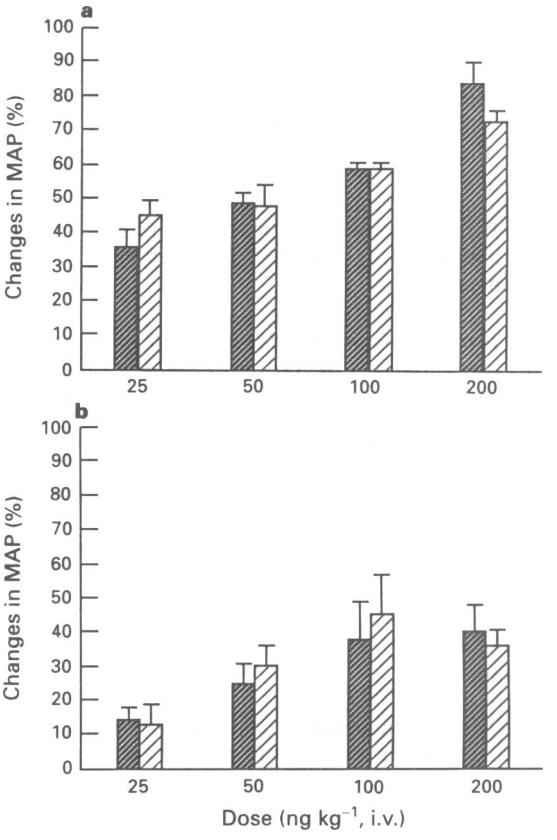

Figure 5 Pressor response to angiotensin II (AII) before (closely hatched columns) and after (widely hatched columns) meclofenamate in anaesthetized, ganglion-blocked (a) non-pregnant rats and (b) pregnant rats in late gestation. The pressor response to AII was unchanged after administration of meclofenamate in both non-pregnant and pregnant animals. Data are expressed as mean (with s.e.mean) percentage change in mean arterial pressure (MAP) of 5 animals in each group.

lofenamate. As plasma catecholamine levels are not altered during pregnancy, unlike levels of AII, we also evaluated whether the pressor responses to PE were modified by NO to a similar magnitude to that observed with AII.

Inhibition of NO synthesis by L-NAME led to an increase in MAP which was of a greater magnitude in pregnant rats in late gestation than that observed in either non-pregnant rats, or in pregnant rats in mid-gestation. This suggests that increased NO synthesis occurs during late pregnancy. Our results differ from those of Umans *et al.* (1990) who failed to observe any differences in the magnitude of rise in MAP between non-pregnant rats and rats in late pregnancy after they were given a single high dose of an NO synthesis inhibitor. Unfortunately, these workers did not perform a dose-response curve to the NO synthesis inhibitor, nor did they abolish autonomic reflexes with ganglion blockade. Thus, inhibition of NO synthesis could have stimulated autonomic reflex changes which would have helped maintain blood pressure at a steady state. A study by Molnár & Hertelendy (1992) also failed to reveal a difference in pressor response between non-pregnant and pregnant animals after administration of an inhibitor of NO synthesis. Again, these animals had intact autonomic reflexes which may have minimized any differences between the pregnant and non-pregnant groups. Using the spontaneously hypertensive rat model, Ahokas *et al.* (1991) saw significantly greater increases in MAP in the pregnant than non-pregnant rats after inhibition of NO synthesis, but they did not observe this difference in the normotensive rats. These investigators also used reflex intact animals which makes comparison to our study difficult.

One possible explanation for the accentuated rise in MAP after L-NAME in animals in late gestation may be the presence of other circulating pressor agents such as AII, the concentration of which is known to increase during pregnancy (Weir *et al.*, 1975). It is conceivable that during late gestation, in addition to increased basal release of NO, AII itself further stimulates NO release, thereby blunting its own pressor response. Therefore, in addition to removing the basal vasodilator mechanism, inhibition of NO synthesis during late gestation may have unmasked the pressor response to circulating AII and caused a significantly greater magnitude of rise in MAP in late pregnancy.

AII produces endothelium-dependent relaxation in the fowl aorta presumably by release of NO (Yamaguchi and Nishimura, 1988). We observed that responses to AII, as well as to PE, were blunted during late pregnancy, but that only the blunted AII response was partially reversed after inhibiting NO synthesis. One possible explanation for this observation is that in the pregnant state AII may stimulate greater release of NO than that observed with PE. However, this aspect needs direct confirmation by actually quantifying NO release *in vivo*. Our findings support the concept that increased NO synthesis plays at least a partial role in modulating vasoconstrictor tone during late pregnancy, but that other factors may be involved such as effects of dilution from increased plasma volume during pregnancy or changes in vasoconstrictor receptor density or affinity. Ahokas & Sibai (1992) found

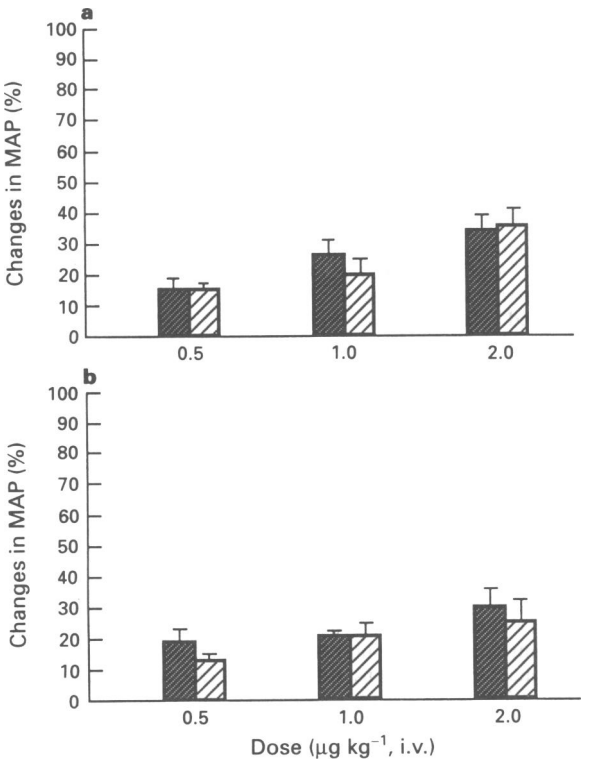

Figure 6 Pressor response to phenylephrine (PE) before (closely hatched columns) and after (widely hatched columns) meclofenamate in anaesthetized, ganglion-blocked (a) non-pregnant rats and (b) pregnant rats in late gestation. The pressor response to PE was unchanged after administration of meclofenamate in both non-pregnant and pregnant animals. Data are expressed as mean (with s.e.mean) percentage change in mean arterial pressure (MAP) of 5 animals in each group.

complete, rather than partial reversal of the blunted AII pressor response in the hindlimb preparation of a pregnant, spontaneously hypertensive rat after inhibition of NO synthesis. One explanation as to why our results may have differed in this aspect is that the AII pressor response in our experiments may have reached its peak value at the lower doses of AII.

The role of vasodilator prostanoids in modulating basal vascular tone during late pregnancy was also explored in this study as it has been shown that pregnancy results in increased prostaglandin production in women (Bay & Ferris, 1979; Goodman *et al.*, 1982) and in laboratory animals (Gerber *et al.*, 1981; Chaudhuri *et al.*, 1982; Paller *et al.*, 1989). Unlike NO, vasodilator prostanoids do not appear to play an important role in the maintenance of vascular tone in late pregnancy since meclofenamate did not change MAP in pregnant and non-pregnant animals. Our observations are similar to those of other investigators who also did not observe any change in MAP in pregnant guinea-pigs (Harrison & Moore, 1989) or any change in the perfusion pressure of the *in situ* blood-perfused mesentery of pregnant rats following inhibition of cyclo-oxygenase (Chu & Beilin, 1993).

In our study, inhibition of NO synthesis only partially restored the attenuated AII pressor response during pregnancy. We therefore investigated whether release of vasodilator protaglandins may at least be partially responsible for this attenuation. However, we were unable to demonstrate a role for prostanoids in mediating the altered response to AII during pregnancy since meclofenamate did not alter the pressor response to AII or PE. Our observations, consistent with those of other investigators (Conrad & Colpoys, 1986; Harrison & Moore, 1989; Chu and Beilin, 1993), do suggest that prostaglandins are not important in the maintenance of vascular tone during pregnancy. The differences that have been observed across studies regarding the relative contributions of NO and protaglandins to the AII pressor response during pregnancy may be explained on the basis of species variations.

We have demonstrated the importance of NO rather than vasodilator prostaglandins in the maintenance of vascular tone during pregnancy. The mechanism underlying this increased release of NO during pregnancy was not explored in this study. However, factors such as increased blood volume leading to increased shear stress, as well as increased oestradiol concentration (Weiner *et al.*, 1994a,b) may be involved. We have also not identified the vascular beds where NO may play an important role in modulating the vascular changes of pregnancy. Identification of these vascular beds would be important in order to understand fully the precise role of NO during pregnancy. Furthermore, the vasoconstrictors used in this study may have direct effects on the heart and venous circulation. Thus, the responses that we observed may not be entirely due to actions on resistance vessels.

Results of our studies have important clinical implications. It has been shown by others that the blunting of the AII response in pregnancy is reversed in patients with pre-eclampsia and in those destined to develop pre-eclampsia (Gant *et al.*, 1973). We have shown that the blunting of the AII response is at least partially modulated by NO. It is, therefore, possible that a disturbance in NO release due to endothelial dysfunction or injury may lead to a rise in systemic vascular resistance and contribute to the pathogenesis of pre-eclampsia.

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Vascular 5-HT_1-like receptors mediating vasoconstriction and vasodilatation: their characterization and distribution in the intact canine cardiovascular system

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1 In anaesthetized dogs, intra-left atrial administration of 5-hydroxytryptamine (5-HT) and selected tryptamine analogues (5-carboxamidotryptamine, 5-CT; 5-methyl tryptamine, 5-MT; α-methyl 5-hydroxytryptamine, α-HT; sumatriptan, Sum) in the presence of ketanserin and MDL72222 (5-HT_2 and 5-HT_3 receptor antagonists, respectively), produced dose-related changes in carotid, coronary and renal vascular conductance mediated by vascular 5-HT_1-like receptors.

2 In the carotid vascular bed, 5-HT, 5-MT, α-HT and Sum were vasoconstrictors with a rank order of potency (comparing ED_{50} values) of 5-HT = Sum > 5-MT > α-HT. By contrast in this vascular bed, 5-CT was a potent vasodilator.

3 In the coronary vascular bed, 5-HT, 5-CT, 5-MT and α-HT were vasodilators with a rank order of potency (comparing ED_{50} values) of 5-CT > 5-HT > 5-MT > α-HT. In this vascular bed, Sum was without effect.

4 In the renal vascular bed, 5-HT, 5-CT, 5-MT, α-HT and Sum were vasoconstrictors with a rank order of potency (comparing ED_{50} values) of 5-CT > 5-HT > Sum > 5-MT > α-HT.

5 The coronary (and carotid) vasodilator responses to 5-CT were antagonized by the 5-HT_1-like receptor antagonists, spiperone (1 mg kg^{-1}) and methiothepin (0.1 mg kg^{-1}), whereas the renal vasoconstrictor responses to this tryptamine analogue were antagonized only by methiothepin.

6 It is concluded from these studies that agonist finger-printing *in vivo*, using tryptamine analogues, identifies and confirms the functional presence of at least two pharmacologically distinct subtypes of the 5-HT_1-like receptor in the intact canine cardiovascular system. These two subtypes are located on the vascular smooth muscle and mediate direct vasoconstriction and vasodilatation responses *in vivo*.

7 In addition, these studies confirm that the distribution of these subtypes within the major vascular beds, shows a marked heterogeneity. The carotid vascular responses to the tryptamine analogues indicate the presence of both the vasodilator and the vasoconstrictor subtypes. The coronary vascular responses to these analogues are, however, consistent with presence of the vasodilator subtype, only. By contrast, the renal vascular responses to these analogues indicates only the presence of the vasoconstrictor subtype.

Keywords: Vascular 5-HT_1-like receptor subtypes; agonist finger-printing; heterogeneous distribution; tryptamine analogues

Introduction

The presence of 5-hydroxytryptamine (5-HT) receptors on vascular smooth muscle, which merit classification as 5-HT_1-like receptors (see Bradley *et al.*, 1986), has been confirmed in *in vitro* and *in vivo* studies. With gene cloning techniques, molecular biology has now identified at least five 5-HT_1-like receptor subtypes; however, there is still insufficient functional or pharmacological data to permit the sub-classification of the 5-HT_1-like receptors located on vascular smooth muscle (see Martin & Humphrey, 1994). For this reason, the vascular 5-HT_1 receptors are best described by the more generic appellation '5-HT_1-like'.

Unlike the 5-HT_2 receptors, which are also located on vascular smooth muscle and mediate vasoconstriction, the vascular 5-HT_1-like receptors appear to mediate both vasodilator and vasoconstrictor responses. 5-Carboxamidotryptamine (5-CT), a tryptamine analogue having a pharmacological profile consistent with that of a 5-HT_1-like receptor agonist, has a potent vasodilator action *in vitro* and *in vivo*, due to its direct action on vascular smooth muscle (Saxena & Verdouw, 1985; Connor *et al.*, 1986; Martin *et al.*, 1987; Whiting & Cambridge, 1990). However, 5-CT has also been shown to elicit vasoconstrictor responses *in vitro*, in particular isolated blood vessels (Martin, 1990; Hamel & Bouchard, 1991; Sahin-Erdemli *et al.*, 1991) and *in vivo*, in specific vascular beds (Saxena & Verdouw, 1985; Cambridge *et al.*, 1991a). The selective, novel 5-HT_1-like receptor agonist, sumatriptan, also elicits vasoconstrictor responses, but *in vitro* these responses are largely confined to specific blood vessels (Humphrey *et al.*, 1988; Martin, 1990; Sahin-Erdemli *et al.*, 1991) and, *in vivo*, to the carotid (cerebral) vascular beds (Feniuk *et al.*, 1989; Saxena & Villalon, 1990; Humphrey *et al.*, 1991).

These experimental observations are therefore consistent with the contention that the vascular smooth muscle 5-HT_1-like receptors mediating vasodilatation and vasoconstriction responses are pharmacologically distinct subtypes. It is also apparent that the vascular 5-HT_1-like receptor subtypes are probably not uniformly distributed throughout the cardiovascular system. Hence the functional demonstration of either the vasoconstrictor or vasodilator subtype in an isolated vascular tissue is not necessarily predictive of the net 5-HT_1-like receptor mediated response *in vivo*. Therefore we undertook the following *in vivo* studies in anaesthetized dogs to characterize further and identify functional populations of these 5-HT_1-like receptor subtypes in the intact cardiovascular system, with particular regard to the major vascular beds. In these studies, we have adopted the agonist finger-printing approach, as previously described for studies in anaesthetized rats (Martin *et al.*, 1987). We have utilized the

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5-HT receptor agonist properties of selected tryptamine analogues to elicit 5-HT$_1$-like receptor-mediated haemodynamic responses and thus to differentiate between the 5-HT$_1$-like receptor subtypes. In addition, we have examined the effects of selected 5-HT$_1$-like receptor antagonists upon these responses.

Preliminary accounts of this work were presented to the IUPHAR meeting in Amsterdam, 1990, the IUPHAR Satellite meeting on Serotonin in Basle, 1990 and the Spring meeting of the British Pharmacological Society at the University of Lyon, France in April 1991 (Whiting & Cambridge, 1990; Cambridge *et al.*, 1991a,b).

Methods

All studies were conducted in accordance with Home Office guidelines and the Animal (Scientific Procedures) Act 1986. Beagle dogs (male, 12–17 kg body wt., Campbell Farms) were anaesthetized with sodium pentobarbitone 30 mg kg^{-1} i.v. (Sagatal, Rhone-Poulenc-Rorer; Dagenham, Essex) and polythene cannulae (Portex Ltd.; Hythe, Kent) inserted into the upper abdominal aorta (via a femoral artery), to measure blood pressure and heart rate, and into a femoral vein, for drug administration. A tracheotomy was also performed and artificial ventilation maintained via a Palmer large animal respiration pump (room air at 20 ml kg^{-1}, 18 breaths min^{-1}). The effectiveness of this ventilation was regularly monitored by analysis of arterial blood samples (ABL-3; Radiometer, Crawley, W. Sussex) to ensure that blood gases were maintained within acceptable limits (Green, 1979). An ultrasonic (Transonic Systems Inc.; New York, U.S.A.) or electromagnetic (Gould-Statham; Hainault, Essex) blood flow probe was then placed on the left carotid (via a neck incision) and left renal (via a left flank incision) arteries to measure carotid and renal blood flow. The chest was opened via a left thoracotomy at rib space 5 to expose the heart, which was then supported by a partial pericardial cradle. An ultrasonic blood flow probe was placed around the left circumflex artery, to measure coronary artery blood flow and a further polythene cannula was inserted into the left atrium to allow systemic administration of 5-hydroxytryptamine (5-HT), and the tryptamine analogues, without first passage through the pulmonary circulation. Each animal was then allowed to equilibrate for at least 30 min before starting the experimental studies. All measured haemodynamic parameters were recorded continuously on a Model 7 polygraph (Grass Instruments Inc., Massachusetts, U.S.A.).

In order to account for any systemic blood pressure changes, carotid, coronary and renal vascular responses were assessed in terms of changes in vascular conductance. Vascular conductance was derived from mean blood flow and systemic blood pressure. All animals were pretreated with ketanserin (0.1 mg kg^{-1}, i.v.) and MDL72222 (0.5 mg kg^{-1}, i.v.) to block 5-HT$_2$ and 5-HT$_3$ receptors, respectively and divided into groups of at least 4 animals for the construction of regional vascular dose-response curves to intra-left atrial (i.l.a.) bolus administration of 5-HT and selected tryptamine analogues. In additional groups of animals, the vascular responses to the most potent agonist, 5-CT, were examined after pretreatment of the animals with a 5-HT$_1$-like receptor antagonist, either spiperone (1 mg kg^{-1}, i.v.) or methiothepin (0.1 mg kg^{-1}, i.v.). The doses of these antagonists were chosen on the basis of results obtained in some preliminary studies.

Drugs

5-Hydroxytryptamine creatinine sulphate was obtained from Sigma Chemicals Co., (Poole, Dorset); MDL72222 (1αH,3α, 5αH-tropan-3-yl-3,5 dichloro-benzoate) was obtained from Merrell-Dow Pharmaceuticals (Strasbourg, France); ketanserin and spiperone were obtained from Janssen Pharmaceutica (Beerse, Belgium); methiothepin was obtained from Hoffman-la Roche Pharmaceuticals (Basle, Switzerland); 5-carboxamidotryptamine, 5-methyltryptamine, α-methyl-5-hydroxytryptamine and sumatriptan were synthesized in the Medicinal Chemistry Laboratories of the Wellcome Research Laboratories at Beckenham, by Drs H.F. Hodson and A.D. Robertson and colleagues.

Doses of all of the above drugs were calculated as the free base, and with the exception of spiperone, were dissolved in 0.9% w/v saline. Spiperone was initially dissolved in 10% w/v polyethylene glycol and then diluted in water.

Analysis of data

Dose-response curves to each tryptamine analogue were constructed by the measurement of haemodynamic responses to i.l.a. administration of ascending doses (dose-volume, 0.01 ml kg^{-1}) of the analogue under test. Each dose was administered only when full recovery of the response to the preceding dose was observed. For some analogues however, full recovery from the highest doses was not achieved, therefore, the subsequent dose was administered, as soon as the measured haemodynamic parameters had stabilized.

The effects of the 5-HT$_1$-like receptor antagonists, spiperone and methiothepin, upon the haemodynamic responses to a specific tryptamine analogue, were assessed by comparing the responses to a range of doses of the analogue in the absence of the antagonist with those obtained following pretreatment with the antagonist.

Data are presented as mean ± s.e. mean. Where the ED_{50} (effective dose to elicit a 50% maximum response) for each tryptamine analogue is shown, this value is the mean of the individual ED_{50} values calculated from each animal in the group. The analytical treatment of the data presented in this paper utilised parametric and non-parametric tests (Student's *t* test and Willcoxon test, respectively) to (i) assess the significance of any differences between the haemodynamic responses to the various analogues and, (ii) the significance of the effects of the 5-HT$_1$-like receptor antagonists upon these responses. A value of $P<0.05$ was taken to be statistically significant.

Results

Systemic haemodynamic effects of tryptamine analogues

In anaesthetized dogs, pretreated with ketanserin and MDL72222, i.l.a. administration of 5-HT, 5-CT, 5-methyltryptamine (5-MT), α-methyl-5-hydroxytryptamine (α-HT) and sumatriptan (Sum) all produced dose-related changes in systemic haemodynamics. 5-HT (0.03–3 μg kg^{-1}), 5-CT (0.003–3 μg kg^{-1}) and 5-MT (100–1000 μg kg^{-1}) produced significant ($P<0.05$) reductions in diastolic blood pressure (up to 25.1 ± 5.0, 60.4 ± 0.8 and 28.2 ± 5.8%, respectively). However, high doses (>3 μg kg^{-1}) 5-HT were also associated with significant ($P<0.05$) increases in diastolic blood pressure (up to 53.0 ± 10.3%). α-HT (30–1000 μg kg^{-1}) or Sum (3–100 μg kg^{-1}) caused significant ($P<0.05$) increases in diastolic blood pressure (up to 93.5 ± 14.7 and 24.6 ± 5.2%, respectively), but neither of these analogues was associated with any reductions.

With the exception of 5-MT and Sum, which caused little or no change in heart rate, the tryptamine analogues also produced some dose-related changes in heart rate. 5-HT (0.03–10 μg kg^{-1}) caused significant ($P<0.05$) reductions (up to 26.4 ± 3.8%), whereas both 5-CT (0.01–3 μg kg^{-1}) and α-HT (1000 μg kg^{-1}) were associated with significant increases in heart rate (up to 24.2 ± 13.7 and 41.4 ± 9.4%, respectively).

The basal values for diastolic blood pressure and heart rate for the animals in each group are summarized in Table 1.

Table 1 Basal values for diastolic blood pressure (DBP), heart rate (HR), carotid arterial blood flow (CarBF), carotid vascular conductance (CarCond), coronary blood flow (CorBF), coronary vascular conductance (CorCond), renal blood flow (RenBF), renal vascular conductance (RenCond) in the 5-hydroxytryptamine (5-HT), 5-carboxytryptamine (5-CT), 5-methyltryptamine (5-MT), α-methyl-5-hydroxytryptamine (α-HT) and sumatriptan (Sum) treatment groups

Parameter	*Treatment group* 5-CT	5-HT	5-MT	α-HT	Sum
DBP (mmHg)	95 ± 10	75 ± 4	93 ± 1	75 ± 2	93 ± 6
HR (b.p.m.)	155 ± 4	156 ± 5	168 ± 4	154 ± 4	183 ± 14
CarBF (ml min^{-1})	186 ± 26	180 ± 30	209 ± 32	163 ± 12	207 ± 32
CarCond (ml min^{-1} mmHg)	1.79 ± 0.33	2.14 ± 0.40	2.03 ± 0.29	1.93 ± 0.54	1.96 ± 0.16
CorBF (ml min^{-1})	23.5 ± 1.0	13.8 ± 2.8	20.8 ± 4.8	12.8 ± 2.3	47.5 ± 8.5
CorCond (ml min^{-1} mmHg)	0.22 ± 0.01	0.17 ± 0.04	0.21 ± 0.05	0.15 ± 0.03	0.48 ± 0.10
RenBF (ml min^{-1})	238 ± 30	189 ± 27	133 ± 15	185 ± 32	215 ± 5
RenCond (ml min^{-1} mmHg)	2.27 ± 0.41	2.21 ± 0.27	1.31 ± 0.16	2.15 ± 0.31	2.09 ± 0.09

Data are shown as the mean ± s.e.mean ($n = 4$ for each analogue).

Table 2 The carotid, coronary and renal vascular conductance responses to 5-hydroxytryptamine (5-HT), 5-carboxytryptamine (5-CT), 5-methyltryptamine (5-MT), α-methyl-5-hydroxytryptamine (α-HT) and sumatriptan (Sum) in anaesthetized dogs

Tryptamine analogue		*Carotid*	*Coronary*	*Renal*
5-HT	ED_{50} (μg kg^{-1})	6.0 ± 5.6	9.0 ± 2.7	0.056 ± 0.017
	max. change (%)	−49.8 ± 4.2	+321 ± 35	−66.0 ± 3.8
5-CT	ED_{50} (μg kg^{-1})	0.12 ± 0.03	0.16 ± 0.07	0.01 ± 0.003
	max. change (%)	+114 ± 25	+424 ± 61	−69.9 ± 7.9
5-MT	ED_{50} (μg kg^{-1})	111.0 ± 29.0	91.3 ± 13.5	2.60 ± 0.34
	max. change (%)	−52.9 ± 5.5	+322 ± 59	−73.9 ± 7.1
α-HT	ED_{50} (μg kg^{-1})	167.0 ± 70	335 ± 94	12.3 ± 5.1
	max. change (%)	−77.2 ± 4.0	+35.2 ± 10.2	−62.8 ± 7.7
Sum	ED_{50} (μg kg^{-1})	6.0 ± 3.4	NE	0.29 ± 0.06
	max. change (%)	−69.8 ± 4.6	NE	−73.9 ± 7.1

Data are derived from the observed changes in vascular conductance for a series of doses of each analogue and shown as the mean ± s.e.mean ($n = 4$ for each analogue) for the ED_{50} and the maximal change achieved. NE no effect observed at doses up to 300 μg kg^{-1}.

Carotid arterial vascular responses to tryptamine analogues

In the carotid arterial vasculature, there was a marked divergence in the vascular response to these analogues. 5-CT (0.01–3 μg kg^{-1}) alone caused a dose-related, significant ($P < 0.05$) increase in carotid arterial blood flow and vascular conductance. By contrast, 5-HT (0.03–100 μg kg^{-1}), Sum (0.03–300 μg kg^{-1}), 5-MT (10–1000 μg kg^{-1}) and α-HT (3–1000 μg kg^{-1}) caused dose-related, significant ($P < 0.05$) reductions in carotid arterial blood flow and vascular conductance. In this vascular bed, Sum and α-HT appeared to be the most efficacious of these analogues as vasoconstrictors. For these analogues, the following rank order of potency was found: 5-HT = Sum > 5-MT > α-HT.

The basal values for carotid arterial blood flow and vascular conductance for the animals in each analogue treatment group are summarized in Table 1. The mean ED_{50} value and maximal change (%) in carotid vascular conductance produced by these analogues are summarized in Table 2, and are derived from their respective dose-response curves (see Figure 1).

Coronary vascular responses to tryptamine analogues

In the coronary vasculature, with the exception of Sum, which had little or no effect in this vascular bed at doses up to 300 μg kg^{-1}, 5-CT (0.01–3 μg kg^{-1}), 5-HT (0.3–100 μg kg^{-1}), 5-MT (10–1000 μg kg^{-1}) and α-HT (10–1000 μg kg^{-1}) caused only dose-related, significant ($P < 0.05$) increases in coronary blood flow and vascular conductance. The apparent efficacies of 5-CT, 5-HT and 5-MT as vasodilators in this vascular bed were similar, whereas that for α-HT appeared to be much lower (approximately one-

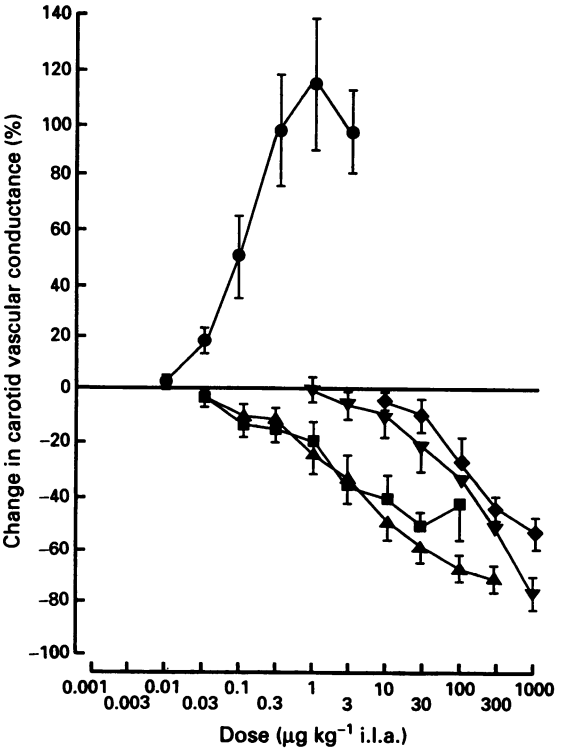

Figure 1 The effects of 5-hydroxytryptamine (■), 5-carboxamidotryptamine (●), 5-methyltryptamine (◆), α-methyl-5-hydroxytryptamine (▼) and sumatriptan (▲) upon carotid vascular conductance in anaesthetized dogs ($n = 4$ for each analogue). Values are shown as mean ± s.e.mean.

tenth). For these analogues the following rank order of potency was found: 5-CT > 5-HT > 5-MT > α-HT >> Sum.

The basal values for coronary blood flow and vascular conductance for the animals in each analogue treatment group are summarized in Table 1. The mean ED_{50} value and maximal change (%) in coronary vascular conductance produced by these analogues are summarized in Table 2, and are derived from their respective dose-response curves (see Figure 2).

Renal vascular responses to tryptamine analogues

In the renal vasculature, 5-CT (0.001–0.3 μg kg^{-1}), 5-HT (0.01–3 μg kg^{-1}), Sum (0.01–30 μg kg^{-1}), 5-MT (0.03–100 μg kg^{-1}) and α-HT (0.1–1000 μg kg^{-1}) caused only dose-related, significant ($P<0.05$) reductions in renal blood flow and vascular conductance. There were no apparent differences between these analogues with respect to their efficacy as vasoconstrictors in this vascular bed and the following rank order of potency was found: 5-CT > 5-HT > Sum > 5-MT > α-HT.

The basal values for renal blood flow and vascular conductance for the animals in each analogue treatment group are summarized in Table 1. The mean ED_{50} value and maximal change (%) in renal vascular conductance produced by these analogues are summarized in Table 2, and are derived from their respective dose-response curves (see Figure 3).

Vascular responses to 5-carboxamidotryptamine in the presence of 5-HT$_1$-like receptor antagonists

Pretreatment with the 5-HT$_1$-like receptor antagonists, spiperone (1 mg kg^{-1}) or methiothepin (0.1 mg kg^{-1}), caused some initial cardiovascular changes which were not sustained at these levels. Although there was a tendency towards lower values for blood pressure, heart rate and blood flows in both the spiperone and the methiothepin-treated groups of animals (see Table 3), the basal values for all measured cardiovascular parameters in the two groups, prior to commencing the 5-CT dose-response, were within the range of basal values recorded for the animals used in the previous tryptamine analogue studies (see above and Table 1).

In the carotid arterial vasculature, the vasodilatation responses to 5-CT (increased vascular conductance) were significantly ($P<0.05$) attenuated by pretreatment with either spiperone or methiothepin. In this vascular bed, the increases in carotid vascular conductance due to the low doses of 5-CT (0.01–1 μg kg^{-1}) were converted to significant ($P<0.05$), dose-related reductions in carotid vascular conductance, indicating vasoconstriction, following pretreatment with spiperone but not with methiothepin (see Figure 4a and b).

In the coronary vasculature, the vasodilatation responses to 5-CT (increased vascular conductance) were also significantly ($P<0.05$) attenuated by pretreatment with either spiperone or methiothepin. However, the inhibitory effects of spiperone on the coronary vascular responses to 5-CT were not as great as those of methiothepin (see Figure 5a and b).

By contrast, in the renal vasculature, the vasoconstrictor responses to 5-CT (reduced vascular conductance) were only marginally affected by pretreatment with spiperone, whereas they were significantly ($P<0.05$) attentuated by pretreatment with methiothepin (see Figure 6a and b).

Discussion

The studies described in this paper demonstrate that, in the presence of 5-HT$_2$ and 5-HT$_3$ receptor blockade, 5-HT and selected tryptamine analogues cause dose-related changes in regional vascular conductance in the dog, *in vivo*. However, these changes comprise both vasoconstriction and vasodilatation responses and there is a marked heterogeneity in the profile of the response of the different vascular beds. In the carotid vasculature, both responses are evident, however, in the coronary vasculature, only vasodilatation is seen, whilst in the renal vasculature, there is only vasoconstriction. The observation that the vasoconstriction (renal) and vasodilatation (coronary/carotid) responses to 5-CT are attentuated by the 5-HT$_1$-like receptor antagonist, methiothepin, in the presence of 5-HT$_2$ and 5-HT$_3$ receptor blockade, confirms that both responses are mediated by 5-HT$_1$-like receptors. However, the rank order of potency for 5-HT and the tryptamine analogues is different with respect to vasoconstriction (renal), when compared with that for vasodilatation (coronary), responses. In addition, the 5-HT$_1$-like receptor antagonist, spiperone, attenuates the vasodilatation, but has

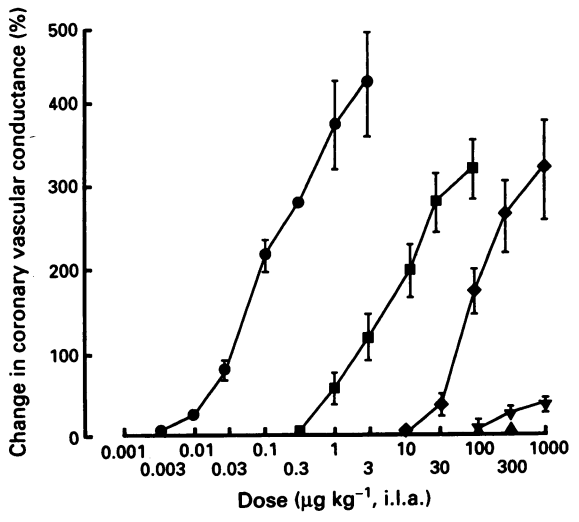

Figure 2 The effects of 5-hydroxytryptamine (■), 5-carboxamidotryptamine (●), 5-methyltryptamine (◆), α-methyl-5-hydroxytryptamine (▼) and sumatriptan (▲) upon coronary vascular conductance in anaesthetized dogs ($n = 4$ for each analogue). Values are shown as mean ± s.e.mean.

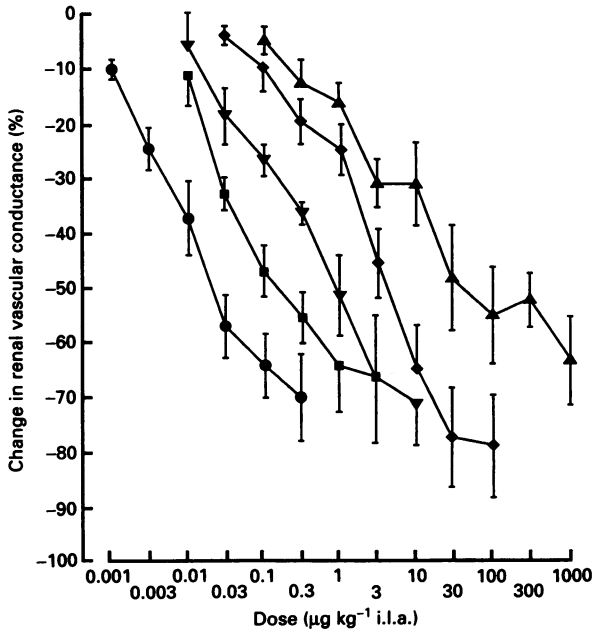

Figure 3 The effects of 5-hydroxytryptamine (■), 5-carboxamidotryptamine (●), 5-methyltryptamine (◆), α-methyl-5-hydroxytryptamine (▲) and sumatriptan (▼) upon renal vascular conductance in anaesthetized dogs ($n = 4$ for each analogue). Values are shown as mean ± s.e.mean.

Table 3 Basal values for diastolic blood pressure (DBP), heart rate (HR), carotid arterial blood flow (CarBF), carotid vascular conductance (CarCond), coronary blood flow (CorBF), coronary vascular conductance (CorCond), renal blood flow (RenBF), renal vascular conductance (RenCond) in the spiperone (Spip: 1 mg kg^{-1}) and methiothepin (Met: 0.1 mg kg^{-1}) pretreatment groups

	Pretreatment group			
Parameter	*pre-Spip*	*post-Spip*	*pre-Met*	*post-Met*
DBP (mmHg)	84 ± 6	72 ± 7	96 ± 7	83 ± 6
HR (b.p.m.)	144 ± 10	133 ± 7	166 ± 8	169 ± 9
CarBF (ml min^{-1})	220 ± 26	191 ± 24	211 ± 16	205 ± 16
CarCond (ml min^{-1} mmHg)	2.19 ± 0.31	2.24 ± 0.31	1.89 ± 0.17	2.12 ± 0.16
CorBF (ml min^{-1})	25 ± 6	22 ± 6	26 ± 6	24 ± 6
CorCond (ml min^{-1} mmHg)	0.25 ± 0.07	0.27 ± 0.08	0.23 ± 0.04	0.25 ± 0.05
RenBF (ml min^{-1})	195 ± 36	170 ± 37	229 ± 48	229 ± 48
RenCond (ml min^{-1} mmHg)	1.94 ± 0.39	1.98 ± 0.39	1.99 ± 0.37	2.27 ± 0.65

Data are shown as the mean ± s.e.mean ($n = 4$ for each antagonist pretreatment group).

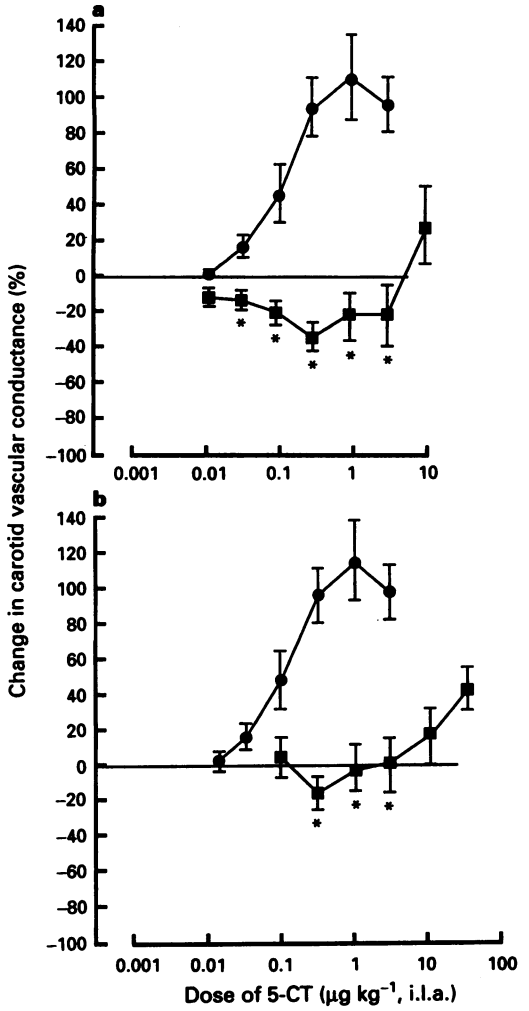

Figure 4 The effects of 5-carboxamidotryptamine (5-CT), alone (●) or following pretreatment with either (a) 1 mg kg^{-1} spiperone (■) or (b) 0.1 mg kg^{-1} methiothepin (■), on carotid vascular conductance in anaesthetized dogs ($n = 4$ for each dose-response curve). Values are shown as mean ± s.e.mean. *Significantly different from response to 5-CT alone ($P < 0.05$).

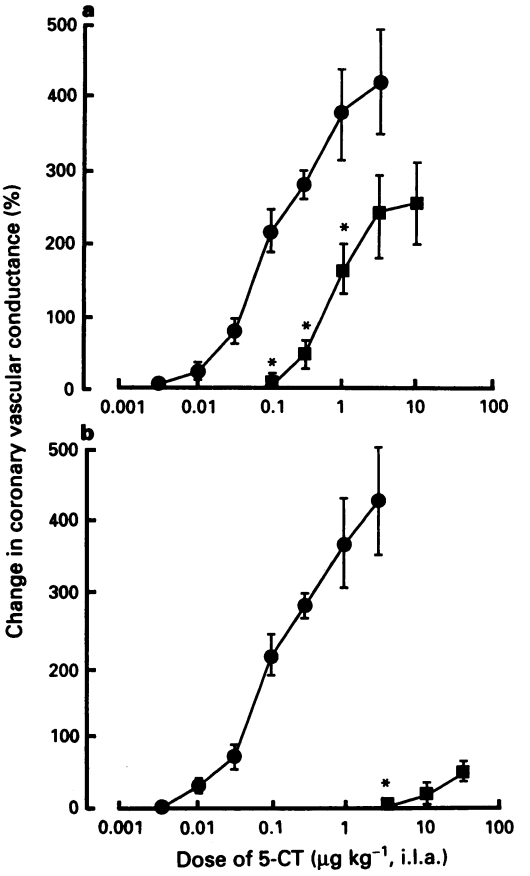

Figure 5 The effects of 5-carboxamidotryptamine (5-CT), alone (●) or following pretreatment with either (a) 1 mg kg^{-1} spiperone (■) or (b) 0.1 mg kg^{-1} methiothepin (■), on coronary vascular conductance in anaesthetized dogs ($n = 4$ for each dose-response curve). Values are shown as mean ± s.e.mean. *Significantly different from response to 5-CT alone ($P < 0.05$).

little or no effect upon the vasoconstriction responses to 5-carboxamidotryptamine.

There is a body of evidence which indicates that there are at least two functional subtypes of the 5-HT$_1$-like receptor located on vascular smooth muscle cells, mediating vasoconstriction and vasodilatation, respectively. The data derived from the studies described here, using a range of tryptamine analogues, to provide a pharmacological finger-print, and selected 5-HT$_1$-like receptor antagonists, clearly support this conclusion.

The vascular 5-HT$_1$-like receptor subtype, which mediates vasoconstriction, has been primarily established through studies with the selective agonist, sumatriptan (Humphrey *et al.*, 1988; Connor *et al.*, 1989; Feniuk *et al.*, 1989; Hamel & Bouchard, 1991; Sahin-Erdemli *et al.*, 1991). However, it has also been observed that the systemic vasodilator tryptamine analogue, 5-CT, is a potent vasoconstrictor in vascular preparations where this same receptor subtype is evident (Saxena & Verdouw, 1985; Martin, 1990; Hamel & Bouchard, 1991). In our studies, the renal vasculature of the dog exhibits only vasoconstrictor responses to the tryptamine

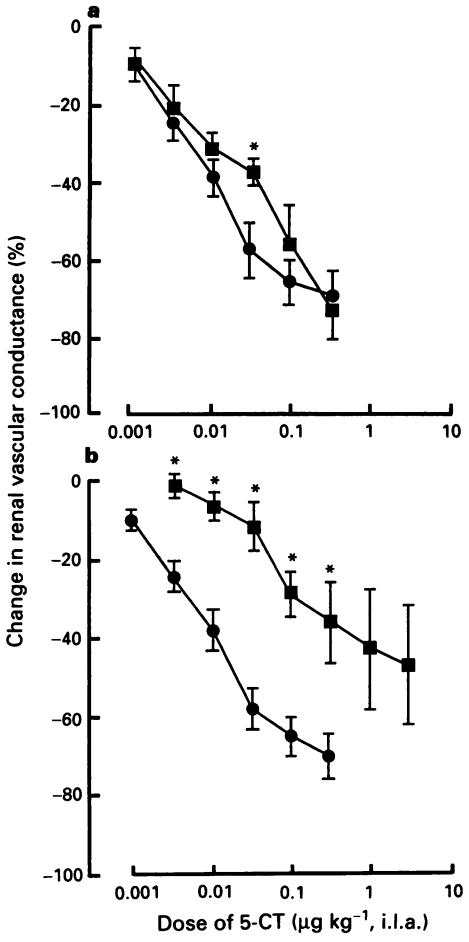

Figure 6 The effects of 5-carboxamidotryptamine (5-CT), alone (●) or following pretreatment with either (a) 1 mg kg^{-1} spiperone (■) or (b) 0.1 mg kg^{-1} methiothepin (■), on renal vascular conductance in anaesthetized dogs ($n = 4$ for each dose-response curve). Values are shown as mean ± s.e.mean. *Significantly different from response to 5-CT alone ($P < 0.05$).

analogues. In this respect 5-carboxamidotryptamine and sumatriptan are potent vasoconstrictors, and the rank order of the tryptamine analogues (5-CT> 5-HT> Sum> 5-MT> α-HT) is entirely consistent with the stimulation of the vasoconstrictor 5-HT$_1$-like receptor subtype. The metabolic fate of these tryptamine analogues could conceivably affect this rank order; however, the experimental design for these studies minimized this influence by using bolus systemic administration of these analogues directly into the left atria. These studies also found the renal vasoconstrictor response to the most potent of the analogues, 5-CT, to be significantly attenuated by methiothepin, but unaffected by doses of spiperone which attenuated the vasodilator (coronary) responses to this agonist. Whilst it is accepted that the 5-HT$_1$-like receptor antagonist profiles of methiothepin and spiperone are not ideal (see Martin & Maclennan, 1990), nevertheless this observation corroborates reports of some *in vitro* studies which finds methiothepin, but not spiperone, to be an effective antagonist at this receptor subtype (Perren *et al.*, 1991; Martin *et al.*, 1991).

5-CT also has a high affinity for the vascular 5-HT$_1$-like receptor subtype mediating vasodilatation (Saxena & Verdouw, 1985; Connor *et al.*, 1986; Leff *et al.*, 1987; Martin *et al.*, 1987; Martin, 1990). In fact, the predominant haemodynamic response to i.v. 5-CT in the intact animal is a blood pressure fall, indicative of extensive vasodilatation (Connor *et al.*, 1986; Martin *et al.*, 1987; Saxena & Villalon, 1990). By contrast, in the intact animal, as confirmed in the studies reported here, i.v. Sum is reported to produce very little change in blood pressure (Feniuk *et al.*, 1989; Humphrey *et al.*, 1991). Sum has also been shown to have little or no direct relaxant effect in isolated tissues, such as the rabbit jugular vein (Martin *et al.*, 1993), the cat saphenous vein (Humphrey *et al.*, 1988) or porcine vena cava (Sumner, 1991). All of these isolated tissues relax in response to 5-CT or 5-HT.

In the studies described here, the coronary vasodilator responses to the tryptamine analogues (rank order of potency 5-CT> 5-HT> 5-MT> α-HT>> Sum) are entirely consistent with stimulation of a vascular 5-HT$_1$-like receptor subtype. However, this subtype clearly differs in its pharmacological profile when compared with the subtype mediating vasoconstriction (see above). Furthermore our studies found that the coronary (and carotid) vasodilatation responses to 5-carboxamidotryptamine were significantly attenuated by the 5-HT$_1$-like receptor antagonists, methiothepin and spiperone. Both methiothepin and spiperone have been shown to act as antagonists of the vascular 5-HT$_1$-like receptor subtype mediating vasodilatation *in vitro* (Martin *et al.*, 1987; Humphrey *et al.*, 1988; Perren *et al.*, 1991). Thus it would appear that our data from the anaesthetized dog are also consistent with the involvement of a different vascular receptor subtype mediating vasodilatation *in vivo*. With respect to these antagonist studies, it is apparent that spiperone can differentiate between these functional receptor subtypes. It is therefore of particular interest that in our studies, spiperone also appeared to unmask the vasoconstrictor activity of 5-CT in the carotid vasculature.

It is possible that the vasodilator responses observed with 5-HT and the tryptamine analogues, could be the result of endothelial stimulation. 5-HT, and more recently, 5-CT and Sum, have been shown to stimulate endothelium-dependent vascular relaxation in a number of different isolated vascular preparations (Cocks & Angus, 1983; Leff *et al.*, 1987; Martin *et al.*, 1987; Schoeffter & Hoyer, 1990; Mankad *et al.*, 1991; Gupta, 1992). From these studies it appears that a 5-HT$_1$-like receptor subtype mediates these endothelial-dependent relaxation responses. However, there is some controversy over the exact nature of this endothelial receptor subtype (Martin *et al.*, 1987; Martin, 1990; Sumner, 1991; Martin *et al.*, 1993). In addition, the rank order of potency for the tryptamine analogues as vasodilators in our studies, and our observation that the inhibition of nitric oxide (NO)-synthesis by N^G-nitro-L-arginine methyl ester had little or no effect upon the coronary vasodilatation responses to 5-CT (Cambridge *et al.*, 1991b), would appear to confirm that these responses are mediated by the subtype located on the smooth muscle.

The pharmacological data generated by this study in intact animals were not intended to provide an unequivocal classification of these functional vascular 5-HT$_1$-like receptor subtypes. Indeed it is evident from the recent review by Martin & Humphrey (1994), that there are anomalies within the current nomenclature for the 5-HT family of receptors which have still to be resolved. Nevertheless, of the 5-HT receptors which have been identified through gene cloning, the vascular 5-HT$_1$-like receptor subtype which mediates vasoconstriction would appear to be most consistent with the 5-HT$_{1D\alpha/\beta}$ receptor. By contrast, the vascular 5-HT$_1$-like receptor subtype which mediates vasodilatation may be the more recently described 5-ht7 receptor. This receptor has been identified on smooth muscle including coronary vasculature and may actually be distinct from the 5-HT$_1$-like receptor class (see also Eglen *et al.*, 1994). However, until more data become available it is probably more appropriate to continue to refer to both the vasoconstrictor and the vasodilator subtypes as 5-HT$_1$-like.

It is evident from our studies that there is a heterogeneous distribution of these two receptor subtypes, which suggests that they contribute to the control of vascular tone at a regional or localized level. In the cardiovascular system of the dog (this paper) and rat (Martin *et al.*, 1987) the subtype

which mediates vasodilatation appears to be much more widely distributed. This probably explains why the most persistent cardiovascular response to exogenously administered 5-HT, in the intact animal, is a reduction in blood pressure through systemic vasodilatation (see review by Saxena & Villalon, 1990). The canine coronary vasculature is an excellent example of a vascular bed which predominantly contains the vasodilator subtype. By contrast, the functional distribution of the subtype mediating vasoconstriction is clearly restricted. Using Sum, it has been shown that the functional distribution of this receptor subtype seems to be almost exclusively confined to the carotid (cerebral) vasculature (Feniuk *et al.*, 1989; Humphrey *et al.*, 1991). Our studies have also confirmed its importance in this vascular bed, although our finding that this bed also contains a substantial population of the vasodilator subtype, probably explains the poor vasoconstrictor activity of 5-HT (and 5-MT), which has affinity for both receptor subtypes. However, we have also identified a significant population of the vasoconstrictor subtype in the renal vascular bed. In this respect the renal vasculature appears to be devoid of the vasodilator subtype, for unlike the carotid vasculature, the renal vasculature exhibits only vasoconstrictor responses to the tryptamine analogues. It should be noted that in the canine renal vasculature we have found evidence for endothelial 5-HT$_1$-like receptors coupled to the endogenous vasodilator, nitric oxide (see the following paper, Whiting & Cambridge, 1995).

Although the vasoconstrictor subtype of the 5-HT$_1$-like receptor appears to be restricted to certain conduit blood vessels, within intact vascular beds it is evident that this subtype is closely associated with arteriovenous-anastomoses (Perren *et al.*, 1989; Martin & MacLennan, 1990; Saxena & Villalon, 1990; Humphrey *et al.*, 1991). The carotid (cerebral) vasculature is rich in these structures, hence it is not surprising that this vascular bed is so responsive to the vasoconstrictor actions of the most selective of the tryptamine analogues, Sum. By contrast, the coronary vasculature is reported to lack arteriovenous-anastomoses (Berne & Rubio, 1979). This probably explains the absence of any evidence from our studies which would be consistent with the presence of a functional population of this subtype in the coronary vascular bed. It is also a matter of debate whether the renal vasculature possesses any arteriovenous-anastomoses (Brod, 1973). However, the anatomy of the renal vasculature has evolved to allow it to redistribute blood flow within the kidney to a remarkable degree, and it is well endowed with a substantial network of arterio-anastomoses (Brod, 1973). It is possible therefore that functional populations of the vasoconstrictor subtype of the 5-HT$_1$-like receptor within the renal vasculature are associated with these networks. Although our studies do not permit us to identify the exact location of this receptor subtype, our data would suggest that they are located at the level of the intra-renal resistance vessels. Our studies also shed no light upon the role of these receptor subtypes within the kidney. However, the complex vascular structure of the mammalian kidney which is integral to the filtration/excretion function of this organ must be particularly susceptible to the effects of aggregating platelets and the formation of intra-renal thrombi. If these 5-HT$_1$-like receptor subtypes are located on the arterio-anastomoses, their stimulation, by the intra-renal release of 5-HT due to platelet activation and aggregation, may serve to redistribute intra-renal blood flow away from those regions of the kidney which would be most at risk from the effects of thrombus formation (i.e. the low flow regions of the medulla).

In conclusion, these studies in the intact cardiovascular system of the dog have clearly shown that the technique of agonist finger-printing can be used effectively *in vivo* to evaluate the pharmacological basis of receptor-mediated vascular events. Using this technique and comparing functional (haemodynamic) responses to a range of tryptamine analogues, in conjunction with some antagonist studies, we have demonstrated the presence of at least two vascular 5-HT$_1$-like receptor subtypes within the mammalian cardiovascular system, and defined their different pharmacological and functional profiles. Furthermore, these studies have demonstrated a marked heterogeneity in the cardiovascular distribution of these receptor subtypes.

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Canine renovascular responses to sumatriptan and 5-carboxamidotryptamine: modulation through endothelial 5-HT_1-like receptors by endogenous nitric oxide

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1 In anaesthetized dogs, intra-left atrial (i.l.a.) administration of the 5-HT_1-like receptor agonists, sumatriptan (1-10 $\mu g\ kg^{-1}$) and 5-carboxamidotryptamine (0.03-0.3 $\mu g\ kg^{-1}$) produced dose-related reductions in renal blood flow and vascular conductance, which were characterized by their rapid onset and recovery.

2 In these animals, i.v. administration of the inhibitor of nitric oxide (NO) synthase, N^G-nitro-L-arginine methyl ester (L-NAME; 10 mg kg^{-1}) significantly augmented the renal vasoconstrictor responses to i.l.a. sumatriptan and 5-carboxamidotryptamine.

3 The effects of L-NAME upon these responses to sumatriptan and 5-carboxamidotryptamine were significantly reversed by subsequent i.v. administration of L-arginine (1000 mg kg^{-1}).

4 L-NAME significantly attenuated the systemic hypotensive responses to i.v. acetylcholine (0.3-3 $\mu g\ kg^{-1}$) and this effect was also reversed by L-arginine.

5 L-NAME had no effect upon the renal vasoconstrictor response to i.l.a. administration of angiotensin II, nor did it affect the renal vascular conductance recovery response to brief mechanical occlusion of the renal artery.

6 These data suggest that sumatriptan and 5-carboxamidotryptamine stimulate the release of NO through the activation of a 5-HT_1-like receptor located on the endothelial cells.

7 It is concluded that in canine renal vasculature, 5-HT_1-like agonists (and presumably endogenous 5-hydroxytryptamine) can cause simultaneous activation of a 5-HT_1-like receptor on both vascular smooth muscle and endothelial cells. The net renal vascular response to these agonists is therefore a function of both the vascular smooth muscle vasoconstriction and the concurrent vasodilator influence of NO released from the endothelium.

Keywords: 5-HT_1-like receptors; endothelium; nitric oxide, sumatriptan; 5-carboxamidotryptamine

Introduction

The current status of the nomenclature used in the classification of 5-hydroxytryptamine (5-HT) receptors confirms that the appellation '5-HT_1-like' remains the most appropriate way to describe those vascular smooth muscle 5-HT receptors which mediate both vasoconstriction and vasodilatation responses and which cannot be classified as 5-HT_2, 5-HT_3 or 5-HT_4 (see Bradley *et al.*, 1988; Martin & Humphrey, 1994). The vasoconstrictor 5-HT_1-like receptors are selectively stimulated by the 5-HT_1-like agonist, sumatriptan (Humphrey *et al.*, 1988; Feniuk *et al.*, 1989; Cambridge *et al.*, 1995), whilst the tryptamine analogue, 5-carboxamidotryptamine, is a potent agonist at the vasodilator 5-HT_1-like receptors (Saxena & Verdouw, 1985; Connor *et al.*, 1986; Leff *et al.*, 1987; Martin *et al.*, 1987; Cambridge *et al.*, 1995).

In previous studies we used selected tryptamine analogues (including sumatriptan and 5-carboxamidotryptamine) to identify the functional presence of vasoconstrictor and vasodilator subtypes of the 5-HT_1-like receptor within the different vascular beds of the dog, and to characterize these subtypes pharmacologically. It was concluded that there was a heterogeneous distribution of these receptor subtypes such that, the coronary vasculature contained only the vasodilator subtype, the renal vasculature contained only the vasoconstrictor subtype, whilst the carotid vasculature contained both subtypes (Whiting & Cambridge, 1990; Cambridge *et al.*, 1991a,b; 1995).

There were, however, marked differences in the profile of the carotid and renal vasoconstrictor responses to these 5-HT_1-like agonists. In the carotid vasculature, systemic bolus injections of sumatriptan produced well-sustained vasoconstrictor responses, reflecting its constrictor action at the extensive arteriovenous anastomoses which characterize this vascular bed (see also Humphrey *et al.*, 1988; Feniuk *et al.*, 1989). In this vascular bed 5-carboxamidotryptamine produced long-lasting vasodilator responses (see also Saxena & Verdouw, 1985; Connor *et al.*, 1986), although this agonist is also know to constrict arteriovenous anastomoses (Saxena & Verdouw, 1985).

By contrast, in the renal vascular beds, sumatriptan and 5-carboxamidotryptamine both produced marked vasoconstrictor responses, which were characterized by a very rapid recovery and evidence of a secondary vasodilatation (Cambridge *et al.*, 1991a,b; 1995). It had been previously concluded that the renal vascular beds possessed very few, if any, of the vasodilator subtype of the 5-HT_1-like receptor, at which 5-carboxamidotryptamine is a potent agonist. Hence, it appeared that sumatriptan and 5-carboxamidotryptamine might elicit additional vasodilatation in the renal vasculature which modulated the primary vasoconstrictor responses to these agonists.

Studies in specific isolated blood vessels (Molderings *et al.*, 1989; Schoeffter & Hoyer, 1990; Gupta, 1992) *in vitro* indicated that sumatriptan and 5-carboxamidotryptamine could elicit endothelial-dependent relaxation through the relese of nitric oxide (NO), the endothelial-derived vasodilator formerly known as EDRF (Khan & Furchgott, 1987; Ignarro *et al.*, 1987; Palmer *et al.*, 1987). We have therefore investigated the possibility that intra-renal NO release could be responsible for modulating the renal vasoconstrictor responses to 5-HT_1-like receptor agonists. A preliminary

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account of this work was presented to the Summer meeting of the British Pharmacological Society at the University of Glasgow in July 1991 (Whiting & Cambridge, 1991) and to the 2nd International Symposium on Serotonin at Houston in September 1992.

Methods

All studies were carried out in accordance with Home Office guidelines and the Animals (Scientific Procedures) Act, 1986. Beagle dogs (male, 12–16 kg body wt; Cambell Farms) were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, i.v.; Sagatal, Rhone-Poulenc, Rorer, Dagenham, Essex) and polythene cannulae (Portex Ltd., Hythe, Kent) inserted into the upper abdominal aorta via a femoral artery, to measure blood pressure and heart rate, and into a femoral vein, for drug administration. A tracheotomy was also performed and artificial ventilation maintained via a Palmer large animal respiration pump (room air at 20 ml kg^{-1}, 18 breaths min^{-1}). The effectiveness of this ventilation was regularly monitored by analysis of arterial blood samples (ABL-3; Radiometer, Crawley, W. Sussex) to ensure that blood gases were maintained within acceptable limits (Green, 1979).

An ultrasonic (Transonic Systems Inc., New York, U.S.A.) blood flow probe was then placed on the left renal artery (via a left flank incision) to measure renal blood flow. As in previous studies (see Cambridge *et al.*, 1991b; 1995), a further polythene cannula was inserted into the left atrium, via a thoracotomy at rib space 5, to allow intra-left atrial (i.l.a.) administration of 5-carboxamidotryptamine and sumatriptan and thus achieve systemic exposure to these agonists without first passage through the pulmonary circulation. Each animal was then allowed to equilibrate for at least 30 min prior to commencing the experimental studies. All measured haemodynamic parameters were recorded continuously on a Model 7 polygraph (Grass Instruments Inc., Massachusetts, U.S.A.). In order to account for any changes in systemic blood pressure, renal vascular responses were measured as changes in renal vascular conductance. Renal vascular conductance was derived from mean renal blood flow and systemic blood pressure.

In preliminary dose-ranging studies using closed-chest, anaesthetized animals ($n = 3$), the haemodynamic effects of the NO-synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME) or the substrate for NO formation, L-arginine, were assessed, in order to identify a suitable single dose of each of these compounds for use in the main part of the study. Ascending doses of either L-NAME (1–30 mg kg^{-1}, i.v.) or L-arginine (10–1000 mg kg^{-1}, i.v.) were administered as bolus injections and the effects on systemic blood pressure and heart rate were measured. In those animals which received L-NAME, the effects of this compound on the systemic blood pressure responses to acetylcholine (0.3–3 mg kg^{-1}, i.v.) were also assessed.

In the open-chest, anaesthetized animals used for the main part of the study, 5-HT$_2$ and 5-HT$_3$ receptor blockade was achieved in each animal for the duration of the experimental protocol by administration of a single dose of ketanserin (0.1 mg kg^{-1}, i.v.) and MDL72222 (0.5 mg kg^{-1}, i.v.), respectively (see Cambridge *et al.*, 1995). Groups of at least 4 animals were then used for the construction of a limited dose-response curve to i.l.a. administration of one or other of the 5-HT$_1$-like receptor agonists sumatriptan (1, 3 and 10 μg kg^{-1}) or 5-carboxamidotryptamine (0.03, 0.1 and 0.3 μg kg^{-1}). The renal vascular responses to these agonists were assessed before and 15 min after i.v. bolus administration of L-NAME (10 mg kg^{-1}), and again 15 min after additional i.v. bolus administration of L-arginine (1000 mg kg^{-1}). In some of these animals the effects of L-NAME and L-arginine treatment upon the systemic blood pressure responses to i.l.a. administration of acetylcholine (0.3, 1 and 3 μg kg^{-1}) were also assessed.

In a further group of 4 open-chest animals, the effects of L-NAME treatment alone upon the renal vascular responses to i.l.a. administration of the vasoconstrictor peptide, angiotensin II (0.1 μg kg^{-1}), and the renal vascular recovery response following a 15 s mechanical occlusion of the left renal artery were also assessed.

Drugs

Ketanserin and MDL72222 (1αH,3α,5αH-tropan-3-yl-3,5-dichloro-benzoate) were obtained from Janssen Pharmaceutica (Beerse, Belgium) and Marion Merrell-Dow (Strasbourg, France), respectively. L-NAME, L-arginine, acetylcholine and antiotensin II were all obtained from Sigma Chemical Co. (Poole, Dorset). Sumatriptan and 5-carboxamidotryptamine were synthesized at the Medicinal Chemistry Laboratories of the Wellcome Research Laboratories at Beckenham by Dr A.D. Robertson and colleagues.

Doses of all the above drugs were calculated as the free base, dissolved in 0.9% w/v saline, and administered in a dose volume of 0.01 ml kg^{-1}.

Analysis of data

To assess the effects of L-NAME and L-arginine upon the renal vascular responses (renal vascular conductance) to sumatriptan, 5-carboxamidotryptamine and angiotensin II, and the systemic haemodynamic responses (mean systemic blood pressure) to acetylcholine, the area under the curve (AUC) for each response was measured by computerised planimetry (Macintosh Software). Data expressed as an AUC (measured in arbitrary units) therefore comprise both the magnitude and the duration of the response. Where appropriate, however, data reflecting simply the magnitude of the response to each agonist, are also expressed as a percentage change from pre-dosing levels. Data for the renal vascular recovery response following brief mechanical occlusion of the renal artery are, however, simply expressed as the mean time to 50% recovery.

Data are presented as mean ± s.e.mean. The statistical analysis of all the data utilised the non-parametric Willcoxon test. A value of $P<0.05$ was taken to be statistically significant.

Results

Preliminary dose-ranging studies with L-NAME and L-arginine

In two closed-chests animals i.v. bolus L-NAME (1–30 mg kg^{-1}) caused a dose-related increase in diastolic blood pressure (+17 to +42 mmHg) and reduction in heart rate (−52 to −85 b.p.m.). In a separate closed-chest animal, i.v. bolus L-arginine (10–1000 mg kg^{-1}) caused little change in diastolic blood pressure or heart rate, except following the 300 and 1000 mg kg^{-1} doses, when it produced short-lived reductions in diastolic blood pressure (−25 and −67 mmHg, respectively) and small falls in heart rate (−10 and −25 b.p.m., respectively). On the basis of these dose-ranging experiments (and the work of Rees *et al.*, 1990), it was concluded that for the main part of the study, L-NAME (10 mg kg^{-1}, i.v.) would provide adequate and sustained inhibition of NO-synthase activity in the anaesthetized dog, and that 1000 mg kg^{-1} L-arginine i.v. would be more than sufficient to reverse its effects without compromising the haemodynamic status of these preparations.

Haemodynamic effects of L-NAME and L-arginine

In anaesthetized animals ($n = 8$) L-NAME (10 mg kg^{-1}) caused a sustained and statistically significant ($P<0.05$) increase in basal systemic blood pressure and decrease in basal

heart rate and basal renal vascular conductance (Table 1). Subsequent administration of L-arginine (1000 mg kg^{-1}) caused a significant ($P<0.05$) reversal of these haemodynamic effects of L-NAME (Table 1).

Effects of L-NAME and L-arginine on the systemic hypotensive response to acetylcholine

Intra-left atrial administration of acetylcholine (0.3–3 μg kg^{-1}; $n=4$), produced significant ($P<0.05$), dose-related reductions in systemic blood pressure. The peak percentage reduction in mean arterial blood pressure seen with the highest dose of acetylcholine (3 μg kg^{-1}) was 58 ± 1%. After the administration of L-NAME (10 mg kg^{-1}) these responses to acetylcholine were attenuated, particularly with respect to the duration of the response. Analysis of each systemic hypotensive response measured as an area under the curve (AUC) confirmed that in the presence of L-NAME, the responses to all doses of acetylcholine were significantly ($P<0.05$) attenuated (Figure 1).

The attenuating effects of L-NAME upon systemic hypotensive responses to acetylcholine were reversed by subsequent administration of L-arginine (1000 mg kg^{-1}). Analysis of each systemic hypotensive response to acetylcholine measured as an AUC, confirmed a significant ($P<0.05$), although not complete, reversal by L-arginine of the L-NAME induced attenuation (Figure 1).

Table 1 Effects of i.v. administration of N^G-nitro-L-arginine methyl ester (L-NAME), and subsequently, L-arginine, upon mean arterial blood pressure, heart rate and renal vascular conductance in anaesthetized dogs

Haemodynamic parameter	*Control*	*L-NAME* (10 mg kg^{-1})	*L-Arginine* (1000 mg kg^{-1})
Mean arterial blood pressure (mmHg)	119 ± 6	150 ± 6*	124 ± 9#
Heart rate (b.p.m.)	170 ± 4	134 ± 10*	148 ± 6#
Renal vascular conductance (ml min^{-1} mmHg)	0.95 ± 0.08	0.64 ± 0.04*	0.85 ± 0.08#

Results are shown as the mean of 8 animals ± s.e.mean. *Significant difference ($P<0.05$) from control value by Willcoxon test; #Significant difference ($P<0.05$) from L-NAME value by Willcoxon test.

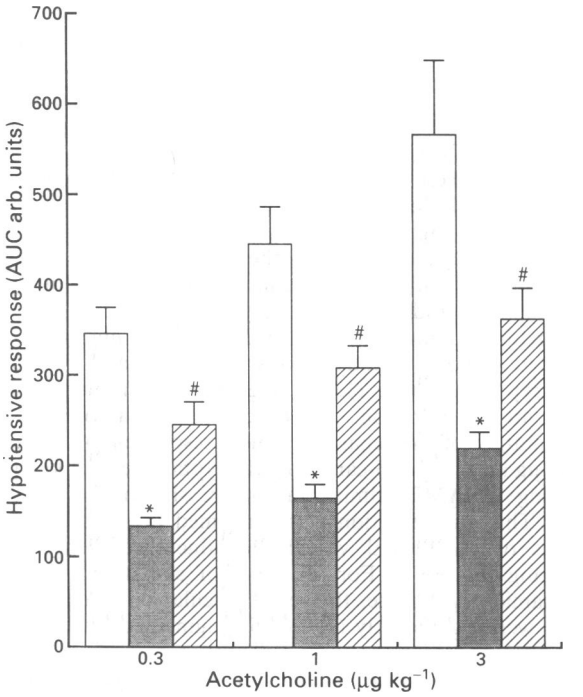

Figure 1 Effects of i.v. administration of N^G-nitro-L-arginine methyl ester (L-NAME, stippled columns, 10 mg kg^{-1}), and subsequently, L-arginine (hatched columns, 1000 mg kg^{-1}), upon the control (open columns) hypotensive responses of anaesthetized dogs to intra-left atrial administration of acetylcholine. Each column represents the mean of 4 animals ± s.e.mean. *Significant difference ($P<0.05$) from control response by Willcoxon test; #Significant difference ($P<0.05$) from response in presence of L-NAME by Willcoxon test.

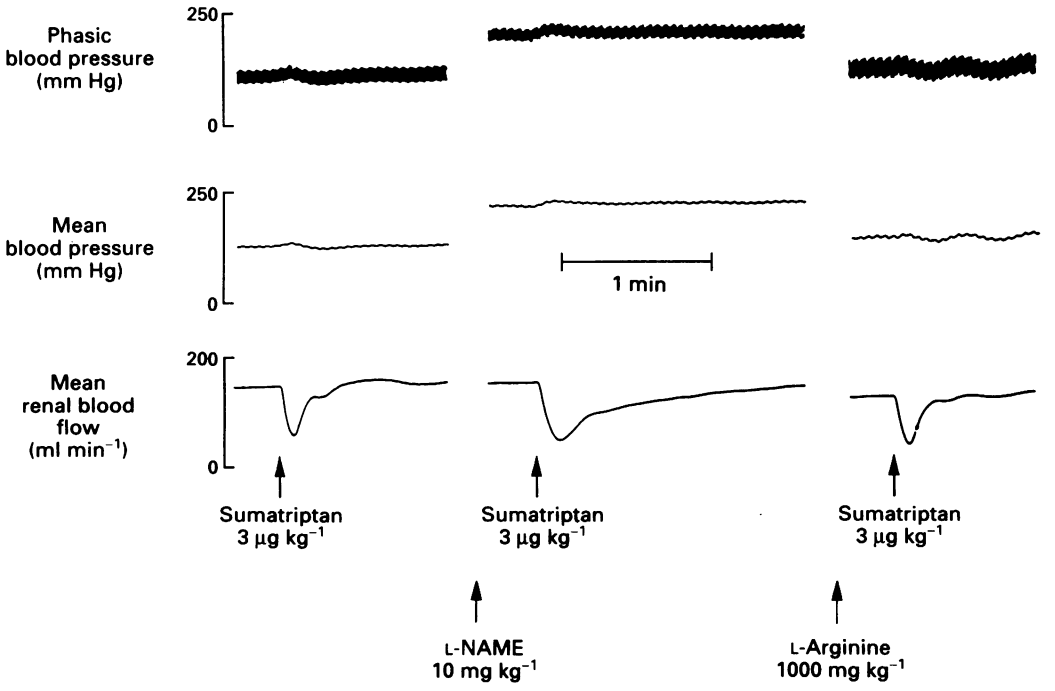

Figure 2 Effects of i.v. administration of N^G-nitro-L-arginine methyl ester (L-NAME, 10 mg kg^{-1}), and subsequently, L-arginine (1000 mg kg^{-1}), upon systemic blood pressure and renal blood flow responses to intra-left atrial administration of sumatriptan, in an anaesthetized dog.

Effects of L-NAME and L-arginine on the renal vascular responses to 5-HT$_1$-like agonists

Intra-left atrial administration of sumatriptan (1–10 μg kg^{-1}; $n = 4$) or 5-carboxamidotryptamine (0.03–0.3 μg kg^{-1}; $n = 4$) caused significant ($P < 0.05$), dose-related, but short-lived reductions in renal blood flow. Examples of the renal blood flow response to sumatriptan (3 μg kg^{-1}) and 5-carboxamidotryptamine (0.1 μg kg^{-1}) are shown in Figures 2 and 3. The doses of 5-carboxamidotryptamine, and to a lesser extent, sumatriptan, used in these studies, did produce some additional changes in systemic blood pressure, although the peak renal blood flow responses to these agonists always preceded any systemic effects. However, the influence of any systemic effects due to these agonists was accounted for by expressing all renal blood flow responses in terms of renal vascular conductance changes. The peak percentage reduction in renal vascular conductance seen with the highest dose of sumatriptan (10 μg kg^{-1}) and 5-carboxamidotryptamine (0.3 μg kg^{-1}) was 66 ± 11% and 53 ± 14%, respectively.

After the administration of L-NAME (10 mg kg^{-1}), the renal blood flow responses to sumatriptan and 5-carboxamidotryptamine were clearly augmented, particularly with respect to the duration of the response. Examples of the augmentation of the renal blood flow responses to sumatriptan (3 μg kg^{-1}) and 5-carboxamidotryptamine (0.1 μg kg^{-1}) following L-NAME administration are shown in Figures 2 and 3. This augmentation was confirmed in the subsequent analysis of each renal vascular conductance response by measuring the AUC. For all doses of sumatriptan and 5-carboxamidotryptamine, the renal vascular conductance responses were significantly ($P < 0.05$) augmented by L-NAME (Figures 4 and 5).

The L-NAME induced augmentation of the renal vascular responses to sumatriptan and 5-carboxamidotryptamine was reversed by subsequent administration of L-arginine (1000 mg kg^{-1}). Examples of the reversal of L-NAME-induced augmentation of the renal blood flow response to sumatriptan (3 μg kg^{-1}) and 5-carboxamidotryptamine (0.1 μg kg^{-1}) following L-arginine administration are shown in Figures 2 and 3. This reversal was confirmed in the subsequent analysis of each renal vascular conductance response measured as an AUC. For all doses of sumatriptan and 5-carboxamidotryptamine, there was a significant ($P < 0.05$), and virtually complete, reversal of the L-NAME-induced augmentation of these responses, following L-arginine administration (Figures 4 and 5).

Effects of L-NAME on the renal vascular response to angiotensin II and brief mechanical occlusion of the renal artery

In a separate group of animals ($n = 4$), an i.l.a. bolus dose of angiotensin II (0.1 μg kg^{-1}) produced a significant ($P < 0.05$) reduction in renal blood flow and vascular conductance (by 74 ± 7%). This response, measured as an AUC, was not significantly altered by 10 mg kg^{-1} L-NAME (AUC, in arbitrary units, was 5597 ± 671, prior to L-NAME and 5087 ± 1384, post-L-NAME). L-NAME also had no effect on its magnitude.

In this same group of animals, a 15 s occlusion of the renal artery, when released, produced a rapid recovery of renal blood flow and vascular conductance. However, the duration of this recovery, assessed by the change in renal vascular conductance was not significantly affected by L-NAME (time, in s, to recover to 50% of the pre-occlusion value was 8.0 ± 0.2, prior to L-NAME and 8.9 ± 0.9, post-L-NAME).

Discussion

In anaesthetized dogs, the renal vasoconstrictor responses to sumatriptan and 5-carboxamidotryptamine, were significantly augmented by the NO-synthase inhibitor, L-NAME, which increased both the magnitude and duration of these responses. This L-NAME-induced augmentation was reversed by subsequent administration of L-arginine. However, L-NAME had no effect upon the renal vasoconstrictor responses to angiotensin II, nor did it affect the renal vascular conductance recovery response following brief mechanical occlusion of the renal artery.

The addition of NO-synthase inhibitors to isolated vascular smooth muscle preparations with an intact endothelium results in a contractile response, due to the inhibition of the basal production and release of NO, and the attenuation of the vasorelaxation response to acetylcholine (Rees *et al.*, 1989; 1990; Kobayashi & Hattori, 1990; Moore *et al.*, 1990). The administration of these inhibitors to intact animals results in a characteristic increase in systemic blood pressure and reduction in regional blood flows and vascular conductance (Whittle *et al.*, 1989; Rees *et al.*, 1990; Gardiner *et al.*, 1990a,b; Vargas *et al.*, 1991), which can be reversed by the administration of an excess of L-arginine (Whittle *et al.*, 1989; Rees *et al.*, 1990). In addition, these inhibitors have

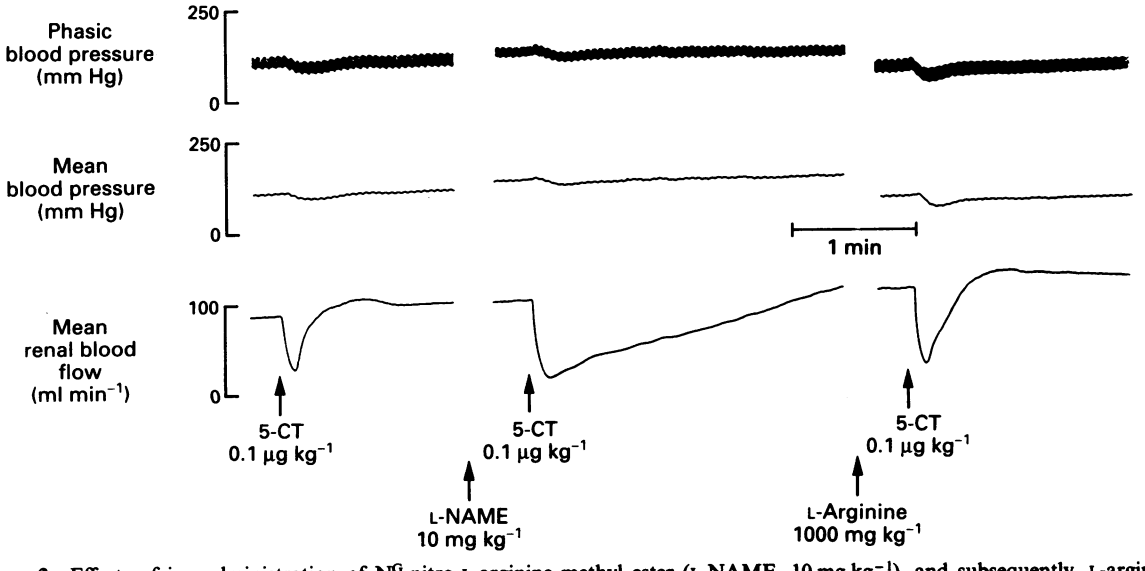

Figure 3 Effects of i.v. administration of N^G-nitro-L-arginine methyl ester (L-NAME, 10 mg kg^{-1}), and subsequently, L-arginine (1000 mg kg^{-1}), upon systemic blood pressure and renal blood flow responses to intra-left atrial administration of 5-carboxamidotryptamine, in an anaesthetized dog.

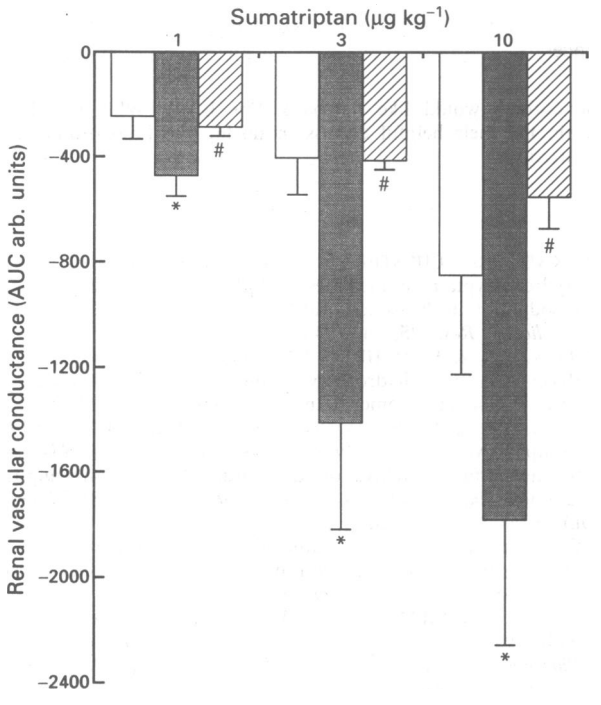

Figure 4 Effects of i.v. administration of N^G-nitro-L-arginine methyl ester (L-NAME, stippled columns, 10 mg kg^{-1}), and subsequently, L-arginine (hatched columns, 1000 mg kg^{-1}), upon the control (open columns) renal vascular conductance responses of anaesthetized dogs to intra-left atrial administration of sumatriptan. Each column represents the mean of 4 animals ± s.e.mean. *Significant difference ($P<0.05$) from control response by Willcoxon test; #Significant difference ($P<0.05$) from response in presence of L-NAME by Willcoxon test.

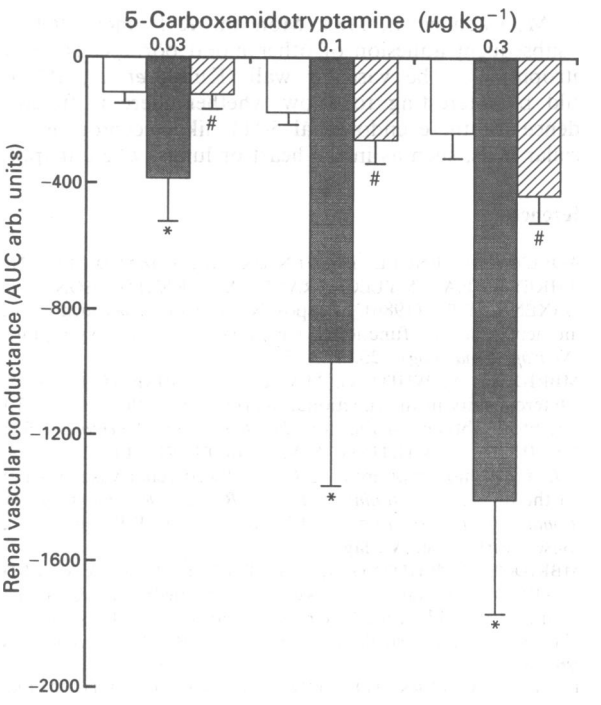

Figure 5 Effects of i.v. administration of N^G-nitro-L-arginine methyl ester (L-NAME, stippled columns, 10 mg kg^{-1}), and subsequently, L-arginine (hatched columns, 1000 mg kg^{-1}), upon the control (open columns) renal vascular conductance responses of anaesthetized dogs to intra-left atrial administration of 5-carboxamidotryptamine. Each column represents the mean of 4 animals ± s.e.mean. *Significant difference ($P<0.05$) from control response by Willcoxon test; #Significant difference ($P<0.05$) from response in presence of L-NAME by Willcoxon test.

been shown to attenuate the NO-mediated vasodilator response to acetylcholine in rats (Whittle *et al.*, 1989; Rees *et al.*, 1990), rabbits (Rees *et al.*, 1990), dogs (Chu *et al.*, 1991) and in man (Vallance *et al.*, 1990).

5-Hydroxytryptamine stimulates 5-HT$_1$-like receptors located on vascular smooth muscle to elicit both vasoconstrictor and vasodilator responses. These functional responses are mediated by two pharmacologically distinct subtypes of the 5-HT$_1$-like receptor (for further discussion see preceding paper, Cambridge *et al.*, 1995). The marked canine renal vascular responses to sumatriptan and 5-carboxamidotryptamine, are consistent with the stimulation of the vasoconstrictor subtype of this receptor (Cambridge *et al.*, 1991a; 1995). This subtype most closely resembles the recently cloned 5-HT$_{1D\alpha/\beta}$ receptor (see Martin & Humphrey, 1994).

5-Hydroxytryptamine has been shown to stimulate endothelium-dependent vascular relaxation, through the release of NO, in a number of different isolated vascular preparations (Cocks & Angus, 1983; Leff *et al.*, 1987; Martin *et al.*, 1987; Mankad *et al.*, 1991). However, the 5-HT receptor which mediates this response is atypical and pharmacologically distinct from the vascular smooth muscle 5-HT$_1$-like receptor subtypes (Martin *et al.*, 1987; Sumner, 1991). Of greater interest is the observation that both sumatriptan and 5-carboxamidotryptamine can elicit endothelial-dependent relaxation responses in the pig isolated coronary artery (Molderings *et al.*, 1989; Schoeffter & Hoyer, 1990) and guinea-pig isolated jugular vein (Gupta, 1992). This suggests that the endothelial cells in some blood vessels may also possess a 5-HT$_1$-like receptor with a pharmacological profile which is actually consistent with the vasoconstrictor subtype found on the vascular smooth muscle (for further discussion see Cambridge *et al.*, 1995).

From the results of the studies in anaesthetized dogs, it appears that the NO-mediated component of the renal vasoconstrictor response to 5-HT$_1$-like receptor agonists is not shared with other vasoconstrictor agents, nor can it be ascribed to reactive hyperaemia, since the responses to angiotensin II and renal artery occlusion were not modified by L-NAME. It is therefore suggested that the renal vasoconstrictor responses to sumatriptan and 5-carboxamidotryptamine observed *in vivo*, reflect the summation of the activation of two separate populations of the 5-HT$_1$-like vasoconstrictor receptor subtype, eliciting two separate events, which together determine the net renal vascular smooth muscle response. The primary response, the renal vasoconstriction, which may reflect constriction of shunt-like arterio-anastomoses within the kidney (see Cambridge *et al.*, 1995), is rapid in onset, and results from the stimulation of the receptor subtype located on vascular smooth muscle cells. However, the simultaneous stimulation of the same receptor subtype located on the endothelial cells, elicits an endothelium-dependent vasodilatation mediated by the release of NO. The released NO modulates both the magnitude and the duration of the primary renal vasoconstriction response.

At present, the vascular distribution and functional significance of these modulating endothelial 5-HT$_1$-like receptors, remains unclear. However, it is feasible that this mechanism has evolved to maintain the integrity of local tissue perfusion in vascular beds which may be at particular risk from the release of 5-hydroxytryptamine from aggregating or activated platelets. The kidney has a complex vascular anatomy which is central to its role in salt and water homeostasis hence the intra-renal distribution of blood flow must be well controlled. Intra-renal endothelial 5-HT$_1$-like receptors may contribute to this control by moderating (through the release of NO) the vasoconstrictor potential of platelet-derived 5-hydroxytryptamine within the kidney. The NO that is released may also provide additional protection to the renal vasculature by preventing further platelet aggrega-

tion (Moncada *et al.*, 1988; Moncada & Palmer, 1990) and the subsequent adhesion of other blood borne cells, such as neutrophils, to the vascular wall (Kubes *et al.*, 1991). It would be interesting to know whether there is functional evidence for these endothelial 5-HT$_1$-like receptors in other vascular beds, such as in the heart or lungs, where inappropriate platelet aggregation can elicit major pathological disturbances.

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Haemostatic mechanism in the endometrium: role of cyclo-oxygenase products and coagulation factors

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1 The primary mechanism of haemostasis in the endometrium of rat was studied and results were compared to that in the mesenteric artery.

2 The bleeding time of the rat endometrium as assessed by haemoglobin output was significantly decreased after pretreatment of the animals with either indomethacin (5 mg kg^{-1}, i.v.) or meclofenamate (3 mg kg^{-1}, i.v.) whereas the bleeding time was significantly increased in the rat mesenteric artery.

3 The bleeding time of the rat endometrium was unchanged from control values following treatment with prostacyclin (0.5 $\mu g\ kg^{-1}\ min^{-1}$, i.v.) or 1-benzylimidazole (50 mg kg^{-1}, i.v.) whereas the bleeding times were increased in the rat mesenteric artery.

4 Administration of heparin (100 units kg^{-1}) increased the bleeding time in the rat mesenteric artery but had no effect on the bleeding time of the endometrium.

5 Superfusion of the endometrium with 16, 16-dimethyl PGE_2 (1 $\mu g\ ml^{-1}$), a vasodilator, increased the bleeding time of the endometrium but superfusion of PGE_2 over the mesenteric artery did not affect the bleeding time from this site.

6 Histological studies of the mesenteric artery and the endometrium following haemostatis revealed that the haemostatic plug in the mesenteric artery was mainly composed of platelets and fibrin whereas in the endometrium it was mainly composed of fibrin.

7 These findings suggest that haemostasis in the endometrium may be mediated by the vascular tone and fibrin whereas formation of the platelet plug may be the primary mechanism for haemostasis in the mesenteric artery.

Keywords: Cyclo-oxygenase inhibitors; prostaglandins; non-steroidal, anti-inflammatory drugs; haemostasis; endometrium; mesenteric artery

Introduction

The primary mechanism of haemostasis in the skin and arterial vessels involves adhesion and aggregation of platelets at the site of injury with subsequent platelet plug formation (Sixma & Wester, 1977; Verstraete & Vermylen, 1984). Thromboxane A_2 (TxA_2), a cyclo-oxygenase pathway product of arachidonic acid (AA) metabolism formed by platelets, has been implicated as an endogenous mediator of platelet aggregation (Whittle & Moncada, 1983). Administration of non-steroidal, anti-inflammatory drugs (NSAIDs) which inhibit the cyclo-oxygenase enzyme (Vane, 1971) can reduce platelet aggregation *in vitro* (Smith & Willis, 1971) and also prolong cutaneous bleeding time in man (Amezcua *et al.*, 1979). Conversely, NSAIDs promote haemostasis in the endometrium and have been successfully used to treat idiopathic menorrhagia (Anderson *et al.*, 1976; Fraser, 1983; Muggeridge & Elder, 1983) as well as menorrhagia associated with the use of the intra-uterine contraceptive device (IUD) (Damarawy & Toppazada, 1976; Guillebaud *et al.*, 1978; Ylikorkala *et al.*, 1978; Roy & Shaw, 1981). The mechanism of this paradoxical effect of NSAIDs in modulating haemostasis in the skin and endometrium is not known.

The primary mechanism of haemostasis may vary by site. Platelet aggregation plays a major role in the haemostatic events in the skin or vasculature whereas the arrest of gastric haemorrhage is brought about largely by a process primarily involving the coagulation system (Whittle *et al.*, 1986). We, therefore, investigated the mechanism of haemostasis in the endometrium. To accomplish this, we slightly modified the technique used for studying the haemostatic mechanism in the gastric mucosa (Whittle *et al.*, 1986) and developed a method for evaluating bleeding from a standard incision in the endometrium of the rat. With this technique, we evaluated the mechanism of haemostasis in the endometrium and compared it with that of the mesenteric artery in the rat. Oophorectomized rats supplemented with oestradiol were used in order to control for the hormonal milieu which may modify the bleeding time.

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Methods

Sprague–Dawley rats (220–260 g) were obtained from Bantin and Kingman Farms (Freemont, CA, U.S.A.). They were housed under controlled conditions. Water and commercial chow were allowed *ad libitum*. The rats were anaesthetized with ketamine hydrochloride (100 mg kg^{-1}, i.m.) and xylazine (10 mg kg^{-1}, i.m.) after which they were oophorectomized through a ventral mid-line incision. The abdominal layers were sutured with 2.0 chromic and the animals were allowed to recover. The animals were then injected daily with 17β oestradiol benzoate (1 mg kg^{-1}, s.c.) in oil for 4 days and were used on the 4th day at least 2 h after the injection. This ensured that the hormonal milieu was the same in all the experimental animals used for the study.

Evaluation of bleeding time

The technique to assess bleeding time in the endometrium and the mesenteric artery was similar to the previously described method for determining bleeding time in the gastric mucosa (Whittle *et al.*, 1986). The animals were anaesthetized with ketamine hydrochloride (100 mg kg^{-1}, i.m.) and xylazine (10 mg kg^{-1}, i.m.) after which the femoral vein was can-

nulated for intravenous drug or vehicle administration. The uterus was exposed by a midline laparotomy. The uterine horn was opened along the anti-mesometrial border and the initial bleeding at the incision site was controlled with microelectrocautery. The exposed endometrium was then placed in a modified plastic chamber ensuring that the vascular supply was intact. The endometrium was gently superfused with isotonic saline solution at 37°C delivered with the aid of an infusion pump at a rate of 2 ml min^{-1}. A standardized 3 mm incision was made with microscissors perpendicular to the rugae of the endometrium to initiate endometrial bleeding. The perfusate was directed away from the site of the lesion so as to minimize any potential disruption of the haemostatic plug and collected in 1 min fractions for determination of haemoglobin output. This technique was found to be highly reproducible for studying bleeding time in the endometrium.

Terminal ileum mesenteric arteriolar vessel bleeding time was also determined in the same animal. A loop of the ileum was placed over the edge of the chamber which provided access to the vascular arcades of the mesentery without disrupting the vascular supply. A binocular dissecting microscope was used to identify a branch of the mesenteric artery close to the ileum and bleeding was produced by puncture with a 25-gauge needle (Whittle *et al.*, 1986). Isotonic saline at 37°C was superfused at a rate of 2 ml min^{-1}, taking care not to disrupt the incision site and the superfusate was collected at 1 min intervals.

Bleeding time was determined as the haemoglobin output (mg min^{-1}) into the superfusate collected at 1 min intervals. Samples were treated with Lys-SII (Coutler, Hialeah FL, U.S.A.) 0.1 ml to lyse erythrocytes and the optical density of haemoglobin in the perfusate was determined spectrophotometrically (540 nm). Haemoglobin output (mg min^{-1}) was calculated with the use of a standard curve constructed with rat heparinized arterial blood diluted with saline. The bleeding time was taken as the time from the incision to the first collection period during which the haemoglobin output was <0.1 mg min^{-1}, corrected for the chamber wash out time.

Effect of cyclo-oxygenase inhibitors on bleeding time

Bleeding time in the mesenteric vessel and the endometrium of one uterine horn was initially determined followed by a repeat bleeding time determination in an equivalent mesenteric vessel and in the endometrium of the contralateral uterine horn performed 30 min after administration of either indomethacin (5 mg kg^{-1}, i.v.) or sodium meclofenamate (3 mg kg^{-1}, i.v.). These doses have been previously demonstrated to inhibit formation of cyclo-oxygenase products (Chaudhuri, 1973; Chaudhuri *et al.*, 1982; Whittle *et al.*, 1986).

Effects of inhibition of platelet aggregation

Bleeding times in the mesenteric artery and the endometrium were determined prior to and 10 min following intravenous infusion of prostacyclin (Epoprostenol; Wellcome) and maintained throughout the observation period at a dose of 0.5 μg kg^{-1} min^{-1} by an infusion pump. This concentration of prostacyclin has previously been demonstrated to produce near maximal inhibition of platelet aggregation (Whittle *et al.*, 1986).

Effects of thromboxane synthase inhibitor

The bleeding time in the mesenteric artery and the endometrium were determined after intravenous administration of 1-benzylimidazole as the fumarate salt (50 mg kg^{-1}, i.v.). This dose was selected as it has been previously demonstrated to inhibit platelet thromboxane synthesis by over 90% (Whittle *et al.*, 1986).

Effects of heparin on bleeding time

In order to assess the effect of inhibition of the coagulation cascade on the haemostatic mechanism in the endometrium, bleeding time was determined in the mesenteric vessel and endometrium after intravenous injection of heparin (100 units kg^{-1}). This dose does not interfere with platelet function in this species (Whittle *et al.*, 1986).

Effect of prostaglandin E_2 (PGE_2) on bleeding time

In order to assess whether the vasodilator effect of PGE_2 modulates the haemostatic mechanism in the endometrium, bleeding time was assessed in the mesenteric artery and the endometrium 10 min after the initiation of superfusion of 16, 16-dimethyl PGE_2 (1 μg ml^{-1}) and which was continued for the duration of the observation period.

Histology

Histological studies were performed on the mesenteric artery and the endometrium after initiation of bleeding and fixation of the tissues following haemostasis. The tissues were embedded in paraffin, sectioned and stained with haematoxylin and eosin, Giemsa stain and Trichrome stain for evaluation by light microscopy.

Chemicals

Indomethacin and sodium meclofenamate were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Prostacyclin (Epoprostenol) and 1-benzylimidizole were gifts from Wellcome Research Laboratories (Beckenham, Kent, U.K.). The stable analogue of prostaglandin E_2, 16, 16-dimethyl PGE_2 was obtained from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.). Heparin was obtained from UCLA Pharmaceutical Services (Los Angeles, CA, U.S.A.).

Statistical analysis

Results are expressed as the mean ± s.e.mean for each point. Differences between groups were assessed by ANOVA with repeated measures and Students' *t* test where appropriate. *P* values <0.05 were considered as significant.

Results

The bleeding time studies were performed by a single individual in order to control for variation related to incisional length and depth that may arise. This also ensured that the haemoglobin output during the initial collection period was similar in the respective blood vessels under study. The initial haemoglobin output was found to vary between vessels. Initial haemoglobin output in the mesenteric artery was 6.82 ± 0.61 mg min^{-1} whereas in the endometrium the initial haemoglobin output averaged 2.07 ± 0.15 mg min^{-1}.

Effects of cyclo-oxygenase inhibition on bleeding time

Haemoglobin output from the mesenteric artery ($n = 6$) and the endometrium ($n = 6$) before and after treatment with indomethacin is shown in Figure 1. Prior to treatment with indomethacin, haemoglobin output diminished rapidly from the time of the incision. Bleeding time in the mesenteric artery was 4.80 ± 0.15 min whereas that of the endometrium was 6.10 ± 0.11 min. The haemoglobin output from the mesenteric artery was prolonged following administration of indomethacin (bleeding time 11.22 ± 0.34 min; $P<0.001$) whereas it was significantly shortened in the endometrium (bleeding time 4.43 ± 0.20 min; $P<0.001$). Following administration of meclofenamate, the bleeding time of the mesenteric artery ($n = 5$) was prolonged (12.06 ± 0.68 min;

$P<0.001$) whereas that of the endometrium ($n=5$) was significantly reduced (3.80 ± 0.19 min; $P<0.001$).

Effects of inhibition of platelet aggregation

Intravenous infusion of prostacyclin prolonged the bleeding time (10.96 ± 0.63 min; $P<0.005$) of the mesenteric artery ($n=5$), whereas in the endometrium there was no significant difference from control values (6.18 ± 0.30 min) ($n=5$). A similar prolongation of the bleeding time (10.08 ± 0.52 min; $P<0.001$) of the mesenteric artery ($n=4$) was observed following administration of BZi as the fumarate salt whereas there was no change in the bleeding time (5.8 ± 0.25 min) of the endometrium ($n=4$). The haemoglobin output from the mesenteric artery and the endometrium before and after treatment with either prostacyclin or BZi are shown in Figure 2.

Effects of heparin

Intravenous infusion of heparin had no effect on the bleeding time (5.53 ± 0.38 min) of the mesenteric artery ($n=6$), whereas it significantly prolonged the bleeding time (>12.0 min; $P<0.001$) of the endometrium ($n=6$). The haemoglobin output from the mesenteric artery and the endometrium before and after treatment with heparin are shown in Figure 3.

Effect of PGE_2 on bleeding time

Superfusion of the stable analogue of PGE_2 over the mesenteric artery ($n=6$) had no effect on the bleeding time of that vessel (4.88 ± 0.12 min) whereas superfusion of the same analogue over the endometrium ($n=6$) significantly prolonged the bleeding time (9.42 ± 0.94 min; $P<0.001$) of that tissue. The haemoglobin output from the mesenteric artery and the endometrium before and after the stable analogue of PGE_2 had been added to the superfusion medium is shown in Figure 4.

Histology

Platelets were the main component in the haemostatic plug of the mesenteric artery as had been demonstrated by others (Whittle *et al.*, 1986) whereas a fibrinous coagulated mass was the main component of the haemostatic plug of the endometrium (Figure 5).

Discussion

It is now well accepted that the primary arrest of bleeding from both the skin and small blood vessels is dependent on platelet adhesion to the cut surface and consequent aggregation. Following this, there is participation of the coagulation system and fibrin strand formation in the stabilization of the initial platelet plug (Jorgensen & Borchgrevink, 1964; Hovig & Stormorken, 1974; Sixma & Wester, 1977; Wester *et al.*, 1978). On the other hand, the mechanism of haemostasis from the endometrium is not known (Aparicio *et al.*, 1979, Christiaens *et al.*, 1980), although vasospasm of endometrial vessels is thought to play a role (Markee, 1940; 1948; Shaw *et al.*, 1972). In the present study, we investigated the role of AA metabolites of the cyclo-oxygenase pathway in controlling haemostasis in the endometrium. We assessed this indirectly by observing the effects of parenteral administra-

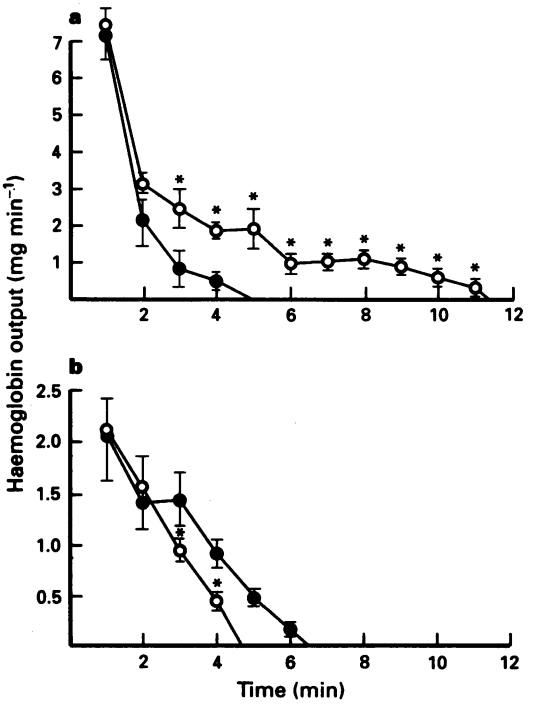

Figure 1 Bleeding from the mesenteric artery following a standard puncture with a 25 g needle (a) and that from the endometrium (b) following a standardized 3 mm incision in the endometrium, when they were encased in a plastic chamber and superfused with isotonic saline (37°C) at 2 ml min^{-1}. Bleeding is expressed as the haemoglobin output (mg min^{-1}) determined spectrophotometrically and shown as mean ± s.e.mean of 6 experiments in each group. Bleeding is shown under control conditions (●) and 30 min following indomethacin (5 mg kg^{-1}, i.v.) administration (○); *significant ($P<0.05$) difference from control values.

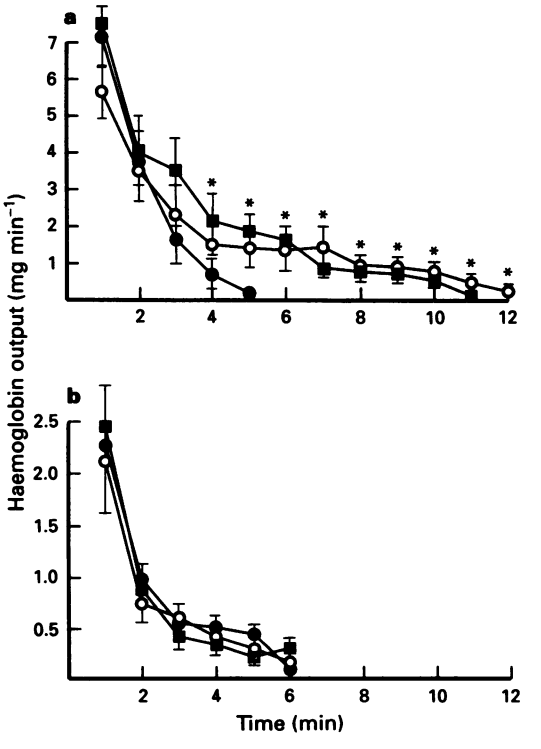

Figure 2 Bleeding time from the mesenteric artery following a standard puncture with a 25 g needle (a) and that from the endometrium (b) following a standardized 3 mm incision in the endometrium, when they were encased in a plastic chamber and superfused with isotonic saline (37°C) at 2 ml min^{-1}. Bleeding time is expressed as the haemoglobin output (mg min^{-1}) determined spectrophotometrically and shown as mean ± s.e.mean of 4–5 experiments in each group. Bleeding is shown under control conditions (●); during prostacyclin infusion (0.5 μg kg^{-1} min^{-1}, ○) or 10 min after 1-benzylimidazole (50 mg kg^{-1}, i.v.; ■); *significant ($P<0.05$) prolongation.

tion of prostacyclin, which directly inhibits platelet aggregation (Whittle *et al.*, 1980); of the thromboxane synthetase inhibitor, BZi (Whittle *et al.*, 1986), as well as indomethacin and meclofenamate, both of which inhibit the enzyme cyclo-oxygenase (Flower *et al.*, 1972) and thereby reduces all AA products formed by this pathway. In order to assess the role of vasospasm in modulating haemostatis in the endometrium, we superfused the vasodilator prostaglandin E_2 (PGE_2) over the endometrium and the mesenteric artery and assessed the bleeding time. In addition, the effects of low doses of heparin, which interfere with the clotting process (Whittle *et al.*, 1986), were also investigated. These studies, therefore, allowed us to evaluate the relative importance of platelet aggregation, blood coagulation and vasospasm in the haemostatic mechanism of the endometrium.

In the present study, the slightly prolonged bleeding time in the endometrium when compared to the rat mesenteric artery indicated that the mechanism of haemostasis at these two sites may be different. Pretreatment of animals with either indomethacin or meclofenamate at dosages which inhibit clyco-oxygenase (Flower *et al.*, 1972) and, more specifically, endometrial prostaglandin production (Chaudhuri, 1973; Chaudhuri *et al.*, 1982), increased the bleeding time from the rat mesenteric artery, but shortened the bleeding time in the endometrium. In this respect, the results on bleeding time of the endometrium were different from similar studies on gastric mucosa, where no changes in bleeding time from control values were observed after the administration of indomethacin (Whittle *et al.*, 1986). This observation suggests that AA products of the cyclo-oxygenase pathway modulate the bleeding time of rat mesenteric artery and endometrium by different mechanisms.

In order to elucidate further the role of platelet aggregation in the haemostatic process of the endometrium, we assessed the effects of inhibition of TxA_2 and administration of prostacyclin on this process. TxA_2 formed by platelets has been implicated as an endogenous proaggregatory mediator and inhibition of its synthesis can reduce platelet aggregation *in vitro* (Whittle & Moncada, 1983) and also prolong cutaneous bleeding time in man (Fitzgerald *et al.*, 1983; Dale *et al.*, 1983). When BZi was administered intravenously at a

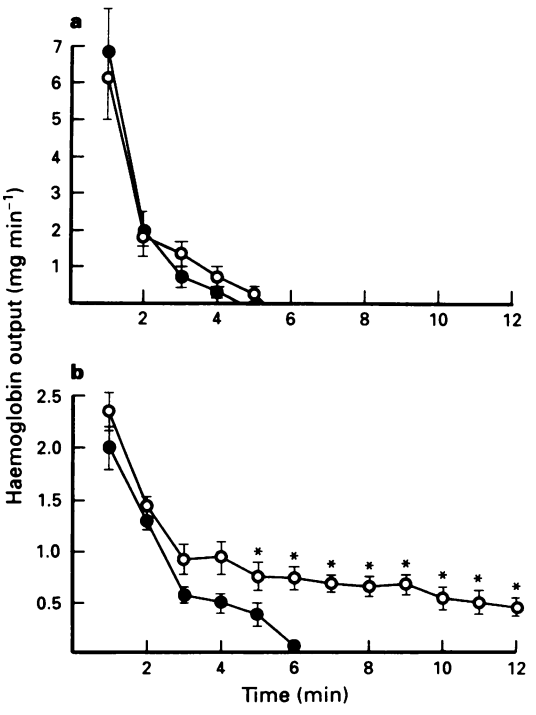

Figure 3 Bleeding from the mesenteric artery following a standard puncture with a 25 g needle (a) and that from the endometrium (b) following a standardized 3 mm incision in the endometrium, when they were encased in a plastic chamber and superfused with isotonic saline (37°C) at 2 ml min^{-1}. Bleeding time is expressed as the haemoglobin output (mg min^{-1}) determined spectrophotometrically and shown as the mean ± s.e.mean of 6 experiments in each group. Bleeding is shown under control conditions (●) and following intravenous heparin (100 units kg^{-1}; ○); *significant ($P<0.05$) difference from control values.

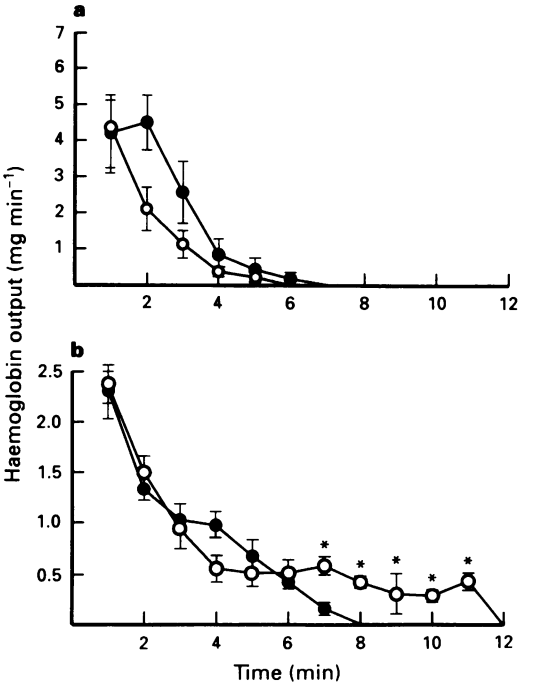

Figure 4 Bleeding from the mesenteric artery following a standard puncture with a 25 g needle (a) and that from the endometrium (b) following a standardized 3 mm incision in the endometrium, when they were encased in a plastic chamber and superfused with isotonic saline (37°C) at 2 ml min^{-1}. Bleeding time is expressed as the haemoglobin output (mg min^{-1}) determined spectrophotometrically and shown as mean ± s.e.mean of 6 experiments in each group. Bleeding is shown under control conditions (●) and 10 min following addition of 16, 16-dimethyl PGE_2 (1 μg ml^{-1}; ○) to the superfusate; *significant ($P<0.05$) prolongation.

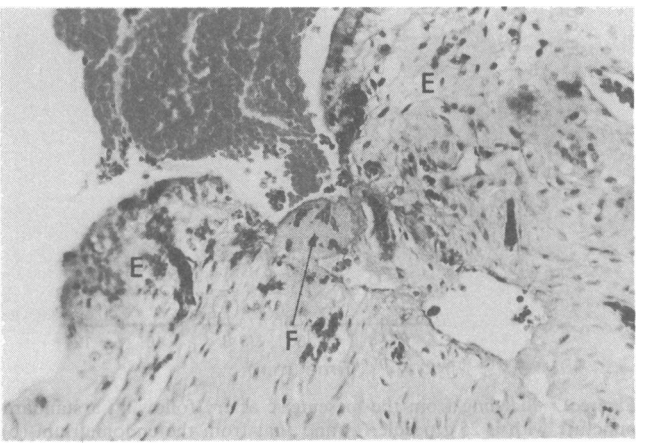

Figure 5 Histological appearance of the rat endometrium (E) after haemostasis following a standardized incision. The tissue was removed and stored in the fixative solution. (10% formalin buffer). By use of routine procedures, the tissues were embedded in paraffin and 2 μm sections were prepared and stained with trichrome stain. The haemostatic plug at the incision site was mainly composed of fibrin (F) (magnification × 199).

dose which caused near-maximal inhibition of TxA_2 (Whittle *et al.*, 1986), the bleeding time assessed on the rat mesenteric artery was significantly increased and was similar to that reported by other investigators (Whittle *et al.*, 1986). However, BZi did not affect the bleeding time of the endometrium. This indicated that the platelet plug formation at the bleeding site plays a less important role in haemostasis in the endometrium. This concept was further supported by our studies with prostacyclin. Prostacyclin inhibits the aggregation of platelets, both *in vivo* and *in vitro* when aggregation is induced by all endogenous agents (Moncada *et al.*, 1976; Whittle *et al.*, 1980). In our studies, intravenous administration of prostacyclin at a dose that has been demonstrated to inhibit, near-maximally, the *ex vivo* platelet aggregation in rats (Whittle *et al.*, 1986), failed to prolong bleeding time in the endometrium, but successfully prolonged bleeding time of the rat mesenteric artery.

Vasoconstriction of the endometrial vessels may play a role in haemostasis of the endometrium (Markee, 1940; 1948). It is therefore conceivable that the presence of vasodilator substances in the endometrium could prolong the bleeding. PGE_2 is a vasodilator and is synthesized by the endometrium (Chaudhuri, 1973) and the concentration of E prostaglandins in the endometrium is increased just prior to menstruation (Downie *et al.*, 1974). In ovulating women with excessively heavy measured menstrual blood loss, there appeared to be a shift in the endometrial synthetic capacity in favour of PGE_2 over $PGF_{2\alpha}$ (Smith *et al.*, 1981; Cameron *et al.*, 1987). It has been suggested that there is a relationship between the total prostaglandin content of the endometrium and measured menstrual blood loss (Cameron *et al.*, 1987). Similarly, there is also an increase in endometrial prostaglandins released by the IUD (Chaudhuri, 1973; Hillier & Kasonde, 1976), and this increased release of prostaglandins could therefore be responsible for the menorrhagia associated with use of the IUD. We, therefore, decided to assess the role of PGE_2 in modulating haemostasis in the endometrium. Superfusion of the endometrium with a stable analogue of PGE_2 led to an increase in endometrial bleeding time, whereas bleeding time was unchanged when similar experiments were performed on the rat mesenteric artery. This indicated that bleeding time in the endometrium may be partly modulated by the vascular tone of the endometrial vessels and that in the presence of excessive amounts of the vasodilator PGE_2 in the endometrium, increased bleeding may be observed. The inhibition of synthesis of PGE_2 by the endometrium following administration of NSAIDs may partly explain the decrease in endometrial bleeding time after the administration of meclofenamate or indomethacin.

Fibrin has been identified in the first day menstrual endometrium obtained by curettage (Salvatore, 1969; Aparicio *et al.*, 1979; Christiaens *et al.*, 1980), suggesting the importance of coagulation factors (Rybo, 1966) in controlling menstrual bleeding. Furthermore, the haemostatic platelet plugs in the menstruating uterus are present only for a very limited time period in early menstruation. The predominance of fibrin in the early menstruating endometrium, therefore, prompted us to assess the role of the clotting factors in endometrial haemostasis. Intravenous infusion of heparin (100 units kg^{-1}) substantially prolonged the bleeding time from the endometrium, whereas no change in bleeding time of the rat mesenteric artery was observed. This indicated that interference of clotting mechanisms with this dose of heparin selectively prolonged endometrial bleeding without affecting mesenteric artery bleeding. This was corroborated by histological findings that fibrin played an important role in endometrial haemostasis, whereas platelets played an important role in controlling bleeding from the mesenteric artery.

The correlation of our findings on the mechanism of haemostasis in the rat endometrium to the human endometrium is not clear at present as rats do not menstruate and shed endometrium. On the other hand, results from our studies, suggesting that vascular tone and coagulation factors may play an important role in endometrial haemostasis in rats may explain many findings in women. The ability of NSAIDs to inhibit the synthesis of the vasodilator PGE_2 may therefore be the explanation for the beneficial effect of this group of drugs in the treatment of idiopathic menorrhagia (Anderson *et al.*, 1976; Fraser, 1983; Muggeridge & Elder, 1983) as well as that associated with the use of the IUD (Ylikorkala *et al.*, 1978; Damarawy & Toppazada, 1981; Roy & Shaw, 1981). Similarly, the importance of the fibrin plug in endometrial haemostasis is supported by the observations that fibrinolytic inhibitors are also highly effective for the treatment of idiopathic menorrhagia and that associated with the use of the IUD (Nilsson & Rybo, 1967; Kasonde & Bonnar, 1975; Bonnar *et al.*, 1976; Ylikorkala & Vinikka, 1983; Milson *et al.*, 1991).

In conclusion, the mechanism of haemostasis can vary in different tissues and more than one mechanism may be involved. In the endometrium, the vascular tone mediated by endometrial prostaglandins and the coagulation system especially fibrin may be more important than platelets, whereas at peripheral sites such as the skin, formation of the platelet plug is the primary mechanism for haemostasis. Results of our study, therefore, explain the paradox as to why NSAIDs increase bleeding time at some peripheral sites, whereas bleeding from the endometrium is diminished.

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Angiotensin-converting enzyme (ACE) inhibitor transport in human intestinal epithelial (Caco-2) cells

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1 The role of proton-linked solute transport in the absorption of the angiotensin-converting enzyme (ACE) inhibitors captopril, enalapril maleate and lisinopril has been investigated in human intestinal epithelial (Caco-2) cell monolayers.

2 In Caco-2 cell monolayers the transepithelial apical-to-basal transport and intracellular accumulation (across the apical membrane) of the hydrolysis-resistant dipeptide, glycylsarcosine (Gly-Sar), were stimulated by acidification (pH 6.0) of the apical environment. In contrast, transport and intracellular accumulation of the angiotensin-converting enzyme (ACE) inhibitor, lisinopril, were low (lower than the paracellular marker mannitol) and were not stimulated by apical acidification. Furthermore, [^{14}C]-lisinopril transport showed little reduction when excess unlabelled lisinopril (20 mM) was added.

3 pH-dependent [^{14}C]-Gly-Sar transport was inhibited by the orally-active ACE inhibitors, enalapril maleate and captopril (both at 20 mM). Lisinopril (20 mM) had a relatively small inhibitory effect on [^{14}C]-Gly-Sar transport. pH-dependent [^{3}H]-proline transport was not inhibited by captopril, enalapril maleate or lisinopril.

4 Experiments with BCECF[2',7',-*bis*(2-carboxyethyl)-5(6)-carboxyfluorescein]-loaded Caco-2 cells demonstrate that dipeptide transport across the apical membrane is associated with proton flow into the cell. The dipeptide, carnosine (β-alanyl-L-histidine) and the ACE inhibitors, enalapril maleate and captopril, all lowered intracellular pH when perfused at the apical surface of Caco-2 cell monolayers. However, lisinopril was without effect.

5 The effects of enalapril maleate and captopril on [^{14}C]-Gly-Sar transport and pH_i suggest that these two ACE inhibitors share the H^+-coupled mechanism involved in dipeptide transport. The absence of pH-dependent [^{14}C]-lisinopril transport, the relatively small inhibitory effect on [^{14}C]-Gly-Sar transport, and the absence of lisinopril-induced pH_i changes, all suggest that lisinopril is a poor substrate for the di/tripeptide carrier in Caco-2 cells. These observations are consistent with the greater oral availability and time-dependent absorption profile of enalapril maleate and captopril, compared to lisinopril.

Keywords: Proton-coupled transport; dipeptide; amino acid; ACE inhibitor; intestine; epithelium; Caco-2 cells; intracellular pH

Introduction

The intestinal di/tripeptide transport is an important membrane transport protein localized at the apical surface of the intestinal epithelium (Matthews, 1975; Matthews & Adibi, 1976) that plays a significant physiological role in the absorption of protein in the form of small peptides (2–3 amino acids in length). Unlike most other ion/solute co-transporters of the gastrointestinal tract, transport via the di/tripeptide carrier is coupled to the movement of protons (Ganapathy & Leibach, 1985). The driving force for this H^+-coupled carrier is provided by the acid microclimate (an area of low pH lying adjacent to the apical surface of the intestinal epithelium) as demonstrated both *in vivo* (McEwan *et al.*, 1988) and *in vitro* (Lucas *et al.*, 1975). Recently, the complementary DNA coding for a 707-amino acid peptide transporter (PepT1) was isolated from rabbit intestine using the *Xenopus laevis* expression cloning system (Fei *et al.*, 1994). The specificity of this cloned H^+-coupled transporter is similar to the specificity of the H^+-coupled di/tripeptide carrier in the human intestine epithelial cell line Caco-2 (Thwaites *et al.*, 1994a). Although the gastrointestinal epithelial cell wall represents a major barrier to drug delivery via the oral route, many peptide-like drugs have significant oral bioavailability (Humphrey, 1986; Humphrey & Ringrose, 1986). The H^+-coupled dipeptide carrier may play an important role in the oral absorption of a number of these peptide-like drugs including the angiotensin-converting enzyme (ACE) inhibitors, enalapril (Friedman & Amidon, 1989a), captopril (Hu & Amidon, 1988) and lisinopril (Friedman & Amidon, 1989b).

The Caco-2 cell system is a suitable model system for intestinal epithelial permeability studies (Hidalgo *et al.*, 1989). This human intestinal epithelial cell line expresses functional H^+-coupled dipeptide carriers at both apical and basolateral membranes (Thwaites *et al.*, 1993a,b). The transepithelial transport of the orally-absorbed cephalosporin cephradine (Inui *et al.*, 1992) and the anti-cancer agent, bestatin (Saito & Inui, 1993) across Caco-2 cell monolayers are mediated via this H^+-coupled transcellular route of absorption. These observations confirm the suitability of this human model system to determine the role of the peptide carrier in the absorption of peptide-like drugs.

The aim of this investigation, therefore, was to identify the role of the intestinal di/tripeptide carrier in the transepithelial transport of three orally-active ACE inhibitors (enalapril maleate, captopril and lisinopril) using a human intestinal epithelial model system (Caco-2).

Methods

Cell culture

Caco-2 cells (passage number 95–114) were cultured in DMEM (with 4.5 g l^{-1} glucose), with 1% non-essential amino acids, 2 mM L-glutamine, 10% (v/v) foetal calf serum

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and gentamicin (60 $\mu g\ ml^{-1}$). Cell monolayers were prepared by seeding at high density ($4.4-5.0 \times 10^5$ cells cm^{-2}) onto tissue culture inserts [Transwell polycarbonate filters (Costar)]. Cell monolayers were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. Cell confluence was estimated by microscopy and determination of transepithelial electrical resistance measured at 37°C.

Transport experiments

Uptake and transport experiments with Gly-Sar, lisinopril, mannitol and proline were performed 16–30 days after seeding and 18–24 h after feeding. Transepithelial flux measurements were performed as described previously (Thwaites *et al.*, 1993a). Briefly, the cell monolayers (24.5 mm in diameter) were washed by sequential transfer through 4 beakers containing 500 ml of modified Krebs buffer (all mmol l^{-1}): NaCl 137, KCl 5.4, $CaCl_2$ 2.8, $MgSO_4$ 1.0, NaH_2PO_4 0.3, glucose 10, HEPES/Tris 10 (pH 7.4, 37°C) and placed in 6-well plates, each well containing 2 ml of modified Krebs buffer. Krebs buffer (pH 7.4), 2 ml, was placed in the upper filter cup (apical solution) and the filters were incubated for 10 min at 37°C. The experimental composition of the buffers in the apical and basal chambers were identical except where stated otherwise. For Na^+-free experiments, NaCl was replaced by choline chloride and NaH_2PO_4 was omitted. The pH 5.5, 6.0 and 6.5 buffers were prepared by replacement of 10 mM HEPES with 10 mM MES and adjustment to the required pH using Tris base. Radiolabelled substrates (0.5 $\mu Ci\ ml^{-1}$) were added to the apical chamber (Gly-Sar (36 μM) or lisinopril (66 μM), and in each case an equivalent concentration of mannitol). In experiments involving high (20 mM) concentrations of substrates iso-osmolarity was maintained by addition of mannitol to control samples. Fluxes in the absorptive (apical-to-basal, J_{a-b}) direction were determined for 1 h and are expressed as pmol $cm^{-2}\ h^{-1}$. The transcellular portion of Gly-Sar transport can be determined by subtraction of the passive (paracellular) component (estimated by mannitol flux). However, since [^{3}H]-mannitol is routinely used in our experiments the paracellular component is likely to be overestimated (i.e. the results in Figure 2 suggest that 20 mM cold Gly-Sar reduces [^{14}C]-Gly-Sar transport below zero). At the end of the incubation period cell monolayers were washed by sequential transfer through 4 beakers containing 500 ml volumes of Krebs buffer (pH 7.4) at 4°C to remove any loosely-associated radiolabel, and removed from the insert. Cell monolayer-associated radiolabel was determined by scintillation counting. Cellular accumulation of Gly-Sar, mannitol, lisinopril and proline are expressed as μM. Cell height was determined by confocal microscopy and this value was used in the determination of intracellular volume. Results are expressed as mean ± s.e.mean.

Experiments with proline were performed in Na^+-free conditions to eliminate any Na^+-coupled proline transport. Flux measurements were performed in two ways. Firstly, bidirectional proline (0.2 $\mu Ci\ ml^{-1}$, 50 μM) fluxes were performed as described previously (Thwaites *et al.*, 1993c). Alternatively, the apical-to-basal (J_{a-b}) transport of [^{3}H]-proline (0.2 $\mu Ci\ ml^{-1}$, 50 μM) was determined and compared with [^{14}C]-mannitol flux (0.2 $\mu Ci\ ml^{-1}$, 50 μM). All other conditions were as described above.

Short-time uptake of Gly-Sar

Cell monolayers were washed by sequential transfer through 4 beakers containing 500 ml Krebs solution (pH 7.4) at 37°C and placed in fresh 6-well plates containing 2 ml pre-warmed Krebs (pH 7.4) in both apical and basolateral compartments. The filters were incubated at 37°C for 30 min. Uptake was initiated by replacing the apical solution with an experimental solution (pH 6.0, 37°C) containing [^{14}C]-Gly-Sar (0.5 $\mu Ci\ ml^{-1}$, 36 μM) and [^{3}H]-mannitol (0.5 $\mu Ci\ ml^{-1}$, 36 μM). After a 30 s incubation the apical solution was rapidly aspirated and the cell monolayer was washed by sequential transfer through 4 beakers containing 500 ml ice-cold Krebs (pH 7.4). Cell-associated radioactivity was determined as above. Residual extracellular marker activity (as determined with mannitol) associated with the cell monolayers was small (0.023 ± 0.001% ($n = 36$) of total label). All uptakes were corrected for this small residual component (Thwaites *et al.*, 1994b).

Intracellular pH measurements

For intracellular pH (pH_i) measurements (Thwaites *et al.*, 1993a), Caco-2 cells grown to confluence (15 days after seeding) on 12 mm diameter Transwell polycarbonate filters (Costar) were loaded by incubation with BCECF-AM (5 μM), in both apical and basal chambers, for 40 min at 37°C. After loading, the inserts were placed in a 24 mm diameter perfusion chamber mounted on the stage of an inverted fluorescence microscope (Nikon Diaphot). Perfusion of the apical and basolateral chambers was accomplished by a compressed air-driven system (flow rate 5 $ml\ min^{-1}$, at 37°C). Intracellular H^+ concentration was quantified by fluorescence (excitation at 440/490 nm and emission at 520 nm) from a small group of cells (5–10) with a photon counting system (Newcastle Photometric Systems). Intracellular BCECF fluorescence was converted to pH_i by comparison with values from an intracellular calibration curve using nigericin (10 μM) and high K^+ solutions (Thomas *et al.*, 1979; Watson *et al.*, 1991). Results are expressed as $\Delta pH_i\ min^{-1}$ [mean ± s.e.mean (n)]. The rate of change of pH_i ($\Delta pH_i\ min^{-1}$) was calculated by linear regression using Photon Counter System 4.7 (Newcastle Photometric Systems). Changes in $\Delta pH_i\ min^{-1}$ (due to a change in the composition of the superfusate) were determined by linear regression, by comparison of the linear portions of the trace over 30–50 s (15–25 data points) periods before and after the change in composition (Thwaites *et al.*, 1994a).

Materials

[^{3}H]-mannitol (specific activity 30 Ci $mmol^{-1}$) was obtained from NEN. [^{14}C]-Gly-Sar (L-glycyl[1-^{14}C]-sarcosine (specific activity 14 mCi $mmol^{-1}$)), D-[1-^{14}C]-mannitol (specific activity 57 mCi $mmol^{-1}$), L-[U-^{14}C]-proline (specific activity >250 mCi $mmol^{-1}$) and L-[2,3-^{3}H]-proline (specific activity 20–40 Ci $mmol^{-1}$) were from Amersham. [^{14}C]-lisinopril (specific activity 8.4 mCi $mmol^{-1}$) was a gift from Zeneca (UK). Lisinopril was a gift from Zeneca (UK) and Merck (UK). Carnosine (β-alanine-L-histidine) was from Peptide Inst. Inc. (Japan). Gly-Sar, captopril, enalapril maleate and L-proline were from Sigma. BCECF, cell culture media, supplements and plastic were supplied by Life Technologies. All other chemicals were from Merck.

Statistics

Results are expressed as mean ± s.e.mean (n). Statistical analysis was performed using one way analysis of variance ANOVA or Student's paired t test.

Results

The transepithelial transport and cellular uptake of the ACE inhibitor lisinopril were determined and compared with transport and uptake of the dipeptide, Gly-Sar and the paracellular marker, mannitol (Figure 1). Figure 1 demonstrates stimulation of transepithelial transport and intracellular accumulation of the dipeptide, Gly-Sar across Caco-2 cell monolayers, by lowering apical medium pH, as reported previously (Thwaites *et al.*, 1993a). At both apical pH 6.0 and 7.4, Gly-Sar transport was significantly greater

($P<0.001$) than transport of the paracellular marker mannitol (Figure 1a). In contrast the apical-to-basal transport of [^{14}C]-lisinopril was significantly lower ($P<0.001$) than mannitol transport (Figure 1a). Apical-to-basal transport (J_{a-b}) of lisinopril was not stimulated on lowering apical pH (Figure 1, Table 1). The dipeptide, Gly-Sar, showed marked intracellular accumulation above medium levels (36 μM) (Figure 1b,) at both apical pH 6.0 (352.7 ± 18.4 μM, $n = 20$) and pH 7.4 (271 ± 18.0 μM, $n = 19$). Lisinopril uptake was significantly reduced compared to Gly-Sar uptake ($P<0.001$) and was similar in magnitude to cell monolayer-associated mannitol at acidic apical pH (Figure 1, Table 1). [^{14}C]-lisinopril transport J_{a-b} at 66 μM (apical pH 6.0, basolateral pH 7.4) was not substantially reduced from 73.4 ± 2.6 pmol cm^{-2} h^{-1} ($n = 4$) when excess unlabelled lisinopril (20 mM) was added (to 63.0 ± 1.9 pmol cm^{-2} h^{-1} ($n = 4$)). This lack of competitive inhibition contrasts with that seen for [^{14}C]-Gly-Sar transport with unlabelled Gly-Sar (Figure 2). The extracellular concentration of lisinopril did not affect intracellular levels of [^{14}C]-lisinopril at 1 h across the apical membrane (11.6 ± 1.1 μM ($n = 4$) at 66 μM (extracellular lisinopril) and 9.5 ± 0.4 μM ($n = 4$) at 20 mM extracellular lisinopril). Under identical experimental conditions lisinopril J_{a-b} was 34.2 ± 6.9 pmol cm^{-2} h^{-1} ($n = 3$) at 66 μM (extracellular lisinopril) and 45.8 ± 6.0 pmol cm^{-2} h^{-1} ($n = 5$) at 20 mM (extracellular lisinopril). Uptake of lisinopril across the basolateral membrane was 12.3 ± 0.5 μM ($n = 4$) at an extracellular concentration of 66 μM and 9.5 ± 0.5 μM ($n = 5$) at 20 mM extracellular lisinopril.

Excess unlabelled Gly-Sar (20 mM) inhibited transcellular [^{14}C]-Gly-Sar transport (Figure 2a). The three ACE inhibitors also inhibited [^{14}C]-Gly-Sar transport, but to varying degrees in the order enalapril > captopril > lisinopril (Figure 2). However, the effect of these substrates on the steady-state uptake of [^{14}C]-Gly-Sar showed a different pattern (Figure 2b). Gly-Sar and enalapril maleate both significantly ($P<0.001$) reduced [^{14}C]-Gly-Sar uptake. Lisinopril also reduced uptake of the dipeptide but the inhibitory effect was less marked ($P<0.05$). However, unlike its effect on transport, captopril did not inhibit [^{14}C]-Gly-Sar uptake at steady-state (there was in fact a small increase). This unexpected effect of captopril is likely to represent an inhibitory effect on the exit of [^{14}C]-Gly-Sar across the basolateral membrane since the initial uptake of the dipeptide (measured at 30 s) was significantly reduced ($P<0.001$) in the presence of cap-

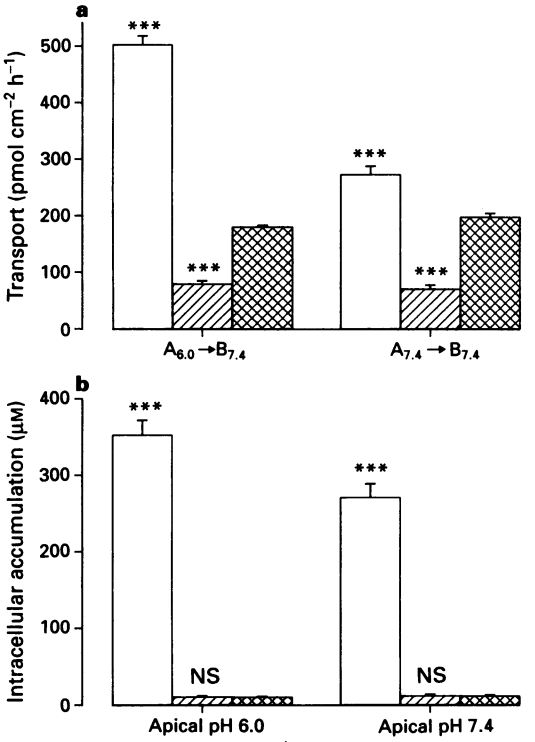

Figure 1 Transepithelial transport and cellular uptake of a dipeptide (Gly-Sar), and ACE inhibitor (lisinopril) and the paracellular marker mannitol. Apical-to-basal (J_{a-b}) transport (a) and cellular accumulation (across the apical membrane, b) of [^{14}C]-Gly-Sar (open columns), [^{14}C]-lisinopril (hatched columns) and [^{3}H]-mannitol (cross-hatched columns) across Caco-2 cell monolayers in the presence and absence of a transepithelial pH gradient. Basolateral pH was maintained at pH 7.4. Results are expressed as mean ± s.e.mean ($n = 5.21$); ***$P<0.001$; NS$P>0.05$, significance of difference from mannitol data. For abbreviations, in this and subsequent legends, see text.

Table 1 Acidic apical media do not stimulate either transport or intracellular accumulation of lisinopril

pH of apical medium	*Apical to basal transport* J_{a-b} (pmol cm^{-2} h^{-1})	*Intracellular accumulation* *Lisinopril* (μM)	*Mannitol* (μM)
7.4	70.7 ± 7.3 (9)	12.0 ± 2.0 (15)	11.8 ± 1.3 (15)
6.5	64.2 ± 5.4 (4)	18.4 ± 0.4 (9)	13.0 ± 1.3 (9)
6.0	79.5 ± 5.8 (11)	10.6 ± 1.4 (15)	10.4 ± 1.0 (10)
5.5	85.7 ± 4.1 (7)	12.5 ± 0.6 (10)	11.4 ± 1.0 (10)

Apical-to-basal (J_{a-b}) transport and cellular accumulation (across the apical membrane) of [^{14}C]-lisinopril (66 μM) and [^{3}H]-mannitol (66 μM) across Caco-2 cell monolayers in the presence and absence of a transepithelial pH gradient. Basolateral pH was maintained at pH 7.4. Results are expressed as mean ± s.e.mean n value given in parentheses. One way analysis of variance (ANOVA) for transport data $F = 1.67$ $P = 0.20$, not significant. Note that neither lisinopril nor mannitol show accumulation above medium values, even at the most acidic apical pH.

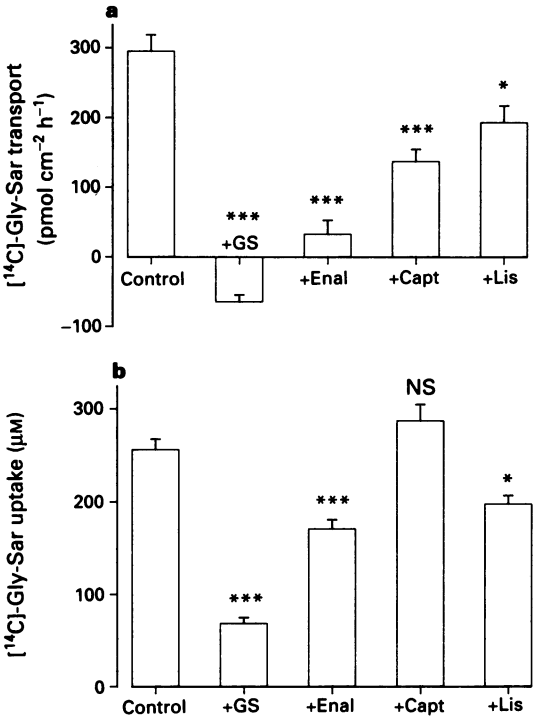

Figure 2 pH-dependent dipeptide (Gly-Sar) transport and cellular uptake in the presence of cold Gly-Sar and three ACE inhibitors. Apical-to-basal (J_{a-b}) [^{14}C]-Gly-Sar (36 μM) transport (a) and cellular uptake across the apical membrane (b) in the presence and absence of 20 mM cold Gly-Sar (GS), enalapril maleate (Enal), captopril (Capt), and lisinopril (Lis) (apical pH 6.0, basolateral pH 7.4). Results are mean ± s.e.mean ($n = 9$–16); ***$P<0.001$; *$P<0.05$; NS$P>0.05$ versus control data.

topril (Figure 3). The inhibitory effects of the compounds (all at 20 mM) on the initial uptake of [^{14}C]-Gly-Sar (Figure 3) showed a similar order of potency (Gly-Sar > enalapril maleate > captopril > lisinopril) as observed with transepithelial [^{14}C]-Gly-Sar transport (Figure 2a). Lisinopril showed no significant inhibition of initial [^{14}C]-Gly-Sar uptake.

The Caco-2 cell line expresses an apically-localized H^+-coupled amino acid transporter (Thwaites *et al.*, 1993c) that is involved in transepithelial proline transport. A possible role for this H^+-coupled transporter in ACE inhibitor transport was investigated (Figure 4). Figure 4a shows that 20 mM excess unlabelled proline markedly reduced transport of [^{3}H]-proline whereas the dipeptide, Gly-Sar, had a smaller inhibitory effect (which most probably represents an effect on the driving force (the H^+ gradient) rather than the transport itself). The three ACE inhibitors failed to inhibit [^{3}H]-proline transport (Figure 4a). However, the levels of proline uptake in the presence of this group of substrates showed a different pattern with only proline inhibiting [^{3}H]-proline uptake (Figure 4b). The most striking effect is that of enalapril maleate (Figure 4b) which more than doubled the uptake of [^{3}H]-proline (1091 ± 98 μM ($n = 10$) compared to 430 ± 31 μM ($n = 11$) under control conditions).

The transapical transport of the dipeptide, Gly-Sar, is associated with the movement of protons across the cell wall that can be detected by the resultant acidification of the intracellular environment (Thwaites *et al.*, 1993a). Figure 5 clearly indicates that after exposure to the dipeptide carnosine (β-Ala-His) at 20 mM at the apical surface (apical pH 6.5, basolateral pH 7.4) the intracellular pH became more acidic due to substrate-induced H^+ flow into the cells. The inclusion of carnosine (20 mM) in the apical superfusate significantly increased ($P < 0.001$) the initial rate of intracellular acidification (ΔpH_i min^{-1}) from 0.009 ± 0.003 pH units min^{-1} ($n = 5$) to 0.115 ± 0.013 pH units min^{-1} ($n = 5$). Similar effects were noted with enalapril maleate and captopril (Figure 5). Enalapril maleate (20 mM) increased ΔpH_i min^{-1} from 0.009 ± 0.003 pH units min^{-1} ($n = 5$) to 0.115 ± 0.017 pH units min^{-1} ($n = 5$) whereas captopril increased ΔpH_i min^{-1} from 0.013 ± 0.004 pH units min^{-1} ($n = 4$) to 0.074 ± 0.026 pH units min^{-1} ($n = 4$). The ACE inhibitor lisinopril, however, failed to induce a significant ($P > 0.05$) change in intracellular pH (0.021 ± 0.002 pH units min^{-1}, $n = 3$) compared to the effect of exposure to pH 6.5 superfusate alone (0.017 ± 0.008 pH units min^{-1}, $n = 3$). The rate of intracellular acidification (ΔpH_i min^{-1}) observed in the presence of enalapril maleate or captopril were both significantly greater than that measured in the presence of lisinopril.

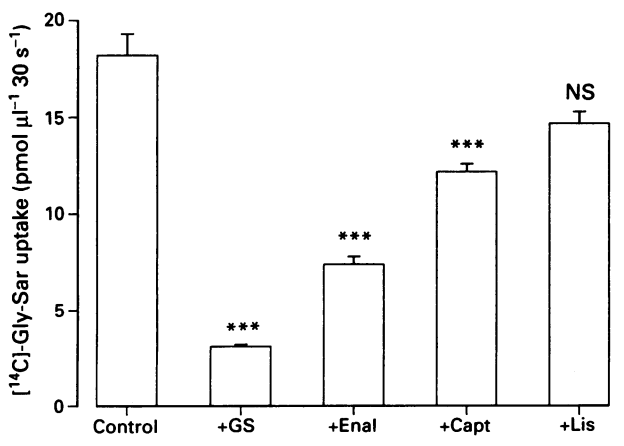

Figure 3 Short-time uptake of Gly-Sar into Caco-2 cell monolayers in the presence or absence of cold compounds. pH-dependent (apical pH 6.0, basolateral pH 7.4) [^{14}C]-Gly-Sar uptake across the apical membrane of Caco-2 cell monolayers in the presence and absence of 20 mM cold Gly-Sar (GS), enalapril maleate (Enal), captopril (Capt), and lisinopril (Lis). Uptake was measured over a 30 s period. Results are expressed as mean ± s.e.mean ($n = 6$, $n = 3$ for lisinopril); ***$P < 0.001$; NS$P > 0.05$, significance of difference from control data.

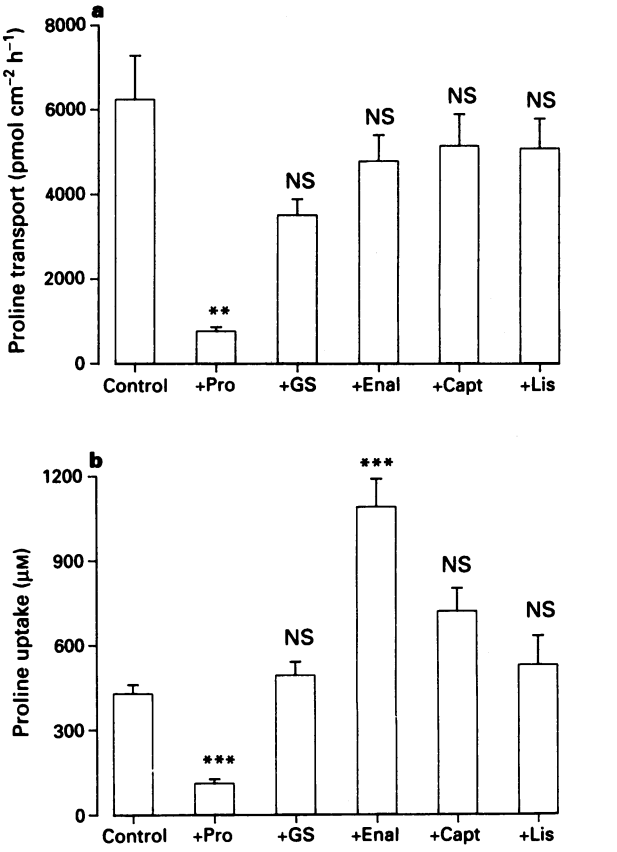

Figure 4 pH-dependent (Na^+-independent) amino acid (L-proline) transport and cellular accumulation in the presence of proline, Gly-Sar and three ACE inhibitors. pH-dependent (apical pH 6.0, basolateral pH 7.4), Na^+-independent proline net transport (a) and cellular uptake across the apical membrane (b) in the presence and absence of 20 mM cold L-proline (Pro), Gly-Sar (GS), enalapril maleate (Enal), captopril (Capt) and lisinopril (Lis). Results are expressed as mean ± s.e.mean ($n = 7$–11); ***$P < 0.001$; **$P < 0.05$; NS$P < 0.05$ versus control data.

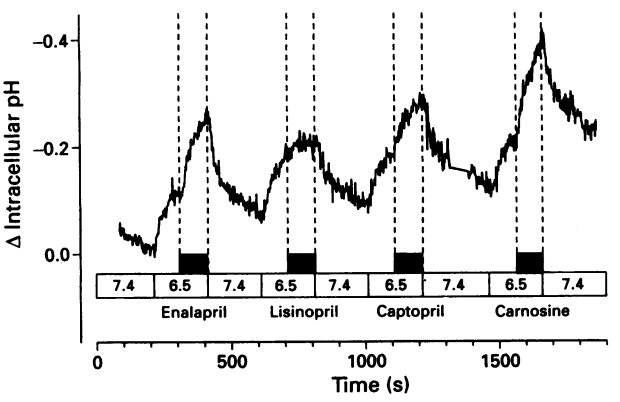

Figure 5 Intracellular pH measured in BCECF-loaded Caco-2 cell monolayers. The effect on intracellular pH of apical exposure to enalapril maleate, lisinopril, captopril or the dipeptide carnosine. All substrates (20 mM) were superfused across the apical surface at pH 6.5. Basolateral pH was maintained at pH 7.4. The figure is a representative trace of 3–5 separate experiments.

Discussion

Oral bioavailability is crucial to the use of angiotensin-converting enzyme (ACE) inhibitors in the clinical treatment of systemic hypertension and congestive heart failure (Humphrey, 1986; Humphrey & Ringrose, 1986). Captopril demonstrates high oral bioavailability (62%) in man with a peak plasma concentration after 1 h (Duchin *et al.*, 1982). Enalapril maleate is a monoethyl ester prodrug of the diacid enalaprilat. This prodrug (enalapril maleate) needs to undergo deesterification *in vivo* to produce its active form, enalaprilat (Wyvratt & Patchett, 1985). Studies in man indicate that absorption of enalapril maleate (61% oral availability) is rapid (peak plasma levels between 0.5–1.5 h after administration) and that enalapril maleate/enalaprilat is recovered intact (94%) from urine and faeces (apart from the bioactivation which is post-absorptive) (Ulm *et al.*, 1982). In animal experiments similar observations were made (Tocco *et al.*, 1981). Enalapril maleate had 61% oral bioavailability in the dog (whereas enalaprilat was 11%) and 34% in the rat (Tocco *et al.*, 1981). Lisinopril is similar in structure to enalaprilat but the alanine residue of enalaprilat has been replaced by a lysine residue (Wyvratt & Patchett, 1985). In human studies, the absorption of lisinopril was low (29%) and slow (peak plasma concentration 6–8 h after administration) compared to enalapril maleate (Ulm *et al.*, 1982). Lisinopril was, however, resistant to metabolism (97% was recovered intact in the urine and faeces, Ulm *et al.*, 1982). The effects of enalapril maleate and lisinopril are both longer lasting than the effects of the less stable captopril (Ulm *et al.*, 1982).

The structures of the first three orally-active ACE inhibitors (captopril, enalapril maleate and lisinopril) are similar or analogous to the dipeptide Ala-Pro (Wyvratt & Patchett, 1985) which is the C-terminal dipeptide of bradykinin-potentiating peptide 5_a ($BPP5_a$), a potent inhibitor of ACE activity isolated from snake venom (Cheung *et al.*, 1980; Wyvratt & Patchett, 1986). Studies in rat jejunum using the single-pass perfusion method have suggested that the significant levels of absorption of these three ACE inhibitors across the intestinal epithelial wall is due to transport via the intestinal di/tripeptide carrier (Hu & Amidon, 1988; Friedman & Amidon, 1989a,b).

We have investigated whether captopril, enalapril maleate and lisinopril are substrates for proton-coupled solute transport using the human intestinal epithelial Caco-2 cell model. This human intestinal epithelial cell line expresses H^+-coupled dipeptide transporters at both apical and basolateral membranes (Thwaites *et al.*, 1993a,b). The specificity of the dipeptide transporter at the apical surface of Caco-2 cells (Thwaites *et al.*, 1994a) is similar to the cloned dipeptide transporter from rabbit intestine (PepT1) when expressed in *Xenopus laevis* oocytes (Fei *et al.*, 1994). Similarly, dipeptide transport is rheogenic in both Caco-2 cell monolayers (Thwaites *et al.*, 1993d) and PepT1-expressing oocytes (Fei *et al.*, 1994). The importance of this H^+-coupled transcellular route in uptake and transepithelial transport of the orally-absorbed cephalosphorins cephalexin (Dantzig & Bergin, 1990) and cephradine (Inui *et al.*, 1992), and the anti-cancer agent, bestatin (Saito & Inui, 1993) has been demonstrated using this cell line. Although it is clear that this model has proved useful in demonstrating both pH-dependency and H^+-coupling of dipeptide/aminocephalosporin transport the present studies are the first to investigate ACE inhibitor transport. An additional advantage of using the Caco-2 cell system rather than a cloned membrane transporter expressed in oocytes is that alternative transport pathways and their relative importance may be studied in the same cell system.

The present studies provide no evidence for pH-dependent transport or accumulation of lisinopril (Figure 1) in the human intestinal cell line, Caco-2. Moreover, lisinopril permeability is lower than mannitol (Figure 1). Nor is lisinopril (20 mM) addition associated with H^+ flow across the apical membrane (Figure 5) as has been observed with both dipeptides (Gly-Sar) and aminocephalosporins (cephalexin) (Thwaites *et al.*, 1993a,b). Lisinopril (20 mM) failed to induce H^+ flow into the cells above levels seen with apical acidity alone. Clearly from these results it appears that the transport of Gly-Sar is via the transcellular route and lisinopril via the paracellular pathway. The weak inhibitory effects of 20 mM lisinopril on Gly-Sar transport and accumulation (Figure 2) suggest that this ACE inhibitor may be a weak non-transported inhibitor, although non-specific effects on cell monolayer-transporter integrity cannot be ruled out.

In contrast to lisinopril, the inhibitory actions of captopril and enalapril maleate on transepithelial Gly-Sar transport (Figure 2a) and the ability of these two ACE inhibitors to stimulate H^+-flow across the apical membrane (Figure 5) suggest that they may be transported across the apical membrane by the di/tripeptide carrier. However, although both captopril and enalapril maleate reduce the initial uptake (Figure 3) of the dipeptide, Gly-Sar, their effects on the steady-state levels of accumulation of Gly-Sar within the cell monolayers show a different pattern (Figure 2b). It is likely that the inhibitory action of captopril on transepithelial dipeptide transport consists of two components. Firstly, the results suggest that although Gly-Sar appears to have a higher affinity for the apical transporter (Figure 3), once inside the cell captropril has a higher affinity for the basolateral efflux mechanism. This accounts for the decrease in transport but increase in accumulation of Gly-Sar in the presence of captopril (Figure 2).

All three ACE inhibitors fail to inhibit the pH-dependent (Na^+-independent) transepithelial transport of the amino acid, proline (Figure 4). However, enalapril maleate caused a large increase in the accumulation of proline within the cell monolayer suggesting that enalapril maleate may inhibit the efflux of proline across the basolateral membrane. This effect of enalapril maleate was not observed with dipeptide accumulation (Figure 2).

These observations regarding the mechanisms involved in the absorption of the ACE inhibitors captopril, enalapril maleate and lisinopril show broad agreement with the available data from man (Duchin *et al.*, 1982; Ulm *et al.*, 1982) and suggest that the Caco-2 cell model may be useful in predictive studies for oral bioavailability. There are however differences with the present data and work using the rat jejunum as the main experimental model (Hu & Amidon, 1988; Friedman & Amidon, 1989a,b). The permeability of all three ACE inhibitors in rat jejunum show saturation and a decrease in permeability in the presence of high concentrations of dipeptides or the aminocephalosporin cephradine. Although our evidence from experiments in this human intestinal epithelial cell-line point to a role for the intestinal dipeptide carrier in the transport of enalapril maleate and captopril, the evidence also suggests that lisinopril is absorbed passively. A passive mechanism of lisinopril absorption is consistent with the slow rate of disappearance observed in the experiments with rat jejunum (Friedman & Amidon, 1989b) and in studies in man (Ulm *et al.*, 1982).

There are two steps involved in transport across the intestinal epithelial barrier. Firstly, movement across the apical membrane into the cell and, secondly, efflux across the basolateral membrane. For Gly-Sar transport it is likely that the rate limiting step for transepithelial transport is in fact exit from the cell across the basolateral membrane (Thwaites *et al.*, 1993b). Nothing is known about the mechanisms involved in ACE inhibitor transport across the basolateral membrane of the intestinal enterocyte. However, our observations in this study suggest that although both captopril and enalapril maleate share a basolateral efflux mechanism with the dipeptide Gly-Sar, the relative affinities for this mechanism are different from the apical dipeptide transporter. Perhaps, more importantly, the results also suggest

that enalpril maleate has a high affinity for a basolateral transporter associated with the Na^+-independent transport of amino acids such as proline. Clearly more work needs to be performed to define the exact mechanisms of exit of ACE inhibitors across the basolateral membrane.

In conclusion it appears that both captopril and enalopril maleate are substrates for the H^+/dipeptide transporter expressed in human intestinal cells. Lisinopril is not a transported substrate. It is likely that the Caco-2 cell system will be useful in predictive studies of oral bioavailability via the H^+-coupled dipeptide transporter.

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Suppression by the sumatriptan analogue, CP-122,288 of c-fos immunoreactivity in trigeminal nucleus caudalis induced by intracisternal capsaicin

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1 The effects of an intravenously administered sumatriptan analogue were examined on c-fos-like immunoreactivity (c-fos-LI), a marker of neuronal activation, evoked within trigeminal nucleus caudalis (TNC) and other brain stem regions 2 h after intracisternal injection of the irritant, capsaicin (0.1 ml, 0.1 mM), in pentobarbitone-anaesthetized Hartley guinea-pigs.

2 C-fos-LI was assessed in eighteen serial sections (50 μm) using a polyclonal antiserum. A weighted average, reflecting total expression within lamina I, II_o of TNC was obtained from three representative levels (i.e., at −0.225 mm, −2.475 mm and −6.975 mm.)

3 Capsaicin caused significant labelling within lamina I, II_o, a region containing axonal terminations of small unmyelinated C-fibres, as well as within the nucleus of the solitary tract, area postrema and medial reticular nucleus. A similar distribution of positive cells was reported previously after intracisternal injection of other chemical irritants such as autologous blood or carrageenin.

4 Pretreatment with a conformationally restricted sumatriptan analogue (with some selectivity for 5-HT_{1B} and 5-HT_{1D} receptor subtypes) CP-122,288, reduced the weighted average by approximately 50–60% ($P<0.05$) in lamina I, II_o at $\geqslant 100$ pmol kg^{-1}, i.v., but did not decrease cell number within area postrema, nucleus of the solitary tract or medial reticular nucleus. A similar pattern was reported previously following sumatriptan, dihydroergotamine or CP-93,129 administration after noxious meningeal stimulation.

5 We conclude that modifications at the amino-ethyl side chain of sumatriptan dramatically enhance the suppression of c-fos expression within TNC, a finding consistent with its remarkable potency against neurogenic plasma protein extravasation within dura mater. CP-122,288 and related analogues may serve as an important prototype for drug development in migraine and related headaches.

Keywords: CP-122,288; trigeminovascular system; capsaicin; c-fos expression; 5-$HT_{1B/D}$ agonist; sumatriptan analogue; migraine; headache; intracisternal

Introduction

Very few animal models have been developed to evaluate compounds of potential importance for the treatment of migraine and related headaches. Moreover, traditional models do not assess the effects of drugs on nociception, but utilize measures of blood flow (Friberg, 1991; L'acombe *et al.*, 1993), blood velocity (Zanette *et al.*, 1992; Zwetsloot *et al.*, 1992) or assess the ability of compounds to constrict or dilate vascular smooth muscle (Feniuk & Humphrey, 1992).

We have utilized the expression of the transcription factor and immediate early gene c-fos to assess the response of postsynaptic cells within the trigeminal nucleus caudalis (TNC) to noxious chemical meningeal stimulation, and the effects of potential treatments on this response (Nozaki, 1992a,b). C-fos expression serves as a marker of functional activity in neurones and has been demonstrated within lamina I and II_o of the dorsal horn of the spinal cord (Hunt *et al.*, 1987; Menetrey *et al.*, 1989; Presley *et al.*, 1990).

We previously reported that the analgesic, morphine, the 5-HT_{1D} receptor agonists, dihydroergotamine or sumatriptan, (which are used as symptomatic migraine therapy), and the selective 5-HT_{1B} receptor agonist, CP-93,129, block c-fos antigen expression in the rat TNC (Nozaki, 1992b) after noxious meningeal stimulation. In this paper, we tested the effects of CP-122,288 on c-fos-LI after intracisternal injection of the irritant, capsaicin. CP-122,288 is a conformationally restricted indole-containing analogue which belongs to a series of analogues which can be used to probe the receptor-ligand recognition elements at the 5-HT_{1D} binding site (Macor *et al.*, 1992a,b). CP-122,288 possesses a chemical structure similar to that of sumatriptan with the exception that a C3-(**R**)-pyrrolidin-2-ylmethyl group has been incorporated into the C3-aminoethyl side chain of sumatriptan (Macor *et al.*, 1992a,b). We now demonstrate that CP-122,288 when administered intravenously to guinea-pigs 30 min prior to capsaicin injection, potently decreases c-fos-like immunoreactivity (c-fos LI) within laminae I and II_o.

Methods

Animal preparation

Male Hartley guinea-pigs (200–300 g; Charles River Laboratories, Wilmington, MA, U.S.A.) were anaesthetized with sodium pentobarbitone (40 mg kg^{-1}, i.p.) and maintained with 10 mg kg^{-1}, i.p., every 1–2 h as needed to suppress the withdrawal response to hindpaw stimulation. A soft catheter (PE-10, 0.28 mm internal diameter; Intramedic, Clay Adams, Parsippany NJ, U.S.A.) was introduced into the cisterna magna after a midline skin incision was made from the occipital protuberance to the cervical area. The animals were then placed in lateral decubitus position for 5.5 h as

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previously described (Nozaki *et al.*, 1992a). The animals then received a femoral vein injection of either the vehicle or drug. Thirty minutes later (6 h after catheter placement), capsaicin solution (100 µl of 0.1 µmol ml^{-1}) was injected into the cisterna magna over 1 min with a tuberculin syringe. (In previously published experiments, it was shown that 100 µl of ≤ 0.1 mM capsaicin produced brisk c-fos expression in TNC; 218 cells per section). Therefore, this dosage was used in experiments described here (Cutrer *et al.*, 1994).

To facilitate capsaicin distribution within the subarachnoid space, animals were placed in the reverse Trendelenberg position (−30 degrees) for 30 min and in lateral position for the next 90 min. Core temperature was maintained at 36–37°C by a homeothermic blanket (Harvard Apparatus No. 551, South Natick MA, U.S.A.). An overdose of pentobarbitone (80 mg kg^{-1}, i.p.) was used for euthanasia 2 h after capsaicin instillation.

Physiological monitoring was carried out in randomly selected animals. Arterial pH, P_{CO_2}, and P_{O_2} (Corning 178 Blood Gas Analyzer, Ciba Corning Diag. Corp. Medford MA, U.S.A.), arterial pressure (MAP), heart rate (HR) and respiratory rate (RR) (MAC Lab ADI Instruments Castle Hill, Australia) were measured.

Animals were perfused via the ascending aorta with saline (0.9% 200 ml), followed by 500 ml formaldehyde (4%) in 0.1 M phosphate buffer (PB, pH 7.3). Perfused brainstems with attached cervical cords were stored overnight in the same fixative and were then placed in a cryoprotectant solution (20% sucrose, 30% ethylene glycol in 0.1 M PB) for 48 h before sectioning. The brainstems and upper cervical spinal cords were sectioned serially with a freezing microtome (Reichert-Jung; 2000 Leica, U.S.A.) from rostral to obex (1 mm) to the C2 level (190 sections ± 10), and every third 50 µm section or 18 selected sections (see below) were saved and processed for immunohistochemistry.

Protocol

Drug vehicle (normal saline; $n = 7$) or CP-122,288, 10 pmol kg^{-1}, ($n = 3$) 100 pmol kg^{-1} ($n = 3$) or 1.0 nmol kg^{-1} ($n = 8$) was injected via the femoral vein 5.5 h after catheter placement and 2.5 h before the animals were killed.

Immunohistochemistry

Tissue sections were processed as free floating sections with the avidin-biotin procedure using commercially available kits (Vectastain ABC; Vector Labs Burlingame, CA, U.S.A.) as described previously (Uemura *et al.*, 1991), with slight modifications (Cutrer *et al.*, 1994). Briefly, sections were incubated in 10% normal goat serum, 0.03% hydrogen peroxide in 0.1 M phosphate buffered saline (PBS; pH 7.3), primary c-fos antibody (kindly provided by Dr Dennis Slamon, the Department of Hematology and Oncology at the University of California, LA, U.S.A.) (1/5000 dilution in PBS with 0.3% Triton X-100) (PBS-T Sigma Labs St. Louis, MO, U.S.A.) (room temperature overnight), secondary antibody, a biotinylated anti-rabbit IgG antiserum (1/200 dilution in PBS-T, Vector Labs, Burlingame, CA, U.S.A.) (room temperature for 2 h), ABC-peroxidase complex (Vector Lab) (room temperature 2 h) and finally a solution of 3,3'-diaminobenzidine tetrahydrochloride (40 mg%; Sigma Labs) and 0.003% hydrogen peroxide in 50 mM Tris-HCl buffer (pH 7.6) (room temperature for 20 min).

After immunohistochemical processing, tissue sections were serially arranged on gelatin slides, air dried overnight and coverslipped.

Cell counting

C-fos positive cells (i.e., stained nuclei) were counted by an observer naive to the treatment group (F.M.C.) and confirmed (in randomly selected sections) by a second investigator (V.L.) also blind to the treatment groups, as described previously (Cutrer *et al.*, 1994).

Weighted average methods

Cell numbers were compared using a weighted average of cells per section. Based on the observation that c-fos expression was maximal at −2.25 to −2.55 mm and decreased *linearly* on either side of this level, a weighted average of the cell number per section was calculated as follows: Six 50 µm sections (every third section) were counted at each of three levels: +0.15 to −0.60 (midpoint: −0.225); −2.10 to −2.85 (midpoint: −2.475); and −6.60 to −7.35 (midpoint: −6.975). The mean values from the three levels were designated x_1, x_2 and x_3 respectively. (The use of six samples at each of three levels was based on established statistical methods; Efron, 1979.) The values x_1, x_2 and x_3 correspond to the heights AB, DC and EF respectively (see Figure 1). The total number of labelled cells within the two trapezoids, ABCD and CDEF (see Figure 1), can be estimated by the formula $15(x_1 + x_2)/2$ for ABCD and $30(x_2 + x_3)/2$ for CDEF. The sum of the two values is then divided by the total number of sections (45) to obtain the weighted average.

To validate the weighted average method, brainstem sections from animals receiving capsaicin (i.c.) plus either vehicle ($n = 5$) or CP-122,288 1 nmol kg^{-1} ($n = 5$), were analyzed by both the weighted average sampling method and the counting of positively stained cells in TNC in every 3rd section within the same sampling area (45 total sections).

Qualitative estimate of c-fos LI

An assessment of the extent of c-fos LI in nuclei other than TNC (present in 18 sections) was performed in: nucleus of the tractus solitarius (NTS, 12 sections), area postrema (AP, 2 sections), medullary reticular nucleus (MRN, 8 sections) and lateral reticular nucleus (LRN, 6 sections) by an observer naive to the treatment protocol. An estimate of c-fos staining in nuclei other than TNC was evaluated qualitatively by a three point scale: 3+ indicating intense expression comparable to the amount seen in TNC (>30 cells); 2+ indicating moderate expression (11-30); 1+ (1-10 cells); and 0 indicating no cells.

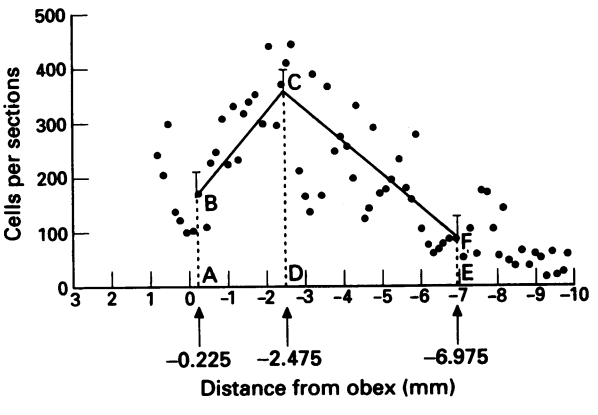

Figure 1 Number of cells in a representative animal expressing c-fos antigen when sampled in every third 50 µm section from just rostral to the obex through C_2 after intracisternal capsaicin injection (0.1 ml, 0.1 mM). Each dot represents the number of cells per section in lamina I, II_o of the entire TNC. For comparison, a weighted average method was used to estimate cell number based on mean values obtained at coordinates AB, CD and EF (6 sections at each of three levels −0.225, −2.475 and −6.975). The weighted average per animal was determined by the total area of the 2 trapezoids (ABCD and DCFE, see Methods) divided by the number of sections.

Drugs

Capsaicin solution (Polyscience Inc. Warrington, PA, U.S.A.) was made fresh weekly. Capsaicin 3.05 mg was diluted in 1 ml of saline:ethanol:Tween 80 (8:1:1) and sonicated for 5 min. The solution was further diluted (1:100) in normal saline and stored at 4°C as a stock solution. Sodium pentobarbitone was obtained from Anthony Products Co. Arcadia, CA, U.S.A. Lignocaine HCl, 2% was from Astra Pharmaceutical Products Inc., Westborough MA, U.S.A. CP-122,288 (Pfizer Inc., Groton, CT, U.S.A.) was diluted in saline from frozen aliquots for each experiment.

Statistics

Data from the TNC are expressed as a weighted average ± standard error of the mean (s.e.mean). The weighted averages were compared by a one sided Williams test which controls for multiple comparisons in a dose-response experiment (Williams, 1972). Statistical comparisons of mean arterial blood pressure (MABP), heart rate (HR) and respiratory rate (RR) were made between vehicle and CP-122,288-treated groups using Analysis of Variance plus Bonferroni/Dunnett *post hoc* tests. Qualitative data were analysed by the Mann-Whitney unpaired *t* test.

Results

Data were obtained from 75 animals.

Physiological parameters after capsaicin

Baseline values did not differ between groups. Mean arterial blood pressure, heart rate, respiratory rate or end tidal CO_2 did not change after CP-122,288 alone (data not shown). Capsaicin caused transient changes in all of the above parameters but differences between groups reached statistical significance at 1 min only (HR and MABP $P<0.05$ see Figure 2).

C-fos expression after capsaicin

Spatial distribution C-fos-like immunoreactivity was present bilaterally and most intensely within dorsal and ventral aspects of laminae I and II_o at each sampled level of TNC. The magnitude of c-fos expression was greatest −2.475 from obex with a mean in the control animals ($n=7$) of 357 ± 24 cells per section. At −0.225 and −6.975 there was a mean of 210 ± 24 and 69 ± 10 cells per section, respectively.

C-fos positive cells were also observed in association with arachnoid and pial blood vessels throughout, but were most prominent in that portion of meninges overlying the trigeminal nucleus caudalis or the dorsal horn of the spinal cord. Their identity was not determined. No evidence of subarachnoid haemorrhage was seen at the time of dissection.

Three plus c-fos-LI was observed in the area postrema (AP) at the level of −0.225 whereas 2+ was seen in the ventral portion of the medullary reticular nucleus (MRN) and NTS; 1+ positive cells were also seen in the lateral reticular nucleus (LRN). At −2.475 mm, 1+ c-fos-LI was present in the NTS and in the MRN. At −6.975 mm, 1+ staining was present in laminae III, IV, V and X. There was sparse labelling in lamina VII (see Figure 3).

Validation of the weighted average method

The numbers obtained by counting every third section (45 sections) were not statistically different from the weighted average values either in the control group ($P<0.35$) or in the CP 122,288-treated group ($P<0.52$) (see Table 1). The percentage reductions were likewise very similar for the two methods (all sections, 51.5% versus the weighted average method, 54.1%).

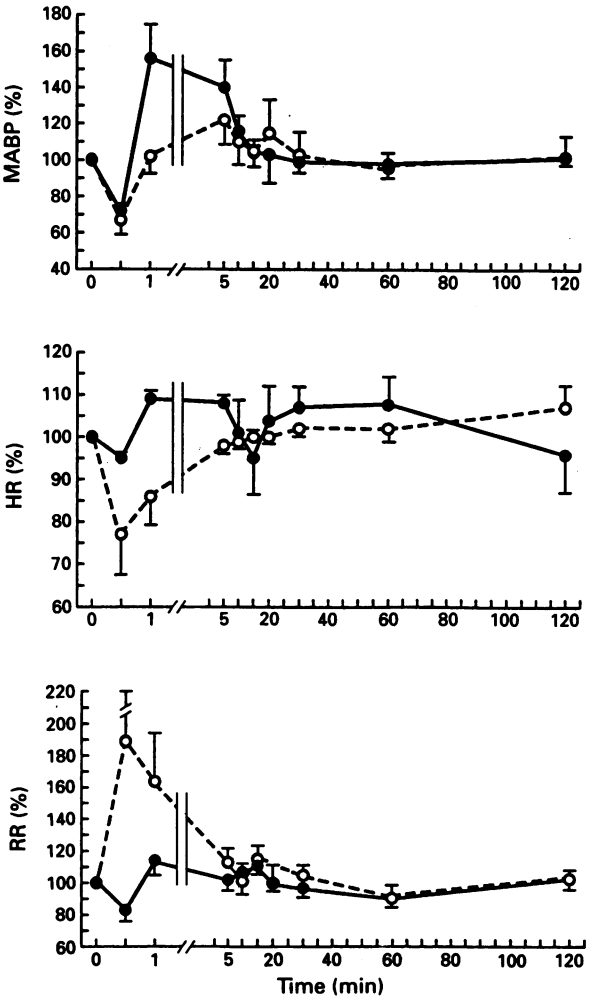

Figure 2 Time-dependent changes in mean arterial blood pressure (MABP), heart rate (HR), and respiratory rate (RR) following intracisternal injection of capsaicin (0.1 ml, 100 nM) in pentobarbitone anaesthetized guinea-pigs pretreated 30 min before with vehicle ($n=4$) or CP-122,288 (1 nmol kg^{-1}) ($n=3$.) (See Methods). Baseline values for MABP, HR and RR were 56 ± 4 mmHg, 257 ± 17 beats per min or 60 ± 7 respirations per min respectively in the vehicle group and 69 ± 9 mmHg, 285 ± 29 beats per min or 48 ± 9 respirations per min respectively after CP-122,288 treatment. Data are expressed as percentage baseline ± s.e.mean.

Drug treatment

Two hundred and twenty eight ± 12 cells were present per 50 μm section after capsaicin ($n=7$). CP-122,288 administration 30 min prior to capsaicin, dose-dependently reduced c-fos expression within TNC. Pretreatment with 10 pmol ($n=3$), 100 pmol ($n=3$) and 1 nmol ($n=8$) kg^{-1} reduced c-fos-LI by 35%, 56% ($P<0.01$) and 59% ($P<0.01$), respectively (see Figure 4). A dose of 10 pmol kg^{-1} did not significantly decrease the weighted average ($P>0.05$) but decreased the number of c-fos positive cells at −6.975 mm by 54% ($P<0.05$). After administering 100 pmol kg^{-1}, cell counts were significantly decreased at −6.975 and −2.475 mm by 57% and 78% respectively ($P<0.05$). At 1 nmol kg^{-1}, significant reductions were found at all three anatomical levels (see Figure 5). Treatment with CP 122,288 appeared to reduce c-fos LI in the ventral portion of TNC more than in the dorsal portion in a few animals; however, this was not a consistent finding. CP 122,288 did not change the estimate of positive cells in area postrema, nucleus of the tractus solitarius, lateral reticular nucleus and medullary reticular nucleus.

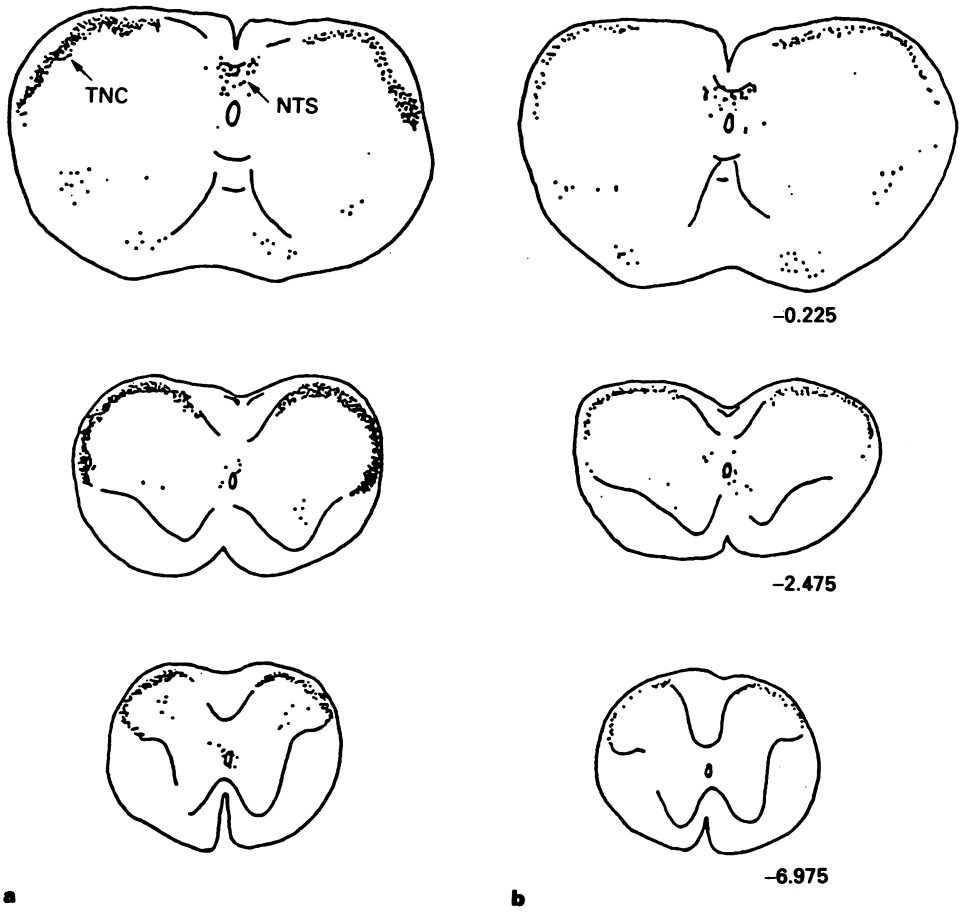

Figure 3 Camera lucida drawings showing the location of c-fos antigen immunostained cells (dots) in coronal brain stem sections taken from vehicle (a) and CP-122,288 (1 nmol kg^{-1}, i.v.) (b)-injected guinea-pigs given capsaicin intracisternally (0.1 ml, 100 nM) and killed 120 min later. The distance (mm) causal or rostral to the obex is given to the right of each drawing. The findings from these 2 animals are representative of the results in 7 animals for each group. Not shown are the c-fos protein-like immunoreactive cells in pia mater, arachnoid and ependyma. TNC, trigeminal nucleus caudalis, NTS, nucleus of the solitary tract.

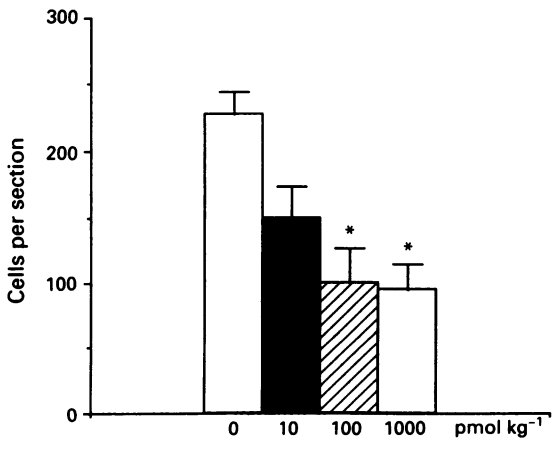

Figure 4 Pretreatment with CP-122,288 dose-dependently decreases the c-fos immunoreactive cells evoked by intracisternal capsaicin injection. Cell numbers are given per 50 μm section within the trigeminal nucleus caudalis (TNC) (lamina I, II_o) as determined by a weighted average method (see Methods). Vehicle ($n = 7$), CP-122,288 10 pmol kg^{-1} ($n = 3$), 100 pmol kg^{-1} ($n = 3$) or 1 nmol kg^{-1} ($n = 8$) was injected 30 min prior to capsaicin and the animals killed 120 min later. $^*P < 0.01$ as compared to vehicle-treated group.

Table 1 Comparison of average cells per section counted in every third section with the weighted average method obtained from eighteen selected sections in the same 10 guinea-pigs (5 NS vehicle and 5 CP-122,288 1 nmol kg^{-1}, i.v.)

Group	*Animal*	*Method I*	*Method II*	
NS	302931	153	193	
NS	302932	284	307	
NS	302935	262	263	
NS	311931	177	262	
NS	316932	152	184	
CP-122 288	302933	64	80	
CP-122 288	302934	102	103	
CP-122 288	311934	97	129	
CP-122 288	316931	94	102	
CP-122 288	316934	143	140	
	NS all cells	*CP all cells*	*NS select*	*CP select*
Mean	206	100	242	111
s.d.	63	29	52	24
s.e.	28	13	23	11
% Reduct.	*All sections*	*Selected sections*		
	51.5%	54.1%		

NS = normal saline; Method I, every third section counted; Method II, weighted average method in which 18 selected sections (6 each from obex, −2.25 and −6.75) were counted.

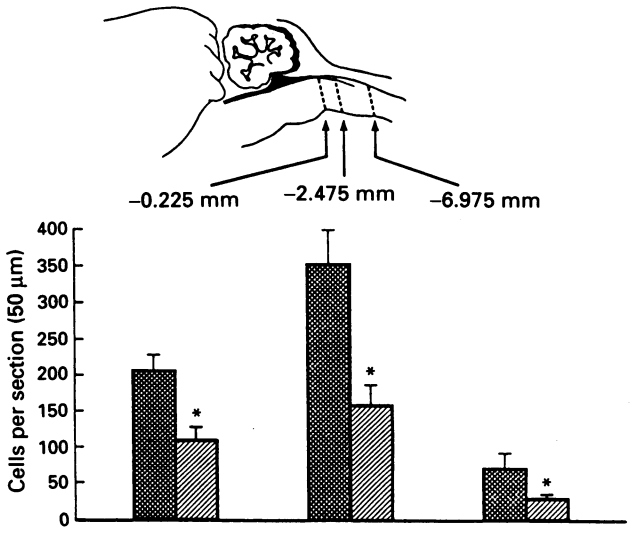

Figure 5 Distribution of c-fos protein-like immunoreactive cells at three different levels within TNC (lamina I, II_o) in animals that received capsaicin injection (0.1 ml, 100 nM) into the cisterna magna 30 min prior to vehicle ($n = 7$) or CP-122,288 (1 nmol kg^{-1}, $n = 8$), and killed 120 min later. Cells were counted in six 50 µm sections sampled every 150 µm at −0.225 (6 sections), −2.475 mm (6 sections) and −6.975 mm (6 sections). $^*P < 0.05$ compared with vehicle-treated animals. Top figure shows a midsagittal section through brain stem and cerebellum. The arrows refer to the brain stem levels from which the analysis was performed.

Discussion

Intracisternal injection of the irritant, capsaicin, evoked c-fos-LI within TNC, particularly lamina I, II_o. The magnitude of expression was greatest −2.475 mm from obex. At more caudal levels, the number of positive cells gradually decreased to amounts comparable to vehicle-injected animals. The rostro-caudal distribution is similar to that previously reported by Nozaki *et al.* (1992a) after intracisternal instillation of autologous blood in the rat. Pretreatment with CP-122,288 ($\geqslant$ 100 pmol kg^{-1}, i.v.) significantly and dose-dependently attenuated the number of positive cells in TNC. The number of stained cells was reduced by a maximum of 59% at 1 nmol kg^{-1}. The reduction was not apparent in AP, MRN, LRN or NTS.

These findings are also consistent with the study of Nozaki *et al.* (1992b) showing that pretreatment with 5-HT_{1B} receptor agonist such as sumatriptan, dihydroergotamine or CP-93,129 significantly attenuated the c-fos response in TNC but not within AP or NTS after intracisternal injection of autologous blood. The 59% reduction after CP-122,288 (1 nmol kg^{-1}, i.v.) is larger than the maximal reduction after pretreatment with sumatriptan (720 nmol kg^{-1}, i.v. × 2) or dihydroergotamine (86 nmol kg^{-1}, i.v. × 2) [31% or 33% respectively], but is smaller than after pretreatment with morphine (15 µmol kg^{-1}, s.c.) [63%]. These reductions approximate the maximum decrease (62% to 67%) reported by others in lamina I, II_o following morphine 10 mg kg^{-1} administration (Presley *et al.*, 1990; Toelle *et al.*, 1990). Although the models used to test sumatriptan and dihydroergotamine are not identical to the capsaicin model described here, the potency of CP-122,288 is remarkably similar.

Cell counting methods

We developed a relatively simple method for estimating the extent of c-fos expression within trigeminal nucleus caudalis. The use of this estimation strategy has several advantages over counting every third section. It allows one to increase the number of animals per treatment group, while also conserving antibody. We sampled six sections at each site because six gave the least variance.

C-fos expression and nociception

It is likely that activation of meningeal afferents is the major source of c-fos LI in TNC. Following the intracisternal instillation of autologous blood or carageenin, c-fos positive cells appear within 1 h, peak at 2 h and decline thereafter (Nozaki *et al.*, 1992a). As in the present study, labelling was found in both the ventral and dorsal aspects of TNC within lamina I, II_o which reflects the termination sites for small unmyelinated C-fibres from all three trigeminal divisions. The destruction of unmyelinated fibres by neonatal capsaicin treatment significantly decreased the number of positive cells as did surgical transection of trigeminal meningeal afferents (Nozaki *et al.*, 1992a). We presume that similar mechanisms are operating after capsaicin instillation.

Capsaicin as a nociceptive stimulus

Capsaicin has been extensively employed as an activating stimulus for c-fos expression (Ceccatelli *et al.*, 1989; Pelto-Huikko *et al.*, 1991). Strassman & Vos showed that injection of capsaicin into the supraorbital region of rats evoked c-fos expression in laminae I, II of the medullary trigeminal nuclear complex (Strassman & Vos, 1993). Capsaicin proved to be a superior irritant to autologous blood when injected into the subarachnoid space. The average number of cells within the TNC after subarachnoid haemorrhage was 37 ± 4 (Nozaki *et al.*, 1992a); whereas in the present study the number of labelled cells after capsaicin was 228 ± 12. Capsaicin specifically activates the predominant population of meningeal nerve fibres which are the small unmyelinated C fibres. The evoked c-fos expression is 20 times that of animals receiving intracisternal vehicle injection (Cutrer *et al.*, 1994). Capsaicin solutions also proved less cumbersome to prepare and inject and unlike blood, its precise composition is known.

CP-122,288

The remarkable potency of CP-122,288 parallels its effect on neurogenic inflammation. CP-122,288 blocks neurogenic plasma protein extravasation within dura mater of guinea-pig with an 1800 fold greater potency than sumatriptan (EC_{50} of 1.66 pmol kg^{-1} versus 3.0 nmol kg^{-1} for CP-122,288 and sumatriptan respectively) following electrical stimulation of the trigeminal ganglion (Lee & Moskowitz, 1993). CP-122,288, like sumatriptan, appears to block neurogenic inflammation via a prejunctional receptor and its effect is reversed partially by metergoline, a non-selective 5-HT_{1D} antagonist. However, the mechanism for its high potency in both the c-fos and plasma extravasation assays is not yet fully understood and is not likely to be accounted for by differences in affinities for the 5-HT_{1D} receptor as determined by autoradiographic ligand binding studies (sumatriptan: 252 ± 40 nM vs CP-122,288: 155 ± 13 nM; EC_{50} to displace 1 nM [^{3}H]-5-CT in guinea-pig substantia nigra; C. Waeber unpublished data). Nevertheless, the existence of the mRNA encoding the 5-$HT_{1D\alpha}$ receptor subtype has recently been identified in trigeminal ganglia using the reverse transcriptase polymerase chain reaction (Rebeck *et al.*, 1993). More than a single receptor subtype or mechanism (perhaps non-5-HT-mediated) may be involved (Lee & Moskowitz, 1993). The development of a potent and selective receptor antagonist would help to clarify the role of the 5-HT_{1D} receptor subtype, as would experiments to determine the affinity of CP-122,288 for the 5-HT_{1F} receptor subtype, which also binds sumatriptan with nanomolar affinity. The high potency of CP 122,288 in this model might also be explained by increased brain penetration. However, information about its lipid partition

coefficient compared to sumatriptan is not at present available. It should be noted that CP 122,288 more potently reduces neurogenic plasma extravasation within the dura mater than sumatriptan (Lee & Moskowitz, 1993), an effect that does not require penetration of the blood brain barrier. We remain uncertain as to the relative importance of central and/or peripheral trigeminovascular receptors as to the effects of CP-122,288 or sumatriptan on the c-fos response following noxious meningeal stimulation. The results are consistent with both or either.

CP-122,288 constricts cat pial vessels with no greater potency than sumatriptan (Wei *et al.*, unpublished data). In fact, topical application of CP-122,288 ($\geqslant 1\ \mu M$) dilated pial vessels (5–10% above baseline). Barring significant species differences or differences due to intravenous versus topical administration, these data strongly suggest that CP-122,288 does not block neurogenic inflammation or c-fos expression within TNC by a mechanism dependent upon vasoconstriction. Modifications at the amino ethyl side chain of sumatriptan may be a useful strategy to enhance the antimigraine properties of 5-HT_{1D} agonists without increasing vasoconstriction.

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5-Hydroxytryptamine (5-HT)$_4$ receptors in *post mortem* human brain tissue: distribution, pharmacology and effects of neurodegenerative diseases

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1 The distribution, pharmacology and effects of neurodegenerative diseases on 5-HT$_4$ receptors in human brain have been characterized *in vitro*.

2 The 5-HT$_4$ receptor in *post mortem* human brain tissue was specifically labelled with [^{3}H]-GR 113808. In human putamen, this ligand labelled a homogeneous population of sites, with an apparent affinity ($-\log K_d$) of 10.1 and a density (B_{max}) of 5.73 fmol mg^{-1} tissue. The pharmacology of this site was characterized by use of a series of displacing ligands, and the following rank order of apparent affinities (with mean ± s.d. $-\log K_i$ values in parentheses) was generated: GR113808 (10.05 ± 0.04)> SDZ 205,557 (8.65 ± 0.08)> DAU 6285 (7.95 ± 0.04)> BIMU-1 (7.81 ± 0.06)> DAU 6215 (7.42 ± 0.23)> tropisetron (7.39 ± 0.23)> 5-HT (7.32 ± 1.00)> BIMU-8 (7.25 ± 0.04)> (**R**)-zacopride (5.82 ± 0.04). The Hill coefficients were not significantly different from unity, consistent with an interaction at a single site. A comparison of the affinities of these compounds with those obtained from guinea-pig striatum indicated no evidence of species differences.

3 The regional distribution of 5-HT$_4$ receptors was assessed by determining the density of binding sites for [^{3}H]-GR 113808. The distribution were as follows (with mean ± s.d. B_{max} values, fmol mg^{-1} tissue, in parentheses): caudate nucleus (8.7 ± 1.5), lateral pallidum (8.6 ± 5.5), putamen (5.7 ± 3.0), medial pallidum (3.8 ± 0.9), temporal cortex (2.6 ± 0.6), hippocampus (2.4 ± 0.8), amygdala (2.3 ± 1.1), frontal cortex (1.7 ± 0.5), cerebellar cortex (< 1.0). In these studies, the affinities of GR 113808 were not significantly different.

4 The density of 5-HT$_4$ receptors selected from regions of *post mortem* brains of patients with Parkinson's disease, Huntington's disease and Alzheimer's disease were compared to age-matched controls. In Parkinson's disease, there was no significant difference between control or patient values (mean ± s.d. B_{max} values, fmol mg^{-1} tissue; putamen, control 4.74 ± 0.07, patient 5.86 ± 1.48; substantia nigra, control 4.21 ± 2.56, patient 5.57 ± 0.10). In Huntington's disease, there was a significant decrease in putamen (control 5.33 ± 1.08, patient 2.68 ± 1.08), while in Alzheimer's disease, there was a marked loss of receptors in hippocampus (control 2.34 ± 0.62, patient 0.78 ± 0.61), in frontal cortex (control, 1.76 ± 0.19, patient 1.30 ± 0.22). Receptor density in temporal cortex showed a decrease, but did not achieve statistical significance (control 2.06 ± 0.21, patient 1.44 ± 0.64).

5 These data suggest a heterogeneous distribution of 5-HT$_4$ receptors in human brain, with high to moderate densities in basal ganglia and limbic structures. These receptors may not be principally co-localized on dopaminergic cell bodies or terminals, given the lack of change observed in Parkinson's disease. The loss of 5-HT$_4$ receptors in the putamen in Huntington's disease raises the possibility of their presence on intrinsic striatal GABAergic or cholinergic neurones. The marked loss of receptors in hippocampal and cortical regions in the brains from patients with Alzheimer's disease is consistent with a role for the 5-HT$_4$ receptor in cognitive processing.

Keywords: Human brain; 5-HT$_4$ receptors; Alzheimer's disease; Parkinson's disease; Huntington's disease; [^{3}H]-GR 113808

Introduction

The 5-hydroxytryptamine (5-HT)$_4$ receptor is a guanine nucleotide binding protein coupled receptor and has been cloned recently from rat. It has seven putative transmembrane spanning domains, and possesses a low homology (< 50%) to other 5-HT receptor subtypes (Gerald *et al.*, 1994). The receptor may exist as a long and a truncated form, each of which, when transfected, augments adenylyl cyclase activity (Gerald *et al.*, 1994). Activation of 5-HT$_4$ receptors in either rat oesophageal muscularis mucosae, mouse colliculi, guinea-pig hippocampus or human frontal cortex results in elevation of adenylyl cyclase activity (Ford *et al.*, 1992; Dumuis *et al.*, 1988; 1989; Eglen *et al.*, 1993; 1994; Monferini *et al.*, 1993). The 5-HT$_4$ receptor is potently stimulated by substituted benzamides or benzimidazalones, such as SC-53116 or BIMU-8 (Flynn *et al.*, 1992; Dumuis *et al.*, 1989), respectively, and antagonized, with subnanomolar affinity, by SB 204070, GR 113808 and GR 125487 (Wardle *et al.*, 1994; Grossman *et al.*, 1993; Gale *et al.*, 1994). [^{3}H]-GR 113808 (Grossman *et al.*, 1993), [^{125}I]-SB 207710 (an analogue of SB 204070; Brown *et al.*, 1993) or [^{3}H]-BIMU-1 (Jakeman *et al.*, 1994) have been used to characterize the pharmacology and distribution of 5-HT$_4$ receptors in mouse, rat, guinea-pig, porcine, bovine, macaque monkey and human brain (Waeber *et al.*, 1993; 1994; Domenech *et al.*, 1994; Jakeman *et al.*, 1994; Schiavi *et al.*, 1994).

The CNS distribution of the receptor is conserved across several species, although some minor differences are apparent between guinea-pig, mouse and rat in the globus pallidus, substantia nigra and interpeduncular nucleus (Waeber *et al.*, 1994). Furthermore, the receptor is found in macaque monkey, but not rat or guinea-pig lateral geniculate nucleus

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thalamus (Waeber *et al.*, 1994; Jakeman *et al.*, 1994). The heterogeneous brain distribution of the receptor implies a specific involvement in CNS function. The localization of the receptor in hippocampus, for example, suggests a role for the receptor in learning and memory (Waeber *et al.*, 1993; 1994; Domenech *et al.*, 1994; Jakeman *et al.*, 1994), while a high density in the nigrostriatal pathway suggests a role in extrapyramidal and motivational behaviour (Jakeman *et al.*, 1994; Domenech *et al.*, 1994). It is possible that regional pathophysiological changes in 5-HT$_4$ receptor density in neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease or Huntington's disease may elucidate their CNS function.

In the present study, the 5-HT$_4$ receptor in human *post mortem* brain tissue obtained from subjects with Alzheimer's, Huntington's and Parkinson's disease have been characterized. In addition, the receptor pharmacology and distribution in normal, age-matched, human brain have been described. The data obtained support previous reports (Waeber *et al.*, 1993; Domenech *et al.*, 1994) that demonstrate a high density of the receptor in human caudate nucleus and intermediate density in hippocampus. A preferential decline of receptor density in hippocampus was seen in patients with Alzheimer's, suggesting an association of the receptor with cholinergic, but not dopaminergic, systems.

Preliminary accounts of these data have been communicated to the British Pharmacological Society (Reynolds *et al.*, 1994a,b) and the third IUPHAR satellite meeting on Serotonin (Wong *et al.*, 1994).

Methods

Radioligand binding studies

Post mortem brain was homogenized in Tris HCl (25 mM) containing 0.5 mM EDTA at pH 7.4, followed by two cycles of centrifugation at 48,000 *g* and resuspension. Guinea-pig striatal homogenates were prepared according to the method of Francis & Burnham (1992). The final homogenate was incubated in Tris buffer at a 400 fold dilution (0.5 mg tissue/assay tube) with [^{3}H]-GR 113808 and competing agents, as appropriate, for 30 min at 21°C in triplicates. Non-specific binding was defined in either the presence of 10 μM tropisetron (ICS 205-930) or 100 μM 5-HT. The incubation was terminated by rapid filtration and washing with ice-cold buffer. The pharmacological specificity of the [^{3}H]-GR 113808-labelled binding sites were assessed by performing displacement studies with 0.05 nM radioligand and appropriate concentrations of inhibitors, using two concentration-ranges per decade. These experiments were performed on putamen samples from at least three different control subjects. In this tissue, the total counts per assay tube were typically between 1800–3000 d.p.m., 95% of which was specific displaceable binding. A single displacement experiment, with most compounds, was also carried out on frontal cortical tissue.

Regional distribution studies were performed by saturation analysis of specific [^{3}H]-GR 113808 binding at seven concentrations over a range of 6.25 pM–0.4 nM. At least three different control subjects were used for these studies. In studies on tissue from subjects with various neurodegenerative disorders, [^{3}H]-GR 113808 binding studies were undertaken at two ligand concentrations, 0.05 and 0.2 nM, due to the shortage of tissue for these studies. The higher concentration therefore related to the 5-HT$_4$ receptor density, while the ratio of binding at the two concentrations provided an estimate of ligand affinity, thereby controlling for artefactual effects of changes in apparent ligand affinity. The mean displaceable binding at 0.2 nM in control putamen was 89% of total.

Determination of the density of muscarinic receptors was performed according to the method of Nordberg *et al.* (1983). Tissues were homogenized in 80 mM sodium/potassium phosphate buffer at pH 7.4, followed by centrifugation at 48,000 *g* and resuspension. The final homogenate was resuspended in phosphate buffer solution at 500 fold dilution with [^{3}H]-quinuclidinyl benzylate (QNB) in a total volume of 0.25 ml for 60 min at 37°C, in triplicate. The incubation was terminated by rapid filtration through GF/B filters (pretreated in 0.05% polyethyleneimine) and washing with ice cold buffer. Non-specific binding was determined in the presence of 10 μM atropine. B_{max} values were determined by saturation analysis employing eight ligand binding concentrations between 0.02 and 2 nM.

Parkinson's, Alzheimer's and Huntington's diseases were confirmed in the patients by standard histopathological diagnostic criteria. Each series of tissue from patients with neurodegenerative disease was compared with tissues from an appropriate age-matched control group.

Analysis of data

Competition binding data were analysed by iterative curve fitting procedures from a four parameter logistic equation. The apparent affinity ($-\log K_i$) values of the competing ligands were calculated from IC_{50} values by the Cheng-Prusoff relationship (Cheng & Prusoff, 1973). Statistically significant differences were assessed by Student's *t* test.

Compounds used

BIMU-1 (endo-N-(8-methyl-8-azabicyclo[3.1.2]oct-3-yl)-2,3-dihydro-3-ethyl-2-oxo-1H-benzimidazole-1-carboxamide); BIMU-8 (endo-N-(8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-2,3-dihydro-3-isopropyl-2-oxo-1H-benzimidazole-1-carboxamide); DAU 6215 (N-(endo-N-(8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-2,3-dihydro-2-oxo-1H-benzimidazole-1-carboxamide hydrochloride); DAU 6285 (endo-6-methoxy-8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-2,3-dihydro-2-oxo-1H-benzimidazole-1-carboxylate hydrochloride); GR 113808 ([1-(2-methane-sulphonamido-ethyl)-piperidin-4-yl]-methyl-indole-3-carboxylate maleate), SDZ 205, 557 (2-methoxy-4-amino-5-chlorobenzoic acid 2-(diethylamino) ethyl ester), tropisetron (ICS 205,930) and (R)-zacopride (4-amino-*N*-(1-azabicyclo[2.2.2]oct-3-yl) 5-chloro-2-methoxybenzamide hydrochloride), were synthesized in the Institute of Organic Chemistry, Syntex Discovery Research, Palo Alto, CA, U.S.A. All remaining compounds were obtained from Sigma Chemical Co., St Louis, MO, U.S.A. [^{3}H]-GR 113808 (87.9 Ci mmol^{-1}) was synthesized in the Institute of Organic Chemistry, Syntex Discovery Research, Palo Alto, CA, U.S.A. [^{3}H]-QNB (quinclidinyl benzylate; 60 Ci mmol^{-1}) was purchased from Dupont New England Nuclear, Boston, MA, U.S.A.

Results

Characterization of 5-HT$_4$ receptors in human brain tissue

The binding of the radioligand [^{3}H]-GR 113808 to various regions of human brain appeared to be saturable and of high affinity (Figure 1). The pharmacological nature of the site was characterized by using a series of displacing ligands (Table 1; Figure 2a,b). All compounds displaced bound ligand with a Hill coefficient not significantly different from unity. The rank order of apparent antagonist affinities was GR 113808> SDZ 205,557> DAU 6285> BIMU-1>DAU 6215> tropisetron> 5-HT> BIMU-8> (R)-zacopride. This rank order was similar to that observed in guinea-pig striatum (Table 1).

The distribution of 5-HT$_4$ receptors in human brain was heterogeneous (Table 2). Thus, high densities were seen in caudate nucleus, lateral pallidum and putamen. Low and

inconsistently detectable levels were seen in cerebellar cortex. Hippocampus, temporal cortex and amygdala expressed intermediate levels of 5-HT_4 receptor density. The apparent affinity of [^{3}H]-GR 113808 was similar throughout all regions studied and the saturation isotherms were consistent with an interaction at a single site (Table 2).

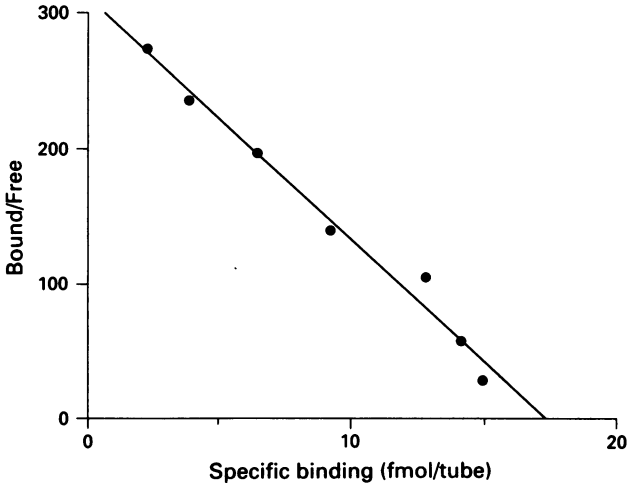

Figure 1 Representative Scatchard analysis of [^{3}H]-GR 113808 binding to membranes from human caudate nucleus. In this experiment the $-\log K_d$ value was 56 pM and the B_{max} was 7.0 fmol mg^{-1} tissue.

Table 1 Pharmacology of 5-HT_4 receptors in human *post mortem* brain tissue and guinea-pig striatum

Ligand	*Putamen*[a] $-\log K_i$	nH	*Cortex*[a] $-\log K_i$	*Striatum*[b] $-\log K_i$
GR 113808	10.05 ± 0.04	1.02 ± 0.12	10.12	10.20 ± 0.07
SDZ 205,557	8.65 ± 0.08	0.96 ± 0.20	8.32	8.63 ± 0.06
DAU 6285	7.95 ± 0.04	0.97 ± 0.06	8.20	7.55 ± 0.11
BIMU-1	7.81 ± 0.06	0.96 ± 0.09	7.75	7.91 ± 0.09
DAU 6215	7.42 ± 0.23	0.87 ± 0.25	7.23	ND
Tropisetron	7.39 ± 0.23	1.08 ± 0.03	7.38 ± 0.21	7.51 ± 0.02
5-HT	7.32 ± 1.00	0.95 ± 0.10	7.46 ± 0.04	7.52 ± 0.06
BIMU-8	7.25 ± 0.04	1.05 ± 0.05	ND	ND
(R)-zacopride	5.82 ± 0.04	1.01 ± 0.12	5.94	6.16 ± 0.02

Values are mean ± s.d., from three subjects, except for the single values obtained from human cortical tissue. The concentration of [^{3}H]-GR 113808 was 0.04–0.06 nM. The $-\log K_i$ values are calculated assuming K_d values of 73 pM for human putamen, 75 pM for human cortex and 30 pM for guinea-pig striatum.
[a]Human; [b]guinea-pig. ND – not determined.

Table 2 Regional density (B_{max}) and apparent affinity (K_d) of [^{3}H]-GR 113808 at 5-HT_4 receptors in human *post mortem* brain

Brain region	B_{max} (fmol mg^{-1} tissue)	K_d (pM)	n
Caudate nucleus	8.7 ± 1.5	68 ± 11	3
Lateral pallidum	8.6 ± 5.5	99 ± 30	8
Putamen	5.7 ± 3.0	94 ± 27	8
Medial pallidum	3.8 ± 0.9	90 ± 41	3
Temporal cortex	2.6 ± 0.6	69 ± 18	3
Hippocampus	2.4 ± 0.8	55 ± 25	3
Amygdala	2.3 ± 1.1	57 ± 25	3
Frontal cortex	1.7 ± 0.5	53 ± 23	3
Cerebellar cortex	< 1.0	ND	3

Values are expressed as mean ± s.d. from *n* subjects. ND, not determined.

Characterization of 5-HT_4 receptor density in neurodegenerative disorders

The levels of binding sites for [^{3}H]-GR 113808 in *post mortem* brains from patients with various degenerative disorders was also evaluated (Table 3). Most striking were decreases in binding of 67, 30 and 26% in hippocampus, temporal cortex and prefrontal cortex, respectively, in confirmed Alzheimer's disease brains. There was no significant deficit observed in the motor frontal cortex. No significant change in muscarinic receptor density, as determined by [^{3}H]-QNB binding, was observed in the same temporal cortex sample from Alzheimer patients reported above (control 56 ± 10; patient 58 ± 12 fmol mg^{-1} tissue).

No deficit in 5-HT_4 receptor binding was seen in putamen and substantia nigra from subjects with confirmed Parkinson's disease. However, a substantial deficit (approximately 50%) in 5-HT_4 receptor binding in putamen was observed in confirmed Huntington's disease patients.

Discussion

The availability of a high affinity and selective radioligand, such as [^{3}H]-GR 113808 (Grossman *et al.*, 1993), has allowed the distribution of 5-HT_4 receptors to be studied in several species. Waeber *et al.* (1993) and Domenech *et al.* (1994) have previously assessed, albeit from a limited number of tissue samples, the 5-HT_4 receptor distribution in human brain. The present study presents a more comprehensive characterization and assesses, for the first time, potential changes in 5-HT_4 receptors in three neurodegenerative disorders.

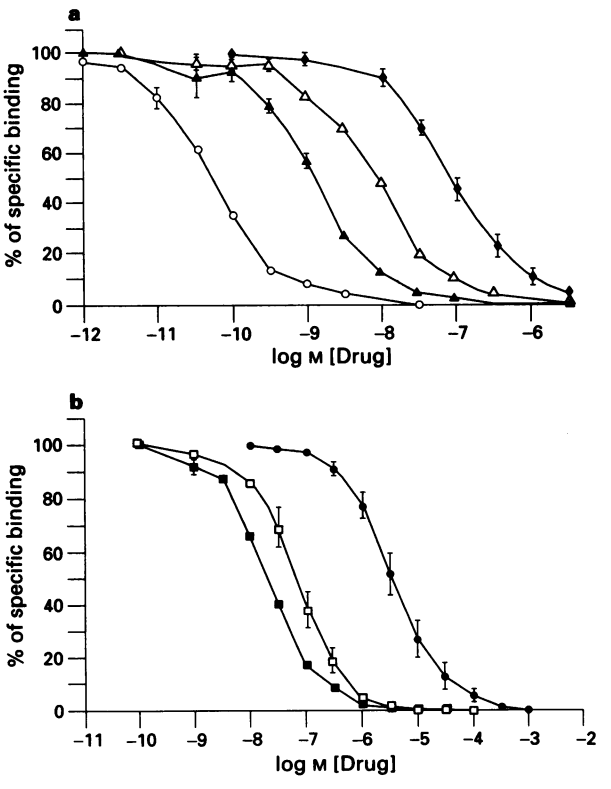

Figure 2 Competition displacement curves of [^{3}H]-GR 113808 binding in membranes from human putamen. The ligands were (a) GR 113808 (○), SDZ 205,557 (▲), BIMU-1 (△), 5-HT (◆) and (b) DAU 6285 (■), tropisetron (□) and (R)-zacopride (●). Values are mean with s.e.mean, from tissues removed, *post mortem*, from at least 3 patients. For abbreviations, see text.

Table 3 Levels of [^{3}H]-GR 113808 binding in *post mortem* brain tissue in various neurodegenerative diseases

Alzheimer's disease	*Binding* (fmol mg^{-1} tissue)	*Ratio*[a]	*Age* (yrs)	*Sex*	Post mortem *delay* (h)
Hippocampus					
Controls	2.34 ± 0.62	0.68 ± 0.25	75 ± 10	4F/4M	28 ± 11
Patients	0.78 ± 0.61***	0.64 ± 0.25	82 ± 8	6F/4M	33 ± 26
Temporal cortex (area 22)					
Controls	2.06 ± 0.21	0.71 ± 0.09	79 ± 5	3F/3M	26 ± 12
Patients	1.44 ± 0.64	0.66 ± 0.17	82 ± 10	3F/3M	25 ± 11
Frontal cortex (area 11)					
Controls	1.76 ± 0.19	0.71 ± 0.06	77 ± 6	3F/3M	23 ± 14
Patients	1.30 ± 0.22**	0.69 ± 0.06	81 ± 9	5F/3M	35 ± 27
Frontal cortex (area 4)					
Controls	1.43 ± 0.25	0.71 ± 0.06	84 ± 3	3F/3M	25 ± 9
Patients	1.18 ± 0.20	0.82 ± 0.10	82 ± 7	3F/2M	22 ± 4
Huntington's disease					
Putamen					
Controls	5.33 ± 1.08	0.60 ± 0.17	55 ± 9	1F/5M	39 ± 22
Patients	2.68 ± 1.08***	0.60 ± 0.06	57 ± 12	3F/3M	42 ± 23
Parkinson's disease					
Putamen					
Controls	4.74 ± 0.07	0.55 ± 0.15	64 ± 13	1F/5M	30 ± 25
Patients	5.86 ± 1.48	0.53 ± 0.10	69 ± 6	2F/4M	32 ± 33
Substantia nigra					
Controls	4.21 ± 2.56	0.57 ± 0.09	68 ± 7	1F/5M	31 ± 25
Patients	5.57 ± 0.10	0.62 ± 0.04	68 ± 6	2F/4M	32 ± 33

Values are mean ± s.d. of specific binding at 0.2 nM [^{3}H]-GR 113808 (fmol mg^{-1} tissue). [a]Ratio equals (specific binding at 0.05 nM)/(specific binding at 0.2 nM). **$P<0.01$, ***$P<0.001$ vs age-matched control group (Student's t test).

The rank order of ligand affinities in human putamen and cortex resembled rank orders observed at 5-HT$_4$ receptors in guinea-pig striatum (Table 1). Similar rank orders have been observed in caudate nucleus membranes from calf and pig (Grossman *et al.*, 1993; Domenech *et al.*, 1994; Schiavi *et al.*, 1994) suggesting limited species variation in the affinity profile of 5-HT$_4$ receptors. However, the absolute affinity values for the antagonists, GR 113808, SDZ 205,557 or DAU 6285, were somewhat higher than those obtained in observed functional studies (Ford & Clarke, 1993). Similar differences with tropisetron and SDZ 205,557 were seen in homogenate binding studies in guinea-pig striatum and hippocampus (Grossman *et al.*, 1993; Jakeman *et al.*, 1994), suggesting that species difference was not the cause. The reason for these discrepancies between functional and binding studies therefore remains unclear. In the case of differences in the apparent affinity of GR 113808, the temperature of the binding assay is an important factor (Waeber *et al.*, 1993; 1994; Wong *et al.*, 1995).

The data obtained in the present study demonstrate a heterogeneous distribution of 5-HT$_4$ receptors in human brain, with the highest densities of receptors in caudate nucleus, pallidum and putamen and intermediate levels in cortex, hippocampus and amygdala. These findings are consistent with those reported in human, rat and primate brain (see Introduction for references). The discrete localization of 5-HT$_4$ receptors, as labelled by [^{3}H]-GR 113808, implies specific CNS functions. In particular, the association of the receptor with septal-hippocampal or nigra-striatal pathways, suggest an involvement in cognitive, emotional and motor function control.

The density of 5-HT$_4$ receptors in regions of human brain, even in caudate nucleus, was low in comparison to the density of 5-HT$_4$ receptors in non-human brain. The effects of agonal state, age or other factors associated with *post mortem* tissue collection and storage may contribute to these low densities. Alternatively, species differences in CNS distribution of 5-HT$_4$ receptors is the most probable reason. For example, in man and guinea-pig, 5-HT$_4$ receptors are localized to the pars reticula of substantia nigra (Waeber *et al.*, 1994), whereas in rat and mouse, they are found only in the pars lateralis. The density of 5-HT$_4$ receptors was also low in comparison to the density of 5-HT$_2$ receptors, although not as low as the density of 5-HT$_3$ receptors (Barnes *et al.*, 1989; Domenech *et al.*, 1994). It is possible that a low density of 5-HT$_4$ receptors reflects a modulatory role of 5-HT$_3$ or 5-HT$_4$ receptors within the CNS (Costall & Naylor, 1994), in comparison to a dominant excitatory or inhibitory role of amino acid or cholinoceptors (Iversen, 1984), that are present within the CNS in higher densities. However, *in lieu* of data regarding the neuronal localization of 5-HT$_4$ receptors, the precise reason for the low density remains speculative.

Insight into 5-HT$_4$ function in these areas can be gained by studying changes in 5-HT$_4$ receptor density in various degenerative disorders. In the present study three such diseases were evaluated, two of which, Huntington's disease and Parkinson's disease, are associated with changes in the nigrostriatal pathway, and a third, Alzheimer's disease, is associated with changes in hippocampal and cortical function. The most striking changes in the present study were seen in the selective loss of hippocampal 5-HT$_4$ receptors in Alzheimer's brain. This reduction was greater than reported for 5-HT$_{1A}$ or 5-HT$_3$ receptors in the same region (Cross *et al.*, 1984; Barnes *et al.*, 1990), although the decrease was similar to that reported for 5-HT$_2$ receptors (see Greenamyre & Maragos, 1993, for review).

Case histories of patients in the present studies indicated that there was no record of the administration of any drug likely to compete for the 5-HT$_4$ receptor. In addition, the absence of any increase in the binding ratio (Table 3) indicated that no significant difference in apparent receptor affinity occurred between neurodegenerative disease and control cases. Therefore, competition of such drugs for the receptor was unlikely to be responsible for the diminished binding in Alzheimer's and other disease tissues. A plausible reason for the reduction in 5-HT$_4$ receptors in Alzheimer's brain is the loss of neurones on which they are expressed. It

is unlikely that these changes reflect a general degeneration of the brain occurring from *post mortem* ischaemia or oxidative insult, since in the temporal cortical area, there was no corresponding change in muscarinic receptors. Moreover, no significant deficit is found in hippocampal [^{3}H]-QNB binding sites in Alzheimer's disease (Nordberg *et al.*, 1983). It is presently unknown upon which neurones the 5-HT$_4$ receptors are located. However, they may reside on cholinergic neurones, given their association with the septal-hippocampal pathway and the well-documented cholinergic deficits in Alzheimer's disease (Katzman, 1989). It is possible, therefore, that the loss of 5-HT$_4$ receptors may provide a marker for degenerating cholinergic terminals, as also suggested for the 5-HT$_2$ receptor (Greenarmyre & Maragos, 1993).

The location of 5-HT$_4$ receptors in hippocampus from several species, including man (see Introduction for references), is also consistent with a role for the receptor in learning and memory (Bockaert *et al.*, 1994a). In support of this hypothesis, several lines of evidence have been reported. Thus, Rowchoudhury & Anderson (1995) have presented preliminary data indicating that activation of 5-HT$_4$ receptors facilitates long term potentiation (LTP) in the CA1 region of rat hippocampus. In mouse colliculi neurones, moreover, 5-HT$_4$ receptor agonism elevates intracellular adenylyl cyclase and, consequently, inhibits voltage-sensitive potassium channels opening time (Dumuis *et al.*, 1989; Andrade & Chaput, 1992; Bockaert *et al.*, 1994a,b). Prolonged closure of potassium channels, and thus neuronal hyperexcitability, was seen, even after very short exposures to 5-HT (Bockaert *et al.*, 1994b). These mechanisms may be involved in the induction of hippocampal CA1 late stage LTP (Frey *et al.*, 1993); a potential mechanism for explicit forms of memory. Finally, RS 66331-190, BIMU-1 or BIMU-8, (potent, but mixed 5-HT$_4$ agonists/5-HT$_3$ antagonists), enhance cognitive performance in rodents (Fontana *et al.*, 1994; Ghelardini *et al.*, 1994).

Huntington's disease is a condition associated with a profound neuronal loss in the basal ganglia, such as the caudate nucleus, putamen and pallidum (Reynolds *et al.*, 1990). In Huntington's disease, there is a decrease in glutamate and GABA levels in the putamen, which may be related to the dementia associated with late stages of the disease (Reynolds *et al.*, 1990). The data obtained in the present study indicate a decrease in 5-HT$_4$ receptors in the putamen, suggesting that these receptors may also be associated with either glutamatergic transmission or intrinsic GABAergic neurones. This suggestion is supported by ontogenic studies of Waeber *et al.* (1994), showing a synchronous, albeit transient, expression of 5-HT$_4$ receptors and glutamatergic innervation in rat globus pallidus. The decrease in putamen 5-HT$_4$ receptors binding may alternatively, or additionally, be due to the presence of receptors on cholinergic interneurones, which also decline in the disease (Forno, 1992; Greenamyre & Maragos, 1993). It is at present unclear, however, whether the reduction in 5-HT$_4$ receptors plays a role in the progression of dementia and/or the appearance of dyskinesias.

The lack of change in the density of binding to 5-HT$_4$ receptors in the basal ganglia areas in patients with confirmed Parkinson's disease indicates that the receptor is not principally expressed on dopaminergic cell bodies or terminals in the substantia nigra or putamen, even though the 5-HT$_4$ receptor is present in high density. Indeed, 6-hydroxydopamine lesions in rat striatum (Jakeman & Fontana, 1994 unpublished observations; Dumuis *et al.*, 1994) further support an absence of the 5-HT$_4$ receptors on dopaminergic neurones.

In conclusion, the distribution and characterization of 5-HT$_4$ receptors in human brain, is well conserved across species. The existence of the receptor in the nigra-striatal pathway is intriguing given the lack of change seen in Parkinson's disease. These data may suggest a dissociation between the 5-HT$_4$ receptor and the dopaminergic system, while the data from Huntington's disease suggest the presence of the receptor on intrinsic striatal GABAergic or cholinergic neurones. The location of the receptor in hippocampus and its decline in terminal Alzheimer's disease highlights a possible association with the cholinergic system and supports the involvement of 5-HT$_4$ receptors in cognition.

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Further investigations into adenosine A_1 receptor-mediated contraction in rat colonic muscularis mucosae and its augmentation by certain alkylxanthine antagonists

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1 The alkylxanthine antagonists, 8-phenyltheophylline (8-PT), 8-*p*-sulphophenyltheophylline (8-SPT) and 1,3,7-trimethylxanthine (caffeine) produced rightward displacements of contractile concentration-effect curves to 5'-N-ethylcarboxamidoadenosine (NECA) in rat isolated colonic muscularis mucosae (RCMM) with concentration-ratios consistent with adenosine receptor blockade. The non-xanthine antagonist, 9 fluro-2-(2-furyl)-5,6-dihydro [1,2,4] triazo to [1,5-c]-quinazin-imine (CGS15943A) also antagonized contractions to NECA with an affinity (pK_B 8.1–8.5) consistent with adenosine A_1 receptor blockade.

2 In addition to producing rightward shifts of the concentration-response curves, the maximum contractions to 5'-N-ethylcarboxamidoadenosine (NECA) were also markedly increased in the presence of 8-PT (by 83 ± 16% at 1 μM), 8-SPT (by 37 ± 7% at 10 μM) and caffeine (by 45 ± 5% at 100 μM) but were unaffected by CGS15943A (at 0.01 and 0.03 μM).

3 As with NECA, the maximum contractions to the adenosine A_1 receptor agonists **R**-phenylisopropyladenosine (**R**-PIA) and N-[(1S, *trans*)-2-hydroxyclopentyl] adenosine (GR79236) were both antagonized and augmented by 8-PT. In addition, the contractions to NECA in the presence of 8-PT (1 μM) were inhibited by nanomolar concentrations of 8-cyclopentyl-1,3-dipropylxanthine (DPCPX).

4 The non-selective phosphodiesterase (PDE) inhibitor, 3-isobutyl-1-methylxanthine (1 μM) produced a marked increase in the NECA maximum without producing a rightward shift in the NECA curve, whereas a higher concentration (10 μM) virtually abolished responses. The PDE type III inhibitor, milrinone (1 μM), the type IV inhibitor, rolipram (10 μM), and the type V PDE inhibitor, zaprinast (3 μM), were all without effect on NECA responses in RCMM.

5 Partial inhibitions of contractions to NECA were produced by indomethacin (at 3 or 10 μM) or piroxicam (at 3 μM). Responses to GR79236 were also partially inhibited by indomethacin. In the presence of indomethacin, 8-PT was still able to enhance markedly the maximum contractions obtained to NECA in RCMM.

6 The present study has shown that certain alkylxanthine antagonists (but not the non-xanthine CGS15943A) produced a marked augmentation of adenosine A_1 receptor-mediated contractions in RCMM. The mechanism of this augmentation is, as yet, not known but is unlikely to result from inhibition of PDE. This study has also shown that adenosine A_1 receptor-induced contractions in RCMM are mediated, in part, via products of the cyclo-oxygenase pathway.

Keywords: Adenosine A_1 receptors; rat colonic muscularis mucosae; contraction; indomethacin

Introduction

Adenosine produces biological effects in many mammalian tissues and, based initially on rank orders of agonist potencies and susceptibility to blockade by certain antagonists and more recently by molecular cloning, adenosine receptors have been characterized into 4 subtypes, namely A_1, A_{2a}, A_{2b} and A_3 (Collis & Hourani, 1993).

We and others (Bailey *et al.*, 1992; Reeves *et al.*, 1993b) have previously reported that adenosine agonists produced contractions in the rat isolated colonic muscularis mucosae (RCMM). Contractions were produced by the non-selective adenosine receptor agonist, 5'-N-ethylcarboxamidoadenosine (NECA); the adenosine A_1-selective agonists, cyclopentyladenosine (CPA), **R**-phenylisopropyladenosine (**R**-PIA) and N[(1S,*trans*)-2-hydroxycyclopentyl]adenosine (GR79236) and by the A_2-selective agonists, metrifudil and 2-[*p*-(2-carboxyethyl) phenylethyl amine)-5'-N-ethylcarboxamido-adenosine (CGS21680). The rank order of agonist potency (CPA ≥ GR79236 = **R**-PIA ≥ NECA > metrifudil > CGS21680; Reeves *et al.*, 1993b) was identical to that obtained for the inhibition of spontaneous rate in rat isolated right atria and the inhibition of lipolysis in rat isolated adipocytes, two adenosine A_1 receptor containing preparations (Gurden *et al.*, 1993). Furthermore, contractions in RCMM were antagonized by nanomolar concentrations of the selective adenosine A_1 receptor antagonist, 8-cyclopentyl-1,3-dipropyl-xanthine (DPCPX, Bruns *et al.*, 1987; Haleen *et al.*, 1987). These data confirmed that the contractions of RCMM induced by adenosine agonists were mediated via adenosine A_1 receptors.

We also found that the non-selective adenosine A_1/A_2 receptor antagonist, 8-phenyltheophylline (8-PT; Collis *et al.*, 1985), not only antagonized concentration-effect curves to NECA but also augmented its maximum response (Reeves *et al.*, 1993b). The purpose of the present study therefore was to investigate further the characteristics of this augmentation by the use of a number of different adenosine receptor agonists and antagonists. In addition, some experiments were performed in an attempt to determine the mechanism of this augmentation. Preliminary accounts of this work have been presented to the British Pharmacological Society (Reeves *et al.*, 1993a; Jarvis *et al.*, 1994).

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Methods

Rat isolated colonic muscularis mucosae (RCMM) were prepared and suspended in Krebs-Henseleit solution at 32°C gassed with 95% O_2/5% CO_2 according to the method previously described by Reeves *et al.* (1993b).

Effects of agonists and antagonists

The RCMM were contracted by a single low concentration of the test agonist. When the peak contractile response had been attained the tissues were washed in fresh Krebs-Henseleit solution and allowed to recover (normally 10 min) before the addition of a higher concentration of agonist. This process was repeated to obtain full concentration-effect curves.

Following the construction of two control concentration-effect curves to the agonist, the test antagonist was added and allowed to equilibrate for 30 min prior to the construction of a third (test) curve to the agonist.

Analysis of results

The contractile responses in RCMM are expressed as a percentage (arithmetic mean ± s.e.mean) of the maximum response obtained in the second (control) concentration-effect curve. EC_{50} values were calculated at the 50% response level for the control (second) concentration-effect curve. Concentration-ratios (CR) were calculated by dividing the EC_{50} of the test curve directly with that of the second control curve. EC_{50} values for agonists are presented as geometric mean values (derived from n experiments), with 95% confidence intervals shown in parentheses. Where appropriate (i.e. where parallel shifts of agonist curves with no effect on the maximum contractions were reproduced), the negative logarithm of the apparent dissociation constant for an antagonist (pK_B) was estimated by calculation of the arithmetic means of the individual result for each concentration of antagonist from the equation: [pK_B = log (concentration-ratio − 1) − log (antagonist concentration)]. pK_B values are presented as the mean value (derived from n experiments), with 95% confidence intervals shown in parentheses.

Drugs

Commercially obtained drugs were: carbachol chloride (BDH), 3-isobutyl-1-methylxanthine (IBMX, Sigma) and 1,3,7 trimethylxanthine (caffeine, Sigma) which were dissolved in distilled water; indomethacin and piroxicam (both Sigma) which were dissolved in 10% $NaHCO_3$ solution; 8-phenyltheophylline (8-PT) and 8-*p*-sulphophenyltheophylline (8-SPT; Research Biochemicals Inc.) which were dissolved in 50 mM NaOH; N^6(R-phenylisopropyladenosine) (R-PIA; Boehringer Mannheim) which was dissolved in 50 mM HCl; and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, Research Biochemicals Inc.) which was dissolved in dimethylsulphoxide.

Drugs synthesized by Glaxo Research and Development were: N-[(1S, trans)-2-hydroxycyclopentyl] adenosine (GR72936), 5'-N-ethylcarboxamidoadenosine (NECA), N-[(2-methylphenyl) methyl] adenosine (metrifudil) and 4-(3'-cyclopentyloxy-4'-methoxyphenyl)-2-pyrrolidine (rolipram) which were dissolved in 50 mM HCl; 1,6-dihydro-2-methyl-6-oxo-(3,4-bipyridine)-5-carbonitrile (milrinone) and 2-O-propoxyphenyl-8-azapurine-6-one (zaprinast) which were dissolved in 50 mM NaOH; and 9-fluro-2-(2-furyl)-5, 6-dihydro [1,2,4] triazolo [1,5-c] quinazin-5-imine (CGS15943A) which was dissolved in dimethylsulphoxide.

Results

Effect of adenosine antagonists on adenosine receptor agonist-induced contractions in RCMM

Following the construction of an initial contractile concentration-effect curve to NECA (0.01–10 μM) in RCMM, second and third curves to NECA were reproducible with mean EC_{50} values of 0.18 (0.14–0.23) and 0.14 (0.11–0.16) μM, and maximum contractions (at 3 μM) of 0.35 ± 0.04 and 0.37 ± 0.05 g (n = 12) respectively.

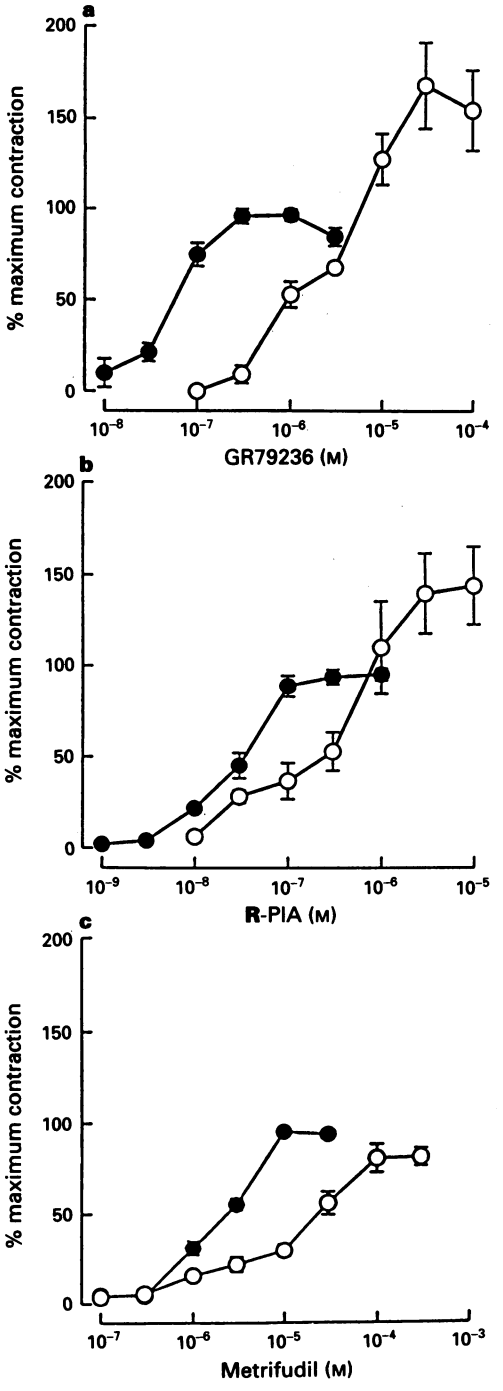

Figure 1 The effect of 8-phenyltheophylline (8-PT) (1.0 μM) on concentration-effect curves to GR79236, R-phenylisopropyladenosine (R-PIA) or metrifudil in rat isolated colonic muscularis mucosae: (a) (●) GR79236 control and (○) GR79236 + 8-PT (n = 3); (b) (●) R-PIA control and (○) R-PIA + 8-PT (n = 5); and (c) (●) metrifudil control and (○) metrifudil + 8-PT (n = 6). Each point represents the mean (± s.e.mean) calculated as a percentage of the control agonist maximum.

We have previously shown that the non-selective A_1/A antagonist, 8-PT, produced both concentration-dependent rightward shifts of the NECA concentration-effect curve and also produced marked increases in the maximum contraction to NECA (Reeves *et al.*, 1993b). Qualitatively similar effects were seen with GR79236 and R-PIA in the present study (Figure 1). 8-PT (1 μM) produced rightward shifts in the concentration-effect curves to both GR79236 [CR = 18.4 (5.4–62.9), $n = 3$] and R-PIA [CR = 7.4(2.7–20.5), $n = 5$] and increased the maximum contraction by 67 ± 23% ($P < 0.05$) and 44 ± 22% ($P < 0.05$), respectively. In contrast, the concentration-effect curve to metrifudil, a weak partial agonist in RCMM (Reeves *et al.*, 1993b), was shifted to the right [CR = 8.0(3.7–24.0), $n = 6$] by 8-PT (1 μM) without increasing the maximum contraction.

The effects of 8-PT on NECA-induced contractions in RCMM were mimicked by 8-SPT and caffeine (Figure 2). 8-SPT (10 μM) and caffeine (100 μM) produced CR values of 8.0(5.4–11.9) and 2.8(1.5–5.1) as antagonists of NECA and increased the NECA maximum by 37 ± 7% ($P < 0.05$, $n = 5$) and 45 ± 5% ($P < 0.05$, $n = 3$), respectively.

In contrast, to the above antagonists, CGS15943A produced concentration-dependent rightward shifts of NECA concentration-effect curves in RCMM without increasing the maximum contraction to NECA (Figure 3). From these data, pK_B values of 8.1 (6.9–9.3) and 8.5 (8.0–9.1) were calculated for CGS15943A at 0.01 μM and 0.03 μM respectively.

Effect of DPCPX on contractions to NECA plus 8-PT in RCMM

The effect of DPCPX on contractions to NECA in the presence of 8-PT (1 μM) in RCMM are shown in Figure 4. DPCPX produced concentration-dependent rightward shifts of the NECA responses with CR values of 2.0 (1.2–3.2) and 6.0 (2.8–16.3) at 3 nM ($n = 6$) and 10 nM ($n = 5$) respectively. In addition, the higher concentration of DPCPX (10 nM) produced a 46 ± 2% reduction in the NECA maxima in the presence of 8-PT.

Effect of phosphodiesterase inhibitors on NECA-induced contractions in RCMM

The phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX; 1 μM) produced an enhancement of the NECA maximum contractile response of 50 ± 13% ($P < 0.05$, $n = 6$) without producing a rightward shift of the concentration-effect curve (Figure 5a). However, a higher concentration of IBMX (10 μM) produced a rapid loss of basal tone in RCMM and virtually abolished contractions to NECA. In contrast, three other phosphodiesterase inhibitors, zaprinast (3 μM), milrinone (1 μM) and rolipram (10 μM) had no effect on the concentration-effect curves to NECA (Figure 5b).

Effect of cyclo-oxygenase inhibitors on contractions to adenosine receptor agonists in RCMM

Indomethacin (3 μM) markedly inhibited contractions to NECA in RCMM (Figure 6a) and reduced the maximum response by 35 ± 6% ($n = 8$). However, a higher concentration of indomethacin (10 μM, $n = 4$) produced no further inhibition. Indomethacin (3 μM) also produced marked reductions in the maximum contractions to GR79236 (by 29 ± 7%, $n = 5$). The effects of indomethacin on contractile responses to NECA were mimicked by piroxicam (3 μM), which produced a 38 ± 4% reduction in the NECA maxima.

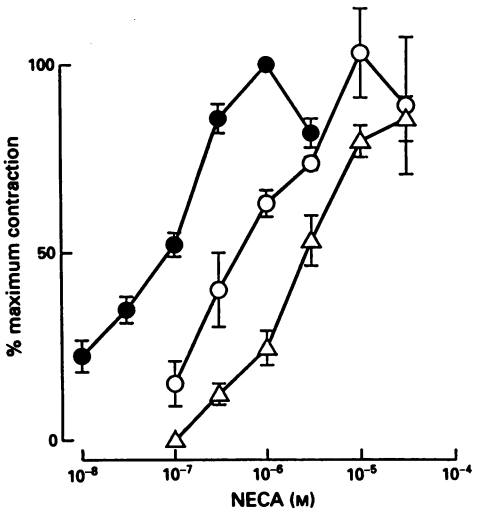

Figure 3 The effect of CGS15943A on concentration-effect curves to 5'N-ethylcarboxamidoadenosine (NECA) in rat isolated colonic muscularis mucosae: (●) control NECA, $n = 7$; (○) NECA + CGS15943A (0.03 μM, $n = 3$); and (△) NECA + CGS15943A (0.1 μM, $n = 4$). Each point represents the mean (± s.e.mean) calculated as a percentage of the control NECA maximum.

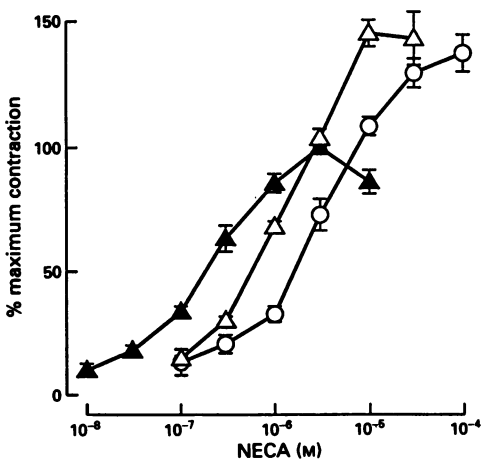

Figure 2 The effect of 8-*p*-sulphophenyltheophylline (8-SPT) or caffeine on concentration-effect curves to 5'N-ethylcarboxamidoadenosine (NECA) in rat isolated colonic muscularis mucosae: (▲) control NECA, $n = 8$; (○) NECA + 8-SPT (10 μM, $n = 5$); and (△) NECA + caffeine (100 μM, $n = 3$). Each point represents the mean (± s.e.mean) calculated as a percentage of the control NECA maximum.

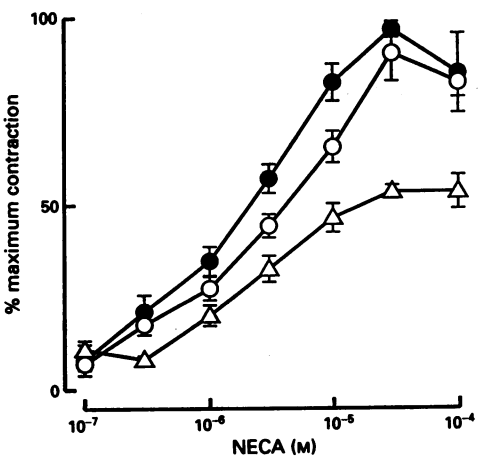

Figure 4 The effect of 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) on contraction to 5'-N-ethylcarboxamidoadenosine (NECA) in the presence of 8-phenyltheophylline (8-PT, 1 μM) in rat isolated colonic muscularis mucosae: (●) NECA + 8-PT, $n = 6$; (○) NECA + 8-PT + DPCPX (3 nM, $n = 6$) and (△) NECA + 8-PT + DPCPX (10 nM, $n = 5$). Each point is the mean (± s.e.mean) calculated as a percentage of the NECA + 8-PT maximum.

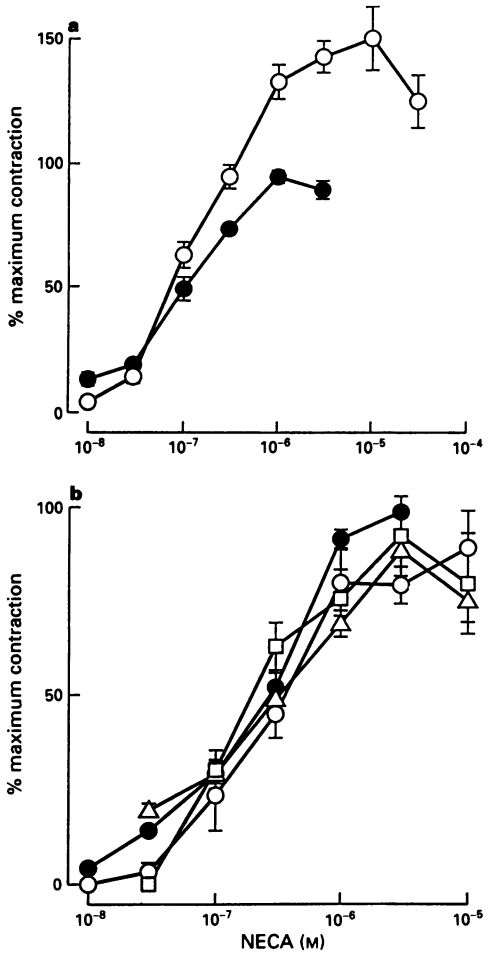

Figure 5 The effect of phosphodiesterase inhibitors on concentration-effect curves to 5'-N-ethylcarboxamidoadenosine (NECA) in rat isolated colonic muscularis mucosae: (a) (●) control NECA and (○) NECA + 3-isobutyl-1-methylxanthine (1 μM, $n = 6$); and (b) (●) control NECA, $n = 14$; (○) NECA + zaprinast, (3 μM, $n = 6$); (△) NECA + milrinone (1 μM, $n = 4$); and (□) NECA + rolipram (10 μM, $n = 4$). Each point represents the mean (± s.e.mean) calculated as a percentage of the control NECA maximum.

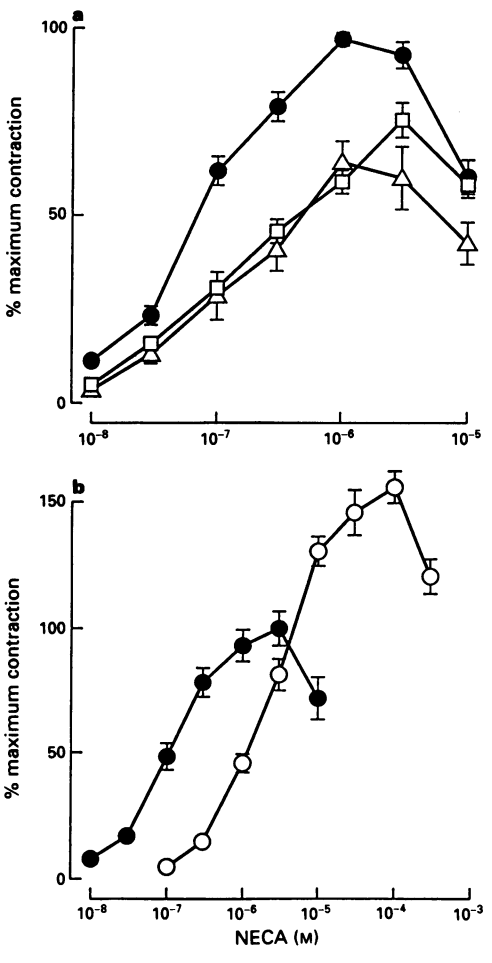

Figure 6 The effect of indomethacin or indomethacin plus 8-phenyltheophylline (8-PT, 1 μM), on concentration-effect curves to 5'-N-ethylcarboxamidoadenosine (NECA) in rat isolated colonic muscularis mucosae: (a) (●) control NECA, $n = 12$; (□) NECA + indomethacin (3 μM, $n = 8$); and (△) NECA + indomethacin (10 μM, $n = 4$); (b) (●) control NECA and (○) NECA + 8-PT ($n = 10$) each in the presence of indomethacin (3 μM). Each point is the mean (± s.e.mean) calculated as a percentage of the control NECA maximum.

In the presence of indomethacin (3 μM), 8-PT (1 μM) still produced both a rightward shift [CR = 19.9 (14.2–27.8)] and increased the maximum contraction to NECA by 55 ± 7% ($n = 7$) in RCMM (Figure 6b).

The augmentation of contractions seen in this study was specific for adenosine receptor stimulation in that none of the antagonists affected contractions to carbachol in RCMM.

Table 1 Augmentation of 5'-N-ethylcarboxamidoadenosine (NECA) responses in the presence of 8-phenyltheophylline (8-PT) in rat colonic muscularis mucosae (RCMM)

8-PT concentration (μM)	n	*% increase in NECA maximum*
0.1	5	36.0 ± 9.4 ($P<0.05$)
0.3	6	71.0 ± 7.6 ($P<0.05$)
1.0	4	83.3 ± 15.6 ($P<0.01$)
3.0	5	99.7 ± 12.3 ($P<0.01$)

Mean (± s.e.mean) increases in the maximum contractions to NECA following 8-PT calculated from previously reported data (Reeves *et al.*, 1993b). Statistical significance (P value) was calculated by comparison of control maxima using Student's paired t test, n = number of experiments. In separate experiments, 8-PT (0.01–100 μM) produced no contractions in RCMM in the absence of NECA.

Discussion

The present study has extended our previous observation that 8-PT not only antagonizes but also augments adenosine A_1 receptor-mediated contractions in rat colonic muscularis mucosae (RCMM; Reeves *et al.*, 1993a,b; Jarvis *et al.*, 1994). We have previously reported that 8-PT produced concentration-dependent rightward shifts of concentration-effect curves to NECA (Reeves *et al.*, 1993b) with CR values consistent with adenosine receptor blockade (i.e. pK_B 6.4–6.6; Collis *et al.*, 1985; Gurden *et al.*, 1993). In addition, we found that 8-PT produced significant and concentration-dependent increases in the maximum contraction to NECA (see Table 1). One possible explanation for this augmentation was that RCMM contains adenosine receptors mediating both contraction (A_1) and relaxation (A_2) and that 8-PT has higher affinity for the relaxant receptor allowing the contractile component to predominate. However, we (Reeves *et al.*, 1993b) were unable to show a relaxant effect to the non-selective adenosine receptor agonist NECA or the A_{2a} agonist, CGS21680 (Hutchinson *et al.*, 1989) in RCMM pre-contracted with carbachol in the presence of the

adenosine A_1 receptor antagonist DPCPX (Bruns *et al.*, 1983; Haleen *et al.*, 1983). Therefore, we could find no evidence for adenosine receptors mediating relaxation in RCMM.

It was observed, both previously (Reeves *et al.*, 1993b) and in the present study, that supramaximal concentrations of NECA produced consistently smaller contractions than maximal concentrations in RCMM. Although the mechanism of this apparently bell-shaped activity of NECA is unknown, the present study and earlier studies (Reeves *et al.*, 1993a,b) have shown that it is shifted to the right in a concentration-dependent manner by both 8-PT and DPCPX, suggesting that it is a phenomenon of adenosine receptor stimulation.

The present study has shown that the augmented contractions to NECA in the presence of 8-PT in RCMM were antagonized by nanomolar concentrations of DPCPX. However, in contrast to the enhanced responses observed following 8-PT, DPCPX produced a marked reduction in the maximum contractions to NECA in RCMM under these conditions. This effect is consistent with earlier observations using DPCPX in this preparation (Bailey *et al.*, 1992; Reeves *et al.*, 1993b), and is possibly the consequence of the relatively slow dissociation rate of DPCPX from its receptor (Bruns *et al.*, 1987). In addition to the augmentation of NECA maximum by 8-PT and its inhibition by DPCPX, the present study has shown that 8-PT also augmented contractions to the selective adenosine A_1 receptor agonist, GR79236 (Gurden *et al.*, 1993) in RCMM. These data suggest that, whatever the mechanism, augmentation is dependent on adenosine A_1 receptor stimulation.

It was of interest that the degree of augmentation of maximum contractions by 8-PT in RCMM was apparently agonist-dependent. For example, contractions to the A_1 receptor agonist, **R**-PIA (Smellie *et al.*, 1979; Gurden *et al.*, 1993) were augmented to a lesser extent than those obtained to NECA or GR79236, whereas responses to metrifudil were not augmented at all by 8-PT.

To investigate the augmentation of adenosine A_1 receptor-mediated contraction in RCMM further, we determined the effects of a number of different adenosine receptor antagonists. As with 8-PT, both 8-SPT and caffeine also produced rightward shifts of NECA concentration-effect curves (with CR values consistent with adenosine receptor blockade) and augmentations of the maximum contractions observed. In contrast, the non-xanthine adenosine receptor antagonist CGS15943A (Ghai *et al.*, 1987) produced concentration-dependent rightward shifts of the NECA responses, but had no effect on the maximum contractions to NECA. CGS15943A is an adenosine receptor antagonist with some degree of selectivity for adenosine A_{2a} receptors (Ghai *et al.*, 1987; Rollins *et al.*, 1994), the pK_B values (8.1–8.5) obtained for CGS15943A in the present study are generally consistent with A_1 receptor blockade. It is known that certain alkylxanthine antagonists such as 8-PT, not only inhibit adenosine receptors but also inhibit phosphodiesterase (PDE, Smellie *et al.*, 1979). However, no known PDE inhibitory properties are associated with CGS15943A (Williams *et al.*, 1987). Therefore, the possibility that augmentation of contractions in RCMM was in some way related to PDE inhibition was explored.

A low concentration (1 μM) of the non-selective PDE inhibitor IBMX did produce a significant increase in the maximum response to NECA. However, at concentrations expected to cause inhibition of PDE, the PDE III inhibitor, milrinone, the PDE IV inhibitor, rolipram, and the PDE V inhibitor, zaprinast (see Torphy & Undem, 1991; Ukena *et al.*, 1993) were all without effect on contractions to NECA suggesting that these isozymes of PDE are not responsible for the augmentation of contractions seen in RCMM. At present, there are no PDE I- and PDE II-selective inhibitors available so inhibition of one or both of these isozymes may be involved in mediating augmentation of contraction in RCMM. However, augmentation occurred with 8-PT at concentrations too low for PDE inhibition (Smellie *et al.*, 1979; Ukena *et al.*, 1993) and a concentration of IBMX (10 μM) expected to inhibit PDE I and II (Ukena *et al.*, 1993) almost abolished NECA-induced contraction in RCMM. This inhibition is not surprising, since PDE inhibition would result in an increase in intracellular cyclic AMP levels which would be expected to reduce, rather than enhance, smooth muscle contraction. It is therefore unlikely that augmentation is the result of PDE inhibition, although the discovery of selective PDE I and PDE II inhibitors is needed to confirm this.

It has been reported that adenosine A_1 receptor-mediated contractions of guinea-pig myometrium results from production of prostaglandins (Schiemann *et al.*, 1991). Similarly, the present study has shown that contractions to NECA in RCMM were partially inhibited by indomethacin, an effect mimicked by the more selective cyclo-oxygenase inhibitor, piroxicam (Carty *et al.*, 1980). Contractions to the A_1 receptor agonist GR79236, were also partially inhibited by indomethacin. These data suggest that adenosine A_1 receptor-mediated contractions in RCMM are also dependent, in part, on the production of prostanoids. However, it is unlikely that an increase in prostanoid production is responsible for the augmentation of contractions following xanthine antagonists in RCMM since marked enhancements of NECA contractions by 8-PT were also seen in the presence of indomethacin.

In conclusion, the present study has shown that certain alkylxanthine adenosine receptor antagonists both inhibit and augment adenosine A_1 receptor-mediated contractions in RCMM. The exact mechanism of this augmentation is not known but is unlikely to be the result of phosphodiesterase inhibition. In addition, this study has shown that adenosine A_1-induced contractions in RCMM are mediated, in part, via products of the cyclo-oxygenase pathway.

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Involvement of B_1 and B_2 receptors in bradykinin-induced rat paw oedema

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1 The mechanisms involved in bradykinin (BK)-induced oedema in the rat paw as well as the interactions between BK and several inflammatory mediators, have been investigated.

2 Intraplantar injection of BK (1 nmol/paw) in rats pretreated with captopril (5 mg kg^{-1}, s.c.) caused a small amount of oedema formation (0.17 ± 0.05 ml). Des-Arg^9-BK (DABK, a selective B_1 receptor agonist) up to 300 nmol/paw caused minimal oedema (0.03 ± 0.01 ml).

3 Co-administration of prostaglandin E_2 (PGE_2), prostaglandin I_2 (PGI_2), calcitonin gene-related peptide (CGRP), 5-hydroxytryptamine (5-HT), substance P (SP) or platelet activating factor (PAF) (1 pmol–1 nmol/paw) with BK (1 nmol/paw) dose-dependently potentiated BK-induced paw oedema. The rank order of potency (mean ED_{50}, pmol/paw) for this effect was: SP (8.1) > PAF (13.7) > PGI_2 (20.5) > 5-HT (23.8) > CGRP (25.7) > PGE_2 (52.0). Co-administration of BK with the various inflammatory mediators resulted in maximal paw oedemas (ml) of: PGE_2 (0.71 ± 0.02); PGI_2 (0.66 ± 0.02); 5-HT (0.65 ± 0.01); SP (0.63 ± 0.05); CGRP (0.60 ± 0.05) and PAF (0.47 ± 0.02) ml. Histamine (up to 1 nmol/paw) was ineffective in potentiating the response to BK.

4 Hoe 140 or NPC 17731 (two selective B_2 receptor antagonists, 0.1–3 nmol/paw) produced dose-dependent inhibition of paw oedema potentiation induced by co-injection of BK with other mediators with the following mean ID_{50}s (nmol/paw): Hoe 140–1.4; 1.3; 1.5 and 1.1 and NPC 17731–1.0; 1.0; 0.9 and 0.7; in the presence of PGE_2, PGI_2, CGRP and SP, respectively. The selective B_1 receptor antagonist des-Arg^9 [Leu^8]-BK (DALBK, up to 300 nmol/paw) had no effect.

5 Daily intraplantar injections of BK (10 nmol/paw) once a day for 7 consecutive days caused a progressive and complete desensitization of the paw oedema, which was specific for BK, since paw oedema induced by PAF, PGE_2, SP or histamine was not affected. In addition, the oedema caused by BK in the paw desensitized to the peptide was almost completely reversed if BK was co-injected with PGE_2, PGI_2 or SP (1 nmol/paw). Injection of PGE_2 or SP (10 nmol/paw) together with the first BK injection (10 nmol/paw), partially prevented BK-induced desensitization.

6 When animals were completely desensitized to BK, DABK (100 nmol/paw) caused paw oedema (0.25 ± 0.03 ml) which was consistently blocked by the B_1 receptor antagonist, DALBK (100 nmol/paw).

7 Treatment of animals with dexamethasone (0.5 mg kg^{-1}, s.c., 24 h previously) antagonized paw oedema induced by DABK (100 nmol/paw) in desensitized paws, but not that induced by BK (3 nmol/paw) in naive paws. The steroid also prevented the recovery of oedema seen after co-injection of BK with PGE_2 or PGI_2 (1 nmol/paw) in desensitized paws.

8 These results suggest that both B_1 and B_2 receptors are involved in BK-induced rat paw oedema. The B_2 receptors are constitutive, but induction of expression of B_1 receptors seems to occur only after complete desensitization of the paw to BK. In addition, very low doses of inflammatory mediators markedly potentiate BK-induced paw oedema and can attenuate BK-induced paw oedema desensitization. Such mechanisms may be relevant for the manifestation of acute and chronic inflammatory processes.

Keywords: Paw oedema (rat); bradykinin; des-Arg^9-bradykinin; inflammatory mediators; synergism; desensitization; B_1 and B_2 kinin antagonists

Introduction

Kinins, including bradykinin (BK) and kallidin (lys-BK) are generated in plasma and in a variety of peripheral tissues in response to tissue injury or infection by the action of kallikreins on low molecular weight kininogen precursors. Kinins are recognized as potent vasoactive peptides which promote venular dilatation, increased vascular permeability, enhanced fluid secretion from epithelia and produced pain and hyperalgesia (Lewis, 1970; Garcia Leme, 1978; Marceau *et al.*, 1983; Proud & Kaplan, 1988; Steranka & Burch, 1991; Dray & Perkins, 1993). In addition, inflammation is associated with increased levels of BK and its metabolites des-Arg^9-BK and des-Arg^{10}-Lys-BK (Regoli & Barabé, 1980; Hargreaves *et al.* 1988; Damas *et al.*, 1990).

The actions of kinins are mediated by activation of two types of specific membrane receptors, denoted B_1 and B_2. The expression of B_1 kinin receptors is usually restricted to some rabbit blood vessels and nonvascular smooth muscle tissues. Importantly, this type of receptor exhibits higher affinity for the kinin metabolites, des-Arg^9-BK and des-Arg^{10}-Lys-BK and can be selectively and competitively antagonized by the B_1 receptor antagonists, des-Arg^9[Leu^8]-BK or des-Arg^{10}[Leu^8]-Lys-BK. In contrast, B_2 kinin receptors are widely distributed both in the peripheral and central nervous systems and show high affinity for BK and Lys-BK, being selectively and competitively antagonized by several potent and selective B_2 receptor antagonists, including among others, Hoe 140 and NPC 17731. While B_2 receptors are constitutive and mediate the majority of kinin actions, B_1 receptors are expressed following *in vitro* incubation for long

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periods or after tissue trauma or infection (Regoli & Barabé, 1980; Burch & DeHaas, 1990; Bathon & Proud, 1991; Marceau & Regoli, 1991; Bhoola *et al.*, 1992; Burch *et al.*, 1993).

It has been demonstrated that most of the actions of kinins, including their pro-inflammatory and algesic properties, are indirectly mediated by production and release of pro-inflammatory mediators derived from the arachidonic acid pathway, especially prostaglandin E_2 (PGE_2) and prostaglandin I_2 (PGI_2), as well as histamine and 5-hydroxytryptamine (5-HT) following mast cell degranulation (Gaginella & Kachur, 1989; Hall, 1992; Burch *et al.*, 1993). In addition, kinins may also stimulate visceral sensory neurones to release pro-inflammatory neuropeptides such as substance P (SP), neurokinin A or calcitonin gene-related-peptide (CGRP) (Bhoola *et al.*, 1992; for review see: Farmer & Burch, 1992; Geppetti, 1993). Therefore, a large part of the pro-inflammatory and algesic actions of kinins is most likely to be due to synergistic actions between these mediators (Brain & Williams, 1989; Buckley *et al.*, 1991; Cruwys *et al.*, 1992; Warren *et al.*, 1993).

The purpose of the present study was to analyse, by use of selective BK receptor agonists and antagonists, the mechanisms involved in BK-induced oedema in the rat paw. In addition, we have also investigated the synergism between BK and co-administration of low doses of several inflammatory mediators.

Methods

Measurement of rat paw oedema

Experiments were conducted on non-fasted male Wistar rats (140–200 g) kept in a room controlled for temperature (22 ± 2°C) and illumination (12 h on and 12 h off). All animals were pretreated with captopril (5 mg kg^{-1}, s.c.) 1 h prior to any given experiment to prevent BK degradation (Corrêa & Calixto, 1993). Under ether anaesthesia animals received 0.1 ml intraplantar injections in one hindpaw of phosphate buffered saline (PBS; composition mmol l^{-1}: NaCl 137, KCl 2.7 and phosphate buffer 10) containing BK (1 nmol/paw) either alone or mixed with PGE_2, PGI_2, CGRP, SP, 5-HT, PAF or histamine (1 pmol/paw to 1 nmol/paw). The contralateral paw received 0.1 ml PBS and was used as a control. Oedema was measured by use of a plethysmometer (Ugo Basile) at several time points (10, 20, 30, 60 and 120 min) or only at the peak (20 min) after injection of inflammatory mediators. Oedema has been expressed in ml as the difference between the test and control paws.

Influence of bradykinin receptor antagonists

In a separate series of experiments in order to assess whether the oedema resulting from co-injection of BK and other inflammatory mediators involved activation of B_1 or B_2

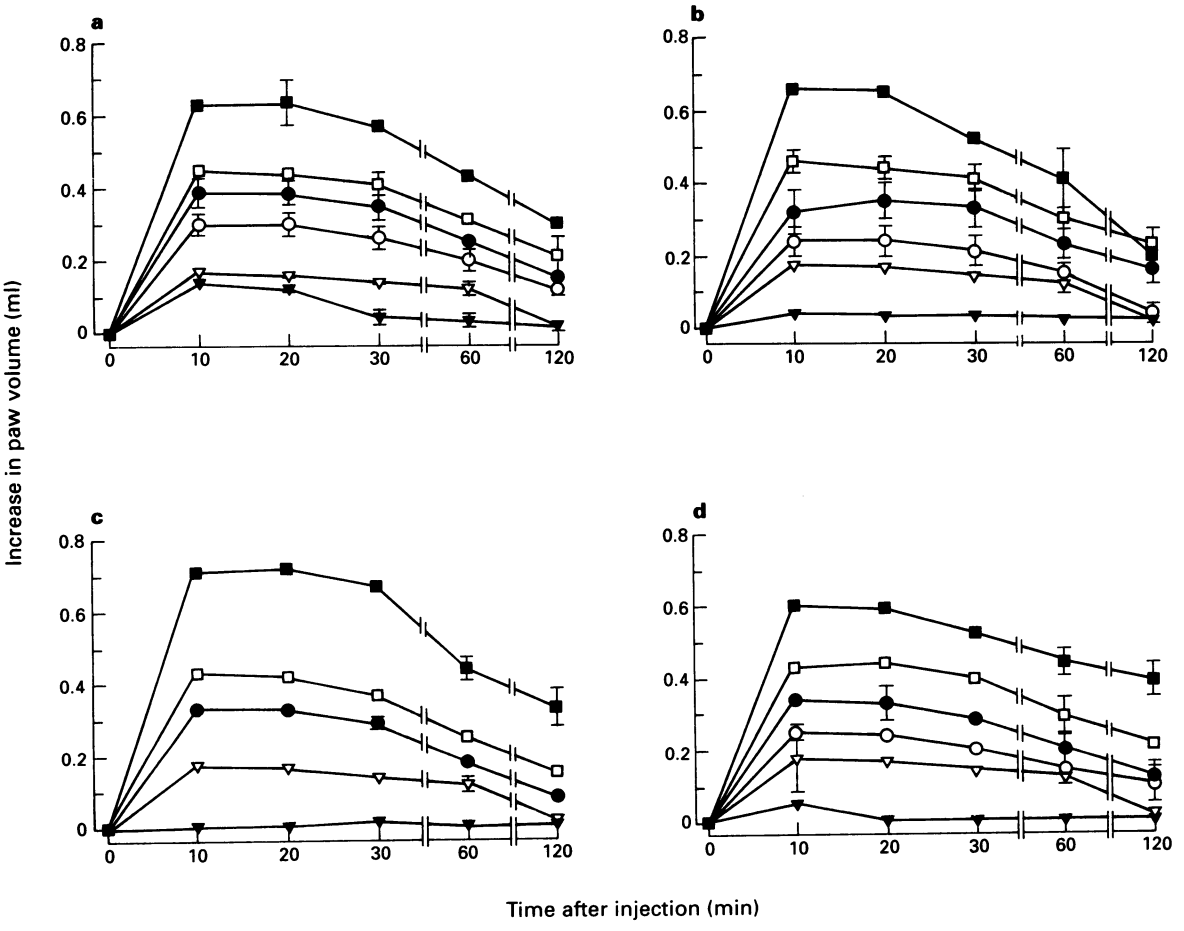

Figure 1 Effect of intraplantar injection of bradykinin (BK), given alone or in combination with other inflammatory mediators, on rat hindpaw volume. Values shown represent the differences between volumes (in ml) or vehicle-treated (0.1 ml of PBS solution) and drug-injected paws. BK was injected either alone (▽, 1 nmol/paw in all panels), or in combination with 1(○), 10 (●), 100 (□), and 1000 (■) pmol/paw of substance P (a), prostaglandin I_2 (PGI_2) (b), PGE_2 (c) or calcitonin gene-related peptide (d). The effects of each mediator alone are also shown in their respective panels (▼, 1 nmol/paw). Each point represents the mean ± s.e.mean of 5 to 6 animals pretreated with captopril (5 mg kg^{-1}, s.c.). In some cases the error bars are hidden within the symbols.

receptors, animals received intraplantar injections of BK (1 nmol/paw) together with the inflammatory mediators in the absence (control group) or in the presence of the selective B_1 receptor antagonist, des-Arg^9[Leu^8]-BK (DALBK) or the B_2 selective receptor antagonists, Hoe 140 or NPC 17731 (0.1 to 3 nmol/paw).

Desensitization to bradykinin

In a further series of experiments, in order to assess the specificity of the synergism between BK and the inflammatory mediators and the possible mechanisms underlying this effect, animals were desensitized with single daily intraplantar injections of BK (10 nmol/paw) for 7 consecutive days. Paw oedema was measured as described above. The contralateral paw received only PBS and was used as a control. In another group of experiments, animals which were completely desensitized to BK (by day 7), received an intraplantar injection of BK (1 nmol/paw) together with PGE_2, PGI_2 or SP (1 nmol/paw).

The possible occurrence of cross-desensitization was also evaluated by intraplantar injection of PGE_2 (10 nmol/paw), SP (3 nmol/paw), PAF (10 nmol/paw) or histamine (100 nmol/paw) in naive or in BK desensitized paws. The doses of mediators were chosen because they were equieffective in increasing the response to BK alone. In other experiments, in order to evaluate the effect of inflammatory mediators on desensitization of BK-induced oedema, animals received an intraplantar injection of BK (10 nmol/paw) together with SP (10 nmol/paw) or PGE_2 (10 nmol/paw) on the first day, but BK (10 nmol/paw) alone was injected once daily throughout days 2 to 7. Other groups of animals which were completely desensitized with BK were treated with dexamethasone (0.5 mg kg^{-1}) 24 h previously or DABK (100 nmol/paw). Control animals received only BK (10 nmol/paw) from day 1 to 7 or PBS solution.

Drugs

The following drugs were used: BK, PGE_2, iloprost, (a stable analogue of PGI_2), CGRP, SP, 5-HT, histamine, captopril, dexamethasone (all from Sigma Chemical Company, St. Louis, U.S.A.), des-Arg^9[Leu^8]-BK (Peninsula Belmont, CA, U.S.A.), PAF (Bachem, Switzerland), Hoe 140 (D-Arg-[Hyp^3, Thi^5, D-Tic^7, Oic^8]-BK) and NPC 17731 (D-Arg^0-Arg^1-Pro^2-Hyp^3-Gly^4-Phe^5-Ser^6-[D-Hype^3 (transpropyl)^7]-Oic^8-Arg^9)h, were kindly supplied by the Department of Pharma Synthesis, Hoechst (Frankfurt Main, Germany) and by SCIOS/NOVA (Baltimore, U.S.A.), respectively. The stock solutions for all peptides used were prepared in PBS (1–10 mM) in siliconized plastic tubes and were kept at −18°C, and diluted to the desired concentration just before use. The other drugs were prepared daily in 0.9% w/v NaCl.

Statistical analysis

The results are presented as the mean ± s.e.mean, except for the ID_{50} or ED_{50} values (i.e. the concentrations of drugs that reduced oedema by 50% relative to control value or concentrations which produced 50% of the maximal oedema increase), which are presented as means accompanied by their respective 95% confidence limits. The ID_{50} or ED_{50} values were determined by the use of the least squares method. Statistical analysis of the data was performed by analysis of variance followed by Dunnett's test or by Student's unpaired t test, as indicated, and differences with $P \leq 0.05$ were considered significant.

Results

The subplantar injection of BK (1 nmol/paw) in captopril-pretreated rats (5 mg kg^{-1}, s.c., 1 h previously) produced modest paw oedema (0.17 ± 0.05 ml). Intraplantar injections of PGE_2, PGI_2, CGRP, SP, 5-HT, PAF or histamine (all 1 nmol/paw) caused even smaller or no increase in paw volume (Figures 1 and 2). However, co-administration of PGE_2, PGI_2, CGRP, SP, 5-HT or PAF (1 pmol to 1 nmol/paw) with BK (1 nmol/paw) resulted in significantly greater paw oedemas (Figures 1 and 2) ($P<0.05$). These effects were dose-dependent, yielding a rank order of potency (mean ED_{50} pmol/paw and 95% confidence limits) for the potentiating effects of these mediators of: SP (8.1; 6.7–9.1) > PAF (13.7; 10.2–16.8) > PGI_2 (20.5; 19.2–22.1) > 5-HT (23.8; 22.9–25.3) > CGRP (25.7; 23.4–26.3) > PGE_2 (52.0; 50.4–57.6). The maximal increases in paw volume (in ml) induced by

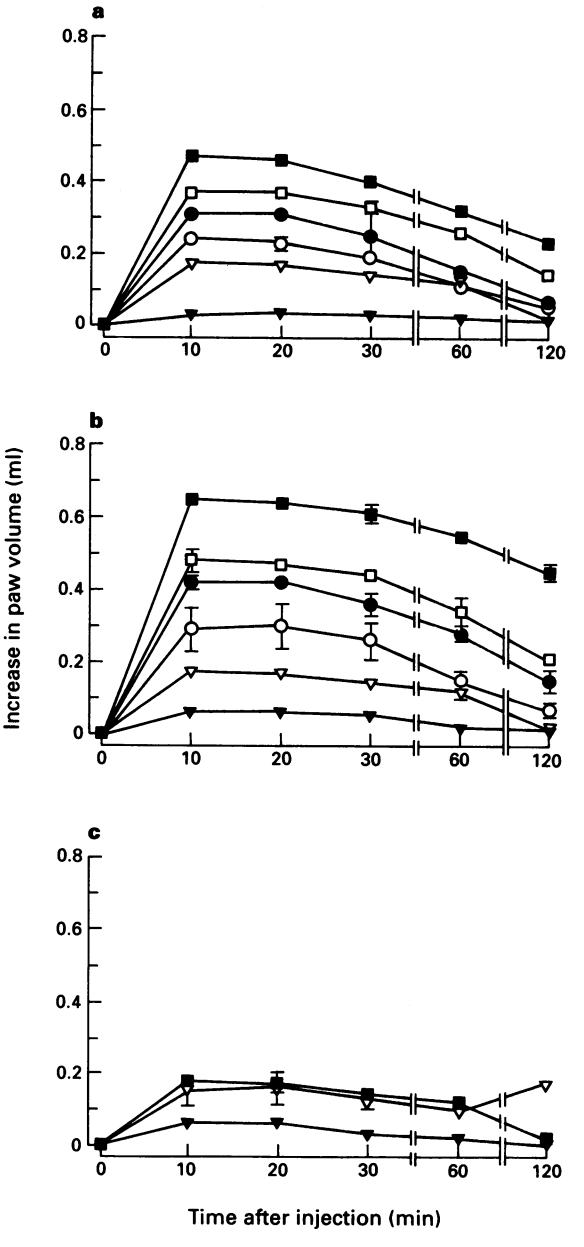

Figure 2 Effect of intraplantar injection of bradykinin (BK), given alone or in combination with other inflammatory mediators, on rat hindpaw volume. Values shown represent the differences between volumes (in ml) of vehicle-treated (0.1 ml of PBS solution) and drug-injected paws. BK was injected either alone (▽, 1 nmol/paw in all panels), or in combination with 1(○), 10 (●), 100 (□), and 1000 (■) pmol/paw of PAF (a), 5-HT (b) or histamine (c). The effects of each mediator alone are also shown in their respective panels (▼, 1 nmol/paw). Each point represents the mean ± s.e.mean of 5 to 6 animals pretreated with captopril (5 mg kg^{-1}, s.c.). In some cases the error bars are hidden within the symbols.

co-injection of BK together with each mediator were (mean ± s.e.mean): PGE_2 (0.71 ± 0.02), PGI_2 (0.66 ± 0.02), 5-HT (0.65 ± 0.01), SP (0.63 ± 0.05), CGRP (0.60 ± 0.05), PAF (0.47 ± 0.02) ml. In contrast, histamine (1 nmol/paw) did not potentiate BK-induced rat paw oedema (Figure 2).

On the other hand, co-injections into the paw of different combinations of PGE_2, PGI_2, SP, CGRP, and 5-HT (e.g. PGE_2 and PGI_2, SP and PGE_2, CGRP and SP, PGI_2 and 5-HT) resulted, at best, in 50% of the oedema produced by combination of any of these mediators with BK. All combinations, except those with BK, caused oedema which appeared to reflect only the summation of the individual effects of each mediator (each given at 1 nmol/paw; $n = 6-7$; results not shown), i.e. their effects were merely additive.

The intraplantar injection of the selective B_2 receptor antagonists, Hoe 140 or NPC 17731 (0.1, 1 and 3 nmol/paw) alone had no effect (results not shown). However, when they were co-injected with BK (1 nmol/paw) plus one of the inflammatory mediators (1 nmol/paw), both caused a dose-dependent and significant inhibition of the size of the response to BK given together with inflammatory mediators ($P<0.05$), with the followings mean ID_{50s} (nmol/paw) (and 95% confidence limits): 1.4 (0.9–2.0), 1.3 (0.8–2.2), 1.5 (1.1–2.0) and 1.1 (0.9–2.2) for Hoe 140 and 1.0 (0.9–1.1), 1.0 (0.8–1.2), 0.9 (0.9–1.5) and 0.7 (0.6–0.7) for NPC 17731 in the presence of PGE_2, PGI_2, CGRP and SP, respectively (Figures 3 and 4). In contrast, the selective B_1 receptor antagonist des-Arg^9[Leu^8]-BK (DALBK) (up to 100 nmol/paw) did not affect the potentiation of paw oedema (control response to BK (3 nmol/paw) (mean ± s.e.mean) of 0.41 ± 0.03 ml; treated with DALBK (100 nmol/paw) 0.44 ± 0.02 ml ($P>0.05$)).

Successive daily intraplantar injections of BK (10 nmol/paw) for 7 days caused progressive desensitization of the oedema response reaching a maximal inhibition of 93 ± 3% on day 7 (Figure 5a). This desensitization of oedema was specific for BK, since intraplantar injections of PGE_2 (10 nmol/paw), SP (3 nmol/paw), PAF (10 nmol/paw) or histamine (100 nmol/paw) into the BK desensitized paws produced oedema of the same magnitude observed in the naive paw, indicating the absence of cross desensitization between BK and these inflammatory mediators (Figure 5b). Co-administration of BK (1 nmol/paw) with PGE_2 (1 nmol/paw), SP (1 nmol/paw) or PGI_2 (1 nmol/paw) in the BK-desensitized paws almost completely restored to control level BK-induced paw oedema (Figure 6). Moreover, the intraplantar co-injection of PGE_2 or SP (10 nmol/paw) together with the first BK (10 nmol/paw) injection (day 1) partially, but significantly, prevented BK-induced desensitization (Figure 7). The maximal reductions in oedema (mean ± s.e. mean) (at the end of 7 days) were: 55 ± 3% and 78 ± 2%, in animals that received PGE_2 or SP respectively, compared with 93 ± 3% inhibition when desensitization was carried out in the absence of these mediators ($P<0.01$). The results in Figure 8 show that prior treatment of animals with dexamethasone (0.5 mg kg^{-1}, s.c., 24 h previously) markedly reduced the size of the response to BK given together with either PGE_2 (1 nmol/paw) or PGI_2 (1 nmol/paw) in the BK desensitized paws ($P<0.01$).

Intraplantar injection of the selective B_1 agonist, DABK (up to 100 nmol/paw) caused modest paw oedema in control

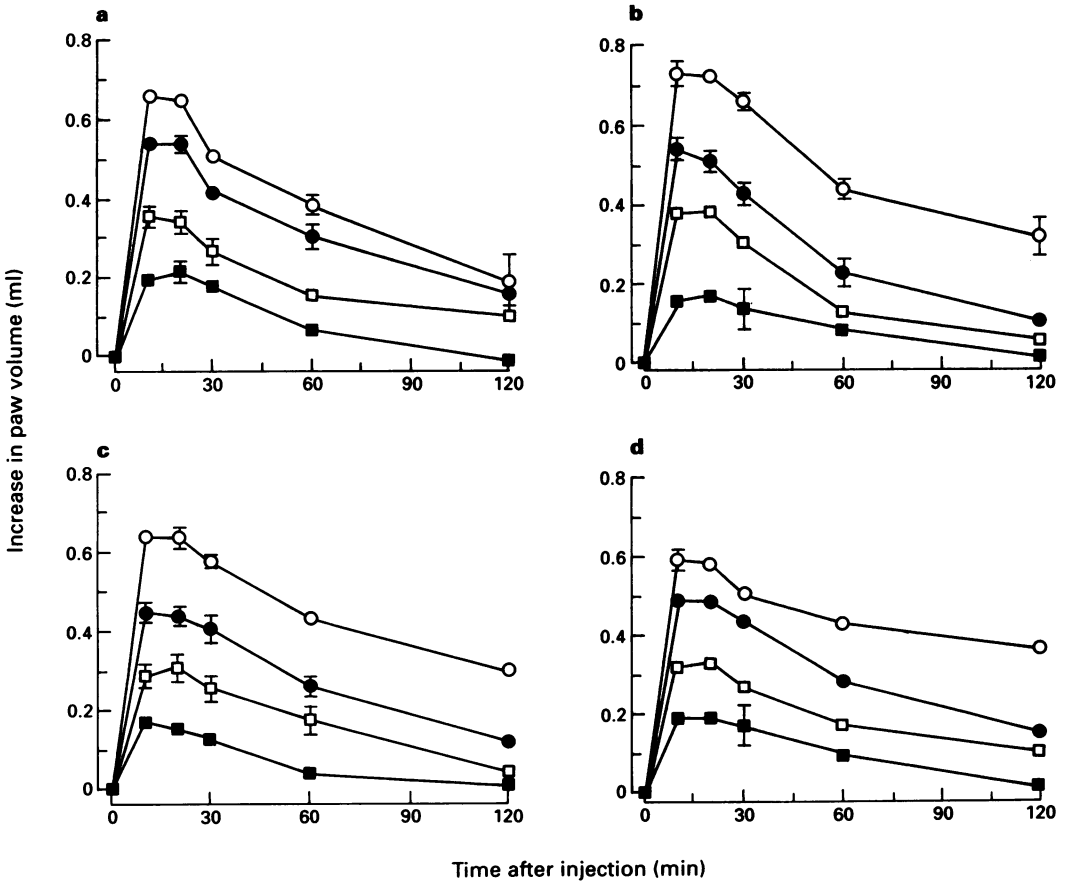

Figure 3 Effect of intraplantar injection of the selective B_2 receptor antagonists Hoe 140 given in combination with BK (1 nmol/paw) and 1 nmol/paw PGI_2 (prostaglandin I_2) (a), PGE_2 (b), substance P (c) or calcitonin gene-related peptide (d), on rat hindpaw volume. Values shown represent the differences between volumes (in ml) of vehicle-treated (0.1 ml of PBS solution) and drug-injected paws. Control responses (○) and responses obtained in the presence of Hoe 140 (nmol/paw): 0.1 (●); 1 (□) and 3 (■). Each point represents the mean ± s.e.mean of 5 to 6 animals pretreated with captopril (5 mg kg^{-1}, s.c.). In some cases the error bars are hidden within the symbols.

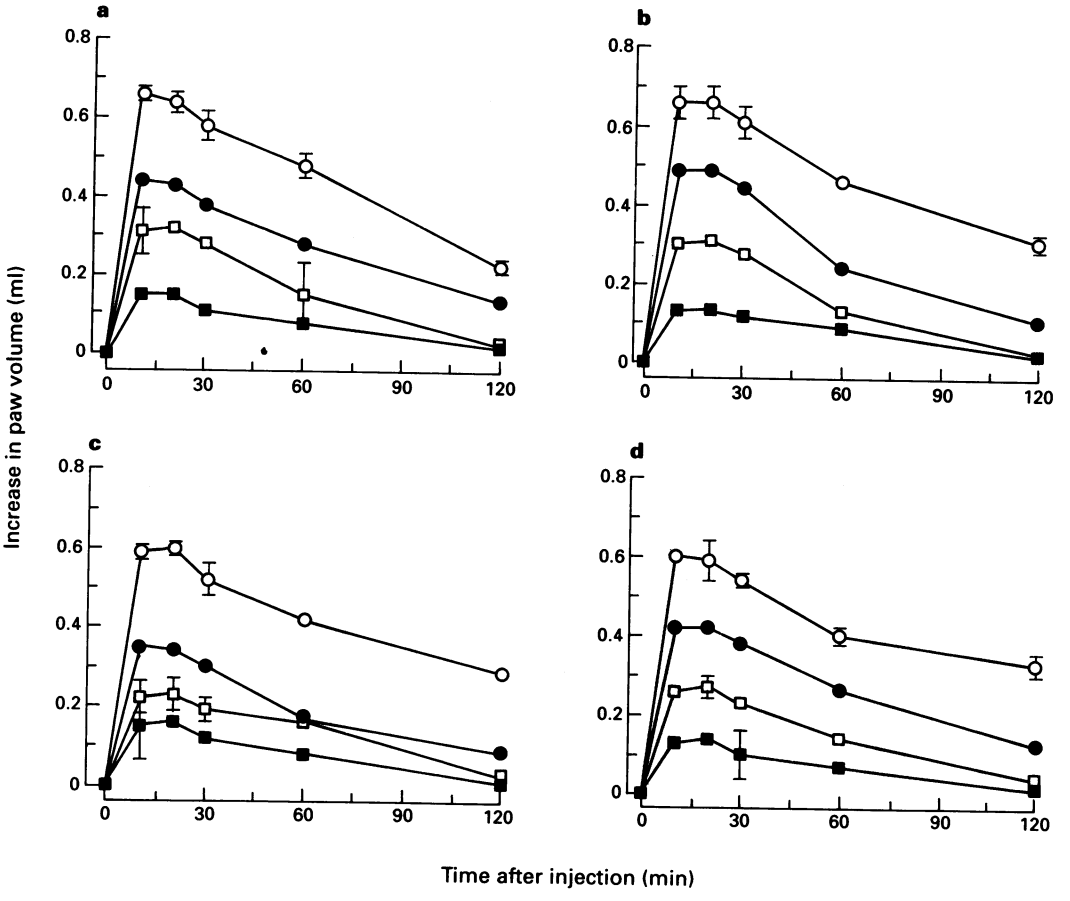

Figure 4 Effect of intraplantar injection of the selective B_2 receptor antagonist, NPC 17731 given in combination with bradykinin (BK) (1 nmol/paw) and 1 nmol/paw prostaglandin I_2 (PGI_2) (a), PGE_2, (b), substance P (c) or calcitonin gene-related peptide (d), on rat hindpaw volume. Values shown represent the differences between volumes (in ml) of vehicle-treated (0.1 ml of PBS solution) and drug-injected paws. Control responses (○) and responses obtained in the presence of NPC 17731 (nmol/paw): 0.1 (●); 1 (□) and 3 (■). Each point represents the mean ± s.e.mean of 5 to 6 animals pretreated with captopril (5 mg kg^{-1}, s.c.). In some cases the error bars are hidden within the symbols.

animals (0.3 ± 0.01 ml). However, when animals were completely desensitized to BK (10 nmol/paw for 7 days), intraplantar injection of the selective B_1 agonist DABK (100 nmol/paw) caused marked paw oedema (0.25 ± 0.03 ml), which correspond to about 60% of the maximal oedema induced by BK (Figure 9a). Paw oedema produced by DABK (100 nmol/paw) was significantly attenuated by co-injection of the selective B_1 receptor antagonist DALBK (100 nmol/paw) (36 ± 6% inhibition) ($P<0.01$) (Figure 9a). In addition, prior treatment of animals with dexamethasone (0.5 mg kg^{-1}, 24 h previously) also consistently antagonized DABK (100 nmol/paw)-induced oedema in desensitized paws (53 ± 4% inhibition) ($P<0.05$) (Figure 9a). In contrast, the same treatment with dexamethasone had no effect on oedema induced by BK (3 nmol/paw) in naive paws (Figure 9b).

Discussion

The present study demonstrated that BK-induced paw oedema in the rat can be markedly potentiated by several mediators of inflammation. Thus, co-injection of very low doses of PGE_2, PGI_2, SP, CGRP, PAF or 5-HT, which alone caused little or no oedema, induced marked dose-dependent potentiation of BK-induced paw oedema, whereas histamine was ineffective. The potentiating actions of all mediators seems to involve an amplification of responses to BK mediated by B_2 receptors, as the oedema caused by co-injection of BK together with each mediator was dose-dependently and similarly blocked by two selective and potent B_2 BK receptor antagonists, Hoe 140 and NPC 17731. Similar inhibition of kinin responses *in vivo* by these B_2 antagonists has been reported (Wirth *et al.*, 1991; Dray *et al.*, 1992; Corrêa & Calixto, 1993; Heapy *et al.*, 1993; Kyle & Burch, 1993).

It is important to mention that co-injections of different combinations of PGE_2, PGI_2, SP, CGRP or histamine always resulted in oedema that was smaller than that caused by any co-injections with BK. These results strongly suggest that BK plays a key role in this process. The mechanisms underlying the potentiating effects of these agents on B_2 receptor-mediated BK-induced paw oedema have yet to be characterized. Nevertheless, it is possible that the influences of prostaglandins, SP and CGRP on BK-induced paw oedema may involve their ability to enhance blood flow, as has been shown for BK-induced increases in vascular permeability (Brain & Williams, 1989; Buckley *et al.*, 1991; Cruwys *et al.*, 1992). Alternatively, there may well be interactions between the distinct second messenger systems activated by BK and these mediators. It has been shown that BK-stimulated PGE_2 synthesis is potentiated by interleukin$_1$ (IL-1) in human synovial fibroblasts (Bathon *et al.*, 1992) and by IL-1 and tumour necrosis factor (TNF_α) in 3T3 fibroblasts (Burch *et al.*, 1988; 1989a,b; Burch & Tiffany, 1989; for review see Burch *et al.*, 1993). Moreover, both IL-1 and TNF_α have been reported to increase BK-induced membrane GTP binding and GTP activity (Burch *et al.*, 1988; Imamura *et al.*, 1988).

Both paw oedema and pleural exudation triggered by BK can be progressively inhibited by repeated daily injections of the peptide (Martins *et al.*, 1992). Confirming this report, we have found that daily intraplantar injections of BK (10 nmol/

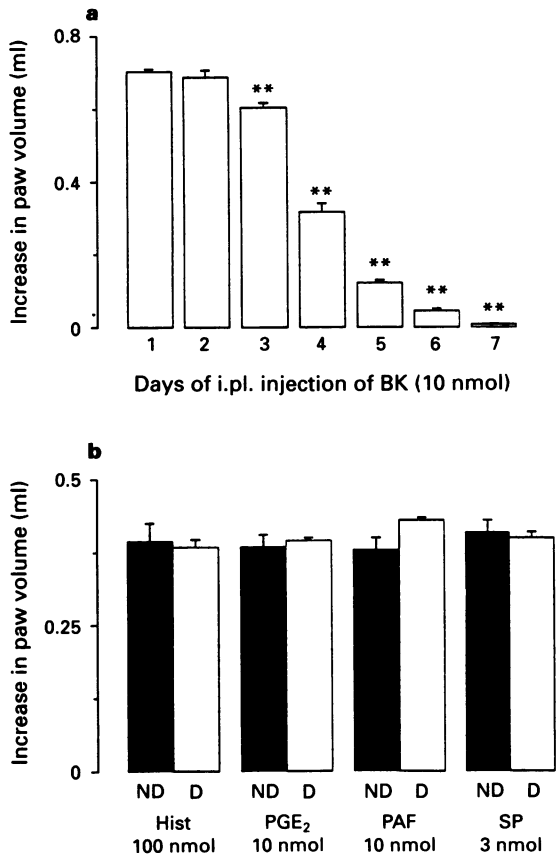

Figure 5 (a) Desensitization of bradykinin (BK)-induced rat paw oedema caused by intraplantar (i. pl.) daily injections of BK (10 nmol/paw) once a day for 7 days. (b) Absence of cross-desensitization between BK and inflammatory mediators. Response of desensitized (D, open columns) or non-desensitized paws (ND, solid columns). Values shown represent the differences between volumes (in ml) of vehicle-treated (0.1 ml of PBS solution) and drug-injected paws. Each column represents the mean ± s.e.mean of 5 to 6 animals pretreated with captopril (5 mg kg^{-1}, s.c.). Significantly different from control: $^{**}P<0.01$. The oedema was measured 20 min after intraplantar injection of inflammatory mediators.

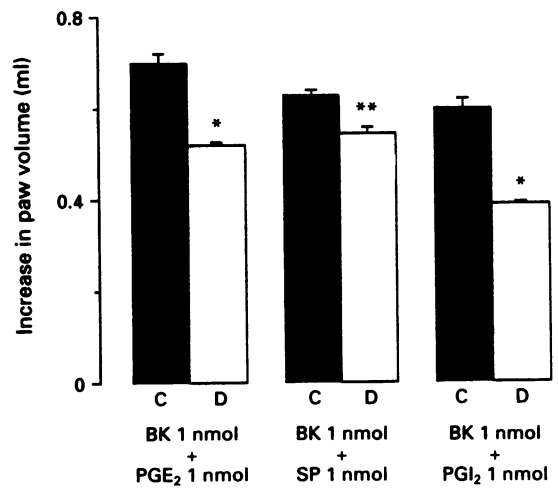

Figure 6 Effect of intraplantar injection of bradykinin (BK) (1 nmol/paw), given in combination with prostaglandin E_2 (PGE_2) (1 nmol/paw), substance P (1 nmol/paw) or PGI_2 (1 nmol/paw) on rat hindpaw volume in naive (solid columns) or in BK-desensitized (seven days) paws (open columns). Values shown represent the differences between volumes (in ml) of vehicle-treated (0.1 ml of PBS solution) and drug-injected paws. Each column represents the mean ± s.e.mean of 5 to 6 animals pretreated with captopril (5 mg kg^{-1}, s.c.). Significantly different from control: $^{*}P<0.05$; $^{**}P<0.01$. The oedema was measured 20 min after intraplantar injection of inflammatory mediators.

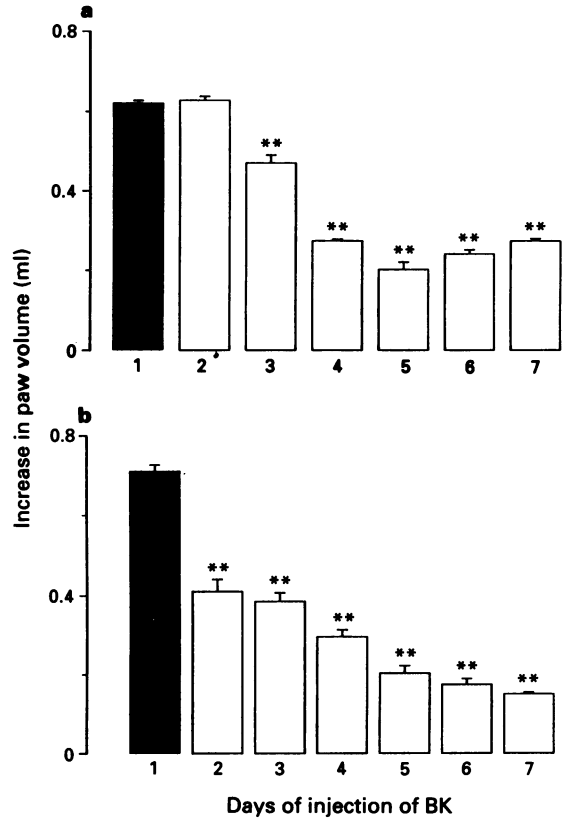

Figure 7 Effect of intraplantar administration of substance P (SP) (a, 10 nmol/paw) or prostaglandin E_2 (PGE_2) (b, 10 nmol/paw) together with the first bradykinin (BK) injection (10 nmol/paw) (solid columns) on BK (10 nmol/paw)-induced rat paw oedema desensitization by repeated injection of the peptide once a day for 7 days (open column). Values shown represent the differences between volumes (in ml) of vehicle-treated (0.1 ml of PBS solution) and drug-injected paws. Each column represents the mean ± s.e.mean of 5 to 6 animals pretreated with captopril (5 mg kg^{-1}, s.c.). Significantly different from control: $^{**}P<0.01$. The oedema was measured 20 min after intraplantar injection of inflammatory mediators.

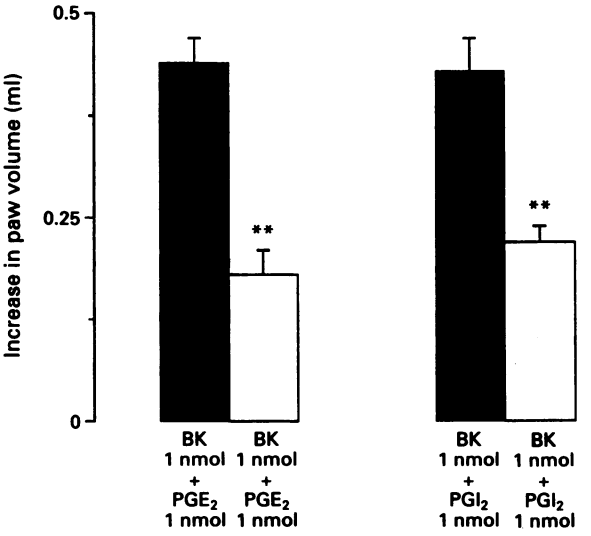

Figure 8 Effect of pretreatment of animals with dexamethasone (0.5 mg kg^{-1}, s.c., 24 h previously, open columns) on the rat paw potentiation caused by intraplantar co-administration of bradykinin (BK) (1 nmol/paw) with prostaglandin E_2 (PGE_2) (1 nmol/paw) or PGI_2 (1 nmol/paw) in desensitized paws (solid columns). Values shown represent the differences between volumes (in ml) of vehicle-treated (0.1 ml of PBS solution) and drug-injected paws. Each column represents the mean ± s.e.mean of 4 animals pretreated with captopril (5 mg kg^{-1}, s.c.). Significantly different from control: $^{**}P<0.01$. The oedema was measured 20 min after intraplantar injection of inflammatory mediators.

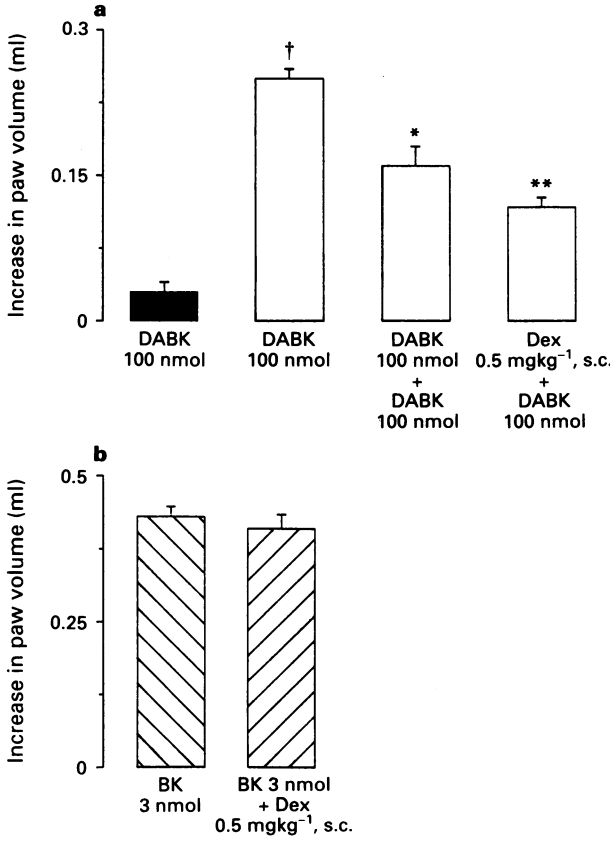

Figure 9 (a) Effect of intraplantar injection of des-Arg^9-BK (DABK, 100 nmol/paw) in naive control paws (solid column) or in bradykinin (BK)-densensitized paws (open columns) in rats pretreated with captopril (5 mg kg^{-1}, s.c.). Values shown represent the differences between volumes (in ml) of vehicle-treated (0.1 ml of PBS solution) and drug-injected paws. Panel (a) also shows the inhibition of DABK-induced paw oedema in BK-desensitized paws caused by co-injection of des-Arg^9[Leu^8]-BK (DALBK; 100 nmol/paw) or by the treatment of animals with dexamethasone (Dex, 0.5 mg kg^{-1}, s.c., 24 h previously). (b) Effect of treatment of animals with Dex (0.5 mg kg^{-1}, s.c., 24 h previously) on BK-induced paw oedema. Each column represents the mean ± s.e.mean of 4 to 5 animals. Significantly different from control (†$P<0.01$) or from DABK-induced oedema (*$P<0.05$; **$P<0.01$). The oedema was measured 20 min after intraplantar injection of the peptides.

paw) for seven days caused a progressive and complete desensitization of paw oedema. This phenomenon was specific for BK, as BK-desensitized paws were equally responsive to intraplantar injections of PAF, PGE_2, SP or histamine. Interestingly, however, intraplantar co-injection of PGE_2 or SP (10 nmol/paw), together with the first BK (10 nmol/paw) injection, significantly attenuated the desensitization caused by daily intraplantar injections of BK. In addition, the oedema response of BK-desensitized paws to the peptide could be almost completely restored to initial levels if BK was co-injected on day 7 together with PGE_2, PGI_2 or SP (1 nmol/paw). These results are consistent with the view that these mediators are important modulators of BK action.

Recent biochemical studies have suggested that BK-induced desensitization involves changes at the receptor level, as well as of second messenger transducer mechanisms. Thus, BK-induced desensitization in cultured sensory neurones is mediated via release of nitric oxide, which, in turn, activates guanylate cyclase to increase cyclic GMP (Burgess *et al.*, 1989; Lang *et al.*, 1990; McGehee *et al.*, 1992). Furthermore, BK-induced desensitization is correlated with a down regulation and internalization of kinin receptors, allied to a decreased coupling of activated receptors to G-proteins (Roscher *et al.*, 1984; 1990; Munoz & Leeb-Lundberg, 1992; Wolsing & Rosenbauum, 1993). Such observations suggest that BK-induced desensitization may be relevant as a mechanism for regulation of its pro-inflammatory properties.

In this regard, the current study shows that the selective B_1 receptor agonist, DABK, which caused a very weak effect in naive paws, produced marked oedema in BK-desensitized paws. It is well known that DABK does not produce any inflammatory or algesic responses in non-traumatized tissues. This is likely to be because the B_1 receptors are not expressed in normal tissues (Regoli & Barabé, 1980; Marceau *et al.*, 1983; Steranka & Burch, 1991). Nevertheless, expression of B_1 receptors can be induced under a variety of conditions, and thus exert important roles in several pathological states, including inflammation and hyperalgesia (Marceau *et al.*, 1983; Farmer *et al.*, 1991; Dray & Perkins, 1993; Perkins & Kelly, 1993). We have attempted to provide more direct evidence that B_2 receptor desensitization induces the expression of B_1 receptors, by pretreating animals with dexamethasone 24 h before intraplantar injection of DABK. Dexamethasone consistently attenuated DABK-mediated oedema in paws desensitized to BK. Interestingly, the same treatment with dexamethasone failed to modify BK-induced paw oedema, which is mediated by activation of constitutive B_2 receptors. Thus, the paw oedema caused by intraplantar injection of DABK in BK-desensitized paws is likely to be associated with a dexamethasone-sensitive induction of B_1 kinin receptor expression. Indeed, dexamethasone has been found to inhibit the induction of B_1 receptor expression *in vitro* (Deblois *et al.*, 1988). Recent findings suggest that BK triggers the release of several cytokines such as IL-1, IL-2 and TNF_α (Tiffany & Burch, 1989; Ferreira *et al.*, 1993). Cytokines can also mediate the expression of B_1 receptors (Deblois *et al.*, 1988; 1991), and their synthesis is blocked by corticosteroids (Roscher & Manganiello, 1984; Deblois *et al.*, 1988). These data suggest that cytokines may be involved in the upregulation of expression of B_1 receptors in BK-desensitized paws. Our results strongly suggest that the desensitization of B_2 receptors may play an important role in expression of B_1 receptors following tissue damage or in inflammatory states. However, it remains to be confirmed whether B_2 receptor desensitization and induction of expression of B_1 receptors may occur in inflamed tissues.

In conclusion, we have shown that both B_1 and B_2 kinin receptors can trigger oedema in the rat paw under different conditions. The B_2 receptors are constitutive and can interact in a synergistic manner with several inflammatory mediators. On the other hand, B_2 receptor desensitization induces the expression of B_1 receptors through a dexamethasone-sensitive mechanism which may involve cytokine production. As BK can stimulate prostanoid synthesis in most tissues and activate sensory neurones to release the pro-inflammatory neuropeptides SP and CGRP, the current findings may have important implications for the manifestation of acute and chronic inflammatory process.

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Sensitization by calyculin A of brain capillary endothelial cells to endothelin-1

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1 Cultured brain capillary endothelial cells of the rat respond to endothelin-1 (ET-1) by an increased activity of the $Na^+,K^+,2Cl^-$, cotransporter and a mobilization of intracellular Ca^{2+} stores.

2 Calyculin A (1-30 nM), but not okadaic acid, sensitizes up to 100 fold the $Na^+,K^+,2Cl^-$ cotransporter to the action of ET-1.

3 Calyculin A (30 nM) does not modify the binding properties of ET-1 to ET_A receptors.

4 Calyculin A (30 nM) inhibits ET-1 induced intracellular Ca^{2+} mobilization.

5 It is concluded that inhibition of protein phosphatase 1 selectively modifies the repertoire of intracellular actions of ET-1 and favours actions that are unrelated to the phospholipase C signalling cascade.

Keywords: Blood brain barrier; endothelin; Na^+; K^+; $2Cl^-$ cotransport; phospholipase C; calyculin A; endothelial cells

Introduction

Endothelins are powerful vasoactive agents (Yanagisawa *et al.*, 1988). They have potent vasoconstrictor and cardiotonic properties and are thought to play an important role in cardiovascular diseases (Masaki & Yanagisawa, 1990; Masaki, 1993). Endothelin-1 (ET-1) has numerous intracellular actions in a variety of cell types (Simonson & Dunn, 1990). Brain capillary endothelial cells (BCEC) respond to ET-1 by activation of phospholipase C and the mobilization of intracellular Ca^{2+} stores (Vigne *et al.*, 1990; Stanimirovic *et al.*, 1994), activation of phospholipase A_2 (Stanimirovic *et al.*, 1994; Vigne & Frelin, 1994), inhibition of adenylate cyclase (Ladoux & Frelin, 1991), increased activities of the Na^+/H^+ antiporter (Vigne *et al.*, 1991) and of the $Na^+,K^+,2Cl^-$ cotransporter (Vigne *et al.*, 1994b). All these effects are triggered by nanomolar concentrations of ET-1. In this paper we show that the specificity of the intracellular actions of ET-1 can be dramatically altered when cells are treated with calyculin A, an inhibitor of protein phosphatases (Ishihara *et al.*, 1985). Calyculin A suppressed the Ca^{2+} mobilizing action of ET-1 and sensitized the $Na^+,K^+,2Cl^-$ cotransporter to the action of ET-1. The functional significance of these results is discussed.

Methods

A clonal population of rat BCEC was prepared as previously described (Vigne *et al.*, 1989) and grown in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% foetal bovine serum (Dutscher, Strasbourg, France), 100 units ml^{-1} penicillin and 100 $\mu g\ ml^{-1}$ streptomycin.

For $^{86}Rb^+$ uptake experiments, cells, seeded into 24 well tissue culture clusters, were washed with an Earle's salt solution (composition, mM: NaCl 140, KCl 5, $CaCl_2$ 1.8, $MgSO_4$ 0.8, glucose 5, buffered at pH 7.4 with N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid-NaOH 25) and further incubated at 37°C in the same solution supplemented with 1 mM ouabain and $^{86}Rb^+$, 1.5-2 $\mu Ci\ ml^{-1}$. After 6 min of incubation at 37°C, the incubation solution was rapidly aspirated off and the cells were rinsed four times with ice cold 0.1 M $MgCl_2$. Cells were then harvested into 0.1 N NaOH and the cell-associated radioactivity was counted. We checked that under the conditions used, $^{86}Rb^+$ uptake was linear with time for at least 10 min. The activity of the $Na^+,K^+,2Cl^-$ cotransporter was defined as the difference in the rate of $^{86}Rb^+$ uptake measured in the absence or the presence of 10 μM bumetanide.

[^{125}I]-ET-1 binding experiments were performed as previously described (Vigne *et al.*, 1990). Briefly, confluent monolayers were incubated at 37°C into an Earle's salt solution supplemented with 10 pM [^{125}I]-ET-1 and different concentrations of unlabelled ET-1. After 45 min of association, cells were washed with an Earle's salt solution and the cell associated radioactivity was determined after digestion of the cells into 0.1 N NaOH.

For intracellular Ca^{2+} measurements, cells were loaded with 5 μM Indo-1/AM for 2 h. Flow cytometric analysis of the Indo-1 fluorescence was then performed as previously described (Vigne *et al.*, 1994a) using a FacStar Plus (Becton-Dickinson).

EC_{50} values were determined by fitting dose-response curves to a logistic function using the SigmaPlot software.

Materials

ET-1 and BQ-123 were from Neosystems (Strasbourg, France). Calyculin A, ouabain and indo-1/AM were from the Sigma Chemical Co. Okadaic acid was from Boehringer Mannheim. Bumetanide was kindly provided by Dr L. Feit (Leo Pharmaceuticals, Ballrup, Denmark). $^{86}RbCl$(18.5-370 MBq mg^{-1}) and [^{125}I]-ET-1 (2000 Ci $mmol^{-1}$) were from Amersham Corp.

Results

$Na^+,K^+,2Cl^-$ cotransport is a major K^+ uptake pathway in rat cultured BCEC. Its activity accounts for about half of the initial rate of $^{86}Rb^+$ uptake, the remaining uptake being mediated by the (Na^+,K^+)ATPase (Vigne *et al.*, 1994b). The pharmacological and kinetic properties of the $Na^+,K^+,2Cl^-$ cotransporter have been analysed previously (Vigne *et al.*, 1994b). Addition of calyculin A, an inhibitor of protein phosphatases 1 and 2A, dose-dependently increased Na^+, $K^+,2Cl^-$ cotransport activity. Its action developed at concentrations >1 nM (Figure 1). Okadaic acid (up to 50 nM), a more selective inhibitor of protein phosphatase 2A had no

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action on $^{86}Rb^+$ uptake (data not shown). This indicated that calyculin A stimulated $Na^+,K^+,2Cl^-$ cotransporter activity by inhibiting protein phosphatase 1.

Figure 1 also shows that, as previously described (Vigne *et al.*, 1994b), ET-1 increased $^{86}Rb^+$ uptake by the $Na^+,K^+,2Cl^-$ cotransporter about 2 fold. The EC_{50} value for the ET-1 action was 1 nM. The action of ET-1 was prevented by BQ-123 (K_i = 17 nM), thus indicating a likely involvement of an ET_A receptor subtype (Vigne *et al.*, 1994b).

Figure 1 presents dose-response curves for ET-1 action in cells that have been exposed to different concentrations of calyculin A 45 min before exposure to ET-1. It shows that calyculin A treatment shifted the dose-response curves for ET-1 action on $Na^+,K^+,2Cl^-$ cotransport to lower concentrations. The EC_{50} value for ET-1 was 300 pM in the presence of 1 nM calyculin A. It was 50 pM in the presence of 10 nM calyculin A and 7 pM in the presence of 30 nM calyculin A (Figure 2). This sensitizing action required a prolonged (> 30 min) exposure of the cells to calyculin A and was not observed when cells were treated at the same time with calyculin A and ET-1 and $^{86}Rb^+$ uptake measured 6 min later.

A possible mechanism to account for this observation is that inhibition of protein phosphatase 1 modified the properties of interaction of ET-1 with its receptor. To test this hypothesis, [^{125}I]-ET-1 binding experiments were performed in intact cells. Figure 2 shows that calyculin A, at a concentration of 30 nM which sensitizes of $Na^+,K^+,2Cl^-$ cotransport to ET-1 100 fold (Figure 1), did not alter the apparent affinity of ET-1 for its receptors. Under the experimental conditions used, [^{125}I]-ET-1 only titrated ET_A receptor sites (Vigne *et al.*, 1991) and [^{125}I]-ET-1 binding was fully sensitive to BQ-123 (K_i = 20 nM) (Vigne *et al.*, 1993). This result suggests that calyculin A acted down stream of the receptor.

We next investigated the action of calyculin A on ET-1-induced intracellular Ca^{2+} mobilization. This action of ET-1 is also mediated by BQ-123-sensitive ET_A receptors (Vigne *et al.*, 1993). Figure 3 shows that calyculin A dose-dependently suppressed the Ca^{2+} mobilizing action of ET-1. Its action developed at concentrations > 10 nM similar to those found to alter $Na^+,K^+,2Cl^-$ cotransport activity (Figure 1). It was not mimicked by 50 nM okadaic acid, hence indicating a likely involvement of protein phosphatase 1, and it required long (> 30 min) pre-exposures to the inhibitor to be observed. Finally, Figure 3 shows that in calyculin A-treated cells, the sensitivity to ET-1 was not altered. Calyculin A by itself had no action on intracellular Ca^{2+} or on the activity of phospholipase C (data not shown).

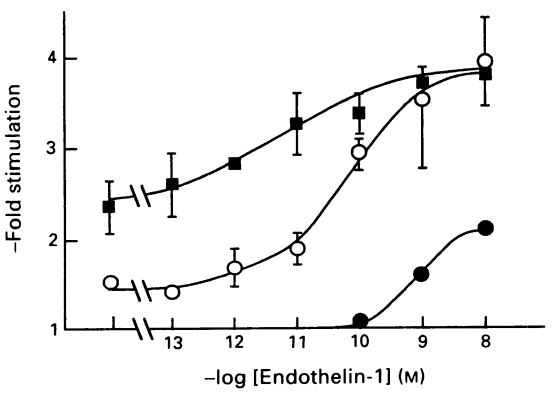

Figure 1 Calyculin A potentiates the action of endothelin-1 (ET-1) on $Na^+,K^+,2Cl^-$ cotransport activity. Dose-response curves for ET-1 activation of $Na^+,K^+,2Cl^-$ cotransport by ET-1 in the absence (●) or the presence of 10 nM (○) or 30 nM (■) calyculin A. In these experiments cells were treated with calyculin A for 45 min prior to the addition of ET-1. Means ± s.e. of 3 to 4 independent experiments performed in duplicate are shown. Activity is expressed relative to the basal activity measured in parallel experiments in the absence of both calyculin A and ET-1.

Discussion

ET-1 has two prominent actions in rat BCEC: (i) it activates the $Na^+,K^+,2Cl^-$ cotransporter (Vigne *et al.*, 1994b) and this action is probably important for promoting K^+ transport across the blood brain barrier. (ii) It activates phospholipase C and thereby induces intracellular Ca^{2+} mobilization (Vigne *et al.*, 1990). This paper shows that a prior treatment of the cells with calyculin A has opposite actions on these two actions of ET-1. It potentiates the action of ET-1 on $Na^+,K^+,2Cl^-$ cotransport activity (Figure 1) and suppresses the action of ET-1 on intracellular Ca^{2+} mobilization (Figure 3). As these actions are not shared by okadaic acid, a more selective inhibitor of protein phosphatase 2A (Ishihara *et al.*, 1985), an obvious conclusion is that protein phosphatase 1 rather than protein phosphatase 2A mediated the actions of calyculin A. The contrasting actions of calyculin A indicate that different intracellular signalling mechanisms control $Na^+,K^+,2Cl^-$ cotransport activity and intracellular Ca^{2+} mobilization in BCEC. Indeed, intracellular Ca^{2+} mobilization is triggered by the activation of phospholipase C by a pertussis toxin-insensitive G protein and the release of

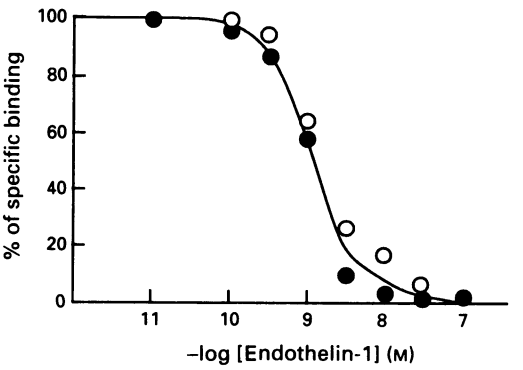

Figure 2 Dose-response curves for the inhibition by endothelin-1 (ET-1) of [^{125}I]-ET-1 binding. Experiments were performed in control (●) and 30 nM calyculin A (○)-treated cells. [^{125}I]-ET-1 was used at 10 pM.

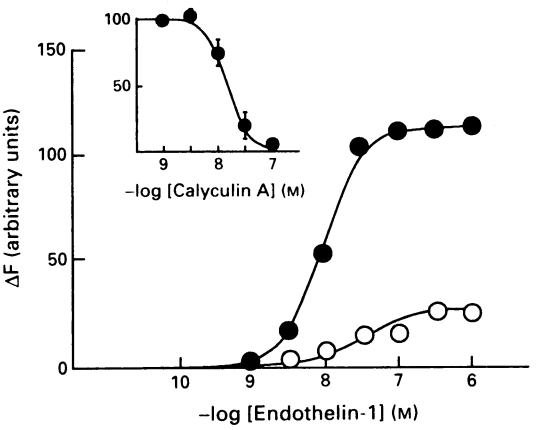

Figure 3 Calyculin inhibits endothelin-1 (ET-1)-induced intracellular Ca^{2+} mobilization. Main panel: dose-response curves for ET-1-induced increase in Indo-1 fluorescence ratio in control cells (●) and cells that had been treated for 45 min with 30 nM calyculin A (○). Changes in Indo-1 fluorescence ratio were measured 15 s after the addition of ET-1 and means of 1000 individual cell measurements collected during a 2 s period were calculated. Means of duplicates in a typical experiments are shown. Inset: dose-response curve for calyculin A inhibition of the Ca^{2+} mobilizing action of ET-1. Cells were treated for 45 min with the indicated concentrations of calyculin A and then with 100 nM ET-1. The peak Ca^{2+} transient was measured 10 s after the addition of ET-1. Means ± s.e. (n = 3) are indicated.

inositol (1,4,5)trisphosphate (Vigne *et al.*, 1990). Activation of $Na^+,K^+,2Cl^-$ cotransport in BCEC involves a yet undefined pathway that is independent of changes in $[Ca^{2+}]_i$ or of protein kinase C and that is unlikely to be a direct consequence of the phospholipase C signalling cascade (Vigne *et al.*, 1994b).

A major difficulty encountered in evaluating the pathophysiological roles of endothelins is that circulating levels of endothelins in normal (1–5 pM) or pathological situations (up to 25 pM) (Battistini *et al.*, 1993) are much lower than the concentrations necessary to occupy receptors (Figure 2) and to induce intracellular actions in isolated cell preparations (Figures 1 and 3). It is also much lower than the concentrations that are necessary to elicit contractions in isolated vessel preparations (Marsault *et al.*, 1991). An important point of this paper is that the cell sensitivity to ET-1 is not a fixed property. It is dependent on the cell state. In the absence of calyculin A treatment, all intracellular actions of ET-1 develop at similar nanomolar concentrations of the peptide. In 30 nM calyculin A-treated cells, intracellular actions of ET-1 are observed over a much larger range of concentrations. $Na^+,K^+,2Cl^-$ cotransport fully activates at low picomolar concentrations of ET-1; intracellular Ca^{2+} mobilization is reduced and can only be observed at concentrations >10 nM. Thus, activating protein phosphatase 1 selectively favours some of the intracellular actions of ET-1 and sensitizes them to the peptide. It is worth noting that in the presence of calyculin A, the concentrations of ET-1 that are active on $Na^+,K^+,2Cl^-$ cotransport (1–30 pM, Figure 1) are close to systemic ET-1 levels in man (1–25 pM) (Battistini *et al.*, 1993).

Calyculin A is an artificial means of inhibiting protein phosphatases and cells *in vivo* rarely encounter calyculin A. The activity of protein phosphatase 1 is however regulated by interaction of the catalytic subunit with endogenous regulatory and inhibitory proteins (Mumby & Walter, 1993). It is of interest that two of these inhibitors (inhibitor 1 and DARPP-32) become active only after phosphorylation by protein kinase A (Mumby & Walter, 1993) and that cyclic AMP is thought to be important for determining the tightness of the blood-brain barrier (Rubin *et al.*, 1991). One possibility is therefore that conditions encountered *in vivo* favour parts of the signalling repertoire of ET-1 and specify actions that are unrelated to the phospholipase C signalling pathway. One of the main functions of endothelial cells of the blood brain barrier is to secrete K^+ into the blood (Bradbury, 1979; Cornford, 1985) and the $Na^+,K^+,2Cl^-$ cotransport is thought to play an important role in this process (Vigne *et al.*, 1994b). The sensitizing mechanism described in this paper would provide a means for the cells to respond to low concentrations of ET-1 and to regulate K^+ secretion by the blood-brain barrier more efficiently in the absence of Ca^{2+} load.

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The interaction of antidepressant drugs with central and peripheral (enteric) 5-HT_3 and 5-HT_4 receptors

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1 A combined study of receptor binding in central neuronal cell membranes and functional responses in isolated segments of guinea-pig small intestine allowed characterization of the interaction of four antidepressant drugs with central and peripheral 5-HT_3 and 5-HT_4 receptors.

2 Clomipramine, paroxetine and fluoxetine inhibited [^{3}H]-DAU 6215 binding to 5-HT_3 recognition sites in NG 108-15 cells with IC_{50} values in the range 1.3–4 μM. Litoxetine had an IC_{50} of 0.3 μM. The specific binding of [^{3}H]-GR 113808 to 5-HT_4 recognition sites in pig striatal membranes was inhibited by all four antidepressants with negligible potency (IC_{50} values $\geqslant$ 20 μM).

3 In whole ileal segments, concentration-response curves to 5-HT were biphasic, with the high- and low-potency phases involving 5-HT_4 and 5-HT_3 receptors, respectively. Curves to 2-methyl-5-hydroxytryptamine (2-methyl-5-HT: a 5-HT_3 receptor agonist) and 5-methoxytryptamine (5-MeOT: a 5-HT_4 receptor agonist) were monophasic. All antidepressants were used at concentrations lacking anticholinoceptor properties, as demonstrated in both electrically stimulated longitudinal muscle-myenteric plexus preparations (LMMPs) and in unstimulated LMMPs following addition of acetylcholine (100 nM).

4 Fluoxetine (0.1–1 μM) and litoxetine (0.3–3 μM) antagonized both the high- and low-potency phases of the 5-HT curve. Schild analysis for the low-potency phase yielded pA_2 estimates of 6.6 ± 0.3 (Schild slope of 1.1) and of 6.6 ± 0.1 (Schild slope of 1.1), respectively. At higher concentrations (3 μM), fluoxetine markedly inhibited the 5-HT response maximum. Clomipramine (10–300 nM) inhibited, by a mechanism independent of concentration, both phases of the 5-HT curve with a reduction of the maximum response. Paroxetine (1 μM) was ineffective on the high-potency phase, but caused a rightward shift of the low-potency phase (pK_B: 6.1 ± 0.01).

5 Responses to 2-methyl-5-HT were inhibited by 1 μM fluoxetine (pK_B: 5.4 ± 0.02). Like clomipramine (30 and 100 nM), litoxetine (1 and 3 μM) produced rightward displacements of 2-methyl-5-HT-induced contractions, which were virtually independent of antidepressant concentration (pK_B values: 6.0 ± 0.02 and 5.5 ± 0.01, respectively). At higher concentrations, fluoxetine (3 μM) and clomipramine (300 nM) markedly reduced the 2-methyl-5-HT response maximum. Paroxetine (1 μM) was ineffective.

6 Responses to 5-MeOT were shifted to the right by fluoxetine (0.1–1 μM) and litoxetine (1 and 3 μM) in a concentration-dependent manner. At higher concentrations, fluoxetine (3 μM) markedly reduced the 5-MeOT response maximum, an effect also observed with 100 and 300 nM clomipramine. Paroxetine (1 μM) was ineffective.

7 In unstimulated LMMPs, the excitatory effects evoked by 5-HT, 2-methyl-5-HT and 5-MeOT and the antagonism produced by 300 nM clomipramine were comparable to those obtained in whole ileal segments. This suggests that 5-HT contained in the mucosa of whole preparations does not interfere with agonist-induced contractile responses and with the inhibitory effect of antidepressant drugs.

8 In conclusion, our results show that clomipramine, fluoxetine, paroxetine and litoxetine possess low to moderate potency/affinity at both central and peripheral (enteric) 5-HT_3 receptors. In contrast, all four antidepressants are virtually ineffective at central 5-HT_4 receptors. Inhibition of 5-HT_4 receptor-mediated ileal contractions by fluoxetine, litoxetine and clomipramine may result from allosteric antagonism or, more likely, from post-receptor blockade of second messenger generation. The interaction of antidepressants with central and peripheral 5-HT_3 and 5-HT_4 receptors may be relevant for both potential therapeutic action and adverse effects at gastrointestinal level.

Keywords: 5-HT_3 receptors; 5-HT_4 receptors; NG 108–15 cells; pig corpus striatum; guinea-pig ileum; antidepressant drugs (clomipramine, fluoxetine, paroxetine, litoxetine)

Introduction

It is generally accepted that central 5-hydroxytryptaminergic pathways are involved in the pathogenesis of depression. By blocking 5-hydroxytryptamine (5-HT) reuptake from nerve terminals, antidepressant drugs, such as clomipramine, fluoxetine and paroxetine, enhance central 5-hydroxytryptaminergic transmission, which can be regarded as an initial step in the therapeutic action of these compounds. In fact, other mechanisms have been reported, including changes in 5-HT_{1A} and 5-HT_2 receptor density and/or sensitivity as a consequence of chronic antidepressant treatment (see Cowen, 1990, for review).

More recently, two additional receptor types, the 5-HT_3 and 5-HT_4 receptors, have been identified in the central nervous system (CNS) (see Peters *et al.*, 1992; Bockaert *et al.*, 1994 for reviews). The 5-HT_3 receptor sites are ligand gated ion channels which mediate the release of a number of neurotransmitters, while 5-HT_4 receptors are positively coupled to adenylyl cyclase and appear to mediate slow excitatory responses to 5-HT in brain (see Zifa & Fillion,

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1992 for review). Although antidepressants possess low to moderate affinity for central 5-HT$_3$ binding sites (Hoyer *et al.*, 1989; Kilpatrick *et al.*, 1989; Schmidt & Peroutka, 1990), potent antagonists at these receptors (tropisetron and ondansetron) have been found, at least in one animal model of depression, to be as active as conventional antidepressants (Martin *et al.*, 1992). This suggests a potential involvement of 5-HT$_3$ receptors in depressive disorders (Greenshaw, 1993). Conversely, the role of 5-HT$_4$ receptors is still obscure.

Both 5-HT$_3$ and 5-HT$_4$ receptors are present in peripheral tissues (Eglen *et al.*, 1990; Tonini *et al.*, 1991; Ford & Clarke, 1993). In the guinea-pig ileum, 5-HT acts mainly by facilitating the neuronal release of acetylcholine (Ford & Clarke, 1993). The resulting concentration-contractile response curve is typically biphasic, with the high (submicromolar) and low (micromolar) potency phase mediated by 5-HT$_4$ and 5-HT$_3$ receptors, respectively (Buchheit *et al.*, 1985; Clarke *et al.*, 1989; Eglen *et al.*, 1990). These receptors, like those in the CNS, may represent a peripheral target for antidepressant drugs.

This study was designed to evaluate whether clomipramine (McTavish & Benfield, 1990), fluoxetine (Benfield *et al.*, 1986), paroxetine (Dechant & Clissold, 1991) and litoxetine (Angel *et al.*, 1993), which block 5-HT reuptake with a noradrenaline/5-HT uptake blocking ratio ranging from 20 to 320 (Benfield *et al.*, 1986; Thomas *et al.*, 1987; Scatton *et al.*, 1988), interact with central and peripheral (enteric) 5-HT$_3$ and 5-HT$_4$ receptors. In particular, binding of these drugs to central receptors was assessed by using two different models, the NG 108–15 neuroblastoma-glioma cells and the pig corpus striatum homogenate, which are suitable for studying 5-HT$_3$ and 5-HT$_4$ receptors, respectively (Giraldo *et al.*, 1992; Rizzi *et al.*, 1994). Functional studies were aimed at investigating the effects of antidepressant drugs on the contractile responses elicited by 5-HT, 2-methyl-5-hydroxytryptamine (agonist at 5-HT$_3$ receptors) and 5-methoxytryptamine (agonist at 5-HT$_4$ receptors) in the guinea-pig isolated ileum.

Methods

5-HT$_3$ receptor binding in NG 108-15 hybrid cells

NG 108-15 neuroblastoma-glioma hybrid cells were cultured as described by Hoyer & Neijt (1987). Crude membrane fractions were prepared according to the method of Bradbury *et al.* (1990), with slight modifications. Briefly, subconfluent cultures were washed twice with phosphate-buffered saline and lysed in 2 mM Tris HCl/1 mM EDTA (pH 7.1) solution for 30 min at 0°C. The suspension was homogenized and centrifuged (400 *g*, 5 min) to remove the nuclei. The supernatant was centrifuged at 30000 *g* for 20 min, and the pellet was resuspended and centrifuged as above. Membranes were suspended in 50 mM HEPES buffer (pH 7.4), divided into 0.5 ml aliquots and stored at −80°C until use.

Displacement experiments were performed by incubating the homogenate, diluted to about 150 μg protein ml^{-1} final concentration, at 30°C for 30 min in the presence of 0.3 nM [^{3}H]-DAU 6215 (Giraldo *et al.*, 1992) and different concentrations of the test compounds dissolved in the assay buffer. Incubation volume was 1.0 ml. The specific binding of [^{3}H]-DAU 6215 (defined as the binding displaceable by 3 μM MDL 72222) was about 95% of total binding. The reaction was terminated by rapid filtration using an IH-110 INOTECH cell harvester (type G7 glass filters, INOTECH). The filters were transferred into plastic vials, 4.0 ml scintillation cocktail (Filter Count, Packard) was added, and radioactivity was counted by liquid scintillation spectrometry (Contron Betamatic V). Protein content was determined by the method of Bradford (1976).

5-HT$_4$ receptor binding in pig corpus striatum

Pig corpora striata were removed and kept on ice for about 2 h before a cold solution of 50 mM HEPES buffer (pH 7.4) was added (w/v 1:10). The tissue was homogenized in an Ultra-Turrax (30 s at full speed) followed by homogenization in a Potter-Elvehjem glass-on-Teflon homogenizer. The homogenate was divided into 5 ml aliquots, and stored at −80°C until use. Displacement experiments were performed by incubating 980 μl of the homogenate (final tissue dilution 1:70) at 30°C for 30 min in the presence of 0.1 nM [^{3}H]-GR 113808 (Grossman *et al.*, 1993) and different concentrations of the test compounds dissolved in the assay buffer. Incubation volume was 1.0 ml. Specific [^{3}H]-GR 113808 binding (defined as the binding displaceable by 10 μM BIMU 1) was about 80% of total binding. The incubation was stopped by rapid filtration as described above.

The inhibition of specific binding by competing ligands was analyzed graphically to estimate IC_{50} values (concentration of antidepressant displacing 50% of specifically bound radioligand) by a nonlinear least squares regression analysis.

Experimental animals

Fasted male Dunkin-Hartley guinea-pigs weighing 480–600 g, were killed by CO_2 asphyxiation. A segment of ileum, 8 cm in length was excised about 1–2 cm from the ileo-caecal junction and the luminal contents were flushed out with warm Krebs-Henseleit solution (composition in mM: NaCl 118, KCl 5.6, $CaCl_2.2H_2O$ 2.5, $MgSO_4.7H_2O$ 1.19, NaH_2PO_4 1.3, $NaHCO_3$ 25, glucose 10; pH 7.4).

Electrically stimulated longitudinal muscle-myenteric plexus preparations (LMMPs)

Longitudinal muscle-myenteric plexus preparations (LMMPs), prepared as described by Paton & Zar (1968), were mounted isometrically (tension 5 mN) in 5 ml organ baths containing oxygenated (95% O_2 + 5% CO_2) Krebs-Henseleit solution. Each preparation was allowed to equilibrate at 37°C for at least 60 min before experiments were started.

Electrical field stimulation was delivered by means of two platinum electrodes placed at the top and the bottom of the chamber. Maximal nerve-mediated acetylcholine contractions were evoked by rectangular pulses with the following parameters: 0.1 Hz, 40–60 V, pulse duration 0.5 ms. After at least 10 min of reproducible 'twitch' contractions, cumulative concentration-response curves to each antidepressant drug were obtained. Drug-induced changes in 'twitch' height were expressed as percentage of the control contractions taken as 100% response.

In a separate set of unstimulated LMMPs, antidepressant drugs were tested against muscarinic contractile responses induced by 100 nM acetylcholine (ACh), the magnitude of which was equivalent to that of the 'twitch' contractions.

The above experiments were carried out to determine the range of antidepressant concentrations devoid of anti-acetylcholine activity, to be used in functional (contractility) studies with 5-HT and 5-HT-related agonists.

Functional studies in whole resting ileal segments and unstimulated LMMPs

Segments of whole ileum (1.5–2 cm long) were set up isometrically (tension 10 mN) in 10 ml organ baths containing oxygenated Krebs-Henseleit solution at 37°C. Tissues were allowed to equilibrate for 60 min with a 15 min wash cycle.

Non-cumulative concentration-response curves to 5-HT, 2-methyl-5-hydroxytryptamine (2-methyl-5-HT) and 5-methoxytryptamine (5-MeOT) were constructed in separate tissues using 0.5 log unit increments at 15 min intervals. Each

agonist concentration was removed as soon as the maximum effect was reached. After completion of concentration-response curves, tissues were washed for 30 min with Krebs-Henseleit solution containing a given antidepressant concentration, which was left in the bath during the construction of subsequent agonist curves. Only one antidepressant concentration was tested in each ileal preparation. Reversibility of the inhibitory effect caused by antidepressants on agonist-induced contractions was tested by repeating concentration-response curves in tissues rinsed with normal Krebs-Henseleit solution for at least 60 min.

In order to allow direct between-agonist comparisons, a series of concentration-response curves to 5-HT, 2-methyl-5-HT and/or 5-MeOT were constructed in single preparations. For between-agonist comparisons, responses were expressed as a percentage of the maximal response to 5-HT.

In separate experiments using unstimulated LMMPs, clomipramine (300 nM) was tested against contractions induced by 5-HT, 2-methyl-5-HT and 5-MeOT. This procedure was designed to evaluate whether the presence (whole ileal segments) or the absence (LMMPs) of mucosal 5-HT may influence the effects of drugs with 5-HT reuptake blocking properties.

Data analysis

Curves were analyzed by fitting them to a logistic equation of the form: $\text{Effect} = E_{maximum}/1 + e^{\{-2.303 \times \text{slope} \times (\log [A] - \log [A_{50}])\}}$ where: $E_{maximum}$ = maximum response; [A] = molar agonist concentration; [A_{50}] = molar agonist concentration inducing 50% of the maximum response. All data were fitted either to a single logistic expression or to the sum of two logistics. Goodness of fit to a single or double logistic expression was evaluated by the F-test of the residual variances using a significance criterion of $P<0.05$ (SAS Institute Inc., 1988).

Agonist potency values were expressed as $-\log$ EC for monophasic curves and as $-\log EC_1$ and $-\log EC_2$ for the first and second phase of biphasic curves, where EC indicates molar agonist concentration inducing 50% of the maximum effect. Antidepressant-induced change of agonist curve was calculated as a percentage of the maximum effect of agonist obtained before antidepressant addition. Antagonist pA_2 estimates were calculated following Schild regression analysis, using agonist concentration-ratios (CR) determined at EC_{50} levels in control and test curves. Confidence limits (CL) at 95% probability for the slope of regression were evaluated by using a computer programme (PHARM/PCS, Version 4.1) based on a manual of pharmacological calculations (Tallarida & Murray, 1986). Apparent affinity estimates (pK_B) from single antagonist concentrations were calculated by the Gaddum (1957) equation. All data in the text are expressed as means ± s.e.mean. Differences between means were analyzed by Student's two-tail t test. Values of $P<0.05$ were taken as statistically significant.

Drugs

5-Hydroxytryptamine hydrochloride and acetylcholine chloride were obtained from Sigma; 2-methyl-5-hydroxytryptamine (2-methyl-5-HT) maleate, 5-methoxytryptamine (5-MeOT) hydrochloride, and clomipramine hydrochloride were obtained from RBI; fluoxetine hydrochloride, paroxetine hydrochloride hemihydrate and litoxetine hydrochloride were kindly donated by Eli-Lilly Italia S.p.A., SmithKline Beecham (Great Britain) and Synthelabo Recherche (LERS) (France), respectively. BIMU 1 (3-ethyl-2,3-dihydro-N-(8-methyl-8-azabicyclo [3.2.1] oct-3-yl)-2-oxo-1H-benzimidazole-1-carboxamide hydrochloride) and MDL 72222 (1αH,3α, 5αH-tropan-3-yl-3,5-dichlorobenzoate) were synthesized by Boehringer Ingelheim, Italia. The radiolabelled ligands [^{3}H]-DAU 6215 (N-[endo-8-methyl-8-azabicyclo (3.2.1) oct-3-yl]-2,3-dihydro-2-oxo-1H-benzimidazol-1-carboxamide, hydrochloride) (83 Ci mmol^{-1}) and [^{3}H]-GR 113808 ([1-[2-[(methylsulphonyl)amino]ethyl]-4-piperidinyl]methyl-1-methyl-1H-indole-3-carboxylate) (82–85 Ci mmol^{-1}) were from Amersham International (UK). All drugs were dissolved in distilled water.

Results

Binding studies

IC_{50} values of antidepressants determined in membranes from NG 108-15 cells and pig striatum are shown in Table 1.

Clomipramine, paroxetine and fluoxetine inhibited [^{3}H]-DAU 6215 binding to 5-HT$_3$ recognition sites in NG 108-15 cells with IC_{50} values in the range 1.3–4 μM. Litoxetine was the most active compound showing an IC_{50} of 0.3 μM. The specific binding of [^{3}H]-GR 113808 to 5-HT$_4$ recognition sites in pig striatal membranes was inhibited by the antidepressant drugs with low potency (IC_{50} values ⩾ 20 μM).

Functional studies

5-HT (1 nM–30 μM), 2-methyl-5-HT (0.1–30 μM) and 5-MeOT (3 nM–10 μM) induced concentration-dependent contractions in whole segments of guinea-pig ileum. Curves to 5-HT were better fitted to a biphasic than to a monophasic model: ($F = 7.004$ $P<0.005$). The first phase occurred at 5-HT concentrations ranging from 1 nM to 0.3 μM, while the second phase in the range of 1 and 30 μM. $-\log EC_1$ and $-\log EC_2$ values for 5-HT were 7.99 ± 0.03 and 5.83 ± 0.02 for the first and second phase, respectively (Figure 1). The concentration-response curve to the selective 5-HT$_3$ receptor agonist, 2-methyl-5-HT was monophasic ($-\log EC = 5.45 \pm 0.01$) and the maximum response was 64.0 ± 2.7% of that

Table 1 Potency values (IC_{50}) of antidepressant drugs at 5-HT$_3$ receptors in NG 108-15 neuroblastoma-glioma cells and at 5-HT$_4$ receptors in pig corpus striatum homogenate

Test substance	*NG 108-15 cells* IC_{50} (nM)	*Corpus striatum* IC_{50} (nM)
Clomipramine	1308 ± 85	31500 ± 1200
Fluoxetine	4000 ± 150	42800 ± 1500
Paroxetine	2154 ± 110	66600 ± 1850
Litoxetine	315 ± 65	19600 ± 850

[^{3}H]-DAU 6215 and [^{3}H]-GR 113808 were used as ligands of 5-HT$_3$ and 5-HT$_4$ receptors, respectively. Data are expressed as means ± s.e.mean of 3 experiments.

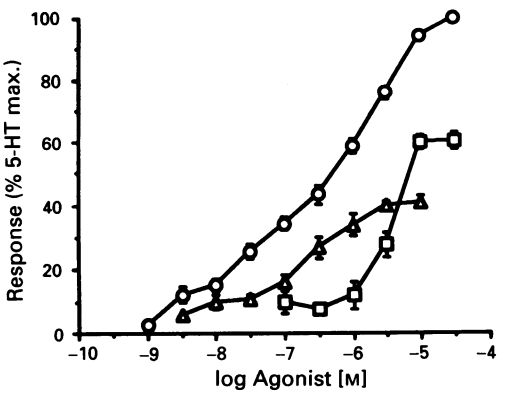

Figure 1 Concentration-response curves to 5-hydroxytryptamine (○), 2-methyl-5-hydroxytryptamine (□), and 5-methoxytryptamine (△) in isolated segments of whole guinea-pig ileum. Values are expressed as means ± s.e.mean, $n = 20$.

obtained with 30 μM 5-HT (Figure 1). The concentration-response curve to the selective 5-HT$_4$ receptor agonist 5-MeOT was also monophasic ($-\log EC = 6.92 \pm 0.02$). The maximum response ($41.0 \pm 1.5\%$) was not significantly different from that obtained with 0.3 μM 5-HT (Figure 1). In time control experiments, no evidence of desensitization was obtained, provided that there were frequent solution changes (every 5–10 min) and 30–60 min recovery periods between subsequent agonist concentration-response curves.

To assess the interaction of antidepressant drugs with 5-HT$_3$ and 5-HT$_4$ receptor-mediated contractions, these drugs were used at concentrations which were ineffective (or slightly effective) on neurogenic cholinergic 'twitch' contractions and on ACh-induced contractions in electrically stimulated and unstimulated LMMPs, respectively. In fact, the highest concentrations of clomipramine (300 nM), fluoxetine (3 μM), paroxetine (1 μM) and litoxetine (3 μM) tested did not reduce the amplitude of both indirect (Figure 2a) and direct ACh (100 nM)-mediated responses (Figure 2b) by more than 15%. None of these drugs incubated with the ileum before agonist administration changed the basal tone of the preparations. Clomipramine (10–300 nM) inhibited, by a mechanism not clearly dependent on concentration, both phases of the 5-HT curve with progressive reduction of maximum response up to 30% of control (Figure 3a). This prevented the evaluation of affinity estimates for the drug. Complete reversibility of the inhibitory effect caused by 300 nM clomipramine was obtained following a 60 min washing period. At low concentrations (30 and 100 nM), clomipramine slightly shifted the 2-methyl-5-HT concentration-response curve to the right in a concentration-independent manner (CR: 2.1 ± 0.02 and 2.1 ± 0.02 at 30 and 100 nM, respectively). At higher concentrations (300 nM), the drug caused a further rightward shift with marked (50%) depression of agonist response maximum (Figure 3b). At 100 and 300 nM, clomipramine concentration-dependently shifted the 5-MeOT concentration-response curve to the right, an effect associated with approximately 50% depression of maximum response (Figure 3c). The antagonist properties of clomipramine (including the reduction of agonist response maximum) were also observed in resting LMMPs. In these preparations, 300 nM clomipramine produced a rightward shift of 5-HT, 2-methyl-5-HT and 5-MeOT curves which was superimposable on that obtained in whole ileal segments (Figure 4).

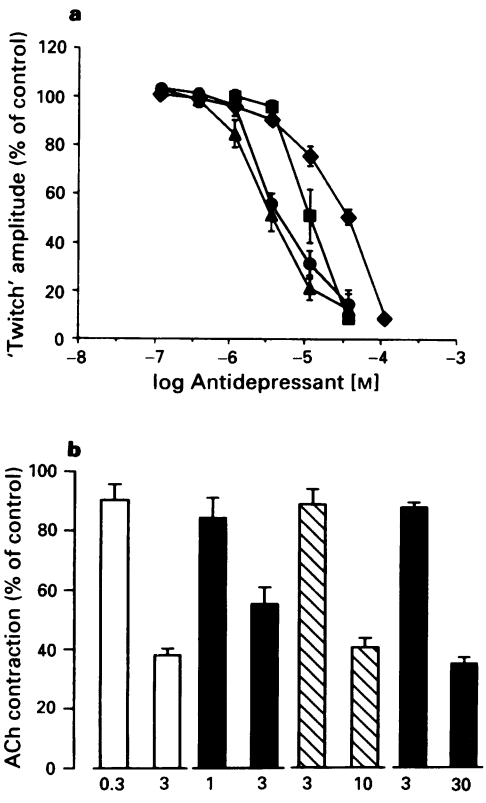

Figure 2 Concentration-response curves for clomipramine (▲), paroxetine (●), fluoxetine (■) and litoxetine (◆) in inhibiting nerve-mediated acetylcholine-mediated contractions to electrical field stimulation ('twitch') in longitudinal muscle-myenteric plexus preparations (LMMPs) from the guinea-pig ileum (a). In (b) the effects of 0.3 μM clomipramine (open column), 1 μM paroxetine (solid column), 3 μM fluoxetine (hatched column) and 3 μM litoxetine (stippled column) on contractile responses caused by 100 nM acetylcholine are shown. Higher clomipramine (3 μM), paroxetine (3 μM), fluoxetine (10 μM) and litoxetine (30 μM) concentrations significantly inhibited ($P < 0.05$) ACh-induced contractions. The latter antidepressant concentrations caused an approximately 50% reduction of twitch contraction amplitude (a). Values are expressed as means ± s.e.mean, $n = 4–6$.

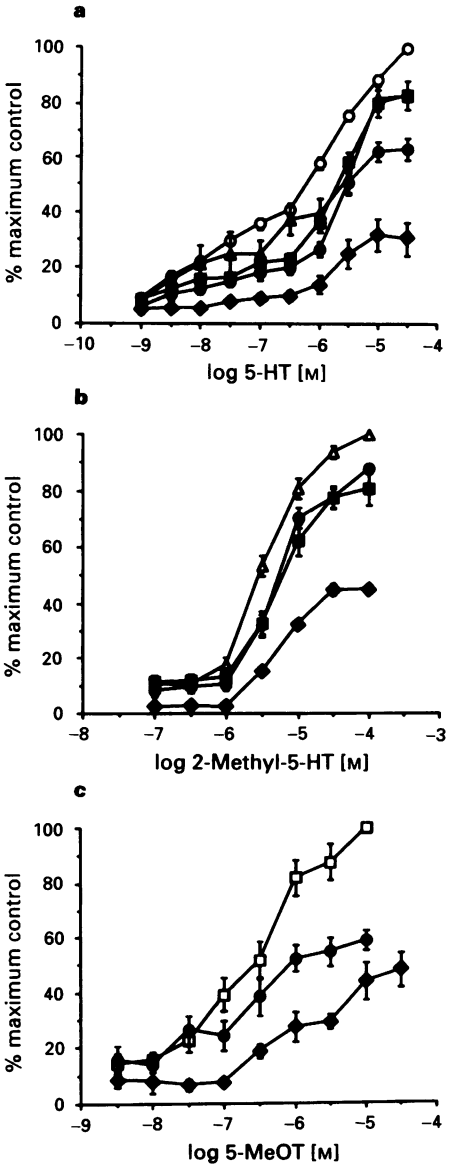

Figure 3 Effect of clomipramine on responses to 5-hydroxytryptamine (5-HT) (a), 2-methyl-5-hydroxytryptamine (2-methyl-5-HT) (b) and 5-methoxytryptamine (5-MeOT) (c) in isolated segments of whole guinea-pig ileum. Control responses (open symbols); responses in the presence of 10 (▲), 30 (■), 100 (●) and 300 nM (◆) clomipramine. Values are expressed as means ± s.e.mean, $n = 6–14$.

In whole ileal segments, fluoxetine (0.1–1 μM) reduced both phases of the 5-HT curve in a concentration-related manner with some depression of maximum response (Figure 5a). By comparison of the responses at the original EC_{50} level for the second phase (EC_2), Schild analysis yielded pA_2 estimates of 6.6 ± 0.3 (Schild slope of 1.1 (0.8–1.2)). At 3 μM, fluoxetine further shifted the 5-HT curve to the right, with marked depression (70%) of maximum effect. The antagonism caused by 3 μM fluoxetine was fully reversed by 60 min washing. In contrast, responses to 2-methyl-5-HT were slightly affected by 1 μM fluoxetine. Use of the Gaddum equation yielded a pK_B value of 5.4 ± 0.02. A higher fluoxetine concentration (3 μM) caused a parallel rightward displacement of the 2-methyl-5-HT curve up to 10 μM. However, this effect was followed by a decreased responsiveness of the preparation (≥ 50%) to higher 2-methyl-5-HT concentrations (Figure 5b). Curves to 5-MeOT were concentration-dependently shifted to the right by fluoxetine (range 0.1–1 μM), with slight (20%) depression of maximum response. However, since these fluoxetine concentrations are 40–400 fold lower than those interacting with central 5-HT$_4$ recognition sites, it is unlikely that the target of fluoxetine action in the ileum is the '5-HT$_4$ receptor'. In fact, affinity estimates of GR 113808 (a 5-HT$_4$ receptor ligand) at both the central and peripheral receptors are superimposable (Grossman *et al.*, 1993; Ford & Clarke, 1993; Bockaert *et al.*, 1994), thus presumably excluding 5-HT$_4$ receptor heterogeneity. Based on this assumption, affinity values for fluoxetine (as well as for litoxetine, see below) were not calculated. When fluoxetine was used at 3 μM, a marked (≥ 80%) depression of maximum agonist response was observed (Figure 5c).

Paroxetine was used at 1 μM only, since higher concentration produced marked inhibitory effects on cholinoceptor-mediated contractions in LMMPs. At this concentration, paroxetine did not alter the first high-potency phase of the

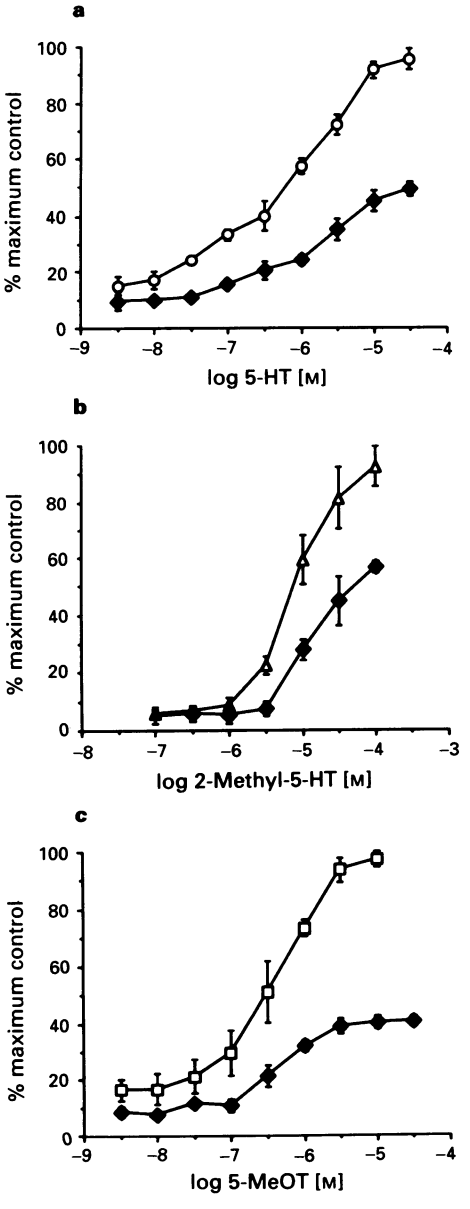

Figure 4 Effect of clomipramine on responses to 5-hydroxytryptamine (5-HT) (a), 2-methyl-5-hydroxytryptamine (2-methyl-5-HT) (b) and 5-methoxytryptamine (5-MeOT) (c) in longitudinal muscle-myenteric plexus preparations (LMMPs) of guinea-pig ileum. Control responses (open symbols); responses in the presence of 300 nM (closed symbols) clomipramine. Note that the inhibitory effect caused by clomipramine is not significantly different from that obtained in whole segments at the same concentration of the antidepressant (Figure 3). Values are expressed as means ± s.e.mean, $n = 4–6$.

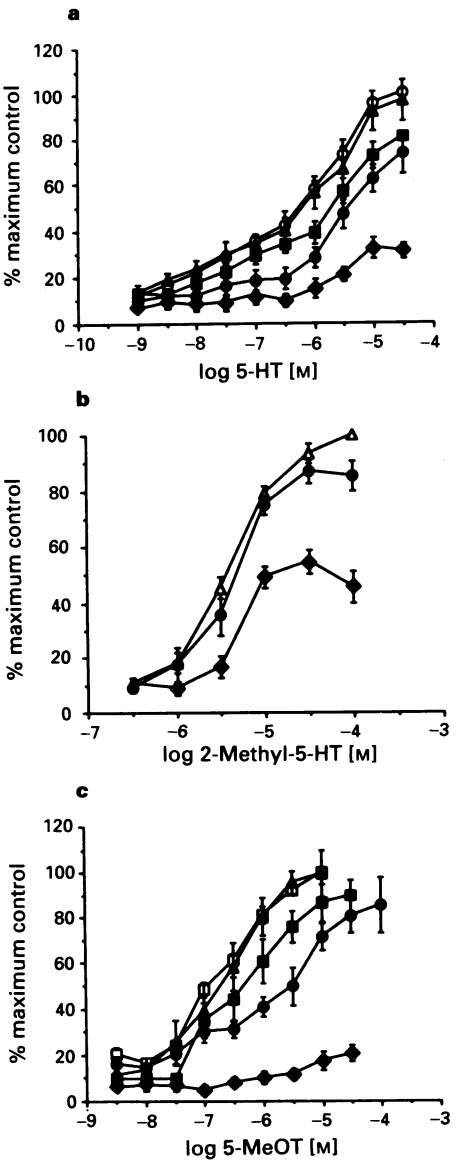

Figure 5 Effect of fluoxetine on responses to 5-hydroxytryptamine (5-HT) (a), 2-methyl-5-hydroxytryptamine (2-methyl-5-HT) (b) and 5-methoxytryptamine (5-MeOT) (c) in isolated segments of whole guinea-pig ileum. Control responses (open symbols); responses in the presence of 0.1 (▲), 0.3 (■), 1 (●) and 3 μM (◆) fluoxetine. Values are expressed as means ± s.e.mean, $n = 6–14$.

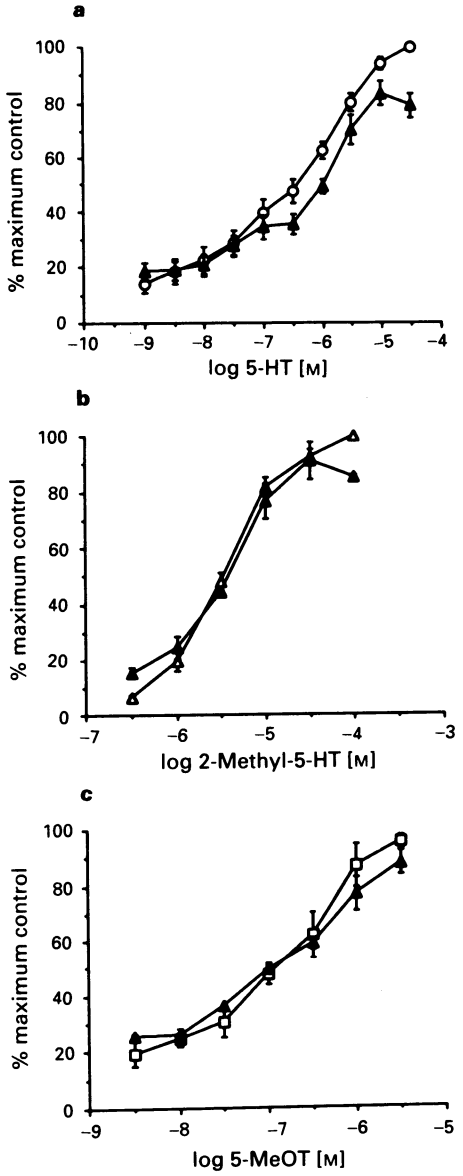

Figure 6 Effect of paroxetine on responses to 5-hydroxytryptamine (5-HT) (a), 2-methyl-5-hydroxytryptamine (2-methyl-5-HT) (b) and 5-methoxytryptamine (5-MeOT) (c) in isolated segments of whole guinea-pig ileum. Control responses (open symbols); responses in the presence of 1 μM paroxetine (▲). Values are expressed as means ± s.e.mean, $n = 4-8$.

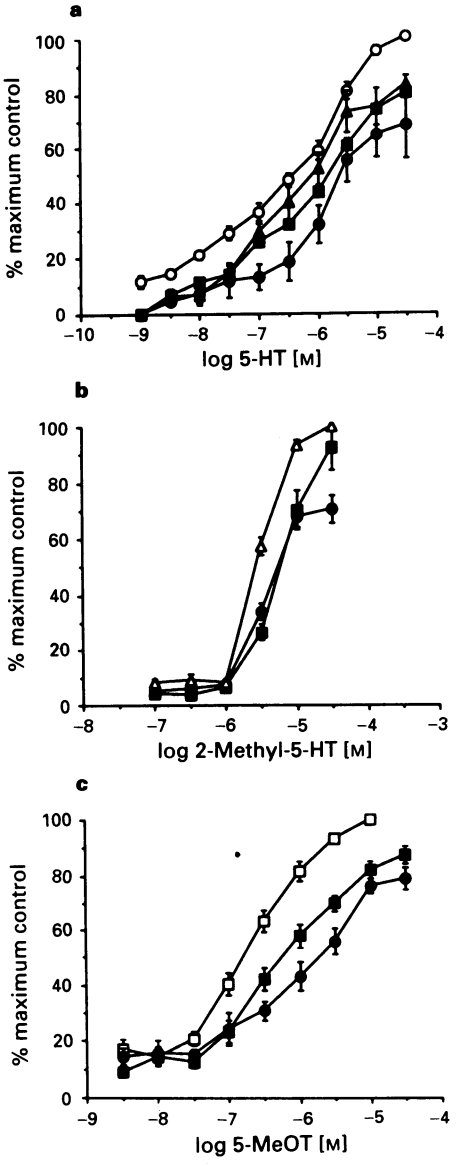

Figure 7 Effect of litoxetine on responses to 5-hydroxytryptamine (5-HT) (a), 2-methyl-5-hydroxytryptamine (2-methyl-5-HT) (b) and 5-methoxytryptamine (5-MeOT) (c) in isolated segments of whole guinea-pig ileum. Control responses (open symbols); responses in the presence of 0.3 (▲), 1 (■) and 3 μM (●) litoxetine. Values are expressed as means ± s.e.mean, $n = 6-18$.

5-HT curve, but caused a slight dextral shift of the second low-potency phase (pK_B: 6.1 ± 0.01). In contrast, the responsiveness of the preparations to 2-methyl-5-HT was unaffected by paroxetine, as was the concentration-response curve to 5-MeOT (Figure 6).

Like fluoxetine, litoxetine (0.3–3 μM) reduced both phases of the 5-HT curve in a concentration-related manner with some depression of maximum response (Figure 7a), an effect reversed by prolonged (60 min) washing. Use of the Schild analysis yielded a pA_2 estimate of: 6.6 ± 0.1 (Schild slope of 1.1 (0.9–1.2)). At 1 and 3 μM, litoxetine produced slight rightward displacements of 2-methyl-5-HT-induced contractions, which were virtually independent of the antidepressant concentration used (pK_B values: 6.0 ± 0.02 and 5.5 ± 0.01, respectively) (Figure 7b). Conversely, these concentrations shifted to the right, in an apparently concentration-dependent manner, the response caused by 5-MeOT (Figure 7c).

Discussion

A combined study of receptor binding in central neuronal cell membranes and functional responses in guinea-pig isolated small intestine preparations allowed this study to characterize the interaction of four antidepressant drugs with central and peripheral 5-HT$_3$ and 5-HT$_4$ receptors.

Receptor binding studies

In NG 108-15 neuroblastoma-glioma cell membranes, clomipramine, fluoxetine, paroxetine and litoxetine displaced tritiated DAU 6215 (a ligand of 5-HT$_3$ receptors) from its binding sites. In agreement with previous evidence, the IC_{50} values for clomipramine, fluoxetine and paroxetine were in the range 1.3–4 μM, suggesting a low potency of these drugs at 5-HT$_3$ receptors (Hoyer *et al.*, 1989; Schmidt & Peroutka,

1990; Angel *et al.*, 1993). Conversely, litoxetine was 4–12 fold more potent than the aforementioned antidepressants ($IC_{50} = 0.3\ \mu M$), suggesting a moderate potency at 5-HT$_3$ receptors, as previously reported by Angel *et al.* (1993) in rat cortical membranes. As far as the influence of antidepressants at central 5-HT$_4$ receptors is concerned, the results of binding experiments in pig striatal membranes indicate that all four bind to central 5-HT$_4$ recognition sites with negligible potency (IC_{50} values $\geqslant 20\ \mu M$). Following this evidence, our study was expanded to assess the influence of antidepressant drugs on neurogenic contractions evoked by 5-HT$_3$ and 5-HT$_4$ receptors in enteric preparations.

Functional studies

As previously reported (Buchheit *et al.*, 1985; Fox & Morton, 1990; Eglen *et al.*, 1990; Butler *et al.*, 1990; Craig *et al.*, 1990), in isolated segments of guinea-pig ileum, the concentration-contractile response curve to 5-HT was biphasic in nature. It is well established that the high- and low-potency phases of the 5-HT curve are mediated by the 5-HT$_4$ and 5-HT$_3$ receptors, respectively. Both the 5-HT$_4$ receptor agonist 5-MeOT and the 5-HT$_3$ receptor agonist 2-methyl-5-HT exhibited monophasic curves with potency values in agreement with previous evidence (Eglen *et al.*, 1990; Fox & Morton, 1990; Butler *et al.*, 1990; Turconi *et al.*, 1991; Buchheit *et al.*, 1992). 5-MeOT behaved as a full agonist (see also Eglen *et al.*, 1990; Turconi *et al.*, 1991), while 2-methyl-5-HT showed partial agonist properties. The latter findings are in agreement with those of Butler *et al.* (1990), but at variance with those of Eglen *et al.* (1990) and Fox & Morton (1990), who reported that in the guinea-pig ileum, 2-methyl-5-HT induces a maximal response comparable to that of 5-HT.

All four antidepressants were devoid of intrinsic activity in ileal segments. At concentrations not exceeding $3\ \mu M$, clomipramine, fluoxetine and litoxetine inhibited both the high- and low-potency phases of 5-HT curve. Conversely, paroxetine slightly affected the low-potency phase only. The effect of antidepressants on each phase of the 5-HT curve will be discussed separately.

The parallel rightward displacement of the low-potency phase caused by antidepressants (with the exception of clomipramine and within low ranges of concentration) allowed the evaluation of their apparent affinity estimates at enteric 5-HT$_3$ receptors. The resulting affinity estimates of fluoxetine (range $0.1-1\ \mu M$, pA_2: 6.6), paroxetine ($-\log K_B$: 6.1) and litoxetine (pA_2: 6.6) are consistent with low to moderate potency of these drugs, as observed at central and peripheral (vagus nerve) 5-HT$_3$ recognition sites (Hoyer *et al.*, 1989; Kilpatrick *et al.*, 1989; Schmidt & Peroutka, 1990; Angel *et al.*, 1993). In contrast, the antagonism exerted by clomipramine on the second phase of 5-HT curve was hardly dependent on concentration, thus preventing the evaluation of affinity estimates of this drug at enteric 5-HT$_3$ receptors. However some discrepancies emerged from data obtained using central and peripheral models. For example, litoxetine was 12 fold more potent than fluoxetine at central 5-HT$_3$ receptors, while it possesses the same affinity as fluoxetine at peripheral 5-HT$_3$ sites. Furthermore, at variance with pure 5-HT$_3$ receptor antagonists, whose affinity is significantly lower in guinea-pig tissues suggesting a species variant of the 5-HT$_3$ receptor (Butler *et al.*, 1990; Kilpatrick & Tyers, 1992; Wong *et al.*, 1992; 1993), antidepressants do not show clear-cut differences between their potency/affinity values in central and peripheral (guinea-pig ileum) tissues.

The antagonism of litoxetine towards 5-HT$_3$ receptor-mediated contraction is in agreement with previous evidence obtained by Galzin *et al.* (quoted by Angel *et al.*, 1993) in the same experimental model. However, it is noteworthy that fluoxetine (like clomipramine and, to a minor extent, litoxetine) at the highest concentration tested, markedly reduced 5-HT response maximum, a feature which suggests unsurmountable antagonism rather than competitive antagonism. As mentioned above, in the guinea-pig ileum both 5-HT$_3$ and 5-HT$_4$ receptors participate in the contractile response of 5-HT, which is mainly dependent on acetylcholine release from cholinergic neurones (Eglen *et al.*, 1990; Tonini *et al.*, 1991; Ford & Clarke, 1993). However, the reduction of 5-HT response maximum caused by antidepressants cannot be ascribed to their potential anti-acetylcholine (antimuscarinic) activity (Thomas *et al.*, 1987), since the highest antidepressant concentrations used in functional studies were previously found to affect only slightly cholinoceptor-mediated contractions to electrical stimulation or to applied ACh in LMMPs. Another possible explanation is that the apparent unsurmountable antagonism of the 5-HT$_3$ and 5-HT$_4$ receptor-mediated responses observed with antidepressant compounds may be due to receptor desensitization. In fact, in the presence of the reuptake blockers, 5-HT, released from the enterochromaffin cells of the mucosa, may remain in contact with the receptors for a sufficiently long time to desensitize them, thus shifting the 5-HT curve in a non-competitive manner. This possibility however, has been ruled out by the observation that in LMMPs, clomipramine reduced responses to 5-HT (as well as to 2-methyl-5-HT and 5-MeOT) to an extent similar to that observed in whole ileal segments. Furthermore, in the latter preparations, paroxetine and litoxetine, which possess the highest potency/affinity in inhibiting 5-HT uptake in rat synaptosomes (Thomas *et al.*, 1987; Scatton *et al.*, 1988; Benfield *et al.*, 1986), were significantly less potent than clomipramine and fluoxetine in inhibiting contractile responses to 5-HT. This would further exclude any 5-HT reuptake blocking mechanism in the inhibition of 5-HT responses caused by antidepressant compounds.

Unsurmountable antagonism, leading to reduction of maximum agonist response, could be expected as a result of pseudoirreversible competitive antagonism (Kenakin, 1987). This type of antagonism, for example, is exerted by the 5-HT$_1$/5-HT$_{2A}$ receptor antagonist, metergoline, on 5-HT-induced vasoconstrictor response in the rat isolated kidney (Bond *et al.*, 1989). Pseudoirreversible antagonism may partly explain our findings, since inhibition of 5-HT contractile responses produced by clomipramine, fluoxetine and litoxetine was reversed very slowly by washing. This may reflect slow dissociation kinetics of antidepressants from the 5-HT$_3$ receptor (and/or slow diffusion from tissues), and not true irreversibility.

To characterize further the antagonism of antidepressants at the 5-HT$_3$ receptor, additional studies were conducted with the selective 5-HT$_3$ receptor agonist 2-methyl-5-HT (Richardson *et al.*, 1985). However, the interaction of antidepressants with 2-methyl-5-HT-induced responses was less clearcut than that obtained with 5-HT as an agonist. In fact, fluoxetine at a concentration ($1\ \mu M$) which effectively antagonized the 5-HT$_3$ receptor-mediated component of 5-HT curve (Figure 4a) was poorly effective on 2-methyl-5-HT responses (pK_B: 5.4), while paroxetine was ineffective. Furthermore, concentrations of litoxetine (1 and $3\ \mu M$) which produced concentration-dependent dextral shift of the 5-HT curve, slightly antagonized 2-methyl-5-HT-induced contractions in an apparent concentration-independent manner (Figure 6b). Concentration-independent antagonism was also produced by clomipramine (30 and 100 nM), as observed in 5-HT experiments. High clomipramine, fluoxetine and litoxetine concentrations reduced 2-methyl-5-HT response maximum by an extent similar to that observed with 5-HT. Based on partial discrepancy of results with 5-HT and 2-methyl-5-HT emerging from our functional studies, other experimental models, such as the 5-HT$_3$ receptor-mediated vagal reflex bradycardia (von Bezold Jarish reflex), are required to assess the potential interaction of antidepressants with peripheral 5-HT$_3$ receptors. Very recently, imipramine and fluoxetine were found to inhibit the inward current mediated by 5-HT$_3$ receptor activation in rat nodose ganglion neurones, thus providing additional evidence for an interaction of

antidepressants with 5-HT$_3$ sites (Fan, 1994).

As mentioned above, all antidepressants, with the exception of paroxetine, inhibited the 5-HT$_4$ receptor-mediated high-potency phase of the 5-HT curve. This was a rather unexpected finding, since antidepressants had negligible potency at central 5-HT$_4$ receptors as measured by radioligand binding ($IC_{50} \geqslant 20\ \mu M$). Antagonism of antidepressant drugs at ileal 5-HT$_4$ receptors was further investigated with the selective agonist, 5-MeOT (Eglen *et al.*, 1990; Hill *et al.*, 1990; Fozard, 1990). Clomipramine, fluoxetine and litoxetine inhibited 5-MeOT-induced ileal contractions at concentrations 6–100 fold lower than those required to bind striatal 5-HT$_4$ recognition sites. In the case of fluoxetine (range 0.1–1 μM) and litoxetine, a concentration-related dextral shift of the 5-MeOT curve with slight reduction (20%) of maximum agonist effect was observed. However, this type of antagonism cannot be ascribed to competitive antagonism for two reasons. First, previous receptor binding and functional studies have demonstrated that central and peripheral 5-HT$_4$ receptors are a homogeneous receptor population, which is recognized with comparable affinity by 5-HT$_4$ receptor ligands (Grossman *et al.*, 1993; Ford & Clarke, 1993; Bockaert *et al.*, 1994). Second, high concentrations of clomipramine (300 nM) and fluoxetine (3 μM) (albeit much lower than those affecting 5-HT$_4$ receptor binding) caused a marked reduction (50–80%) of agonist response maximum. Therefore, the peripheral target of antidepressants might not be the 5-HT$_4$ receptor, but rather an allosteric site in the receptor macromolecule (never demonstrated, however, in central binding studies) or, more likely, post-receptor site(s), involving inhibition of transduction signalling pathways. In this respect, antidepressants have been found to modulate, at least after chronic treatment, post-receptor transduction mechanisms (i.e. G proteins) leading either to increased (Menkes *et al.*, 1983) or decreased cyclic AMP generation (Lesch *et al.*, 1991; 1992).

In conclusion, results from binding studies in NG 108-15 neuroblastoma-glioma cells suggest that the antidepressants clomipramine, fluoxetine, paroxetine possess low and litoxetine moderate potency at central 5-HT$_3$ receptors. Although less homogeneous data have been obtained in the guinea-pig ileum, antidepressants seem to possess comparable affinity also for peripheral enteric 5-HT$_3$ receptors. Binding experiments in pig striatum homogenates demonstrated that antidepressant drugs are virtually ineffective at central 5-HT$_4$ receptors. Nevertheless, in functional studies, submicromolar or micromolar concentrations of clomipramine, fluoxetine and litoxetine effectively inhibited 5-MeOT-induced ileal contractions, through a mechanism which may reflect either allosteric antagonism or post-receptor blockade of second messenger (i.e. cyclic AMP) generation.

Based on our findings, the interaction of antidepressants with central and peripheral 5-HT$_3$ and 5-HT$_4$ receptors may be relevant to their therapeutic action (Greenshaw, 1993; Angel *et al.*, 1993; Bockaert *et al.*, 1994) and may explain their constipating effect (Sanger *et al.*, 1991), which can be further exacerbated by anticholinoceptor properties (Benfield *et al.*, 1986; McTavish & Benfield, 1990; Dechant & Clissold, 1991; Leonard, 1992).

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Pharmacological characterization of α_1-adrenoceptor subtypes in rat heart: a binding study

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1 The α_1-adrenoceptor subtypes of rat heart were characterized in binding experiments performed with [^{3}H]-prazosin as the radiolabel. The specific binding to the α_1-adrenoceptors was determined with 0.3 μM prazosin, because phentolamine (10 μM) was insufficient to inhibit completely the specific binding of high concentrations of [^{3}H]-prazosin.

2 In saturation experiments, [^{3}H]-prazosin bound to two distinct affinity sites (pK_D = 10.39 and 8.19). The proportion of the low affinity sites was approximately 84% of total specific binding. Membranes pretreated with chloroethylclonidine (CEC, 10 μM) also showed two distinct affinity sites for [^{3}H]-prazosin, although the maximum numbers of high and low affinity sites were reduced by 86 and 64%, respectively.

3 In competition experiments, [^{3}H]-prazosin (100 pM) binding was inhibited by WB4101 (2-(2,6-dimethoxy-phenoxyethyl)aminomethyl-1,4-benzodioxane) and 5-methylurapidil. The inhibition curves displayed shallow slopes which could be subdivided into high and low affinity components (pK_I = 10.43 and 8.36 for WB4101, 8.62 and 6.61 for 5-methylurapidil). However, unlabelled prazosin or HV723 (α-ethyl-3,4,5-trimethoxy-α-(3-((2-(2-methoxyphenoxy)-ethyl)amino)propyl)benzeneacetonitrile fumarate) competed for [^{3}H]-prazosin binding monophasically (pK_I = 10.34 and 8.28, respectively). In CEC-pretreated membranes, prazosin, WB4101, 5-methylurapidil and HV723 antagonized the [^{3}H]-prazosin (100 pM) binding monophasically (pK_I = 9.70, 9.56, 8.60 and 8.82, for each antagonist).

4 On the other hand, 1000 pM [^{3}H]-prazosin binding was inhibited by unlabelled prazosin biphasically (pK_I = 10.49 and 8.49). HV723 did not discriminate both prazosin-high and low affinity sites (pK_I = 8.18).

5 These results suggest the presence of at least three distinct α_1-adrenoceptor subtypes in rat hearts (two prazosin-high affinity sites and one prazosin-low affinity site). According to the recent α_1-adrenoceptor subclassifications, one of the former two sites corresponds to the α_{1B} subtype with low affinities for WB4101 and 5-methylurapidil and sensitive to CEC, while another site with relatively high affinities for WB4101 and 5-methylurapidil may be classical α_{1A}, cloned α_{1C}, α_{1D} subtypes or their mixture. The prazosin-low affinity site corresponds to putative α_{1L} subtype with low affinity for HV723, which may be predominantly involved in the positive inotropic response to phenylephrine.

Keywords: α_1-adrenoceptor subtype; α_{1B}-adrenoceptor; α_{1L}-adrenoceptor; rat heart; [^{3}H]-prazosin binding

Introduction

α_1-Adrenoceptors were originally subdivided into two subclasses (classical α_{1A} and α_{1B} subtypes on the basis of binding experiments) (Morrow & Creese, 1986; Han *et al.*, 1987a). However, subsequent molecular biological studies have revealed the existence of at least three α_1-adrenoceptor subtypes (cloned α_{1A} or α_{1D}, α_{1B} and α_{1C}) with high affinity for prazosin (1993 Receptor Nomenclature supplement, Trends In Pharmacological Sciences; Lomasney *et al.*, 1991a; García-Sáinz, 1993). Pharmacological features of the classicial α_{1B} subtype are essentially similar to those of the cloned α_{1B} subtype; high affinity for prazosin but low affinity for WB4101 (2-(2,6-dimethoxy-phenoxyethyl)aminomethyl-1, 4-benzodioxane) and 5-methylurapidil (Pimoule *et al.*, 1992; Ford *et al.*, 1994). The classicial α_{1A} subtype shows high affinities for WB4101 and 5-methylurapidil, while the cloned α_{1A} subtype is less sensitive to both the antagonists (Han *et al.*, 1987a; Hanft & Gross, 1989; Lomasney *et al.*, 1991b; Schwinn & Lomasney, 1992). The cloned α_{1C} and α_{1D} subtypes are also highly sensitive to WB4101 but the α_{1D} subtype may be distinguished by its lower sensitivity to 5-methylurapidil than the cloned α_{1C} subtypes (Lomasney *et al.*, 1991a; Perez *et al.*, 1991). Recently, it was suggested that the classicial α_{1A} and cloned α_{1C} subtypes are the same on the basis of evidence from molecular biological and pharmacological studies (Ford *et al.*, 1994; Forray *et al.*, 1994). On the other hand, another subclassification has been proposed from functional and binding studies, whereby the α_1-adrenoceptors can be separated into three subtypes (α_{1H}, α_{1L} and α_{1N}) (Muramatsu *et al.*, 1990; 1994; Oshita *et al.*, 1991; 1993; Ohmura *et al.*, 1992; Bylund *et al.*, 1994). Prazosin has a higher affinity for the α_{1H} subtype than for the α_{1L} and α_{1N} subtypes. The third α_{1N}-adrenoceptor subtype is distinguished by its higher affinity for HV723 (α-ethyl-3, 4, 5-trimethoxy-α-(3-((2-(2-methoxyphenoxy) ethyl)-amino)propyl)benzeneacetonitrile fumarate) from the α_{1L} subtype. Although the three subtypes have not yet been identified by molecular cloning technique, the classical α_{1A} and cloned α_{1A}, α_{1B}, α_{1C}, α_{1D} subtypes mentioned above may be included in the α_{1H} group of the latter subclassification because of their high affinity for prazosin (Muramatsu *et al.*, 1994).

The α_1-adrenoceptors occur not only in blood vessels but also in heart and circulatory system and are involved in the positive inotropic response in hearts (Endoh & Blinks, 1988; Puceat *et al.*, 1992; Terzic *et al.*, 1993). Recently, the cardiac α_1-adrenoceptors have been suggested to be related to certain pathological conditions such as congestive heart failure, cardiomyopathy, hypertension and hypothyroidism (Terzic *et al.*, 1993). The stimulation of cardiac α_1-adrenoceptors activates protein synthesis (Meidell *et al.*, 1986) and induces cardiac hypertrophy (Simpson *et al.*, 1990). However, it is

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not known what kinds of α_1-adrenoceptor subtypes occur in heart and are involved in the physiological responses. Recently, we have demonstrated that the positive inotropic response to phenylephrine in rat atria may be mediated through an α_1-adrenoceptor subtype which has low affinity for prazosin (putative α_{1L} subtype) (Noguchi *et al.*, 1993). In the present study, we have characterized the α_1-adrenoceptor subtypes of rat heart by use of binding techniques.

Methods

Binding study

Male adult Wistar rats weighing 250–350 g were used. The hearts were removed immediately after exsanguination and were perfused through the aorta with ice-cold saline. After connective tissue and atria had been removed, the ventricle was blotted, weighed and homogenized in 40 vol. of buffer (Tris HCl 50 mM, NaCl 100 mM, EDTA 2 mM, pH 7.4) with a polytron (setting 8, 15 s × 3). The homogenates were filtered through four layers of gauze (0.83 mm mesh) and centrifuged at 80,000 *g* for 20 min at 4°C. The pellets were resuspended in the same volume of assay buffer (Tris HCl 50 mM, EDTA 1 mM, pH 7.4), incubated 10 min at 37°C, and centrifuged again as described above. All procedures to prepare membranes were conducted at 4°C except preincubation of the membranes, and ice cold buffers were used. The final pellet was resuspended in assay buffer and used for the binding assay. The membranes were incubated with [^{3}H]-prazosin for 45 min at 30°C. Incubation volume was 1 ml (approximately 140 μg protein/tube), but displacement experiments with CEC-treated membranes were carried out in a final volume of 2 ml (approximately 250 μg protein/tube). Reactions were terminated by rapid filtration in a Brandel M30 cell harvester on to Whatman GF/C filters. The filters were then washed three times with 4 ml of ice cold 50 mM Tris-HCl buffer (pH 7.4), dried and the filter-bound radioactivity determined. Non-specific binding was defined as binding in the presence 0.3 μM prazosin unless mentioned elsewhere. Assays were conducted in duplicate.

Chloroethylclonidine (CEC) treatment

Membrane preparations were incubated 20 min at 37°C with 10 μM CEC and centrifuged at 80,000 *g* for 30 min. The pellets were washed once with assay buffer before the binding experiment.

Calculations

Binding data were analysed by the weighted Least-squares interactive curve fitting programme, LIGAND (Munson & Rodbond, 1980). The data were first fitted to a one- and then a two-site model, and if the residual sums of squares was statistically less for a two-site fit of the data than for a one-site, as determined by *F*-test comparison, then the two-site model was accepted. *P* values less than 0.05 were considered significant. Proteins were assayed according to the method of Bradford with bovine serum albumin used as standard (Bradford, 1976).

Drugs

Drugs used were: [^{3}H]-prazosin (specific activity 76.2 Ci mmol^{-1}, NEN, Boston, U.S.A.), (±)-propranolol hydrochloride (Nacalai, Kyoto, Japan), prazosin hydrochloride (Sigma, St. Louis, U.S.A.), WB4101 hydrochloride, 5-methylurapidil, chloroethylclonidine dihydrochloride (CEC) (Funakoshi, Tokyo, Japan), HV723 (α-ethyl-3,4,5-trimethoxy-α-(3-(2-(2-methoxyphenoxy) ethyl))amino)propyl) benzeneacetonitrile fumarate, Hokuriku Seiyaku, Katsuyama, Fukui, Japan), noradrenaline bitartrate (Sigma, St. Louis, U.S.A.), phentolamine mesylate (Ciba, Basel, Switzerland).

Results

Saturation experiments with [^{3}H]-prazosin

[^{3}H]-prazosin at concentrations ranging from 10–7000 pM was used to label α_1-adrenoceptors of rat ventricles. When nonspecific binding was determined with 0.3 μM prazosin, the specific binding was approximately 80% of the total binding at 300 pM [^{3}H]-prazosin and showed a saturable tendency at the concentrations of 6000–7000 pM (Figure 1). Scatchard plots of the binding data were curvilinear (Figure 2a), suggesting the presence of more than a single class of binding site. LIGAND analysis fitted the data to a two site model. The pK_D values of high and low affinity sites were 10.39 ± 0.07 and 8.19 ± 0.04, and the B_{max} values for both sites were 77 ± 16 and 391 ± 178 fmol mg^{-1} protein, respectively ($n = 4$) (Table 1). In contrast, using 10 μM phentolamine as non specific binding, the amount of specific binding was much lower than that determined by 0.3 μM prazosin and reached a plateau at 1000–2000 pM [^{3}H]-prazosin (Figure 1). Scatchard analysis of the binding data resulted in a linear plot (Figure 2b), indicating binding of [^{3}H]-prazosin to a one site model under these conditions. The pK_D value was 9.94 ± 0.01 and B_{max} value was 100 ± 31 fmol mg^{-1} protein ($n = 3$). This suggests either that 10 μM phentolamine may not be a high enough concentration to detect the specific [^{3}H]-prazosin binding to all subclasses of α_1-adrenoceptors in this preparation, or that 0.3 μM prazosin may detect additional binding sites of [^{3}H]-prazosin besides α_1-adrenoceptors. These possibilities were examined by comparing the ability of various α_1-adrenoceptor antagonists to inhibit the binding of a high concentration (5000 pM) of [^{3}H]-prazosin. As shown in Figure 3, phentolamine at 10 μM was less potent than unlabelled prazosin (0.1, 0.3 and 1 μM), WB4101 (10 μM) and HV723 (10 μM) in inhibiting the [^{3}H]-prazosin binding. The inhibitory potency of prazosin (0.3 and 1.0 μM) was equal to that of WB4101 and HV723. Thus, the specific binding of [^{3}H]-prazosin to the α_1-adrenoceptors of rat heart membranes seemed to be suitable to be determined by prazosin, and 0.3 μM prazosin was used to determine the non specific binding in the following experiments.

The membranes pretreated with 10 μM CEC also showed two distinct affinity sites for [^{3}H]-prazosin. Although both the affinities were not significantly different from the corresponding values estimated in CEC-untreated membranes, the maximum numbers of high and low affinity sites reduced by

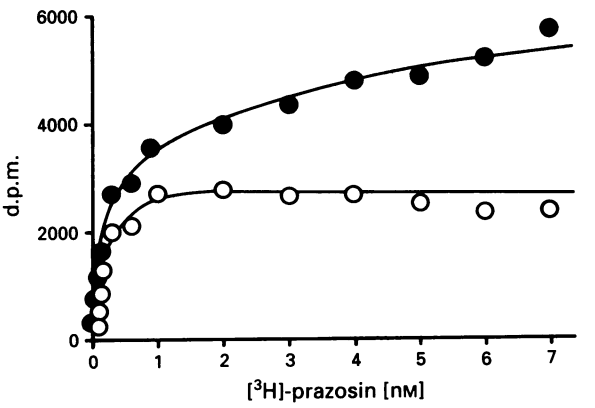

Figure 1 Saturation curves of [^{3}H]-prazosin binding to rat ventricle membranes. Specific binding was determined by 0.3 μM prasozin (●) or 10 μM phentolamine (○). The data were obtained from a single experiment with the same membranes, where each point is the mean of duplicate determinations.

86 and 64%, respectively, by pretreatment with 10 μM CEC (Table 1).

Displacement experiments

The pharmacological profile of high and low affinity sites for prazosin was examined in displacement experiments. [^{3}H]-prazosin at 100 pM was used to label the high affinity sites for prazosin. Unlabelled prazosin displaced the binding in a monophasic manner with a high pK_I value (10.34 ± 0.10) (Table 2). On the other hand, computerized analysis of the displacement curves for WB4101 or 5-methylurapidil revealed the presence of two distinct affinity sites, suggesting heterogeneity of the high affinity site for prazosin. The proportion of the high affinity sites for WB4101 and 5-methylurapidil was approximately 35% of specific binding at prazosin-high affinity sites. HV723 antagonized the [^{3}H]-prazosin binding monophasically with a single pK_I value (8.28) (Table 2).

The binding of 100 pM [^{3}H]-prazosin in CEC-pretreated membranes was monophasically inhibited by all the drugs used. The pK_I values for prazosin, WB4101 and 5-methylurapidil were close to the values of the high affinity sites in CEC-untreated membranes (Table 3).

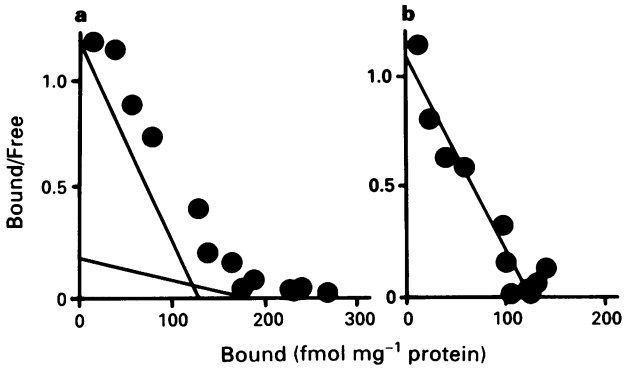

Figure 2 Scatchard plots for specific [^{3}H]-prazosin binding to rat ventricle membranes in saturation experiments. Specific binding was determined with 0.3 μM prazosin (a) and 10 μM phentolamine (b).

Table 1 Binding of [^{3}H]-prazosin to α_1-adrenoceptors of rat ventricle membranes

Treatment	n	*pK_D, high*	*pK_D, low*	*R_{high}*	*R_{low}*
None	4	10.39 ± 0.07	8.19 ± 0.04	77 ± 16	391 ± 178
CEC	4	10.63 ± 0.08	8.53 ± 0.20	11 ± 2	141 ± 24

Data shown are means ± s.e.mean. n = number of experiments. p$K_{D,high}$ and p$K_{D,low}$ are negative log of the equilibrium dissociation constants at the high and low affinity sites, respectively. R_{high} and R_{low}: maximal number of prazosin high and low affinity sites (fmol mg^{-1} protein). CEC, membranes pretreated with chloroethylclonidine (CEC, 10 μM) for 20 min and then washed.

The low affinity sites for [^{3}H]-prazosin were characterized in the displacement experiments with a higher concentration of [^{3}H]-prazosin (1000 pM) and unlabelled prazosin as competitor. LIGAND analysis fitted the data to a two site model (slope factor = 0.58 ± 0.09). The pK_I values of high and low affinity sites were 10.49 ± 0.19 and 8.49 ± 0.20, respectively (n = 3). The proportion of the high affinity sites for prazosin was 52% of total binding sites. HV723 inhibited 1000 pM [^{3}H]-prazosin binding monophasically (pK_I = 8.18 ± 0.20, n = 3).

Discussion

The present study demonstrates that prazosin binds to two distinct populations of binding sites in the rat heart. The finding of two distinct affinity sites for prazosin is new, because only a single affinity site has been demonstrated for

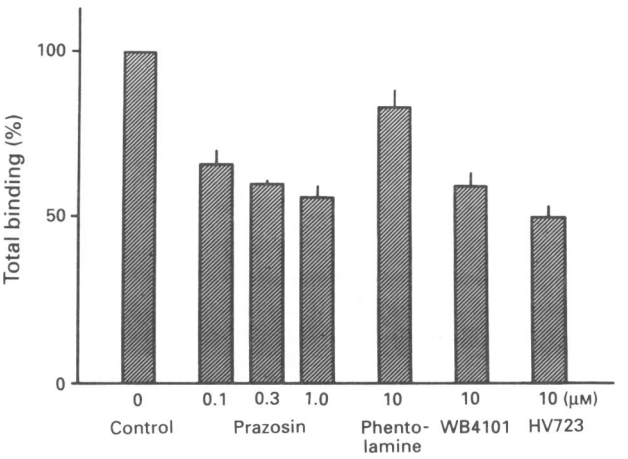

Figure 3 Potency of various α_1-adrenoceptor antagonists in inhibiting [^{3}H]-prazosin (5000 pM) binding to rat ventricle membranes. The total binding of the same membrane preparations in the absence of antagonist is represented as 100% on the ordinate scale. Mean ± s.e.mean of 5 experiments.

Table 3 Inhibition by α-adrenoceptor antagonists of [^{3}H]-prazosin (100 pM) binding to α_1-adrenoceptors of rat ventricles pretreated with chloroethylclonidine (CEC)

Antagonist	n	*pK_I*	*Slope factor*
Prazosin	3	9.70 ± 0.12	1.04 ± 0.08
WB4101	3	9.56 ± 0.1	0.93 ± 0.05
5-Methylurapidil	3	8.60 ± 0.17	0.96 ± 0.01
HV723	3	8.82 ± 0.14	0.91 ± 0.03

Data shown are means ± s.e.mean. n = number of experiments. Displacement experiments were done with 100 pM [^{3}H]-prazosin. pK_I: negative log of the equilibrium dissociation constant (− log M).

Table 2 Inhibition of [^{3}H]-prazosin (100 pM) binding to α_1-adrenoceptors of rat ventricle by α-antagonists

Antagonist	n	*pK_I, high*	*pK_I, low*	*% high*	*Slope factor*
Prazosin	4	10.34 ± 0.10	–	100	0.86 ± 0.05
WB4101	3	10.43 ± 0.12	8.36 ± 0.14	35 ± 5	0.60 ± 0.05[a]
5-Methylurapidil	4	8.62 ± 0.30	6.61 ± 0.06	34 ± 5	0.63 ± 0.02[a]
HV723	4	8.28 ± 0.04	–	100	0.92 ± 0.05

Data shown are means ± s.e.mean. n = number of experiments. Displacement experiments were done with 100 pM [^{3}H]-prazosin. p$K_{I,high}$ and p$K_{I,low}$: negative log of the equilibrium dissociation constants (− log M) at prazosin-high and low affinity sites for antagonists tested. % high: population binding at the high affinity site compared to the total specific binding sites. [a]Significantly different from unity (P<0.05).

prazosin in previous studies (Hanft & Gross, 1989; Michel *et al.*, 1994). This discrepancy may be in part associated with the radioligand concentrations used, as the low affinity sites may not have been detected when only low concentrations of [^{3}H]-prazosin are used (Muramatsu *et al.*, 1994). Another reason for this conflicting result may be a difference in the antagonists used to determine specific binding. This point was first checked, and we found that phentolamine at 10 μM is insufficient to inhibit completely the specific binding of high concentrations of [^{3}H]-prazosin to α_1-adrenoceptors. These results strongly suggest that the specific binding of [^{3}H]-prazosin to α_1-adrenoceptors in rat heart muscle must be determined by prazosin or other antagonists (such as WB4101 and HV723) instead of phentolamine. We used 0.3 μM prazosin in the present study.

The prazosin-high affinity sites were characterized in the displacement experiments with WB4101 or 5-methylurapidil. Both the antagonists bound to two distinct (high and low) affinity sites in a 35:65 ratio, suggesting heterogeneity of the high affinity sites for prazosin. Recently, Michel *et al.* (1994) also reported that prazosin-high affinity sites of rat ventricle could be subdivided by 5-methylurapidil or (+)-nigludipine into high and low affinity sites in a 20:80% ratio.

At present, 5 subtypes in α_1-adrenoceptors with high affinity for prazosin (classical α_{1A} and cloned α_{1A}, α_{1B}, α_{1C}, α_{1D}) are reported, where the cloned α_{1A} subtype may be different from the classical α_{1A} subtype determined in binding experiments (Schwinn & Lomasney, 1992). Rather, a possibility that classical α_{1A} and cloned α_{1C} subtypes are the same has been proposed (Ford *et al.*, 1994; Forray *et al.*, 1994). The classical α_{1A} and the cloned α_{1C} and α_{1D} subtypes are more sensitive to WB4101 ($pK_B \geqslant 9$) as compared with the α_{1B} subtype. 5-Methylurapidil shows higher affinity ($pK_B \geqslant 9$) towards the classical α_{1A} and cloned α_{1C} subtypes and an intermediate affinity (pK_B close to 8) for the α_{1D} subtype. 5-Methylurapidil also shows a low affinity (pK_B close to 7) towards the cloned α_{1A} and α_{1B} subtypes (Han *et al.*, 1987a,b; Cotecchia *et al.*, 1988; Schwinn *et al.*, 1990; Lomasney *et al.*, 1991a,b; Perez *et al.*, 1991; Ford *et al.*, 1994). CEC produces a remarkable inactivation of the α_{1B} subtype although a partial inactivation occurs in other subtypes. According to these pharmacological criteria, the characteristics of α_1-adrenoceptor of rat ventricle obtained in the present study show that the prazosin-high affinity sites are subdivided into at least two subclasses. The one is α_{1B} which is remarkably inactivated by CEC and is low sensitive to WB4101 ($pK_B = 8.4$) and 5-methylurapidil ($pK_B = 6.6$), while another may be classicial α_{1A} (or cloned α_{1C}) and/or cloned α_{1D} because of relatively high affinity for WB4101 ($pK_B > 9.5$) and for 5-methylurapidil ($pK_B = 8.6$). Michel *et al.* (1994) recently reported that the prazosin- and 5-methylurapidil-high affinity sites of rat ventricle were the classical α_{1A} subtype because the sites showed a high affinity for (+)-nigludipine, a high affinity probe for classicial α_{1A}. However, molecular biological studies have demonstrated the existence of mRNA of α_{1B}, α_{1C} and α_{1D} (or cloned α_{1A}) subtypes in rat heart (Cotecchia *et al.*, 1988; Lomasney *et al.*, 1991a; Perez *et al.*, 1991; Rokosh *et al.*, 1994), suggesting the coexistence of α_1-subtypes with high affinity for prazosin.

On the other hand, α_1-adrenoceptors with low affinity for prazosin are classified into α_{1L} and α_{1N} subtypes by their different affinities for HV723. However, WB4101 and 5-methylurapidil cannot discriminate between α_{1L} and α_{1N} subtypes (Ohmura *et al.*, 1992; Muramatsu, 1992; Muramatsu *et al.*, 1994). It is likely that the prazosin-low affinity sites detected in the present study correspond to the α_{1L} subtype because of low affinity for HV723.

Phenylephrine and other α_1-adrenoceptor agonists produce positive inotropic responses in hearts of various species (Benfey, 1982; Puceat *et al.*, 1992; Terzic *et al.*, 1993). Recently, we found that the positive inotropic response of rat hearts to phenylephrine was inhibited by prazosin, WB4101 and HV723 with relatively low pA_2 values ranging from 7.90 to 8.79, suggesting a possible involvement of prazosin-low affinity sites in the positive inotropic response (Noguchi *et al.*, 1993). A good correlation between the pA_2 values for antagonists in the functional study and the low pK_D values in the present binding study strongly supports our previous view that the positive inotropic response to the α_1-adrenoceptor agonist is predominantly mediated through the α_{1L} subtype in the rat heart.

CEC was originally reported to produce a selective and complete inactivation of the α_{1B} subtype (Han *et al.*, 1987b). However, a partial inactivation is produced at the other subtypes by CEC (present study; Schwinn *et al.*, 1990; Oshita *et al.*, 1991; Lomasney *et al.*, 1991b; Ohmura *et al.*, 1992). We previously reported a lack of inhibitory effect of CEC on the positive inotropic response to phenylephrine (Noguchi *et al.*, 1993). This may be related to a possible occurrence of spare receptors with the α_{1L} subtype. Indeed, the prazosin-low affinity sites of rat heart were more resistant to CEC and were larger in density as compared with the high affinity sites.

In conclusion, the present study first indicates the occurrence of prazosin-low affinity α_1-adrenoceptor subtypes (presumably α_{1L}) in addition to prazosin-high affinity sites in the rat ventricles. The α_{1L} subtype may be predominantly involved in the positive inotropic response to phenylephrine.

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Inhibition of morphine withdrawal by the association of RB 101, an inhibitor of enkephalin catabolism, and the CCK_B antagonist PD-134,308

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1 The effects induced in rats on naloxone-precipitated morphine withdrawal syndrome by the new mixed inhibitor of enkephalin catabolism able to cross the blood-brain barrier RB 101 (N-{(**R,S**)-2-benzyl-3[(S)(2-amino-4-methylthio)butyl dithio]-1-ox-opropyl-L-phenylalanine benzyl ester) given alone or associated with the selective CCK_B antagonist, PD-134,308, were investigated.

2 The systemic administration of RB 101 (5, 10 and 20 mg kg^{-1}, i.v.) elicited a significant decrease in 8 of the 14 withdrawal signs evaluated. PD-134,308 (3 mg kg^{-1}, i.p.) did not modify the expression of morphine abstinence when given alone, but induced a strong facilitation of RB 101 responses (12 of 14 withdrawal signs were decreased). This potentiation was particularly intense in peripherally mediated withdrawal signs.

3 In order to clarify the biochemical mechanisms implicated in these responses, the effects induced by the association of RB 101 and PD-134,308 on the occupation of brain opioid receptors by endogenous enkephalins were also investigated in mice. PD-134,308, as well as RB 101, inhibited [^{3}H]-diprenorphine binding to opioid receptors. These results suggest that an increase in endogenous enkephalin levels induced by PD-134,308 could participate in the facilitation of RB 101 behavioural responses.

4 RB 101 has a promising potential role in the management of the opiate withdrawal syndrome. CCK_B antagonists, such as PD-134,308 may be useful in potentiating this anti-withdrawal effect.

Keywords: Morphine withdrawal; RB 101; PD-134,308; endogenous enkephalins; cholecystokinin; CCK_B receptors; [^{3}H]-diprenorphine; *in vivo* binding.

Introduction

Neutral endopeptidase 24.11 'enkephalinase' (Malfroy *et al.*, 1978) and aminopeptidase N (Hambrook *et al.*, 1976; Waksman *et al.*, 1985) are the two main enzymes responsible for the *in vivo* degradation of the enkephalins. Several inhibitors of these enzymes, selective for one enzyme or with dual selectivity, have been designed. These compounds have been shown to protect enkephalins from catabolism *in vitro* and *in vivo*, and to elicit pharmacological properties deriving from this protection (review in Roques *et al.*, 1993). The administration of the specific neutral endopeptidase inhibitors, thiorphan and phosphoramidon, into the lateral ventricle (Dzoljic *et al.*, 1986; Haffmans & Dzoljic 1987) were shown to minimize the severity of the morphine withdrawal syndrome in rats. The intensity of this syndrome was also reduced by the peripheral administration of acetorphan (prodrug of thiorphan) or the selective neutral endopeptidase inhibitor, SCH 34828 (Livingston *et al.*, 1988; Dzoljic *et al.*, 1992). However, the attenuating effect of these selective neutral endopeptidase inhibitors was limited since several signs remained unaffected, and in the case of peripheral injection of acetorphan or SCH 34828, some signs were even increased (Dzoljic *et al.*, 1992). A more intense inhibition of the withdrawal syndrome was induced by central administration of the complete inhibitors of enkephalin catabolism, kelatorphan or RB 38 A (Maldonado *et al.*, 1989, 1992a). These inhibitors also induced much higher antinociceptive responses than selective neutral endopeptidase compounds, but were unable to enter the brain following systemic administration (Fournié-Zaluski *et al.*, 1984; Schmidt *et al.*, 1991).

Recently, a new mixed inhibitor of enkephalin catabolism, able to cross the blood-brain barrier, RB 101 (N-{(**R,S**)-2-benzyl-3[(S)(2-amino-4-methylthio)butyl dithio]-1-ox-opropyl}-L-phenylalanine benzyl ester) has been synthesized (Fournié-Zaluski *et al.*, 1992). This compound elicits potent antinociceptive responses in mice and rats after systemic administration (Noble *et al.*, 1992), by elevating the extracellular levels of enkephalins (Ruiz-Gayo *et al.*, 1992). The opportunity to use this compound by a peripheral route is a promising step towards the development of new therapeutic tools.

On the other hand, the cholecystokinin octapeptide (CCK-8) is discretely distributed in several areas of the central nervous system (CNS) (Vanderhaegen *et al.*, 1975; Gall *et al.*, 1987) and it has been suggested that it plays an opposite role to endogenous opioids in the control of several CNS functions. Thus, the administration of cholecystokinin octapeptide (CCK-8) or CCK agonists antagonized the antinociceptive effects induced by opioids, whereas the CCK antagonists potentiated this opioid response (review in Baber *et al.*, 1989). Two subtypes of CCK receptors have been pharmacologically identified and recently cloned: the CCK_A receptor, located mainly in peripheral tissues, and the CCK_B receptor, predominantly found in the CNS (Moran *et al.*, 1986; Kopin *et al.*, 1992; Wank *et al.*, 1992). The interaction between CCK and opioids seems to be selectively mediated through CCK_B receptors at least on the control of nociceptive stimuli (Dourish *et al.*, 1988; 1990; Maldonado *et al.*, 1993; Valverde *et al.*, 1994) and emotional effects (Derrien *et al.*, 1994b). Thus, we have recently demonstrated that the CCK_B antagonists, L-365,260, PD-134,308 and RB 211, but not the CCK_A antagonist, lorglumide, were able to potentiate

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strongly the antinociceptive responses induced by RB 101 (Maldonado *et al.*, 1993; Valverde *et al.*, 1994). The strongest antinociceptive effect was obtained with the association of PD-134,308 (Hughes *et al.*, 1990) and RB 101 (Valverde *et al.*, 1994). The biochemical mechanisms involved in the facilitatory effect induced by CCK_B antagonists on endogenous enkephalin-induced pharmacological responses remain unclear.

The aim of this study was, first, to investigate the effects induced on naloxone-precipitated morphine withdrawal syndrome by systemic administration of RB 101; second, to evaluate the possible facilitatory effect induced by the CCK_B antagonist, PD-134,308, on the response induced by the peptidase inhibitor; and third, to clarify the biochemical mechanisms implicated in these behavioural responses.

Methods

Animals

Male Sprague-Dawley rats (Depré, France), ranging in weight from 200 to 220 g at the beginning of the experiment, were used in the behavioural morphine withdrawal studies. Male albino mice (Depré, France) weighing 20–22 g were used in the *in vivo* binding studies. Animals were housed in cages with water and food made available *ad libitum*, and maintained in a controlled environment.

Induction of physical dependence and morphine withdrawal

Rats were divided into 16 groups ($n = 10$) corresponding to morphine treatment groups (8) and saline control groups (8). Saline and morphine HCl were injected i.p. twice daily. The morphine dose was progressively increased from 8 mg kg^{-1} to 30 mg kg^{-1} over a period of 5 days. The first and second number in parentheses represent the dose of morphine (mg kg^{-1}) injected at 09 h 00 min and 19 h 00 min, respectively on consecutive days: 1st day (8, 10), 2nd day (15, 20), 3rd day (25, 30), 4th day (30, 30), 5th day (30; only at 09 h 00 min). The withdrawal syndrome was precipitated by injection of naloxone HCl (1 mg kg^{-1}, s.c.) 2 h after the final morphine administration. RB 101 was administered i.v. at the doses of 5, 10 and 20 mg kg^{-1}, 10 min before precipitation of withdrawal syndrome. PD-134,308 was injected i.p. at a dose of 3 mg kg^{-1}, 30 min before naloxone administration. The doses of these compounds were selected from antinociceptive responses induced on the rat tail-flick test (Valverde *et al.*, 1994).

Immediately after naloxone administration, animals were placed individually in test chambers consisting of round boxes (30 cm diameter × 35 cm height) and withdrawal signs were evaluated during a 25 min period. Intensity of withdrawal was tested by measuring several classical signs of morphine abstinence as reported by Bläsig *et al.* (1973). Two classes of behavioural signs were measured. The number of bouts of teeth chattering, mastication, wet dog shakes and jumping were counted. Ptosis, rhinorrhea, lacrimation, eye twitch and diarrhoea were evaluated over 5 min periods with one point being given for the presence of each sign during each period. The number of periods showing the sign were then counted (maximum score: 5). Salivation, piloerection and locomotor activity were also evaluated over 5 min periods giving a value between 0 and 2 for each period. Here the values were added for the whole 10 min period (maximum score 10).

Colonic temperature was determined by inserting a lubricated temperature probe 5 cm into the rectum of rats. The temperature was recorded 1 min later with a thermometer (Model TE 3, Ellab instruments, Carrieri). Body weight and colonic temperature were determined two min before, and 30 and 60 min after naloxone injection.

In vivo *binding studies*

The rapid membrane filtration technique was used to separate bound and free radioactivity in the brain (Pert and Snyder, 1975). [^{3}H]-DPN was injected i.v. at a dose of 15 pmol per mouse. RB 101 (1, 2.5, 5 and 10 mg kg^{-1}, i.v.) and PD-134,308 (1 and 3 mg kg^{-1}, i.p.) were administered 5 and 15 min respectively before the injection of [^{3}H]-DPN. Animals were killed 15 min after RB 101 injection and their brains were quickly removed. Total brains (minus cerebellum) were homogenized for 10 s in 10 ml ice cold 50 mM Tris-HCl buffer, (pH = 7.4) with a Brinkman Polytron. Aliquots of 0.15 ml were immediately filtered through Whatman GF/B glass filters and rinsed twice with ice-cold buffer. Four filters were placed in a scintillation vial containing 15 ml of Biofluor, and the radioactivity was counted. Each point was made in triplicate. Total radioactivity in the homogenate was determined by counting 0.6 ml of the preparation as previously described (Ruiz-Gayo *et al.*, 1992).

Drugs

RB 101 (mesylate salt), the inhibitor of enkephalin-degrading-enzymes (Fournié-Zaluski *et al.*, 1992), and PD-134,308, the CCK_B antagonist (Horwell *et al.*, 1991) were synthesized in our laboratory as described. Naloxone HCl, morphine HCl and cremophor EL were obtained from Sigma laboratories, and [^{3}H]-diprenorphine ([^{3}H]-DPN) (31 Ci $mmol^{-1}$) from Amersham. RB 101 was dissolved in the following vehicle: ethanol (10%), cremophor EL (10%) and distilled water (80%). PD-134,308 was suspended in carboxymethylcellulose 0.5%. Naloxone HCl, morphine HCl and [^{3}H]-DPN were dissolved in saline (0.9% NaCl). [^{3}H]-DPN was administered in a constant volume of 0.1 ml. All the other drugs were administered to rats in a volume of 1 ml kg^{-1} and to mice in a volume of 1 ml 100 g^{-1}.

Statistical analysis

Pharmacological results in rats are expressed as absolute values. Changes in colonic temperature and weight losses were analyzed by two-way analysis of variance. The factors of variation were group (between subjects) and time (within subjects). If a significant effect of treatment or interaction with treatment was observed, one way analysis of variance was used to determine the significance at each time point. Behavioural signs of withdrawal were analyzed by one-way analysis of variance between subjects. In all the cases, individual comparisons were made by the Newman-Keuls test. The level of significance was $P < 0.05$.

In vivo binding results were expressed as the ratio between specific bound radioactivity (B) and free radioactivity (F). The rate of displacement of [^{3}H]-DPN binding was measured as a percentage of B/F found in treated mice vs B/F found in controls. Non specific binding was defined as the B/F ratio found in mice receiving the same dose of [^{3}H]-DPN co-injected with 300 nmol of naloxone. Data were analyzed by an one-way analysis of variance followed by Newman Keuls's test comparisons. The level of significance was $P < 0.05$.

Results

Behavioural changes

Non-dependent animals Control rats chronically treated with saline and injected with saline or PD-134,308 (3 mg kg^{-1}) did not show any behavioural signs of opiate withdrawal after naloxone (1 mg kg^{-1}). RB 101 (5, 10 and 20 mg kg^{-1}) induced a non-significant trend to induce wet dog shake behaviour. When PD-134,308 was associated with RB 101 this effect was facilitated, and a significant presence of wet

dog shakes was observed with the highest dose of RB 101 (20 mg kg^{-1}). A trend to increase locomotor activity was also observed by the co-administration of PD-134,308 plus RB 101 (Table 1).

Morphine dependent animals After naloxone administration (1 mg kg^{-1}), morphine-dependent rats acutely treated with saline showed a withdrawal syndrome characterized by numerous behavioural changes. PD-134,308 (3 mg kg^{-1}), given alone, did not modify any sign of withdrawal (Figures 1, 2 and 3). RB 101 (5, 10 and 20 mg kg^{-1}) reduced significantly the frequency of several withdrawal signs. Jumping and teeth chattering were significantly reduced at all the doses used (Figure 1). Wet dog shakes and eye twitch were decreased after the administration of 10 and 20 mg kg^{-1} of RB 101 (Figures 1 and 2). Mastication, lacrimation and hyperlocomotor activity were also reduced, but only with the highest dose (20 mg kg^{-1}) (Figures 1, 2 and 3).

The co-administration of PD-134,308 (3 mg kg^{-1}) facilitated the effects induced by RB 101 (5, 10 and 20 mg kg^{-1}) on behavioural signs of withdrawal. Thus, jumping, wet dog shakes, teeth chattering, salivation, rhinorrhea and lacrimation were significantly reduced at all the doses of RB 101 used (Figures 1, 2 and 3). Mastication, piloerection, hyperlocomotor activity, diarrhoea and eye twitch were reduced when PD-134,308 was associated with the doses of 10 and 20 mg kg^{-1} of RB 101 (Figures 1, 2 and 3). The only sign not significantly decreased was ptosis, but a tendency (45% of reduction) was observed when PD-134,308 was co-administered with the highest dose of RB 101 (20 mg kg^{-1}) (Figure 2).

Table 1 Wet dog shakes behaviour and locomotor activity: effects induced by the administration of saline, PD-134,308 (3 mg kg^{-1}, i.p.), RB 101 (5, 10 and 20 mg kg^{-1}, i.v.) or the association of PD-134,308 (3 mg kg^{-1}, i.p.) plus RB 101 (5, 10 and 20 mg kg^{-1}, i.v.) on control animals chronically treated with saline (5 days, twice a day, i.p.) and injected with naloxone (1 mg kg^{-1}, s.c.)

	Wet dog shakes	*Locomotor activity*
Saline (i.v.) + saline (i.p.)	0.26 ± 0.26	2.03 ± 0.44
Saline (i.v.) + PD-134,308 (3 mg kg^{-1}, i.p.)	0.33 ± 0.33	1.83 ± 0.16
RB 101 (5 mg kg^{-1}, i.v.) + saline (i.p.)	0.50 ± 0.34	1.55 ± 0.34
RB 101 (5 mg kg^{-1}, i.v.) + PD-134,308 (3 mg kg^{-1}, i.p.)	0.33 ± 0.21	1.83 ± 0.47
RB 101 (10 mg kg^{-1}, i.v.) + saline (i.p.)	0.66 ± 0.21	1.83 ± 0.47
RB 101 (10 mg kg^{-1}, i.v.) + PD-134,308 (3 mg kg^{-1}, i.p.)	1.66 ± 0.76	3.66 ± 0.80
RB 101 (20 mg kg^{-1}, i.v.) + saline (i.p.)	4.16 ± 2.00	2.50 ± 0.61
RB 101 (20 mg kg^{-1}, i.v.) + PD-134,308 (3 mg kg^{-1}, i.p.)	5.83 ± 0.98*	4.33 ± 0.66

Colonic temperature

Non-dependent animals No significant changes in colonic temperature were observed after naloxone administration (1 mg kg^{-1}) in non-dependent rats treated with saline, PD-134,308 (3 mg kg^{-1}), RB 101 (5, 10 and 20 mg kg^{-1}) or the association of PD-134,308 plus RB 101 (data not shown).

Morphine-dependent animals Naloxone injection (1 mg kg^{-1}) induced a hypothermia in morphine-dependent rats acutely treated with saline. This hypothermia was not modified by the pretreatment with PD-134,308 (3 mg kg^{-1}). RB 101 administration (5, 10 and 20 mg kg^{-1}) reduced significantly the temperature loss measured 60 min after naloxone at all the doses used. No significant effect was observed in the temperature measured 30 min after naloxone. When PD-134,308 was associated with RB 101, a significant antagonism of the hypothermia was observed 30 and 60 min after nalox-

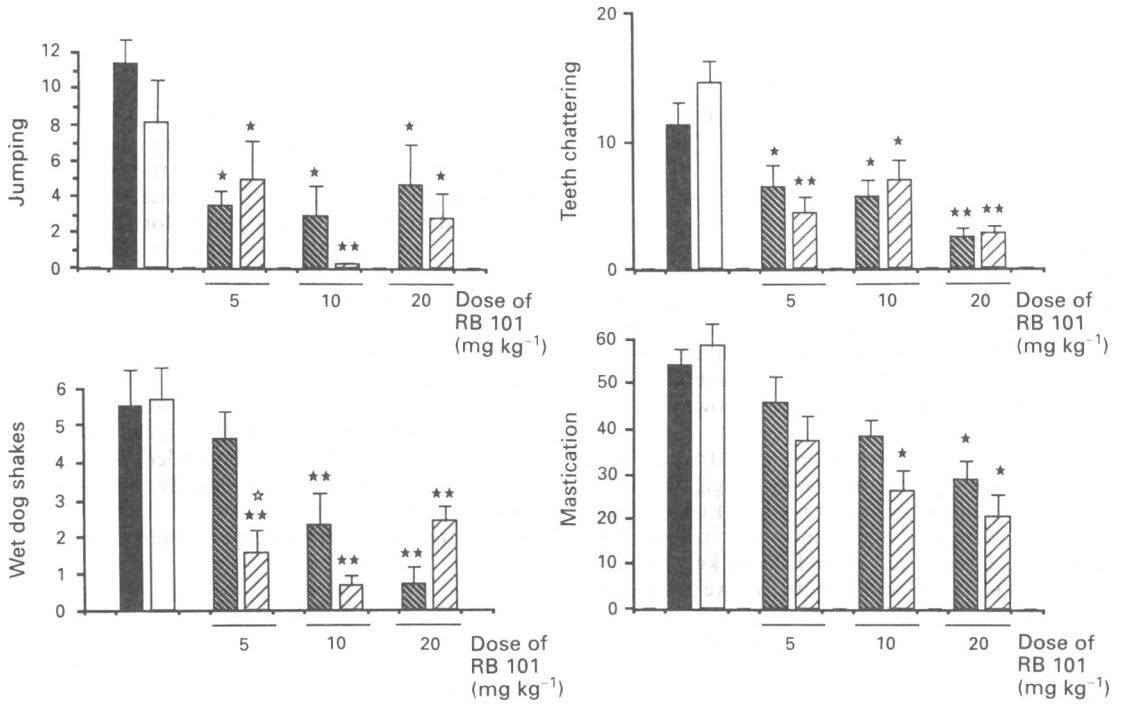

Figure 1 Signs of withdrawal syndrome (jumping, teeth chattering, wet dog shakes, mastication) after naloxone administration (1 mg kg^{-1}) in rats treated chronically with morphine (from 8 mg kg^{-1} to 30 mg kg^{-1} for 5 days): effect of acute pretreatment with saline, PD-134,308 (3 mg kg^{-1}, i.p.), RB 101 (5, 10 and 20 mg kg^{-1}, i.v.) or PD-134,308 (3 mg kg^{-1}, i.p.) plus RB 101 (5, 10 and 20 mg kg^{-1}, i.v.). (■) Saline (i.v.) + saline (i.p.); (□) saline (i.v.) + PD-134,308 (3 mg kg^{-1}, i.p.); (▨) RB 101 (i.v.) + saline (i.p.); (▨) RB 101 (i.v.) + PD-134,308 (3 mg kg^{-1}, i.p.). Values are mean ± s.e.mean. Number of animals per group = 10. *, $P<0.05$; **, $P<0.01$: vs saline + saline group. *, $P<0.05$: vs RB 101 + saline group (Newman-Keuls test).

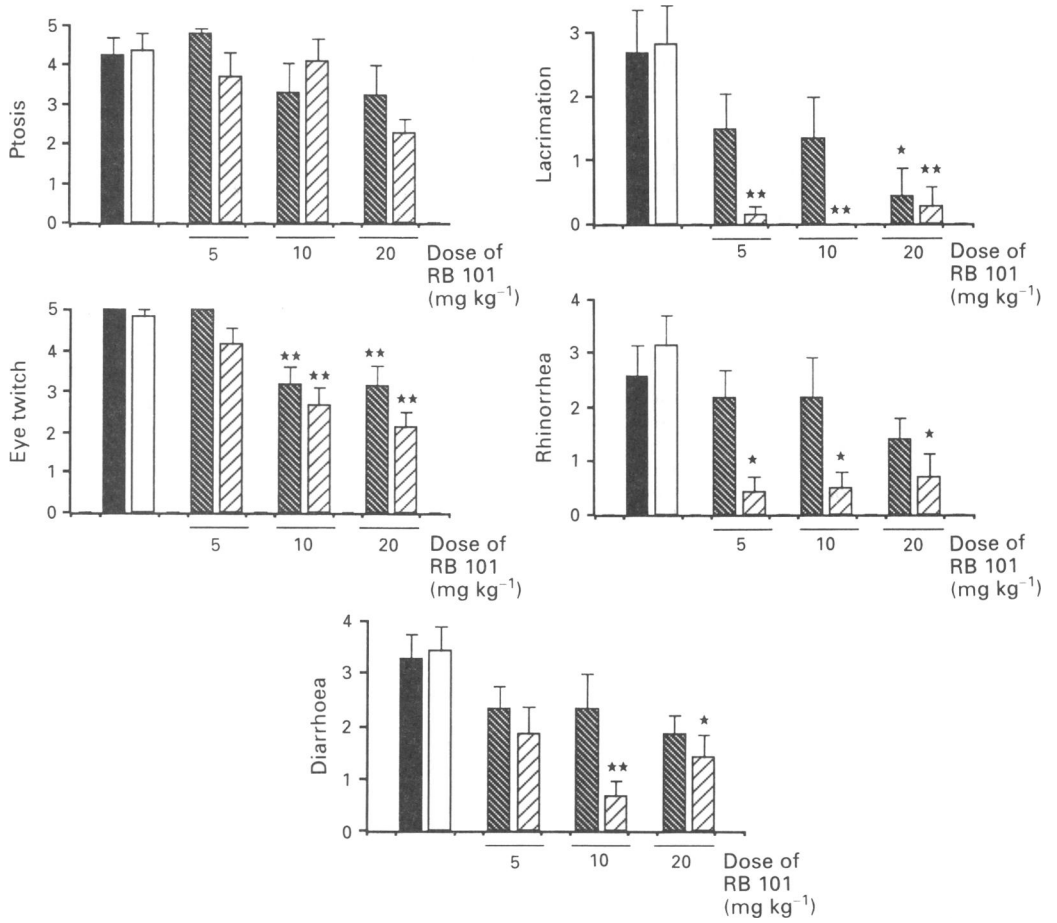

Figure 2 Signs of withdrawal syndrome (ptosis, lacrimation, eye twitch, rhinorrhea, diarrhoea) after naloxone administration (1 mg kg^{-1}) in rats treated chronically with morphine (from 8 mg kg^{-1} to 30 mg kg^{-1} for 5 days): effect of acute pretreatment with saline, PD-134,308 (3 mg kg^{-1}, i.p.), RB 101 (5, 10 and 20 mg kg^{-1}, i.v.) or PD-134,308 (3 mg kg^{-1}, i.p.) plus RB 101 (5, 10 and 20 mg kg^{-1}, i.v.). (■) Saline (i.v.) + saline (i.p.); (□) saline (i.v.) + PD-134,308 (3 mg kg^{-1}, i.p.); (▨) RB 101 (i.v.) + saline (i.p.); (▨) RB 101 (i.v.) + PD-134,308 (3 mg kg^{-1}, i.p.). Values are mean ± s.e.mean. Number of animals per group = 10. *, $P<0.05$; **, $P<0.01$: vs saline + saline group (Newman-Keuls test).

one, but only at the highest dose of RB 101 (20 mg kg^{-1}) (Figure 4).

Weight loss

Non-dependent animals Small weight losses (less than 1% of body weight within 60 min) were observed in non-dependent rats after naloxone injection (1 mg kg^{-1}). No significant modification was induced after treatment with PD-134,308 (3 mg kg^{-1}), RB 101 (5, 10 and 20 mg kg^{-1}) or the association of PD-134,308 and RB 101 (data not shown).

Morphine-dependent animals Dependent rats treated with acute saline lost 3.5 and 4% of their body weight within 30 and 60 min after naloxone injection (1 mg kg^{-1}) respectively. These weight losses were not significantly modified by PD-134,308 (3 mg kg^{-1}), RB 101 (5, 10 and 20 mg kg^{-1}) nor by the association of PD-134,308 and RB 101. However, a trend towards a decrease in weight loss was observed in groups receiving the association of PD-134,308 plus RB 101 (data not shown).

Global withdrawal score

A global withdrawal score was individually calculated for each rat by using a range of possible score from 0 to 80 for the observed withdrawal signs, as previously reported (Maldonado *et al.*, 1992b). To obtain this global value and to give all the signs proportional weighting, the score obtained for each sign was multiplied by a constant. Weight loss and hypothermia contributed 8 points each (4 points for the value measured at 30 min and 4 points for the value measured at 60 min). Teeth chattering, jumping, salivation, rhinorrhea and lacrimation contributed 7 points each. Mastication, wet dog shakes, piloerection and locomotor activity contributed 5 points each. Ptosis, eye twitch and diarrhoea contributed 3 points each. Morphine-dependent rats treated with acute saline elicited a high score of withdrawal after naloxone injection (1 mg kg^{-1}). This score was not modified by the administration of PD-134,308 (3 mg kg^{-1}). RB 101 (5, 10 and 20 mg kg^{-1}) induced a dose-dependent decrease of the global score. This effect was significantly potentiated, at all the doses of RB 101 used (5, 10 and 20 mg kg^{-1}), by the co-administration of PD-134,308 (3 mg kg^{-1}) (Figure 5).

In vivo *binding experiments*

Effect of RB 101 administration on [^{3}H]-diprenorphine in vivo binding in mice The i.v. administration of RB 101 induced a reduction in [^{3}H]-DPN binding, as previously reported (Ruiz-Gayo *et al.*, 1992). RB 101 at a dose of 1 mg kg^{-1} induced only a not significant decrease in [^{3}H]-DPN binding (% of specific binding = 89.2 ± 15.3). The lowest dose producing a significant effect was 2.5 mg kg^{-1} (% of specific binding = 58.8 ± 6.2, $P<0.05$). Higher doses or RB 101 (5 and

10 mg kg^{-1}) also reduced [^{3}H]-DPN binding, but the magnitude of the response was almost similar with the three effective doses (% of specific binding: 5 mg kg^{-1} = 49.1 ± 4.6, $P<0.05$; 10 mg kg^{-1} = 53.0 ± 5.1, $P<0.05$).

Effect of the association of PD-134,308 and RB 101 on [^{3}H]-diprenorphine in vivo binding in mice A non-significant trend in reducing [^{3}H]-DPN binding was observed after i.p. injection of 1 mg kg^{-1} of PD-134,308, and this effect became significant at a dose of 3 mg kg^{-1}. A non-effective dose of RB 101 (1 mg kg^{-1}) was associated with PD-134,308. The response induced by PD-134,308 at a dose of 3 mg kg^{-1}, but not at 1 mg kg^{-1}, was increased by RB 101 (Figure 6).

Discussion

In this study, systemic administration of the complete inhibitor of enkephalin catabolism, RB 101, elicited a significant decrease in 8 of the 14 withdrawal signs evaluated. Pretreatment with the CCK_B antagonist, PD-134,308, did not induce any modification in the expression of morphine withdrawal when given alone, but produced a strong facilitatory

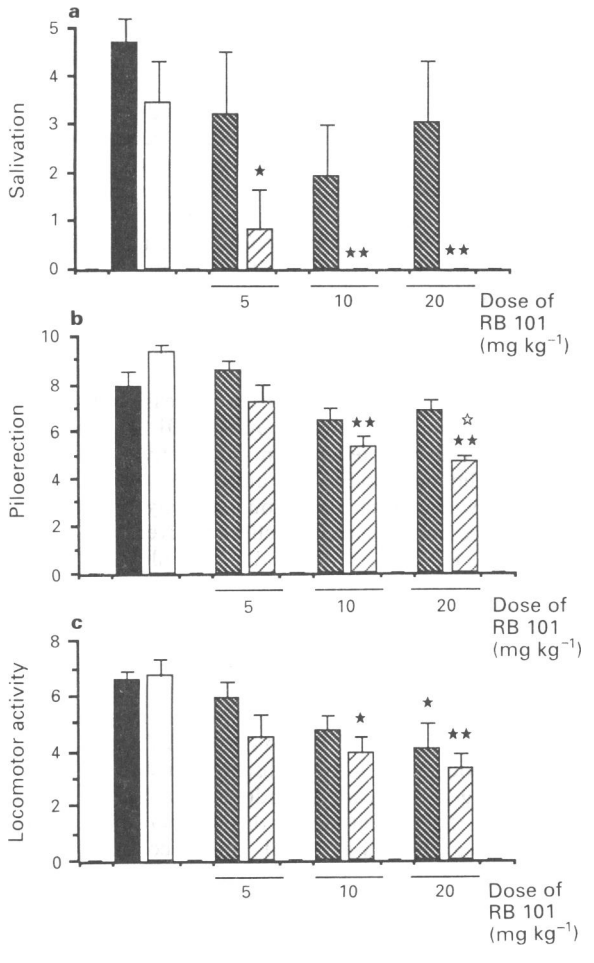

Figure 3 Signs of withdrawal syndrome (salivation, piloerection, locomotor activity) after naloxone administration (1 mg kg^{-1}) in rats treated chronically with morphine (from 8 mg kg^{-1} to 30 mg kg^{-1} for 5 days): effect of acute pretreatment with saline, PD-134,308 (3 mg kg^{-1}, i.p.), RB 101 (5, 10 and 20 mg kg^{-1}, i.v.) or PD-134,308 (3 mg kg^{-1}, i.p.) plus RB 101 (5, 10 and 20 mg kg^{-1}, i.v.). (■) Saline (i.v.) + saline (i.p.); (□) saline (i.v.) + PD-134,308 (3 mg kg^{-1}, i.p.); (▩) RB 101 (i.v.) + saline (i.p.); (▨) RB 101 (i.v.) + PD-134,308 (3 mg kg^{-1}, i.p.). Values are mean ± s.e.mean. Number of animals per group = 10.*, $P<0.05$; **, $P<0.01$: vs saline + saline group. *, $P<0.05$: vs RB 101 + saline group (Newman-Keuls test).

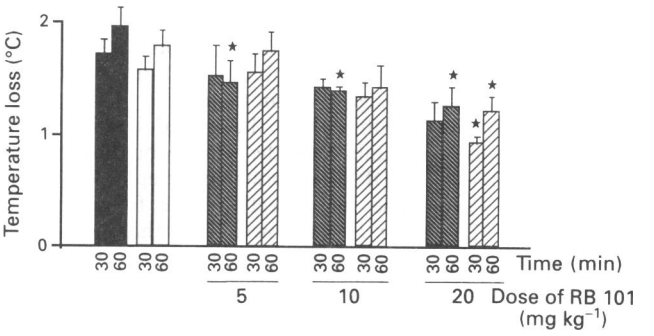

Figure 4 Temperature losses 30 and 60 min after naloxone administration (1 mg kg^{-1}) in morphine-dependent rats: effect of acute pretreatment with saline, PD-134,308 (3 mg kg^{-1}, i.p.), RB 101 (5, 10 and 20 mg kg^{-1}, i.v.) or PD-134,308 (3 mg kg^{-1}, i.p.) plus RB 101 (5, 10 and 20 mg kg^{-1}, i.v.). (■) Saline (i.v.) + saline (i.p.); (□) saline (i.v.) + PD-134,308 (3 mg kg^{-1}, i.p.); (▩) RB 101 (i.v.) + saline (i.p.); (▨) RB 101 (i.v.) + PD-134,308 (3 mg kg^{-1}, i.p.). Values are mean ± s.e.mean. Number of animals per group = 10.*, $P<0.05$; **, $P<0.01$: vs saline + saline group at the same time interval (Newman-Keuls test).

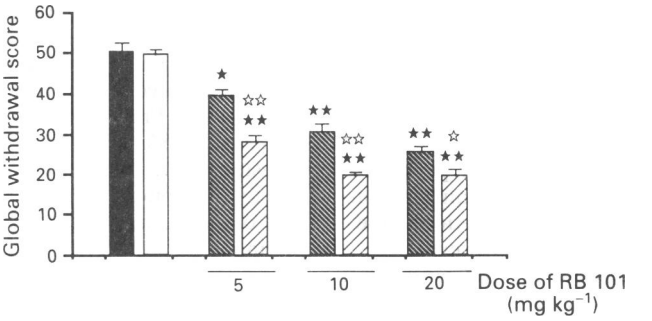

Figure 5 Global withdrawal score after naloxone administration (1 mg kg^{-1}) in morphine-dependent rats: effect of acute pretreatment with saline, PD-134,308 (3 mg kg^{-1}, i.p.), RB 101 (5, 10 and 20 mg kg^{-1}, i.v.) or PD-134,308 (3 mg kg^{-1}, i.p.) plus RB 101 (5, 10 and 20 mg kg^{-1}, i.v.). (■) Saline (i.v.) + saline (i.p.); (□) saline (i.v.) + PD-134,308 (3 mg kg^{-1}, i.p.); (▩) RB 101 (i.v.) + saline (i.p.); (▨) RB 101 (i.v.) + PD-134,308 (3 mg kg^{-1}, i.p.). Values are mean ± s.e.mean. Number of animals per group = 10.*, $P<0.05$; **, $P<0.01$: vs saline + saline group. *, $P<0.05$; **, $P<0.01$: vs RB 101 + saline group (Newman-Keuls test).

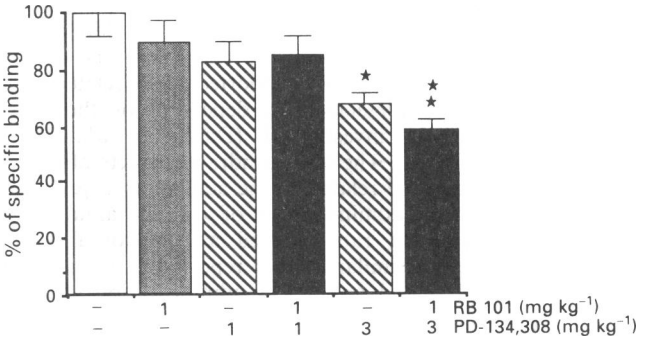

Figure 6 Effect of PD-134,308 (1 and 3 mg kg^{-1}, i.p.) administered alone or with RB 101 (1 mg kg^{-1}, i.v.) on [^{3}H]-diprenorphine [^{3}H]-DPN binding. PD-134,308 and RB 101 were administered 15 and 5 min respectively before [^{3}H]-DPN. Radioactivity was measured in mouse brain 15 min after tracer injection. Values are mean ± s.e.mean. Number of animals per group = 10. ★, $P<0.05$; ★★, $P<0.01$: vs saline + saline group (Newman-Keuls test).

effect on RB 101 responses. Indeed, 12 of 14 withdrawal signs were decreased by the co-administration of the CCK_B antagonist and the peptidase inhibitor, and the inhibitory effect induced on each sign was more severe.

Although devoid of affinity for the opioid binding sites, RB 101 administration inhibited the *in vivo* binding of the opioid antagonist [^{3}H]-DPN, as previously reported (Ruiz-Gayo *et al.*, 1992). The first effective dose found in the present study (2.5 mg kg^{-1}) was lower than that previously reported (5 mg kg^{-1}), probably as a consequence of the use of the mesylate salt, instead of acetate, which has been shown to improve the pharmacokinetic properties of RB 101 by almost 50% (Fournié-Zaluski *et al.*, unpublished observations). These biochemical results suggest that the decrease in the severity of morphine abstinence induced by RB 101 was at least in part induced by the enhancement of the opioid receptor occupation by the endogenous opioid peptides, protected from peptidase inactivation. Accordingly, these peptides also inhibit the binding to opioid receptors of the competitive antagonist, naloxone, injected to precipitate the morphine withdrawal syndrome. Due to the selective involvement of neutral endopeptidase and aminopeptidase N in the inactivation of [Met^5] and [Leu^5]enkephalins and of [Met^5]enkephalin-Arg-Phe (Turner *et al.*, 1985), but not in the degradation of β-endorphin (review in Roques *et al.*, 1993), it appears likely that the endogenous enkephalins released during abstinence are responsible for the reduction in the withdrawal syndrome. In agreement with this, a phasic release of β-endorphin and endogenous enkephalins has been reported in rats at the moment of naloxone-precipitated morphine withdrawal (Gudehithlu *et al.*, 1991). Previous results indicated that the central (Dzoljic *et al.*, 1986; Haffmans & Dzoljic, 1987) or peripheral (Livingston *et al.*, 1988; Dzoljic *et al.*, 1992) administration of selective inhibitors of neutral endopeptidase reduced the severity of naloxone-precipitated abstinence. However, the attenuating effect observed with these compounds was limited, since several signs of withdrawal remained unaffected or even increased. This limited response could be probably due to an insufficient increase in the level of endogenous enkephalins. In agreement with this hypothesis, the inhibition of the withdrawal syndrome was more intense and widespread after central administration of complete inhibitors of enkephalin catabolism (Maldonado *et al.*, 1989), and particularly in the present study, after the peripheral administration of the complete inhibitor, RB 101. Mixed inhibitors have also been found to produce stronger antinociceptive responses than selective neutral endopeptidase or aminopeptidase N inhibitors (Fournié-Zaluski *et al* 1984; Schmidt *et al.*, 1991; Noble *et al.*, 1992). In addition an extensive inhibition of the withdrawal syndrome, similar to that induced in the present study, was observed when enkephalin catabolism inhibitors were directly injected into the periaqueductal gray matter, a region involved in the expression of physical dependence on opioids (Maldonado *et al.*, 1992a). The effects induced by the inhibitors of the enkephalin catabolism have been reported to be related to μ and/or δ opioid receptor activation, depending on the pharmacological response investigated (Daugé *et al.*, 1988; Baamonde *et al.*, 1991). The anti-withdrawal effects observed in this study seem to be related to the activation of μ opioid receptors. Indeed, the administration of μ opioid antagonists to morphine-dependent rats induced a behavioural withdrawal response very similar to that reported in the present study, whereas δ opioid antagonist administration did not induce any relevant behavioural changes in opiate-dependent animals (Maldonado *et al.*, 1992b). A predominant involvement of μ opioid receptors in the precipitation of physical signs of opiate withdrawal has also been proposed in other studies (Cowan *et al.*, 1988; Suzuki *et al.*, 1988).

Several non-opioid peptides, such as substance P, CCK and neurotensin, are also degraded by neutral endopeptidase and/or aminopeptidase N *in vitro* (reviewed in Roques, 1991). The possibility cannot be excluded that an increase in the concentration of these non-opioid peptides is involved in the responses induced by the peptidase inhibitors. However, several findings suggest that the participation of these peptides is not important. Thus, the level of substance P in cerebrospinal fluid did not significantly change after injection of selective neutral endopeptidase inhibitors whereas enkephalin levels were increased 9 fold (Yaksh *et al.*, 1991). Likewise, no modification of the extracellular concentration of CCK in the rat striatum was observed after bestatin and phosphoramidon administration (Butcher *et al.*, 1989). Moreover, the effects of these peptides are usually opposite to those of opioids (Faris *et al.*, 1983; Kalivas *et al.*, 1984; Vaught, 1988), and an increase in their levels is improbable considering the strong inhibition induced on withdrawal by RB 101.

Acute administration of PD-134,308 alone did not modify naloxone-precipitated morphine abstinence, in agreement with previous studies showing no modification of the development of morphine-dependence after chronic treatment with CCK_B antagonists (Panerai *et al.*, 1987; Dourish *et al.*, 1988; 1990; Xu *et al.*, 1992). In addition, the peripheral (Pournaghash & Riley, 1991) or central (Maldonado *et al.*, 1994) administration of CCK-8 in morphine-dependent rats did not precipitate any sign of withdrawal, whereas the central injection of the selective CCK_B agonist, BC 264, induced behavioural changes that represent a very weak degree of withdrawal syndrome (Maldonado *et al.*, 1994). Taken together, these data indicate that CCK plays only a minor role in the development and expression of morphine physical dependence. Indeed, even if the antiopioid effect of CCK seems to implicate preferentially μ opioid receptor-mediated responses, the blockade of the endogenous CCK tonus was not enough by itself to modify morphine withdrawal. PD-134,308, which has no affinity for opioid binding sites (Hughes *et al.*, 1990) was found to inhibit slightly the *in vivo* binding of [^{3}H]-DPN. However, this effect was less potent than that induced by RB 101, and probably the increase in endogenous enkephalin levels induced by the CCK_B antagonist was not strong enough to modify the severity of withdrawal syndrome.

PD-134,308 strongly facilitated the decrease induced in the severity of morphine abstinence by RB 101. A similar result has already been reported for antinociceptive studies. Thus, PD-134,308 did not modify the antinociceptive threshold in rat tail-flick when given alone, but strongly potentiated the RB 101-induced antinociceptive effect (Valverde *et al.*, 1994). In this study, PD-134,308 facilitated the inhibition of the *in vivo* binding of [^{3}H]-DPN induced by RB 101. This result suggests that an increase in endogenous enkephalin levels induced by the CCK-B antagonist could participate in the facilitation of RB 101 behavioural responses. Accordingly, a modification in the release of opioid peptides after treatment with CCK compounds has already been reported (Millington *et al.*, 1992). However, it would be difficult to explain the strong behavioural facilitation observed in these studies only by considering an increase in endogenous enkephalin levels, first, because the magnitude of this facilitatory response at the biochemical level was small, and second, because the inhibition of the *in vivo* [^{3}H]-DPN binding was not associated with any modification of morphine withdrawal when PD-134,308 was given alone. The interaction between endogenous CCK and opioids could also be due to direct or indirect changes in the μ opioid receptor transduction processes. Thus, the modulation of endogenous opioid-induced antinociception by CCK was restricted to μ opioid receptors (Maldonado *et al.*, 1993). These changes in the transduction processes could also participate in the facilitation observed in the present study since μ opioid receptors are the most important in the development and expression of physical opiate dependence (Cowan *et al.*, 1988; Maldonado *et al.*, 1992b).

RB 101 induced the presence of wet dog shake behaviour in non-dependent rats, and this response was facilitated by

PD-134,308. Accordingly, wet dog shakes were observed after treatment with enkephalins (Drust *et al.*, 1981; Cowan & Tortella, 1982) or with other enkephalin catabolism inhibitors (Ukponmwan *et al.*, 1985; Maldonado *et al.*, 1989), and this effect was attenuated but not suppressed by naloxone (Drust *et al.*, 1981; Ukponmwan *et al.*, 1985). The CCK_B antagonist also seems to facilitate the effects of RB 101 on locomotor activity in non-dependent animals, in agreement with previous results showing that the administration of the CCK_B agonist, BC 264, reversed morphine-induced hyperlocomotion (Maldonado *et al.*, 1994).

Changes observed in dependent animals after administration of RB 101 alone or associated with PD-134.308 correspond to a decrease in the severity of withdrawal syndrome. Indeed, many of the signs designated as 'dominant' (jumping, teeth chattering, mastication, hyperactivity, salivation, lacrimation, rhinorrhea and hypothermia) (Bläsig *et al.*, 1973) were found to be significantly decreased by these treatments. The association of PD-134,608 and RB 101 was particularly efficient in decreasing the presence of secretory signs, such as salivation, lacrimation and rhinorrhea. Interestingly, the administration of RB 101 alone had a very slight effect on these signs, and only the appearance of lacrimation was decreased after the injection of the highest dose. The decrease of these secretory signs reflects a facilitatory action induced by the CCK_B antagonist on RB 101 responses, since these signs are present only in a severe degree of withdrawal and their inhibition clearly expresses a decrease in the degree of abstinence (Bläsig *et al.*, 1973; Dzoljic *et al.*, 1992). Otherwise, these secretory signs, as well as diarrhoea and weight loss, are mainly mediated through a peripheral mechanism (Maldonado *et al.*, 1992c). The lack of effect on these peripheral signs after administration of RB 101 alone (only 1 out of 5 was significantly decreased) and the extensive inhibition induced on the other signs (7 out of 9 were significantly reduced) suggest that the effect of this peptidase inhibitor was mainly mediated through central mechanisms. However, an effective blockade of peripherally mediated signs was induced by the association of PD-134,308 and RB 101. Consequently, the CCK_B antagonist probably induced a considerable increase in the tonic release of endogenous opioids at the peripheral level. This result is apparently in contrast with the predominant localisation of CCK_B receptors on the central nervous system. However, the behavioural effects previously reported after the intraperitoneal administration of the selective CCK_B agonist, BC 264 at doses as low as $3\ \mu g\ kg^{-1}$, also suggest the participation of peripheral mechanisms on CCK_B-mediated responses (Derrien *et al.*, 1994a).

Ptosis and weight loss were the only signs not modified in the present study by any treatment. These two signs were reported to be unchanged after i.c.v. injection of peptidase inhibitors (Maldonado *et al.*, 1989), and ptosis was not modified even during the widespread decrease in withdrawal syndrome observed after local administration into the periaqueductal gray matter (Maldonado *et al.*, 1992a).

To summarize all these results and give a quantitative value of the effects induced by each treatment on withdrawal syndrome, a global score of withdrawal was calculated by attributing to all the signs a proportional weight (Maldonado *et al.*, 1992b). This score was dose-dependently decreased by RB 101 administration, and a reduction of almost 50% was obtained with the highest dose ($20\ mg\ kg^{-1}$). The facilitation induced by PD-134,308 ($3\ mg\ kg^{-1}$) was observed with all the doses, but was maximum for $10\ mg\ kg^{-1}$ of the peptidase inhibitor. Under these conditions the reduction in global score reached 60%. The ceiling effect observed with $20\ mg\ kg^{-1}$ of RB 101 could be due to the different responses induced on each sign when PD-134,308 was associated with the inhibitor. Indeed, some withdrawal signs such as mastication, hyperlocomotor activity, salivation, eye twitch and hypothermia were decreased in a dose-dependent manner, whereas a bell-shaped curve was observed with other signs as jumping, wet dog shakes, lacrimation, rhinorrhea and diarrhoea. A bell-shaped dose-response curve for CCK antagonist enhancement of opioid responses has also been reported for analgesia (Watkins *et al.*, 1984; 1985; Dourish *et al.*, 1988; 1990). Consequently, all these results indicate that complex interactions, such as compensatory re-regulation between CCK and/or opioid systems could participate in the interactions between these two systems.

In conclusion the present findings indicate that the co-administration of RB 101, the systemically active complete inhibitor of enkephalin catabolism, and the CCK_B antagonist, PD-134,308, has a promising potential role in the management of opiate withdrawal syndrome in man. In particular PD-134,308 could reduce the effective dose of the peptidase inhibitor.

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Interaction of β-carboline inverse agonists for the benzodiazepine site with another site on $GABA_A$ receptors

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1 We examined the effects of methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM), a β-carboline inverse agonist for the benzodiazepine site, on γ-aminobutyric acid (GABA)-induced Cl^- currents in several cloned rat $GABA_A$ receptor subtypes expressed in human embryonic kidney cells. The Cl^- currents were measured in the whole cell configuration of patch clamp techniques.

2 DMCM at low concentrations (<0.5 μM) occupying only the benzodiazepine site decreased GABA-induced Cl currents in the α1β2γ2 and α3β2γ2 subtypes as expected from an inverse agonist, but produced no change in the α6β2γ2 subtype (perhaps a neutral antagonist). The drug at higher concentrations (>0.5 μM) enhanced Cl^- currents in all the subtypes, with a half maximal concentration of 6 to 20 μM, depending on the α isoform. In the α1β2 subtype, which is without the benzodiazepine site, DMCM monophasically increased Cl^- currents with a half maximal concentration of 1.9 μM.

3 Ro 15-1788 (a classical benzodiazepine antagonist) had no effect on Cl^- current enhancement by DMCM and, in fact, increased the current level through blocking current inhibition by DMCM via the benzodiazepine site. Also, Cl^- current enhancement by pentobarbitone or by 3α, 21-dihydroxy-5α-pregnan-20-one was additive to that by DMCM at saturating doses. It appears that the agonist site for DMCM is distinct from those for benzodiazepines, barbiturates and neurosteroids.

4 Among β-carboline analogues, methyl-β-carboline-3-carboxylate and propyl-β-carboline-3-carboxylate markedly enhanced GABA-induced Cl currents in the α1β2γ2 subtype, while N-methyl-β-carboline-3-carboxamide and 1-methyl-7-methoxy-3,4-dihydro-β-carboline did not. It appears that the 3-carboxyl ester moiety is necessary for β-carbolines to interact with a novel site on $GABA_A$ receptors as agonists.

Keywords: β-Carbolines; cloned $GABA_A$ receptors; methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM); agonist action of DMCM

Introduction

β-Carbolines are known to interact with the benzodiazepine site on $GABA_A$ receptors, the chloride channel-receptor complex consisting of multiple subunits (Sieghart, 1992). Several β-carboline derivatives inhibit the receptor function (inverse agonists) (Braestrup *et al.*, 1982). Methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM), in particular, has been frequently employed as a prototype inverse agonist and shown to induce convulsions in experimental animals (Braestrup *et al.*, 1982). However, in our preliminary studies DMCM at micromolar concentrations marginally decreased GABA-induced Cl^- currents in cloned $GABA_A$ receptors (unpublished observations). This led us to examine full dose-response profiles for DMCM in several subtypes of cloned rat $GABA_A$ receptors. The drug at low concentrations (<0.5 μM) inhibited GABA-induced Cl^- currents as expected from an inverse agonist, but at higher concentrations (>0.5 μM) enhanced Cl^- currents. A further competition study indicated that the low affinity site for DMCM appears to be distinct from those for benzodiazepines, barbiturates and neurosteroids, and accommodates various β-carbolines containing the 3-carboxylate moiety.

Methods

The stable cell-lines expressing α1β2, α1β2γ2, α3β2γ2 and α6β2γ2 subtypes of $GABA_A$ receptors were derived by transfection of plasmids containing cDNA for respective $GABA_A$ receptor subunits and a plasmid encoding G418 resistance into the human kidney cells (HEK 293 cells) as described elsewhere (Hamilton *et al.*, 1993). GABA-induced Cl^- currents were recorded in the whole cell configuration of patch clamp techniques (Hamill *et al.*, 1981), using the pipette and extracellular solutions as detailed elsewhere (Im *et al.*, 1993). Currents were recorded at a holding potential of −60 mV at room temperature, by use of an Axopatch 1D amplifier, a CV-4 headstage (Axon Instrument Co.) and a Gould Recorder 220. A submaximal concentration of GABA, at which allosteric ligands are highly effective, varied for each subtype of $GABA_A$ receptors. In the α1β2γ2 and α3β2γ2 subtypes, we chose 5 μM GABA at which diazepam (5 μM) increased Cl^- currents by 125 ± 20 and 441 ± 96%, respectively. In the α1β2 and α6β2γ2 subtypes (diazepam-insensitive subtypes), we chose 1 μM GABA at which U-92813 (5 μM), a substituted pyrazinone (Im *et al.*, 1993), increased Cl currents by 160 ± 27 and 350 ± 47%, respectively.

Results

Biphasic effect of DMCM on GABA-induced Cl^- currents in the α1β2γ2 subtype

We examined the effect of DMCM at various concentrations on GABA-induced Cl^- currents in the α1β2γ2 subtype of $GABA_A$ receptors expressed in HEK 293 cells (Figure 1). DMCM displayed a biphasic effect; the drug at low concentrations (<0.2 μM) reduced Cl^- currents (−33 ± 8% at 0.1 μM), but at higher concentrations (>0.5 μM) enhanced the currents (+45 ± 18% at 20 μM). From analysis of the latter phase with a logistic equation ($E = E_{max} * [DMCM]^n / (K_{0.5}^n + [DMCM]^n)$), we obtained a half maximal DMCM concentration ($K_{0.5}$) of 6 ± 1.2 μM, a slope factor (^{n}) of 0.9 ± 0.2, and a maximal net current increase (E_{max}) of 115 ± 19%. Furthermore, as shown in Figure 2, the current

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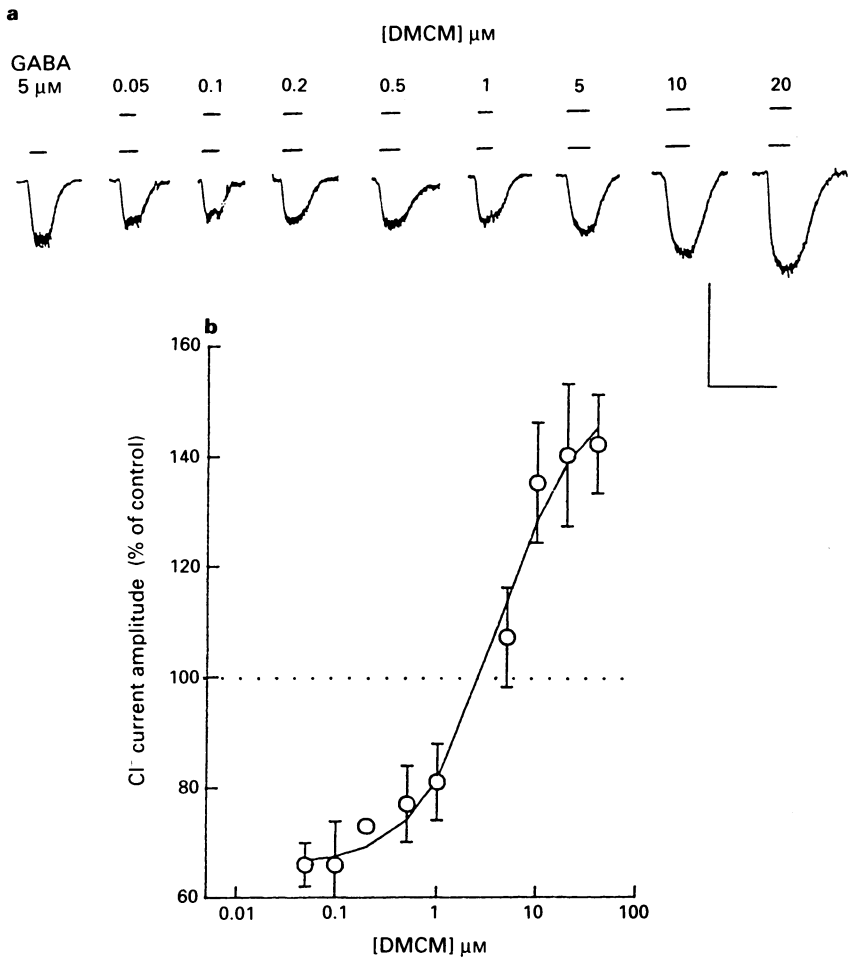

Figure 1 Dose-response profile for the action of DMCM on GABA-induced Cl^- in the α1β2γ2 subtype of $GABA_A$ receptor expressed in HEK293 cells. The traces (a) represent GABA (5 μM)-induced Cl^- currents in the whole cell configuration at a holding potential of −60 mV, in the presence of a symmetrical Cl^- gradient. DMCM was applied at the indicated concentration with 5 μM GABA for 10 s. The amplitude of the currents was normalized to that observed with 5 μM GABA alone. The data fit the logistic equation given in the text (b) and represent the mean ± s.e. from three experiments. The horizontal calibration bar represent 30 s, and the vertical bar 500 pA.

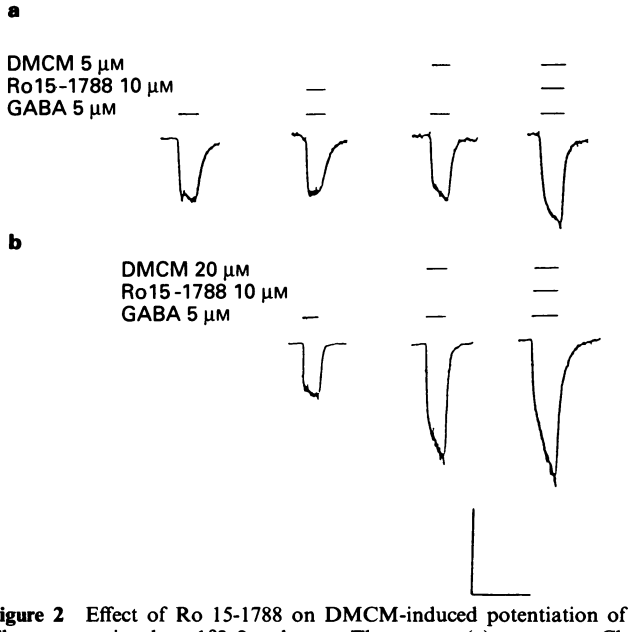

Figure 2 Effect of Ro 15-1788 on DMCM-induced potentiation of Cl^- currents in the α1β2γ2 subtype. The traces (a) represent Cl^- currents induced by 5 μM GABA in the presence of 10 μM Ro 15-1788, 5 μM DMCM, or the combination of the two drugs. Panel (b) shows similar traces obtained with 20 μM DMCM. The average increase in the current response in the presence of Ro 15-1788 was 34 ± 7%, regardless of DMCM concentration. The horizontal calibration bar represents 30 s, and the vertical bar 500 pA.

amplitude observed with DMCM increased upon addition of Ro 15-1788, a classical benzodiazepine antagonist which has been shown to block the inhibitory effect of DMCM. For instance, DMCM at 5 and 20 μM increased GABA-induced Cl^- currents to 105 and 174% of control (5 μM GABA alone), respectively, but in the presence of Ro 15-1788 (ethyl-8-fluoro-5, 6-dihydro-5-methyl-6-oxo-4H-imidazo-[1,5-a] [1,4] benzodiazepine-3-carboxylate; 10 μM) these were increased to 136 and 204%, respectively. The average increase in the presence of Ro 15-1788 was 34 ± 7%, regardless of DMCM concentration (i.e., 5, 10, 20 and 40 μM), which coincided with the amount blocked by DMCM at low concentrations (0.2 μM or less) via the benzodiazepine site. Thus, the two opposing effects of DMCM on Cl^- currents appears to be additive. These results indicate that DMCM at low concentrations acts as an inverse agonist via the benzodiazepine site, but at higher concentrations interacts with another independent site on the $GABA_A$ receptor as an agonist.

Effect of DMCM on GABA-induced currents in the α3β2γ2, α6β2γ2 and α1β2 subtypes

The α1β2γ2 subtype examined above represents Type 1 $GABA_A$ receptors, the most wide-spread subtype in mammalian brains. We also examined DMCM action in Cl^- currents in the α3β2γ2 subtype (Type 2 receptor), the α6β2γ2 subtype (the cerebellar granule cell-specific receptor), and the α1β2 subtype (without the benzodiazepine site) (Figure 3). In the α3β2γ2 subtype, DMCM also displayed a biphasic effect; the drug at low concentrations (<0.5 μM) decreased GABA

(5 μM)-induced Cl^- currents (−35 ± 9% at 0.5 μM), but at high concentrations (>0.5 μM) enhanced the currents with a maximal current increase of 346 ± 28%, a half maximal concentration of 20 ± 4 μM, and a slope factor of 1.3. In the α6β2γ2 subtype, on the other hand, DMCM at concentrations up to 1 μM showed no appreciable effect, but at higher concentrations enhanced the currents with a maximal current increase of 96 ± 47%, a half maximal concentration of 8.5 ± 3 μM, and a slope factor of 1.2. No detectable functional effect of DMCM at 1 μM suggests the drug may be a neutral antagonist at the benzodiazepine site on the α6β2γ2 subtype. DMCM has been reported to inhibit [^{3}H]-Ro 15-4513 binding with a K_i value of 210 ± 50 nM (Luddens *et al.*, 1990). In the α1β2 subtype, which is without a benzodiazepine site (Prichett *et al.*, 1989), DMCM monophasically increased GABA (1 μM)-induced currents (Figure 6) with a maximal current increase of 113 ± 8%, a half maximal concentration of 1.9 ± 0.3 μM, and a slope factor of 1.2. This indicates that the γ subunit is not an integral part for the second site for DMCM, unlike the benzodiazepine site (Prichett *et al.*, 1989). Nevertheless, quaternary interactions involving γ seem to influence the second site judging from the decrease in the half maximal DMCM concentration, for 6 to 1.9 μM, upon removal of the subunit.

The agonist site for DMCM being distinctive from those for barbiturates and neurosteroids

Besides the benzodiazepine site, there are another two major agonist modulatory sites on $GABA_A$ receptors, the barbiturate and the neurosteroid sites. DMCM appeared not to interact with these sites, because enhancement of GABA-induced Cl^- currents by pentobarbitone or 3α, 21-dihydroxy-5α-pregnan-20-one (5α-THDOC) was additive to that by DMCM at saturating concentrations (i.e. 20 μM) (Figure 4). For example, pentobarbitone at 5 μM and 5α-THDOC at 20 nM increased Cl^- currents by 75 ± 18 and 121 ± 33%, respectively, as normalized to the control (5 μM GABA alone). A combination of pentobarbitone (5 μM) and DMCM (20 μM) enhanced the current response by 76 ± 20% above the level observed with DMCM alone. Similarly, a combination of 5α-THDOC (20 nM) and DMCM (20 μM) produced an increase of 133 ± 35% above the level observed with DMCM alone. This additiveness indicates that the agonist site for DMCM (low affinity site) is distinctive from that for barbiturates or neurosteroids.

Effect of various β-carboline analogues on GABA-induced Cl^- currents in the α1β2γ2 subtype

We examined the effects of methyl-β-carboline-3-carboxylate (β-CCM), propyl-β-carboline-3-carboxylate (β-CCP), N-methyl-β-carboline-3-carboxamide (FG-7142), and 1-methyl-7-methoxy-3,4-dihydro-β-carboline (harmaline) on GABA-induced Cl^- currents in the α1β2γ2 subtype (Figure 5). β-CCM at 0.5 μM reduced the currents to 69 ± 6% of control, but at higher concentrations enhanced currents, i.e., at 40 μM by nearly 300%. β-CCP at 0.05, 0.1 and 0.2 μM, on the other hand, produced no appreciable effect on Cl^- currents, but at higher concentration increased the currents, i.e., at 10 μM by nearly 400%. FG-7142 monophasically reduced the currents to 67 ± 7% of control at concentrations ranging from 0.1 to 40 μM. Harmaline had no appreciable effect on Cl^- currents (91 to 100% of control) at concentrations ranging from 0.1 to 20 μM. The dose-response profiles for β-CCM and β-CCP were analysed with the logistic equation (Figure 6). For β-CCM, we obtained a half maximal concentration of 22.6 ± 3 μM, a slop factor of 0.9, and a maximal net current increase of 342 ± 26%. For β-CCP, the corresponding values were 4.3 ± 1.4 μM, 1.4 and 388 ± 48%, respectively.

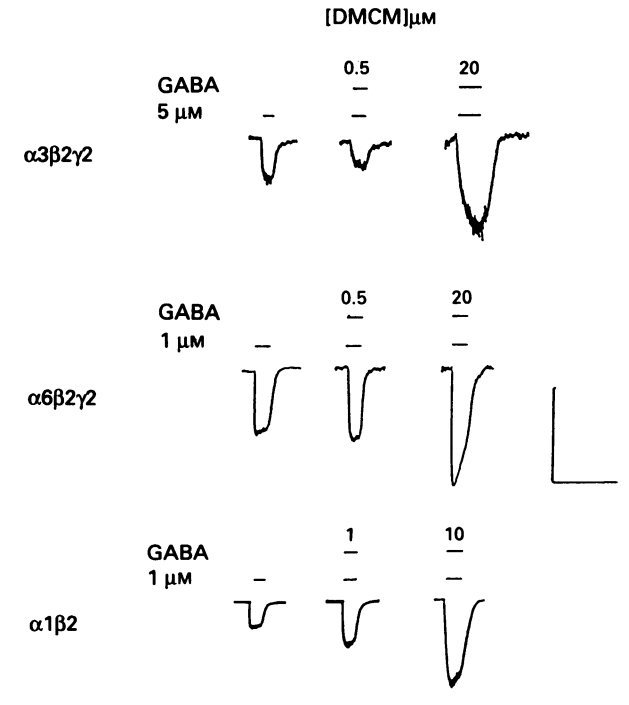

Figure 3 Dose-dependent potentiation of GABA-induced Cl^- currents by DMCM in the α3β2γ2, α6β2γ2 and α1β2 subtypes of $GABA_A$ receptors. The traces represent Cl^- currents evoked by 5 μM GABA in the α3β2γ2, and by 1 μM GABA in the α6β2γ2 and α1β2 subtypes in the whole cell configuration. The holding potential was −60 mV under a symmetrical Cl^- gradient. DMCM was applied at the indicated concentrations with GABA. The horizontal calibration bar represents 30 s, and the vertical bar 500 pA.

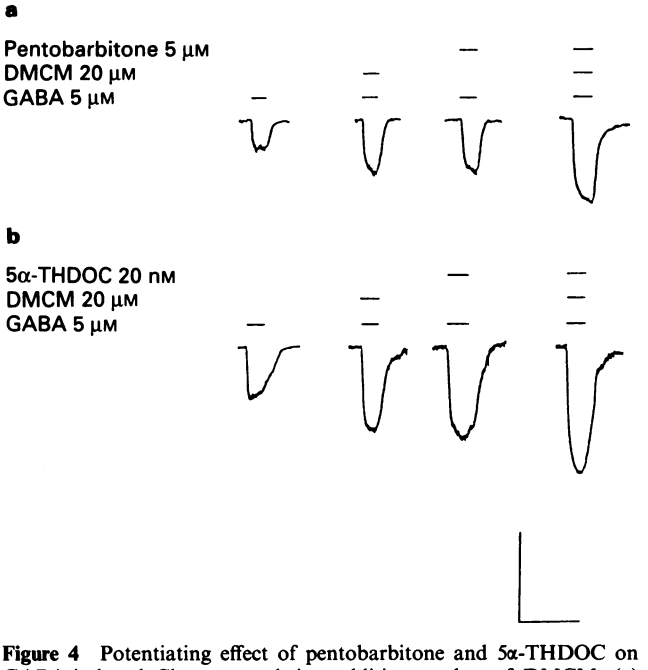

Figure 4 Potentiating effect of pentobarbitone and 5α-THDOC on GABA-induced Cl^- currents being additive to that of DMCM. (a) Shows the current traces obtained in the presence of 5 μM GABA without or with 20 μM DMCM, 5 μM pentobarbitone or the combination of the two. Similar traces obtained with 20 nM 5α-THDOC instead of pentobarbitone are shown in (b). Pentobarbitone at 5 μM and 5α-THDOC at 20 nM increased GABA-induced Cl^- currents by 75 ± 1 and 121 ± 33%, respectively, and their level of potentiation was not altered in the presence of DMCM at 20 μM, a saturating concentration. The horizontal calibration bar represents 30 s, and the vertical bar 1000 pA.

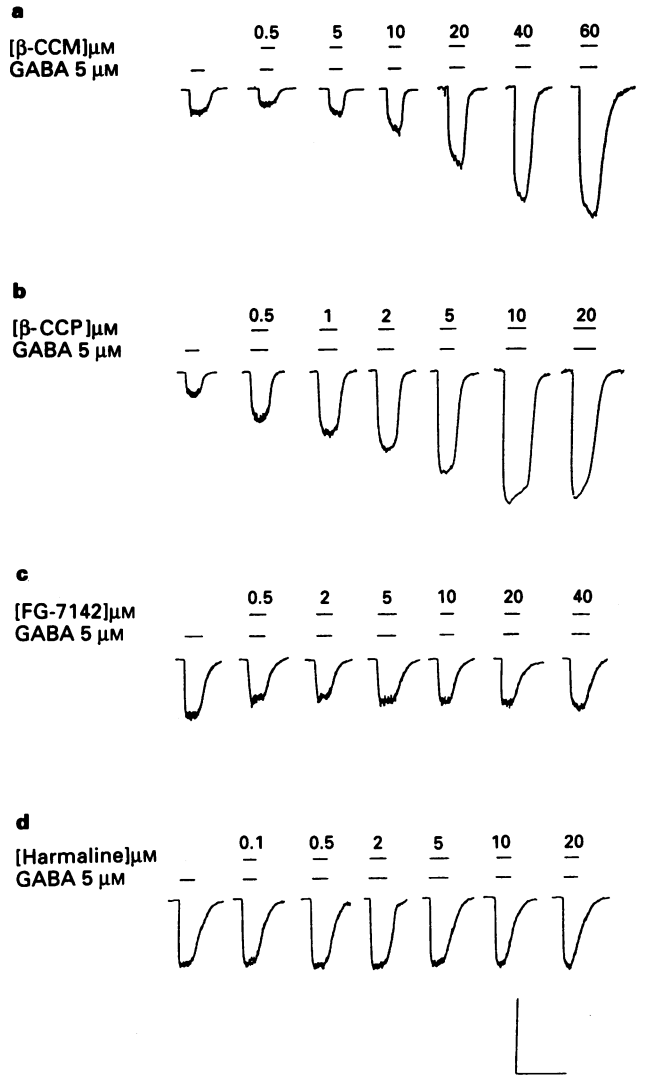

Figure 5 Effect of various β-carbolines on GABA-induced Cl$^-$ currents in the α1β2γ2 subtype. The current traces represent 5 μM GABA-induced Cl$^-$ currents in the α1β2γ2 subtype of cloned GABA$_A$ receptor in the presence of β-CCM (a), β-CCP (b), FG-7142 (c), and harmaline (d) at various concentrations. β-CCM and FG-7142 at 0.5 μM or less reduced GABA-induced Cl$^-$ currents by about 32%, but β-CCP and harmaline at 0.05 or 0.1 μM had no appreciable effect on the currents. Furthermore, only β-CCM and β-CCP at micromolar concentrations markedly enhanced the currents. The horizontal calibration bar represents 30 s, and the vertical bar 1000 pA for (a) and (b), 500 pA for panel (c) and (d).

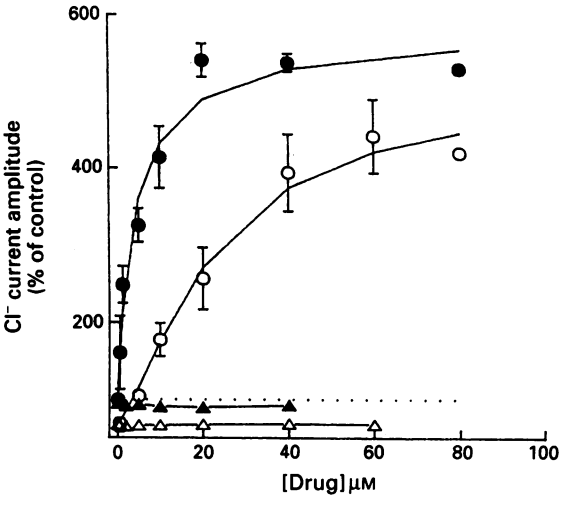

Figure 6 Plots showing concentration-dependent enhancement of Cl$^-$ currents by β-CCM (○) and β-CCP (●) in the α1β2γ2 subtype; effects of FG-7142 (△) and harmaline (▲) are also shown. The amplitude of Cl$^-$ currents obtained in the presence of GABA (5 μM) and β-CCM or β-CCP at indicated concentrations was normalized to that for GABA alone (dotted line). The data for β-CCM and β-CCP were fitted with the logistic equation as described in the text (solid lines). For β-CCM, the half maximal concentration was 22.6 ± 3 μM, with a maximal current enhancement of 442 ± 26% and a slope factor of 0.9. The corresponding values for β-CCP were 4.3 ± 1.4 μM, 488 ± 48%, and 1.4, respectively. Each data point represents the mean ± s.e. from three separate measurements.

Discussion

In this study, we have shown a biphasic effect of DMCM on GABA-induced Cl$^-$ currents; the drug at low concentrations acted as an inverse agonist via the benzodiazepine site (its most well known action), but at higher concentrations interacted with another site on the GABA$_A$ receptor as an agonist. The agonist site for DMCM appears to be distinct from that for benzodiazepines, barbiturates and neurosteroids (the three major positive modulatory sites on GABA$_A$ receptors), on the basis of several observations presented in this study. (1) Ro 15-1788, a classical benzodiazepine antagonist, did not block the agonist action of DMCM at high concentrations, but rather boosted the agonist action by abolishing the inhibitory effect of DMCM via the benzodiazepine site. (2) DMCM potentiated GABA-induced Cl$^-$ currents in the α1β2 subtype which is without a benzodiazepine site. (3) The agonist action of DMCM at a saturating concentration was additive to that of the barbiturate and neurosteroid (5α-THDOC). We propose that DMCM at high concentrations interacts with a novel site on GABA$_A$ receptors, which could possibly be exploited as a novel therapeutic target.

The current study with several key analogues of β-carbolines showed that the 3-carboxyl ester moiety is necessary for their agonist action. For example, β-CCM and β-CCP containing the 3-methyl or propyl ester group, respectively, markedly enhanced GABA-induced Cl$^-$ currents, while FG-7142, containing the 3-carboxamide group, and harmaline, a 3,4 dihydro β-carboline without a C3 carboxyl substituent, did not appreciably enhance the currents. Among the analogues containing the 3-carboxyl ester moiety, β-CCP showed the highest potency and intrinsic efficacy, suggesting a key interaction of the alkyl chain of the ester group with GABA$_A$ receptors.

In view of its newly discovered agonist activity, we need to re-evaluate the use of DMCM as a prototype inverse agonist for GABA$_A$ receptors. So far, central nervous system depressant activity of DMCM has not been reported in experimental animals. Even at high doses of DMCM, its depressant activity could be transient and easily overlooked because of the subsequent appearance of its powerful convulsant activity, as drug occupancy of the low affinity site gradually decreases. Careful examination of animal behaviours will be needed following DMCM administration. *In vitro*, DMCM at concentrations less than 0.5 μM behaves as an inverse agonist for the benzodiazepine site, but at concentrations greater than 1 μM will not be effective as an inverse agonist. FG-7142 appears to be a more reliable inverse agonist because of its failure to interact with the low affinity site for DMCM. In summary, DMCM and several β-carbolines containing the 3-carboxyl ester moiety interact with a novel, low affinity site on GABA$_A$ receptors as an agonist besides its interaction with the benzodiazepine site as an inverse agonist.

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Functional effects of long-term activation on human β_2- and β_3-adrenoceptor signalling

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1 The functional effects of long-term activation of β-adrenoceptors were investigated by measuring adenylyl cyclase activity, cyclic AMP accumulation and cyclic AMP-dependent protein kinase activity in CHW and L cells expressing either human β_2- or β_3-adrenoceptors.

2 Pre-incubation of CHW and L cells expressing β_2-adrenoceptors with 10 μM isoprenaline for 24 h produced a marked reduction in the total receptor number and dramatically reduced the capacity of the receptor to stimulate adenylyl cyclase maximally.

3 In contrast, the ability of β_3-adrenoceptors to stimulate adenylyl cyclase maximally was not affected by pre-incubation with the agonist in either cell type. However, a significant reduction of isoprenaline potency and a sustained down-regulation of β_3-adrenoceptor number was observed in L but not in CHW cells.

4 Maximal levels of intracellular cyclic AMP concentrations were reached during the first hour of receptor activation with isoprenaline in all four cell lines. In the absence of phosphodiesterase inhibitors, cyclic AMP decreased to basal levels within 24 h of continuous stimulation. This phenomenon occurred more rapidly in cells expressing the β_2- than the β_3-adrenoceptors.

5 These results confirm that, at the level of adenylyl cyclase stimulation and cyclic AMP accumulation, the β_3-adrenoceptor is more resistant than the β_2-adrenoceptor to long-term desensitization. However, when cyclic AMP-dependent protein kinase activity was considered, a 24 h stimulation of β_2- and β_3-adrenoceptor expressing cells led to the desensitization of the kinase in L but not in CHW cells.

6 In conclusion, long-term desensitization may have distinct functional effects on cell signalling depending on the receptor subtype and the cell type considered. These findings might have practical implications for future strategies involving long-term therapies with receptor agonists.

Keywords: Atypical β-adrenoceptors; β_3-adrenoceptors; desensitization; down-regulation; adenylyl cyclase; cyclic AMP

Introduction

Based on the clinical observation that bronchodilator β_2-sympathomimetic drugs, prescribed to asthmatic patients, may induce down-regulation of leukocyte β-adrenoceptors (Galant *et al.*, 1978), and that contractility improvement produced by β-adrenoceptor agonists in patients with cardiomyopathy wanes with time (Packer, 1990), it is generally admitted that the loss of pharmacological effect is a potential shortcoming for long-term treatments with receptor agonists (Hausdorff *et al.*, 1990). Indeed, signal transduction of most G protein-coupled receptors is tightly controlled by regulatory processes which, on the one hand, prevent the hormonal overload (desensitization) and which, on the other hand, reset the signalling pathways for further hormonal stimuli (resensitization). However, clinically important tachyphylaxis has not always been observed in asthmatic patients upon sustained treatment with β-adrenoceptor agonists (Tattersfield, 1985). More recently, sustained treatment with β-agonists has been shown to promote tolerance to their non-bronchodilator actions but not to their direct bronchorelaxant effects (O'Connor *et al.*, 1993). This raises the intriguing possibility that the clinical manifestation of agonist-promoted desensitization may be tissue-specific.

Desensitization is a multifactorial process which limits the effects of receptor activation by impairing the signal-transmission pathway at receptor and/or post-receptor levels. The β_2-adrenoceptor (β_2AR) is one of the most thoroughly investigated models of receptor desensitization (Lefkowitz, 1993). After a few minutes of incubation with an agonist, the β_2ARs is phosphorylated by the adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent protein kinase A (PKA) and by the β-adrenoceptor kinase (βARK), causing the functional uncoupling of the receptor from the signalling pathway (Benovic *et al.*, 1988; Hausdorff *et al.*, 1989). Phosphorylation of the β_2AR by PKA, activated by the increase of intracellular cyclic AMP levels, constitutes a heterogeneous negative feedback loop affecting all β_2AR present while βARK phosphorylates only those receptors occupied by the agonist (Benovic *et al.*, 1986). Resensitization of the β_2AR-mediated response occurs following the sequestration of the phosphorylated β_2AR and its subsequent dephosphorylation and recycling to the cell surface (Yu *et al.*, 1993). When receptor activation is sustained for longer periods of time (h), receptor down-regulation contributes to the reinforcement of desensitization through the degradation of pre-existing receptors (Doss *et al.*, 1981; Homburger *et al.*, 1984) and the destabilization of receptor mRNA (Hadcock *et al.*, 1988; Bouvier *et al.*, 1989). Under these conditions, *de novo* synthesis of receptor proteins is necessary for cellular responsiveness to be fully recovered (Doss *et al.*, 1981).

In rodents, β_3-adrenoceptors (β_3AR) play a major role in the control of white adipose tissue lipolysis and brown adipose tissue thermogenesis (Arch & Kaumann, 1993). In man, it has been shown that β_3AR distribution is mostly restricted to adipose tissues and gallbladder (Krief *et al.*, 1993) and that β_3-selective agonists may promote lipolysis in

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samples of human adipose tissues (Lönnqvist *et al.*, 1993). The β_3AR thus represents a target for anti-obesity therapy using adrenoceptor agonists (Connacher *et al.*, 1988; MacLahan *et al.*, 1991; Howe, 1993). However, desensitization of the cellular response might hamper the therapeutic effect of these compounds.

In contrast to the well documented rapid desensitization of the β_2AR, recent studies have shown the β_3AR to be almost completely resistant to short-term desensitization (Granneman, 1992; Liggett *et al.*, 1993; Nantel *et al.*, 1993), presumably because this receptor does not contain PKA or βARK phosphorylation sites (Liggett *et al.*, 1993; Nantel *et al.*, 1993). Several observations also suggest that the β_3AR could be less prone that the β_2AR to desensitization which occurs following longer activation with agonists. Indeed, little or no agonist-induced down-regulation of the β_3AR was found in CHW cells (Liggett *et al.*, 1993; Nantel *et al.*, 1994), in 3T3-F442A adipocytes (Thomas *et al.*, 1992) and in human SK-N-MC cells (F.N. and S.M., unpublished observation). Similarly, β_3AR-mediated adenylyl cyclase activation in hamster adipocytes was unaffected by *in vivo* infusion of adrenaline for up to six days (Carpéné *et al.*, 1993).

While long-term desensitization has been extensively investigated at the level of the receptor, little attention has been given to the functional consequences of this regulation on distal signalling events such as cyclic AMP accumulation and PKA activation. Therefore, in the present study, we have compared the adenylyl cyclase activation, the intracellular cyclic AMP levels and the PKA activity, upon sustained agonist activation, in cells expressing βAR subtypes with different desensitization profiles.

Methods

Cell culture

The coding regions of human β_2AR cDNA and human β_3AR genomic DNA were cloned into the pBC12BI plasmid and transfected into either chinese hamster fibroblasts (CHW-β_2AR and CHW-β_3AR) or murine L cells (L-β_2AR and L-β_3AR) as previously described (Nantel *et al.*, 1993). Cells were grown in 75 cm^2 Corning flasks at 37°C in an atmosphere of 95% air/5% CO_2. The cell culture medium consisted of Dulbecco's modified Eagles medium (DMEM) supplemented with 10% (v/v) FBS, 100 u ml^{-1} penicillin, 100 $\mu g\ ml^{-1}$ streptomycin, 0.25 $\mu g\ ml^{-1}$ fungizone and 1 mM glutamine. Geneticin, 150 or 450 $\mu g\ ml^{-1}$, was added to the medium for CHW or L cell clones, respectively.

Membrane preparation

Nearly confluent cells, grown in 75 cm^2 flasks, were washed twice with ice-cold PBS, mechanically detached and resuspended in 10 ml of 5 mM Tris, 2 mM EDTA, pH 7.4 supplemented with protease inhibitors (5 $\mu g\ ml^{-1}$ soybean trypsin inhibitor, 5 $\mu g\ ml^{-1}$ leupeptine, 10 $\mu g\ ml^{-1}$ benzamidine). Cells suspensions were homogenized with a polytron homogenizer (Janke & Undel Ultra-Turrax T25) for 5 s at maximal setting. The lysate was centrifuged at 500 *g* for 5 min at 4°C (to eliminate the nucleus and unbroken cells). The supernatant was centrifuged at 43,000 *g* for 20 min at 4°C, and the pellet was resuspended in 10 ml of 5 mM Tris, 2 mM EDTA, pH 7.4. After an additional centrifugation at 43,000 *g* for 20 min at 4°C the pelleted membranes were resuspended in 75 mM Tris (pH 7.4), 5 mM $MgCl_2$, 2 mM EDTA supplemented with protease inhibitors (as above).

Radioligand binding

To determine total β_2AR number, 150 ml of membrane preparation (10 μg protein) was incubated in the presence of 250 pM [^{125}I]-cyanopindolol ([^{125}I]-CYP) in the absence or presence of 10 μM (−)-alprenolol (to define non-specific binding). In β_3AR-expressing cells, 50 μg protein were used with 1 nM [^{125}I]-CYP as previously described (Nantel *et al.*, 1993). The binding assays were conducted for 90 min at 25°C in a final volume of 500 ml of 75 mM Tris (pH 7.4), 5 mM $MgCl_2$, 2 mM EDTA supplemented with protease inhibitors (as above). The reaction was terminated by rapid filtration through Whatman GF/C glass fibre filters previously soaked for 30 min in 25 mM Tris (pH 7.4), 0.3% polyethyleneimine (to reduce non-specific binding). Protein concentrations were determined by the method of Bradford (1976) using the Bio-Rad protein assay system. Bovine serum albumin was used as standard.

Adenylyl cyclase assays

Adenylyl cyclase activity was measured by the method of Salomon *et al.* (1974). Briefly, 0.02 ml membrane preparation (as above, 2–6 μg protein), 45 mM Tris (pH 7.4), 3 mM $MgCl_2$, 1.2 mM EDTA, 0.12 mM ATP, 0.053 mM GTP, 0.1 μM cyclic AMP, 0.1 mM isobutylmethylxanthine, 1 μCi [α-^{32}P]-ATP, 2.8 mM phosphoenolpyruvate, 0.2 u pyruvate kinase and 1 u myokinase were mixed in a final volume of 50 μl. Enzymatic activity was determined in the presence of 0–100 μM isoprenaline for 15 min at 37°C. The reactions were terminated by the addition of 1 ml ice-cold stop solution containing 0.4 mM ATP, 0.3 mM cyclic AMP and 25,000 c.p.m. [^{3}H]-cyclic AMP. Cyclic AMP was then isolated by sequential chromatography on a Dowex cation exchange resin and aluminium oxide.

Determination of intracellular cyclic AMP levels

Cells grown in 75 cm^2 flasks were exposed to 10 μM isoprenaline for 1–24 h at 37°C. The cells were washed twice with 5 ml of ice-cold PBS and resuspended in 6 (CHW) or 1.5 (L cells) ml of ice-cold 50 mM Tris (pH 7.4), 5 mM EDTA. A 1 ml aliquot was boiled for 3 min and centrifuged in a microfuge at maximum speed for 5 min. The supernatant was used for cyclic AMP determination using an [^{3}H]-cyclic AMP radio-immunoassay system (Amersham).

Protein kinase A assay

PKA activity was measured by using a variant of the protocol described by Corbin & Reimann (1974) as modified by Giembycz & Diamond (1990). Briefly, cells grown to near confluency were exposed, or not, to 10 μM isoprenaline for 10 min or 24 h. The cells were washed thrice with 5 ml ice-cold PBS and homogenized in 5 ml of ice-cold buffer A (5 mM KH_2PO_4, 5 mM K_2PO_4 pH 6.8, 10 mM EDTA, 10 mM dithiothreitol, 0.5 mM isobutylmethylxanthine (IBMX), 500 mM NaCl) for 5 s with a polytron homogenizer. The homogenate was centrifuged at 50,000 *g* for 30 min at 4°C and the supernatant was used for the determination of soluble protein kinase A activity. PKA activity was measured in duplicate using kemptide as substrate for the enzyme. The enzymatic reaction was conducted using 3–5 μg of soluble protein in a final volume of 100 μl containing 10 mM KH_2PO_4, 10 mM K_2HPO_4, 10 mM $Mg(CH_3COO)_2$, 0.5 mM IBMX, 500 mg ml^{-1} BSA, 100 μM ATP, 71 μM kemptide and 100 c.p.m. per pmol [α-^{32}P]-ATP, in the presence or absence of 10 μM cyclic AMP. The reaction was continued for 20 min at 30°C and terminated by cooling the samples on ice. Aliquots of 70 μl were spotted onto 2.5 × 2.5 cm phosphocellulose papers (Whatman, P81) which were subsequently washed four times in 75 mM phosphoric acid (4 × 5 min), once in ethanol and once in ether. The paper squares were allowed to dry and the incorporation of ^{32}P were determined in a scintillation counter. The ratio of ^{32}P incorporation in the absence over that observed in the presence of excess cyclic AMP was used as an index of PKA activity.

Data analysis

The adenylyl cyclase activity data were fitted by non-linear least squares regression analysis with the computer programme ALLFIT (De Léan *et al.*, 1978). Differences between data were evaluated by the Bonferroni *t* test with the computer programme PRIMER. Differences were considered statistically significant with $P<0.05$.

Materials

[α-^{32}P]-ATP and [^{3}H]-cyclic AMP were from DuPont-New England Nuclear. [^{125}I]-CYP was either from DuPont-New England Nuclear or Amersham. Isoprenaline, (−)-alprenolol, ATP, GTP, cyclic AMP, BSA, kemptide, and phosphoenolpyruvate were from Sigma. Pyruvate kinase was from Calbiochem. DMEM, PBS, trypsin-EDTA, geneticin (G418), penicillin, streptomycin and fungizone were from GIBCO/BRL. The FBS was purchased either from GIBCO/BRL or from Immunocorps.

Results

CHW and L cells, which do not express endogenous βARs and are unresponsive to catecholamines, were transfected with either the human β_2 AR cDNA or the human β_3 AR gene as previously described (Nantel *et al.*, 1993). Cell lines expressing an equivalent number of receptors (200–350 fmol mg^{-1} membrane protein) were used throughout this study.

Effect of sustained receptor activation on adenylyl cyclase activity and receptor number

In membrane preparations from CHW-β_2AR and L-β_2AR cells, the binding of the agonist isoprenaline to the β_2AR induced the activation of adenylyl cyclase. Pre-incubation of both CHW and L cells with 10 μM isoprenaline induced a time-dependent decrease in the ability of the β_2AR to stimulate the enzyme maximally (Figure 1 and Table 1). This desensitization was rapid as most of the decrease in adenylyl cyclase stimulation over the 24 h period occurred within the first 3 h of isoprenaline treatment. Following 24 h of continuous incubation with the agonist, the ability of the β_2AR to stimulate the adenylyl cyclase activity was reduced by $\approx$85% in both cell lines. The agonist pretreatment also induced a time-dependent down-regulation in the total number of [^{125}I]-CYP binding sites: after 24 h of continuous stimulation, β_2AR levels decreased by 68% and 83% in CHW-β_2AR and L-β_2AR cells respectively (Table 1). Post-receptor desensitization was evident in CHW-β_2AR cells as a significant reduction in the capacity of NaF and of forskolin to activate the adenylyl cyclase (Table 1) was observed following sustained incubation with the agonist. In contrast, no

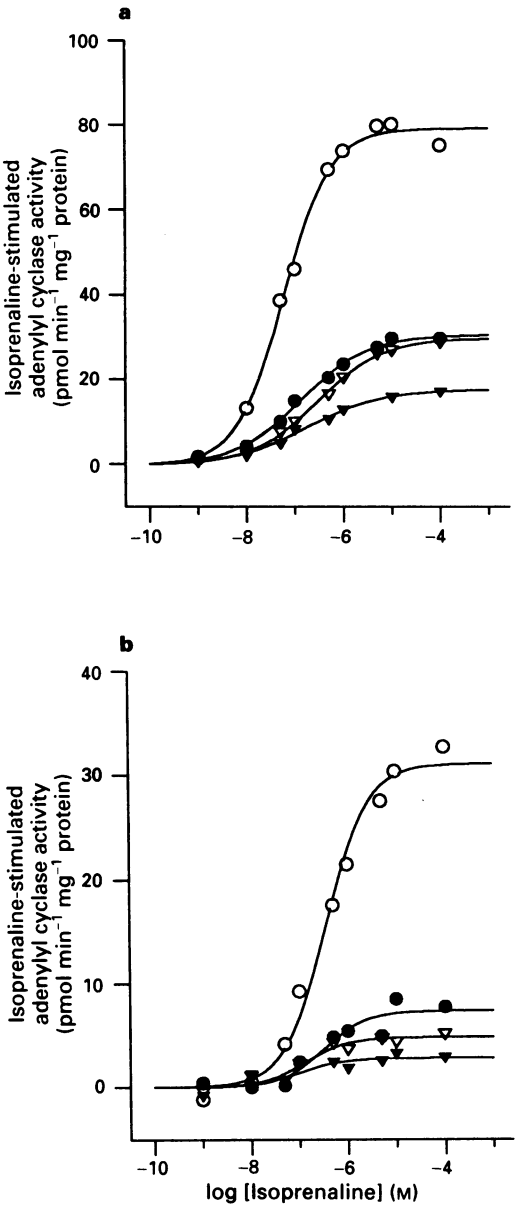

Figure 1 Isoprenaline-induced stimulation of adenylyl cyclase activity in cells expressing β_2-adrenoceptors (β_2AR). Membranes were prepared from CHW-β_2AR (a) or L-β_2AR (b) previously incubated in the presence of 10 μM isoprenaline for 0 (○), 3 (●), 6 (▽) or 24 h (▼). The isoprenaline-stimulated adenylyl cyclase activity is expressed as pmol of cyclic AMP produced min^{-1} mg^{-1} membrane protein. Data represent the mean of 3–4 experiments done in duplicate.

Table 1 β_2-Adrenoceptor (β_2AR) number and stimulation of adenylyl cyclase

Cell line	*Time* (h)	*β_2AR number* (fmol mg^{-1} prot.)	*AC basal activity* (pmol min^{-1} mg^{-1} prot.)	*Iso-stimulated AC activity* (pmol min^{-1} mg^{-1} prot.)	*Iso EC_{50}* (nM)	*NaF-stimulated AC activity* (pmol min^{-1} mg^{-1} prot.)	*FK-stimulated AC activity* (pmol min^{-1} mg^{-1} prot.)
CHW-β_2AR	0	354 ± 7	20.2 ± 10	120.9 ± 18.2	58 ± 22	102 ± 13	241 ± 52
	3	264 ± 36	5.4 ± 1.3	36.1 ± 14.9*	110 ± 50	50 ± 11*	154 ± 42*
	6	186 ± 28*	3.9 ± 2.7	29.8 ± 2.4*	310 ± 20	57 ± 10*	152 ± 39*
	24	113 ± 6*	3.2 ± 1.0	17.7 ± 2.9*	300 ± 100	56 ± 11*	142 ± 34*
L-β_2AR	0	202 ± 6	6.8 ± 1.9	38.3 ± 1.8	340 ± 90	47 ± 6	63 ± 8
	3	52 ± 2*	3.9 ± 1.5	11.7 ± 2.9*	950 ± 880	52 ± 5	68 ± 5
	6	48 ± 5*	1.5 ± 0.7	6.8 ± 1.2*	250 ± 110	44 ± 8	55 ± 7
	24	26 ± 0*	1.5 ± 0.8	5.8 ± 0.6*	983 ± 790	45 ± 9	53 ± 11

Cells were incubated with 10 μM isoprenaline for 0–24 h and membranes were prepared as described under the Methods section. Data represent the mean ± s.e.mean of 3–4 experiments done in duplicate. *$P<0.05$.

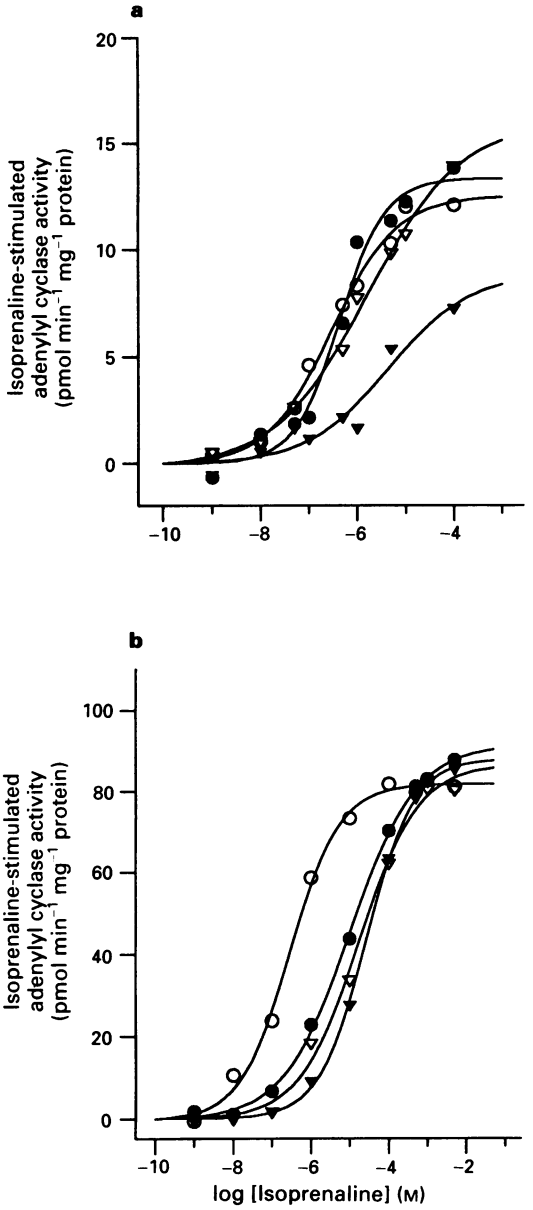

Figure 2 Isoprenaline-induced stimulation of adenylyl cyclase activity in cells expressing β_3-adrenoceptors (β_3AR). Membranes were prepared from CHW-β_3AR (a) or L-β_3AR (b) previously incubated in the presence of 10 μM isoprenaline for 0 (○), 3 (●), 6 (▽) or 24 h (▼). The isoprenaline-stimulated adenylyl cyclase activity is expressed as pmol of cyclic AMP produced $min^{-1} mg^{-1}$ membrane protein. Data represent the mean of 3–4 experiments done in duplicate.

such reduction was observed in L-β_2AR cells, suggesting that the agonist-promoted desensitization occurred mainly at the level of the receptor in these cells.

Contrary to what was observed in the β_2AR-expressing cells, incubation with isoprenaline for 3, 6 and 24 h did not significantly affect the maximal β_3AR-mediated adenylyl cyclase stimulation in either CHW-β_3AR or L-β_3AR cells (Figure 2 and Table 2). It should be pointed out, however, that although it did not reach statistical significance, the 24 h pretreatment caused an apparent modest reduction of the β_3AR-mediated adenylyl cyclase stimulation in CHW cells. This was accompanied, in that cell type, by a significant decrease in NaF- and forskolin-stimulated adenylyl cyclase activity (Table 2) suggesting that the blunted responsiveness might reflect changes occurring down-stream of the receptor. A significant reduction in the potency of isoprenaline in stimulating adenylyl cyclase was found in L-β_3AR cells at the three time points studied. This agonist-promoted reduction in isoprenaline potency may result, in part, from the time-dependent down-regulation of the β_3AR number observed. No such down-regulation was observed in CHW-β_3AR cells.

Effect of sustained receptor activation on intracellular cyclic AMP concentrations

Intracellular concentrations of cyclic AMP were measured following 1, 3, 6 and 24 h of β-adrenoceptor stimulation (Table 3). In the four cell lines studied, a significant elevation in cyclic AMP levels was found following a 1 h isoprenaline treatment. Subsequently, cyclic AMP levels decreased progressively toward basal concentrations despite the continuous presence of the agonist. This decrease can be considered as a cellular manifestation of desensitization since a sustained stimulation fails to maintain a constant level of second messenger. Thus, this decrease was expressed as a percentage of desensitization considering that reaching pre-stimulation levels would represent 100% desensitization. As illustrated in Figure 3, the desensitization tended to be faster and more pronounced in the β_2AR than in the β_3AR expressing cells for the two cell types studied.

Effects of sustained receptor activation on PKA activity

Phosphorylation of target substrates by PKA is, ultimately, one of the major intracellular events leading to the proper cell response following β-adrenoceptor activation. To evaluate the consequences of β-adrenoceptor/adenylyl cyclase pathway desensitization on the subsequent step of signal transduction, PKA activity was assessed *in vitro* following short-term or sustained cell stimulation with isoprenaline (Figure 4). In the four cell lines, a 10 min stimulation led to a 2–3 fold increase in the activity of PKA. In CHW cells, irrespective of the receptor subtype being expressed, this level of activity was maintained for at least 24 h in the presence of

Table 2 β_3-Adrenoceptor (β_3AR) number and stimulation of adenylyl cyclase

Cell line	*Time* (h)	*β_2AR number* (fmol mg^{-1} prot.)	*AC basal activity* (pmol min^{-1} mg^{-1} prot.)	*Iso-stimulated AC activity* (pmol min^{-1} mg^{-1} prot.)	*Iso EC_{50}* (nM)	*NaF-stimulated AC activity* (pmol min^{-1} mg^{-1} prot.)	*FK-stimulated AC activity* (pmol min^{-1} mg^{-1} prot.)
CHW-β_3AR	0	264 ± 63	27.9 ± 3.5	39.8 ± 5.6	440 ± 160	104 ± 16	247 ± 52
	3	232 ± 26	19.6 ± 2.9	34.1 ± 8.1	452 ± 110	95 ± 16	231 ± 42
	6	188 ± 34	19.4 ± 3.5	33.0 ± 7.2	618 ± 20	92 ± 23	224 ± 42
	24	217 ± 19	18.5 ± 5.6	27.4 ± 8.3	2900 ± 200*	73 ± 16*	176 ± 37*
L-β_3AR	0	192 ± 32	9.0 ± 2.7	82.2 ± 2.9	297 ± 57	93 ± 13	81 ± 25
	3	102 ± 6*	7.8 ± 2.0	94.8 ± 3.8	10400 ± 2380*	101 ± 10	67 ± 8
	6	83 ± 1*	7.7 ± 1.7	86.9 ± 3.6	17600 ± 3890*	97 ± 15	55 ± 5
	24	47 ± 5*	7.7 ± 1.4	88.1 ± 3.0	27500 ± 4770*	110 ± 18	54 ± 3

Cells were incubated with 10 μM isoprenaline for 0–24 h and membranes were prepared as described under the Methods section. Data represent the mean ± s.e.mean of 3–4 experiments done in duplicate. *$P<0.05$.

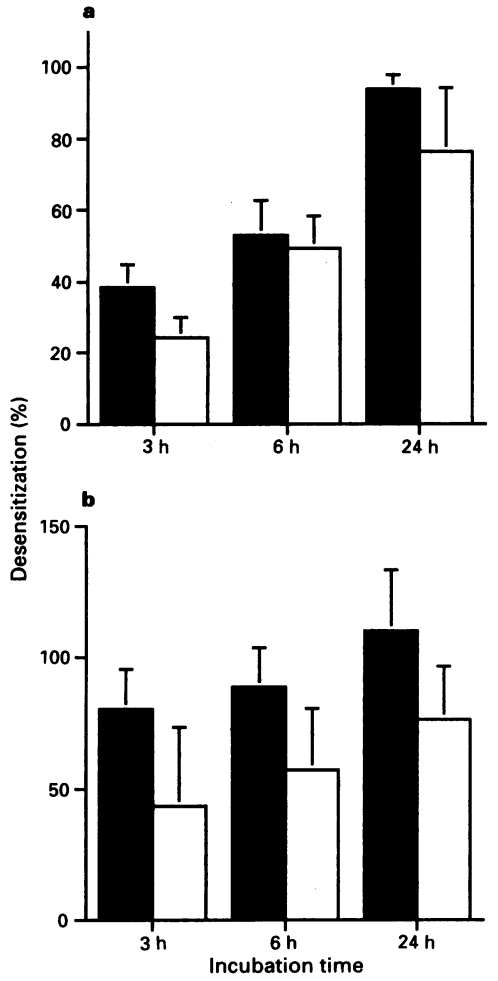

Figure 3 Reduction of intracellular cyclic AMP concentrations in CHW (a) and L cells (b) expressing β_2-adrenoceptors (β_2AR) (solid columns) or the β_3AR (open columns). The reduction in cyclic AMP levels, observed between 3–24 h of continuous stimulation with 10 μM isoprenaline, is expressed as a percentage of desensitization of the maximal cyclic AMP levels observed after 1 h of stimulation (see Table 3) and considering that reaching pre-stimulation levels would represent 100% desensitization. Data represent the mean ± s.e.mean of 3–4 experiments done in duplicate.

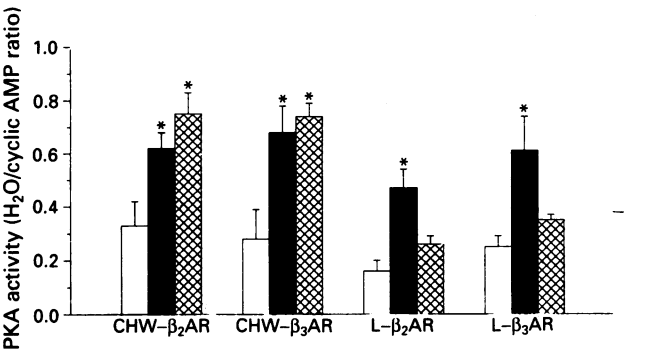

Figure 4 Protein kinase A (PKA) activity in cells incubated with 10 μM isoprenaline for 0 (□), 10 min (■) or 24 h (▩). PKA activity is expressed as the ratio of ^{32}P incorporation in the absence over that observed in the presence of excess cyclic AMP. Data represent the mean ± s.e.mean of 3–5 experiments done in duplicate.

Table 3 Intracellular cyclic AMP concentrations

	Cell type			
Time	*CHW-β_2AR*	*CHW-β_3AR*	*L-β_2AR*	*L-β_3AR*
Control	23.2 ± 8.5	26.5 ± 5.5	7.6 ± 1.7	7.9 ± 0.9
Iso 1 h	82.6 ± 7.4*	60.0 ± 5.0*	18.3 ± 5.1*	20.3 ± 6.8*
Iso 3 h	59.8 ± 3.8*	51.9 ± 1.9*	9.7 ± 1.6	14.9 ± 3.7*
Iso 6 h	51.2 ± 5.8*	43.5 ± 3.0*	8.8 ± 1.6	13.2 ± 2.9*
Iso 24 h	26.8 ± 2.5	34.4 ± 6.0	6.5 ± 2.5	10.8 ± 2.5

Cells were incubated with 10 μM isoprenaline (Iso) for 0–24 h and intracellular cyclic AMP concentrations were determined as described under Methods. Data are expressed in pmol mg^{-1} protein and represent the mean ± s.e.mean of 6–7 experiments done in duplicate.

a saturating concentration of agonist, suggesting that no desensitization could be detected at this level. This contrasted with the kinase activity profile observed in L cells. Indeed, the PKA activity returned to near basal values in both β_2AR and β_3AR expressing cells following 24 h of sustained stimulation. This indicates that, in this cell type, desensitization was also reflected at the PKA level.

Discussion

Previous studies have shown that desensitization processes which occur at the level of the receptor affect more readily the β_2AR than the β_3AR (Granneman, 1992; Liggett *et al.*, 1993; Nantel *et al.*, 1993; 1994). The results presented here indicate that, following long-term treatment with agonists, β-adrenoceptor-mediated stimulation of adenylyl cyclase reflects primarily the changes in βAR number and activity and, thus, that β_3AR-mediated adenylyl cyclase stimulation undergoes only very modest desensitization. Despite this lack of desensitization at the level of the signal transducing apparatus, the level of second messenger did not increase steadily during long-term stimulation of the β_3AR. Instead, the intracellular cyclic AMP levels reached a peak and then gradually declined, suggesting that processes acting downstream of the second messenger production are also involved in blunting the cellular response. When a more distal event in the signalling cascade, the PKA activity, was monitored, desensitization was evident for the two receptor subtypes but only in one of the two cell types being studied. This suggests that distal manifestation of the various desensitization processes are highly dependent on the cell type being considered.

The modest desensitization of isoprenaline-stimulated adenylyl cyclase activity observed in the β_3AR expressing cells, following 24 h of stimulation, contrasted with the 85% decrease in maximal β_2AR-dependent adenylyl cyclase activation seen under the same conditions. The relative resistance of the β_3AR-stimulated adenylyl cyclase to long-term desensitization probably reflects two intrinsic properties of the β_3AR: unlike β_2AR, (1) sustained stimulation of the β_3AR does not promote its functional uncoupling resulting from receptor phosphorylation (Granneman, 1992; Liggett *et al.*, 1993; Nantel *et al.*, 1993), (2) long-term stimulation of this receptor subtype leads to slower and more modest reduction in receptor number. The agonist-induced down-regulation of the β_3AR has recently been shown to result exclusively from a cyclic AMP-mediated reduction of receptor mRNA content (Nantel *et al.*, 1994). The absence of desensitization of the maximal isoprenaline-stimulated adenylyl cyclase activity suggests that the number of receptors remaining following down-regulation still exceeds that required to stimulate the enzyme maximally. In CHW cells, no statistically significant down-regulation of the β_3AR was observed and, thus, no change in either the efficacy or the potency of isoprenaline was observed following stimulation for 3 or 6 h. This observation is consistent with the recent finding that no sustained reduction in β_3AR mRNA content is observed upon long-

term stimulation in that cell line (Nantel *et al.*, 1994). The modest desensitization, observed after 24 h of continued stimulation in CHW-β_3AR cells, probably reflects post-receptor regulation (Unelius *et al.*, 1993) since both NaF- and forskolin-stimulated activity were significantly reduced at that time point.

The difference in response between the two receptor subtypes to agonist-promoted desensitization was also evident, although to a lesser extent, at the level of cyclic AMP accumulation. Indeed, cyclic AMP levels returned toward control values more rapidly in cells expressing the β_2AR than in cells expressing the β_3 subtype. However, in contrast to what was observed for the β_3AR-stimulated adenylyl cyclase activity measured in membrane preparations, the β_3AR-promoted cyclic AMP accumulation clearly became desensitized. This suggests that, even in the absence of desensitization at the level of the receptor itself, other cellular processes may contribute to the reduction of cyclic AMP content during prolonged stimulation. Activation of an inducible cyclic AMP phosphodiesterase (Barber *et al.*, 1992a; Houslay *et al.*, 1992) could contribute to the rapid decrease in cyclic AMP concentrations, despite the continued stimulation of the adenylyl cyclase. The presence of a phosphodiesterase with a strong positive cooperativity toward cyclic AMP in L cells (Barber *et al.*, 1992b) is consistent with such an hypothesis.

The functional consequences of long-term stimulation observed at the level of PKA activity were unexpected. After 24 h of continuous stimulation with a saturating concentration of isoprenaline, although intracellular cyclic AMP fell to near basal levels in all cell-lines, PKA was still fully activated in both CHW-β_2AR and CHW-β_3AR cells. These results indicate that, in these cells, a modest elevation of cyclic AMP concentrations above basal values is sufficient to maintain the dissociation and the activation of the catalytic subunit of the PKA (Cadd *et al.*, 1990). Maximal PKA activation has been shown to occur at relatively low levels of cyclic AMP in some cellular systems (Feldman, 1989; Lohse *et al.*, 1990). The existence of multiple catalytic and regulatory PKA subunits, which may combine to form different isoforms of the holoenzyme (Tasken *et al.*, 1993), could provide variable levels of sensitivity to the system. The functional consequences of this diversity are not completely understood. However, judging from the PKA activity patterns observed in the present study, it is clear that regulation of the kinase activity varies among cell lines and may lead to distinct patterns of cellular responsiveness regulation. Indeed, under identical conditions, the 24 h stimulation which led to no desensitization of the isoprenaline-stimulated PKA activity in CHW cells almost completely desensitized this activity in L-β_2AR and L-β_3AR cells.

This nearly complete desensitization of the agonist-stimulated PKA activity, in L-β_3AR cells, occurred despite no detectable desensitization of the receptor-stimulated adenylyl cyclase activity in membrane preparations. This suggests that, in these cells, the other processes contributing to the reduction in cyclic AMP levels are sufficient to promote desensitization. Therefore, the final phenotype of desensitization appears to be highly dependent on the cell type being considered. The precise nature of the mechanisms leading to such desensitization remain largely unknown. Whether it reflects a heterologous desensitization of all the PKA stimulating pathways or is restricted to the β-adrenoceptor-mediated activation of this kinase remains to be determined.

In the present study, the βAR coding regions were under the control of a viral promoter. For native receptors, modulation of gene transcription might increase further the diversity of βAR regulation: in DTT-MF$_2$ cells, positive PKA-mediated regulation of βAR genes drives a transient (1–2 h) increase of β_2AR mRNA (Collins *et al.*, 1989) while, in 3T3-F442A cells, elevated levels of β_3AR are still measured following 30 h of exposure to the agonist.

Our results provide biochemical support for previous observations of clinical pharmacology suggesting that receptor desensitization does not occur in all tissues. For example, β_2-adrenoceptor agonists are effective in the treatment of asthma by causing bronchodilatation and by protecting against the release of bronchoconstrictor stimuli. The former effect is due to the direct relaxation of airways smooth muscle, while the latter results from the stabilization of mast cells. Long-term exposure to β-agonists does not seem to induce a loss of their airway-specific effects, although tolerance to their protective effects against bronchoconstriction may blunt their beneficial effects in asthma, thus suggesting that βAR desensitization may occur in mast cells (O'Connor *et al.*, 1993) and in polymorphonuclear leukocytes (Galant *et al.*, 1978) but not in the airway smooth muscle cells. Lack of receptor desensitization in various tissues is also illustrated by the observation that in several diseases, normal G protein-coupled receptors are chronically activated by abnormal hormone (or hormone-like) overload, with deleterious consequences. Chronic autoimmune thyrotropin receptor activation in Graves-Basedow disease (McGregor, 1990) and sustained adrenoceptor activation by catecholamines in phaeochromocytoma (Bravo & Gifford, 1984) are well known examples. These observations indicate that some receptor-mediated pathways of signal transmission may escape long-term desensitization in man.

In conclusion, the desensitization of βAR following long-term activation may have distinct functional effects on cell signalling depending on both the receptor subtype and on the cell type considered. The physiological relevance, in man, of variable signalling regulation requires further studies, since these findings might have practical implications for future strategies involving long-term therapies with receptor agonists.

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Regulation of transepithelial ion transport by two different purinoceptors in the apical membrane of canine kidney (MDCK) cells

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1 The effect of extracellular nucleotides on the transepithelial ion transport of Madin Darby canine kidney cells (MDCK) was investigated. Cells were grown up to confluency on permeable supports and the short circuit current (I_{sc}) was measured with an Ussing chamber-like mini-perfusion system.

2 Apical ATP stimulated a biphasic I_{sc} increase consisting of a first rapid and transient peak followed by a broader one.

3 The first peak evoked by ATP was reversibly blocked by basilen blue (BB) in a concentration-dependent fashion, with an EC_{50} of 7.5 μM.

4 The P_{2Y} receptor agonist, 2-methylthioATP (2-MeSATP) caused a single transient I_{sc} increase that was completely blocked by pretreatment with BB. On the contrary, the P_{2x} agonist, α,β-methylene ATP (α,β-meATP) was almost completely ineffective on I_{sc}. UTP essentially induced a monophasic response the time-course of which resembled that of the second peak stimulated by ATP. The agonist potency order was 2-MeSATP $\geqslant$ ATP $>>$ UTP, α,β-meATP for the first peak and UTP $\geqslant$ ATP $>$ 2-MeSATP $>$ α,β-meATP for the second peak.

5 Monolayer incubation with the membrane permeable calcium chelator [*bis-o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′,-tetraacetic acid, tetra(acetoximethyl)-ester] (BAPTA/AM) inhibited the ATP-evoked first peak.

6 The non-hydrolyzable ATP analogue, adenosine-5′-*O*-(3-thio)-trisphosphate (ATP-γ-S) elicited a biphasic response similar to that of ATP. The P_1 receptor agonist, 2-chloroadenosine and CGS-21680, were almost unable to induce an I_{sc} increase. These results rule out the involvement of ATP hydrolysis and P_1 receptor activation as responsible for I_{sc} increase.

7 Inhibition of prostaglandins synthesis by indomethacin abolished the second ATP-evoked peak.

8 Chloride replacement with gluconate on both sides of the epithelium completely inhibited the second peak induced by ATP but only reduced the amplitude of the first spike.

9 The results suggest that ATP stimulates I_{sc} increase by two mechanisms. The first one is mediated by a P_{2Y} receptor and by intracellular calcium increase. The second induces prostaglandin synthesis probably through a P_{2U} receptor activation.

Keywords: MDCK cells; ATP; UTP; P_2 purinoceptors; transepithelial ion transport; short circuit current

Introduction

By interacting with specific membrane receptors termed P_2, extracellular ATP elicits a variety of responses in a large number of cell types (Benham & Tsien, 1987; Okajima *et al.*, 1987; Gerwins & Fredholm, 1992; Rugolo *et al.*, 1993; Galietta *et al.*, 1994). In particular, purinoceptor stimulation results in activation of transepithelial chloride transport in human airway epithelia (Mason *et al.*, 1991), human intestinal T84 cells (Dho *et al.*, 1992), and Madin-Darby canine kidney (MDCK) cells (Simmons, 1981a,b). Although two ATP receptor genes have been cloned recently (Lustig *et al.*, 1993; Webb *et al.*, 1993), the number and characteristics of P_2 purinoceptors are still unclear. Recent classification, based on the potency order of ATP analogues and response to UTP, postulates the presence of at least 5 receptors termed P_{2X}, P_{2Y}, P_{2U}, P_{2T} and P_{2Z} (O'Connor *et al.*, 1991; Watson & Girdlestone, 1994). While the P_{2X} type seems to be a ligand-gated cation channel, P_{2Y} receptors are commonly associated with phospholipase C activation, inositol phosphates cascade and cytosolic free Ca^{2+} increase. P_{2U} receptors seem also to elicit inositol phosphate turnover, but besides ATP they are also activated by UTP for which reason they have been called nucleotide receptors (O'Connor *et al.*, 1991). P_{2Z} purinoceptors seem to have limited distribution since they are mainly found in mast cells and other immune cells where they form a large membrane channel. P_{2T} purinoceptors are essentially ADP receptors and are found on platelets.

Simmons (1981a,b) reported the ability of extracellular nucleotides to affect transepithelial ion transport in MDCK cells. ATP, ADP, UTP, and ITP applied to the basolateral side were all effective in increasing Cl^- secretion through a mechanism possibly involving prostaglandin synthesis. Simmons (1981a) also mentioned that apical application of ATP was equally effective but, in contrast with the basolateral stimulation, it was characterized by a biphasic response. We investigated whether this feature was due to the presence of two separate transduction mechanisms. The data obtained in this study demonstrate the presence of at least two ATP receptors distinguished by their sensitivity to basilen blue, selectivity to different nucleotides and mechanism of signal transduction.

Methods

Cell culture

MDCK-1 cells were routinely cultured on plastic culture flasks in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 supplemented with 10%

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foetal calf serum, 2 mM L-glutamine, 100 u ml^{-1} penicillin, 100 $\mu g\ ml^{-1}$ streptomycin. Aliquots of 2×10^6 cells were stored in liquid nitrogen. Experiments were prepared by thawing and seeding cells on Costar's Transwell-COL cell culture chamber inserts (diameter 24.5 mm) at a density of 75,000 cells cm^{-2}. Cells were allowed to grow to confluency with change of medium every 2 days.

Electrical measurements

After 3 to 7 days in culture, Transwell cups were mounted in a modified Ussing chamber (miniperfusion system Trans-24, World Precision Instruments). The transepithelial potential difference was short-circuited with a voltage clamp (Mod. 558-C5, Bioengineering, The University of Iowa) connected to apical and basolateral chambers via Ag/AgCl electrodes. Potential sensing electrodes were connected to the experimental solution through agar bridges containing 150 mM NaCl. The potential difference and the fluid resistance between these electrodes were compensated. Transepithelial resistance was calculated from the ratio of the potential difference measured in open-circuit conditions to the short circuit current. Epithelia with resistances higher than 600 Ωcm^{-2} were considered for analysis. Experiments were performed at room temperature (20–24°C).

Solutions

Where not otherwise specified, the experiments were carried out in a symmetrical solution containing (in mM): NaCl 126, KH_2PO_4 0.38, K_2HPO_4 2.13, $MgSO_4$ 1, $CaCl_2$ 1, $NaHCO_3$ 24, glucose 10 and phenol red 0.04. The solution was continuously bubbled with 5% CO_2:95% air. In those experiments where the role of intracellular calcium was evaluated, the cell monolayer was incubated for 30 min in the experimental solution containing 20 μM *bis*-(*o*-aminophenoxy-ethane-*N*, *N*, *N'*, *N'*-tetraacetic acid, tetra (acetoximethyl)-ester) (BAPTA/AM). Thereafter the epithelium was washed with BAPTA/AM-free solution and used for electrical measurements.

Statistics

We observed that the magnitude of the ATP-evoked I_{sc} increase displayed some variability between separate batches of MDCK monolayers. Nevertheless, the shape of the response and the ratio of the first to the second peak were constant. Given this variability of current amplitude, already noted by Simmons (1981b), the comparison between different experimental conditions was performed by collecting data from the same cell preparation. Data are presented as raw current measurement or as mean ± standard error of the mean (s.e.mean). Significance of differences between mean values was tested by Student's two-tailed *t* test. Rank potency orders of the agonists were obtained by comparing the estimated EC_{50}s.

Materials

2-Methylthio ATP (2-MeSATP), α,β-methylene ATP (α,β-meATP), basilen blue (BB), 2-chloroadenosine, and CGS-21680 were purchased from Research Biochemicals International. [*bis*-(*o*-aminophenoxy)-ethane-*N*,*N*,*N'*,*N'*,-tetraacetic acid, tetra(acetoximethyl)-ester] (BAPTA/AM) and UTP were from Calbiochem. Adenosine-5'-O-(3-thio)-trisphosphate (ATP-γ-S) was obtained from Boehringer Mannheim. Indomethacin (Liometacen) was from Chiesi Farmaceutici. ATP and other chemicals were from Sigma.

Results

When MDCK monolayers were mounted in the perfusion chamber and voltage clamped, low values of short circuit current (I_{sc}) were recorded (0.5 ± 0.05 $\mu A\ cm^{-2}$, $n = 127$). The epithelia exhibited a relatively high transepithelial resistance of 3372 ± 276 Ωcm^{-2}. Addition of ATP (50–1000 μM) to the apical side of MDCK cells elicited a biphasic activation of I_{sc}. As depicted in Figure 1a, a sharp transient peak at 15–30 s was followed by a second smoother response that peaked at about 2 min. For clarity, these two phases of the ATP response will be indicated as the first peak and the second peak throughout the test. ATP concentrations giving 50% of the maximum response (EC_{50}) were 80 μM and 116 μM for the first and the second peak respectively (Figure 2). In the presence of ATP, the I_{sc} did not completely return to basal level even after 20 min. However, a complete recovery was attained by extensively washing the apical chamber with a volume of fresh solution at least ten times that of the chamber. A second application of ATP, 15 to 25 min after the first one, was again effective but the current amplitude of the second peak was reduced to 45% ($n = 5$).

Interestingly, when basilen blue (BB, 100 μM) was administered to the apical side of the epithelium, the first peak evoked by ATP was dramatically reduced (Figure 1a). The inhibitory effect of BB was completely reversible since ATP was able to elicit a large biphasic response when again administered after washing (not shown). Application of BB to the basolateral side of the epithelium did not affect the response to apical ATP ($n = 3$; not shown). The inhibitory effect of apical BB was dose-dependent, higher molar additions resulting in diminished peak response. The EC_{50} of BB on the ATP-evoked first peak was 7.5 μM, as shown in Figure 1b.

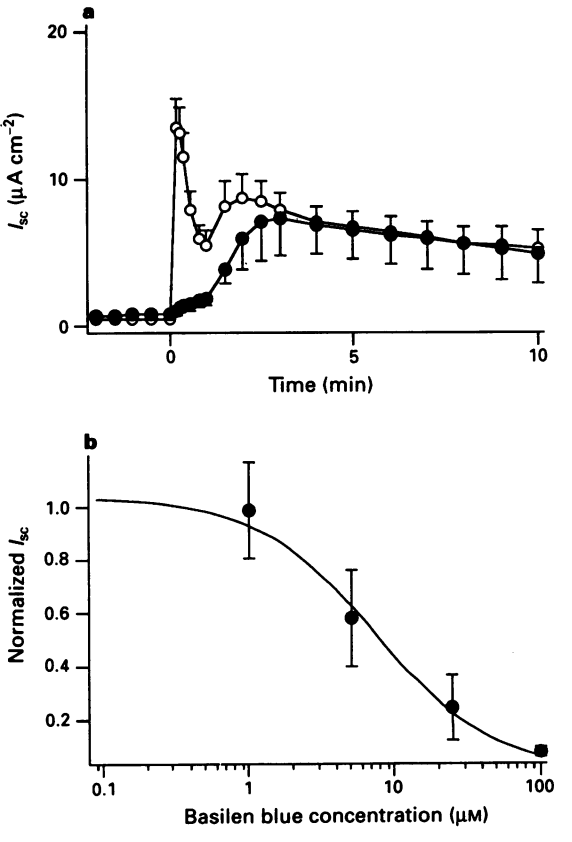

Figure 1 (a) Stimulation of I_{sc} by apical ATP in the absence (○, $n = 13$) and in the presence (●, $n = 5$) of 100 μM basilen blue (BB). In contrast with the second peak, the first peak elicited by ATP was significantly inhibited ($P < 0.001$) by BB. (b) Dose-dependent inhibition of the ATP-induced I_{sc} by apical BB. The peak obtained at 15–20 s from the application by 100 μM ATP was plotted against BB concentration. Each individual value was normalized to the mean I_{sc} peak obtained with ATP alone on the same day. Each point is the mean of 4 to 9 experiments. The fit with a Hill plot yields an EC_{50} of 7.5 μM.

Different ATP analogues and nucleotides were tested in the apical solution. The P_{2Y} receptor agonist, 2-MeSATP (O'Connor *et al.*, 1991) at concentrations up to 100 μM, evoked a single transient peak that slowly decreased in a monotonic way toward control values (Figure 3a). At a higher concentration (500 μM) 2-MeSATP was also able to induce a second peak. Interestingly, the 2-MeSATP response at 100 μM was completely inhibited by BB (see Figure 3a). Addition of ATP after 2-MeSATP elicited a large delayed response that was devoid of the initial transient peak (Figure 3b).

We also investigated the response of MDCK monolayers to apical UTP (Figures 2 and 4a). This nucleotide induced a slow I_{sc} increase the time-course of which mimicked that of ATP, except that the first peak was very small even at the highest concentrations. In this context, it is worth noting that application of ATP after UTP elicited only a single transient peak (Figure 4b). The P_{2X} agonist α,β-meATP was poorly effective since high concentrations (> 100 μM) produced only minor changes of I_{sc}. Summarising, the agonist potency order was 2-MeSATP ≥ ATP >> UTP,α,β-meATP for the first peak and UTP ≥ ATP > 2-MeSATP > α,β-meATP for the second peak (see Figure 2).

The possible role of intracellular Ca^{2+} in the ATP-induced I_{sc} increase was investigated by incubating cell monolayers with 20 μM of the membrane permeable Ca^{2+} chelating agent, BAPTA/AM for 30 min before electrical measurements. This treatment significantly inhibited the first peak elicited by ATP but potentiated the second one (Figure 5).

It is well known that membrane ecto-nucleotidases are able to breakdown ATP rapidly to ADP, AMP and adenosine. Adenosine is a ubiquitous modulator acting *via* specific P_1 purinoceptors. In this context, we asked whether part of the

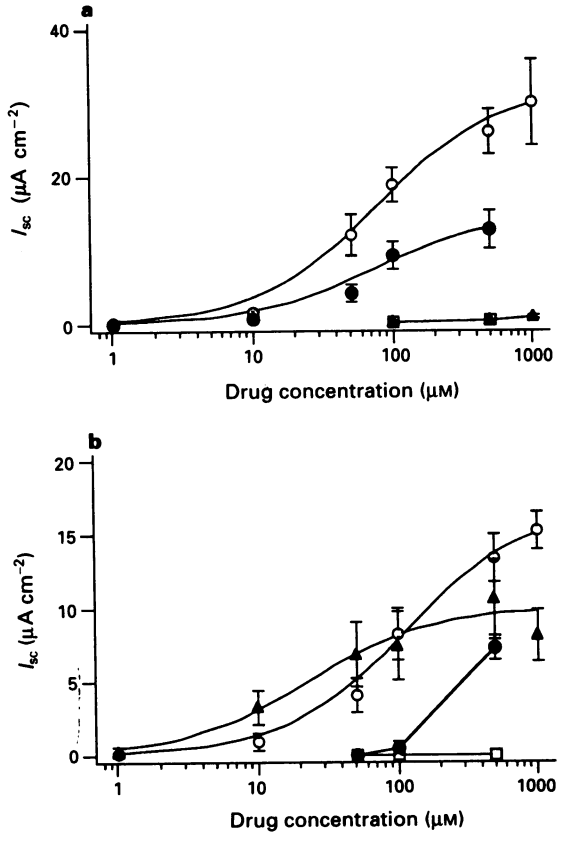

Figure 2 Dose-response relationships of various agonists, ATP (○), UTP (▲), 2-MeSATP (●) and α,β-meATP (□), for the first (a) and the second (b) peak. Each point is the mean of 3 to 7 experiments. EC_{50} for the first peak was 76 and 80 μM for 2-MeSATP and ATP respectively; for the second peak the EC_{50} was 22.5 for UTP and 116 μM for ATP. For abbreviations, see text.

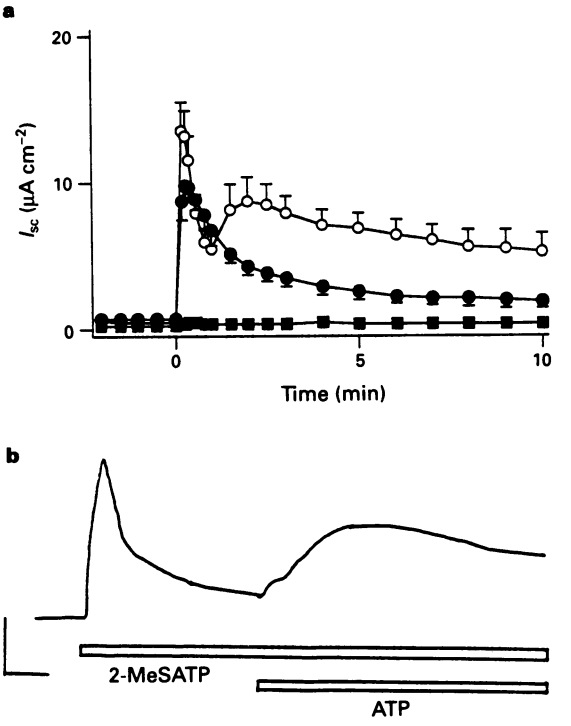

Figure 3 (a) Comparison between the time-course of I_{sc} increase induced by 100 μM ATP (○, $n = 13$), 100 μM 2-MeSATP (●, $n = 6$) and 100 μM BB plus 100 μM 2-MeSATP (■, $n = 3$) applied apically. The first peak evoked by ATP and 2-MeSATP was not statistically different. All data were statistically different from each other from the second minute up to the end of the experiment ($P < 0.05$). (b) Effect of consecutive addition of 2-MeSATP and ATP. The trace is representative of three similar experiments. Vertical and horizontal scale bars represent 5 μA cm^{-2} and 2 min, respectively.

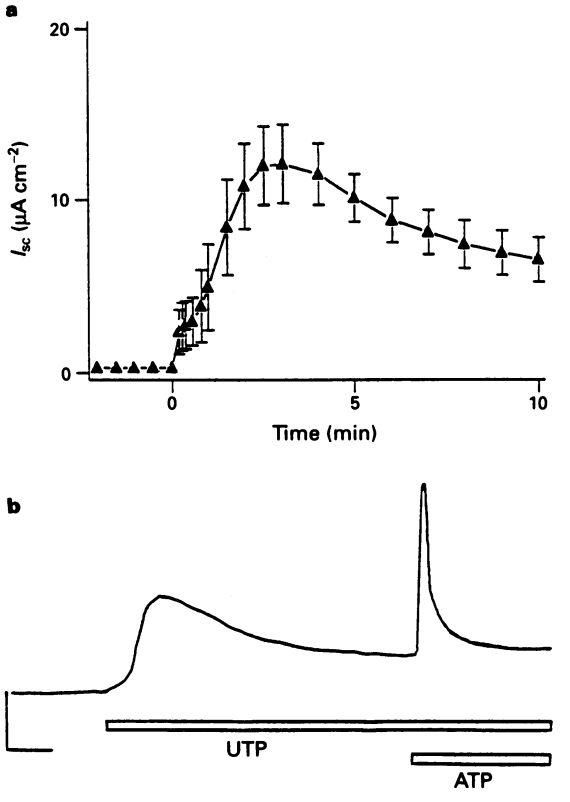

Figure 4 (a) I_{sc} increase evoked by 100 μM apical UTP ($n = 4$). (b) Consecutive addition of UTP and ATP. The trace is representative of three similar experiments. Vertical and horizontal scale bars represent 5 μA cm^{-2} and 2 min respectively.

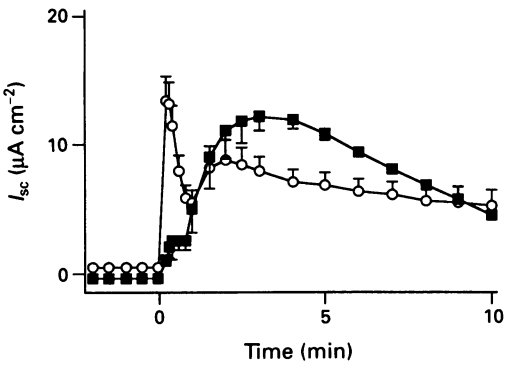

Figure 5 Time-course of I_{sc} increase induced by 100 μM apical ATP in control conditions (○, $n = 13$) and after the incubation of the monolayer with 20 μM BAPTA/AM (■, $n = 6$). The first peak was significantly inhibited ($P < 0.001$) by BAPTA/AM incubation. Values obtained with BAPTA/AM at 4–6 min were significantly higher than those obtained with ATP ($P < 0.05$).

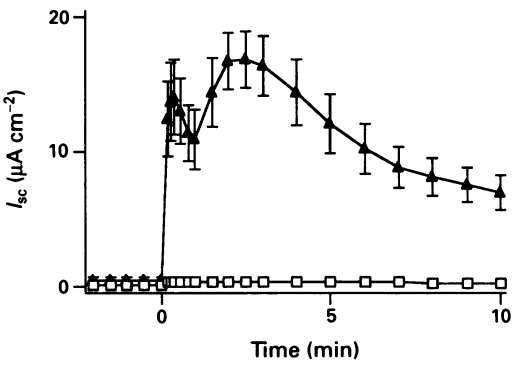

Figure 6 I_{sc} increase evoked by 100 μM apical ATP-γ-S (▲, $n = 4$) and 20 μM 2-chloroadenosine (□, $n = 3$).

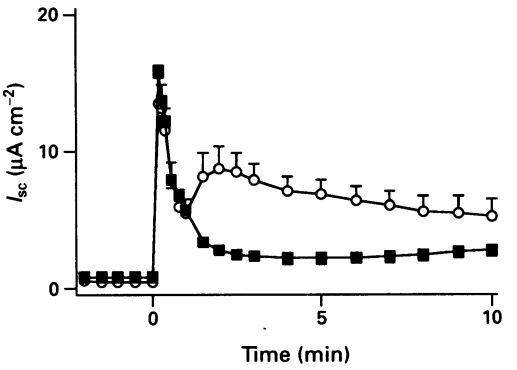

Figure 7 Inhibition of the ATP-evoked I_{sc} increase by indomethacin. Addition of 20 μM indomethacin 2 to 4 min before application of 100 μM apical ATP (■, $n = 3$) significantly inhibited ($P < 0.05$) the second peak evoked by ATP in control conditions (○, $n = 13$).

ATP effect could be mediated by P_1 purinoceptors through ATP hydrolysis and rapid accumulation of adenosine. Accordingly, we tested adenosine analogues CGS-21680 and 2-chloroadenosine in the apical chamber. While the former compound was completely ineffective ($n = 3$), the latter caused an I_{sc} increase of less than 0.2 μA cm^{-2} ($n = 3$, Figure 6). To further test the possibility of adenosine involvement, we also investigated the activity of ATP-γ-S: 100 μM of this non hydrolyzable ATP analogue elicited a strong I_{sc} increase the time-course of which was comparable to that produced by ATP (Figure 6). The first peak was not statistically different from that obtained with ATP at the same concentration, whereas the more delayed current increment was even higher.

Indomethacin has been found to block fully the response to basolateral nucleotide application in MDCK cells (Simmons, 1981a). We tested the ability of this compound to affect the activity of apical ATP. Addition of 20 μM indomethacin to both sides of the epithelia 2–6 min before application of ATP completely inhibited the second part of the ATP response (Figure 7) leaving the first peak unaffected. This indomethacin concentration did not significantly modify I_{sc} by itself.

Isosmotic replacement of NaCl with Na-gluconate and of 2 mM $CaCl_2$ with 5 mM $CaSO_4$ completely abolished the second peak stimulated by ATP but only reduced the amplitude of the first spike to 39% of the control ($n = 3$, not shown). Apical addition of the epithelial sodium channel blocker amiloride (100 μM) did not modify the current amplitude.

Discussion

We have analysed the response of MDCK cells to apical nucleotide stimulation using various agonists and inhibitors. Apical ATP elicited a biphasic I_{sc} response consisting of a first transient spike followed by a second broader current increase. Our study clearly demonstrated that BB is able to inhibit in a dose-dependent fashion the initial peak of the ATP effect. Since BB has been described as an antagonist of P_{2Y} purinoceptors (Burnstock & Warland, 1987), its partial effect in MDCK cells suggests that apical ATP increases I_{sc} by stimulation of two separate receptors one of which belongs to the P_{2Y} group. This seems confirmed by the lack of activity of α,β-meATP and by the fact that 2-MeSATP, a selective agonist of P_{2Y} receptors, at concentrations up to 100 μM elicited a single transient response that could be completely blocked by BB. Cross desensitization experiments indicated that 2-MeSATP and ATP act on a common receptor, since the latter agonist evoked only a monophasic delayed response when applied after the former one. The agonist potency order for the first peak (2-MeSATP ≥ ATP >> UTP, α,β-meATP) is actually different from that of classical P_{2Y} receptors on which 2-MeSATP is sensibly more active than ATP. Nevertheless, the near equipotency of 2-MeSATP and ATP in the present work is similar to that described by Webb *et al.* (1993) for a cloned ATP receptor, blocked by BB and designated as P_{2Y1}. Another interesting observation regarding 2-MeSATP is that it produces a maximum response lower than ATP, suggesting a partial agonist behaviour. The order of potency for the second peak (UTP > ATP > 2-MeSATP > α,β-meATP) is instead consistent with a P_{2U} purinoceptor. Cross-desensitization experiments indicated that UTP and ATP share a common receptor. Indeed, ATP elicited only the first peak when applied following UTP.

Chelation of intracellular Ca^{2+} confirmed that the biphasic response to ATP is due to two different transduction mechanisms. Indeed, BAPTA/AM incubation precluded only the first part of the ATP response. This finding indicates that the putative P_{2Y} receptor in the apical membrane of MDCK cells causes a rapid and transient activation of transepithelial ion transport through an increase in intracellular Ca^{2+}. This could happen *via* phospholipase C stimulation and Ca^{2+} release from intracellular stores, although we cannot exclude an alternative or additive role of extracellular Ca^{2+} influx. The remainder of the ATP response is mediated by a different receptor type and probably by a Ca^{2+}-independent transduction mechanism. We tested whether this response is related to ATP degradation since hydrolysis to adenosine and stimulation of P_1 receptors has been indicated as one of the mechanisms causing ATP-induced Cl^- secretion in T84 cells

(Dho *et al.*, 1992). Nevertheless, this mechanism has been ruled out by our experiments. Adenosine analogues were not able to stimulate significantly I_{sc}. On the other hand, the non hydrolysable ATP-γ-S gave a biphasic effect similar to that of ATP. In addition, UTP, the hydrolysis of which does not produce adenosine, elicited a large response with a time-course resembling that of the second ATP-dependent peak.

The inhibition of the second ATP-evoked peak by indomethacin indicates the involvement of prostaglandins synthesis (Slivka & Insel 1988). Actually, prostaglandins are able *per se* to stimulate transepithelial ion transport in MDCK cells (Simmons, 1991). A mechanism through which prostaglandins can be generated is the Ca^{2+}-dependent activation of phospholipase A_2. Nevertheless, this possibility seems excluded by the finding that intracellular Ca^{2+} buffering by BAPTA/AM did not prevent the delayed response to ATP. It is possible to speculate that prostaglandin production is independent from P_{2Y}-mediated activation of the phospholipase C pathway. Separate activation of both phospholipase pathways has been indeed demonstrated following stimulation of α_1-adrenoceptors and bradykinin receptors in MDCK cells (Slivka & Insel, 1987; 1988). Parallel activation of phospholipase A_2 and phospholipase C has also been observed in neutrophils and in HL60 cells stimulated by extracellular ATP (Cockcroft & Stutchfield, 1989).

Another interesting finding regarding the ionic basis of the ATP response was that although the second peak was entirely dependent on the presence of extracellular chloride and therefore consistent with a mechanism of chloride secretion, the first peak was only partially affected by chloride substitution. This probably indicates the presence of a different mechanism of ion transport the basis of which needs further investigation.

In conclusion, apical ATP stimulates I_{sc} by two different mechanisms. The first one is mediated by a P_{2Y} receptor and by intracellular Ca^{2+} increase. The second is instead mediated by prostaglandins, to some extent is independent of cytosolic calcium and is probably triggered by a P_{2U} purinoceptor, although a conclusive characterization of this receptor is limited by the absence of effective antagonists.

MDCK cells display functional and morphological properties similar to those of the distal tubule and the collecting duct (Valentich, 1981; Herzlinger *et al.*, 1982; Garcia-Perez & Smith, 1983). Our work shows a complex mechanism of regulation of transepithelial ion transport mediated by two purinoceptors in the apical membrane of MDCK cells. This localization poses the important question of the *in vivo* source of luminal nucleotides which should bind to apical receptors. An autocrine mechanism can be postulated through release of ATP and UTP from the same epithelial cells.

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Evidence for the involvement of different receptor subtypes in the pre- and postjunctional actions of angiotensin II at rat sympathetic neuroeffector sites

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1 The effects of the nonpeptide angiotensin II receptor (AT) antagonists losartan and PD 123319 on actions of angiotensin II in the rat caudal artery and rat vas deferens preparations were investigated.

2 Angiotensin II (1.0 μM) increased perfusion pressure in isolated segments of the rat caudal artery. This increase in perfusion pressure was prevented by the AT_1-antagonist, losartan (0.1 μM) but was not affected by the AT_2-antagonist, PD 123319 (0.1 μM).

3 Angiotensin II (0.1–3.0 μM) produced a concentration-dependent enhancement of the stimulation-induced (S-I) efflux of [^{3}H]-noradrenaline from isolated segments of rat caudal artery in which the noradrenergic transmitter stores had been labelled with [^{3}H]-noradrenaline. The maximum enhancement of S-I efflux was approximately 60% with 1.0 μM angiotensin II.

4 Losartan (0.01 and 0.1 μM) reduced the enhancement of S-I efflux produced by 1.0 μM angiotensin II in the caudal artery.

5 PD 123319 (0.01 μM) did not affect the enhancement of S-I efflux produced by angiotensin II (1.0 μM) in the caudal artery. However, in a higher concentration (0.1 μM), PD 123319 reduced the enhancement of S-I efflux produced by 1.0 μM angiotensin II.

6 Angiotensin II produced concentration-dependent enhancement of the purinergic twitch responses (1 pulse/60 s) in the rat vas deferens,

7 Losartan (0.03 μM) and PD 123319 (0.03 μM) each reduced the angiotensin II-induced enhancement of the twitch responses in the rat vas deferens.

8 These findings indicate that the enhancement of sympathetic neuroeffector transmission in both the caudal artery and vas deferens of the rat involves angiotensin receptor subtype(s) sensitive to both losartan and PD 123319. In contrast, the direct vasoconstrictor effect of angiotensin II in the rat caudal artery involves activation of a receptor subtype sensitive only to losartan.

Keywords: Angiotensin II; losartan; PD 123319; vasoconstriction; sympathetic neuroeffector transmission

Introduction

The renin-angiotensin system plays a central role in cardiovascular homeostasis by influencing vascular tone, extracellular fluid and electrolyte balance, and the sympathetic nervous system (Sealey & Laragh, 1989). The interactions of the renin-angiotensin system with the cardiovascular system are predominantly mediated by the octapeptide, angiotensin II. Angiotensin II influences cardiovascular function by several mechanisms, including direct constriction of resistance and capacitance vessels and direct cardiac inotropic and chronotropic activity (Sealey & Laragh, 1989). In addition, angiotensin II has been shown to facilitate noradrenergic neuroeffector transmission by enhancing stimulation-induced release of noradrenaline from sympathetic nerves, although it has also been reported to increase the rate of synthesis of noradrenaline and to inhibit neuronal uptake of the transmitter (Story & Ziogas, 1987). More recently, angiotensin II has been shown to modulate the release of co-transmitters from sympathetic nerve terminals. Thus, in the guinea-pig vas deferens, with low frequencies of stimulation, angiotensin II enhances noradrenergic and purinergic transmission (Ellis & Burnstock, 1989; Ziogas & Cunnane, 1991). With higher frequencies of stimulation, although the noradrenergic component is still enhanced, the purinergic component is not affected (Ellis & Burnstock, 1989). Trachte (1988a) reported that, in the rabbit vas deferens, angiotensin II produces the usual enhancement of noradrenergic transmission, but that the peptide inhibits the purinergic component.

The receptors through which angiotensin II acts to produce its diverse effects were considered homogeneous; however, in the past five years evidence has been obtained for the existence of distinct receptor subtypes (Whitebread *et al.*, 1989; Chiu *et al.*, 1989; Smith *et al.*, 1992). This advance was stimulated by the development of non-peptide receptor antagonists such as DuP 753 (now know as losartan), and PD 123319 and the related PD 123177. Based on numerous radioligand receptor binding and autoradiographic studies, angiotensin receptors are now divided into two major subtypes, subtype 1 (AT_1) and subtype 2 (AT_2). The angiotensin II receptor subtype sensitive to losartan has been designated AT_1, while the receptor sensitive to either PD 123319 or PD 123177 has been designated AT_2. Recent cloning studies suggest that there are two subtypes of AT_1 receptors; termed AT_{1A} and AT_{1B} (Iwai & Inagami, 1992; Murphy *et al.*, 1991).

In functional studies *in vivo* and *in vitro*, the AT_1 receptor antagonist, losartan, effectively attenuates many actions of angiotensin II, including contraction of isolated vascular and nonvascular smooth muscle and cardiac muscle (Smith *et al.*, 1992), stimulation of aldosterone and catecholamine release, its dipsogenic effect, and potentiation of responses to sympathetic nerve stimulation (Wong *et al.*, 1990a). The AT_2 antagonists PD 123177 and PD 123319 are generally without

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effect. Therefore, despite overwhelming evidence for two major subtypes of receptors, the functional responses to angiotensin II seem to be mediated exclusively by activation of the AT_1 receptor. An important physiological consequence of angiotensin II binding to AT_2 receptor sites remains to be established.

In the present study, the non-peptide angiotensin II receptor antagonists, losartan and PD 123319, were used to characterize the receptor subtypes through which angiotensin II mediates its effects on sympathetic neuroeffector function in the rat isolated caudal artery and vas deferens.

Methods

Rat caudal artery preparations

Sprague-Dawley rats (250–350 g) of either sex were killed by a blow to the head and exsanguinated. A segment of the central caudal artery (3–4 cm) was dissected free from the connective tissue and transferred to physiological salt solution (PSS) which had been bubbled with 95% O_2 and 5% CO_2 and maintained at 37°C. Artery segments (1–2 cm) were cannulated at the proximal end and mounted longitudinally under 0.5 g tension, with the proximal end lowermost. The distal end was tied off and a small incision was made through the artery wall, close to the upper tie around the distal end to enable the perfusate to superfuse the adventitial surface. The segments were perfused and superfused at a constant flow rate at 4 ml min^{-1} with PSS by use of a Gilson Minipuls-2 peristaltic pump. Luminal perfusion pressure was monitored with a Grass P23 I.D. blood pressure transducer (Gould Inc., Oxnard, California, U.S.A.) and recorded on a Macintosh IIci computer using a MacLab/4 data acquisition system and chart v3.2.6. software (Analogue Digital Instruments, Sydney, Australia). The A/D sampling was usually between 250–1000 Hz. Circular, bipolar, platinum electrodes were placed around the segments to enable periarterial sympathetic nerve stimulation. Artery segments were allowed to equilibrate for 15 min before the start of experimental procedures.

In the first series of experiments, 20 min after the initial equilibration period, the vasoconstrictor responses to angiotensin II were investigated by introducing 1.0 μM angiotensin II into the PSS perfusing/superfusing the artery preparations. Each artery preparation was exposed only once to angiotensin II since preliminary experiments demonstrated that there was marked desensitization to the peptide with second and subsequent exposures. In experiments in which the effect of losartan and PD 123319 on vasoconstrictor responses to angiotensin II were investigated, the antagonists were introduced to the perfusion fluid in a concentration of 0.1 μM, 20 min before the introduction of angiotensin II.

In the second series of experiments, after the 15 min equilibration period artery segments were stimulated (5 Hz, 10 s) to assess their viability. Suitable preparations were then removed from the organ baths and incubated for 30 min in 2 ml of PSS containing [^{3}H]-noradrenaline (10 μCi ml^{-1}, 0.67 μM). After incubation with [^{3}H]-noradrenaline, artery segments were again set up for perfusion and superfusion, at a constant flow rate of 4 ml min^{-1}, with PSS. After 60 min of perfusion and superfusion, a 30 s train of 1 ms square wave pulses at a frequency of 5 Hz was applied to the preparations in order to facilitate the removal of non-specifically bound radioactivity from the tissues. After a further 30 min of perfusion/superfusion, the artery segments were subjected to two periods of electrical field stimulation (5 Hz, 30 s), delivered 30 min apart. In each stimulation period, 6 consecutive 1-min collections (4 ml volume) of the perfusate/superfusate solution were taken for measurement of the efflux of [^{3}H]-noradrenaline from the artery segments. In each period, stimulation was given after the second collection. In experiments in which the effects of angiotensin II and non-peptide angiotensin II antagonists were investigated, on resting and S-I effluxes of [^{3}H]-noradrenaline, these drugs were introduced to the perfusion/superfusion fluid 20 min before the second period of stimulation.

The perfusate/superfusate was collected in ice-cold glass vials containing 10 μl Na_2SO_3 (1 μM) and 10 μl ethylenediaminetetraacetic acid disodium salt (0.3 M) to limit oxidation of the catecholamines. These samples were frozen for subsequent chromatographic separation of [^{3}H]-noradrenaline and its tritiated metabolites.

Separation of [^{3}H]-noradrenaline from its metabolites

[^{3}H]-noradrenaline and its metabolites were separated by column chromatography with alumina and Dowex -50W ion-exchange resin, using a method adapted from that of Graefe *et al.* (1973). The pH of each collection of perfusate/superfusate was adjusted to pH 8.3–8.5 with 1 M Tris-acetate buffer pH 8.7. Each sample was first passed through an alumina column which adsorbed the catechol compounds noradrenaline (NA) and 3,4-dihydroxyphenylethylene-glycol (DOPEG). NA and DOPEG were then eluted from the alumina columns with two 1 ml aliquots of acetic acid (0.4 M) and NA was then separated from DOPEG by adsorption on Dowex -50W columns. NA was eluted from the Dowex -50W columns with two 0.5 ml aliquots of 6 M HCl/ethanol (1:1, v/v). To determine the recovery of [^{3}H]-noradrenaline from the columns, a known quantity of purified [^{3}H]-noradrenaline was routinely passed through the columns. The values of resting and stimulation-induced effluxes given have not been corrected for recovery (73 ± 2.9%).

Determination of [^{3}H]-noradrenaline

Fractions (1 ml) eluted from the Dowex -50W columns with HCl/ethanol (see above) were mixed with 12 ml of scintillation fluid (Packard, Ultima Gold XR) and the radioactivity present was determined by liquid scintillation counting (Packard Tri-carb 2000). Corrections for counting efficiency were made by external automatic standardisation and the results expressed as disintegrations per minute (d.p.m.).

The resting efflux (R_1 and R_2) of [^{3}H]-noradrenaline preceding each of the two periods of stimulation was determined as the mean content of [^{3}H]-noradrenaline in the two 1-min collections of the perfusate/superfusate solution taken immediately before each period of stimulation. The S-I efflux of [^{3}H]-noradrenaline for each stimulation period was calculated by subtracting the corresponding resting efflux from the content of [^{3}H]-noradrenaline in each of the four consecutive 1-min collections of superfusate, taken from the onset of stimulation, if the radioactivity present exceeded the mean resting efflux, and summing the differences. In each experiment, the resting and S-I effluxes of [^{3}H]-noradrenaline for the second period of stimulation were each expressed as a percentage of their corresponding value for the first period of stimulation, (% R_2/R_1) and (% S_2/S_1), respectively.

Rat vas deferens preparation

Male Sprague-Dawley rats (250–350 g) were killed by decapitation. Whole vasa deferentia were removed and carefully stripped of connective tissue and blood vessels. Segments of 1–2 cm were dissected from the prostatic end and placed longitudinally in tissue baths. A pair of platinum ring eletrodes was placed around the prostatic end of each preparation and the epididymal end was attached to a Ugo Basile isometric transducer. The preparations were perfused at 2 ml min^{-1} with PSS by use of a Gilson Minipuls-2 peristaltic pump. An initial resting tension of 1 g was added to each preparation and changes in tension were recorded on a Rikadenki double channel pen recorder. The preparations were allowed to equilibrate for 30 min.

After the equilibration period, the preparations were stimulated electrically with 1 pulse every 60 s. The voltage was supra-maximal (30 V) and the pulse duration was 0.3 ms. After 10 min of stimulation the responses had stabilized, at which time the PSS solution was changed to a solution containing angiotensin II (3–600 nM) and the tissue was then stimulated for a further 10 min. A further 10 responses were obtained after the washout of angiotensin II. The tissue was then perfused without stimulation for 10 min and the above procedures were repeated using the next higher concentration of angiotensin II. In a separate series of experiments, either losartan or PD 123319 was introduced into the PSS after the initial 10 min stimulation period and a series of twitch responses were evoked in the presence of the antagonist over 10–15 min. The effect of angiotensin II on the twitch responses was then re-examined, in the presence of the antagonist, as described above.

Drugs and radiochemicals

The PSS had the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 0.45, $NaHCO_3$ 25, KH_2PO_4 1.03 and D-(+)-glucose 11.1. Ethylenediaminetetraacetic acid disodium salt (0.067 M) and ascorbic acid (0.14 M) were also present to prevent the oxidation of noradrenaline.

The following drugs were used: angiotensin II (synthetic, human sequence, Sigma, U.S.A.); losartan (gift from Du Pont Merck Pharmaceuticals, Wilmington, Delaware), PD 123319 ((S)1-[[4-dimethylamino)-3-methylphenyl]methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazo[4,5-c] pyridine-6-carboxylic acid, ditrifluoroacetate, dihydrate; gift from Parke Davis, Ann Arbor, Michigan); prazosin hydrochloride (Pfizer, U.S.A.); and α, β-methylene-adenosine-5'-triphosphate (α, β-meATP) (Sigma, U.S.A.).

Angiotensin II stock solutions were prepared in concentrations of 1 mM in 5% acetic acid and stored in 50 μl aliquots at 0°C. Stock solutions of losartan and PD 123319 (1.0 mM) were prepared in deionised water and stored at 5°C. Prazosin was prepared by dissolution in deionised water, containing 5% dextrose and 10% glycerol to give a 1 M stock solution. A 1 mM stock solution of α, β-meATP was prepared in deionised water and stored at 0°C. All stock solutions were diluted in PSS and added to the reservoirs supplying the organ bath.

Tritiated noradrenaline (1-[7,8-^{3}H]-noradrenaline) was supplied by the Radiochemical Centre, Amersham, U.K., with a specific activity of 15 Ci mmol^{-1} and a radioactive concentration of 1.0 mCi ml^{-1}.

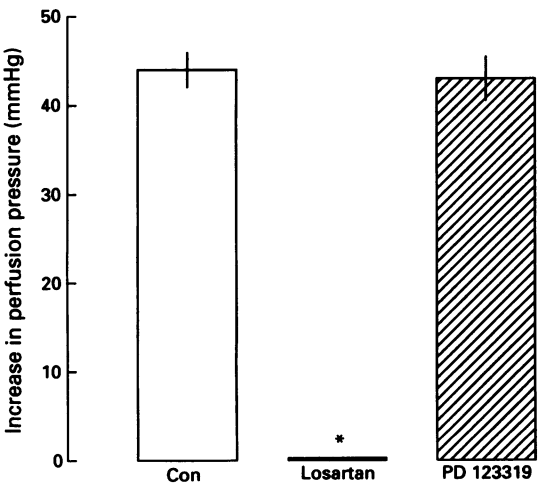

Figure 1 The effects of losartan (0.1 μM) and PD 123319 (0.1 μM) on the vasoconstrictor response to 1.0 μM angiotensin II in isolated perfused/superfused rat caudal artery preparations. The antagonists were introduced 20 min prior to addition of angiotensin II. The vertical axis represents increases in perfusion pressure in mmHg. The columns represent the mean increases in perfusion pressure produced by angiotensin II in the absence (Con) and presence of the antagonists losartan and PD 123319. The vertical lines represent the standard errors of the means from 4–8 experiments. Losartan, but not PD 123319 significantly ($P<0.05$) reduced the vasoconstrictor responses to angiotensin II (ANOVA, unpaired t tests).

Statistical analysis

Data are expressed as means ± standard error of the mean (s.e.mean); n represents the number of experiments. For the rat caudal artery experiments, groups of means were tested for significant differences by using one-way analysis of variance (ANOVA) and planned comparisons (Student's unpaired t test) between predetermined pairs of means. For the concentration-effect curves obtained in the rat vas deferens, analysis of co-variance (ANCOVA) tests were performed with the computer software Regression and Empirical Analytical Procedures (Gamma Research Systems). Data within time-control or angiotensin II concentration-effect groups were tested by ANOVA to determine if there were any time-or concentration-dependent effects. In all cases probability levels less than 0.05 ($P<0.05$) were taken to indicate significant differences between means.

Results

Effects of angiotensin II on perfusion pressure in the rat caudal artery in the absence and presence of losartan and PD 123319

When isolated caudal artery segments were perfused and superfused at a constant flow rate of 4 ml min^{-1}, the luminal perfusion pressure stabilised to between 20 and 40 mmHg within 10–15 min and, in the absence of drugs, remained stable. Addition of angiotensin II (1.0 μM) to the perfusion/superfusion solution produced an increase in perfusion pressure of 44 ± 2 mmHg ($n = 6$). The peak increase in perfusion pressure occurred within 20–30 s and the perfusion pressure declined to basal over the next 2–3 min in the continued presence of angiotensin II.

The receptor antagonists, losartan (0.1 μM) and PD 123319 (0.1 μM), did not significantly alter the basal perfusion pressure ($P>0.05$, ANOVA, unpaired t tests). The AT_1-antagonist, losartan (0.1 μM), when introduced into the perfusion/superfusion fluid 20 min before the addition of angiotensin II (1.0 μM), abolished the vasoconstrictor response to angiotensin II (Figure 1). In contrast, as shown in Figure 1, the vasoconstrictor response to angiotensin II (1.0 μM) was unaltered by the presence of the AT_2-antagonist PD 123319 (0.1 μM).

Effects of angiotensin II on the resting and S-I effluxes of [^{3}H]-noradrenaline in the rat caudal artery

Rat caudal artery segments loaded with [^{3}H]-noradrenaline were given two periods of electrical field stimulation (5 Hz, 30 s) at 30 min intervals. In control experiments, the mean resting efflux of [^{3}H]-noradrenaline, preceding the first period of stimulation, was 207 ± 20 d.p.m. ($n = 7$). There was a progressive decline in the resting efflux of [^{3}H]-noradrenaline between the two periods of stimulation. The mean value of the resting efflux of [^{3}H]-noradrenaline preceding the second period of stimulation, expressed as a percentage of that preceding the first (% R_2/R_1) was 83 ± 6% ($n = 7$). Stimulation of the periarterial sympathetic nerves (5 Hz, 30 s) of the artery segments produced an increased efflux of [^{3}H]-noradrenaline. The mean absolute stimulation-induced (S-I) efflux of [^{3}H]-noradrenaline evoked by the first period of stimulation was 670 ± 154 d.p.m. ($n = 7$). The mean value of the S-I efflux of [^{3}H]-noradrenaline with the second period of stimulation, expressed as a percentage of that for the first (% S_2/S_1), was 96 ± 7% ($n = 7$).

Introduction of angiotensin II (0.1–3.0 μM) to the perfusion/superfusion fluid, 20 min before the second period of stimulation, did not significantly alter the resting efflux of [^{3}H]-noradrenaline ($P>0.05$ ANOVA). In contrast, as shown in Figure 2, angiotensin II (0.1–3.0 μM) produced concentration-dependent increases in the S-I efflux of [^{3}H]-noradrenaline. The maximum enhancement of S-I efflux of 60–70% was produced by 1.0 μM angiotensin II. Further increases in concentration of angiotensin II actually produced less enhancement (Figure 2).

Effect of losartan on the enhancement of S-I efflux by angiotensin II in the rat caudal artery

Losartan in concentrations of 0.01 and 0.1 μM, added to the perfusion/superfusion fluid 20 min before the second period of stimulation, did not significantly alter the resting or S-I effluxes of [^{3}H]-noradrenaline ($P>0.05$, ANOVA). As shown in Figure 3, the enhancement of S-I efflux produced by 1.0 μM angiotensin II was reduced by 0.01 μM losartan and abolished by 0.1 μM losartan.

Effect of PD 123319 on the enhancement of S-I efflux by angiotensin II in the rat caudal artery

PD 123319 in concentrations of 0.01 and 0.1 μM, added to the perfusion/superfusion fluid 20 min before the second period of stimulation, did not significantly alter the resting or S-I effluxes of [^{3}H]-noradrenaline ($P>0.05$, ANOVA). As shown in Figure 4, the enhancement of S-I efflux produced by 1.0 μM angiotensin II was not significantly altered by 0.01 μM PD 123319. In contrast, the enhancement of S-I efflux by 1.0 μM angiotensin II was significantly reduced by 0.1 μM PD 123319 (Figure 4).

Effect of angiotensin II on twitch responses of the rat vas deferens evoked by electrical stimulation

Electrical stimulation with 1 pulse every 60 s of prostatic segments of the rat vas deferens generally produced rapid, monophasic responses. Addition of 1 μM α, β-meATP produced an initial enhancement of these twitch responses, lasting for 4–5 min. However, prolonged exposure desensitized the P_2-purinoceptors and abolished the twitch responses. In some experiments, the responses were biphasic and the effect of the α_1-adrenoceptor antagonist prazosin, was assessed on these responses. Prazosin, in a concentration of 1 μM, completely abolished the second phase of these biphasic responses, leaving a large twitch response to nerve stimulation. In preliminary experiments, angiotensin II clearly enhanced both phases of the biphasic twitch responses. However, for the present study, the effect of angiotensin II was assessed only on the monophasic purinergic twitch responses.

In 9 experiments, the mean control response prior to the addition of the first concentration of angiotensin II was 1.1 ± 0.2 g. By allowing a 10 min rest period without stimulation after washout of each concentration of angiotensin II (see methods), the responses were well maintained and there were no significant differences between the control responses obtained prior to each addition of angiotensin II ($P>0.05$, ANOVA). As shown in Figures 5 and 6, in the absence of antagonists, addition of angiotensin II (3–300 nM) produced concentration-dependent enhancement of the twitch responses to nerve stimulation.

Effect of losartan on the enhancement of twitch responses by angiotensin II in the rat vas deferens

When losartan (0.01 and 0.03 μM), was added to the perfusion solution there was an initial enhancement in the twitch responses (10–15%). Once the responses had stabilized, losartan (0.01 and 0.03 μM) produced a concentration-dependent reduction in the enhancement of twitch responses produced by angiotensin II (Figure 5).

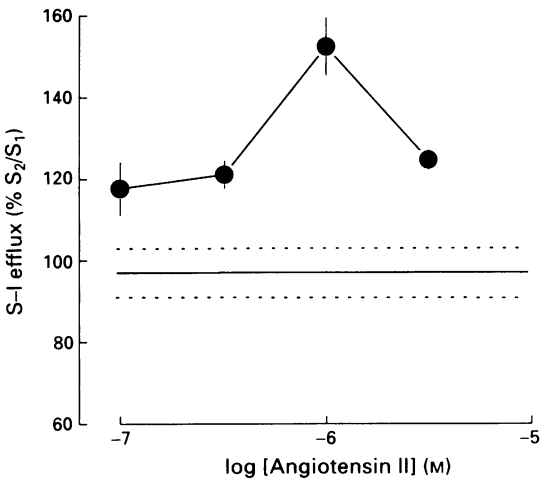

Figure 2 Log concentration-response relationship for the effect of angiotensin II on the enhancement of stimulation-induced (S-I) efflux of [^{3}H]-noradrenaline from rat isolated caudal artery preparations in which the noradrenergic transmitter stores had been radiolabelled with [^{3}H]-noradrenaline. The periarterial sympathetic nerves were stimulated for 2 periods (5 Hz, 30 s) at 30 min intervals. In each experiment, the S–I efflux with the second period of stimulation was expressed as a percentage of that with the first period (% S_2/S_1). The unbroken horizontal line represents the mean S–I efflux in the absence of angiotensin II and the broken lines the standard error of the mean. The filled circles (●) represent the effects of angiotensin II (0.1–3.0 μM) when introduced to the perfusion fluid 20 min before the second period of stimulation. The points plotted represent the means ± s.e.means from 4–8 experiments.

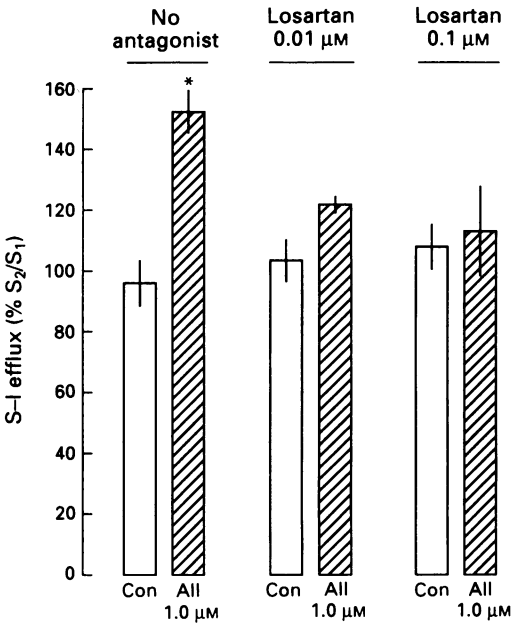

Figure 3 Effect of 1.0 μM angiotensin II in the absence and presence of losartan on the stimulation-induced (S–I) efflux of radioactive noradrenaline from [^{3}H]-noradrenaline-labelled rat caudal artery preparations. The periarterial sympathetic nerves were stimulated for 2 periods (5 Hz, 30 s), at 30 min intervals. In each experiment, the S–I efflux with the second period of stimulation was expressed as a percentage of that with the first period (% S_2/S_1). Angiotensin II, losartan, or a combination of angiotensin II and losartan (0.01 or 0.1 μM), was introduced 20 min before the second period of stimulation. Open columns represent the respective control (Con) experiments and the hatched columns, experiments in which angiotensin II (AII) was added in either the absence or presence of losartan. Columns represent the means ± s.e.means from 4–6 experiments. *Significant differences cf. respective control ($P<0.05$, ANOVA, unpaired t tests).

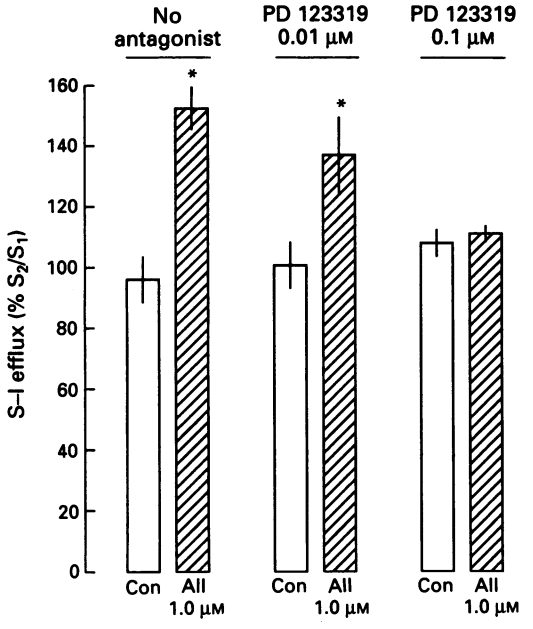

Figure 4 Effect of 1.0 μM angiotensin II in the absence and presence of PD 123319 on the stimulation-induced (S–I) efflux of radioactive noradrenaline from [^{3}H]-noradrenaline-labelled rat caudal artery preparations. The periarterial sympathetic nerves were stimulated for 2 periods (5 Hz, 30 s), at 30 min intervals. In each experiment, the S–I efflux with the second period of stimulation was expressed as a percentage of that with the first period (% S_2/S_1). Angiotensin II, PD 123319, or a combination of angiotensin II and PD 123319 (0.01 or 0.1 μM), was added 20 min before the second period of stimulation. Open columns represent the respective control (Con) experiments and the hatched columns, experiments in which angiotensin II (AII) was added in either the absence or presence of PD 123319. Columns represent the means ± s.e.means from 4–6 experiments. *Significant differences cf. respective control ($P<0.05$, ANOVA, unpaired t tests).

Effect of PD 123319 on the enhancement of twitch responses by angiotensin II in the rat vas deferens

Exposure of the tissue to PD 123319 had no significant effect on the twitch responses to nerve stimulation ($P<0.05$, t test). As shown in Figure 6, PD 123319 (0.01 and 0.03 μM), reduced the enhancement of twitch responses by angiotensin II in a concentration-dependent manner.

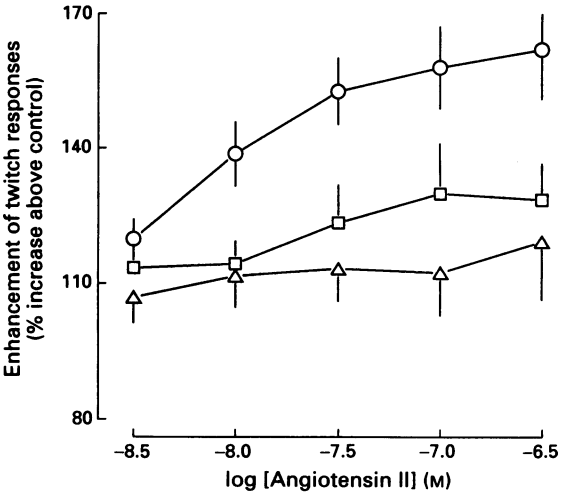

Figure 5 Pooled data showing enhancement of single pulse twitch responses of rat vas deferens preparations by angiotensin II (○), and inhibition of these responses by losartan 0.01 μM (□) and 0.03 μM (△) ($P<0.05$, ANCOVA). The points plotted represent means with s.e.means of 4–6 experiments.

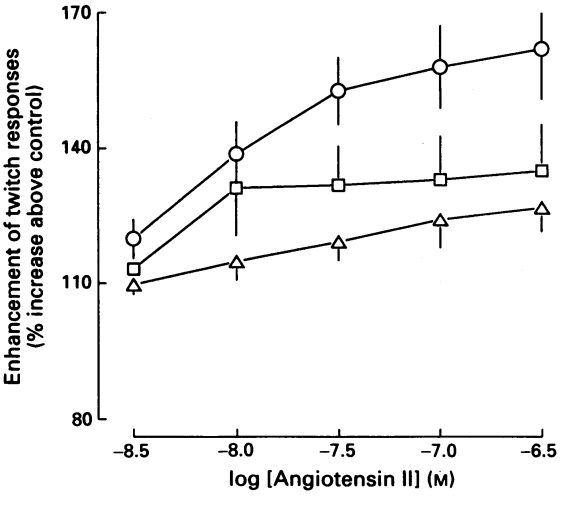

Figure 6 Pooled data showing enhancement of single pulse twitch responses of rat vas deferens preparations by angiotensin II (○), and inhibition of these responses in the presence of PD 123319 0.01 μM (□) and 0.03 μM (△) ($P<0.05$, ANCOVA). The points plotted represent means of 4–6 experiments ± s.e.means.

Discussion

Inhibition of the vasoconstrictor action of angiotensin II by the AT_1-antagonist losartan has been demonstrated in various isolated tissue preparations, including rabbit aorta and uterus (Dudley *et al.*, 1990), rat pulmonary artery (Chang & Lotti, 1989) and rat portal vein (Rhaleb *et al.*, 1991). Similarly, in the present study, in the rat caudal artery, the increase in perfusion pressure produced by angiotensin II was blocked by losartan and not affected by PD 123319. Such *in vitro* findings are consistent with those of *in vivo* studies which have demonstrated that increases in blood pressure produced by angiotensin II are mediated through AT_1 receptors (Wong *et al.*, 1990b). Whereas it is clear that the postjunctional vasoconstrictor effect of angiotensin II is subserved by AT_1 receptors, the receptor(s) involved in the action of angiotensin II on transmitter noradrenaline release is not as well defined.

In rat isolated caudal artery preparations, in which transmitter stores had been radiolabelled with [^{3}H]-noradrenaline, angiotensin II produced concentration-dependent enhancement of the S-I efflux of [^{3}H]-noradrenaline. The maximum enhancement occurred with 1 μM angiotensin II, and further increases in concentration to 3 μM produced less enhancement. Such a 'bell shaped' concentration-effect relationship has also been reported for the modulation of sympathetic transmission by angiotensin II in guinea-pig atria (Ziogas *et al.*, 1984) and rabbit vas deferens (Trachte *et al.*, 1990; Hedge & Clarke, 1993). The enhancement of S-I [^{3}H]-noradrenaline efflux by angiotensin II was blocked by losartan (0.01 and 0.1 μM) and by PD 123319 (0.1 μM). Similar findings were also observed in the rat vas deferens where angiotensin II produced a concentration-dependent enhancement of the twitch responses to sympathetic nerve stimulation. This enhancement was also blocked by both losartan and PD 123319. These findings suggest that the prejunctional angiotensin II receptor has different characteristics from the postjunctional receptor.

Previous studies in which the nature of the prejunctional angiotensin II receptor has been investigated, have given rise to differing conclusions. Several reports suggest that the prejunctional receptor is of the AT_1 subtype. For example, in

guinea-pig isolated atria, the enhancement of noradrenaline release by angiotensin II was blocked by losartan but unaffected by PD 123319 (Brasch *et al.*, 1993). Similarly, the enhancement of noradrenergic transmission in the rat mesenteric vascular bed by angiotensin II was abolished by losartan (Parrish & Cassis, 1991; Tofovic *et al.*, 1991) but not influenced by PD 123177 (Tofovic *et al.*, 1991). The prejunctional enhancement of renal noradrenergic function in the anaesthetized dog was also blocked by losartan (Wong *et al.*, 1990c). In contrast, in the rabbit isolated vas deferens, both losartan and PD 123177 failed to inhibit the enhancement of noradrenergic transmission by angiotensin II (Trachte *et al.*, 1990; 1991). Thus, Trachte *et al.* (1991) suggested that neither AT_1 nor AT_2 receptors are involved in the enhancement of noradrenergic transmission by angiotensin II. In the present study, the enhancement by angiotensin II, of noradrenergic transmission in the rat caudal artery, was inhibited by both losartan and PD 123319, suggesting that the prejunctional receptor differs from the major AT_1 and AT_2 subtypes.

Several studies have suggested that the angiotensin II receptors involved in modulation of sympathetic noradrenergic and purinergic transmission in vas deferens preparations are not homogeneous. Thus, in the guinea-pig vas deferens, with 2 Hz stimulation, angiotensin II enhanced both noradrenaline and ATP release, but at 20 Hz stimulation, only noradrenaline release was enhanced by angiotensin II (Ellis & Burnstock, 1989). The heptapeptide angiotensin III enhanced noradrenaline release, but inhibited ATP release; however, no antagonists were used to determine the subtypes of angiotensin receptors involved in these actions of angiotensin II and angiotensin III (Ellis & Burnstock, 1989). In the rabbit vas deferens, angiotensin II and angiotensin III each enhanced the noradrenergic component of the biphasic response to sympathetic nerve stimulation and inhibited the purinergic component (Trachte, 1988a). Losartan did not prevent the enhancement of the noradrenergic component but it did prevent the inhibition of the purinergic component by angiotensin II and also the release of prostaglandin E evoked by angiotensin II (Trachte *et al.*, 1990). In the guinea-pig vas deferens, angiotensin II usually enhanced purinergic excitatory junction potentials; however, following reserpine treatment, angiotensin II inhibited purinergic transmission (Ziogas & Cunnane, 1991). Interestingly, the inhibition was prevented by pretreatment with the cyclo-oxygenase inhibitor, indomethacin (Ziogas & Cunnane, 1991). Several studies have demonstrated that endogenously released prostaglandins can interfere with the prejunctional enhancement of sympathetic transmission by angiotensin II in a number of preparations. Thus, Lanier & Malik (1982) demonstrated that the enhancement of noradrenaline release in the rat heart by angiotensin II was attenuated by prostaglandins released from the myocardium. In the rabbit ear artery the inhibition of sympathetic neurotransmission by angiotensin II was reversed by indomethacin pretreatment (Rónai, 1991). Trachte (1988b) reported that indomethacin and eicosatetraenoic acid, but not acetylsalicylic acid, could prevent the inhibition of purinergic transmission by angiotensin II in the rabbit vas deferens. Therefore, it is likely that the inhibition of the purinergic response by angiotensin II in the rabbit vas deferens may involve stimulation of a postjunctional AT_1 receptor which subserves release of prostaglandins. An inhibitory action involving prostaglandins does not appear to interfere with the enhancement of purinergic transmission reported in this study with the rat vas deferens or in the guinea-pig vas deferens (Ellis & Burnstock, 1989). Whether these differences relate to species or differences in other experimental conditions remains to be determined. None the less, the findings of this study indicate that the enhancement of sympathetic purinergic transmission by angiotensin II may involve a prejunctional receptor that is sensitive to both losartan and PD 123319.

As mentioned in the Introduction, the original studies on which the AT_1 and AT_2 classifications were based, indicated that AT_2 antagonists are generally ineffective in blocking the multitude of actions of angiotension II in the cardiovascular system (Smith *et al.*, 1992). However, recent studies have demonstrated that the AT_2 antagonist used in the present study, PD 123319, can inhibit the actions of angiotensin II in certain preparations. Thus, Kunert-Radek *et al.* (1994) have recently shown that the stimulatory effect of angiotensin II on the proliferation of mouse spleen lymphocytes was blocked by both losartan and PD 123319. Similarly, the intracerebroventricular injection of angiotensin II in anaesthetized rats (Widdop *et al.*, 1993a) and the angiotensin II-mediated drinking response in rats (Rowland *et al.*, 1992) are both blocked by PD 123319. In contrast, the related AT_2 antagonist, PD 123177, was without effect on the intracerebroventricular injection of angiotensin II (Widdop *et al.*, 1993b; Rowland *et al.*, 1992). Therefore, it is likely that PD 123319 is not as selective for the AT_2 receptor as initially reported and that functional subtypes of the AT_1 and AT_2 receptors may exist.

Cloning studies have identified at least two subtypes of the AT_1 receptor which have been termed AT_{1A} and AT_{1B} (Murphy *et al.*, 1991; Iwai & Inagami, 1992). Moreover, Zhou *et al.* (1993) have identified two distinct binding sites in rat mesangial cells, coupled to G proteins, which are sensitive to both losartan and PD 123319. One binding site, which showed a much higher affinity for losartan was termed AT_{1A} by Zhou *et al.* (1993). The other binding site which showed a higher affinity for PD 123319 was termed AT_{1B} (Zhou *et al.*, 1993). The AT_{1A} and AT_{1B} subtypes are involved in the phospholipase C-mediated increase in intracellular calcium and inhibition of adenylyl cyclase evoked by angiotensin II in the rat mesangial cells (Madhun *et al.*, 1993). Only the AT_{1A} receptor is involved in the angiotensin II-induced increase in protein synthesis (Madhun *et al.*, 1993). Therefore, the original classification of AT_1 and AT_2 receptors may need to be extended to include AT_{1A} and AT_{1B} receptor subtypes.

In conclusion, the receptor involved in the enhancement of sympathetic neuroeffector transmission in the rat caudal artery and the rat vas deferens is blocked by both losartan and PD 123319. This suggests that the prejunctional receptor may be of the AT_{1B} subtype as described by Zhou *et al.* (1993). In contrast, the vasoconstrictor effect of angiotensin II in the rat caudal artery is only blocked by losartan and may be regarded as the AT_{1A} subtype.

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Evidence for lack of modulation of μ-opioid agonist action by δ-opioid agonists in the mouse vas deferens and guinea-pig ileum

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1 There is evidence from *in vivo* studies for an interaction of μ- and δ-opioid ligands. In the present work this concept has been investigated using the mouse vas deferens and guinea-pig ileum myenteric plexus-longitudinal preparations.

2 In field stimulated vasa deferentia of the mouse, co-administration of sub-effective concentrations of the δ-opioid agonist [D-Pen^2,D-Pen^5]enkephalin (DPDPE) and [Met^5]- or [Leu^5]enkephalin had no effect on the dose-response curves of the μ-agonists [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (DAMGO) and morphine. Similarly, the δ-opioid agonists did not alter the potency of morphine and DAMGO when added at different times prior to the μ-opioid agonists, or when EC_{50} concentrations of δ-opioid ligands were co-administered. Compounds with preferred activity for the putative δ_1-(DPDPE) or δ_2- ([D-Ala^2,Glu^4]deltorphin II (Delt II)) opioid receptors were ineffective in this respect.

3 The guinea-pig ileum contains δ-opioid receptors. No function of these receptors in mediating blockage of field-stimulated contractions was observed with ligands having affinity for the putative δ_1 or δ_2 subtypes nor were the agonists able to modulate responses to μ-opioid ligands in this tissue.

4 The results demonstrate that modulation of μ-opioid agonists by δ-opioid agonists does not occur in the isolated peripheral tissues examined. Thus the findings do not support the concept of a functional coupling of opioid receptors, though the results may be explained by differences between opioid systems in the brain and peripheral tissues examined.

Keywords: μ-Opioid receptor; δ-opioid receptor; opioid receptor subtypes; μ/δ interactions; β-endorphin; mouse vas deferens; guinea-pig ileum

Introduction

The ability of δ-opioid agonists to modulate the *in vivo* actions of μ-opioid agonists to promote or decrease their activity has been demonstrated (for review see Traynor & Elliott, 1993). The interactions have been reported for antinociceptive[4] tests (Vaught & Takemori, 1979; Lee *et al.*, 1980; Jiang *et al.*, 1990a) but also extend to other activities such as bladder contraction (Sheldon *et al.*, 1989), gut propulsion (Heyman, 1987), EEG and EEG spectral power (Stamidis & Young, 1992) and antitussive activity (Kamei *et al.*, 1993). These effects are observed following the administration of a sub-effective dose of certain δ-opioid agonists. Thus the δ-opioid [D-Pen^2,D-Pen^5]enkephalin (DPDPE) affords an increase in the potency of μ-opioid ligands whilst the δ-opioid agonist [Met^5]enkephalin shifts dose-response curves for the μ-opioid agonists to the right. Both the positive and negative modulatory actions of δ-opioid agonists are prevented by pretreatment with the selective δ-opioid antagonist, ICI 174,864 (N,N-diallyl-Tyr-Aib-Phe-Leu-OH) (Jiang *et al.*, 1990a).

Interestingly not all μ-opioid ligands are responsive to modulation. Thus using the mouse tail-flick assay DPDPE, administered either i.c.v. or s.c., increases the potency of morphine, normorphine, levorphanol, methadone and codeine but not the generally more efficacious μ-opioid agonists [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (DAMGO), [NMePhe^3,D-Pro^4]morphiceptin (PLO17), phenazocine, sufentanil or etorphine (Vaught & Takemori 1979; Heyman *et al.*, 1989). On the other hand, potentiation of the activity of the μ-opioid agonists PLO17 and DAMGO by use of higher (ED_{50}) levels of δ-opioid ligands, administered intrathecally, has been reported in the rat hot plate test (Malmberg & Yaksh, 1992). The reasons for this apparent discrepancy between observations is unknown but may be related to species differences and/or the route of drug administration employed.

The concept of interacting μ- and δ-opioid systems has received support from mathematical analyses of ligand binding assays (Barrett & Vaught, 1983; Demoliou-Mason & Barnard, 1986; Rothman *et al.*, 1988). Following these studies δ-opioid receptors have been divided into so-called μ-complexed and μ-non-complexed types. Biochemical evidence has been provided by the ability of both μ- and δ-opioid ligands to inhibit the covalent linking of β-endorphin to rat striatal membranes (Schoffelmeer *et al.*, 1990). In addition recent work with newly developed antagonists lends support to the presence of a heterogeneous δ-opioid receptor population, leading to a division into so-called δ_1 and δ_2 subtypes (Jiang *et al.*, 1991). However, the relationship between the two definitions of δ-opioid subtypes is unclear (Traynor & Elliott, 1993).

Interactions between co-administered μ- and δ-opioid agonists could be of major clinical importance. It may be possible to overcome tolerance and the ceiling effects of certain lower efficacy μ-opioid ligands. For example it has been shown that while DPDPE and [Leu^5]enkephalin increase the potency of morphine, if either compound is co-administered with morphine then it is only the amount of morphine administered that determines the rate of development of tolerance (Jiang *et al.*, 1990b). To investigate the phenomenon further it would be helpful if such interactions could be observed *in vitro*. This study examines whether two tissue preparations which respond to opioid agonists by an inhibition of electrically-induced contractions, namely the mouse vas deferens and the guinea-pig ileum myenteric plexus-longitudinal muscle, might be suitable models to address this issue. The mouse vas deferens contains a predominantly δ-opioid receptor population, which recent studies

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suggest may be the putative δ_2 subtype (Wild *et al.*, 1993), with μ- and κ-opioid receptors also present (Lord *et al.*, 1977). In this tissue evidence for an interaction between μ-and δ-opioid receptors has been obtained by examining the ability of various ligands to protect the δ-site from alkylation by the affinity ligand β-chlornaltrexamine (Sheehan *et al.*, 1986). The guinea-pig ileum myenteric plexus-longitudinal muscle contains functional μ- and κ-opioid receptors (Chavkin *et al.*, 1982). Whilst δ-opioid receptors have been shown in ligand binding (Leslie *et al.*, 1980) and electrophysiological studies (Egan & North, 1981) ligand occupation of these sites does not lead to a blockade of electrically-induced contractions. Therefore we have considered whether this δ-opioid receptor population might be able to modulate the actions of μ-opioid agonists. Some of the findings of this study have appeared in preliminary form (Elliott & Traynor, 1994).

Methods

Bioassays

Male Dunkin-Hartley guinea-pigs (300–450 g) (David Hall, Burton-on-Trent) were killed by cervical dislocation and segments of ileum were removed and placed in Krebs solution containing (mM): NaCl 118, KCl 4.7, $CaCl_2.2H_2O$ 2.6, KH_2PO_4 1.2, $MgSO_4.7H_2O$ 1.2, $NaHCO_3$ 25 and glucose 11. Male CSI mice (25–30 g) (Nottingham University Medical School) were killed by decapitation and vasa deferentia removed and placed in Krebs solution minus $MgSO_4.7H_2O$.

Mouse vasa deferentia and myenteric plexus-longitudinal muscle preparations of the guinea-pig ileum were set up for field stimulation as previously described (Traynor *et al.*, 1987). Concentration-effect curves for the inhibition of electrically-induced contractions were constructed by cumulative addition of agonists to the bathing fluid. Initially concentration-effect curves for the δ-opioid agonists were obtained. Concentration-effect curves for μ-opioid agonists were then constructed in the absence and presence of a predetermined concentration (either sub-effective or EC_{50}) of δ-opioid agonist. Antagonist equilibrium dissociation constants (K_e nM) were determined from the ratios of EC_{50} values for agonists in the absence or presence of antagonist (added 15 min prior to the redetermination of agonist concentration-response curves) using the formula K_e = [antagonist]/dose ratio − 1 (Kosterlitz & Watt, 1968). Statistical comparisons were made where appropriate by Student's *t* test, where $P<0.05$ was considered significant.

Materials

The following drugs and peptides were used: morphine, naloxone, β-endorphin, [Met^5]- and [Leu^5]enkephalin, [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (DAMGO) and [D-Pen^2,D-Pen^5]enkephalin (DPDPE) (Sigma); [D-Ala^2,Glu^4]deltorphin II (Delt II) (Peninsula); cyprodime and naltrindole (Research Biochemicals).

Results

Mouse vas deferens

The δ-opioid receptor selective ligands, DPDPE (Mosberg *et al.*, 1983) and [D-Ala^2,Glu^4]deltorphin II (Delt II) (Erspamer *et al.*, 1989) and [Met^5]- and [Leu^5]enkephalin were full agonists in the field stimulated mouse vas deferens preparation with potencies (EC_{50} values) of 0.70 ± 0.06 nM, 0.28 ± 0.03 nM, 5.7 ± 1.2 nM and 6.6 ± 0.9 nM respectively (Figure 1). Concentrations of δ-opioid agonists affording < 10% inhibition were chosen as sub-effective concentrations, i.e., 0.1 nM for DPDPE and Delt II or 1.0 nM for [Met^5]- and [Leu^5]enkephalin for subsequent experiments. Concentration-effect curves were obtained for the μ-agonists DAMGO and morphine and repeated in the presence of a co-administered δ-opioid ligand at the chosen sub-effective concentration. There was no significant difference in the potencies for the μ-opioid ligands under the two conditions (Table 1), nor in the slope of the concentration-effect curves (Figure 2). Similarly no attenuation or enhancement of the EC_{50} for the μ-agonists was observed when the δ-opioid agonists were added 2, 4 or 15 min prior to the re-determination of the μ-opioid agonist concentration-effect curve (Table 1).

DPDPE added at a concentration causing 50% inhibition of the electrically evoked twitch also failed to alter significantly the potency of the μ-opioid agonists, morphine (EC_{50} 180 ± 13 nM in the absence of DPDPE compared to 212 ± 22 nM in the presence of DPDPE) and DAMGO (EC_{50}

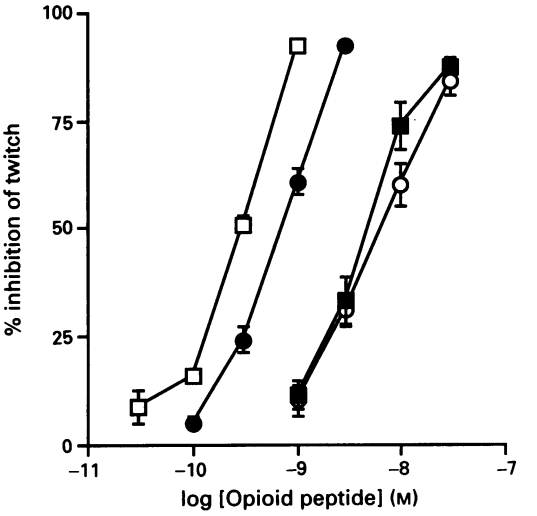

Figure 1 Inhibition of electrically-induced contractions of the mouse vas deferens by DPDPE (●), Delt II (□), [Met^5]enkephalin (○) and [Leu^5]enkephalin (■). Values are means ± s.e.mean where $n \geq 3$. For abbreviations in this and subsequent legends, see text.

Table 1 Potency of μ-ligands in the mouse vas deferens in the absence and presence of sub-effective concentrations of δ-agonists added at different times prior to the μ-agonist

μ-Opioid agonist	*δ-Opioid agonist*	*Time* (min)	IC_{50} (mM)
DAMGO			15.3 ± 1.7
DAMGO	DPDPE	0 (coadministered)	12.1 ± 1.1
		2	12.1 ± 0.9
		4	11.4 ± 1.6
		15	18.3 ± 1.3
	Delt II	0 (coadministered)	16.0 ± 2.4
		2	10.5 ± 1.4
		4	10.5 ± 0.6
		15	13.2 ± 0.7
Morphine			180 ± 13
Morphine	DPDPE	0 (coadministered)	200 ± 16
		2	130 ± 21
		4	122 ± 17
		15	195 ± 30
	DeltII	0 (coadministered)	150 ± 6.5
		2	192 ± 44
		4	161 ± 24
		15	248 ± 60
	[Leu^5]enkephalin	0 (coadministered)	233 ± 26
	[Met^5]enkephalin	0 (coadministered)	182 ± 12

Values are means ± s.e.mean where $n \geq 3$. None of the results was significantly different from additive where $P<0.05$.

15.3 ± 1.7 nM in the absence of DPDPE compared to 14.2 ± 3.6 nM in the presence of DPDPE) when co-administered. The same lack of effect was observed with Delt II and also [Met^5]- and [Leu^5]enkephalin.

β-Endorphin has similar affinity for μ- and δ-opioid receptors (Kosterlitz *et al.*, 1986). This compound was a full agonist in the mouse vas deferens affording an EC_{50} of 31.3 ± 2.7 nM. The dose-effect curve for β-endorphin was shifted to the right in the presence of the non-selective opioid antagonist naloxone (200 nM), having an apparent K_e for naloxone of 26.0 ± 4.3 nM, indicative of an action at δ-opioid receptors (Figure 3). This was confirmed by the effectiveness of the δ-opioid receptor selective antagonist naltrindole (Rogers *et al.*, 1990) in antagonizing the β-endorphin response, affording a K_e of 0.86 ± 0.10 nM, and the lack of antagonism by cyprodime, a selective μ-opioid antagonist (Schmidhammer *et al.*, 1989), affording a $K_e > 300$ nM (Figure 3).

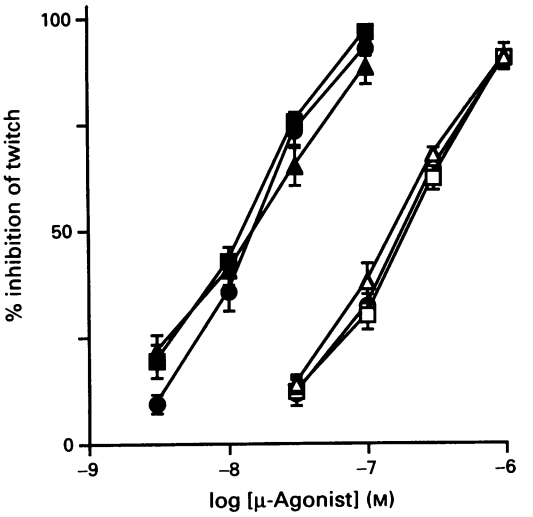

Figure 2 Inhibition of electrically-induced contractions of the mouse vas deferens by DAMGO (closed symbols) and morphine (open symbols) alone (circles) or coadministered with sub-effective concentrations of DPDPE (squares) or Delt II (triangles). Values are means ± s.e.mean where $n \geq 3$.

Guinea-pig myenteric plexus-longitudinal muscle

DPDPE (EC_{50} = 4740 nM) and Delt II (EC_{50} = 2180 nM) were very weakly active in inhibiting the electrically-evoked twitch of the longitudinal muscle in this preparation compared to DAMGO (EC_{50} = 8.3 nM) and morphine (EC_{50} = 100 nM) (Figure 4). Co-administration of 0.3 μM DPDPE, a concentration which was just sub-effective, with the μ-opioid ligands caused no alteration in the potency of DAMGO (IC_{50} 8.3 ± 2.0 nM in the absence of DPDPE and 7.3 ± 1.2 nM in the presence of DPDPE) or morphine (EC_{50} 100 ± 34 nM in the absence of DPDPE and 92 ± 37 nM in the presence of DPDPE). A sub-effective concentration of Delt II was also unable to modulate the potency of the μ-opioid ligands. Similarly no change was observed when either DPDPE or Delt II was used at a concentration which inhibited 50% of the twitch response (data not shown).

Discussion

The results show that in the mouse vas deferens, modulation of the action of μ-opioid agonists by δ-opioid agonists cannot be demonstrated. This applies to both peptide and non-peptide μ-opioid agonists and with either sub-effective levels of δ-opioid agonists or concentrations of δ-opioid agonists which by themselves cause 50% inhibition of the electrically-induced twitch. This is perhaps surprising since both μ- and δ-opioid receptors exist in this tissue and indeed have been shown to exist on the same neurone (Rogers & Henderson, 1990). On the other hand, the results do support the suggestion of an anatomical but not functional coupling of the receptors (Sheehan *et al.*, 1986). In contrast, in the brain μ- and δ-opioid receptors may be functionally coupled since interactions have been shown to occur in the inhibition of the release of neurotransmitters from brain slice preparations. Thus the selective δ-opioid, DPDPE, blocks the morphine (μ-mediated) inhibitions of D_1 dopamine receptor-stimulated cyclic AMP efflux (Schoffelmeer *et al.*, 1987). The results may suggest that differences exist in opioid receptor systems between the brain and periphery. Indeed there is evidence that the δ-opioid receptor populations in the brain and periphery are different. For example, the conformationally constrained peptide [D-Ala^2,(2R,3S)-∇^{E}Phe^4,Leu^5]enkephalin (CP-OH) is very weakly active in the mouse vas deferens

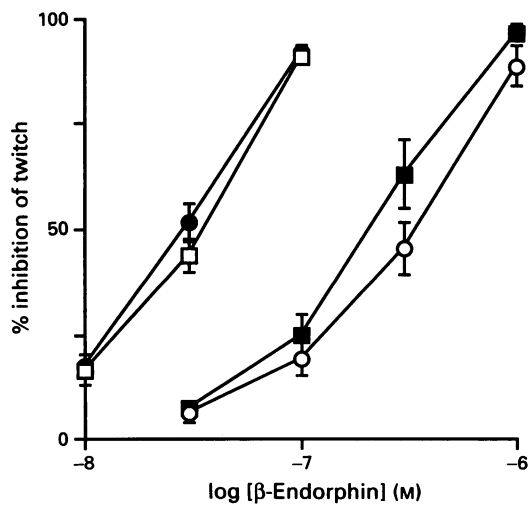

Figure 3 Concentration-effect curves for β-endorphin in the electrically stimulated mouse vas deferens in the absence (●) or presence of 200 nM naloxone (■), 300 nM cyprodime (□) or 6 nM naltrindole (○). Values are means ± s.e.mean where $n \geq 3$.

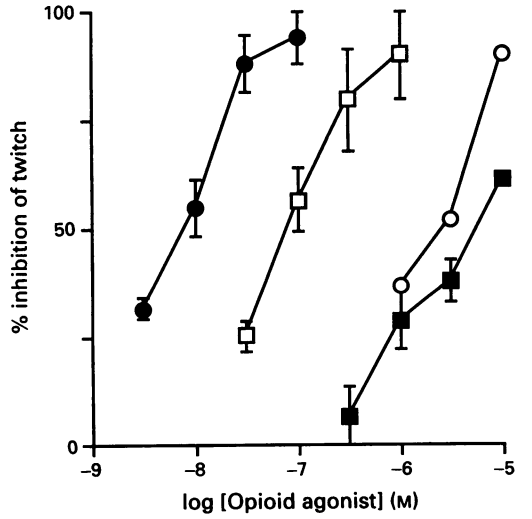

Figure 4 Concentration-effect curves for selective μ- and δ-opioid agonists in field stimulated guinea-pig myenteric plexus-longitudinal muscle, DAMGO (●), morphine (□), Delt II (○) and DPDPE (■). Values are means ± s.e.mean where $n \geq 3$.

although it has high affinity for the δ-opioid receptor in rat brain and can modulate the antinociceptive effects of morphine (Shimohigashi *et al.*, 1987; 1988). Additionally, whereas the tritiated ligand [^{3}H]-*p*-Cl-DPDPE has similar affinity for δ-opioid receptors in the mouse vas deferens and rat brain, the methyl ester of CP-OH (CP-OMe) has a 33-fold lower affinity for the δ-opioid receptors in the mouse vas deferens than those in brain (Vaughn *et al.*, 1990).

Recent *in vivo* evidence has suggested a division of the δ-opioid receptor population in the central nervous system into putative δ_1 and δ_2 subtypes. DPDPE and [D-Ala^2,D-Leu^5]enkephalin (DADLE) are thought to activate the δ_1 subtype, and are antagonized by 7-benzylidenenaltrexone (BNTX) and [D-Ala^2,Leu^5,Cys^6]enkephalin (DALCE). Conversely, Delt II and [D-Ser^2,Leu^5,Thr^6]enkephalin (DSLET) are agonists at the δ_2 subtype while naltriben (the benzofuran analogue of naltrindole) and naltrindole-5′-isothiocyanate (5′-NTII) are antagonists (for example see Sofuoglu *et al.*, 1991; Jiang *et al.*, 1991). Furthermore, a lack of cross-tolerance between DPDPE and Delt II or DSLET is consistent with this classification (Sofuoglu *et al.*, 1991; Mattia *et al.*, 1991). In addition, ligand-binding experiments add credence to the existence of multiple δ-opioid subtypes (Negri *et al.*, 1991; Sofuoglu *et al.*, 1992; Portoghese *et al.*, 1992). There is evidence to suggest that the δ-opioid receptor involved in modulating centrally μ-opioid mediated effects is of the putative δ_2 subtype (Porreca *et al.*, 1992; Kamei *et al.*, 1993). However, in the mouse vas deferens, experiments with selective δ-opioid subtype antagonists point to a single homogeneous δ-opioid receptor population (Wild *et al.*, 1993). Our finding that no difference in effect is seen with either a putative δ_1-opioid agonist (i.e., DPDPE) or a putative δ_2-opioid agonist (i.e., Delt II) would support this.

The endogenous opioid peptide, β-endophin, has similar affinity for μ- and δ-opioid receptors (Kosterlitz *et al.*, 1986). The peptide has been demonstrated to bind to both μ- and δ-sites simultaneously in rat brain membrane preparations (Schoffelmeer *et al.*, 1990) and can be blocked by both μ- and δ-opioid antagonists, providing evidence for receptor interaction. However, this does not appear to be the case in the mouse vas deferens since our studies with antagonists indicate that β-endophin is exerting its actions solely through activation of δ-opioid receptors.

In the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum the studies confirm the lack of potency of δ-opioid ligands using both putative δ_1 preferring and putative δ_2 preferring opioid ligands. The δ-opioid ligands do have agonist properties but only at high concentrations that suggest an action *via* μ-opioid receptors in this tissue. The lack of antagonism of the μ-opioid agonists in the presence of δ-opioid ligands confirms the very low affinity of δ-opioid ligands for μ-opioid receptors. Any δ-opioid receptors present in the tissue are also unable to modulate the actions of μ-opioids since with either DPDPE or Delt II, at doses well in excess of these expected to occupy fully δ-opioid receptors, no changes in the μ-opioid agonist concentration-response curves were observed.

In conclusion the results indicate a lack of interaction between μ- and δ-opioid agonists at any level in the pathway from receptor to effector in either the mouse vas deferens or the guinea-pig ileum myenteric plexus-longitudinal muscle. Although the range of ligands studied was not exhaustive, these results lend support to the proposal (Shimohigashi *et al.*, 1987; Vaughn *et al.*, 1990; Portoghese *et al.*, 1992) that differences exist between types of opioid receptors, or opioid systems, in the periphery and those involved centrally in antinociception (Vaught *et al.*, 1979; Lee *et al.*, 1980; Jiang *et al.*, 1990), inhibition of gut propulsion (Heyman, 1987), volume-induced contractions of the bladder (Sheldon *et al.*, 1989) and antitussive activity (Kamei *et al.*, 1993).

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The effect of morphine on formalin-evoked behaviour and spinal release of excitatory amino acids and prostaglandin E_2 using microdialysis in conscious rats

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1 In the present study, the object was to examine the effects of morphine on spinal release *in vivo* of excitatory amino acids (EAA), prostaglandin E_2 (PGE_2), and a marker for nitric oxide (NO) synthesis, citrulline (Cit), evoked by a protracted noxious stimulus produced by the injection of formalin into the paw. Spinal release was monitored in conscious rats using a microdialysis probe implanted into the subarachnoid space with the active site placed at the level of the lumbar enlargement. In split dialysate samples, EAAs were measured by high performance liquid chromatography (h.p.l.c.) and PGE_2 was determined by radioimmunoassay.

2 Resting concentrations in nmol ml^{-1} for the amino acids (mean ± s.e.mean, $n = 21$) were: 4.8 ± 0.4 for glutamate (Glu), 0.8 ± 0.1 for aspartate (Asp), 8.8 ± 0.8 for taurine (Tau), 24 ± 3 for glycine (Gly), 19 ± 3 for serine (Ser), 5.2 ± 0.8 for asparagine (Asn), 64 ± 4 for glutamine (Gln) and 5.2 ± 0.4 for Cit. Mean basal release for PGE_2 was 12 ± 1 pmol ml^{-1}.

3 Subcutaneous (s.c.) injection of 5% formalin evoked a biphasic flinching behaviour (phase 1: 0–9 min and phase 2: 10–60 min) of the injected paw. Corresponding to phase 1 behaviour, there was a significant increase (50–100%) in spinal levels of Glu, Asp, Tau, Gly, Cit and PGE_2, but not Ser, Asn and Gln. A significant ($P < 0.01$) second phase increase in release was observed only for Cit and PGE_2. However, Glu and Asp levels were increased by approximately 45%.

4 Injection of morphine sulphate (3 mg kg^{-1}, s.c.) had no effect on resting release, but produced a significant suppression of the formalin-evoked behaviour and release of Glu, Asp, Tau, Gly, Cit and PGE_2. The effect of morphine was reversed by pretreatment with 1 mg kg^{-1} naloxone. Naloxone by itself did not change the release or behaviour of the formalin test.

5 This study demonstrates that both spinal EAA and PGE_2 release patterns correlate with behavioural nociceptive responses in the formalin test and that morphine suppresses the formalin-evoked behaviours and spinal release. The reversal by naloxone of the morphine effect indicates mediation via an opioid receptor.

Keywords: Glutamate; aspartate; citrulline; prostaglandin E; formalin test; morphine; naloxone; intrathecal microdialysis; spinal cord

Introduction

When formalin is injected subcutaneously, there is an initial strong activation of small primary afferents that subsides over the next 10 min to a low, but continued level of activity (Heapy *et al.*, 1987). In man, an immediate burning pain is experienced, followed by a throbbing pain over a 30–60 min period (Dubuisson & Dennis, 1977). In rats, formalin injection produces a characteristic biphasic flinching, shaking and licking behaviour of the injected paw (Wheeler-Aceto *et al.*, 1990). The first phase is a result of direct activation of peripheral nociceptors, while the second phase is believed to be mediated by a combination of low ongoing activity in primary afferents and increased sensitivity of spinal cord neurones (Dickenson & Sullivan, 1987; Heapy *et al.*, 1987; for discussion see also Malmberg & Yaksh, 1993a; Yaksh, 1993a). Investigations of the spinal pharmacology of the formalin test have revealed several specific characteristics. First, intrathecal delivery of glutamate (Glu) receptor antagonists of the N-methyl-D-aspartate (NMDA) subtype prior to but not after, the first phase reduce the second phase response (Haley *et al.*, 1990; Yamamoto & Yaksh, 1992; Coderre & Melzack, 1992). This suggests that Glu is release during the first phase and serves to initiate the second phase behaviour. Second, intrathecal cyclo-oxygenase and nitric oxide (NO) synthase inhibitors, administered either before or after the first phase, suppress the magnitude of the second phase response (Chapman & Dickenson, 1992; Haley *et al.*, 1992; Malmberg & Yaksh, 1992a; 1993b). This is consistent with a role for cyclo-oxygenase products and NO in the maintenance of the second phase response and also with studies showing that spinal Glu receptor activation induces a hyperalgesia that is blocked by intrathecal cyclo-oxygenase and NO synthase inhibitors (Malmberg & Yaksh, 1992b; 1993b). This hypothesis is also supported by studies showing that NMDA receptor activation evokes spinal release of prostaglandin E_2 (PGE_2) (Sorkin, 1993a) and citrulline (Cit) (Sorkin, 1993b), a co-product of NO formation (Bredt & Snyder, 1989). Formalin injection has previously been shown to evoke Glu and aspartate (Asp) release in the lumbar spinal cord (Skilling *et al.*, 1988). However, in that study, peak Glu and Asp concentrations were shown only within 50 min after formalin injection and no time course of release was presented.

Systemic morphine inhibits both phases of the formalin test (Wheeler-Aceto & Cowan, 1991; Yamamoto & Yaksh, 1992; Malmberg & Yaksh, 1993a), by activating opioid receptors in the brainstem and spinal cord (see Yaksh, 1993b). At the brainstem level, opiate receptors have been shown to activate bulbospinal pathways which are able to suppress spinal nociceptive processing. At the spinal level, opioid receptors reduce neuronal activity by hyperpolarization of postsynaptic wide dynamic range (WDR) neurones,

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through increased potassium conductance (Fleetwood-Walker *et al.*, 1985; North *et al.*, 1987) and also exert a presynaptic effect upon C-fibre terminals by inhibiting substance P release (Yaksh *et al.*, 1980; Pang & Vasko, 1986; Go & Yaksh, 1987). Morphine has been shown to reduce the release of Glu and Asp following high-intensity stimulation in rat spinal cord slices *in vitro* (Kangrga & Randic, 1991). However, the effect of morphine on noxious-evoked spinal release of EAA or on the release of PGE_2 or NO *in vivo*, using a physiological stimulus such as in the formalin test, has not been examined.

In the present study, the objective was to examine whether formalin injection into the paw induced spinal release of amino acids and PGE_2, and whether a behaviourally effective dose of morphine can modulate the spinal release of neurotransmitters evoked by formalin injection. These studies were performed in conscious animals to obviate any potential effects of anaesthetics on spinal neurotransmitter release.

Methods

Animal preparation

Experiments were carried out according to protocols approved by the Institutional Animal Care Committee of the University of California, San Diego, U.S.A. Male Spraque-Dawley rats (320–350 g; Harlan Industries, Indianapolis, IN, U.S.A.) were implanted with the dialysis system under halothane anaesthesia according to Marsala *et al.* (1995). The dialysis probe was inserted through an incision in the cisternal membrane and extended to the rostral edge of the lumbar enlargement. The two PE-10 ends of dialysis system were externalized on the top of the head. The dialysis catheter was then flushed with artificial cerebrospinal fluid (ACSF; see below). Dialysis experiments were performed 3 to 6 days after the surgery. Only rats with normal neurological functions were used in the study and the rats were used only once.

Construction of dialysis probe

The dialysis system was constructed from a 19 cm Cuprophan hollow fibre with an inner diameter of 200 μm, an outer diameter of 300 μm and 45 kDa molecular weight cut-off (Filtral, AN 69-HF). The dialysis fibre was coated with epoxy glue (Devcon Corporation, Danvers, MA, U.S.A.), except for a 4 cm region in the middle of the 19 cm fibre. A Nichrome-Formvar wire with a 0.0026 inch inner diameter (A-M systems, Inc., Everret, WA, U.S.A.) was passed through the fibre in order to make the dialysis probe firm. The two ends of the fibre were attached to polyethylene catheters (PE-10) with cyanoacrylate (Borden, Inc., Columbus, OH, U.S.A.). An U-shaped loop was formed by gently bending the whole fibre in the middle and the two pieces were bound together at the fibre-PE-10 connection with epoxy glue.

Dialysis and sample collection

For the dialysis experiment, one of the externalized PE-10 tubings on the rat was connected to a microinfusion pump with a length of PE-50 tubing (inflow). The other PE-10 was attached to approximately 20–30 cm of PE-10 catheter that served as outflow. The dialysis system was perfused with ACSF consisting of (mM NA^+ 151.1, K^+ 2.6, Mg^{2+} 0.9, Ca^{2+} 1.3, Cl^- 122.7, HCO_3^- 21.0, HPO_4^- 2.5 and dextrose 3.5. The ACSF was bubbled with 5% CO_2 in 95% O_2 to pH 7.2 and heated to 37°C prior to the experiment. The flow rate was 10 μl min^{-1} in all experiments. Samples were collected at 10-min intervals in polypropylene tubes on ice and frozen at −20°C until analysis of amino acids by h.p.l.c. and PGE_2 by radioimmunoassay (RIA). Information about formalin-evoked behaviour and release of both amino acids and PGE_2 were obtained in all animals. For each 10 min sample (100 μl), 25 μl of the perfusate was used for amino acids detection and 50 μl for PGE_2 analysis.

Chemical analysis

The amino acids Glu, Asp, taurine (Tau), glycine (Gly), serine (Ser), asparagine (Asn), glutamine (Gln) and Cit were analysed with a Waters h.p.l.c with a reverse phase C18 and a u.v. detector, using a phenyl isothiocyanate precolumn derivitization method (Cohen & Strydom, 1988). Sensitivity was 5–10 pmol per injection. Methionine sulphone was added to each sample as an internal standard. External standard containing 40, 400 and 4000 pmol of authentic amino acids were run at the beginning and the end of each sample group. The amino acid peak heights were initially normalized to the methionine sulphone peak and then quantified based on a linear relationship between peak height and amounts of corresponding standards.

The concentration of PGE_2 in the perfusion samples was quantified with commercially available PGE_2 ^{125}I radioimmunoassay kit (Advanced Magnetics Inc., Cambridge, MA, U.S.A.) in accordance with the manufacturer's protocol. A standard curve was constructed between 10 and 2800 fmol of PGE_2 per assay tube for analysis of the perfusate samples. According to the manufacturer, the rabbit anti-PGE_2 in the kit is specific for PGE_2 with cross-reactivity for other prostaglandins and metabolites being less than 1% for 6-keto-PGF_1, PGD_2, dihydro-keto-PGE_2, dihydro-keto-PGF_2, arachidonic acid, thromboxane B_2, 15-keto-PGE_2, 15-keto-PGF_2, 5-hydroxyarachidonic acid (HETE), 12-HETE and 15-HETE, less than 1.9% for PGB_1, PGB_2, 6-keto-PGE_1, PGF_1, PGF_2, 1.9% for PGA_2, 6% for PGA_1, and 50% for PGE_1. In the present study, it is unlikely that the immunoreactivity detected represents PGE_1 to any significant extent, since the PGE_2 precursor, arachidonic acid, is far more abundant in CNS of mammals than the PGE_1 precursor, eicosatrienoic acid. In addition, PGE_1 has not been detected in brain tissue (Steinhauer *et al.*, 1979).

Behavioural assessment

Systematic observation of general behaviour was carried out. Allodynia was determined by assessing the agitation (escape/vocalization) evoked by lightly stroking the rats flank with a pencil. Motor function was examined by the placing/stepping reflex, where a normal behaviour is a stepping reflex when the hindpaws are drawn across the edge of a table. Righting and ambulation were assessed by placing the rat horizontally with its back on the table which normally gives rise to an immediate coordinated twisting of the body to an upright position. Catalepsy was assessed by placing the forepaws on a horizontal bar kept at 4 cm from a table surface. Failure to move from the bar within 30 s was defined as a positive cataleptic response. Changes in spontaneous behaviour were noted during the experiments.

The formalin test and experimental paradigm

The formalin test was performed approximately 1 h after perfusion was initiated. This first period consisted of an initial 30–40 min wash-out period to obtain perfusion equilibrium followed by 20 min of sample collection to obtain resting release levels. In order to perform the formalin injection, the rats were restrained and 50 μl of 5% formalin solution injected s.c. into the dorsal surface of the right hind paw with a 30 gauge needle. The rat was then returned to the plexiglas chamber for observation of the formalin-injected paw. Perfusate collection was started with a delay (2 to 2.5 min depending on the length of the out-flow catheter) in order to sample spinal perfusate from the time of formalin injection and thereafter at 10-min periods. Because the flinch-

ing evoked by the formalin injection is the most consistent behaviour (see also Wheeler-Aceto *et al.*, 1990), pain-related behaviour was quantified by counting the incidence of spontaneous flinching of the injected paw. The number of flinches were counted every min for the first 6 min after the formalin injection. Thereafter, starting at 10 min after the formalin injection, the incidence of paw flinching was counted at 5 min intervals in periods of 1 min. After the 60 min observation period, animals were killed with an overdose of barbiturate mixture (Beuthanasia, 50 mg kg^{-1}, intraperitoneally, Schering-Plough Animal Health Corp., Kenilworth, NJ, U.S.A.).

Drugs and injections

Morphine sulphate (Merck, Sharpe and Dohme; West Point, PA, U.S.A.) and naloxone HCl salt (Dupont) were dissolved in preservative-free physiological saline (0.9% w/v NaCl; Abbott Laboratories, North Chicago, IL, U.S.A.). All drugs were administered s.c. and the doses was based on the salt (e.g., 3 mg kg^{-1} morphine sulphate). The agents were mixed in a concentration such that a 300 g rat would receive a s.c. injection of 300 µl of the drug solution or 300 µl saline. Morphine or saline was administered 20 min before the formalin injection and naloxone was delivered s.c. 2 min before the injection of morphine.

Data analysis and statistics

Behaviour time-response data are presented as the mean number of flinches ± s.e.mean per min for every min during the first 6 min and thereafter at 5 min intervals throughout the 60 min observation period. Statistical analysis of the behavioural data employed the one-way ANOVA followed by the Student Newman-Keuls multiple comparisons test. The temporal release data of the control group was analysed with repeated measures ANOVA on raw data. However, for illustration purposes, all release data are presented as mean ± s.e.mean % change from resting release. The resting release was determined from two subsequent sample collections before the formalin test and after the wash-out period. Comparisons between treatment groups were carried out by the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons test using transformed data (% change from resting release). Analysis of the covariance between behaviour and release was accomplished by linear regression analysis plotting the behaviour versus the release for individual animals.

Results

General behaviour and motor effects

All rats showed normal behaviour and motor function after catheter placement and on the day of experimentation. Spinal dialysis did not influence the normal behaviour of the rat.

Resting release conditions

A pilot study showed that dialysis equilibrium was obtained after 30 min with a perfusion rate at 10 µl min^{-1} and that resting release was stable for at least 210 min (A.B. Malmberg, unpublished observations). The rats were therefore allowed a 40 min washout period before samples were collected for resting levels. Resting release concentrations of the different amino acids and PGE_2 are presented in Table 1.

Effect of formalin injection into the paw

Formalin injection produced a characteristic biphasic flinching behaviour of the injected paw (Figure 1). The levels of Glu, Asp, Tau and Gly were significantly ($P<0.01-0.001$; repeated measures ANOVA) elevated during the first phase, while a less marked increase occurred during the second phase (Figure 2). The amino acids Ser, Asn and Gln remained unchanged ($P>0.05$) after paw formalin injection (Figure 3). Like the behavioural profile, spinal release of Cit and PGE_2 showed a significant ($P<0.01$; repeated measures ANOVA) biphasic release pattern (Figure 4). The significant second phase of formalin-evoked release was found in the 20–30 min period for Cit and the 20–40 min period for PGE_2.

Effect of morphine and naloxone

Systemic injection of 3 mg kg^{-1} morphine sulphate 20 min before the formalin test produced a significant ($P<0.001$; ANOVA) inhibition of both phases of flinching behaviour (Table 2). Similarly, morphine significantly suppressed the formalin-evoked release of the Glu, Asp, Tau, Gly, Cit and PGE_2 (Table 3) without influencing the resting release of any of the amino acids or PGE_2 (data not shown). Pretreatment with 1 mg kg^{-1} naloxone before morphine administration reversed both the behavioural effects of morphine and the suppressant effects on release (Tables 2 and 3). Injection of naloxone alone slightly increased the behavioural response and the amino acid and PGE_2 release evoked by formalin injection, but this was not significantly different from the control response (Tables 2 and 3).

Correlation between release and behaviour

The release of amino acids and PGE_2 appeared to correlate well with the magnitude of behavioural responses in the

Table 1 Resting release ($n = 21$) of amino acids and prostaglandin E_2 (PGE_2) in the lumbar intrathecal space as measured by microdialysis

	Mean ± s.e.mean[a] (nmol ml^{-1})	*Median* (nmol ml^{-1})
Glu	4.8 ± 0.4	4.8
Asp	0.8 ± 0.1	0.8
Tau	8.8 ± 0.8	7.2
Gly	24 ± 3	22
Ser	19 ± 3	18
Asn	5.2 ± 0.8	4.4
Gln	64 ± 4	68
Cit	5.2 ± 0.4	4.8
PGE_2	12 ± 1 pmol ml^{-1}	8.0 pmol ml^{-1}

[a]Mean concentration of the substance at diffusion equilibrium, e.g., 40 min after the perfusion was initiated. Glu, glutamate; Asp, aspartate; Tau, taurine; Gly, glycine; Ser, serine; Asn, asparagine; Gln, glutamine; Cit, citrulline.

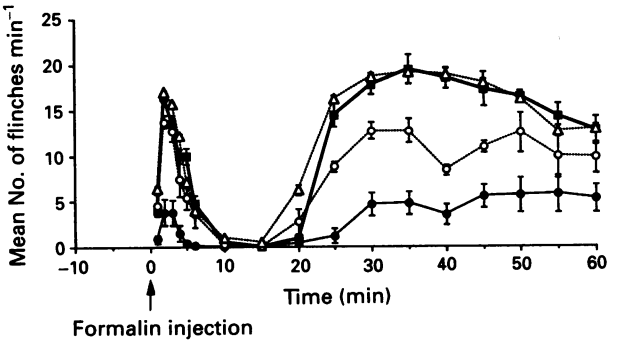

Figure 1 Time-effect curve of saline control (■, $n = 6$), 3 mg kg^{-1} morphine sulphate (●, $n = 6$), 1 mg kg^{-1} naloxone (○, $n = 5$), and naloxone administered 2 min before the morphine injection (△, $n = 4$), which was delivered 20 min before the formalin test. The data are presented as the mean ± s.e.mean number of flinches min^{-1} versus the time after the formalin injection.

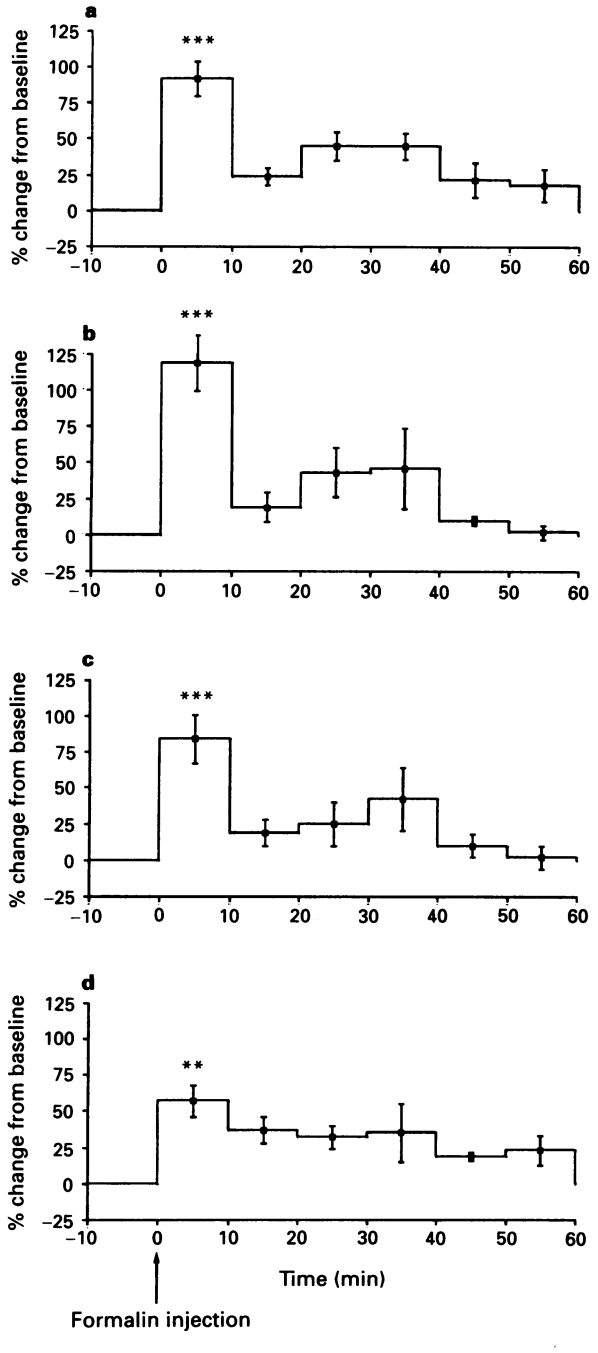

Figure 2 Spinal release of (a) glutamate (Glu), (b) asparate (Asp), (c) taurine (Tau) and (d) glycine (Gly) following formalin injection into the paw. Significant release was shown during the first behavioural phase of the formalin test. The asterisks indicate significantly increased concentrations compared to resting release (repeated measures ANOVA using raw data; $^{**}P<0.01$; $^{***}P<0.001$). The data are presented as the mean ± s.e.mean of the % change from basal release versus the time after formalin injection.

formalin test. There was a significant correlation (regression analysis; $P<0.01-0.001$) between first phase behaviour and first phase release of Glu, Asp, Tau, Gly, Cit or PGE_2, but not for Ser, Asn or Gln (Table 4). Cit and PGE_2, which were significantly elevated during the second phase displayed a positive correlation with behavioural responses during the second phase in addition to the first phase. However, the release of Glu, Asp and Tau, but not Gly, Ser, Asn or Gln, also covaried significantly with the second phase behaviour (Table 4), although these substances were not significantly elevated during phase 2.

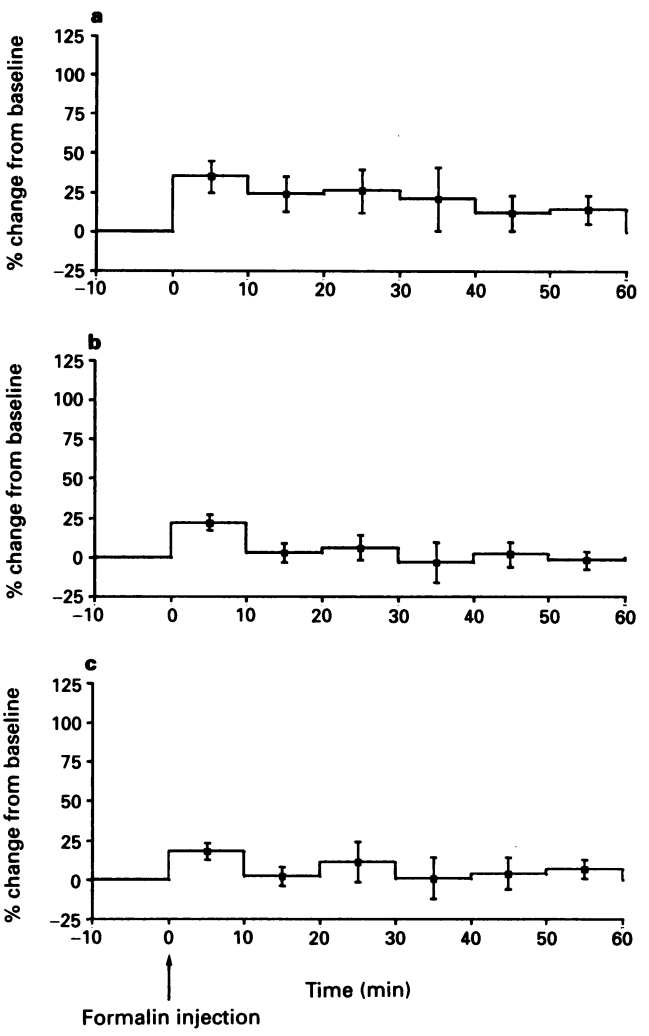

Figure 3 Spinal release of (a) serine (Ser), (b) asparagine (Asn) and (c) glutamine (Gln) did not change following the formalin test (repeated measures ANOVA using raw data). The data are presented as the mean ± s.e.mean of the % change from basal release versus the time after formalin injection.

Discussion

Intrathecal dialysis

Using an intrathecal microdialysis system, we were able to measure the release of amino acids and PGE_2 into the CSF from the lumbar spinal cord in awake, freely-moving rats. This model has several advantages. First, the loop probe is sufficiently robust as to permit the ongoing dialysis in the unanaesthetized rat. Spinal superfusion or transversal spinal dialysis probes can be used to assess release, but these models are difficult to maintain in the unanaesthetized rat (Skilling *et al.*, 1988; Sorkin *et al.*, 1988). Second, the ability to perform the studies in the absence of anaesthesia permits a direct assessment of release without the suppressant action of these agents. In recent studies, it has been shown that spinal amino acid release is markedly depressed by anaesthetic concentrations of halothane (Sanghvi & Yaksh, 1994). Third, because the studies are carried out in the unanaesthetized animal, we are able to draw close correlations between spinal release and the behaviour of the rat.

Detectable levels of amino acids in the spinal cord by use of microdialysis have previously been reported in anaesthetized (Paleckova *et al.*, 1992; Sorkin, 1993a,b) and in conscious rats (Skilling *et al.*, 1988; Sluka & Westlund, 1993). Also, spinal PGE_2 has been detected by microdialysis studies

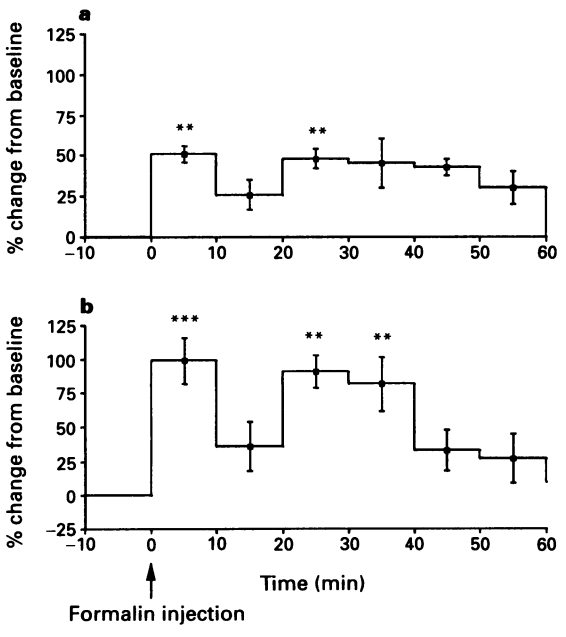

Figure 4 Spinal release of (a) citrulline (Cit) and (b) prostaglandin E_2 showed a biphasic profile corresponding to the flinching behaviour of the formalin test. The asterisks indicate significantly elevated concentrations compared to resting release (repeated measures ANOVA using raw data $^{**}P<0.01$; $^{***}P<0.001$). The data are presented as the mean ± s.e.mean of the % change from basal release versus the time after formalin injection.

(Sorkin 1992; 1993b). The perfusate concentrations in the present study were higher probably because of better recovery when the probe is immersed in the subarachnoid fluid compared to when the probe is placed in the spinal cord tissue, in which the diffusion is impeded by the tortuosity of the extracellular space (see Benveniste & Hüttemeier, 1990, for review). In a separate study, we examined the *in vitro* and *in vivo* recovery of PGE_2, which was found to be 30% and 10%, respectively (Malmberg & Yaksh, unpublished observations). These values indicate that in the subarachnoid space, parts of the dialysis fibre may be in contact with spinal cord tissue, thus decreasing the recovery, compared to the ideal situation *in vitro* where the dialysis membrane is only surrounded by fluid.

Noxious-evoked EAA release

Formalin injection into the paw has previously been demonstrated to elevate the levels of Glu and Asp in the lumbar spinal cord using the microdialysis technique where the catheter is inserted transversely in spinal grey matter (Skilling *et al.*, 1988). However, only the peak concentration over the 50 min observation period was shown. In the present study, we found the maximal formalin-evoked Glu and Asp release to be during the first 10 min after formalin injection. During the second phase Glu and Asp were never significantly increased at any particular 10-min interval, although the mean levels were elevated to approximately 45% in the period 20–40 min after formalin injection. It is possible that this slightly delayed increase is essential in generating the behavioural activity during the second phase. This notion is supported by the positive correlation between Glu or Asp

Table 2 Effect of s.c. injection of morphine sulphate (3 mg kg^{-1}) and naloxone (1 mg kg^{-1}) on phases 1 and 2 of the nociceptive behaviour of the formalin test

		Formalin-evoked behavioural response			
		Phase 1 (0–9 min)		*Phase 2* (10–60 min)	
Treatment	n	*Flinches*[a]	*Statistics*[b]	*Flinches*[a]	*Statistics*[b]
Control	6	59 ± 3	–	133 ± 8	–
Morphine	6	10 ± 3	***	37 ± 4	***
Naloxone + morphine	5	48 ± 3	***	89 ± 7	***
Naloxone	4	63 ± 3	NS	141 ± 15	NS

[a]The mean ± s.e.mean of the sum of paw flinches in the period 10–60 min after the paw formalin injection. [b]The asterisks indicate statistical significance comparing morphine vs control, naloxone + morphine vs morphine, or naloxone vs control (ANOVA followed by Student Newman-Keuls test; $^{***}P<0.001$).

Table 3 Effect of morphine and naloxone on evoked release of Glu, Asp, Tau, Gly, Cit and prostaglandin E_2 (PGE_2) in the formalin test

	% change from baseline and statistical significance[a]			
Substance	*Control (saline)*	*Morphine + saline*	*Naloxone + morphine*	*Naloxone + saline*
Phase 1 (0–9 min)				
Glu	92 ± 12	17 ± 11**	94 ± 9*	123 ± 23
Asp	119 ± 20	35 ± 14**	111 ± 20*	95 ± 17
Tau	84 ± 17	18 ± 12*	97 ± 17*	98 ± 16
Gly	57 ± 11	15 ± 6*	51 ± 6*	60 ± 17
Cit	51 ± 5	26 ± 6*	40 ± 6*	50 ± 12
PGE_2	99 ± 17	15 ± 10**	86 ± 11*	138 ± 29
Phase 2 (10–60 min)[b]				
Cit	157 ± 19	57 ± 7**	115 ± 5*	103 ± 25
PGE_2	300 ± 20	2 ± 8**	204 ± 40*	390 ± 60

[a]Data represent the mean % change from baseline ± s.e.mean. The asterisks indicate the level of statistical significance comparing the morphine with the control group or the naloxone + morphine vs the morphine group (Kruskal-Wallis test followed by Dunn's multiple comparisons test $^{*}P<0.05$; $^{**}P<0.01$). The naloxone group was compared to the control group, but no differences were found ($P>0.05$). [b]Only Cit and PGE_2 showed significant second phase release. Phase 2 data are presented as the cumulative release (% change from baseline) during the interval 10 to 60 min after formalin injection.
Abbreviations as in Table 1.

Table 4 Covariance between behaviour and release in the formalin test

	Correlation coefficient	*Regression analysis*	
Phase 1 (0–9 min)			
Flinching vs Glu release	0.70	$P = 0.0005$	***
Asp release	0.76	$P = 0.0001$	***
Tau release	0.67	$P = 0.001$	**
Gly release	0.61	$P = 0.004$	**
Ser release	0.40	$P = 0.07$	NS
Asp release	0.17	$P = 0.44$	NS
Gln release	0.34	$P = 0.12$	NS
Cit release	0.59	$P = 0.005$	**
PGE_2 release	0.73	$P = 0.0002$	***
Phase 2 (10–60 min)			
Flinching vs Glu release	0.60	$P = 0.004$	**
Asp release	0.46	$P = 0.036$	*
Tau release	0.47	$P = 0.032$	*
Gly release	0.42	$P = 0.057$	NS
Ser release	0.24	$P = 0.28$	NS
Asn release	0.04	$P = 0.85$	NS
Gln release	0.02	$P = 0.91$	NS
Cit release	0.53	$P = 0.013$	*
PGE_2 release	0.87	$P = 0.0001$	***

Regression analysis of the number of flinches in relation to release.
Abbreviations as in Table 1.

release during phase 2 of the formalin test. Pharmacological (Yamamoto & Yaksh, 1992; Coderre & Melzack, 1992) and electrophysiological (Haley *et al.*, 1990) studies have emphasized the importance of EAA receptor activation during facilitated nociceptive processing, as occurs during the second phase of the formalin test. EAA receptor antagonists, particularly of the NMDA subtype, have minimal effect on the first phase activity, but produce significant suppression of the second phase of the formalin test (Haley *et al.*, 1990; Yamamoto & Yaksh, 1992; Coderre & Melzack, 1992). It is possible that Glu released during the first phase is acting mainly through non-NMDA receptors (Näsström *et al.*, 1992), although this possibility remains equivocal (Coderre & Melzack, 1992).

Paw formalin injection also significantly elevated spinal Tau and Gly levels during phase 1 of the formalin test. The release of these two inhibitory amino acids may be an early protection mechanism against excitability and neurotoxicity, which has been previously postulated for Tau release (Menedez *et al.*, 1989). Low concentrations of Tau have also been shown to suppress substance P-induced behaviours (Smullin *et al.*, 1990), while Gly had a less noteable modulatory activity. In contrast to our study, Skilling and colleagues showed no significant release of Tau and Gly following formalin injection using the transverse spinal dialysis probe (Skilling *et al.*, 1988). The reason for this is not known, but is possibly due to differences in the methods. We found no elevation in the concentrations of Ser, Asn and Gln following the formalin test. These amino acids are not considered to be neurotransmitters, and the lack of their release further implies the 'specificity' of the increased dialysate concentrations of the other substances associated with the stimulation.

Second phase release: Cit and PGE_2

Behavioural and electrophysiological studies have indicated that spinal cyclo-oxygenase products (Malmberg & Yaksh, 1992a; Chapman & Dickenson, 1993) and NO (Haley *et al.*, 1992; Malmberg & Yaksh, 1993b) are involved in the second phase of the formalin test. In agreement with these observations is the significant release of PGE_2 and Cit, an indicator of NO production (see Sorkin, 1993a), during the second phase of the formalin test. However, more surprising was the greater release of PGE_2 and Cit during the first phase of the formalin test, where pharmacological inhibition of the enzymes involved in prostaglandin and NO synthesis has little effect on nociceptive behaviour. This discrepancy emphasizes the importance of both release and pharmacological studies in evaluating the importance of an endogenous substance. It is possible that during the first phase of the formalin test, the activation of primary afferents to release Glu and Asp drives the behavioural response to a maximum. We suspect that facilitating substances, such as NO and PGE_2, contribute only minimally to the behavioural response during phase 1 because maximal behavioural effect is already induced by the local EAA receptor occupancy.

Effects of morphine on behaviour and release

Systemic morphine administration was shown, in a naloxone-sensitive manner, to suppress significantly the behavioural effects and the elevated spinal levels of amino acids and PGE_2, following formalin injection into the rat's paw. Morphine had no effect on resting release levels of any of the measured substances. Several studies, using different spinal superfusion systems, have shown that morphine inhibits peptide (substance P) release from primary afferents (Yaksh *et al.*, 1980; Pang & Vasko, 1986; Go & Yaksh, 1987). To our knowledge, this study is the first to show that morphine also reduces spinal nociceptive-evoked *in vivo* release of Glu, Asp, Tau, Gly, Cit and PGE_2 in awake animals. Previously, μ-opioid agonist effects on spinal Glu and Asp release from primary afferents have only been demonstrated *in vitro* (Kangrga & Randic, 1991).

The mechanisms by which systemic morphine reduces EAA release cannot be defined in these studies. In rat spinal cord, morphine is principally an agonist at the μ-opioid receptor (see Yaksh, 1993b). Glu has been shown to coexist with substance P in primary afferent terminals in the superficial dorsal horn (De Biasi & Rustioni, 1988), and the inhibitory effect of μ-opioid receptors on substance P is noted above. Glu and Asp are also contained in and probably released from dorsal horn interneurones. If these cells are driven by small afferent input, then decrease by morphine of small afferent terminal release will yield a lesser activation of these neurones.

With regard to Cit and PGE_2, these substances are believed to originate from postsynaptic cell systems, including interneurones (Valtschanoff *et al.*, 1992), noradrenergic terminals (Coderre *et al.*, 1990), glial cells (Marriott *et al.*, 1991), and from neurones activated by NMDA receptors (Sorkin, 1993a, b). Accordingly, blockade of afferent evoked excitation would diminish the evoked release of these products as well.

Conclusion

Our studies demonstrate that noxious stimulation results in the spinal release of direct excitatory substances (Glu and Asp), inhibitory amino acids (Tau and Gly) and mediators that have been postulated to facilitate the cascade of nociceptive processing (PGE_2 and NO). These substances and other nociceptive transmitters not measured, such as the neuropeptide substance P, are all likely to contribute to the behavioural manifestation of acute and prolonged pain behaviours, with possibly different temporal patterns. Acute morphine administration reduced the evoked release of these putative mediators of nociception. The suppressant effect of morphine may be explained by its inhibitory action on the release from primary afferents, thereby blocking the cascade of events in spinal nociceptive processing.

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Cyclic GMP-mediated inhibition of L-type Ca^{2+} channel activity by human natriuretic peptide in rabbit heart cells

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1 Effects of atrial natriuretic peptide (ANP) on the L-type Ca^{2+} channels were examined in rabbit isolated ventricular cells by use of whole-cell and cell-attached configurations of the patch clamp methods. ANP produced a concentration-dependent decrease (10–100 nM) in amplitude of a basal Ca^{2+} channel current.

2 The inactive ANP (methionine-oxidized ANP, 30 nM) failed to decrease the current.

3 8-Bromo-cyclic GMP (300 μM), a potent activator of cyclic GMP-dependent protein kinase (PKG), produced the same effects on the basal Ca^{2+} channel current as those produced by ANP. The cyclic GMP-induced inhibition of the Ca^{2+} channel current was still evoked in the presence of 1-isobutyl-3-methyl-xanthine, an inhibitor of phosphodiesterase. ANP failed to produce inhibition of the Ca^{2+} channel current in the presence of 8-bromo-cyclic GMP.

4 In the single channel recording, ANP and 8-bromo-cyclic GMP also inhibited the activities of the L-type Ca^{2+} channels. Both agents decreased the open probability (NP_o) without affecting the unit amplitude.

5 The present results suggest that ANP inhibits the cardiac L-type Ca^{2+} channel activity through the intracellular production of cyclic GMP and then activation of PKG.

Keywords: Atrial natriuretic peptide; cyclic GMP; L-type Ca^{2+} channel; cardiomyocyte; patch clamp

Introduction

Atrial natriuretic peptide (ANP) is well known as a potent vasodilator. The underlying mechanism of its vasodilator action is related to an increase in the intracellular concentration of guanosine 3′: 5′-cyclic monophosphate (cyclic GMP) in the vascular smooth muscles (Needleman *et al.*, 1989). However, it remains unsettled whether ANP is able to influence directly cardiac function. Some reports suggested that ANP directly affected cardiac performance *in vivo* (Lappe *et al.*, 1985; Seymour *et al.*, 1987), while others denied such direct cardiac actions of ANP (Bergey & Kotler, 1985; Wangler *et al.*, 1985; Criscione *et al.*, 1987). In such a controversy, recent studies on isolated cardiomyocytes clearly showed a directly depressive effect of ANP on contractility (Neyses & Vetter, 1989; Vaxelaire *et al.*, 1989; McCall & Fried, 1990).

The effect of ANP on cardiac Ca^{2+} channels is also controversial. In frog ventricular myocytes, ANP decreased a Ca^{2+} channel current that had been increased by β-adrenoceptor stimulation, but did not affect the basal Ca^{2+} channel current (Gisbert & Fischmeister, 1986). It was suggested that this reduction by ANP of the increased Ca^{2+} channel current may be mediated by activation of cyclic GMP-dependent phosphodiesterase (PDE II) (Hartzell & Fischmeister, 1986; Fischmeister & Hartzell, 1987). However, later work showed that activation of PDE II by cyclic GMP might play only a minor role in cyclic GMP-induced decrease in Ca^{2+} channel current in mammalian (guinea-pig and rat) heart cells (Levi *et al.*, 1989; Méry *et al.*, 1991; Ono & Trautwein, 1991). Furthermore, cardiomyocytes of rat (Sorbera & Morad, 1990), chick embryos (Bkaily *et al.*, 1993) and human atrial cells (LeGrand *et al.*, 1992) were reported to respond to ANP with a decrease in the basal Ca^{2+} channel current.

Sperelakis and his colleagues (Wahler *et al.*, 1990; Tohse & Sperelakis, 1991) reported that basal activities of L-type Ca^{2+} channels in embryonic chick cardiomyocytes were inhibited by 8-bromo-cyclic GMP, a potent activator of cyclic GMP-dependent protein kinase (PKG) (Lincoln & Corbin, 1983). It is very possible that ANP inhibits the basal activity of L-type Ca^{2+} channels through activation of PKG. However, no investigations has been made into a causal relation between ANP, cyclic GMP, and the Ca^{2+} channels in mammalian cardiomyocytes.

In the present study, we examined effects of α-human natriuretic peptide (α-hANP, 1–28) on the whole-cell current and single-channel activities of L-type Ca^{2+} channels in rabbit ventricular cells. The present study suggests that activation of PKG mediates the inhibition by ANP of basal activities of Ca^{2+} channels.

Methods

Cell preparation

Single ventricular cells were enzymatically isolated from hearts of New Zealand White rabbits (1.5–2.0 kg), according to essentially the same methods as previously described (Tohse *et al.*, 1990). In brief, collagenase (0.01% wt./vol.; Wako Chemicals, Osaka Japan) dissolved in nominally Ca^{2+}-free (<60 μM) Tyrode solution was retrogradely perfused into the aorta of isolated hearts suspended in a Langendorff apparatus. The single cells obtained were suspended in a high K^+, low Cl^- solution [Kraftbrühe (KB) solution] (Isenberg & Klöckner, 1982) and stored in a refrigerator (4°C) until later use.

Patch clamp recording

Whole-cell and cell-attached (single channel) patch clamp recordings were made with a patch clamp amplifier (EPC-7, List, Germany, or CEZ 2300, Nihon Kohden, Japan) according to the standard techniques. The patch pipettes had tip resistances of 2–4 MΩ (whole-cell recording) and 5–10 MΩ

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(cell-attached recording). The cells were placed in a perfusion chamber attached to an inverted microscope (IMT-2, Olympus, Japan), and constantly superfused with Tyrode solution or one of the external solutions (their compositions are described later) at 35–37°C. Current signals were filtered at 1 kHz (four-pole Bessel low-pass filter), digitized at 2.5 (whole-cell recording) and 5 kHz (single channel recording), and analysed on a personal computer (PC-98 RL, NEC, Japan) having an AD converter (ADX-98E, Canoopus Electronics, Japan).

Calcium channel current recording

In whole-cell recording, L-type Ca^{2+}-channel current was measured in an external solution containing 2 mM Ba^{2+}. The Ca^{2+} channel current carried by Ba^{2+} was stable, and did not exhibit the 'run-down' phenomenon during 30 min after a start of voltage clamp. The Ba^{2+} current was suitable for observing an inhibitory effect of ANP and cyclic GMP derivatives on the Ca^{2+} channel activity.

The liquid junction potential between intra-pipette solution and external normal Tyrode solution was −8 mV. Therefore, all values of potentials given in whole-cell experiments were corrected.

Single-channel recording

In cell-attached recording of single-channel activities, the cells were perfused with an external depolarizing solution containing 150 mM K^+. The membrane potential of the cells should be approximately 0 mV in this solution. This allowed us to approximate the potential of the patch membrane from the value of the pipette potential.

Activities of a single Ca^{2+} channel were measured as Ba^{2+} current through the channel, by use of a pipette solution containing 110 mM Ba^{2+}. However, the basal activity was too low to observe any inhibitory effect on the Ca^{2+} channel activity of ANP and cyclic GMP derivatives. Therefore, Bay K 8644 (1 μM), a Ca^{2+} channel activator, was used to enhance the channel activities in almost all experiments. In this condition, the inhibitory effect of tested compounds was consistently observed.

Single-channel currents were elicited by depolarization test pulses to 0 mV from a holding potential of −80 mV. The duration of the test pulse was 150 ms. The test pulses were applied to cells at a rate of 0.5 Hz.

Channel activity is represented by the product NP_o, i.e. number of channels in the patch (N) times a proportion of channel open time to the total time (150 ms) of sampled test-depolarization (P_o). An ensemble-averaged current was digitally calculated from sampled current traces (60–120 traces).

Experimental solutions and drugs

The composition of Tyrode solution was (mM): NaCl 143, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, NaH_2PO_4 0.33, glucose 5.5 and HEPES-NaOH buffer 5. The composition of KB solution was (mM): KCl 40, KOH 70, KH_2PO_4 20, L-glutamic acid 50, taurine 20, $MgCl_2$ 3, EGTA 1, glucose 10, and HEPES-KOH buffer 10. The external solution for the whole-cell recording contained (mM): choline Cl 143, $CsCl_2$ 5.4, $BaCl_2$ 2, $MgCl_2$ 0.5, 4-aminopyridine 3, glucose 5.5, and HEPES-HCl buffer 5. In order to eliminate a weak cholinoceptor effect of choline Cl, atropine (5 μM) was added to the external solution for whole-cell recording. Recently, atropine was shown to exert an inhibitory effect on activation of inhibitory G-proteins, which was observed even in the absence of agonists to muscarinic receptors (Hanf *et al.*, 1993). Therefore, it is possible that, in the presence of atropine, adenylyl cyclase activity is altered from the basal state and thus the measured Ca^{2+} currents are not accurately indicative of its basal activity. However, having confirmed the essentially similar effect of ANP on the Ca^{2+} channel current in the absence of atropine ($n = 2$, data not shown), we assumed that the influence of atropine would be minimum in our present study. The composition of the pipette solution for whole-cell recording was (mM): L-glutamic acid 110, CsCl 20, CsOH 110, $MgCl_2$ 3, Na_2ATP 5, Na_2 creatine phosphate 5, EGTA 10 and HEPES-CsOH buffer 5. For the single-channel recording, the external depolarizing solution containing (mM): KCl 140, $MgCl_2$ 0.5, glucose 5.5 and EGTA 10; HEPES-KOH buffer 5, was perfused extracellularly. The pipette solution for the single-channel recording contained (mM): $BaCl_2$ 110, Bay K 8644 0.001, and HEPES-Tris base buffer 5. The pH of all experimental solutions used in the present study was adjusted to 7.4.

α-hANP (1–28) and methionine-oxidized ANP were obtained from Peptide Institute Inc. (Osaka, Japan), and were dissolved in 10 mM Na-phosphate buffer (pH 7.0) to provide a 10 μM stock solution. Bay K 8644 (methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate, Bayer, Germany) was dissolved in ethanol to provide a 10 mM stock solution. 8-Bromo-cyclic GMP and 3-isobutyl-1-methyl-xanthine (IBMX) were obtained from Sigma (St. Louis, U.S.A.). These two compounds were directly dissolved in the external solutions to provide solutions at desired concentration.

Statistics

All values are presented as mean ± s.e.mean. Statistical analyses were performed with Student's paired t test; $P < 0.05$ was defined as significant. Time courses of inactivation of the Ca^{2+} channel current were fitted by the method of least squares.

Results

Effects of human natriuretic peptide (ANP)

Whole-cell Ba^{2+} currents carried through L-type Ca^{2+} channels were elicited by depolarizing steps of 300 ms duration from a holding potential of −78 mV in rabbit ventricular cells. In these cells, K^+ currents were blocked with intra- and extracellular Cs^+, and fast Na^+ current was blocked by a substitution of extracellular Na^+ with choline. ANP at a concentration of 30 nM decreased the Ca^{2+} channel currents elicited by the depolarizations above −28 mV (Figure 1a). Figure 1b shows relations between the peak current and the potential of depolarising pulse. ANP did not shift the potentials for the threshold (−40 mV) and the maximum peak current (−10 mV). Figure 2 shows concentration-response relation between ANP and the Ca^{2+} channel current. ANP at concentrations above 10 nM decreased the peak current at the depolarization steps to −8 mV. We could not observe a maximal response to ANP even at concentrations of 100 nM. Methionine-oxidized ANP ($Met(O)^{12}$-ANP, 30 nM), an inactive analogue of ANP (Watanabe *et al.*, 1988), did not decrease the current (−1.5 ± 4.1%, $n = 4$). This finding strongly indicates that the ANP-induced decrease in the Ca^{2+} channel current may be mediated by ANP receptors (A- or B-receptor) (Koller & Goeddel, 1992).

Effects of 8-bromo-cyclic GMP

ANP increases intracellular production of cyclic GMP in rabbit isolated ventricular cells (Cramb *et al.*, 1987). Therefore, it is possible that the ANP-induced inhibition of the Ca^{2+} channel current is mediated by intracellular cyclic GMP. Figure 3 shows the effects of 8-bromo-cyclic GMP, a membrane-permeable derivative of cyclic GMP, on the Ca^{2+} channel current of rabbit ventricular cells. 8-Bromo-cyclic GMP at a concentration of 300 μM decreased the Ca^{2+} channel current elicited by 300 ms depolarizations from the hold-

ing potential of −78 mV. Current-voltage relations of the Ca^{2+} channel current (Figure 3b) indicate that 8-bromo-cyclic GMP decreased the current without affecting the threshold potential and the potential for the maximum current. In 10 cells, 8-bromo-cyclic GMP decreased the peak current at the depolarization steps to −8 mV by 23.8 ± 3.1%. Increasing its concentration to 1 mM did not result in a further increase of its suppressive effect on the Ca^{2+} channel current (15.1 ± 3.5%, $n = 3$). Therefore, 300 μM 8-bromo-cyclic GMP exerted the maximal suppressive effect on the Ca^{2+} channel current in the rabbit ventricular cells. This finding was different from the data obtained in chick embryonic heart cells (Tohse & Sperelakis, 1991) in which 1 mM 8-bromo-cyclic GMP abolished Ca^{2+} channel activity.

In order to exclude a possible contribution of phosphodiesterase (PDE) to the inhibition of the Ca^{2+} channel current by 8-bromo-cyclic GMP, we examined the effect of 8-bromo-cyclic GMP on the Ca^{2+} channel current in the cells pretreated with 3-isobutyl-1-methyl-xanthine (IBMX). IBMX is well known to inhibit non-selectively all types of PDE (Simmons & Hartzell, 1988). IBMX (100 μM) alone markedly increased the Ca^{2+} channel current (Figure 4a), indicating that IBMX inhibited PDE, and then increased cyclic AMP. In the presence of IBMX, 8-bromo-cyclic GMP (300 μM) still decreased the Ca^{2+} channel current (Figure 4a). In 4 cells, 8-bromo-cyclic GMP decreased the peak current by 29.8 ± 8.1% of the IMBX-stimulated current. These findings suggest that increase in intracellular cyclic GMP inhibits the Ca^{2+} channel current without involving activation of PDE. 8-Bromo-cyclic GMP is reported to be a potent activator of cyclic GMP-dependent protein kinase (PKG) (Lincoln & Corbin, 1983). It is thus quite possible that 8-bromo-cyclic GMP inhibits the Ca^{2+} channel current through activation of PKG.

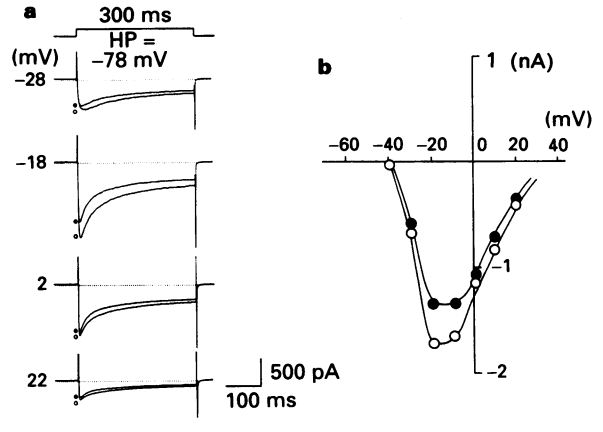

Figure 1 Effects of 30 nM atrial natriuretic peptide (ANP) on whole-cell Ba^{2+} current through the L-type Ca^{2+} channels of freshly isolated rabbit ventricular myocytes. (a) Superimposed current tracings elicited by 300 ms depolarizing test pulses to −28, −18, 2 and 22 mV from a holding potential (HP) of −78 mV. (○) Control currents at each potential; (●) current after external application of 30 nM ANP. (b) Current-voltage relation of the Ca^{2+} channel current (Ba^{2+} current). Amplitude of peak currents at each potential were plotted in absence (○) and presence (●) of 30 nM ANP.

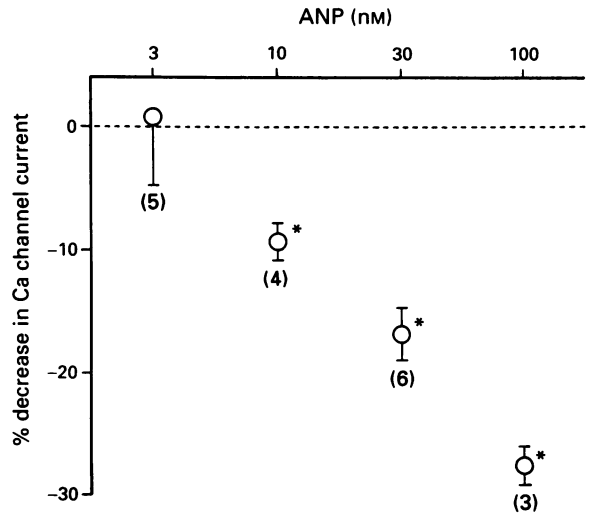

Figure 2 Concentration-response relation between atrial natriuretic peptide (ANP) and the decrease in Ca^{2+} channel current. Numbers in parentheses give the number of cells. Data are mean ± s.e.mean. *Difference from control is statistically significant at $P < 0.05$.

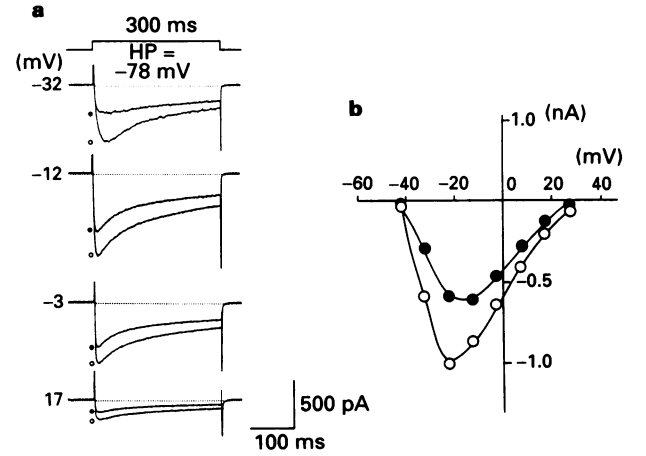

Figure 3 Effects of 300 μM 8-bromo-cyclic GMP on whole-cell Ca^{2+} channel current. (a) Superimposed current tracings elicited by 300 ms depolarizing test pulses from a holding potential (HP) of −78 mV. (○) Control current at each potential; (●) current after external application of 300 μM 8-bromo-cyclic GMP. (b) Current-voltage relation of the Ca^{2+} channel current. Amplitude of peak currents at each potential were plotted in absence (○) and presence (●) of 300 μM 8-bromo-cyclic GMP.

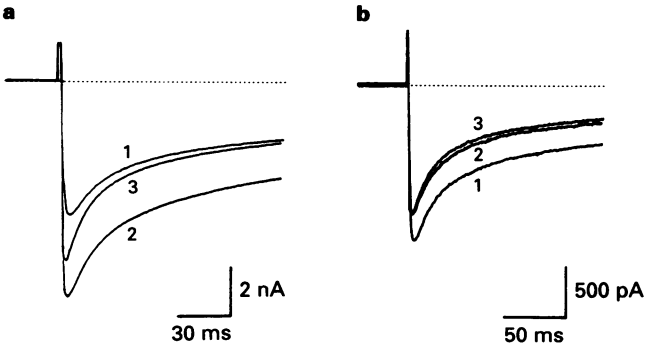

Figure 4 (a) Effects of 8-bromo-cyclic GMP (300 μM) on the Ca^{2+} channel current in the presence of 100 μM 3-isobutyl-1-methylxanthine (IBMX). Current tracings (1), (2) and (3) indicate control, IBMX, and 8-bromo-cyclic GMP with IBMX, respectively. 8-Bromo-cyclic GMP decreased the Ca^{2+} channel current even in the presence of the inhibitor of phosphodiesterase (IBMX). (b) Effects of ANP (30 nM) on the Ca^{2+} channel current decreased by 300 μM 8-bromo-cyclic GMP. Current tracings (1), (2) and (3) indicate control, 8-bromo-cyclic GMP, and atrial natriuretic peptide (ANP) with 8-bromo-cyclic GMP, respectively. ANP did not produce further decrease in the decreased Ca^{2+} channel current. The current was elicited by 300 ms depolarizing pulse to −8 mV from the holding potential of −78 mV, at a rate of 0.1 Hz.

Relationship between ANP and 8-bromo-cyclic GMP

To confirm the involvement of cyclic GMP in the ANP-induced inhibition of the Ca^{2+} channel current, we examined whether ANP was able to exert its suppressive effect on the Ca^{2+} channel current which was maximally inhibited by 8-bromo-cyclic GMP (Figure 4b). ANP at a concentration of 30 nM failed to suppress further the Ca^{2+} channel current inhibited by 300 μM 8-bromo-cyclic GMP (3.2 ± 3.1% decrement of the cyclic GMP-decreased current, $n = 6$). This finding indicated that ANP and 8-bromo-cyclic GMP share a common pathway for the inhibition of the Ca^{2+} channel current, and that the ANP-induced inhibition of the Ca^{2+} channel current is likely to be mediated by cyclic GMP production and resulting PKG activation.

Effects of ANP on single-channel activity

In order to examine the effects of ANP and cyclic GMP on single-channel activities of the L-type Ca^{2+} channels, the cell-attached patch clamp recording was performed by use of patch pipettes containing 110 mM Ba^{2+} and 1 μM Bay K 8644. Unitary Ba^{2+} currents through the channels were elicited by 150 ms-depolarization to 0 mV from a holding potential of −80 mV, at a rate of 0.5 Hz (Figure 5). Extracellular perfusion with 30 nM ANP markedly inhibited the activities of the Ca^{2+} channel without affecting the unit amplitude of channels. Figure 5 also shows that the channel openings were rarely observed in the later period during the depolarizations. The lowest traces show the ensemble-averaged current of the Ca^{2+} channels. ANP decreased the peak current and accelerated decay of the current. Similar effects were observed in another 4 patches (Table 1). On the other hand, the inactive ANP ($Met(O)^{12}$-ANP, 30 nM) did not inhibit the channel openings, and did not change the ensemble-averaged current ($n = 5$, data not shown). Figure 6a shows a time course of the NP_o value during each depolarizing pulse. The NP_o values decreased within 2 min of application of 30 nM ANP, and recovered gradually after washout of ANP. Therefore, the decrease by ANP in NP_o was not caused by the 'run-down' phenomenon.

Effects of 8-bromo-cyclic GMP on single-channel activity

Figure 7 shows effects of 300 μM 8-bromo-cyclic GMP on the single-channel activities of the Ca^{2+} channels. 8-Bromo-cyclic GMP also inhibited the channel openings, as observed in the

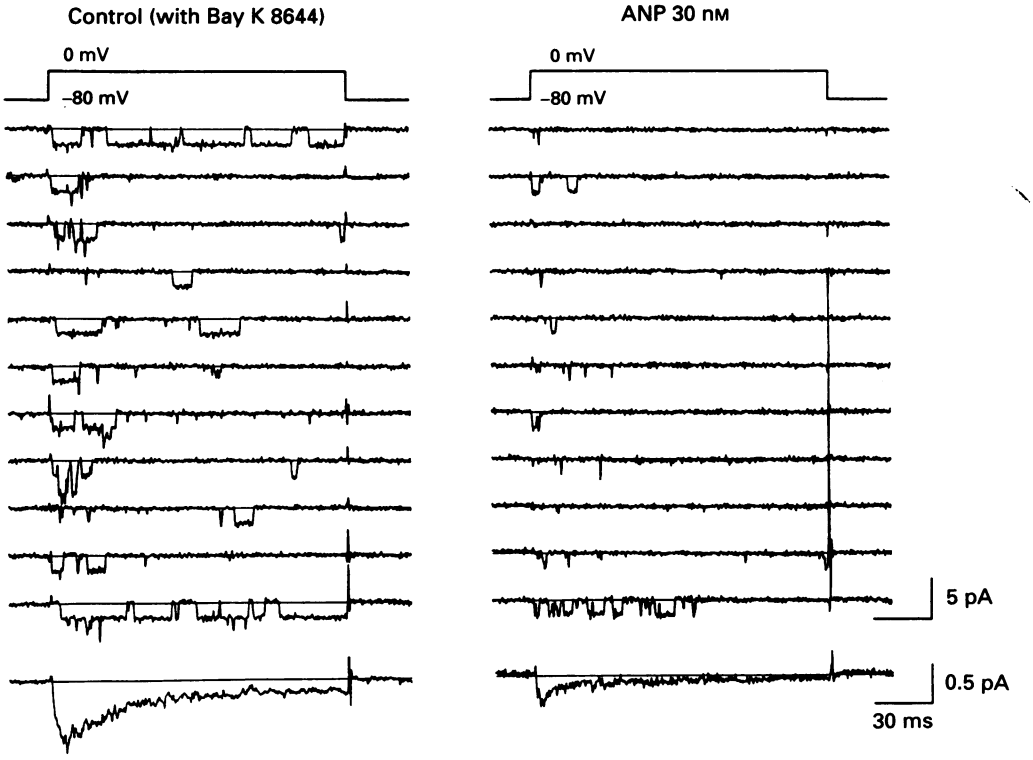

Figure 5 Effects of atrial natriuretic peptide (ANP) on single Ca^{2+} channel activity in rabbit cardiomyocytes. The channel activities were elicited by 150 ms-depolarizing pulses to 0 mV (from a holding potential of − 80 mV) every 2 s in the cell-attached patch configuration. Patch pipettes contained 110 mM Ba^{2+} and 1 μM Bay K 8644. Pulse protocol is shown above the set of tracings. Representative current tracings were chosen. Linear leak and capacity currents have been subtracted. The ensemble-averaged Ca^{2+} current from 90 tracings (including tracings shown in this figure) are shown at the bottom of the tracings. The thin horizontal line indicates the zero current level.

Table 1 Effects of atrial natriuretic peptide (ANP) and 8-bromo-cyclic GMP on characteristics of the single channel activities of the Ca^{2+} channel

	Averaged NP_o (%decrease)	*Peak Amp.* (%decrease)	τ (ms) Before drug	τ (ms) After drug
ANP (30 nM)	57.9 ± 11.1*	49.5 ± 6.4*	41.8 ± 11.7	21.3 ± 2.9**
8-Bromo-cyclic GMP (300 μM)	54.1 ± 9.7*	36.7 ± 9.4*	77.9 ± 7.7	48.2 ± 7.3**

NP_o averaged from 60–120 depolarizations, Peak Amp.: peak amplitude of the ensemble-averaged current; τ: time constant of decay of the ensemble-averaged current. $n = 5$, mean ± s.e. *$P < 0.05$ vs. control, **$P < 0.05$ vs. before drug.

presence of ANP. The channel openings were rarely observed in the later period of the depolarizations in the presence of 8-bromo-cyclic GMP. 8-Bromo-cyclic GMP decreased the peak amplitude of ensemble-averaged current shown in the lowest panels. A decay of the ensemble-averaged current was accelerated by 8-bromo-cyclic GMP. Similar effects were observed in another 4 patches (Table 1). All the changes produced by 8-bromo-cyclic GMP were very similar to those produced by ANP (Figure 5). Figure 6b shows the time course of the 8-bromo-cyclic GMP-induced suppressive effects on the NP_o value during each depolarizing pulse. The effect of 8-bromo-cyclic GMP was also reversible after washing of the drug.

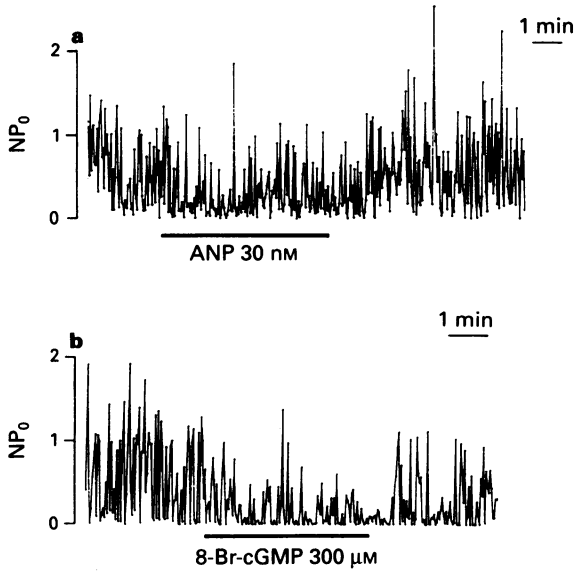

Figure 6 Time course of effects of atrial natriuretic peptide (ANP) and 8-bromo-cyclic GMP on NP_o value of each depolarization pulse. The depolarization pulses were elicited at a rate of 0.5 Hz.

Comparison of effects of ANP and cyclic GMP on single-channel activity

Table 1 summarizes the effects of ANP and 8-bromo-cyclic GMP on averaged NP_o from 60–120 depolarization pulses, the peak amplitude of the ensemble-averaged current, and the time constant of decay of the ensemble-average current. ANP and 8-bromo-cyclic GMP decreased the NP_o value. The peak amplitude of the ensemble-averaged current was also decreased by ANP and 8-bromo-cyclic GMP. The decay of the ensemble-averaged current was well-fitted by a single exponential curve. ANP and 8-bromo-cyclic GMP decreased the time constant of decay of the ensemble-averaged current. However, ANP and 8-bromo-cyclic GMP did not affect the time constant of the whole-cell Ca^{2+} channel current (data not shown). At the present time, we cannot explain this discrepancy.

Discussion

In the present study, we have examined the effects of ANP on the cardiac L-type Ca^{2+} channels, and compared them with those of 8-bromo-cyclic GMP. The results obtained clearly showed that ANP inhibited the Ca^{2+} channel activities in both the whole-cell voltage-clamp experiments and the singe-channel current recordings, and that the ANP-induced inhibition of the Ca^{2+} channel activity had similar characteristics to those of the cyclic GMP-induced one. Furthermore, the inhibitory effects of ANP and cyclic GMP were not additive, suggesting that the ANP-induced inhibition is mediated by an increase in intracellular cyclic GMP.

Relationship between ANP and cyclic GMP

ANP is well known to increase the intracellular concentration of cyclic GMP in various types of cells (Needleman *et al.*, 1989) including cardiomyocytes of rabbits (Cramb *et al.*, 1987), chick embryos (Vaxelaire *et al.*, 1989), and rats (Neyses & Vetter, 1989; McCall & Fried, 1990). Detection of

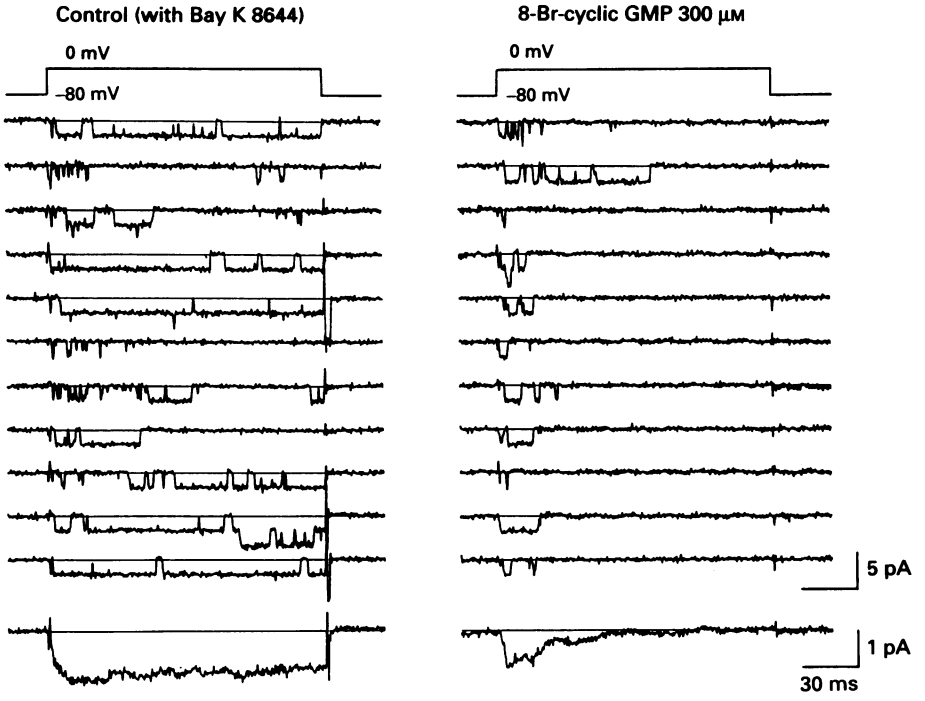

Figure 7 Effects of 8-bromo-cyclic GMP on single Ca^{2+} channel activities by use of a pipette containing 1 μM Bay K 8644. Pulse protocol is shown above the set of tracing. The representative current tracings are chosen. Linear leak and capacity currents have been subtracted. The ensemble-averaged Ca^{2+} currents from 60 tracings (including tracings shown in this figure) are shown at the bottom of the tracings.

guanylate cyclase activities in molecular form of a subtype of the ANP receptor (Leitman *et al.*, 1986; Chinkers *et al.*, 1989; Koller & Goeddel, 1992) implied that the ANP-induced increase in cyclic GMP occurs through activation of its specific receptor mechanism.

The present study provides pharmacological evidence demonstrating a causal relation between ANP and cyclic GMP in rabbit ventricular cells. Firstly, both ANP and 8-bromo-cyclic GMP inhibited the Ca^{2+} channel activity. In addition, both agents produced similar effects on the kinetics of the Ca^{2+} channels in the macroscopic and microscopic current levels. Secondly, Met(O)^{12}-ANP, which is less potent in increasing the cyclic GMP content (Watanabe *et al.*, 1988), failed to inhibit the channel activity. Finally, in the presence of 8-bromo-cyclic GMP, ANP failed to decrease further the Ca^{2+} channel current. All these findings indicate that ANP inhibits the Ca^{2+} channel activity through an intracellular increase in cyclic GMP.

Target molecule for ANP-increased cyclic GMP

Hartzell & Fischmeister (1986), using frog ventricular cells, reported that intrapipette cyclic GMP suppressed the β-agonist-stimulated Ca^{2+} current. This suppression was not observed in the cells pretreated with IBMX. In addition, the Ca^{2+} current amplified by IBMX was not suppressed by intracellular perfusion of 8-bromo-cyclic GMP, which is reported to be ineffective in activating PDE II (Lincoln & Corbin, 1983). From these findings, they concluded that intracellular cyclic GMP might decrease the stimulated Ca^{2+} current through activation of PDE II. However, the situation is quite different in mammalian cardiac cells. It seems unlikely that reduction of cyclic AMP through the activation of PDE II is the major pathway for regulation of the Ca^{2+} channel by cyclic GMP. Levi *et al.* (1989), Méry *et al.* (1991) and Ono & Trautwein (1991) observed that intracellular application of 8-bromo-cyclic GMP decreased the stimulated Ca^{2+} current, and that pretreatment with IBMX did not inhibit the cyclic GMP-induced decrement of Ca^{2+} current. These findings and ours are inconsistent with those of Hartzell & Fischmeister (1986), and deny the possibility of involvement of PDE II in cyclic GMP-induced suppression of Ca^{2+} current.

8-Bromo-cyclic GMP has been reported to be a potent activator of PKG (Lincoln & Corbin, 1983; White *et al.*, 1993). Ono & Trautwein (1991) and Méry *et al.* (1991) pointed out a similarity between suppressive effects induced by 8-bromo-cyclic GMP and PKG on the β-receptor-stimulated Ca^{2+} channel current. Therefore, it is possible that 8-bromo-cyclic GMP activates PKG and inhibits the Ca^{2+} channel activity in rabbit ventricular cells. In the present study, we found that pretreatment of rabbit cells with 8-bromo-cyclic GMP completely abolished the ANP-induced inhibition of the Ca^{2+} channel current. This finding strongly suggests that ANP inhibits the Ca^{2+} channel activity through the activation of PKG.

PKG-induced inhibition of basal activity

Méry *et al.* (1991) and Ono & Trautwein (1991) reported that intracellular administration of PKG did not affect the basal activity of Ca^{2+} channels, but was effective in inhibiting the β-stimulated Ca^{2+} channel current. In contrast with these reports, it was found that the basal Ca^{2+} channel activity was inhibited by 8-bromo-cyclic GMP in chick embryonic heart cells (Wahler *et al.*, 1990; Tohse & Sperelakis, 1991). The present study revealed that ANP and 8-bromo-cyclic GMP inhibited the basal Ca^{2+} channel current in rabbit ventricular cells. The inhibition of the basal Ca^{2+} channel activity by cyclic GMP-PKG system was also observed in pituitary tumour cells (White *et al.*, 1993) and vascular smooth muscular cells (Clapp & Gurney, 1991).

One possible explanation of this discrepancy between the present study and those of Méry *et al.* (1991) and Ono & Trautwein (1991) is that the rabbit and chick cardiomyocytes may have very high basal (non-stimulated) cyclic AMP activity. With high activity of cyclic AMP, the Ca^{2+} channels may be already activated. Actually, the chick cell was reported to have a high level of intracellular cyclic AMP (Thakkar & Sperelakis, 1987). It is noteworthy that inhibition of the basal Ca^{2+} channel activity was clearly observed in the cell-attached patch clamp, in which the intracellular environment was not modified, as shown in the present study and by Tohse & Sperelakis (1991).

Another explanation is that native PKG may be very effective in inhibiting the basal Ca^{2+} channel activity. As mentioned above, ANP inhibited the basal Ca^{2+} channel current in mammalian cells (Sorbera & Morad, 1990; LeGrand *et al.*, 1992). Therefore, the native PKG activated by ANP may be more potent in inhibiting basal activity than the PKG perfused intracellularly through the patches pipettes (Méry *et al.*, 1991; Ono & Trautwein, 1991). Further experiments are required to clarify the discrepancy concerning the cyclic GMP-induced inhibition of the basal Ca^{2+} channel activity. Recently, Han *et al.* (1993) reported that cyclic GMP and 8-bromo-cyclic GMP increased the basal Ca^{2+} current in rabbit ventricular myocytes. At the present time, we cannot explain the discrepancy between their results and ours.

Significance of ANP-induced inhibition of Ca^{2+} channels

Recently, several studies showed that ANP directly decreased contractility in isolated cardiomyocytes (Neyses *et al.*, 1989; Vaxelaire *et al.*, 1989; McCall & Fried, 1990). This negative inotropic action of ANP was also reported *in vivo* (Lappe *et al.*, 1985; Seymour *et al.*, 1987). Therefore, it is possible that the ANP-induced inhibition of Ca^{2+} channel activity contributes to the negative inotropic action of ANP. The negative inotropic action of ANP may be important for pathogenesis of chronic heart failure (CHF) in addition to the physiological regulation of cardiac function. Plasma ANP was reported to be increased in the dog with experimental heart failure (Perrella *et al.*, 1992) and patients with CHF (Shenker *et al.*, 1985; Burnett *et al.*, 1986). Furthermore, CHF patients with high levels of ANP had a significantly low rate of survival (Gottlieb *et al.*, 1989). The ANP-induced inhibition of the Ca^{2+} channel activity should be investigated in some model animals with CHF.

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Copper chelation-induced reduction of the biological activity of S-nitrosothiols

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1 The effect of copper on the activity of the S-nitrosothiol compounds S-nitrosocysteine (cysNO) and S-nitrosoglutathione (GSNO) was investigated, using the specific copper chelator bathocuproine sulphonate (BCS), and human washed platelets as target cells.

2 Chelation of trace copper with BCS (10 μM) in washed platelet suspensions reduced the inhibition of thrombin-induced platelet aggregation by GSNO; however, BCS had no significant effect on the anti-aggregatory action of cysNO. BCS inhibited cyclic GMP generation in response to both cysNO and GSNO.

3 The effect of BCS was rapid (within 30 s), and could be abolished by increasing the platelet concentration to $500 \times 10^9\ l^{-1}$.

4 In BCS-treated platelet suspensions, the addition of Cu^{2+} ions (0.37–2.37 μM) led to a restoration of both guanylate cyclase activation and platelet aggregation inhibition by GSNO.

5 The anti-aggregatory activity of GSNO was reduced in a concentration-dependent manner by the copper (I)-specific chelators BCS and neocuproine, and to a smaller extent by desferal.No effect was observed with the copper (II) specific chelator, cuprizone, the iron-specific chelator, bathophenanthroline sulphonate, or the broader-specificity copper chelator, D-penicillamine.

6 In both BCS-treated and -untreated platelet suspensions, cys NO was more potent than GSNO as a stimulator of guanylate cyclase. In BCS-treated platelet suspensions there was no significant difference between the anti-aggregatory potency of cysNO and GSNO; however, in untreated suspensions, GSNO was significantly more potent than cysNO. Thus, when copper was available, GSNO produced a greater inhibition of aggregation than cysNO, despite being a less potent activator of guanylate cyclase.

7 The breakdown of cysNO and GSNO was measured spectrophotometrically by decrease in absorbance at 334 nm. In Tyrode buffer, cysNO (10 μM) broke down at a rate of 3.3 μM min^{-1}. BCS (10 μM) reduced this to 0.5 μM min^{-1}. GSNO, however, was stable, showing no fall in absorbance over a period of 7 min even in the absence of BCS.

8 We conclude that copper is required for the activity of both cysNO and GSNO, although its influence on anti-aggregatory activity is only evident with GSNO. The stimulatory effect of copper is unlikely to be explained solely by catalysis of S-nitrosothiol breakdown. The enhancement by copper of th anti-aggregatory activity of GSNO, relative to cysNO, suggests that copper may be required for biological activity of GSNO which is independent of guanylate cyclase stimulation.

Keywords: S-nitrosothiol; S-nitrosoglutathione; S-nitrosocysteine; nitric oxide; copper; bathocuproine sulphonate; platelet aggregation; cyclic GMP

Introduction

Nitric oxide (NO) is highly reactive and will combine with a wide array of biological molecules (Stamler *et al.*, 1992b). The reaction of NO with biological thiols to form S-nitrosothiol intermediates is thought to be an important mechanism in the action of nitrovasodilator drugs (Ignarro *et al.*, 1981). Claims that endothelium-derived relaxing factor (EDRF), formed under pharmacological bioassay conditions, is S-nitrosocysteine (cysNO) (Ignarro, 1990), have not been substantiated (Feelisch *et al.*, 1994). Nevertheless, under physiological conditions the stabilization of NO by thiol is likely, and in the blood (Stamler *et al.*, 1992a) and tissues such as the lung (Gaston *et al.*, 1993) NO is found predominantly as S-nitrosothiol adducts of proteins and peptides. The formation of S-nitrosothiols may serve both to stabilize NO during delivery to its target, and to mitigate the toxicity of NO at its site of production.

S-nitrosothiols are effective anti-platelet compounds, inhibiting fibrinogen binding, aggregation and secretion by platelets via an increase in cellular guanosine 3′:5′-cyclic monophosphate (cyclic GMP) (Mellion *et al.*, 1983; Mendelsohn *et al.*, 1990; Lieberman *et al.*, 1991). The most abundant and physiologically relevant S-nitroso adducts are likely to be S-nitrosoproteins, for example, S-nitrosoalbumin and low molecular weight forms such as cysNO and S-nitrosoglutathione (GSNO). There is evidence that the anti-platelet effects of S-nitrosoproteins may be mediated via low molecular weight forms following thiol-nitrosothiol exchange (Simon *et al.*, 1993).

Most S-nitrosothiols are unstable, but measurements of their half-lives depend on the experimental conditions employed. Ignarro and co-workers (1981), reported the half-life of cysNO to be 15 min in oxygenated solutions, compared with more recent estimates of between 4–83 s (Ignarro, 1990; Feelisch *et al.*, 1994; Mathews & Kerr, 1993). The use of Tris-HCl, a metal binding agent (Dawson, 1986), might account for the stabilization of cysNO in the earlier study. Recently, the chemical breakdown of S-nitroso-N-acetyl D,L penicillamine (SNAP) has been shown to be catalysed by Cu^{2+} and Fe^{2+} ions (McAninly *et al.*, 1993), and the stabilization of S-nitrosothiols by EDTA, EGTA and L-cysteine (McAninly *et al.*, 1993; Feelisch *et al.*, 1994) is explained by the ability of these compounds to bind metal ions.

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GSNO is both stable and highly potent as an anti-platelet agent (Radomski *et al.*, 1992) and a smooth muscle relaxant (Kowaluk & Fung, 1990; Jansen *et al.*, 1992). These characteristics suggest that GSNO might not only be an important physiological molecule, but also a useful pharmacological agent. We have therefore chosen to study the low molecular weight S-nitrosothiols GSNO and cysNO. We have used the specific copper (I) chelator bathocuproine sulphonate (BCS) (2,9-dimethyl-4,7-diphenyll,10-phenanthroline) (Blair & Diehl, 1961) to investigate the influence of copper on both the stability and the biological activity of GSNO and cysNO.

Methods

Reagents

Modified Tyrode buffer with the following composition (in mM) was used for suspending washed platelets: NaCl 137.0, glucose 5.55, $CaCl_2$ 1.0, $NaHCO_3$ 11.9, $MgCl_2.6H_2O$ 1.05, $NaH_2PO_4.2H_2O$ 0.36, KCl 2.68 and HEPES 10.0. Using manufacturer's data, the approximate concentrations of copper and iron in the buffer were estimated to be less than 0.37 μM and 0.43 μM respectively. All components of the buffer, and also trisodium citrate and trichloroacetic acid were obtained from Merck (Lutterworth, UK). Bovine thrombin was obtained from Ortho Diagnostics (High Wycombe, UK), cyclic GMP radioimmunoassay kits from Amersham plc (High Wycombe, UK), and prostacyclin was a kind gift from Dr M. Radomski (Wellcome Laboratories, Beckenham, UK). All other reagents were obtained from Sigma (Poole, UK). MilliQ grade water (resistivity 18 MΩ.cm) was used for all solutions.

Preparation of S-nitrosothiols

Fresh S-nitroso-L-cysteine (cysNO) for use in platelet experiments was prepared daily by a modification of the method of Hart (1985), by mixing one volume of 40 mM L-cysteine/40 mM HCl/5 μM BCS with one volume of 44 mM $NaNO_2$ (stock) at 0–4°C for 10 min, followed by dilution with two volumes of 50 mM NaCl, 40 mM sodium phosphate, 5 μM BCS and 5 μM deferoxamine mesylate (desferal), at a pH of 7.4. For experiments to be performed in the absence of BCS, the nitrosating mixture was not neutralised with buffer until the time of dilution and assay.

S-nitrosoglutathione (GSNO) was prepared by mixing one volume of 40 mM glutathione/40 mM HCl with one volume of 44 mM $NaNO_2$ for 120 min at 0°C. GSNO was then precipitated with ice-cold acetone, filtered, and washed three times with further ice-cold acetone. The precipitate was then dried under vacuum and stored at −70°C.

Concentration of each S-nitrosothiol was estimated from the absorbance at 334 nm (extinction coefficients (334 nm): 0.96 and 0.87 mM cm^{-1} for GSNO and cysNO respectively (D.J. Meyer, unpublished data)). Yields were 90–99%.

Platelet preparation

Blood was obtained from healthy volunteers and anticoagulated with trisodium citrate (10.6 mM final concentration). Platelet rich plasma (PRP) was prepared by centrifugation of blood for 15 min at 20°C and 150 *g*. Residual leucocytes and erythrocytes were removed from PRP by centrifugation at 20°C and 110 *g* for 10 min, after which prostacyclin (300 ng ml^{-1}) was added and platelets were pelleted by centrifugation at 20°C and 600 *g* for 10 min. Following resuspension in Tyrode buffer containing 300 ng ml^{-1} prostacyclin, platelets were again pelleted, and finally resuspended at a count of 200 × 10^9 l^{-1} in Tyrode buffer. Washed platelets were stored at room temperature in sealed syringes (to prevent change in pH) for at least 2 h before use to allow recovery from the effects of prostacyclin treatment.

Measurement of cyclic GMP formation

Platelets (180 μl) were equilibrated at 37°C for 5 min before addition of cysNO or GSNO, in a volume of 20 μl. Stimulation of guanylate cyclase was allowed to proceed for 2 min, after which the reaction was stopped by the addition of 200 μl of ice-cold 10% v/v trichloroacetic acid. Samples were mixed, chilled on ice for 5 min then centrifuged at 13 000 *g* for 2 min. Supernatant was removed and stored at −20°C. Prior to assay samples were further extracted using tri-n-octylamine in 1,1,2-trichlorotrifluoroethane, and cyclic GMP measured by radioimmunoassay as previously described (Gordge & Neild, 1992).

Measurement of platelet aggregation inhibition

Aggregation of washed platelets was induced with a submaximal concentration of bovine thrombin (0.012–0.025 u ml^{-1}); 240 μl of platelet suspension was equilibrated for 2 min at 37°C, then treated with either nitrosothiol or vehicle control in a volume of 30 μl for 15 s, after which 30 μl of thrombin was added. Platelet aggregation was measured at 37°C at a stirring rate of 1000 r.p.m., with a Payton turbidometric aggregometer, as the maximum change in light transmission occurring within 3 min, expressed as a percentage of the difference in light transmission between Tyrode buffer and starting platelet suspension. Inhibition of this reaction by cysNO and GSNO was assessed by comparison with the aggregation response obtained in the presence of vehicle alone.

Effect of copper chelation with BCS on the action of GSNO and CysNO

Platelet guanylate cyclase stimulation by GSNO and CysNO (10^{-9}–10^{-5} M) was measured, as described above, in the presence of BCS (10 μM final concentration). Platelets were suspended in Tyrode buffer containing BCS, and all reagents used in the experiments were diluted in water containing BCS. Parallel assessments of the action of GSNO and cysNO were made in the absence of BCS, and their potencies compared in the presence and absence of the copper chelator. The effect of BCS on the anti-aggregatory action of the two S-nitrosothiol compounds was also assessed in a similar way.

Time-course of the action of BCS to reduce the anti-aggregatory activity of GSNO

In order to assess the time required for BCS to reduce the activity of GSNO, platelets were pre-incubated at room temperature with BCS (10 μM) or vehicle for periods of 30 s to 60 min, after which the ability of GSNO (1 μM) to inhibit thrombin-induced platelet aggregation was assessed as described above.

Effect of increasing platelet count on the action of BCS to reduce the anti-aggregatory action of GSNO

Platelet suspensions with counts varying from 100–500 × 10^9 l^{-1} were prepared in Tyrode buffer. BCS (10 μM) was then pe-incubated for 3 min with aliquots of these suspensions, after which the ability of GSNO (1 μM) to inhibit thrombin-induced aggregation was assessed as described above.

Effect of different chelating agents on the action of GSNO

The effects on the anti-aggregatory action of GSNO of the following chelating agents were investigated: the copper (I) specific agents BCS (Blair & Diehl, 1961) and neocuproine (2,9-dimethyl 1,10-phenanthroline) (Diehl & Smith, 1958);

the copper (II) specific agent cuprizone (*bis*cyclohexanone-oxalyldihydrazone) (Peterson & Bollier, 1955), the iron specific agent bathophenanthroline sulphonate (BPS) (4,7-diphenyl, 1,10 phenanthroline) (Blair & Diehl, 1961), and the less specific agents D-penicillamine and desferal. Stock solutions of chelating agents (1 mM) were prepared in water, except for cuprizone, which was dissolved in 50% (v / v) ethanol. Platelets were pre-incubated for 3 min with chelator (100 μM final concentration) or vehicle, prior to the addition of either GSNO (1 μM final concentration) or vehicle. After 15 s platelet aggregation was induced with thrombin and the inhibitory action of GSNO was assessed as described above. Control experiments were also performed in which GSNO was excluded, in order to assess the direct effects of each chelator on platelet aggregation.

Dose-response experiments were performed with BCS, neocuproine and desferal, in which the final concentration of each chelator was increased from 10^{-7} to 10^{-4} M. The effect of this on the action of GSNO (1 μM) both to inhibit platelet aggregation and to elevate platelet cyclic GMP was assessed as described above.

Effect of addition of copper to BCS-treated platelets on the action of GSNO

In further experiments, the effect on the activity of GSNO of restoring Cu^{2+} ions to BCS-chelated platelet suspensions was assessed. Platelets were pelleted from PRP as described above, after which they were washed twice in Tyrode buffer, the first wash in the presence of 10 μM BCS and the second in the presence of 1 μM BCS. Platelets were finally suspended in BCS-free Tyrode in which the concentration of copper from contamination of the buffer salts was estimated to be <0.37 μM. Aliquots of the platelet suspension were then supplemented with 10 μM BCS (to remove all free copper), or $CuSO_4$ to give final concentrations of Cu^{2+} of approximately 0.37–2.37 μM. After 10 min equilibration at 37°C the effect of GSNO (1 μM) on both stimulation of soluble guanylate cyclase and inhibition of platelet aggregation were measured, as described above. In these experiments, platelets used for measurement of guanylate cyclase stimulation were treated wtih isobutyl methyl xanthine (IBMX) (1 mM) for 15 min prior to use in order to inhibit phosphodiesterase activity.

In a similar way the influence of Fe^{3+} ions on the activity of GSNO was assessed. Platelet suspensions were washed in the presence of 10 μM and 1 μM desferal, an iron chelator, and then supplemented with either desferal (10 μM) or $FeCl_3$. The concentration of iron in the Tyrode buffer used in the experiments was estimated to be <0.43 μM.

Effect of copper chelation on the anti-aggregatory action of prostacyclin, sodium nitroprusside and dibutyryl cyclic GMP

In control experiments, the inhibitory effect of prostacyclin (10^{-2}–10^{2} ng ml^{-1}), sodium nitroprusside (10^{-7}–10^{-3} M) and dibutyryl cyclic GMP (10^{-5}–10^{-3} M) on thrombin-induced aggregation of washed platelets was assessed in the presence and absence of BCS (10 μM), as described above.

Effect of BCS on the rate of breakdown of cysNO and GSNO in Tyrode buffer

CysNO and GSNO (both 10 μM) were prepared as described above, but without the addition of BCS. Under acid conditions, both were stable for at least 2 h (less than 0.001 units fall in absorbance at 334 nm). Each compound was then diluted to a final concentration of 10 μM in Tyrode buffer, in the presence and absence of BCS (10 μM) and their breakdown monitored over a period of 7 min by the fall in absorbance at 334 nm, with a Cary 1E spectrophotometer.

Statistics

Dose-response curves were compared by two-way analysis of variance. In experiments in which the action of GSNO was assessed after restoration of Cu^{2+} and Fe^{3+} ions to BCS- or desferal-washed platelets, variation in intra-platelet cyclic GMP and aggregation inhibition obtained at different metal ion concentrations was analysed by Kruskal Wallis one-way analysis of variance.

Results

Effect of copper chelation on platelet cyclic GMP concentrations following stimulation with cysNO and GSNO

BCS caused a significant shift to the right in the dose-response curves of cyclic GMP generation in response to both cysNO ($P=0.027$) and GSNO ($P<0.001$), The influence of copper chelation was greater with GSNO than with cysNO (Figure 1). Comparison of dose-response data obtained with the two different S-nitrosothiols showed that, both in the presence and absence of BCS the elevation of cyclic GMP in platelets was significantly greater following stimulation with cysNO than with GSNO ($P<0.001$).

Effect of copper chelation on the inhibition of platelet aggregation by cysNO and GSNO

BCS caused a significant shift to the right in the anti-aggregatory dose-response curve of GSNO ($P<0.001$), indicating a loss of inhibitory activity. The anti-aggregatory activity of cysNO, however, was not significantly altered by BCS ($P=0.455$) (Figure 2). Comparison of the dose-response data obtained with the two different S-nitrosothiols showed that in the presence of BCS there was no significant difference between their anti-aggregatory potency. In the absence of BCS, however, GSNO was significantly more potent than cysNO ($P<0.001$).

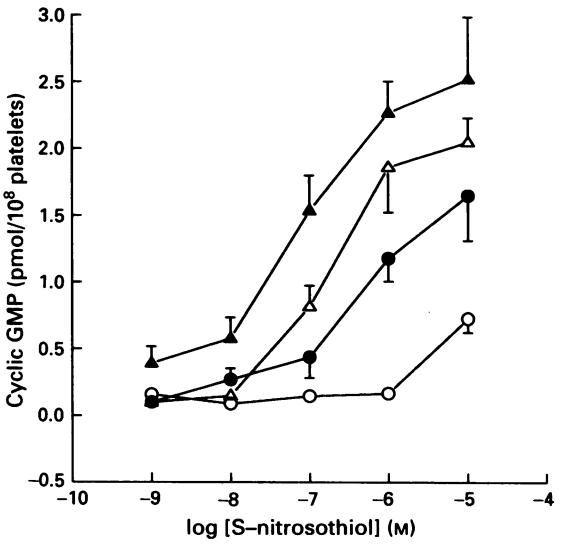

Figure 1 Intraplatelet cyclic GMP concentrations following stimulation for 2 min with cysNO (10^{-9}–10^{-5} M) in the presence (Δ) and absence (▲) of BCS (10 μM), and GSNO (10^{-9}–10^{-5} M) in the presence (○) and absence (●) of BCS (10 μM). Copper concentration in the absence of BCS was estimated to be <0.37 μM. The shift to the right of the dose-response curves in the presence of BCS, analysed by two-way ANOVA, was significant for both cysNO ($P=0.027$) and GSNO ($P<0.001$). Results are mean (±s.e.mean) from four experiments. For abbreviations, see text.

Time course of the action of BCS, and the effect of platelet count on the action of BCS to reduce the anti-aggregatory activity of GSNO

BCS acted rapidly; inhibition of the anti-aggregatory action of GSNO was maximal after pre-incubation of platelets with BCS for 30 s. There was no further increase in the effect of BCS when pre-incubation periods were prolonged up to 60 min ($n=2$; data not shown).

The effectiveness of BCS was diminished by increasing platelet concentration. At a platelet count of $100 \times 10^9\ l^{-1}$ BCS reduced the platelet inhibitory action of GSNO from 92% to 57%. Increasing the platelet count led to a progressive loss of the ability of BCS to reduce the action of GSNO (Figure 3).

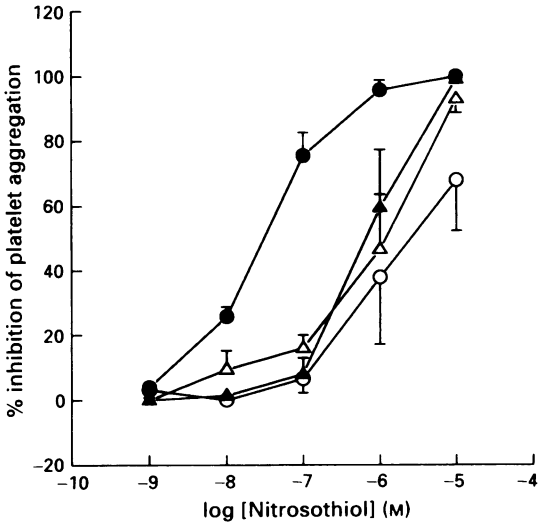

Figure 2 Inhibition of thrombin-induced platelet aggregation by cysNO (10^{-9}–10^{-5} M) in the presence (Δ) and absence (▲) of BCS (10 μM), and by GSNO (10^{-9}–10^{-5} M) in the presence (○) and absence (●) of BCS (10 μM). Copper concentration in the absence of BCS was estimated to be <0.37 μM. BCS caused no significant alteration in the dose-response data for cysNO, but the dose-response curve for GSNO was significantly shifted to the right in the presence of BCS ($P<0.001$) (two-way ANOVA). Results are mean (±s.e.mean) from four experiments. For abbreviations, see text.

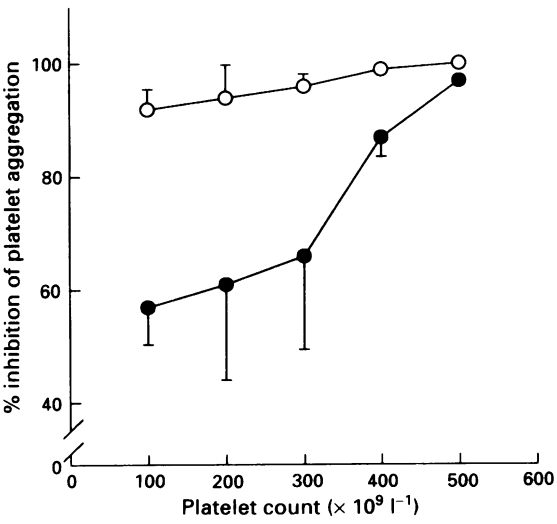

Figure 3 Effect of increasing platelet count on the action of BCS. Platelet suspensions were prepared with counts ranging from $100-500 \times 10^9\ l^{-1}$. The ability of GSNO (1 μM) to inhibit thrombin-induced platelet aggregation was then tested in the presence (●) and absence (○) of BCS (10 μM). Results are mean (±s.e.mean) from four experiments. For abbreviations, see text.

Effect of different chelating agents on the action of GSNO

At concentrations up to 100 μM, none of the chelating agents used in these experiments had any direct effect on platelet aggregation, except for neocuproine, which at concentrations >10 μM, inhibited aggregation by approximately 30%.

In the absence of chelating agents, 1 μM GSNO produced 93–99% inhibition of platelet aggregation. Pre-incubation of platelets with BPS, penicillamine or cuprizone (all at 100 μM) failed to alter this anti-aggregatory activity (mean aggregation inhibition by GSNO 100%, 99% and 96% respectively) ($n=4$). The action of GSNO was, however, reduced by 100 μM concentrations of BCS, neocuproine and, to a smaller extent, desferal (mean aggregation inhibition by GSNO reduced to 43%, 71% and 79% respectively) ($n=4$).

Dose-response experiments showed a progressive reduction in the aggregation inhibitory action of GSNO in the presence of increasing concentrations of BCS, neocuproine and desferal, although in the case of neocuproine this trend was reversed at concentrations above 10 μM (Figure 4). This reversal was probably due to the direct inhibitory effect on platelet aggregation of high concentrations of neocuproine.

Cyclic GMP measurements showed that both BCS and neocuproine, but not desferal, inhibited the elevation of cyclic GMP by GSNO in a dose-dependent fashion (Figure 5). The baseline level of cyclic GMP formation was lower in the experiments with desferal. The reason for this is uncertain.

Effect of restoring Cu^{2+} and Fe^{3+} ions to platelets washed in the presence of either BCS or desferal

The presence of BCS (10 μM) was associated with a significant reduction of both the anti-aggregatory activity and stimulation of intra-platelet cyclic GMP by GSNO (1 μM) ($P<0.05$). Restoration of Cu^{2+} ions to platelets which had been washed in the presence of BCS resulted in a return to full activity of GSNO.

Iron chelation with desferal (10 μM) resulted in only a small, and statistically insignificant reduction in the activity of GSNO (Figure 6a and b).

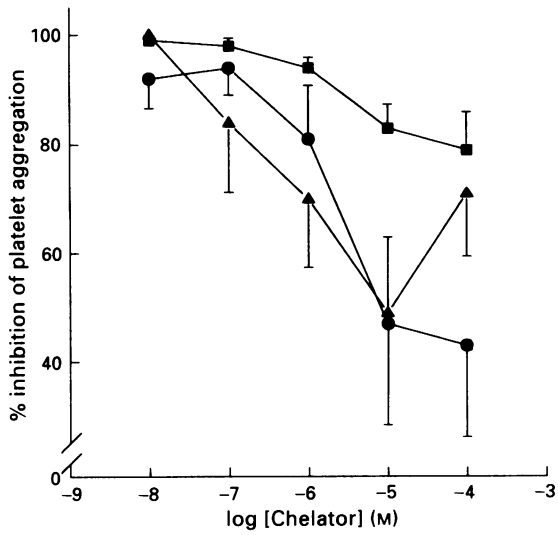

Figure 4 Inhibition of the anti-aggregatory action of GSNO by different chelating agents. The ability of GSNO (1 μM) to inhibit thrombin-induced aggregation was tested in platelet suspensions pre-incubated with increasing concentrations of either BCS (●), neocuproine (▲) or desferal (■). (Note that neocuproine at a concentration $>10^{-5}$ M showed a direct inhibitory effect on platelet aggregation, independent of GSNO). Results are mean (±s.e.mean) of four experiments. For abbreviations, see text.

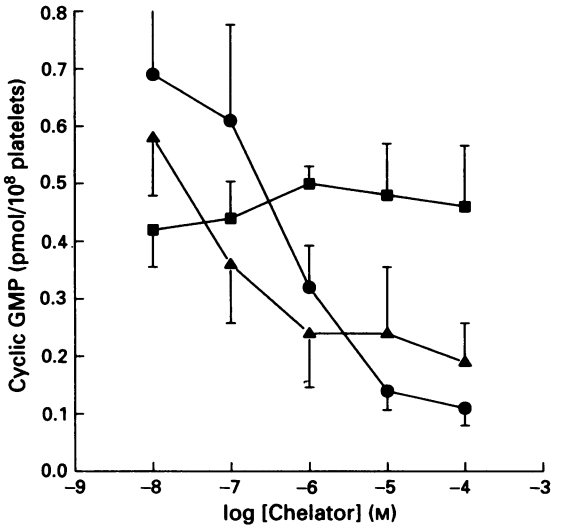

Figure 5 Inhibition of GSNO-stimulated cyclic GMP formation by different chelating agents. The ability of GSNO (1 μM) to stimulate cyclic GMP formation was tested in platelet suspensions pre-incubated with increasing concentrations of either BCS (●), neocuproine (▲) or desferal (■). Results are mean (±s.e.mean) of four experiments. For abbreviations, see text.

Effect of copper chelation on the anti-aggregatory action of prostacyclin, sodium nitroprusside and dibutyryl cyclic GMP

There was no significant alteration in the anti-aggregatory action of either prostacyclin ($n=3$), sodium nitroprusside ($n=3$), or dibutyryl cyclic GMP ($n=2$), following treatment of washed platelet suspensions with 10 μM BCS (data not shown).

Effect of BCS on the breakdown of cysNO and GSNO in Tyrode buffer

CysNo (10 μM) was unstable in Tyrode buffer, breaking down at a rate of 3.3 μM min^{-1}. In the presence of BCS (10 μM), however, this rate of breakdown was reduced to 0.5 μM min^{-1}. In contrast GSNO was stable under these conditions, showing no change in absorbance over a period of 7 min whether or not BCS was present.

Discussion

We have shown that specific chelation of copper with BCS in human platelet suspensions significantly inhibits the anti-aggregatory activity of GSNO. In addition, BCS significantly reduces the stimulation of platelet guanylate cyclase by both cysNO and GSNO. Copper is therefore required for the full activity of these S-nitrosothiol compounds.

What is the mechanism by which copper augments S-nitrosothiol action? The simplest possibility is via catalysis of S-nitrosothiol breakdown, leading to an increase in the availability of NO. Our data do not, however, support such an explanation. We measured the chemical breakdown of both cysNO and GSNO in Tyrode buffer identical to that used in our platelet experiments. We observed no spontaneous breakdown of GSNO, but the breakdown of cysNO under these conditions was rapid, and could be inhibited by copper chelation with BCS. In contrast, BCS had a profound effect on the anti-aggregatory activity of GSNO but did not significantly alter that of cysNO. There was therefore a discrepancy between the effects of copper chelation on the chemical breakdown and the biological activity of the two compounds. Other authors have demonstrated a discrepancy between the

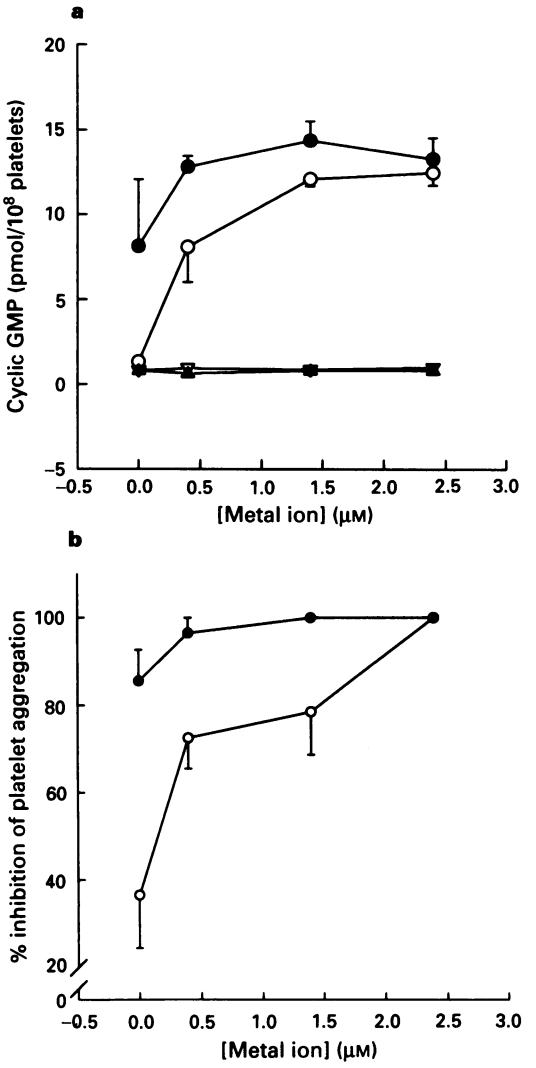

Figure 6 Intraplatelet cyclic GMP concentrations (a), and inhibition of thrombin-induced platelet aggregation (b) induced by GSNO (1 μM) in platelet suspensions containing different concentrations of Cu^{2+} (○) and Fe^{3+} ions (●). Basal levels of cyclic GMP were unaffected by either Cu^{2+} (△) or Fe^{3+} ions (▲).

Metal ions were removed from platelet suspensions by washing in either BCS or desferal, and then restored at the concentrations indicated. For values of zero free Cu^{2+} or Fe^{3+}, experiments were performed in the presence of BCS (10 μM) or desferal (10 μM) respectively. The variation in both cyclic GMP response and percentage inhibition of aggregation associated with alteration of Cu^{2+} concentration was statistically significant ($P<0.05$) (Kruskal Wallis one-way ANOVA). Variation in responses associated with different Fe^{3+} concentrations was not significant. Results are mean (±s.e.mean) from four experiments. Note that, in these experiments, measurements of cyclic GMP were made with platelet suspensions pretreated with IBMX (1 mM). For abbreviations, see text.

biological activity of S-nitrosothiols and their rate of spontaneous liberation of NO (Kowaluk & Fung, 1990; Mathews & Kerr, 1993). Kowaluk & Fung (1990), using bovine smooth muscle cells, found evidence of a membrane-associated enzyme catalysing the generation of NO from SNAP. A similar, membrane-associated activity accelerating the release of NO from GSNO has also been described in human platelets (Radomski *et al.*, 1992). It is not yet known whether copper influences this enzyme activity.

Copper might alter the redox properties of NO released from S-nitrosothiol carriers. It has recently been shown that optimal activation of guanylate cyclase via its haem group requires NO to be in its oxidised (NO^+, nitrosonium) form

(Severina *et al.*, 1992). Such an oxidation could occur during the conversion of Cu(II) to Cu(I).

Our findings using a range of chelating agents suggest that copper in its reduced form (Cu(I)) is required for the full activity of GSNO. Concentration-dependent inhibition of both the anti-aggregatory and the guanylate cyclase-stimulating activity of GSNO was shown by the Cu(I) specific agents BCS and neocuproine, but not by the Cu(II) specific agent cuprizone, nor by the iron-specific agent BPS (structurally very similar to BCS) (Diehl & Smith, 1958; Blair & Diehl, 1961). Desferal produced a small, concentration-dependent inhibition of the anti-aggregatory action of GSNO, but this was not matched by changes in cyclic GMP formation. Desferal may display effects unrelated to its metal-binding properties (Halliwell, 1989), and its influence on the anti-aggregatory action of GSNO may therefore be non-specific. D-Penicillamine was ineffective, despite the fact that it can complex Cu(I) ions. This might be explained by its inability to mobilize protein-bound copper (Laurie & Prime, 1979), or by the fact that copper bound to D-penicillamine is rapidly exchangeable and can participate in cellular processes such as uptake on a transport system (McArdle *et al.*, 1989). Thus the Cu(I)-binding properties of D-penicillamine may be inadequate to interfere with the action of GSNO.

It is unlikely that the action of BCS is mediated via chelation of free Cu(I), since such ions are unstable and would be oxidized to copper (II) in solution. In addition, the concentration of BCS required to bind $<0.37\ \mu M$ free copper in Tyrode buffer would be lower than the maximally effective concentration observed ($10-100\ \mu M$). Instead, the target for BCS may be a copper (I)-containing protein or enzyme on the platelet. Consistent with this, the effect of a fixed concentration BCS could be 'diluted out' by increasing the platelet concentration, suggesting that BCS was interacting with a platelet, rather than a buffer, component. Protein-bound copper can be held in the form of reduced Cu(I) (Poillon & Dawson, 1963), and this may explain our finding that incubation of BCS-washed platelets with Cu(II) ions (as $CuSO_4$) could increase the action of GSNO.

BCS failed to influence the action of either sodium nitroprusside or dibutyryl cyclic GMP. The effect of BCS on the activity of S-nitrosothiols cannot therefore be explained by interference with redox regulation of soluble guanylate cyclase (White *et al.*, 1976) or cyclic GMP-dependent protein kinase (Landgraf *et al.*, 1991), nor by binding of copper in the guanylate cyclase enzyme (Gerzer *et al.*, 1981). In addition, the likelihood that BCS is acting by some non-specific mechanism is weakened by its failure to influence platelet inhibition by prostacyclin.

Our study has also shown that the anti-aggregatory potency of GSNO was greater than that of cysNO, despite the fact that cysNO appeared to be a more efficient stimulator of guanylate cyclase. This augmentation of the inhibitory action of GSNO depended upon the availability of copper, since it was abolished by BCS. In a recent comparison of the properties of eight S-nitrosothiol compounds, Mathews & Kerr (1993) found that the biological activities of these agents did not correlate with their different abilities to stimulate soluble guanylate cyclase. They found GSNO to be the second most potent inhibitor of platelet aggregation but the least active stimulator of guanylate cyclase. These findings raise the possibility that the biological activity of GSNO (and other S-nitrosothiols) may be mediated in part via pathways independent of cyclic GMP. Covalent modification of protein sulphydryl groups by rapid transnitrosation may contribute to the activity of GSNO and other S-nitrosothiols (Park, 1988). In human platelets NO-donors inactivate the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GADPH) by S-nitrosylation and subsequent NAD-dependent covalent modification of a specific cysteine residue (cys-149). This modification appears to influence the transport of NO into platelets from some, but not all NO-donors (McDonald *et al.*, 1993). Thus differences between the biological activities of different S-nitrosothiols (and other NO-donors) might arise from variation in the pattern of protein modification. Our observations of the influence of BCS on the relative activities of cysNO and GSNO suggest that copper participates in these cyclic GMP-independent interactions.

Despite the fact that S-nitrosothiols are potent mediators of NO signalling, the mechanism(s) by which they donate NO and alter cellular function are poorly understood. Our results indicate that copper is a cofactor which increases the biological activity of these compounds. It is important to note that nitrosothiols as generally prepared may contain significant levels of copper (from contamination of reagents such as HCl) which may alter their stability compared with nitrosothiols occurring *in vivo*. It is also of interest that rats fed a copper-deficient diet develop a defect in NO-mediated vasodilatation (Schuschke *et al.*, 1992), an observation which supports the physiological relevance of our findings. Although the mechanism of action of copper is still unclear, it appears that both cyclic GMP-dependent and -independent pathways may be involved.

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Differential effects of OPC-18790, amrinone and dobutamine on cardiac function and energy metabolism in the guinea-pig isolated ischaemic heart

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1 The effects of OPC-18790, a novel positive inotropic agent, on cardiac function and myocardial energy metabolism in the guinea-pig isolated heart with ischaemia were studied by ^{31}P-magnetic resonance spectroscopy (MRS) and compared with those of amrinone and dobutamine.

2 Cardiac ischaemia was induced by intracoronary infusion of 15 μm microspheres to reduce coronary perfusion flow (CPF) by 50%. Microsphere embolisation caused a 40% decrease in left ventricular systolic pressure (LVSP), cardiac contractility measured by peak of ventricular pressure development (LV*dP/dt*) and slightly reduced heart rate. There was also a decrease in ATP and creatine phosphate (PCr) by 20%, an increase in inorganic phosphate (P_i) by 25% and an acidic shift of intracellular pH in the ischaemic heart.

3 In the ischaemic heart, OPC-18790, amrinone and dobutamine were applied at concentrations which increased LV*dP/dt* by about 60%. These compounds increased LVP by 15% to 30% and increased CPF by about 10%. Amrinone and dobutamine but not OPC-18790 increased heart rate. When these drugs produced the haemodynamic changes described above, amrinone and dobutamine reduced ATP and PCr, increased P_i and produced further intracellular acidosis, whereas, OPC-18790 did not change these parameters.

4 Cardiac pacing at 285 beats min^{-1} produced decreases in LVP, LV*dP/dt* and CPF by about 30%, 20%, 5%, respectively and an increase in P_i, decreases in PCr and ATP, and intracellular acidosis.

5 These results suggest that degradation of high energy phosphate compounds closely relates to increase in heart rate in the ischaemic heart. Positive inotropic agents without chronotropic action seem to be beneficial in support of the ischaemic heart.

Keywords: ^{31}P-magnetic resonance spectrometry; high energy phosphates; Langendorff's preparations; ischaemic heart; positive inotropic agents; OPC-18790; amrinone; dobutamine

Introduction

Myocardial energetics and cardiac function are closely related because the heart is a highly anaerobic organ. Cardiac ischaemia produces cardiac dysfunction, however, positive inotropic intervention in heart failure originating from coronary heart disease or cardiac ischaemia is still controversial (Katz, 1986; 1988). An increase in contractile force requires a greater myocardial oxygen supply and many inotropic agents have chronotropic and vasodilator or vasoconstrictor actions as well as positive inotropic actions. Therefore, the usefulness of these drugs in the ischaemic heart is determined by the balance of these actions. In fact, deleterious effects of positive inotropic stimulation in ischaemic hearts have been reported (Rude *et al.*, 1980; Pozen *et al.*, 1981).

Catecholamines such as dobutamine and dopamine are the most popular positive inotropic agents in the clinical stage, but the usefulness of catecholamines is limited by their positive chronotropic and arrythmogenic actions. In the last two decades, many efforts have been made to develop new types of positive inotropic agents. Of these new positive inotropic agents (Taira, 1987), many are guanosine 3':5'-cyclic monophosphate (cyclic GMP)-inhibited phosphodiesterase (cGI-PDE) inhibitors such as amrinone (Alousi *et al.*, 1979). Recently, we described a new compound, OPC-18790 [(±)-6-[3-(3,4-dimethoxybenzylamino)-2-hydroxypropoxy]-2-(1*H*)-quinolinone] (Fujioka *et al.*, 1992) which is a positive inotropic agent with a moderate coronary vasodilator action, lacking a direct chronotropic action (Hosokawa *et al.*, 1992). The cardiovascular profile of OPC-18790 is different from that of the pure cGI-PDE inhibitor, amrinone (Sato *et al.*, 1986) and other cGI-PDE inhibitors (Taira, 1987). It has been shown that the mechanism of positive inotropic action of OPC-18790 involves prolongation of the duration of the action potential in ventricular myocytes as well as inhibition of cGI-PDE (Hosokawa *et al.*, 1992). Lack of direct chronotropic action and weaker vasodilator action compared to positive inotropic action are thought to be beneficial in inotropic therapy for the ischaemic heart.

Recent progress of ^{31}P magnetic resonance spectroscopy (^{31}P-MRS) enables simultaneous measurement of cardiac functions and cardiac energetics. In this study, we used the ^{31}P-MRS to evaluate the effects of OPC-18790 on energy metabolism during positive inotropic treatment in the guinea-pig isolated ischaemic heart in comparison with amrinone and dobutamine.

Methods

Isolated heart preparations

The experiment was conducted on guinea-pig isolated heart preparations. Male guinea-pigs (Hartley strain, SLC, Shizuoka, Japan) weighing 300–400 g were anaesthetized with pentobarbitone (50 mg kg^{-1}, i.p.). The chest was opened

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and the heart immediately isolated. A catheter was inserted retrogradely from the aorta, and the heart was perfused by Langendorff's method at a constant pressure of 80 cmH_2O. The perfusate used was modified Krebs-Henseleit solution (composition in mM: NaCl 119, $CaCl_2$ 2.5, KCl 4.8, $MgSO_4$ 1.2, KH_2PO_4 0.12, $NaHCO_3$ 24.9, glucose 10) bubbled with a gas mixture of 95% O_2 and 5% CO_2, and maintained at 37°C.

Magnetic resonance spectroscopy

The heart preparation was placed in a magnetic resonance probe (20 mm in diameter). ^{31}P-magnetic resonance spectra (^{31}P-MRS) were obtained using a magnetic resonance spectrometer Fx 200 (JEOL, Tokyo, Japan) operating at 80.76 MHz in the Fourier transform mode. For each spectrum 100 free induction decays (FIDs) were acquired with a 5 kHz spectral width. Repetition time was 3 s. Data point for the FID acquisition was 1024, and zero-filled to 4096 before Fourier transformation. Tissue levels of ATP, creatine phosphate (PCr) and inorganic phosphate (P_i) were estimated by integrating areas under individual peaks. The intracellular pH was estimated from the chemical shift (S) of P_i peak from PCr peak by the following equation postulated by Flaharty *et al.* (1982); pH = 6.90 – log [(S-5.85)/(3.29-S)]

Cardiac function

A Latex balloon was introduced into the left ventricle chamber from the left atrium appendage to measure left ventricular pressure (LVP). The first derivative (LV*dP/dt*) was determined from the LVP signals with electronic differentiator (Polygraph EQ-45764, Nihon Kohden, Tokyo, Japan) and used as an index of cardiac contractility. Heart rate was determined with a cardiotachometer (AF601G, Nihon Kohden) triggered by the LVP signals. Coronary perfusion flow (CPF) was determined by a volume of effluent measured every min. Recordings were made on charts with a thermal pen recorder (Recticorder, Nihon Kohden).

Production of cardiac ischaemia

After the haemodynamics of the heart preparation became stable, patchy ischaemia was produced by injecting microspheres (15 μm in diameter) into the coronary artery until the CPF decreased to 50% of the basal level (4–8 × 10^6 beads). After the haemodynamic changes due to ischaemia had stabilized, OPC-18790, amrinone or dobutamine was continuously infused into the coronary artery at an infusion rate of 1% of CPF for 15 min with an infusion pump. Each preparation was treated with one of the test compounds.

Cardiac pacing

Cardiac pacing was performed in another six preparations after patchy ischaemia was produced by injecting microspheres. A pacing electrode was placed on the right atrium and the hearts were electrically driven by a rectangular pulse wave (duration 3.0 ms and intensity 120% of threshold) using an electronic stimulator (SEN-3201, Nihon Kohden) to maintain a rate of 285 beats min^{-1}.

Drugs

The test compounds used were OPC-18790 (Lot. 2D94M, Otsuka Pharmaceutical Co. Ltd., Tokyo, Japan), amrinone (Lot. 921106, synthesized in Otsuka Pharmaceutical Co. Ltd.) and dobutamine HCl (Lot. 1001, Shionogi Phar-

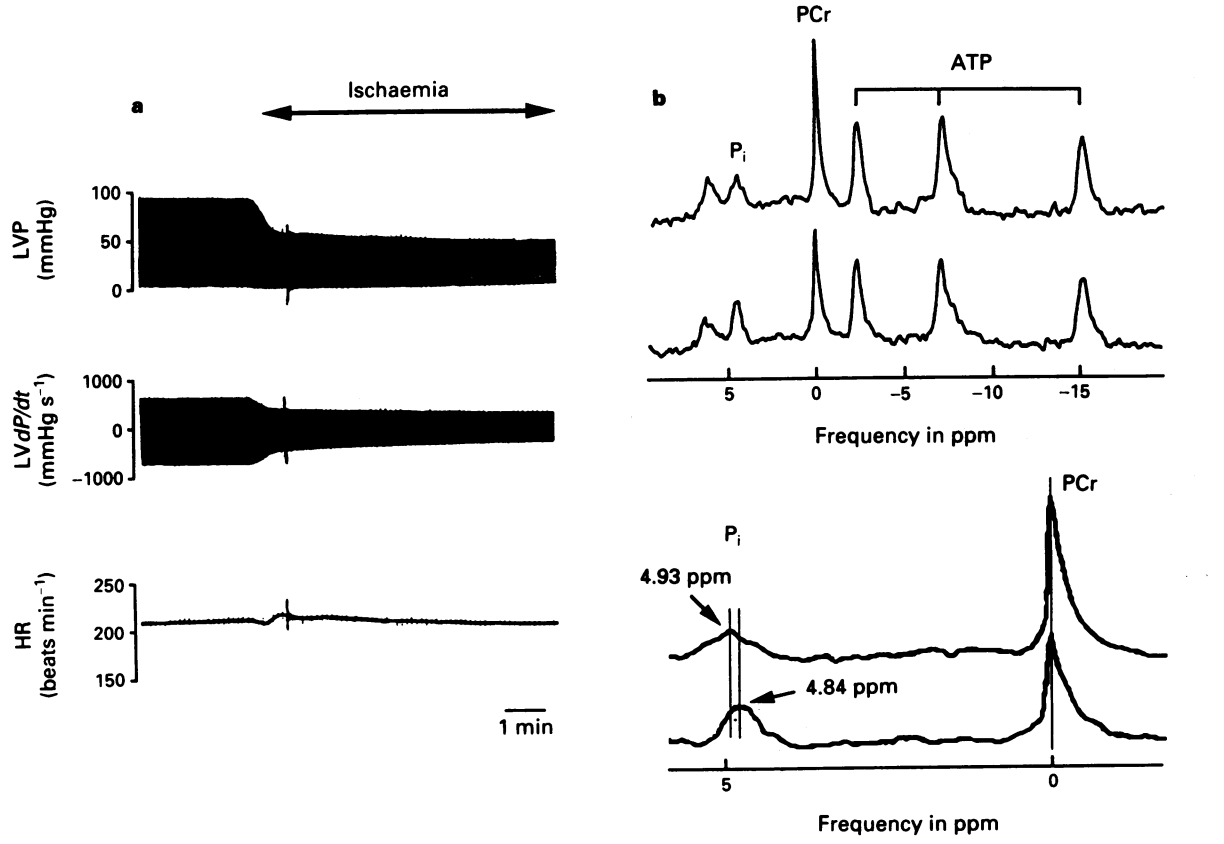

Figure 1 Representative traces of ischaemic changes in cardiac functions and energy metabolism in Langendorff preparations of guinea-pig heart. The traces show left ventricular pressure (LVP), the first derivative of the LVP (LV*dP/dt*) and heart rate (HR), and ^{31}P-magnetic resonance spectra illustrate the inorganic phosphate (P_i), creatine phosphate (PCr) and adenosine triphosphate (ATP). The horizontal bar represents 1 min.

maceutical Co., Osaka, Japan). OPC-18790, amrinone and dobutamine were dissolved in 1.5 mM lactate containing modified Krebs-Henseleit solution at a concentration of 1, 20 and 0.03 mM, respectively.

Statistics

Values are expressed as mean ± s.e.mean. The difference between mean values before and after ischaemia was analysed by Student's paired *t* test. Differences of drug effects or basal values of each groups after ischaemia were analysed by non-parametric 1 way ANOVA then multiple comparison with Tukey's method using SAS. When $P<0.05$, differences were considered significant.

Results

Energetic and functional changes produced by cardiac ischaemia

Coronary perfusion flow (CPF), left ventricular systolic pressure (LVSP), the first derivative of the left ventricular pressure (LV*dP/dt*), heart rate (HR), and the double product (DP) between the LVP and HR were measured after stabilization. Basal values in the normal heart were 13.7–14.8 ml min^{-1}, 88.7–94.5 mmHg, 1190–1330 mmHg s^{-1}, 217–242 beats min^{-1} and 19230–23030 mmHg beats min^{-1}, respectively ($n=24$). Injection of microspheres produced cardiac ischaemia with decreases in LVP, CPF, LV*dP/dt* and DP (typical traces are shown in Figure 1a). There were also decreases in the PCr and ATP signals and an increase in the P_i signal with the chemical shift moved upward, indicating intracellular acidosis (typical spectrograms are shown Figure 1b). Ischaemia caused LVP, CPF, LV*dP/dt*, HR and DP by 60.8%, 50.4%, 55.1%, 94.2% and 56.5% of their respective basal values ($n=24$, Figure 2a). In addition adenosine triphosphate (ATP) and creatine phosphate (PCr) decreased to 85.7% and 92.5% of their respective basal values whilst inorganic phosphate (P_i) increased to 123.6% (Figure 2b) in the ischaemic heart. The intracellular pH showed the development of acidosis (7.16 in the normal heart and 7.04 in the ischaemic heart).

Energetic and functional changes by positive inotropic intervention with OPC-18790, amrinone and dobutamine in the ischaemic hearts

After the induction of cardiac ischaemia, these 24 hearts were randomly divided into four groups, and OPC-18790, amrinone, dobutamine or vehicle injected into the perfusion circuit. As shown in Table 1 and Figure 2, basal values in the various groups prior to drug treatment were not statistically different from each other. Typical traces of effects on cardiac functions of OPC-18790, amrinone and dobutamine are shown in Figure 3. As shown in Figure 4, with almost equal increases in LV*dP/dt*, an index of cardiac contractility, OPC-18790, amrinone and dobutamine increased CPF by 13.6 ± 3.3%, 12.4 ± 1.2% and 10.6 ± 2.2%, respectively from the basal values after ischaemia (Figure 4a). The concentrations of OPC-18790, amrinone and dobutamine in the per-

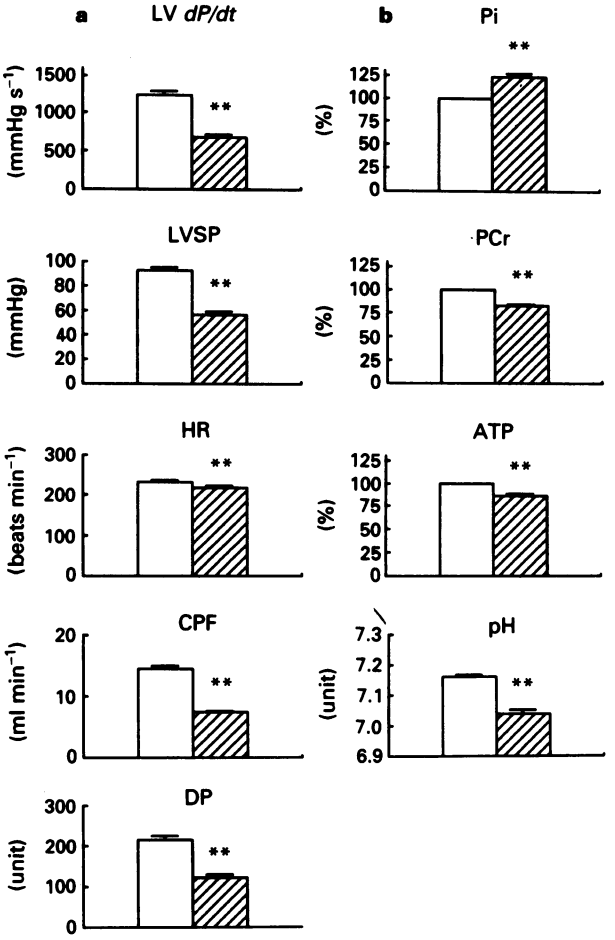

Figure 2 Ischaemic changes in cardiac functions and parameters of energy metabolism in Langendorff preparations of guinea-pig heart. The peak of the first derivative of the LVP (LV*dP/dt*), left ventricular systolic pressure (LVSP), heart rate (HR), coronary perfusion flow (CPF), double product (DP), inorganic phosphate (P_i), creatine phosphate (PCr) and adenosine triphosphate (ATP) and intracellular pH (pH) levels are shown before (open columns) and after (hatched columns) ischaemia induced by microsphere embolisation. Each column represents mean ± s.e.mean of 24 preparations. $*P<0.05$, $**P<0.01$ compared with values before iscahemia. Unit for DP is mmHg beats $min^{-1} \times 10^2$ and unit for pH is log unit.

Table 1 Basal values of cardiac function and energy metabolism in the Langendorff preparation of guinea-pig ischaemic hearts

		OPC-18790	*Vehicle*	*Dobutamine*	*Amrinone*
LVSP	(mmHg)	55.5 ± 7.3	57.8 ± 3.6	59.7 ± 5.4	52.0 ± 2.5
LV *dP/dt*	(mmHg s^{-1})	680 ± 130	680 ± 60	750 ± 100	630 ± 40
HR	(beats min^{-1})	218 ± 5.2	220 ± 8.9	225 ± 5.1	209 ± 14.9
CPF	(ml min^{-1})	7.50 ± 0.27	6.65 ± 0.37	7.60 ± 1.0	7.48 ± 0.50
DP	(unit)	120.3 ± 15.2	126.9 ± 8.6	133.4 ± 10.1	107.7 ± 7.3
P_i	(%)	25.4 ± 8.6	23.2 ± 6.4	17.8 ± 2.8	28.0 ± 6.1
PCr	(%)	−20.9 ± 3.5	−16.1 ± 3.8	−14.6 ± 0.9	−18.2 ± 2.6
ATP	(%)	−14.3 ± 5.6	−17.4 ± 3.8	−9.9 ± 3.1	−15.5 ± 1.4
pH	(unit)	7.00 ± 0.02	7.06 ± 0.03	7.02 ± 0.03	7.08 ± 0.02

The values shown are those prior to drug treatment. LVSP: left ventricular systolic pressure, LV*dP/dt*: peak of the first derivative of left ventricular pressure, HR: heart rate, CPF: coronary perfusion flow, DP: double product, P_i: inorganic phosphate, PCr: creatine phosphate, ATP: adenosine triphosphate, pH: intracellular pH. Unit for DP is mmHg beats $min^{-1} \times 10^2$ and unit for pH is log unit.

fusion fluid estimated from CPF were 14.4 ± 1.6 μM ($n = 6$), 180 ± 14 μM ($n = 6$) and 0.42 ± 0.04 μM ($n = 6$), respectively. The potency of positive inotropic action determined by LV*dP/dt* increase of dobutamine was about 34 times greater than that of OPC-18790 and that of amrinone was 12 times less potent than that of OPC-18790. At the same positive inotropic concentrations of these drugs, OPC-18790 did not exert any change in HR (−3.0 ± 5.9 beats min^{-1}) but amrinone and dobutamine increased HR by 41.5 ± 4.3 beats min^{-1} and 92.2 ± 8.3 beats min^{-1}, respectively. Therefore, OPC-18790, amrinone and dobutamine increased DP by 27.9 ± 6.7%, 46.4 ± 7.7% and 63.5 ± 7.3%, respectively.

When OPC-18790 increased LVSP, CPF and LV*dP*/dt, the compound did not change the levels of P_i, PCr and ATP and slightly reversed cardiac acidosis (Figure 4b). On the other hand, amrinone and dobutamine increased P_i and decreased PCr and ATP. Intracellular pH was lowered further by amrinone and dobutamine treatment (Figure 4b). The degree of aggravation of high energy phosphate metabolism and acidosis by amrinone and dobutamine seemed to correlate with HR increase.

Energetic and functional changes by cardiac pacing in the ischaemic hearts

To clarify the relationship between changes in energetic parameters and heart rate increase, cardiac pacing at 285 beats min^{-1} was performed in the other ischaemic heart preparations. The rate of 285 beats min^{-1} is about 50 beats min^{-1} higher than the baseline rate. Typical traces of LVSP, LV*dP/dt* and HR are shown in Figure 5. The cardiac pacing produced decreases in LVSP, LV*dP*/dt, DP and CPF of about 30%, 20%, 20%, 5%, respectively (Figures 5 and 6). Although these parameters of cardiac function were decreased, at that time parameters of cardiac energetics were aggravated with increases in P_i and decreases in PCr, ATP and intracellular pH (Figures 5 and 6).

Discussion

In these experiments, we used the cardiac ischaemic model induced by 15 μm microsphere embolisation. As reported previously (Hosokawa *et al.*, 1986), this model is characterized by milder ischaemia than that induced by global ischaemia. Changes in high energy phosphate compounds, inorganic phosphate and changes in cardiac function were smaller in microsphere-induced ischaemia than after global ischaemia. As the aim of these experiments was to compare the effects of several positive inotropic agents on cardiac function and energetics simultaneously in ischaemic hearts, we chose the mild ischaemic model and evaluated effects by the ^{31}P-MRS method.

When OPC-18790, amrinone and dobutamine produced almost the same increase in contractility (LV*dP/dt* by about 60%), amrinone and dobutamine increased HR while OPC-18790 did not (Figure 4). Increases in HR induced by amrinone (Alousi *et al.*, 1979; Zannad *et al.*, 1983; Sato *et al.*, 1986) and dobutamine (Tuttle & Mills, 1975; Sonnenblick *et al.*, 1979) have been reported previously and lack of direct chronotropic effect of OPC-18790 is also consistent with a previously reported action in canine isolated right atria (Hosokawa *et al.*, 1992). From the parameters of cardiac energetics elucidated by ^{31}P-MRS signals, it is shown that amrinone and dobutamine aggravate the cardiac energetic state, namely increase P_i, decrease PCr and ATP and cause further acidification of intracellular pH, with an increase in LV*dP/dt* (Figure 4). In contrast to amrinone and dobutamine, OPC-18790 did not change those parameters of energy metabolism when producing the same increase in LV*dP/dt* (Figure 4). These results suggest that the increase in HR and/or DP produced by amrinone and dobutamine are correlated with the changes in cardiac energetics (Figure 4). Therefore, we examined the influences of cardiac pacing at 285 beats min^{-1} on the cardiac function and energetics in the ischaemic hearts. The increase in HR by cardiac pacing

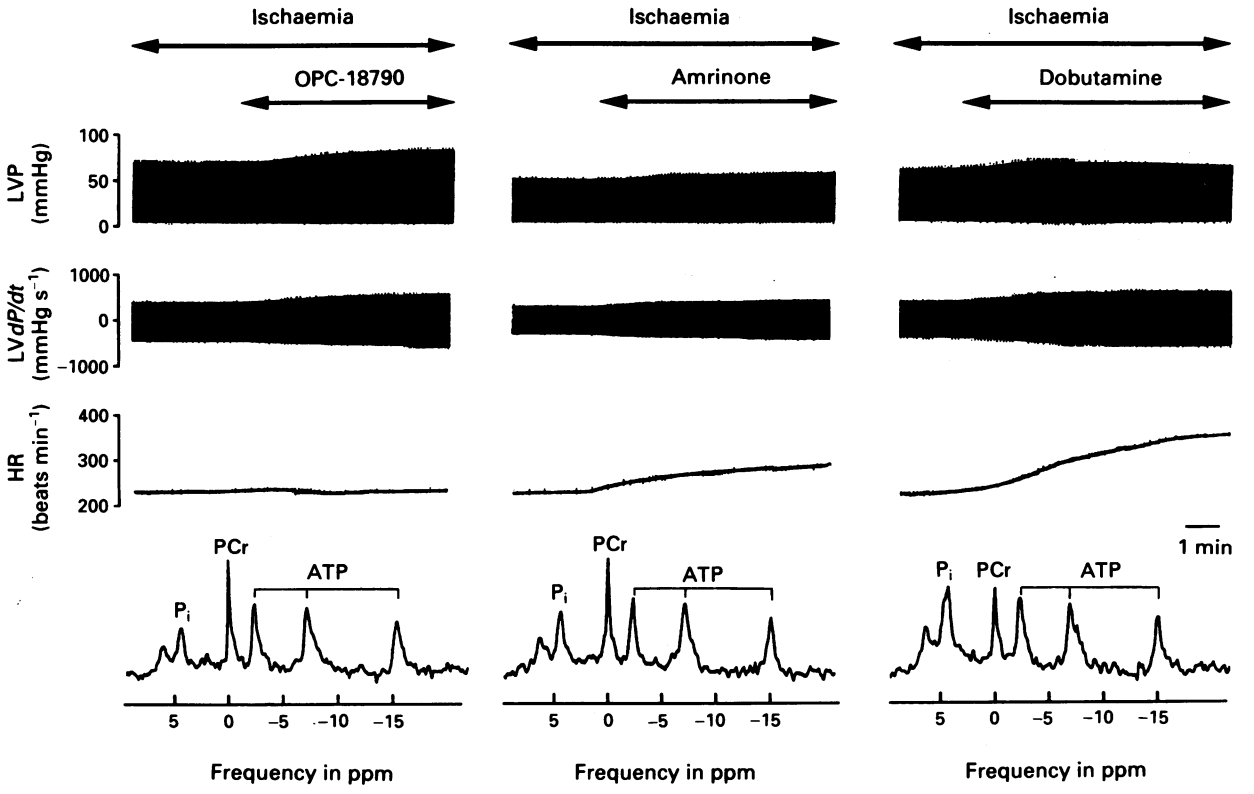

Figure 3 Representative traces of the effects of OPC-18790, amrinone, and dobutamine on cardiac functions and energy metabolism in Langendorff preparations of guinea-pig heart with ischaemia. The traces show the left ventricular pressure (LVP), the first derivative of the LVP (LV*dP/dt*) and heart rate (HR), and ^{31}P-magnetic resonance spectra illustrate the inorganic phosphate (P_i), creatine phosphate (PCr) and adenosine triphosphate (ATP). The horizontal bar represents 1 min.

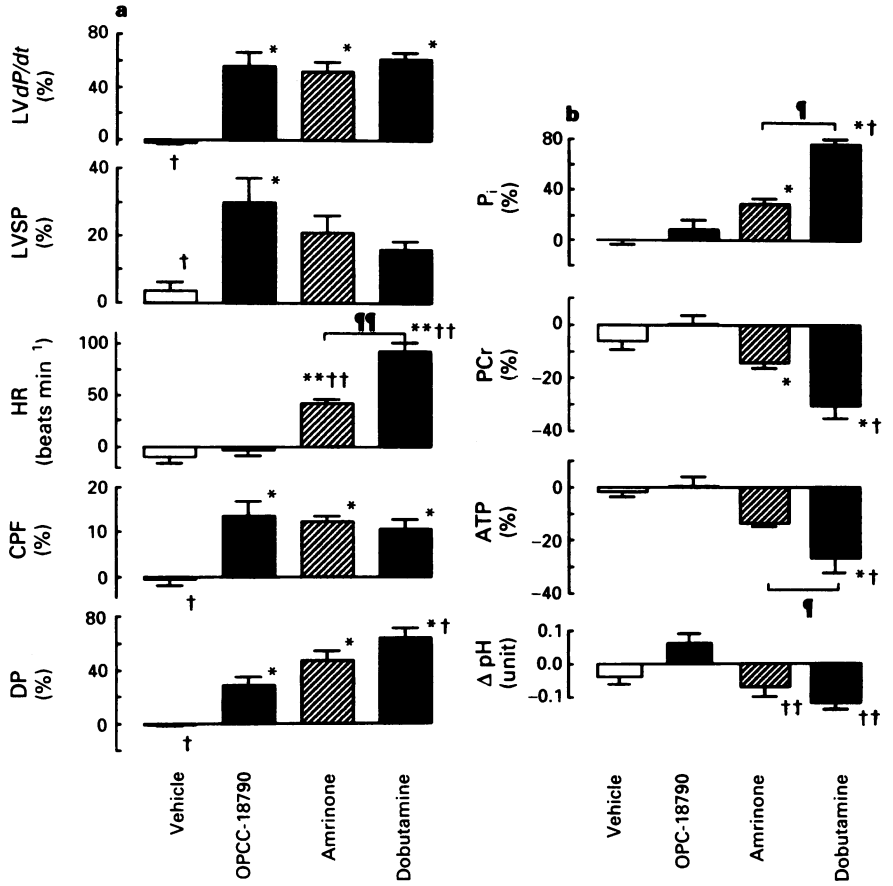

Figure 4 Effects of OPC-18790, amrinone, dobutamine and their vehicle on cardiac functions and energy metabolism in Langendorff preparations of guinea-pig heart with ischaemia. Changes in the peak of the first derivative of LVP (LV*dP/dt*), left ventricular systolic pressure (LVSP), heart rate (HR), coronary perfusion flow (CPF), double product (DP), inorganic phosphate (P_i), creatine phosphate (PCr), adenosine triphosphate (ATP) and intracellular pH (pH) from base line after ischaemia are shown. Each column represents mean ± s.e.mean of 6 preparations. $*P<0.05$, $**P<0.01$ compared with values before drug treatment. †$P<0.05$, ††$P<0.01$ compared with response of OPC-18790. ¶$P<0.05$, ¶¶$P<0.01$.

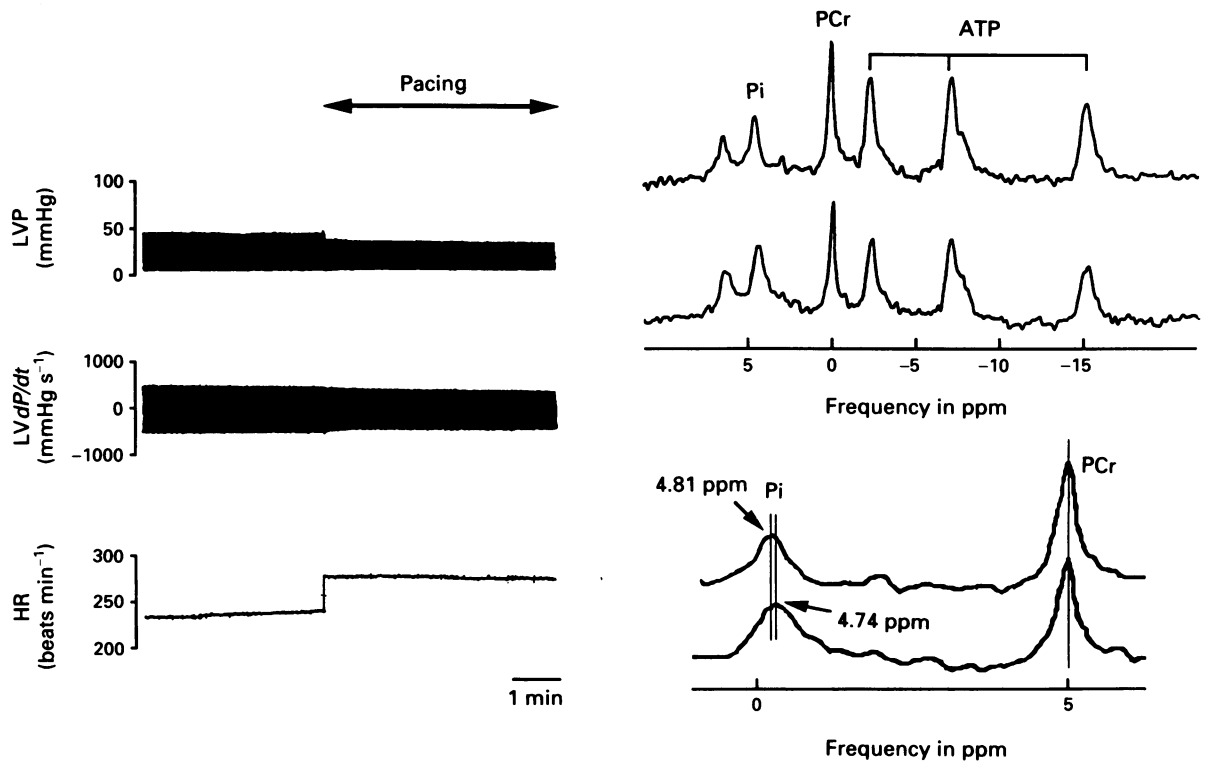

Figure 5 Representative traces of the effects of cardiac pacing at 285 beats min^{-1} on cardiac functions and energy metabolism in Langendorff preparations of guinea-pig heart with ischaemia. The traces show the left ventricular pressure (LVP), the first derivative of the LVP (LV*dP/dt*) and heart rate (HR), and ^{31}P-magnetic resonance spectra illustrate the inorganic phosphate (P_i), creatine phosphate (PCr) and adenosine triphosphate (ATP). The horizontal bar represents 1 min.

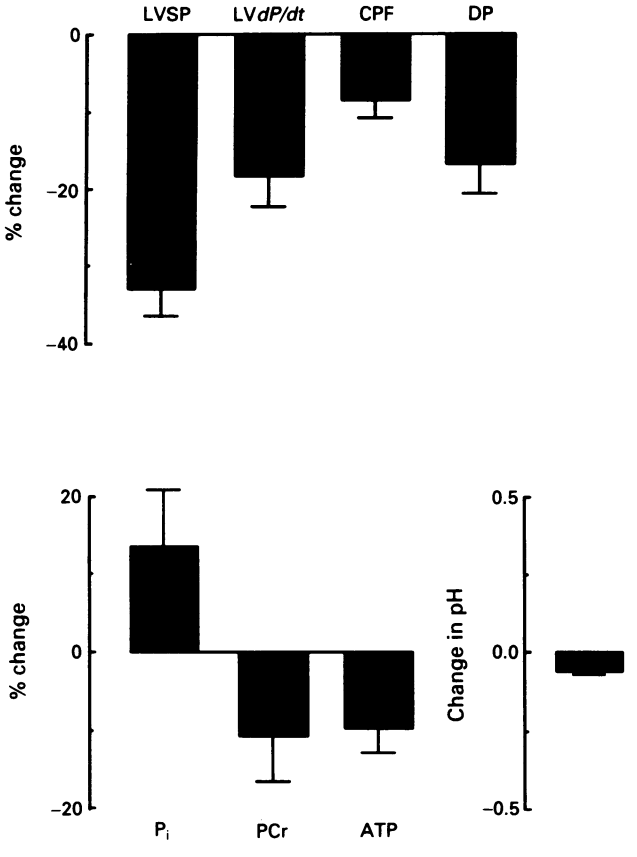

Figure 6 Effects of cardiac pacing at 285 beats min^{-1} on cardiac functions and energy metabolism in Langendorff preparations of guinea-pig heart with ischaemia. Changes in the left ventricular systolic pressure (LVSP), the peak of the first derivative of the left ventricular pressure (LV*dP/dt*), coronary perfusion flow (CPF), double product (DP), inorganic phosphate (P_i), creatine phosphate (PCr), adenosine triphosphate (ATP) and intracellular pH (pH) after rapid atrial pacing are shown. Each column represents mean ± s.e.mean of 6 preparations.

aggravates cardiac energetics and decreases cardiac functions such as LVSP, LV*dP/dt* and DP (Figure 5). From these results, energetic aggravation seems to correlate closely with an increase in HR rather than DP in the ischaemic heart model.

Furthermore, OPC-18790 (Hosokawa *et al.*, 1992) and amrinone (Sato *et al.*, 1986) have direct coronary vasodilator actions, but dobutamine has a vasoconstrictor action via an α-adrenoceptor partial agonist effect (Vatner *et al.*, 1974; Kenakin, 1981; Ruffolo *et al.*, 1981) as well as a β-adrenoceptor agonist action. Dobutamine produced a weaker increase in CPF in proportion to a large increase in DP and this also contributed to the aggravation of cardiac energetics by dobutamine (Figure 4). It is reported that amrinone has cGI-PDE inhibiting action (Endoh *et al.*, 1982; Honerjäger, 1981); the inhibition of cGI-PDE may explain positive inotropic, positive chronotropic and vasodilator actions of amrinone. It has also been demonstrated that OPC-18790 has a cGI-PDE inhibiting action (Hosokawa *et al.*, 1992) and an increase in intracellular cyclic AMP level may be involved in the inotropic effect of OPC-18790 (Endoh *et al.*, 1994). In contrast to amrinone, the positive inotropic action of OPC-18790 was exerted rather than the vasodilator action and it also has no chronotropic effect (Figure 4). Another mechanism other than inhibition of cGI-PDE, for example, potassium channel inhibition (Takase *et al.*, 1994) may be involved in the expression of the whole haemodynamic action of OPC-18790.

One of the explanations of the finding that OPC-18790 improved the cardiac function without changes in cardiac energetics is the well-balanced cardiovascular profile of OPC-18790, i.e., positive inotropic action with little chronotropic action and moderate coronary vasodilator action. Recently, we also showed that OPC-18790 improved cardiac function without changing cardiac energetics in a ^{31}P-MRS study using open-chest dog with cardiac ischaemia (Ishikawa *et al.*, unpublished data).

In conclusion, effects of positive inotropic agents on cardiac energetics are different from each other and energetic aggravation seems to correlate closely to HR increase in the ischaemic heart. A positive inotropic agent without direct chronotropic action may be beneficial in ischaemic hearts.

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Effect of the NMDA-antagonist, MK 801, on benzodiazepine-opioid interactions at the spinal and supraspinal level in rats

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1 Benzodiazepines potentiate morphine antinociception at the spinal level via GABAergic mechanisms. At the supraspinal level, the inhibitory effect of midazolam on morphine antinociception cannot be easily explained by $GABA_A$ receptor activation. Since excitatory amino acids play a role in central transmission, we investigated the effect of dizocilpine (MK 801) on this interaction in spinal cord and brain.

2 In rats with an intrathecal or intracerebroventricular catheter, the mecahnisms of the antinociceptive effect of benzodiazepine-morphine combinations were tested during thermal nociceptive tests.

3 The principal findings of this study were that at the spinal level, midazolam potentiation of morphine antinociception can be antagonized by the NMDA antagonist, MK 801 (10 μg), as assessed by hot-plate and tail-flick tests. When drugs were administered supraspinally, midazolam inhibited morphine antinociception only in the hot-plate test, an effect also inhibited by MK 801. In the tail-flick assay, midazolam failed to influence the morphine response.

4 The NMDA antagonist significantly affected midazolam antinociception at the spinal level, but was not effective following i.c.v. administration of the drugs. MK 801 had no effect on morphine antinociception after i.t. and i.c.v. administration of the drugs.

5 The paradoxical effect of midazolam on morphine antinociception and its reversal by MK 801 might be due to modulation at various levels of the neuraxis and/or modulation of different pathways mediated via both $GABA_A$ and NMDA receptor mechanisms.

Keywords: Benzodiazepines; opioids; NMDA antagonists; dizocilpine (MK 801); nociceptive tests; intrathecal; intracerebroventricular

Introduction

Many neurotransmitters are involved in the modulation of incoming pain information. Opioid drugs can produce analgesia at supraspinal and spinal sites by inhibiting nociceptive input (Yaksh, 1984). These two sites of opioid actions may be influenced either directly or indirectly by non-opioids in an additive or antagonistic manner (Luger *et al.*, 1992; 1993). A growing body of evidence suggests there are mechanisms whereby interactions between two major neurotransmitter systems, excitatory amino acids (EAAs) γ-aminobutyric acid (GABA), can significantly modulate signal transmission in the brain (Watkins & Evans, 1981; Wilcox, 1991).

Several lines of investigation have indicated that in laboratory animals, benzodiazepine agonists injected intrathecally (i.t.) potentiate opioid antinociception in the spinal cord (Yanez *et al.*, 1990; Luger *et al.*, 1993; 1994), while other studies indicate that benzodiazepines given intracerebroventricularly (i.c.v.) reduce the antinociceptive effects of systemic opioids (Mantegazza *et al.*, 1979; 1982; Luger *et al.*, 1993; 1994). GABA is well established as the main inhibitory neurotransmitter found at synapses throughout the brain and spinal cord (Goodchild, 1993). Benzodiazepine agonists exert their effects by potentiation of GABA at a specific receptor site on a benzodiazepine $GABA_A$-ionophore complex in various parts of the CNS (Haefely *et al.*, 1993). Since the inhibitory effect of midazolam on morphine antinociception at the supraspinal level (Mantegazza *et al.*, 1982) cannot be sufficiently explained by $GABA_A$ergic mechanisms (Lorenz *et al.*, 1993; Luger *et al.*, 1994), non-GABA receptors, like N-methyl-D-aspartate (NMDA) receptors, might be involved in this interaction in the brain. EAAs are accepted as the main neurotransmitters mediating synaptic excitation in the CNS (Watkins & Evans, 1981; Wilcox, 1991). The role of EAAs in central nociceptive transmission is based first on neurochemical studies. These show that EAAs such as glutamate are present in the superficial dorsal horn C fibre central terminals, in coexistence with substance P containing C fibres (Greenmayre *et al.*, 1984). Secondly, electrophysiological studies have shown glutamate to activate superficial dorsal horn neurones in the spinal cord (Schneider & Perl, 1988). NMDA has been implicated in the phenomenon called 'wind up', which is thought to be responsible for central hyperalgesia, and which can be abolished by NMDA antagonists (Goodchild, 1993). Thirdly, behavioural studies investigating the spinal administration of NMDA or NMDA agonists show hyperalgesia in the hot-plate and tail-flick assay in mice and rats (Aanonsen & Wilcox, 1987; Raigorodsky & Ucra, 1987; Malmberg & Yaksh, 1993). However, this hyperalgesic effect of NMDA-agonists can be reversed by selective NMDA antagonists (Cahusac *et al.*, 1984; Raigorodsky & Ucra, 1987) and antagonized by muscimol (Aanonsen & Wilcox, 1989). Intrathecal NMDA antagonists at doses below those that resulted in motor dysfunction produced no alterations in the acute response to thermal nociceptive stimuli (Cahusac *et al.*, 1984; Aanonsen & Wilcox, 1987; Yaksh, 1989; Aanonsen *et al.*, 1990). In contrast, they abolish the 'wind up' phenomenon (Yaksh, 1989; Goodchild, 1993). They also inhibit the response to noxious stimuli in dorsal horn neurones in electrophysiological studies (Haley *et al.*, 1990; Woolf & Thompson, 1991). Synaptic excitation can also be antagonized by NMDA antagonists in other regions of the CNS, e.g. in the

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cerebral cortex, cuneate nucleus or hippocampus (Watkins & Evans, 1981). Besides these direct effects of EAAs on nociception, NMDA agonists and antagonists may alter opioid antinociception. NMDA antagonists injected into the PAG antagonized morphine and NMDA antinociception, indicating that morphine produces analgesia in the PAG by disinhibition of neurones that contain NMDA receptors (Jacquet, 1988). Morphine antinociception, but not NMDA-induced antinociception, was antagonized by an NMDA antagonist administered in the nucleus raphe magnus (van Praag & Frenk, 1990). In the majority of reports investigating the acute effects of the noncompetitive NMDA antagonist, MK 801, on morphine antinociception, no effect was observed when it was administered i.t. and i.c.v. (Marek *et al.*, 1991a,b; Trujillo & Akil, 1991b; Yamamoto & Yaksh, 1992). The data of Lipa & Kavaliers (1990) indicate, however, that intraperitoneal administration of MK 801 reduces morphine-induced antinociceptive response in male mice. Additionally, diazepam probably exerts some effect on amino acid-induced responses in spinal motorneurones of the frog (Evans *et al.*, 1977).

Benzodiazepines potentiate morphine antinociception at the spinal level via GABAergic mechanisms. At the supraspinal level, the inhibitory effect of midazolam on morphine antinociception cannot be easily explained by $GABA_A$ receptor activation (Lorenz *et al.*, 1993; Luger *et al.*, 1993; 1994). Since excitatory amino acids may play a role in central transmission and in the light of the above-mentioned facts, our study aimed to investigate the effect of MK 801 on midazolam-morphine interaction at both spinal cord and supraspinal level.

Methods

Animals

For these experiments we used male Sprague-Dawley rats (250–320 g; 5–8 animals/dose). All animals were housed in individual cages on a 12 h-light/12 h-dark cycle at a constant temperature of 23° ± 1°C with free access to food and water. All studies were performed during the light cycle. The study was approved by the 'Kommission für Tierversuchsangelegenheiten' (Tierschutzgesetz BGBI. Nr.501/1989: GZ 66.009/253-I/A/2/92) of the Federal Ministry of Science and Research, Vienna, Austria.

Intrathecal and intracerebroventricular catheter implantation

Intrathecal and intracerebroventricular catheters were implanted under halothane anaesthesia and aseptic conditions with the rats placed in a stereotaxic frame with the head flexed forward. Separate groups of rats received different catheter implantations. An intrathecal catheter (polyethylene tube PE – 10; length 8.5 cm) was inserted through a slit in the cisternal membrane and passed rostrally to the thoracolumbar level. The external part of the catheter was then tunnelled subcutaneously to exit on top of the skull (see Yaksh & Rudy, 1976). For implantation of an intracerebroventricular catheter (polyethylene tube PE – 10; length 4.5 cm), the rat was placed in a stereotaxic frame and a hole trephined in the skull at coordinates overlaying the left ventral ventricle; 1 mm from the bregma and 1 mm from the midline (as described by Paxinos & Watson, 1986). The catheter was inserted 3 mm into the lateral ventricle, fixed by attachment to a stainless steel screw placed in the skull bones, and then tunnelled subcutaneously to exit on top of the skull. Animals with normal motor function and behaviour post surgery were used 4–7 days later for the experiment. Each animal was used only once.

Drug administration

In subseries 1, we compared the effects of saline, midazolam in doses not effective in nociceptive assessments (i.t.: 2.5 μg, i.c.v.: 4 μg), morphine (i.t.: 1 μg, i.c.v.: 4 μg), midazolam and morphine in combination (as fixed doses: 2.5 μg midazolam and 1 μg morphine i.t.; 4 μg midazolam and 4 μg morphine i.c.v.) and the effect of MK 801 (i.t. 10 μg, i.c.v.: 10 μg) on all combinations. All doses of morphine sulphate (Mallinckrodt), midazolam (Hoffman – La Roche) and MK 801 (Dizocilipine, Research Biochemicals Incorporated, RBI), were calculated as the free base. Drugs for i.t. administration were dissolved in 0.9% sodium chloride, so that 10 μl contained the desired dose of the agents. After each injection, the catheter was flushed by an i.t. injection of 10 μl 0.9% sodium chloride. Drugs for i.c.v. administration were dissolved in 0.9% sodium chloride to a volume of 5 μl and injected into the ventricle, followed by 5 μl of 0.9% sodium chloride to flush the catheter. All drugs were administered over a period of 20 s with a microinjector. The NMDA antagonist, (+) MK 801 [(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohep-ten-5,10-imine hydrogenmaleate] was dissolved in 0.9% sodium chloride to provide doses of 10 μg 10 μl^{-1}, i.t. and 10 μg 5 μl^{-1}, i.c.v.

For subseries 2, saline or a fixed dose of MK 801 (i.t.: 10 μg, i.c.v.: 10 μg) were administered with various doses of midazolam and morphine for dose-response curves.

Behavioural tests

Time course The day before each study, animals were placed on an unheated hot-plate for 1–2 min for adaptation. Baseline assessments, including nociceptive and motor function tests, as well as tail skin temperature, were performed on the morning of the testing day. The NMDA antagonist or saline was administerd 5 min before the agonists or saline (0 time) and each of the antinociceptive and motor function tests, as well as the tail skin temperature measurements, were repeated at 5, 15, 30, 60, 90 and 120 min. All tests were performed by one of the investigators, who was unaware of the drug or dosage administered.

Nociceptive tests For the hot-plate test, the animals were placed on a metal plate maintained at 52.5° ± 0.5°C within a plexiglass enclosure. The behavioural endpoint was defined as the time at which the animal jumped off the plate or licked a hindpaw. The cutoff time to prevent tissue damage was 60 s. There was one trial per test per time interval. For the tail-flick test, each animal was placed on a rectangular metal plate under an illuminated lamp (100W), and the light beam focussed on the ventral surface at 1.5 and 3 cm from the tip of the tail. The endpoint was defined as the time when the rat flicked its tail out of the beam. If an animal failed to respond, the test was terminated at 6 s to avoid tissue damage. Two trials were conducted per test per time interval. Tail-skin temperature was measured concurrently on the ventral surface at the proximal part of the tail and recorded immediately after the tail-flick occurred. Changes in tail skin temperature were ruled out as a possible confounding factor in the tail-flick test.

Motor function All rats were observed and scored for motor dysfunction (behavioral index: 0 = no change; 1 = complete loss of hindlimb function). To evaluate catalepsy, animals were placed with their front paws on a step (height 9 cm) and the time measured until the rat either climbed to the top with both hindpaws or removed both front paws from the step. Animals showing neither response within 30 s were scored maximum positive for catalepsy. Additional side effects, like spontaneous agitation and vocalization, were observed and noted.

Data analyses

Hot-plate, tail-flick and catalepsy response latencies are expressed as the area under the time-effect curve (AUC) from zero time to 120 min using the trapezoidal rule, and also as a percentage of the maximum possible effect (%MPE) calculated as follows:

$$\%MPE = \frac{(\text{postdrug time} - \text{predrug time})}{(\text{cutoff time} - \text{predrug time})} \times 100$$

The time-effect courses were analysed by analysis of variance (ANOVA) for repeated measures with the Bonferroni correction (MDPD statistical manual, procedure V4). To compare the maximum antinociceptive effect (peak effect %MPE) and area under the curve (AUC) for the various groups, factorial analysis of variance (ANOVA) was used. In order to construct dose-response curves for the effect of MK 801 on the saline/midazolam and saline/morphine curves, MK 801 dose was plotted against peak %MPE and analysed by least square linear regression analysis. From the curves, values were calculated for ED_{50} (95% confidence intervals [CI]) and slope values (95% CI) when testing for parallelism. Statistical significance was accepted at $P \leqslant 0.05$.

Results

In dose-ranging studies for MK 801 given both i.t. ($n = 4-7$/dose) and i.c.v. ($n = 4$/dose), the highest doses of the NMDA antagonist used for these experiments were those which did not produce catalepsy, and did not show behavioural effects, such as spontaneous agitation and/or vocalization, in more than 15% of the animals (MK 801 = 10 μg i.t. and 10 μg i.c.v., respectively) (data not shown, but described below). All other drugs administered i.t. and i.c.v. had no effect on the behavioural index except those short-lasting episodes of agitation and vocalization.

Intrathecal administration

Figure 1 shows the time course of the antinociceptive effects of i.t. administration of saline/morphine, saline/midazolam and morphine in combination and the EAA antagonist MK 801 (10 μg) injected prior to midazolam plus morphine (%MPE plotted against time). The mean baseline hot-plate (HP) and tail-flick (TF) latency for all animals measured before intrathecal injection was in the normal range and did not differ for the various groups (HP: 8.5 ± 2.2 s; TF 2.82 ± 0.44 s). Combined administration of saline/midazolam 2.5 μg and morphine 1 μg, i.t., significantly increased response latencies within 15 min in both nociceptive tests as compared to saline/morphine 1 μg. Preadministration of 10 μg MK 801 i.t. significantly reduced this potentiating antinociceptive effect of the midazolam-morphine interaction

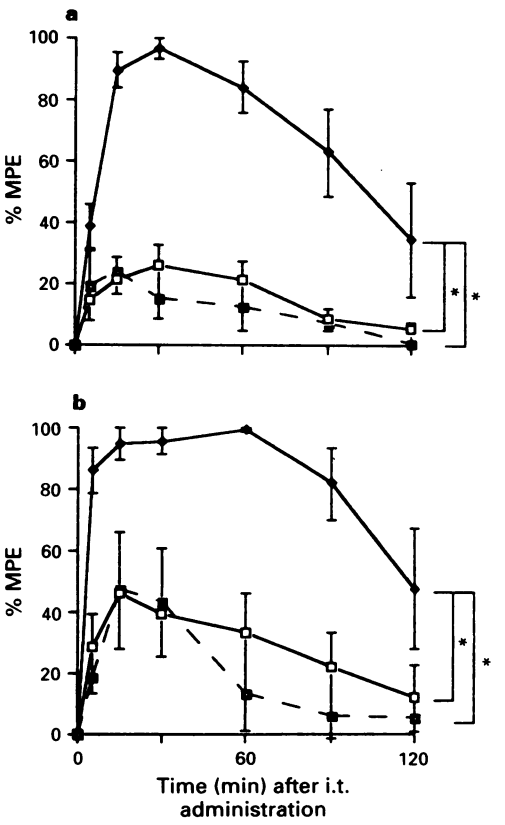

Figure 1 The antinociceptive effect, percentage maximum possible effect (%MPE), in (a) the hot plate (HP) and (b) tail flick test (TF), at different times after intrathecal administration of saline/morphine 1 μg (□), saline/midazolam 2.5 μg + morphine 1 μg in combination (◆) and MK 801 10 μg/midazolam + morphine (■). Mean ± s.e.mean, ANOVA: drug effects: (HP) $P = 0.001$, (TF) $P = 0.0009$; time effect: (HP) $P = 0.0001$, (TF) $P = 0.0009$, $*P < 0.05$.

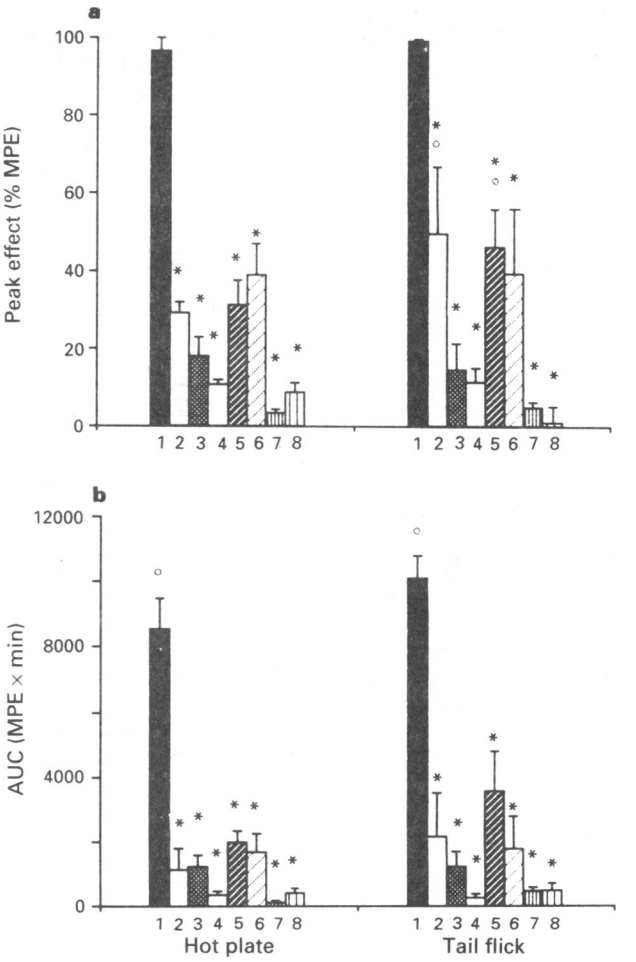

Figure 2 Peak effect (peak %MPE) and area under the time-effect curve (MPE × min) of agonists given i.t. alone and in combination with MK 801 (10 μg), showing that the potentiation and prolongation of the midazolam (2.5 μg) morphine (1 μg) interaction and the effect of saline/midazolam (2.5 μg) is significantly reversed by the antagonist MK 801. Saline/saline and MK 801/saline were ineffective. Saline/midazolam + morphine (1), MK801/midazolom + morphine (2), saline/midazolam (3), MK 801/midazolam (4), saline/morphine 1 μg (5), MK 801/morphine (6), saline/saline (7), MK 801/saline (8). Mean ± s.e.mean, ANOVA: peak %MPE (HP) $P = 0.0001$, (TF) $P = 0.0001$; AUC: (HP) $P = 0.0001$, (TF) $P = 0.0001$, $*P < 0.05$ compared to saline/midazolam + morphine; $°P < 0.05$ compared to saline/midazolam.

to near saline/morphine values over the observation period (ANOVA for repeated measures: drug-effect: (HP) $P = 0.0001$, (TF) $P = 0.0009$; time-effect: (HP) $P = 0.0001$, (TF) $P = 0.0009$; Figure 1). Maximum antinociceptive effects in tail-flick and hot-plate tests (peak %MPE) independent of the time of peak effect and AUC for both tests and for each drug treatment also demonstrate that i.t. coadministration of a minimally effective (effect in less than 15%MPE) dose of midazolam (2.5 μg) with 1 μg morphine potentiated the opoid effects in nociceptive tests (Figure 2). Spinal injection of MK 801 (10 μg) before the respective agonists significantly blocked potentiation by midazolam and prolongation of morphine antinociception in tail-flick and hot-plate tests (ANOVA: peak %MPE (HP) $P = 0.0001$, (TF) $P = 0.0001$; AUC: (HP) $P = 0.0001$, (TF) $P = 0.0001$; Figure 2). This reversal occurred at doses of MK 801 which did not alter response latency when injected alone. It should be noted that the nociceptive tests employed were not designed and not able to detect any significant reduction in latency (e.g. hyperalgesic effect). A lack of effect of MK 801 administered alone was reported previously (Trujillo & Akil, 1991a,b; Malmberg & Yaksh, 1992). Results of subseries 2 are shown in Figure 3. The dose-response curve for peak effect (peak %MPE) of MK 801/midazolam shifted to the right of the saline/midazolam curve in nociceptive tests. The area under the curve (AUC) was decreased by MK 801 as compared to saline/midazolam (Figure 3). There was no shift in the MK 801/morphine dose-response curve in comparison to the saline/morphine curve, showing that MK 801 had no influence on morphine antinociception. It should be noted that in 14.2% of the animals, injection of 10 μg MK 801 caused spontaneous agitation and vocalization for 3 to 5 min. These short-lasting behavioural changes ended before the administration of the agonists and did not influence the catalepsy tests. Single injections of 10 μg MK 801/saline and saline/saline did not produce antinociception (Figure 2).

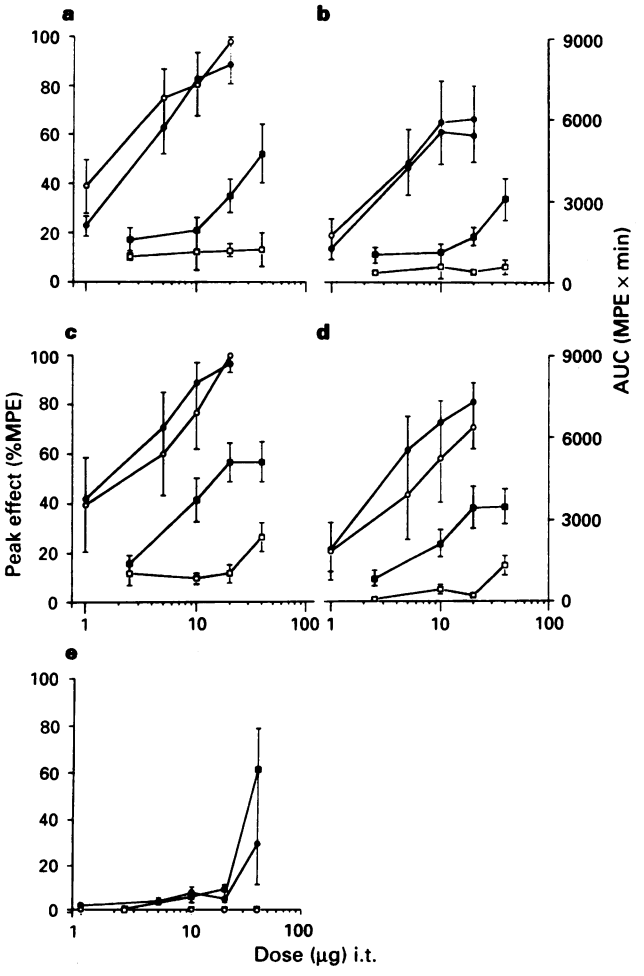

Figure 3 Dose-response curves (peak %MPE) and AUC (MPE × min) for antinociceptive effects in hot plate (a,b) and tail flick (c,d) tests and the incidence of catalepsy (e) after i.t. administration of saline/morphine (●), saline/midazolam (■), MK 801/morphine (○) and MK 801/midazolam (□) demonstrating a right shift of the saline/midazolam curve by MK 801 without affecting saline/morphine.

Intracerebroventricular administration

The time course of antinociception following injection of midazolam and morphine in combination and the influence of the NMDA antagonist MK 801 on effects of the agonist combination are presented in Figure 4. Baseline measurements for the hot-plate and tail-flick test were in normal range for all animals, and there was no between-groups difference (HP: 7.9 ± 3.5 s; TF 2.84 ± 0.46 s). Over the observation period, the administration of saline/morphine (4 μg) resulted in an increase in %MPE, that was significantly reduced by coadministration of midazolam (4 μg) in the hot-plate assay only. This inhibitory effect of midazolam on morphine antinociception was abolished by pretreatment with MK 801 at the dose given (ANOVA for repeated measures: (HP) $P = 0.0044$, (TF) $P = 0.0029$; time-effect: (HP) $P = 0.0001$, (TF) $P = 0.0001$; Figure 4). The maximum antinociceptive effect (peak %MPE), AUCs of the midazolam-morphine treatment and the influence of MK 801 are shown in Figure 5. MK 801 (10 μg), administered prior to the agonists, significantly abolished the inhibition produced by saline/midazolam + morphine at the supraspinal level in the hot-plate test only. In the tail-flick assay, MK 801 was

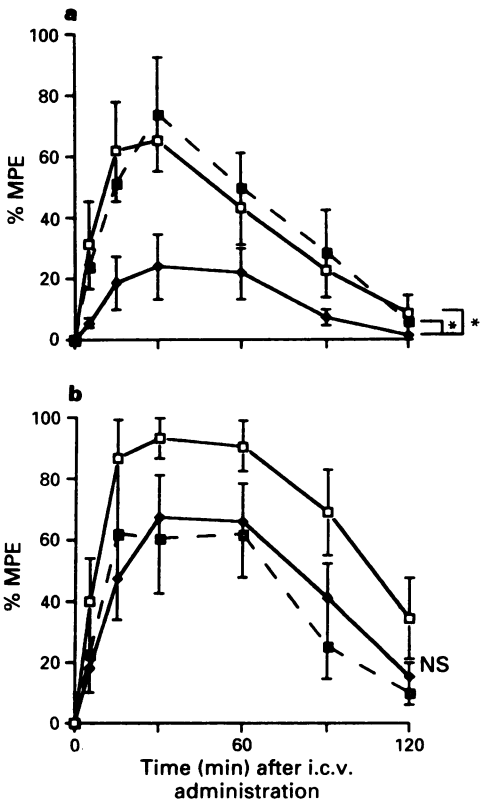

Figure 4 The antinociceptive effect (%MPE) on (a) hot plate and (b) tail flick tests across time after i.c.v. administration of saline/morphine 4 μg (□), saline/morphine 4 μg + midazolam 4 μg (◆), MK 801 (10 μg)/midazolam + morphine (■) in rats. Mean ± s.e.mean, ANOVA: drugs effect: (HP) $P = 0.044$, (TF) $P = 0.0029$; time effect: (HP) $P = 0.0001$, (TF) $P = 0.0001$, $*P < 0.05$. NS, not significant.

not effective at the dose used in altering the midazolam-morphine interaction (Figures 4 and 5). In subseries 2, the dose-response curve for peak %MPE and AUC of saline/midazolam (1–4 μg) was not affected by MK 801 (Figure 6). The dose-response curve for saline/morphine (1–4 μg) was not shifted by MK 801 10 μg (Figure 6), indicating that MK 801 at the dose administered does not influence morphine antinociception in the brain. Injection of 10 μg MK 801 alone was without effect when compared to saline controls in both antinociceptive tests. Rats given the maximum dose of morphine i.c.v. and midazolam i.c.v. in combination with MK 801 showed no motor dysfunction, although 6.25% of the rats produced short-lasting spontaneous agitation and vocalization over 3 to 5 min after i.c.v. administration of the NMDA antagonist. This did not affect the catalepsy test results.

Discussion

The principal findings of this study show that (1) the (potentiating) midazolam-morphine interaction can be antagonized by the NMDA antagonist, MK 801, at the spinal level as assessed by hot-plate and tail-flick nociceptive tests. (2) When the drugs were administered supraspinally MK 801 blocked this (inhibitory) interaction in the hot-plate test only. (3) MK 801 had no effect on morphine antinociception when given i.t. and i.c.v. and (4) MK 801 significantly affected midazolam antinociception at the spinal level, but had a lack of effect following i.c.v. administration of the drugs.

Several recent findings suggest that interactions between the two major neurotransmitter systems, EAAs and GABA, are involved in nociceptive transmission and processing in the spinal cord (Watkins & Evans, 1981; Cahusac *et al.*, 1984; Aanonsen & Wilcox, 1987; Wilcox, 1991; Woolf & Thompson, 1991). EAAs are co-released with substance P in response to noxious stimuli (Battaglia *et al.*, 1986; Wilcox, 1991). There is no direct effect of glutamate, the endogenous ligand of the EAA receptor subtype NMDA (Wilcox, 1991) on binding to the benzodiazepine recognition site on the $GABA_A$ receptor ionophore complex (Schatzki *et al.*, 1990). Therefore it is unlikely that MK 801 affected midazolam antinociception at the spinal level due to direct interaction at the $GABA_A$ receptor. It thus appears reasonable to suggest that the influence of MK 801 on midazolam antinociception in the spinal cord might be due to indirect alteration at the $GABA_A$ ionophore complex. So far, it is unclear through what system (peptidergic) this interaction might take place. In the mouse spinal cord NMDA-induced hyperalgesia is dose-dependently inhibited by muscimol in the tail-flick test (Aanonsen & Wilcox, 1989). In primary cultures of striatal neurones the NMDA receptor system was coupled to the evoked release of GABA (Weiss, 1990). These observations

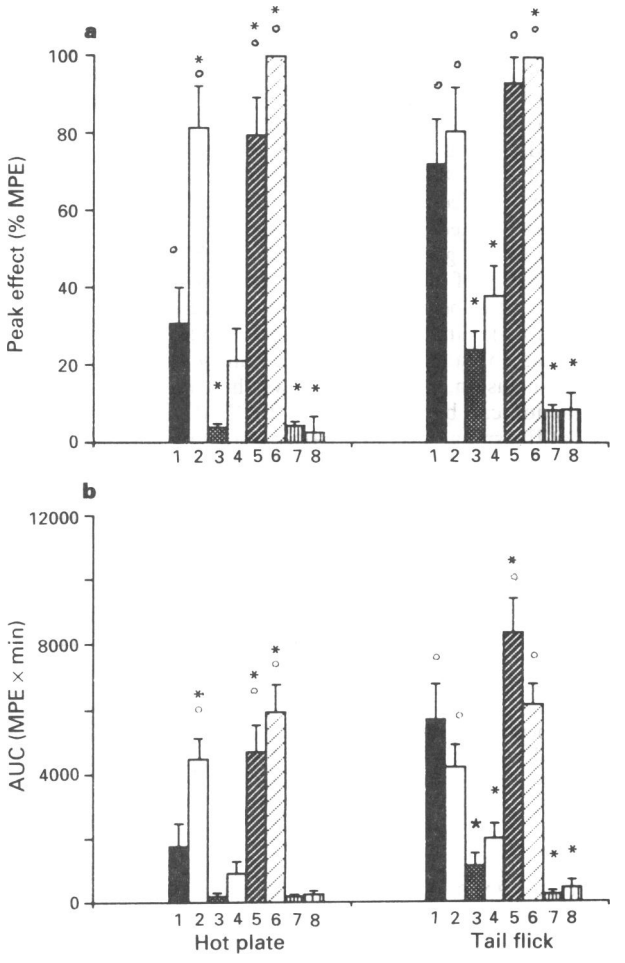

Figure 5 Peak effect of %MPE and area under the time-effect curve (MPE × min) of agonists and MK 801 injected i.c.v., showing that the inhibitory effect midazolam on morphine antinociception is reversed by MK 801 in the hot plate test. Saline/midazolam + morphine (1), MK801/midazolom + morphine (2), saline/midazolam (3), MK 801/midazolam (4), saline/morphine 1 μg (5), MK 801/morphine (6), saline/saline (7), MK 801/saline (8). Mean ± s.e.mean, ANOVA: peak %MPE (HP) $P = 0.0001$, (TF) $P = 0.0001$; AUC: (HP) $P = 0.0001$, (TF) $P = 0.0001$, $^*P < 0.05$ compared to saline/midazolam + morphine; $^\circ P < 0.05$ compared to saline/midazolam.

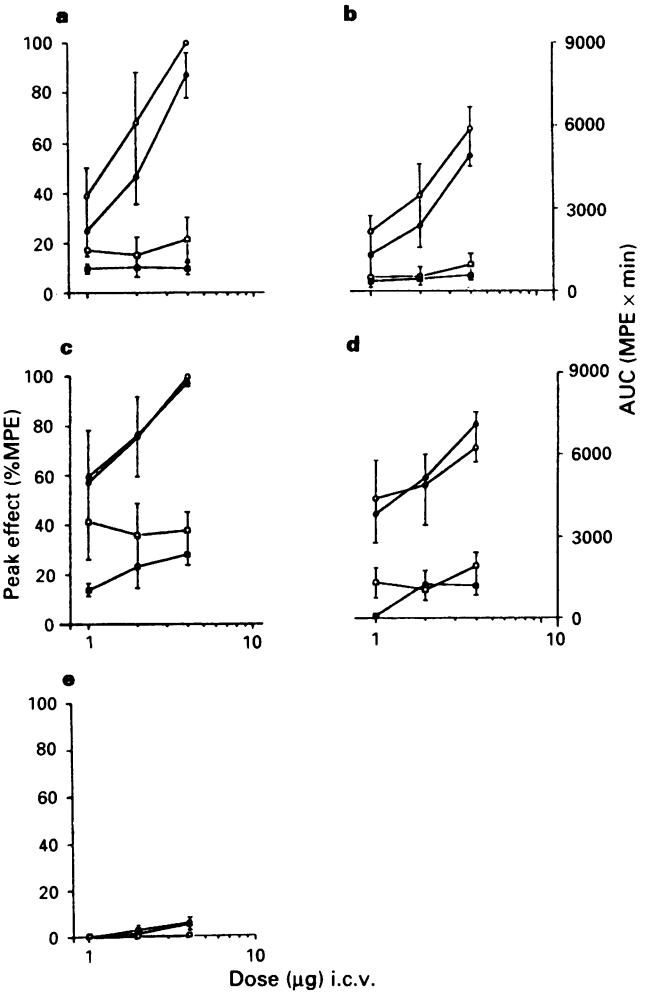

Figure 6 Dose-response curves (peak %MPE) and AUC (MPE × min) for antinociceptive effects in hot plate (a,b) and tail flick (c,d) and the incidence of catalepsy (e) after i.c.v. administration of saline/morphine (■), saline/midazolam (●), MK 801/morphine (□) and MK 801/midazolam (○).

in different areas of the central nervous system indicate that $GABA_A$ receptors may modulate EAA-induced behaviour. Furthermore, NMDA antagonist neurotoxicity studies in animals demonstrate that blockade of the NMDA receptor results in cessation of GABA release (Olney *et al.*, 1991). Benzodiazepine agonists exert their action by increasing the opening frequency of the chloride ion channel at the $GABA_A$ receptor complex (Haefely, 1985). MK 801 acts at the NMDA receptor-operated ion channel as an open channel blocker (Wilcox, 1991). Independent postsynaptic selective modulation of the ion channel of the different receptors may be an explanation for our finding that the antinociceptive effects of midazolam alone were diminished by MK 801 in both nociceptive tests at the spinal level. MK 801 had no effect on midazolam-induced changes in thermal nociceptive latencies following i.c.v. administration of the drugs. This lack of supraspinal effect by MK 801 on midazolam justifies the assumption that this interaction takes place postsynaptically only in the spinal cord. It is conceivable that higher doses of MK 801 would have affected midazolam at the supraspinal level in both acute nociceptive tests. However, this does not seem to be the case, because the doses used were already the highest that did not appear to produce motor dysfunction.

The NMDA antagonist MK 801 showed a lack of effect on morphine antinociception when administered i.t. and i.c.v. The absence of an effect agrees with observations reported previously in rats (Marek *et al.*, 1991a; Trujillo & Akil, 1991a,b; Yamamoto & Yaksh, 1992). In fact, low doses of MK 801, like other NMDA antagonists, did not affect acute morphine antinociception, but do alter the 'wind up' phenomenon and tolerance development to the antinociceptive action of morphine (Yaksh, 1989; Marek *et al.*, 1991b; Trujillo & Akil, 1991b; Goodchild, 1993). These findings stand in contrast to the recent data of Lipa & Kavaliers (1990) where intraperitoneal administration of MK 801 reduced morphine-induced antinociceptive responses in male mice, indicating that NMDA receptors are involved in the mediation of opioid analgesia. This divergence from our observation might be due to the different species used for these experiments (mice versus cats), the high dose used by these authors, and/or the different means of administering the test drugs.

Since morphine antinociception is not influenced by MK 801 at both spinal and supraspinal level, and in light of the proposed mechanism of the noncompetitive NMDA antagonist on benzodiazepine antinociception, this study demonstrates that the effect of the benzodiazepines on morphine antinociception can be modulated by MK 801. Most investigators have found that the two major neurotransmitter systems, EAAs and GABA, may be involved in nociception in the spinal cord (Watkins & Evans, 1981; Cahusac *et al.*, 1984; Aanonsen & Wilcox, 1987; Wilcox, 1991; Woolf & Thompson, 1991) and that the two receptor systems may operate in concert to amplify signals in various regions of the brain (Aanonsen & Wilcox, 1988; Weiss, 1989, Walden *et al.*, 1990). Thus, the assumption is justified that the paradoxical effect of midazolam on morphine antinociception and its reversal by MK 801 might be due to modulation at various levels of the neuraxis and/or modulation of different pathways, signifying a general physiological rather than a pharmacological receptor-receptor interaction. Thus, preadministration of MK 801 might influence the midazolam-morphine interaction postsynaptically, thereby altering the effect of the benzodiazepine on morphine at a spinal and probably supraspinal level. NMDA receptors play an important role in descending pain inhibition from the brainstem (Jacquet, 1988; Aanonsen *et al.*, 1990; Van Praag & Frenk, 1990) and it appears that changes in this system may contribute to alteration of the morphine-midazolam interaction in the spinal cord.

Even the highest doses of morphine, midazolam and their combination with 10 μg MK 801 did not affect catalepsy. This is in agreement with the data of Trujillo & Akil (1991a) who reported that MK 801 potentiated morphine catalepsy and lethality only when high doses were administered systemically. Moreover, the present observation of an immediate and short-lasting spontaneous vocalization after i.t. and i.c.v. administration of 10 μg MK 801 may demonstrate that the neuroprotective potency of delayed application of MK 801, seen in rats and mice after various excitatory stimuli (Turski *et al.*, 1990; Kehne *et al.*, 1991), occurs following initial activation of the receptor (Marek *et al.*, 1991a). Therefore, 10 μg MK 801 administered before an agonist, may initially result in short-lasting motor dysfunction seen as agitation and vocalization followed by a neuroprotective effect in both spinal cord and brain. Other investigators have observed similar behavioural effects (Yamamoto & Yaksh, 1992; Malmberg & Yaksh, 1992), and found that higher doses of MK 801 result in a higher incidence of these behavioural effects. The noncompetitive NMDA antagonist was thus injected i.t. and i.c.v. at the selected dose level in our study to give an incidence of behavioural signs in $\leqslant$ 15%MPE of the rats. As 10 μg MK 801 did not affect catalepsy, it appears that our findings were not influenced by these short-lasting spontaneous behavioural episodes in such a way as to cause false positive interpretations.

In conclusion, this study shows that midazolam-morphine interaction can be modulated by the NMDA antagonist, MK 801, at the spinal level and partly also supraspinally. Furthermore, we observed that MK 801 dose-dependently affected midazolam antinociception at the spinal level, but was without effect on morphine antinociception in either the spinal cord or the brain.

In memory of our friend and teacher Harlan F. Hill, Ph.D., Seattle. Our thanks to Prof. Sandor Agoston, M.D., Ph.D., Research Group for Clinical Pharmacology and Experimental Anaesthesia, University of Groningen, Groningen, The Netherlands, for his help in preparing the manuscript and Mag. Markus Falk, University of Innsbruck, Department of Statistics and Documentation, for his assistance in statistical analysis.

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